THE NATURAL HISTORY OF THE CRUSTACEA

Physiology

VOLUME 4

EDITED BY

Ernest S. Chang

Martin Thiel

Physiology

The Natural History of the Crustacea Series

Functional Morphology and Diversity (Volume 1)
Edited by Les Watling and Martin Thiel

Lifestyles and Feeding Biology (Volume 2)
Edited by Martin Thiel and Les Watling

Nervous Systems and Control of Behavior (Volume 3)
Edited by Charles Derby and Martin Thiel

Physiology (Volume 4)
Edited by Ernest S. Chang and Martin Thiel

Physiology

The Natural History of the Crustacea
Volume 4

EDITED BY ERNEST S. CHANG AND MARTIN THIEL

OXFORD
UNIVERSITY PRESS

OXFORD
UNIVERSITY PRESS

Oxford University Press is a department of the University of Oxford. It furthers the University's objective of excellence in research, scholarship, and education by publishing worldwide.

Oxford New York
Auckland Cape Town Dar es Salaam Hong Kong Karachi
Kuala Lumpur Madrid Melbourne Mexico City Nairobi
New Delhi Shanghai Taipei Toronto

With offices in
Argentina Austria Brazil Chile Czech Republic France Greece
Guatemala Hungary Italy Japan Poland Portugal Singapore
South Korea Switzerland Thailand Turkey Ukraine Vietnam

Oxford is a registered trademark of Oxford University Press in the UK and certain other countries.

Published in the United States of America by
Oxford University Press
198 Madison Avenue, New York, NY 10016

CIP data is on file at the Library of Congress
ISBN 978–0–19–983241–5

9 8 7 6 5 4 3 2 1
Printed in the United States of America
on acid-free paper

PREFACE

This is the fourth volume of a ten-volume series on *The Natural History of the Crustacea*. Our volume is on *Physiology*. It follows *Volume 1: Functional Morphology and Diversity, Volume 2: Life Styles and Feeding Biology*, and *Volume 3: Nervous Systems and Control of Behavior*. The next six volumes will focus on various aspects of the lives of crustaceans, including reproduction, development, life history and behavioral ecology, evolution and biogeography, fisheries and aquaculture, and ecology and conservation biology.

A comprehensive overview of the comparative physiology of crustaceans is essential to the understanding of many aspects of their biology. General overviews of specific aspects of the physiology of crustaceans can be found in some of the major invertebrate zoology textbooks. However, due to space limitations, those textbook chapters can only present a very general introduction to this diverse group. The last extensive overview on this topic was provided in three volumes (vols. 5, 8, and 9) of the 10-volume series on *The Biology of Crustacea*, which was published more than 29 years ago. Those volumes were the successors to the two-volume treatise *The Physiology of Crustacea* published in 1960–1961. Since then, several reviews about particular aspects of crustacean physiology have been published, but these are dispersed in the specialized primary literature. Thus, we believe the time is ripe to synthesize present knowledge about the physiology of crustaceans in a new volume where the information will be presented in a form that is attractive and accessible to a wide general audience of comparative physiologists.

In this volume, Webster details the distinct hormones that regulate molting and metabolism and also describes the functional overlap of these hormones in the first two chapters. McNamara and Mantelatto then present a review of the physiological control of pigmentation. Muscle structure, function, and development are covered in two chapters by Medler and Mykles and Mykles and Medler. The intriguing ability of crustaceans to drop a limb and subsequently regenerate it is reviewed by Hopkins and Das. The various types of hearts and circulatory systems are described in a chapter by McGaw and Reiber, and that is followed by a review of osmoregulation and excretion by Lignot and Charmantier. Nutrition and digestion are described by Saborowski. Many of the chapters in this volume describe not only normal physiology, but also physiological responses to environmental stress. These include responses to varying oxygen, temperature, and pH by Whiteley and Taylor, respiration and oxygen transport by Terwilliger, and metabolic regulation by Jimenez and Kinsey. The cellular and molecular responses to various stressors are reviewed by Stillman and Hurt. The volume concludes with discussions of endocrine disrupters by deFur and Williams and various other environmental pollutants by Weis.

We hope that this volume will not only provide a synthetic overview for scholars interested in the physiology of crustaceans and other arthropods, but also encourage further studies. As becomes evident from many of the contributions, crustaceans are ideal model organisms to study physiological responses to environmental factors, the understanding of which is of utmost importance in a world that is continuously changing. Crustaceans as model organisms will become all the more significant due to their functions as keystone members of food webs, dominance in a multitude of habitats, and economic value as major components of world fisheries and aquaculture.

ACKNOWLEDGMENTS

Foremost, we thank our contributors for the time they have taken in synthesizing the knowledge in their respective fields of research—their efforts and enthusiasm made this volume possible. Special thanks to our editorial assistants, Lucas Eastman and Annie Mejaes, who provided outstanding help in organizing, managing, and editing. The generous contribution from Universidad Católica del Norte was essential for this project—we are grateful for the continuous support that allowed us to focus on the task. The vision and foresight of the university authorities made this project possible, and we hope that this and the upcoming volumes fulfill their expectations. We thank our external referees who provided valuable comments to our authors and us about their chapters. E.S.C. thanks Sharon A. Chang for her laboratory and personal support during this project. Finally, we also recognize our publisher, Oxford University Press, for its commitment to the project.

Editing of this book was generously supported by Universidad Católica del Norte, Chile.

CONTRIBUTORS

EDITORS

Ernest S. Chang
Bodega Marine Laboratory
University of California-Davis
PO Box 247
2099 Westshore Road
Bodega Bay, CA 94923
USA

Martin Thiel
Facultad Ciencias del Mar
Universidad Católica del Norte
Larrondo 1281
Coquimbo
Chile

AUTHORS

Guy Charmantier
AEO Team
UMR 5119, cc 92
Université Montpellier 2
Pl. E. Bataillon
34095 Montpellier cedex 05
France

Penny M. Hopkins
Department of Biology
University of Oklahoma
730 Van Vleet Oval, Room 314
Norman, OK 73019
USA

Sunetra Das
Department of Biology
University of Oklahoma
730 Van Vleet Oval, Room 314
Norman, OK 73019
USA

David A. Hurt
Department of Integrative Biology
University of California, Berkeley
1005 Valley Life Sciences Building
Berkeley, CA 94720
USA

Peter L. deFur
Center for Environmental Studies
Virginia Commonwealth University
1000 West Cary St
Richmond, VA 23284
USA

Ana Gabriela Jimenez
Department of Biology and Marine Biology
University of North Carolina Wilmington
601 South College Road
Wilmington, NC 28403
USA

Stephen T. Kinsey
Department of Biology and Marine Biology
University of North Carolina Wilmington
601 South College Road
Wilmington, NC 28403
USA

Jehan-Hervé Lignot
AEO Team
UMR 5119, cc 92
Université Montpellier 2
Pl. E. Bataillon
34095 Montpellier cedex 05
France

Iain J. McGaw
Department of Ocean Sciences
Memorial University of Newfoundland
0 Marine Lab Road
St John's, NL A1C 5S7
Canada

John Campbell McNamara
Departamento de Biologia
Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto
Universidade de São Paulo
Ribeirão Preto, 14040-901 São Paulo
Brasil

Scott Medler
Department of Biology
State University of New York Fredonia
Fredonia, NY 14063
USA

Sarah Ribeiro Milograna
Departamento de Biologia
Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto
Universidade de São Paulo
Ribeirão Preto 14040-901 São Paulo
Brasil

Donald L. Mykles
Department of Biology
Colorado State University
Fort Collins, CO 80523
USA

Carl L. Reiber
School of Life Sciences
University of Nevada, Las Vegas
4505 Maryland Parkway
Las Vegas, NV 89154
USA

Reinhard Saborowski
Alfred Wegener Institute for Polar and Marine Research
PO Box 120161
27515 Bremerhaven
Germany

Jonathon H. Stillman
Romberg Tiburon Center and Department of Biology
San Francisco State University
3150 Paradise Drive
Tiburon, CA 94920
USA

Department of Integrative Biology
University of California, Berkeley
5041 Valley Life Sciences Building
Berkeley, CA 94720
USA

Edwin (Ted) W. Taylor
Emeritus Professor of Animal Physiology
School of Biosciences
University of Birmingham
Birmingham B15 2TT
UK

Nora B. Terwilliger
Oregon Institute of Marine Biology
University of Oregon
PO Box 5389
Charleston, OR 97420
USA

Simon G. Webster
School of Biological Sciences
Bangor University
Deiniol Road, Bangor
Gwynedd LL57 2UW
UK

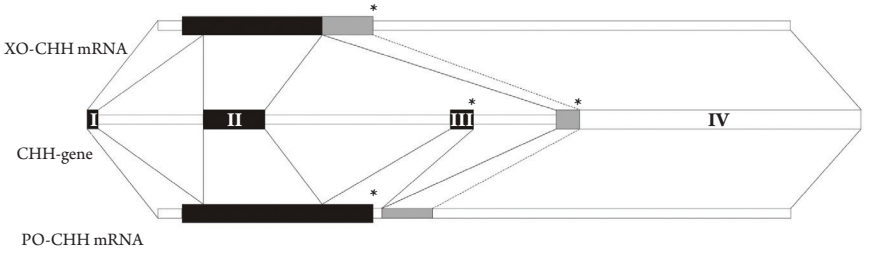
Judith S. Weis
Department of Biological Sciences
Rutgers University
195 University Avenue
Newark, NJ 07102
USA

Nia M. Whiteley
School of Biological Sciences
Bangor University
Deiniol Road, Bangor
Gwynedd LL57 2UW
UK

Laura E. Williams
Center for Environmental Studies
Virginia Commonwealth University
1000 West Cary Street
Richmond, VA 23284
USA

CONTENTS

Physiology



MYSKTIPAMLAIITVAYLCALPHAHARSTQGYGGRMDRILAALKTSPMEPSAALAVEHGTTHPLEKR

QIYDTSCKGVYDRALFNDLEHVCDDCNLYRSYVASACRSNCYSNLVFRQCMDDLLMMDEFDQYARKVQMVGRKK

NNCFENEVFDVCVYELYFPDHEEYLRSRDGLKG

Fig. 2.1.
Schematic representation (not to scale) of crustacean hyperglycemic hormones (CHH) gene, transcript, and peptide structure, illustrated by the *Carcinus maenas* CHH prototype. X-organ (XO)-CHH encoded by exons I, II, IV is expressed by eyestalk neurons and gut paraneurons, whereas pericardial organ (PO)-CHH encoded by exons I–IV is expressed by intrinsic neurons in the POs. Asterisks show positions of stop codons. CHH precursor related peptide (CPRP) is shown outlined in green, XO-CHH in blue, the PO-CHH variant C-terminal (which differs from residues 41–72, and which is unamidated) in yellow. Modified from Dircksen et al. (2001), with permission from The Biochemical Society.

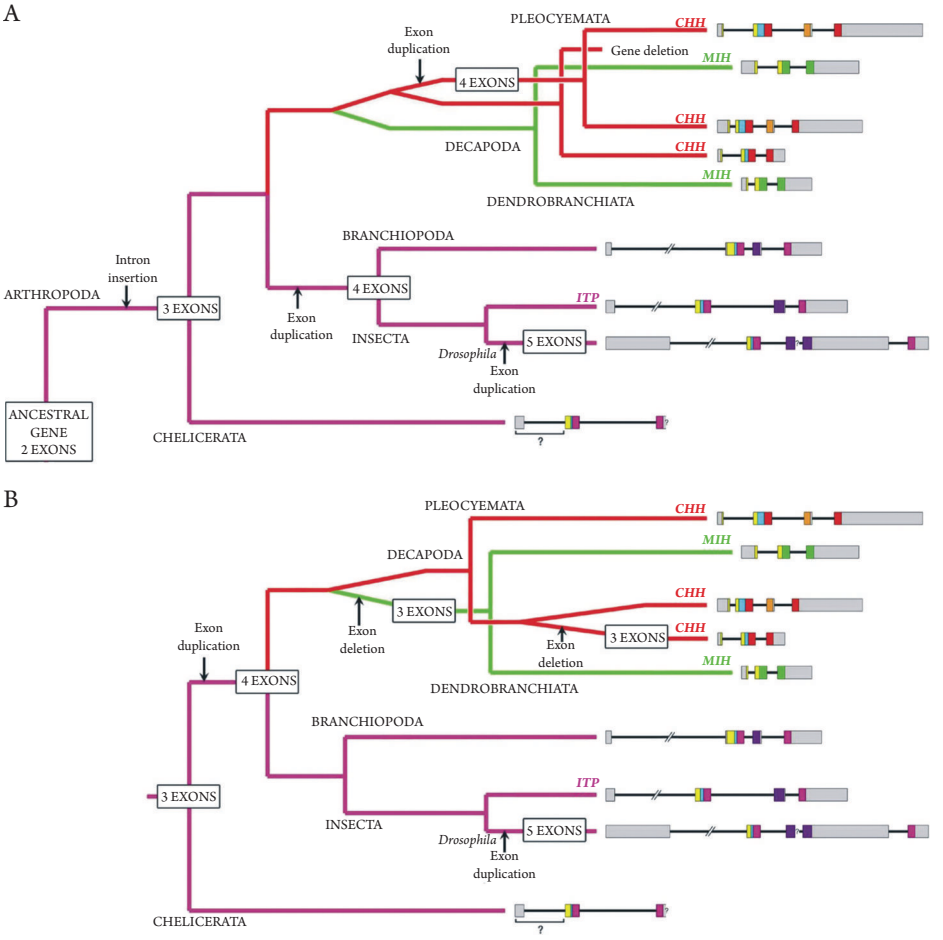


Fig. 2.2.

Evolutionary scenarios of the crustacean hyperglycemic hormones (CHH) family genes in the Arthropoda. (A) An intron insertion in an ancestral 2 exon gene (asterisk) and two independent exon duplications resulted in 4 exons for CHH, and a single-exon duplication gave 3 exon molt-inhibiting hormone (MIH) genes. (B) A single exon duplication accounts for CHH, and exon deletion for MIH gene structure. Note that a further exon duplication can account for the 5 exon ITP gene in *Drosophila.* The scaled schematics show exons represented by boxes, untranslated regions (UTR) (gray), signal peptides (yellow), precursor-related peptides (blue), ITPs (purple), CHHs (red), MIHs (green), alternatively spliced exons encoding ITP-L (violet), and CHH (orange) C-terminus regions. Question marks indicate UTRs whose precise borders are unknown. Figure redrawn from Montagné et al. (2010).

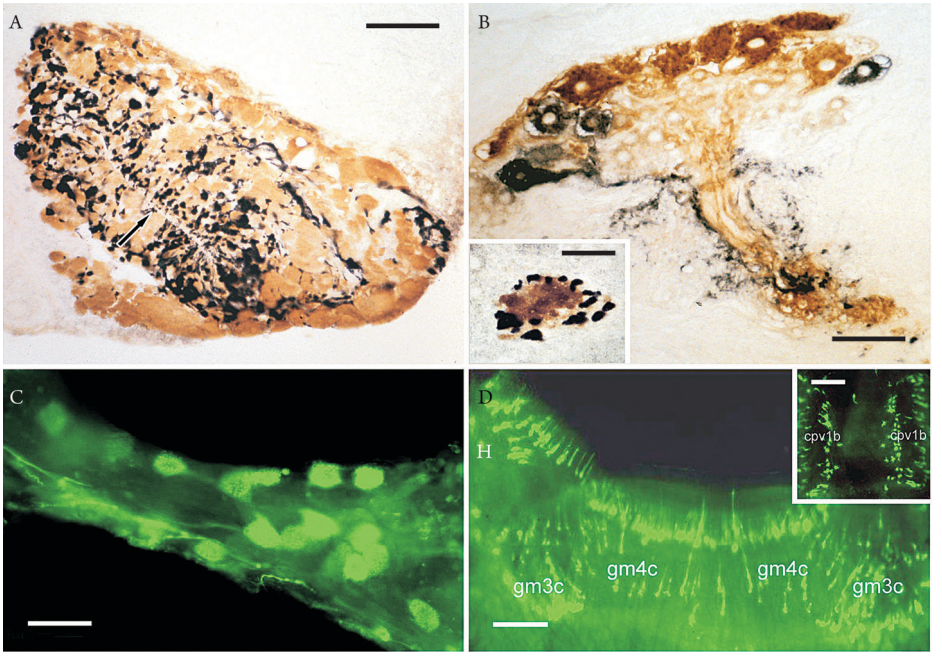


Fig. 2.6.
Immunohistochemical localization of crustacean hyperglycemic hormones (CHH) and molt-inhibiting hormone (MIH) in neural and non-neural tissues of crustaceans. (A) Sinus gland (SG) section of *Carcinus maenas* double immunostained for CHH (brown, peroxidase antiperoxidase/diamainobenzidine) and MIH (black, silver-enhanced immunogold). Arrow points to (collapsed) hemal sinus. (B) Double immunostained (as in A) section of X-organ (XO) of *C. maenas*. Inset shows transverse section of XO-SG tract. (C) Intrinsic CHH neurons in pericardial organ of *C. maenas*. (D) Endocrine cells immunoreactive (whole-mount FITC immunofluorescence) to CHH surrounding the insertions of the gastric and cardiopyloric muscles at the mesocardiac and pterocardiac ossicles of premolt (stage D_2) *C. maenas*, dorsal view. Abbreviations: gm3c, lateral posterior gastric muscles; gm4c, cardiopyloric muscles. Inset shows dorsal view of cells surrounding muscle insertions of the anterior dorsal pyloric dilators (cpv1b) at the posterior mesopyloric and anterior uropyloric ossicles.

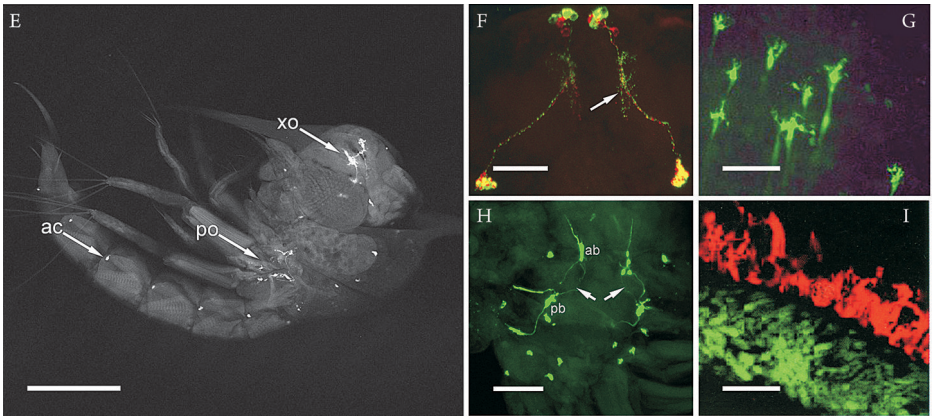


Fig. 2.6 (*Continued*)
(E) Structures immunoreactive to CHH (whole-mount immunofluorescence) in a recently hatched *C. maenas* zoea larva. Arrows point to segmentally iterated abdominal cells (ac) close to the insertions of the abdominal flexor muscles, pericardial organs (po) and associated immunopositive cells, and XO-SG neurosecretory system (xo). (F) Double labeled preparation showing localization of two pairs of CHH (red, Cy3) and MIH (green, FITC) immunoreactive neurons in the brain of a *C. maenas* embryo at the mid-eye stage (70–85% development). Arrow indicates extensive arborizing dendrites. Both peptides are not colocalized, but the SG appears yellow due to stacking of the confocal image. (G) CHH immunoreactive cells (whole-mount FITC) in the hindgut of *C. maenas*, imaged from the basal (hemolymph) side, where the cells exhibit a branching morphology. (H) CHH immunoreactive structures in the PO and adjacent areas in a *C. maenas* embryo just prior to hatching; arrows point to the PO nerve trunks. Abbreviations: ab, anterior bar; pb, posterior bar. (I) Dual-labeled section of the retina of juvenile crayfish (*Procambarus clarkii*). Red structures (Texas red) show CHH immunoreactive tapetal cells, green structures (FITC) show 5-HT immunopositive retinular cell axons. Upper cell layer shows CHH immunoreactive tapetal cells; lower layer, 5-HT immunopositive retinular cell axons. Scale bars: 50 μm (A, B, F, G, H), 100 μm (C), 200 μm (D) and insert (E), 20 μm (I), 25 μm insert (B). Images: (A, B) adapted from Dircksen et al. (1988), with permission from Wiley and Sons, Inc.; (C) courtesy of H. Dircksen; (D, E, F, H) adapted from Chung and Webster (2004), with permission from The Company of Biologists; (G) Webster, unpublished; (I) adapted from Escamilla-Chimal et al. (2001), with permission from The Company of Biologists.

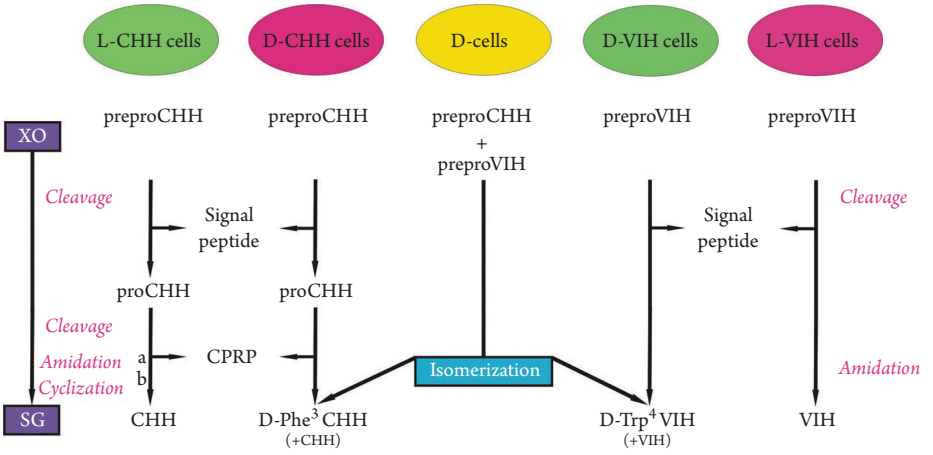


Fig. 2.7.
Diagrammatic representation of cell type specific precursor processing of crustacean hyperglycemic hormones (CHH) and vitellogenesis-inhibiting hormone (VIH) isomers in the X-organ-sinus gland complex. CPRP, CHH precursor-related peptide. Amidation (a) can occur before, during, or after cleavage of CPRP. Cyclization of glutamate to pyroglutamate (b) can only occur after CPRP cleavage. Some cells secrete L-CHH, L-VIH, whereas D-CHH, D-VIH cells, while producing mainly the D-isomers, also secrete variable amounts of L-isomers. However, a small population of cells exclusively secrete the D-isomers of both hormones. Adapted from Ollivaux et al. (2009), with permission from John Wiley and Sons, Inc.

Fig. 3.1.
The Indo-Pacific harlequin shrimp, *Hymenocera elegans*, exhibits one of the most stunning chromogenic adaptations found among the Crustacea. This gnathophyllid coral reef shrimp displays a fairly fixed, sex- and species-specific coloration pattern that consists of irregular, bluish to purple spots and markings, placed fairly symmetrically on a cream-white background to form a complex pigmentary system. Here, a pair of *H. elegans* (seen in frontal view) explores a prey species, the Indo-Pacific blue star, *Linckia laevigata*. With permission from Adriano Morettin, all rights reserved.

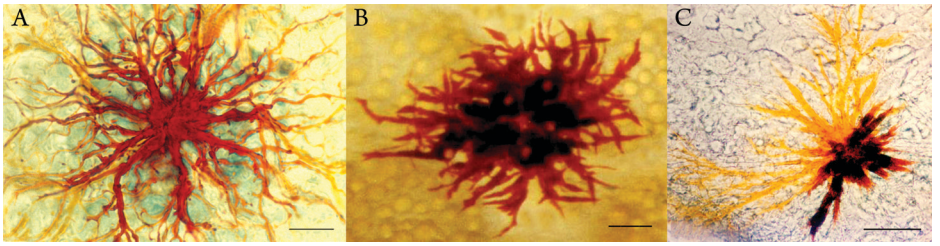


Fig. 3.2.
Chromatosomes consist of multicellular aggregates of highly asymmetrical, single-celled, monochromatic chromatophores. Their intimately apposed, semispherical perikarya contain the pigment granules and form the chromatosome center from which radiate the long chromatophore extensions through which the granules migrate. (A) Monochromatic, red, epidermal chromatosome from the neotropical freshwater shrimp, *Macrobrachium olfersi*. (B) Monochromatic, red chromatosome, showing the nuclei of individual chromatophores (round clear areas within the dark red perikarya) on the dorsal surface of the fibrous capsule containing the ovary of *Macrobrachium olfersi*. These particular pigmentary effectors have been employed with success as models in deciphering the mechanisms of membrane signal transduction, intracellular second-messenger cascades, and regulation of the molecular motors that translocate the pigment granules through the cytosol. (C) Dichromatic, epidermal chromatosome from the New Zealand intertidal shrimp, *Palaemon affinis*, consisting of separate monochromatic yellow (left) and monochromatic red/brown chromatophores (right) containing fully dispersed and partially dispersed pigments, respectively. Scale bars = 50 μm.

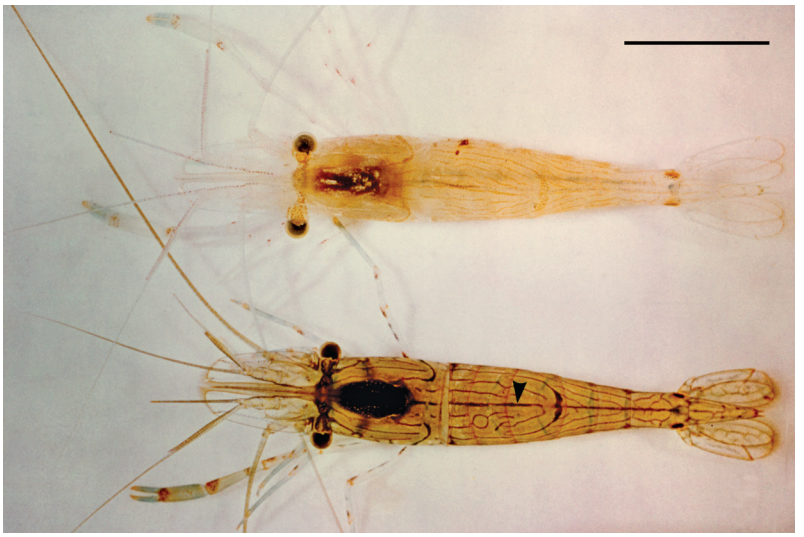


Fig. 3.3.
Chromomotor adaptation in *Palaemon affinis*. The nearly transparent upper shrimp was exposed to an illuminated white background for 2 h, and the brown pigments in its epidermal chromatosomes are fully aggregated; the white somatogastric chromatosome pigments are fully dispersed. The lower shrimp, in which the species' typical chromogenic pattern is clearly visible, was placed on an illuminated black background and displays fully dispersed brown epidermal chromatosome pigments; arrowhead indicates fully dispersed hindgut chromatosomes. Such rapid translocations of the pigment granules through the chromatophore cytosol are regulated by blood-borne neuropeptides called *chromatophorotropins*, such as red pigment concentrating hormone, released from the eyestalk and other neurosecretory centers. Scale bar = 1 cm.

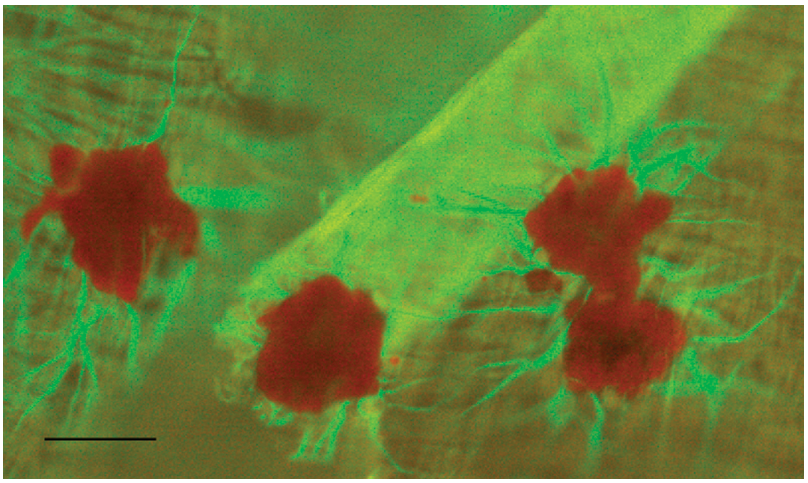


Fig. 3.4.
Epifluorescence microscopy (Leica DM5500B, λ_{ex}=495 nm, λ_{em}=519 nm) showing several red chromatosomes with fully aggregated pigments from the ovary of *Macrobrachium olfersi*. Elongated microtubule bundles (green) lie along the length of the pigment-free cell extensions that project from the perikarya of the individual constituent chromatophores among the surrounding fibroblasts. Monoclonal, anti-β-tubulin primary antibody followed by an Alexa-488 conjugated goat anti-mouse IgG. Scale bar = 100 μm.

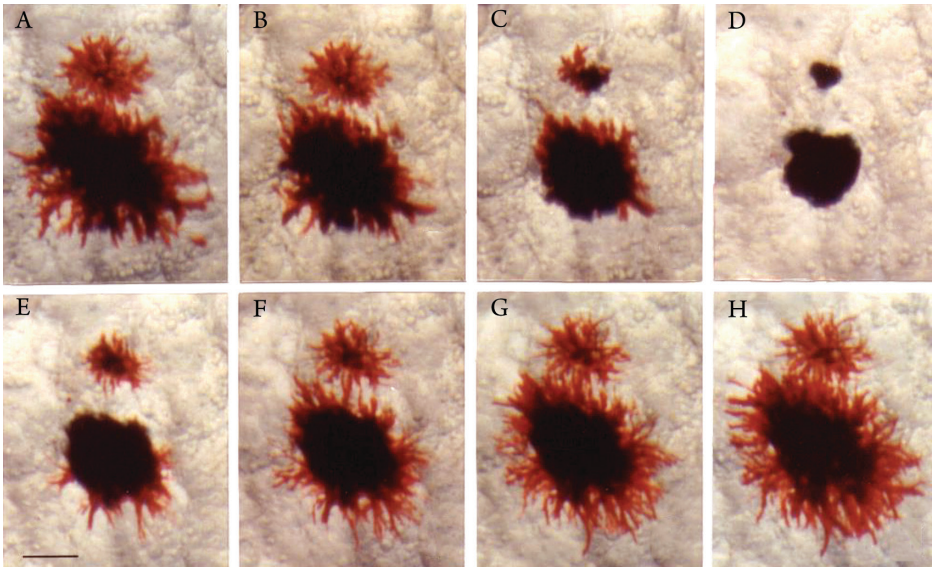


Fig. 3.5.
Sequence of pigment aggregation (A–D) in two red chromatosomes on the ovary of *Macrobrachium olfersi* induced by 30 min perfusion in vitro with 25 μM Ca^{2+}-ionophore A23187 in a 2.5 mM $NaHCO_3$-buffered physiological saline. The pigments were subsequently dispersed (E–H) by 30 min perfusion with a Ca^{2+}-free (10^{-11} M Ca^{2+}), 2 mM EDTA-chelated saline. The pigment translocation rates are not constant (see Fig. 3.6). Scale bar = 50 μm.

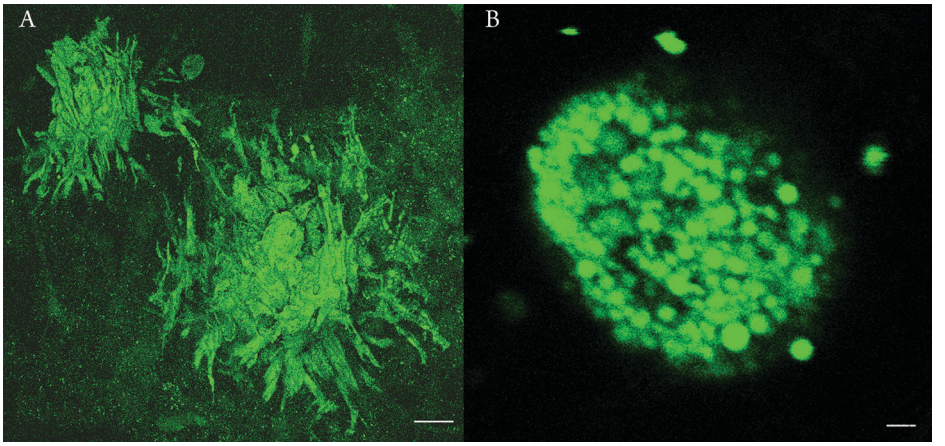


Fig. 3.9.
Myosin molecular motors, intimately associated with the pigment granule membranes, play an important role in granule translocation along the actin cytoskeleton during pigment aggregation and possibly in limiting pigment dispersion brought about by opposing motors. (A) Confocal fluorescence microscopy (Leica TCS SP5, λ_{ex} = 495 nm, λ_{em} = 519 nm) showing the distribution of granule-associated, nonmuscle myosin II throughout the cytoplasm of *M. olfersi* ovarian chromatophores with fully dispersed pigments, revealed using a polyclonal, nonmuscle myosin II primary antibody followed by an Alexa-488 conjugated goat anti-rabbit IgG. (B) Distribution of a skeletal muscle pan-myosin clearly associated with the large membrane-bounded pigment granules in an ovarian chromatophore extension, shown employing a polyclonal, anti-skeletal muscle pan-myosin primary antibody conjugated with an Alexa-488 goat anti-rabbit IgG. Scale bars A = 20 μm, B = 1 μm.

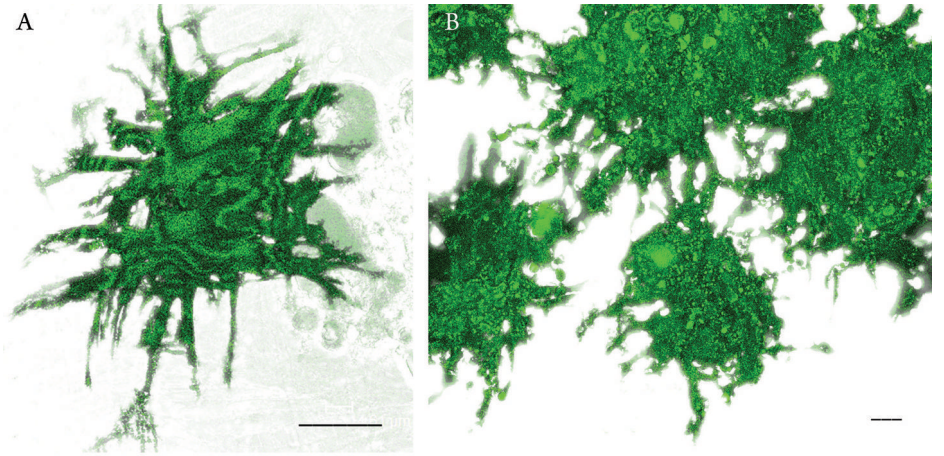


Fig. 3.10.
Molecular motors like kinesin and dynein that respectively transport cargos to the chromatophore periphery or perikaryon along the microtubular component of the cytoskeleton are also associated with the pigment granules. (A) Confocal fluorescence microscopy (Leica TCS SP5, λ_{ex}= 495 nm, λ_{em}= 519 nm) showing the distribution of granule-associated kinesin throughout the cytoplasm of a chromatosome with fully dispersed pigments, revealed using a monoclonal, anti-kinesin primary antibody and an Alexa-488 conjugated goat anti-mouse IgG. (B) Dynein distribution is clearly associated with the large membrane-bound pigment granules in a chromatosome with fully dispersed pigments, shown here employing a monoclonal, anti-dynein primary antibody followed by an Alexa-488 conjugated goat anti-mouse IgG. Bright field and fluorescence images overlaid with background subtraction, scale bars = 20 μm.

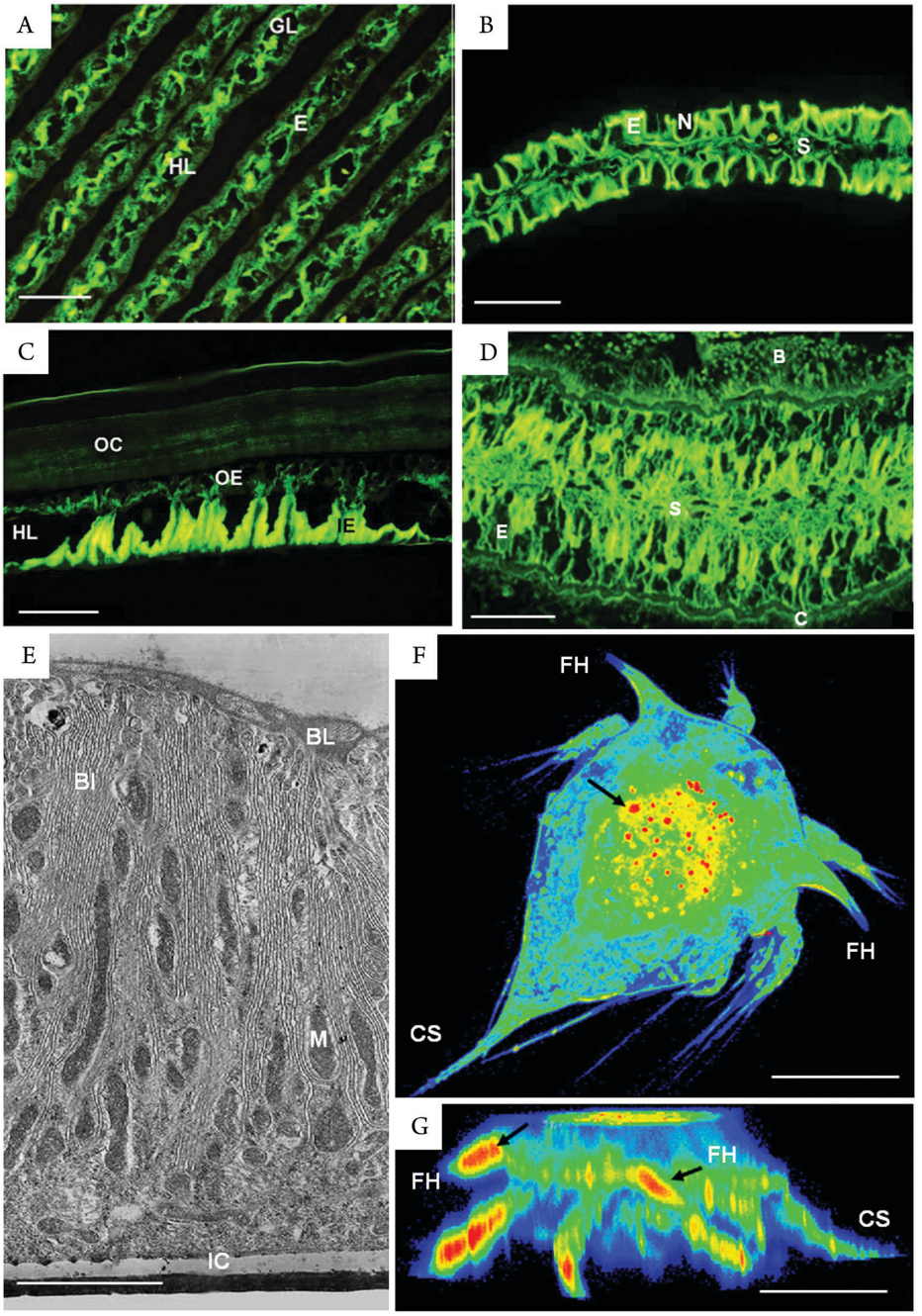


Fig. 8.3.
(A)–(D) Immunolocalization of Na^+/K^+-ATPase in the gills (A), epipodite (B), and branchiostegite (C) of the epibenthic *Palaemon adspersus* and in the epipodite of the deep-sea hydrothermal *Rimicaris exoculata* (D). Scale bars: 50 µm. (E) Transmission electron micrograph of the branchiostegite of *Palaemon adspersus*. Scale bar: 2.5 µm. (F) three-dimensional confocal stack showing the dorsal side of the *Amphibalanus amphitrite* nauplius. Black arrow indicates areas of intense fluorescent labeling in the naupliar tissue below the dorsal shield. (G) Lateral view of the nauplius. Black arrows denote the frontal horns where fluorescence intensity is high. Scale bars: 100 µm (F–G). Abbreviations: B, bacteria; BI, basal infoldings; BL, basal lamina; C, cuticle; CS, caudal spine (posterior); E, epithelium; FH, frontal horns; GL, gill lamellae; HL, hemolymph lacuna; IC: internal cuticle; IE, inner epithelium; M, mitochondria; N, nucleus; OC, outer cuticle; OE, outer epithelium; S, septum. Adapted from Martinez et al. (2005), with permission from Elsevier, and from Gohad et al. (2009), with permission from Elsevier.

1

ENDOCRINOLOGY OF MOLTING

Simon G. Webster

Abstract

Molting processes in all arthropods are ultimately regulated by ecdysteroids produced by Y-organs (YO) from cholesterol via a common biosynthetic pathway. Increases in ecdysteroid hemolymph titer initiate premolt. YO ecdysteroid synthesis is regulated by (inhibitory) peptides produced by neurosecretory cells in the eyestalk. These hormones are defined as molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH). Recent research has shown that molt control (at the level of processes controlling ecdysteroid synthesis in the YO) is complex, first by release of CHH from endocrine cells in the fore- and hindgut, then by release of crustacean cardioactive peptide (CCAP) from the pericardial organs. Finally, insect cuticle hardening hormone is released (together with CCAP), resulting in tanning and sclerotization of the cuticle in immediate postmolt. In silico analyses of genomes and peptidomes predict the existence of other peptide hormones in crustaceans known to be centrally important in insect molting endocrinology.

INTRODUCTION

For all arthropods, a thick, inflexible exoskeleton has inevitable consequences for almost every aspect of their life history. In order for growth to occur, the exoskeleton must be periodically molted. This process, called *ecdysis*, is followed by uptake of water (or air in the case of the majority of insects) and redistribution of hemolymph. This results in the expansion of the new, flexible exoskeleton to its final postmolt dimensions before the cuticle is made inflexible and tough via quinone tanning and sclerotization and, for many malacostracan crustaceans, subsequent calcification. The structure and function of the crustacean cuticle and its

elaboration and mineralization is described in detail by Dillaman et al. (2013) (see Chapter 5 in volume 1).

Given the large number of processes, both somatic and behavioral, that must be precisely coordinated during ecdysis, this process has been the subject of intense research, particularly in insects, where a complex series of steroid and terpenoid hormones, neuropeptides, and neurohormones are involved (Truman 2005, Kim et al. 2006, Ewer 2007, Žitňan et al. 2007). Apart from the well-studied roles of the molting hormones (ecdysteroids) in directing processes such as cell division, differentiation, and the synthesis of new cuticle in insects (Riddiford 1989), the synthesis of which is stimulated by prothoracicotropic hormone (PTTH; Gilbert et al. 1996, 2002), a variety of peptide hormones such as pre-ecdysis triggering hormone (PETH), ecdysis triggering hormone (ETH), kinins, diuretic hormones (DHs), myoinhibitory peptides (MIPS), eclosion hormone (EH), crustacean cardioactive peptide (CCAP), and bursicon are central to the insect ecdysis program (Truman 2005, Arakane et al. 2008). Perhaps unsurprisingly, given the rich genetic resources and tools available in *Drosophila*, far more is known concerning the hormonal control of molting in insects than (genetically intractable) crustaceans. Moreover, as discussed later, not only are there some well-established fundamental differences in the hormonal control of molting in insects and crustaceans, but there are also some fascinating glimpses of recognizably similar, yet rather different, endocrine mechanisms involved in molting in crustaceans as compared to insects. In this review, recent advances in our knowledge of the endocrine control of molting in crustaceans will be highlighted, and the development of the field based on early experiments and observations will be explored.

THE CLASSICAL MODEL OF THE HORMONAL CONTROL OF MOLTING

It has long been known that bilateral eyestalk ablation often (but not invariably) leads to accelerated molting in malacostracan crustaceans (Zeleny 1905, Smith 1940). Such observations, repeated many times, have led to the hypothesis that the eyestalk—or more specifically, the X-organ sinus gland (XO-SG) neurosecretory system—is the source of a molt-inhibiting hormone (MIH) that negatively regulates production of molting hormones (Passano 1953). These observations were supported by broadly contemporaneous observations showing that ablation of the molting glands (Y-organs, YO) prevented molting in intact and eyestalk-ablated crabs (Gabe 1953, Echalier 1959). More recent studies have repeatedly shown that eyestalk removal leads to increases in circulating ecdysteroids consequent upon increased ecdysteroid synthesis by the YO (Chang et al. 1976, Keller and Schmid 1979, Jegla et al. 1983). Reciprocal experiments, although rather infrequently reported, have clearly shown that eyestalk or sinus gland extract injection leads to reduction in ecdysteroid titer (Hopkins 1982, Bruce and Chang 1984, Nakatsuji and Sonobe 2004). The finding that the YO of crayfish (*Orconectes limosus*) taken from animals injected with sinus gland extracts produced rather less ecdysteroids compared to saline-injected controls (Gersch et al. 1980), as well as the finding that the production of YO cultured in vitro could be inhibited by eyestalk and sinus gland extracts or sinus gland-conditioned media (*Pachygrapsus crassipes*, Soumoff and O'Connor 1982; *Cancer antennarius*, Mattson and Spaziani 1985a; *Carcinus maenas*, Webster 1986) further supported the classical model of molt control. The development of the YO bioassay, in which one of a pair of YO was exposed to high-performance liquid chromatography (HPLC)-purified SG fractions followed by estimation (by radioimmunoassay, RIA) of repression of ecdysteroid synthesis, was instrumental in the purification and isolation of MIH, as described later.

HORMONES INVOLVED IN MOLTING

Ecdysteroids

Levels During the Molt Cycle

The identification of ecdysteroids as the molting hormones of both insects and crustaceans was achieved almost 50 years ago (Horn et al. 1966), and, with the development of sensitive immunoassays (e.g., RIA, Borst and O'Connor 1972, 1974, Porcheron et al. 1976; enzyme immunoassay, EIA, Porcheron et al. 1989), estimations of total circulating ecdysteroids have been made in many species. For decapods, examples include crabs (*Callinectes sapidus*; Soumoff and Skinner 1983, Lee et al. 1998, Chung 2010), *C. maenas* (Lachaise et al. 1976, Styrishave et al. 2008), *Emerita asiatica* (Gunamali et al. 2004), *Gecarcinus lateralis* (McCarthy and Skinner 1977), *P. crassipes* (Chang et al. 1976, Chang and O'Connor 1978), *Uca pugilator* (Hopkins 1983, 1986), crayfish and lobsters (*Astacus leptodactylus*; Durlait et al. 1988), *Orconectes sanborni* (Stevenson et al. 1979), *Homarus americanus* (Chang and Bruce 1980, Snyder and Chang 1991a), shrimps (*Palaemon serratus*; Baldaia et al. 1984), *Penaeus japonicus* (Okumura et al. 1989), and *Macrobrachium nipponense* (Okumura et al. 1992). Circulating ecdysteroid levels invariably peak during premolt (stage D_2) before rapidly declining to low levels during late premolt (stages D_{3-4}), which is essential for ecdysis: manipulative elevation of ecdysteroid levels by injection at this time delays this process in *H. americanus* (Cheng and Chang 1991). Low ecdysteroid levels are also seen in immediate postmolt, but in late postmolt (stages $B–C_1$) a small but significant peak in ecdysteroid levels is seen, and this may be correlated with increased carbonic anhydrase activity in the epidermis at this time, which is also coincident with the large premolt peak in ecdysteroids, perhaps suggesting causality (Baldaia et al. 1984). Repeated measurement of circulating ecdysteroids from individual animals has shown that levels can fluctuate at a very fine temporal scale as shown for *H. americanus* (Snyder and Chang 1991a) or that there are well-defined peaks during premolt as determined for *U. pugilator* (Hopkins 1983, 1986). For animals displaying a terminal anecdysis, the pubertal molt is followed by reduced activity or degeneration of the YO, as for example in *Sphaeroma serratum* (Charmantier and Trilles 1979) and, in consequence, low ecdysteroid levels in mature animals (e.g., in *Libinia emarginata*, Laufer et al. 2002; *Chionoecetes opilio*, Tamone et al. 2005). For other malacostracans, for example, isopods (*Helleria brevicornis*, Hoarau and Hirn 1978), *Armadillidium vulgare* (Suzuki et al. 1996), *Ligia oceanica* (Girard and Maissat 1983), amphipods (*Orchestia gammarellus,* Blanchet et al. 1976, 1979), *O. cavimana* (Graf and Delbeque 1987), and mysids (*Siriella armata*, Cuzin-Roudy et al. 1989), ecdysteroid levels peak during premolt, decline before molting, and remain low during postmolt and intermolt. For non-malacostracan crustaceans, measurements of ecdysteroid profiles are destructive due to their small size. In *Daphnia magna*, levels of ecdysteroids peak around 40 h after molting and are associated with the release of first- and second-clutch neonates (Martin-Creuzberg et al. 2007). In *Calanus pacificus,* ecdysteroid levels peak in early premolt (Johnson 2003). Ecdysteroid concentrations have been measured during the principal molt stages in the meiofaunal harpactacoid copepod *Amphiascus tenuiremis* and the amphipod *Leptocheirus plumulosus* (Block et al. 2003). Examples of ecdysteroid profiles during the molt cycle of diverse crustacean are shown in Fig. 1.1.

The major (free) ecdysteroids observed in the hemolymph are ecdysone, 20-hydroxyecdysone, ponasterone A, and 3-dehydro-20-hydroxyecdysone, and ratios of these vary during the molt cycle. For example, in *C. maenas*, only ecdysone and 20-hydroxyecdysone are found during intermolt, yet the large peak of ecdysteroid observed during premolt is mainly ponasterone A, reflecting a switch to 25-deoxyecdysone synthesis by the YO at this time (Lachaise et al. 1986, 1988); during the precipitous fall in ecdysteroid levels just prior to molting, the reduction in concentration of this hormone is relatively greater than the observed reduction in ecdysone and 20-hydroxyecdysone concentrations

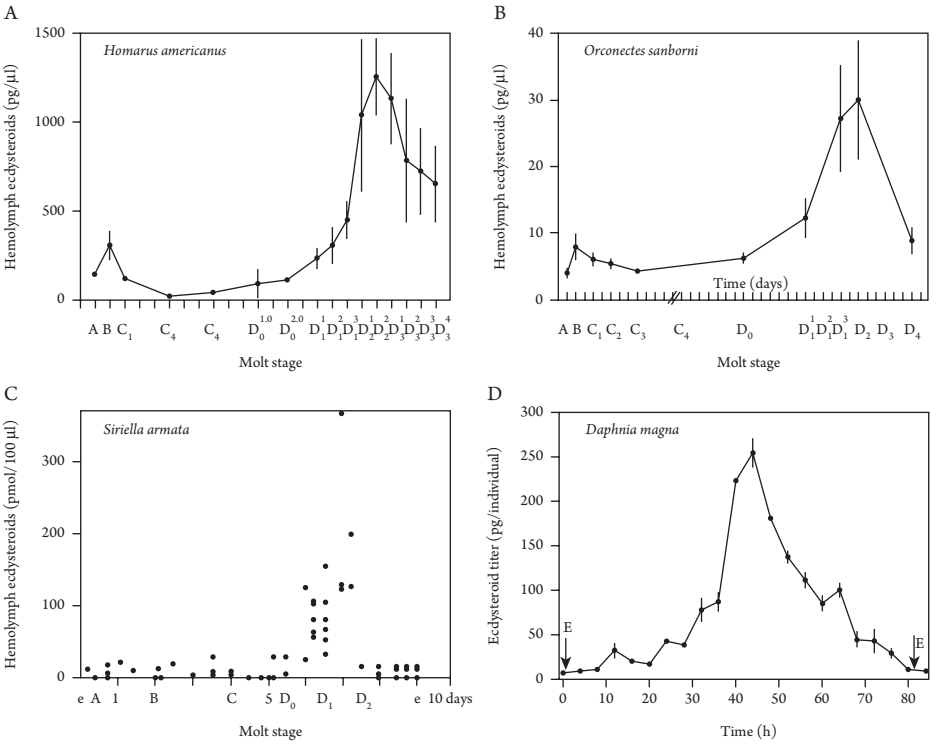


Fig. 1.1.
Profiles of ecdysteroids during the molt cycle of selected crustaceans. (A) Lobster, *Homarus americanus*, males (Snyder and Chang 1991a). Each point represents a mean of 3–7 determinations, error bars, ± SD. Molt stages according to Aiken (1973), and, in late premolt, according to Cheng and Chang (1991). (B) Crayfish, *Orconectes sanborni* (Stevenson et al. 1979). Each point represents a mean of 5–40 determinations, error bars, ± SD. Molt stages according to Drach and Tchernigovtzeff (1967). (C) Mysid, *Siriella armata*, males (Cuzin-Roudy et al. 1989). Molt stages according to Cuzin-Roudy and Tchernigovtzeff (1985). Ecdysis (e). (D) Water flea, *Daphnia magna* (Martin-Creuzberg et al. 2007). Each point represents a mean of three determinations, error bars ± SE. Carcass ecdysteroids were determined every 4 h between two successive molts associated with release of first- and second-clutch neonates. Redrawn from originals.

(Lachaise and Lafont 1984, Styrishave et al. 2008). Conversely, in *Penaeus* (*Marsupenaeus) japonicus*, 20-hydroxyecdysone and ponasterone A are the major ecdysteroids during post- and intermolt, whereas 20-hydroxyecdysone is the major circulating ecdysteroid during premolt (Okumura et al. 1989). Eyestalk ablation also changes the composition of ecdysteroids in the hemolymph: in *C. sapidus*, this operation results in 20-hydroxyecdysone becoming the major ecdysteroid during premolt, rather than ponasterone A (Chung 2010), whereas in *U. pugilator,* eyestalk removal results in a higher ponasterone A-to-20-hydroxyecdysone ratio than for crabs in which molting is induced by multiple limb autotomy (Hopkins 1992). Taken together, these results indicate that where eyestalk ablation is used to induce molting, a normal endocrinological status of the animal cannot be tacitly assumed, and the significance of eyestalk-induced perturbations of ecdysteroid synthesis relative to completion of natural molt cycles remains to be explored.

Biosynthesis

Ecdysteroids are synthesized by the YO—small, epithelioid tissues found at the anterior margin of the branchial chamber that were first described by Gabe (1953) in *C. maenas*. For a review of YO

structure and function, see Lachaise et al. (1993). Because arthropods cannot synthesize the precursor (cholesterol) from acetate or mevalonate, this is acquired from the diet, transported in the hemolymph via high-density lipoproteins, and taken up by the YO via receptor-mediated endocytosis (Spaziani and Kater 1973, Watson and Spaziani 1985). The biosynthetic pathway of ecdysteroid synthesis, which has essentially been determined using in vivo injection of ^{3}H-25-hydroxycholesterol (which is much more soluble than cholesterol or 7-dehydrocholesterol) and subsequent isolation of metabolites via HPLC and mass spectrometry (MS) (Böcking et al. 1994, Wang et al. 2000) has still not been entirely elucidated in crustaceans and seems to be rather diverse compared to insects because a number of ecdysteroids can potentially be synthesized. However, normally only two are released from the YO of a particular species, as shown in Table 1.1. A detailed review of our current understanding of ecdysteroid synthesis by the crustacean YO has recently been published (Mykles 2011), but salient features are mentioned here.

The first stage of ecdysteroid biosynthesis is the conversion of cholesterol to 5β-diketol (3-dehydro-2, 22, 25-deoxyecdysone). This occurs via conversion of cholesterol to 7-dehydrocholesterol (Rudolph and Spaziani 1992, Rudolph et al. 1992) via 7,8-dehydrogenase and a series of as yet incompletely determined reactions involving 3-oxo-Δ^4 intermediates, referred to as the "black box" (Blais et al. 1996), producing Δ^4-diketol, which is then reduced to 5β-diketol (2,22,25-deoxyecdysone) by 3-dehydroecdysteroid-3β-reductase (Böcking et al. 1993). The second stage of biosynthesis involves successive hydroxylations of 5β-diketol resulting in four potential secretory products from the YO (3-dehydro-25-deoxyecdysone, 3-dehydroecdysone, 2-deoxyecdysone, 25-deoxyecdysone) that can undergo metabolism in peripheral tissues to ponasterone A (25-deoxy-20-hydroxyecdysone) and 20-hydroxyecdysone. A current model summarizing the ecdysteroid biosynthetic pathway in crustaceans is shown in Fig. 1.2. The enzymes involved in ecdysteroid biosynthesis from 5β-diketol have been identified in *Drosophila* as the P450 mono-oxygenases (Halloween genes) *Phantom* (*phm*), *Disembodied* (*dib*), *Shadow* (*sad*), and *Shade* (*shd*) (Rewitz et al. 2007). Although these enzymes have broad substrate specificity, it seems that the mandatory order of hydroxylations (C25→C22→C2→C20) is maintained. Early stages

Table 1.1. Ecdysteroids secreted by the Y-organs of decapod crustaceans

Species	E	3DE	25dE	3D25dE	References
Pachygrapsus crassipes	•				Chang and O'Connor 1977
Cancer antennarius	•	•			Watson and Spaziani 1985, Spaziani et al. 1989
Orconectes limosus	•	•			Böcking et al. 1993
Procambarus clarkii	•	•			Sonobe et al. 1991
Penaeus vannamei	•	•			Blais et al. 1994
Macrobrachium rosenbergii	•	•			Okumura et al. 2003
Carcinus maenas	•		•		Lachaise et al. 1989
Callinectes sapidus	•		•		Unpub. Cited in Wang et al. 2000
Uca pugilator	•		•		Hopkins 1986, 1992*
Menippe mercenaria		•		•	Rudolph and Spaziani 1992, Wang et al. 2000

Abbreviations: E, ecdysone; 3DE, 3-dehydroecydsone; 25dE, 25-deoxyecdysone; 3D25dE, 3-dehydro-25-deoxyecdysone.
* Secretion of 25dE inferred by presence of ponasterone A in premolt.
Adapted from Mykles 2011, with permission from Elsevier.

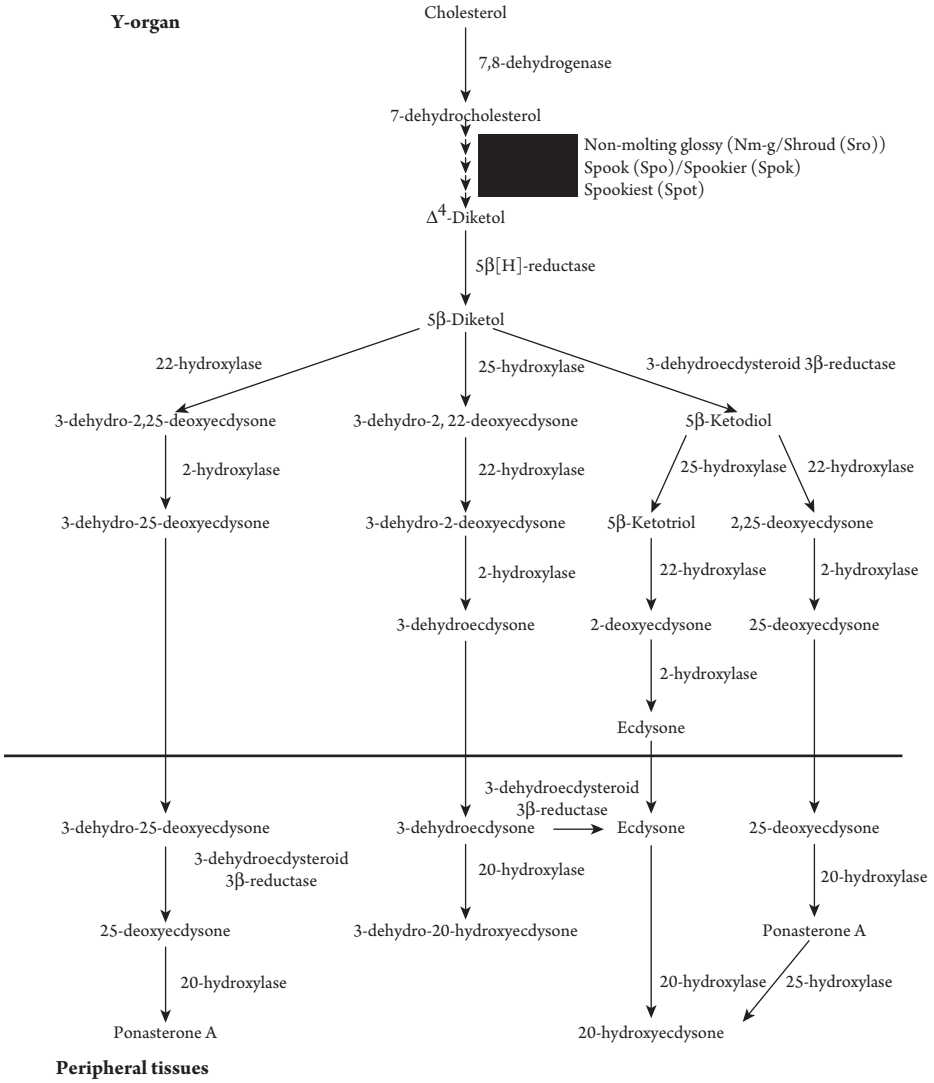


Fig. 1.2.
The ecdysteroid biosynthetic pathway in the crustacean Y-organ (YO). Cholesterol is first converted to 7-dehydrocholesterol by a 7,8-dehydrogenase encoded by the *Neverland* (*nvd*) gene in insects. Subsequent conversion to Δ^4-diketol occurs via a series of as yet incompletely understood reactions named the "black box" involving 3-oxo-Δ^4 intermediates, which are catalyzed by enzymes encoded by *Nonmolting glossy* (*nm-g*)/*Shroud* (*sro*), *Spook* (*spo*)/*Spookier* (*spok*), and *Spookiest* (*spot*) genes in insects. Reduction of Δ^4-diketol by 5β[H]-reductase to 5β-diketol is followed by hydroxylation by enzymes encoded by *Disembodied* (22-hydroxylase, *Phantom* (25-hydroxylase), *Shadow* (2-hydroxylase), and 3-dehydroecdysteroid-3β-reductase, which potentially results in four ecdysteroid products, but only two are secreted by YO (Table 1.1). In peripheral tissues, further hydroxylations occur catalyzed by 20-hydroxylase, encoded by *Shade*, a 25-hydroxylase; and a 3-dehydroecdysteroid-3β-reductase, resulting in a maximum of three final ecdysteroid products. Modified from Mykles (2011), with permission from Elsevier and with information from Böcking et al. (1993), Lachaise et al. (1993), Wang et al. (2000), Gilbert and Rewitz (2009).

of synthesis are controlled by *Neverland* (*Nvd*) (7,8-dehydrogenase; Yoshiyama et al. 2006) and black-box intermediates are catalyzed by *Nonmolting glossy* (*nm-g*)/*shroud* (*sro*) and several Halloween genes: *Spook* (*spo*), *Spookier* (*spok*), and *Spookiest* (*spot*) (Rewitz et al. 2007, Niwa et al. 2010). Because orthologs of all the above-mentioned genes have been identified in the *Daphnia pulex* genome (Rewitz and Gilbert 2008), as well as *phm* in *Marsupenaeus japonicus* (Asazuma et al. 2009), it seems probable, indeed unsurprising, that the genes encoding the enzymes involved in ecdysteroid biosynthesis are highly conserved across the phylum, even if the pathways involved in species-specific ecdysteroid secretion patterns are somewhat diverse.

Metabolism

Hormonally active ecdysteroids are metabolized to more polar compounds and ecdysonoic acids, which are excreted in the urine (the antennal gland is the principal site of excretion of ecdysteroids), or apolar conjugates are produced by the midgut gland, and these conjugates are excreted in feces. A comprehensive review of metabolism of ecdysteroids is included in Mykles (2011); only salient features are mentioned here.

Conversion of ecdysteroids to polar products and ecdysonoic acids involves conversion of 20-hydroxyecdysone to 20, 26-dihydroxyecdysone and conversion of ponasterone A to 25-deoxy-20, 26-dihydroxyecdysone. Oxidation at C26 converts 20-hydroxyecdysone to 20-hydroxyecdysonoic acid and ponasterone A to 25-deoxy-20-hydroxyecdysonoic acid (Lachaise and Lafont 1984, Lafont et al. 1986, Snyder and Chang 1991b,c, 1992). In the crayfish *Procambarus clarkii*, epidermal 3α-reductase converts ecdysone to 3α-hydroxyecdysone and 20-hydroxyecdysone (Ikeda and Naya 1993). Excretion of ecdysteroids as highly polar conjugates in the urine is a very important inactivation mechanism during the precipitous drop in ecdysteroids seen characteristic of late premolt, for example, in *H. americanus* (Snyder and Chang 1991a,b), and conversion is molt-stage specific (Snyder and Chang 1991c).

Conversion of ecdysteroids to apolar conjugates is an important route for sequestration of dietary ecdysteroids. For example, more than 99% of ingested ^{3}H-labelled ecdysone is excreted as conjugated apolar metabolites in *H. americanus*, and, in this species, excretion of these compounds is highest during premolt (Snyder and Chang 1992).

Molt-inhibiting Hormone

Peptide Structures, Genes, and Expression

Although the existence of an MIH had been postulated 60 years ago (Passano 1953), full chemical characterization was only realized 20 years ago (Chang et al. 1990, Webster 1991) with the full structural identification of peptides with activities that functionally defined the hormone. These were the ability to (i) reduce circulating ecdysteroid levels and (ii) inhibit ecdysteroid synthesis by the YO. In the former case, a hormone with MIH activity was isolated from SG of *H. americanus* (Chang et al. 1990), which also had crustacean hyperglycemic hormone (CHH) activity and was clearly an authentic type I (Lacombe et al. 1999) peptide—an authentic CHH (see Chapter 2 in this volume). A peptide that inhibited ecdysteroid synthesis by the YO in *C. maenas* (Webster 1986, Webster and Keller 1986) was fully microsequenced by Edman degradation (Webster 1991), which, although clearly structurally related to CHH, was distinctive in that it was somewhat larger (78 amino acids) and possessed unblocked N- and C-termini while retaining identical disulphide bridge arrangements. Additionally, in this species, CHH also exhibited some MIH activity in the YO bioassay, although about 10–20 times less potent than MIH (Webster and Keller 1986). This

observation, when considered together with that of *Homarus* MIH (a CHH), clearly pointed not only to the multifunctional nature of CHH-superfamily peptides, but also to the existence of a family of type II peptides (Lacombe et al. 1999), with *Carcinus* MIH as the prototype, and also to their often overlapping biological activities.

To date, more than 30 type II peptides have been identified (comprising MIH, mandibular organ-inhibiting hormone [MOIH], and vitellogenesis-inhibiting hormone [VIH] peptides), of which about 21 have been classified as authentic MIHs based on their precursor and primary structures (for accession numbers of authentic MIHs and other structurally related type II peptides, see Table 1.2). These peptides (which have been functionally identified by their ability to repress ecdysteroid synthesis in vitro) seem to be universal in crabs but less common in other decapod groups (see Chapter 2 in this volume). However, it is unfortunate that many of these have not yet been fully identified in terms of functionality by YO bioassays. Structurally, type II peptides are always somewhat larger than type I (CHH) peptides—77–83 versus 72 amino acids. Unlike type I peptides, the preprocessed precursor for type II peptides never includes a precursor-related peptide (PRP). None has N-terminal modification (pGlu), which is commonly seen for CHHs, and all have characteristic Gly and Val residues between the first and second Cys residues (in positions 5 and 13 relative to the first Cys). The Gly residue may be important in conferring specificity in biological activity because insertion of this residue into the CHH of *M. japonicus* reduces biological activity by at least 10-fold (Katayama and Nagasawa 2004). This residue lies within the first predicted α-helix of MIH in this species (Katayama et al. 2003), which seems to be absent in type I peptides. Thus, it seems possible that this structure is critical in conferring specificity between related peptides. Although some type II peptides have C-terminal amidation (e.g., MIH: *O. limosus*, Bulau et al. 2005; *P. clarkia*, Nagasawa et al. 1996; and VIH: *Homarus gammarus, H. americanus*, Soyez et al. 1991, Ollivaux et al. 2006; *Rimicaris kairei*, Qian et al. 2009), the majority do not, in contrast to CHHs where this modification is very common. Intriguingly, no recognizable MIH genes have been found in *D. pulex* despite the identification of CHH (or more correctly ITP-like) genes in this species (Dircksen et al. 2011).

Gene structures for MIH invariably include 3 exons (Chan et al. 1998, Udomkit et al. 2000, Chen et al. 2005, Montagné et al. 2010), which all contain a first phase-0 intron that interrupts the signal peptide-coding region, and a second phase-2 intron. As with CHH, the second exon always terminates in the codon for the 40th or 41st amino acid of the mature MIH. Although multiple copies of CHH genes seem to be common in all crustaceans so far examined—up to nine copies in penaeid shrimp (Gu and Chan 1998)—maximally, only two MIH genes seem to occur in the species thus far examined (Lu et al. 2000, Chen et al. 2007, Nakatsuji et al. 2009). Interestingly, in cancrid crabs, these cluster with MOIH genes, which are further highly similar members of the type II peptide family, thus clearly illustrating a gene duplication event (Lu et al. 2000). A recent review includes details of the structures of CHH superfamily hormone genes (Webster et al. 2012). An overview of MIH peptide, precursor, and gene structures is shown in Fig. 1.3.

Immunohistochemical studies have repeatedly shown that MIH is only present in the XO-SG system of decapod crustaceans (crabs: Dircksen et al. 1988, Lee and Watson 2002, Hsu et al. 2006; penaeid shrimp: Shih et al. 1998, Gu et al. 2001, 2002; palinurids: Marco and Gäde 1999). In species where a clearly defined MOIH peptide exists, for example *Cancer pagurus*, MIH immunoreactive (ir) profiles in the SG always colocalize with MIH (Webster et al. 2012). Only rarely does MIH-ir colocalize with CHH-ir (*M. japonicus*, Shih et al. 1998), although another type II peptide (VIH) often colocalizes with CHH in lobsters (De Kleijn et al. 1992, Rottlant et al. 1993, Ollivaux et al. 2009; see also Fig. 2.6. in Chapter 2 in this volume). mRNA encoding MIH has been observed in the brain of *C. pagurus* (Lu et al. 2001) and in several neural tissues of *Metapenaeus ensis* (Gu et al. 2002), but the presence of expressed peptide has not yet been investigated in these species. The limited distribution of MIH peptides to the XO-SG contrasts vividly with the situation for CHH, where

Table 1.2. Molt-inhibiting hormone prepro-peptide sequences and other members of the type II crustacean hyperglycemic hormone (CHH) superfamily. Accession numbers (Protein) conceptually determined by cDNA, gDNA cloning and sequencing, or directly determined by microsequencing (gray highlights indicate mature peptide sequence information only). MIH, molt-inhibiting hormone; MOIH, mandibular organ-inhibiting hormone; VIH (GIH), vitellogenesis (gonad)-inhibiting hormone; SGP, sinus gland peptide.

Species	Tissue source	Peptide or conceptual translation	Accession No. (Protein)	Reference
Decapoda, Brachyura				
Callinectes sapidus	Eyestalk ganglia	MIH	AAA69029	Lee et al. 1995
Cancer magister	Eyestalk ganglia	MIH	AAC38984	Umphrey et al. 1998
Cancer pagurus	Sinus glands, X-organ, gDNA	MIH	P55846, CAC39425	Chung et al. 1996, Lu et al. 2001
Cancer pagurus	Sinus glands, X-organ, gDNA	MOIH1, MOIH2	P81034, P81025 CAB61424, CAB61425	Wainwright et al. 1996, Tang et al. 1999, Lu et al. 2000
Carcinus maenas	Sinus glands, X-organ	MIH	CAA53591	Webster 1991, Klein et al. 1993
Charybdis feriatus	gDNA	MIH	AAC64785	Chan et al. 1998
Charybdis japonica	Eyestalks gDNA	MIH	ACD11361, ACO90023	Zhu et al. 2008, unpub.; Yang et al. 2009, unpub.
Discoplax celeste	X-organ	MIH	AEM45616	Turner et al. 2011, unpub.
Eriocheir sinensis	Eyestalks, gDNA	MIH	AAQ81640, ABC68517	Song et al. 2003, unpub; Wang et al. 2005 unpub.
Gecarcinus lateralis	Eyestalk ganglia	MIH	ABF06632	Lee et al. 2007
Portunus trituberculatus	Eyestalk ganglia, gDNA	MIH	ABZ04547, ACF77140	Zhu and Shen, unpub. 2007, Zhu et al. 2008 unpub.
Decapoda, Astacura				
Cherax quadricarinatus	Eyestalk ganglia	MIH	ACX55057	Pamuru et al. 2009, unpub.
Homarus americanus	Sinus glands, X-organ	VIH	P55320	Soyez et al. 1991, de Kleijn et al. 1994
Homarus gammarus	X-organ	VIH	ABA42181	Ollivaux et al. 2006

(*continued*)

Table 1.2. *(Continued)*

Species	Tissue source	Peptide or conceptual translation	Accession No. (Protein)	Reference
Orconectes limosus	X-organ	MIH	P83636	Bulau et al. 2005
Procambarus clarkii	Sinus glands	MIH	P55848	Nagasawa et al. 1996
Nephrops norvegicus	Eyestalk ganglia	GIH	AF163771	Edomi et al. 2002
Decapoda, Penaeoidea				
Fenneropenaeus chinensis	X-organ	MIH	AAL55258	Wang et al. 2003
Litopenaeus vannamei	Eyestalk ganglia, gDNA	MIH1, MIH2	AAR04348, AAR04349	Chen et al. 2007
Marsupenaeus japonicus	Eyestalk ganglia	MIHA (SGP-IV)	BAA20432	Ohira et al. 1997
	Eyestalk ganglia	MIHB, MIHC	BAD36757, BAE78494	Ohira et al. 2005
Metapenaeus ensis	Eyestalk ganglia	MIH (MIH-A)	AAC27452	Gu and Chan 1998
	gDNA	GIH (MIH-B)	AAL33882	Gu et al. 2002
Penaeus monodon	Eyestalk ganglia, gDNA	MIH1	ACS88073 AAR89516	Vrinda et al. 2009 unpublished Yodmuang et al. 2004
	gDNA	MIH2	AAR89517	Yodmuang et al. 2004
	X-organ	GIH	ABG33898	Treerattrakool et al. 2008
Decapoda, Caridea				
Rimicaris kairei	Carapace tissues	VIH	ACS535348	Qian et al. 2009
Macrobrachium nipponense	Eyestalk ganglia	VIH	AEJ54623	Wang et al. 2010 unpub.
Macrobrachium rosenbergii	Eyestalk ganglia	SGP-A, SGP-B	AAL37948, AAL37949	Yang and Rao 2001
Isopoda				
Armadillidium vulgare	Sinus glands	VIH	P83627	Grève et al. 1999

expression commonly occurs in other parts of the nervous system; for example, in intrinsic neurons of the pericardial organs of crabs (Dircksen and Heyn 1998, Dircksen et al. 2001, Chung and Zmora 2008), the second roots of the thoracic ganglia of lobsters (Chang et al. 1999, Basu and Kravitz 2003), and in non-neural tissues such as the gut endocrine paraneurons of *C. maenas* (Chung et al. 1999). The distribution of CHH peptides is discussed in Chapter 2 in this volume.

During embryonic development of *C. maenas*, MIH-expressing neurons (two in each eye) are seen at about 75–80% development (Chung and Webster 2004), and, during larval life, each eyestalk contains four MIH-expressing neurons (Webster and Dircksen 1991). Subsequently, many

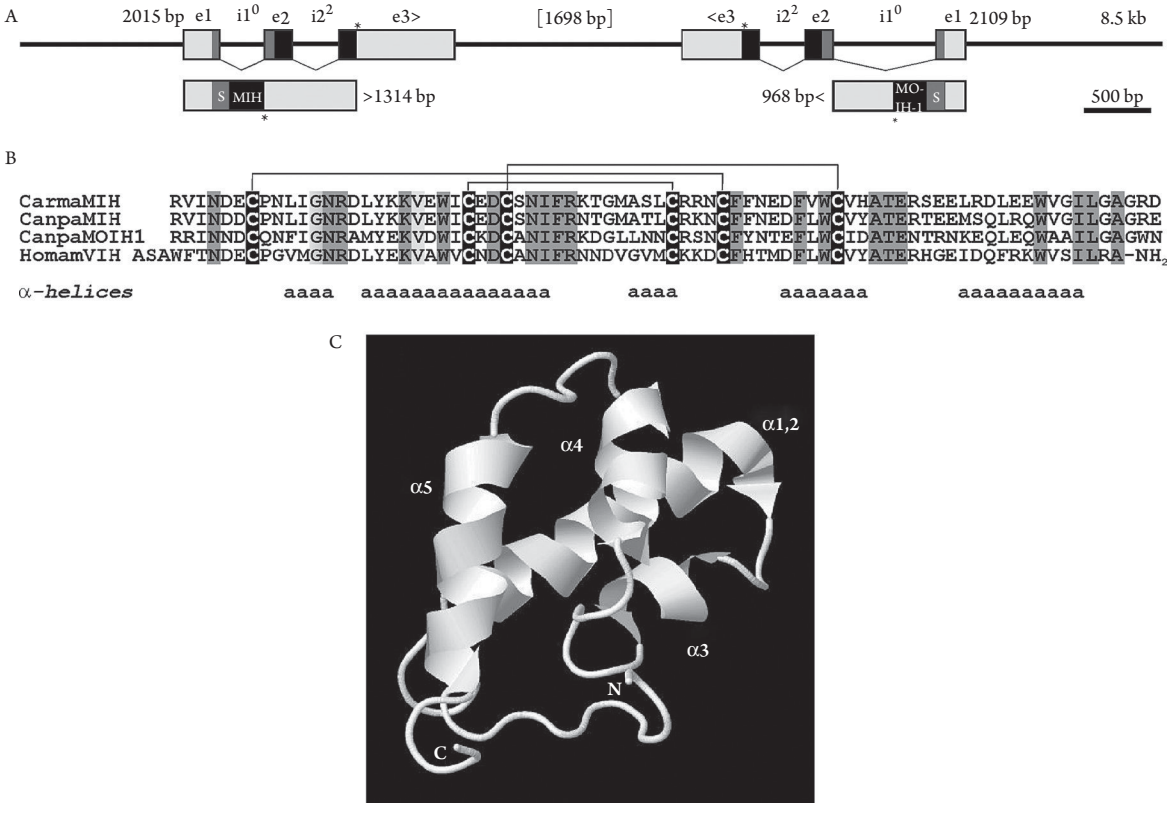


Fig. 1.3.
Gene, precursor, and primary structures of molt-inhibiting hormone (MIH) and related type II peptides. (A) *Cancer pagurus mih/moih1*-gene cluster (Lu et al. 2000). Light gray, UTRs; dark gray, signal peptide; black, MIH or MOIH. Asterisks shows positions of stop codons. Figure redrawn from Webster et al. (2012). (B) Mature peptide structures for the prototype crab MIHs from *Carcinus maenas* (Webster 1991) and *Cancer pagurus* (Chung et al. 1996) and prototype type II peptides: MOIH, from *Cancer pagurus* (Wainwright et al. 1996) and VIH from *Homarus americanus* (Soyez et al. 1991). Cysteine residues, which are in invariant positions in all crustacean hyperglycemic hormone (CHH) family members are marked in black, and disulphide bridges are shown. Invariant residues typical of type II peptides marked in pale gray. Other common residues are shown in dark gray. Five predicted α-helical regions are shown. (C) Ribbon model of energy minimized average structure of *Marsupenaeus japonicus* recombinant MIH, showing the predicted α-helical regions, determined as a solution structure via nuclear magnetic resonance (NMR; Katayama et al. 2003). Data from the Protein Database entry (1JOT.pdb) was remodeled using Jmol V12, which results in fusion of the first two predicted α-helices indicated in B.

more neurons are recruited to give the adult complement of 28–36 MIH-ir neurons in each eyestalk (Dircksen et al. 1988).

Measurement of circulating levels of MIH in the crayfish *P. clarkii* using a highly sensitive, time-resolved fluoroimmunoassay (TR-FIA) showed that intermolt levels of around 6 fmol/mL decline to 1.3 fmol/mL during premolt but increase to intermolt levels during late premolt and postmolt (Nakatsuji and Sonobe 2004). However, in *C. maenas*, levels of MIH measured by RIA do not decline during premolt and are typically less than 5 fmol/mL. There is, however, evidence of episodic release, where hemolymph levels rise to 20–40 fmol/mL, and such events seem to be more common during darkness (Chung and Webster 2005). Steady-state transcription of MIH mRNA (measured by Northern blotting) seems to decline during premolt in *C. sapidus* (Lee et al. 1998), and levels of two putative MIH transcripts measured by quantitative reverse transcription polymerase chain reaction (RT-PCR) declined during premolt in *Litopenaeus vannamei* (Chen et al. 2007). Conversely, no changes in MIH transcript abundance (Northern blotting) have been observed during the molt cycle of *P. japonicus* (Ohira et al. 1997), and similar results were reported using quantitative RT-PCR in *C. maenas* (Chung and Webster 2003). Clearly, these results are not entirely in agreement with the accepted model of molt control in which a decline in MIH secretion permits increased ecdysteroid synthesis by the YO, and because it has been repeatedly shown that premolt YOs become entirely refractive to the inhibitory influence of MIH (Chung and Webster 2003, Nakatsuji and Sonobe 2004, Nakatsuji et al. 2006b), it seems likely that the accepted model of molt control is rather incomplete, as discussed later.

Second Messengers and Signal Transduction Pathways

Two approaches have been used to determine the relevant second-messengers used in MIH signaling in the YO: either (i) by a pharmacological approach using membrane permeant/slowly hydrolyzable cyclic nucleotide analogues followed by measurement of repression of ecdysteroid synthesis by YO or (ii) by a physiological approach; that is, measurement of intracellular cyclic nucleotides in YO extracts following administration of MIH. Because these experiments have been performed on a variety of decapod crustaceans, it is perhaps unsurprising that a rather disparate literature exists (Spaziani et al. 1999, 2001, Covi et al. 2009, Nakatsuji et al. 2009). The approach using exogenous membrane permeant analogues has implicated the involvement of both cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in crayfish (Sedlmeier and Fenrich 1993, Nakatsuji et al. 2006b) and crabs (Saïdi et al. 1994, Covi et al. 2008). Exceptions include *C. antennarius*, where only cAMP analogues are effective (Mattson and Spaziani 1985b, 1986a) and *C. sapidus,* where only cGMP analogues are effective (Nakatsuji et al. 2006a,b). Physiologically based approaches, in which cyclic nucleotides have been measured following addition of MIH, show that increases in cAMP are rapid and transient (1–4 min, twofold) and are followed by impressive increases of cGMP (20- to 60-fold within 30–60 min; Baghdassarian et al. 1996, Chung and Webster 2003, Nakatsuji et al. 2006b). Because nitric oxide (NO) donors such as SNAP and SE175 and the guanylate cyclase agonist YC-1 can inhibit ecdysteroid synthesis in *C. maenas* and *G. lateralis* (Covi et al. 2008, Mykles et al. 2010), and given that YO express the catalytic (β) subunit of guanylate cyclase (GC-1) and a Ca^{2+}/calmodulin-dependent NO synthase in these crabs (H. W. Kim et al. 2004, Lee and Mykles 2006, Lee et al. 2007a,b), a scenario whereby MIH activates a soluble GC-1 seems eminently feasible. Taking earlier observations concerning transient increase in cAMP into account, an attractive and intellectually pleasing model of MIH signaling in crab YO (Fig. 1.4) has been proposed by Chang and Mykles (2011) based on that proposed for cardioacceleratory peptide 2b signaling in *Drosophila* Malpighian tubules (Beyenbach et al. 2010). An interesting peripheral feature of this model involves termination of the cGMP

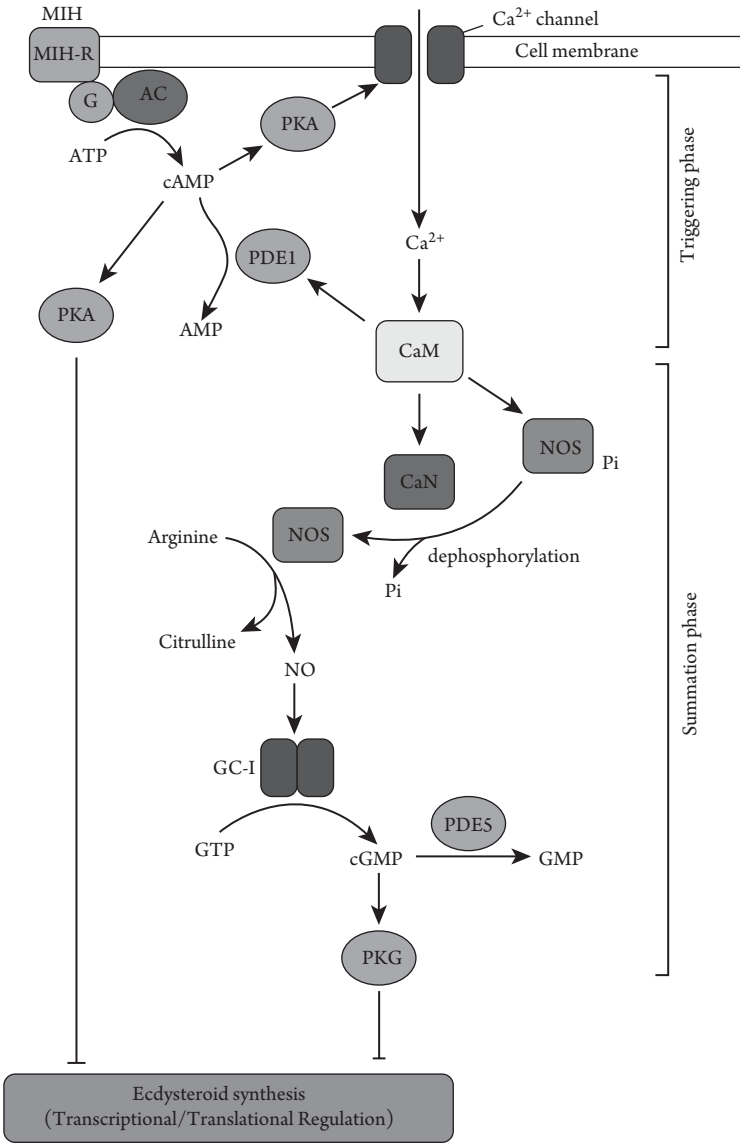


Fig. 1.4.
Model of molt-inhibiting hormone (MIH) signaling in crab Y-organs (YOs). MIH binds to a G-protein coupled receptor (MIH-R), activating adenylate cyclase (AC), resulting in cAMP production and protein kinase A (PKA) activation. Phosphorylation of enzymes via PKA may be important in constitutive control of ecdysteroid synthesis but also by facultative regulation by phosphorylation of Ca^{2+} channels, leading to calmodulin (CaM) activation of nitric oxide synthase (NOS), either directly or indirectly via calcineurin (CaN). Calmodulin can additionally abrogate increases in cAMP via activation of phosphodiesterase 1 (PDE1). A nitric oxide sensitive soluble guanylyl cyclase (GC-1) then increases cGMP synthesis, activating protein kinase G (PKG), which inhibits ecdysteroid synthesis. Phosphodiesterase 5 (PDE5) can abrogate increases in cGMP, potentially modulating this inhibitory pathway during, for example, premolt, when the YO becomes refractory to MIH. From Chang and Mykles (2011), with permission from Elsevier.

signal by phosphodiesterase 5 (PDE5) because upregulation of PDE expression/synthesis could abrogate the MIH signal, which has in fact been observed: during premolt YOs become entirely unresponsive to the inhibitory actions of sinus gland peptides (Sefiani et al. 1996) and notably MIH (Chung and Webster 2003, Nakatsuji and Sonobe 2004, Nakatsuji et al. 2006b), which is associated with a notable reduction in cGMP accumulation in premolt YO following exposure to MIH (Chung and Webster 2003). Furthermore, addition of the nonselective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) to premolt YO preparations restores the inhibitory activity of MIH (Nakatsuji et al. 2006b).

Regarding long-term constitutive control of ecdysteroid synthesis (transcriptional and translational control), rather than the short-term (facultative) allosteric regulation detailed earlier, a variety of studies suggest that this is relevant.

It has long been known that eyestalk ablation dramatically increases protein and RNA synthesis in YO (Simione and Hoffman 1975, Gersch et al. 1977) and that this also occurs during premolt (Toullec and Dauphin-Villemant 1994). During premolt, there are dramatic changes in patterns of gene expression (Lee and Mykles 2006). Because sinus gland extracts inhibit incorporation of amino acids into protein (Gersch et al. 1977, Mattson and Spaziani 1986a, Dauphin-Villemant et al. 1995), and in view of the observations that cAMP, IBMX, or forskolin inhibit in vitro protein synthesis by the YO, it has been proposed that constitutive action of MIH might act via cAMP signaling (Mattson and Spaziani 1986a,b, Han et al. 2006). However, in the absence of critical studies involving administration of pure MIH, this remains speculative because effects unrelated to MIH (or indeed CHH) cannot be disregarded.

A proteomic study in which *C. maenas* YO protein profiles from 2D polyacrylamide electrophoresis were compared following exposure to physiologically relevant doses of MIH showed that a transaldolase was specifically downregulated (Lachaise et al. 1996). Because this enzyme is part of the pentose phosphate pathway involved in nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) production, necessary for cytochrome P450 synthesis, it is entirely feasible that this may be an important control mechanism in constitutive regulation of the YO.

cDNA-encoding P450 enzymes that may be involved in ecdysteroidogenesis have been cloned in *O. limosus* CYP4C15 (Dauphin-Villemant et al. 1999) and *C. maenas* CYP4C39 (Rewitz et al. 2003). The former is expressed in the YO, upregulated during premolt, and differentially downregulated by MIH. However, the sequence similarity of both proteins to *nvd* and *phm* is low, and, for the latter, expression in the midgut gland would preclude a direct role in ecdysteroid synthesis. Nevertheless, a homologue of *phm* with a high sequence identity to the insect homolog is expressed in the YO of *M. japonicus*. Quantitative RT-PCR suggests that this transcript is upregulated during premolt and downregulated by in vitro incubation of YOs with MIH (Asazuma et al. 2009).

Clearly, in view of the recent proteomics revolution in, for example, nanoscale identification of proteins via in-gel digestion of Coomassie-stained 2D polyacrylamide gels followed by MS-based sequencing and homology-based database interrogation to identify candidate proteins, there are attractive possibilities for more fully defining constitutive mechanisms, regulated via MIH, that control the long-term ecdysteroidogenic capacity of the YO.

Binding Sites and Receptors

Receptor binding studies using ^{125}I-labeled MIH have shown high-affinity, saturable binding sites (K_D 1.2×10^{-10} M, B_{MAX} 1.3×10^{-10} M) indicative of receptor binding on YO plasma membrane preparations from *C. maenas* (Webster 1993). Interestingly, receptor binding characteristics remain unchanged during the molt cycle (Chung and Webster 2003), thus the receptor is presumably not involved (via changes in receptor number or affinity) in dramatic changes

in responsiveness of the YO to MIH during the molt cycle, as detailed earlier. Binding of ^{125}I-recombinant MIH to a 70 kDa YO membrane protein has been shown for *M. japonicus* (Asazuma et al. 2005) and a 51 kDa protein from *C. sapidus* (Zmora et al. 2009). Although these masses are incongruent with those of a receptor guanylate cyclase (rGC) with a mass of ca. 151 kDa that is supposedly a likely candidate CHH receptor (Webster et al. 2012), some studies give evidence supporting such a scenario. An rGC has been cloned and sequenced from *C. sapidus* YO cDNA (Zheng et al. 2006), and immunochemical studies using antisera raised against the predicted extracellular domain of this protein show that it is expressed in YO plasma membranes and that pretreatment of YOs with this antibody blunts the suppressive effect of MIH (Zheng et al. 2008). However, because this rGC is widely distributed in many tissues and is not specific to the YO (the only tissue that is known to bind MIH), the significance of these interesting observations is unclear.

In view of the uncertainties regarding the nature of the MIH receptor and those involved in suggested models of second-messenger signal transduction, a very important and much needed result would be the determination of the MIH (and CHH) receptors. Ideally, candidate cDNAs should be expressed in heterologous cells with suitable reporters. In a broader context, such an approach will allow screening of a number of CHH superfamily peptides and would give much-needed information on target tissues of individual peptides.

Crustacean Hyperglycemic Hormone

In many species of crustaceans, CHHs can also act as functional MIHs; indeed, the first functionally identified MIH, found in *H. americanus,* was a CHH (Chang et al. 1990). Furthermore, because CHH is active in the YO bioassay in *C. maenas* (Webster and Keller 1986), although at least 10× less potent with respect to MIH, and considering the recent report showing that a recombinant CHH from *G. lateralis* inhibits ecdysteroidogenesis in *C. maenas* YOs (but had no hyperglycemic activity; Zarubin et al. 2009), it seems possible that CHH might be biologically relevant in molt control, particularly because clearly identifiable CHH binding sites are found on YO plasma membranes in crabs (Webster 1993, Chung et al. 2010). However, because it has been shown that simultaneous release of MIH and CHH does not occur and because both have very short half-lives in the hemolymph (ca. 5 min.; Chung and Webster 2005), the functional significance of CHH in molt control remains uncertain.

A novel action of CHH concerns its role in stimulating water uptake during ecdysis. As detailed in Chapter 2, synthesis and release of CHH from gut paracrine cells during late premolt leads to dipsogenesis, water absorption, and subsequent swelling of crabs to postmolt dimensions (Chung et al. 1999; see Fig. 1.5). Further studies are now needed to see whether similar intrinsic gut cells (Webster et al. 2000) and CHH release patterns occur in other crustaceans.

Crustacean Cardioactive Peptide and Bursicon

CCAP was first isolated from the pericardial organs of *C. maenas* (Stangier et al. 1987), where its defining biological activity was in the chronotropic and inotropic acceleration of heart rate in semi-isolated preparations. Subsequently, it has been found in a variety of crustacean and arthropod species, and it is undoubtedly ubiquitous in its occurrence in the phylum. Notably, the structure and distribution of CCAP immunoreactive neurons and their principal central and projection patterns are remarkably similar in all arthropods, suggesting highly conserved roles (Dircksen 1998). Transcript structure of several crustacean CCAP cDNAs has been shown to be similar to those of a variety of insect counterparts (Chung et al. 2006).

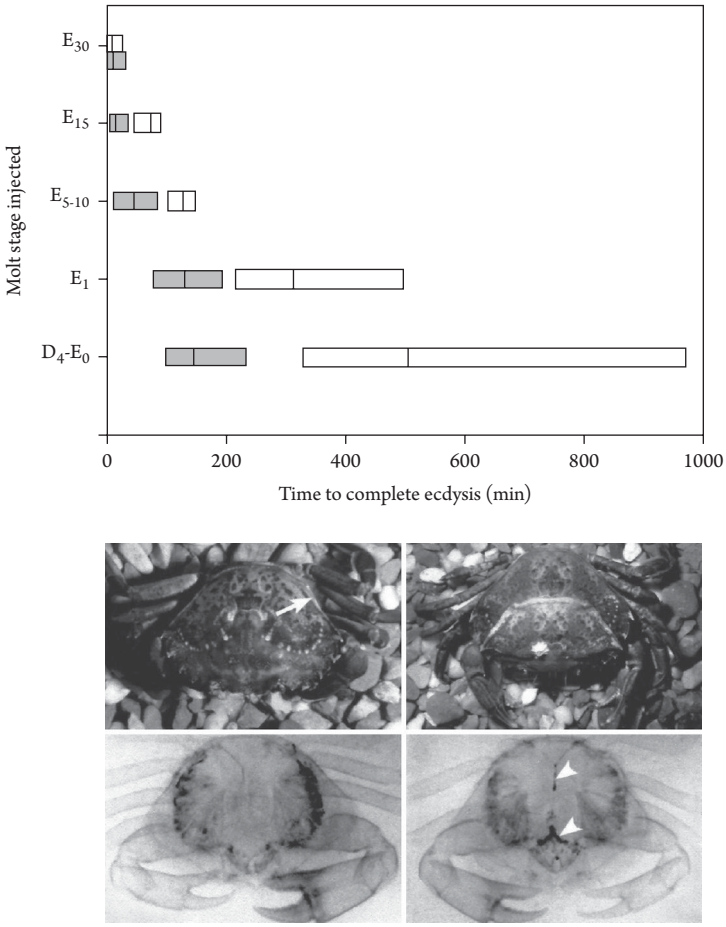


Fig. 1.5.
The effect of crustacean hyperglycemic hormone (CHH) injection on ecdysis. Graph shows effect of CHH injection (100 pmol) on time to ecdysis. Filled bars, treated crabs; open bars, saline injected controls (n = 3–10); range is shown. Vertical bars indicate means. Molt stages are shown as percentage of ecdysis. Images show the effect of a single 300 pmol injection of CHH, upper right, 2 h after injection, compared to a saline-injected control, 2 h after injection. Arrow indicates epimeral suture line. Lower images show radiograms of a crab injected with 100 pmol CHH, followed by immersion in 15% barium sulphate in seawater, autoradiography 30 min later, right, and corresponding saline-injected control. Arrowheads show considerable accumulation of radio-opaque barium sulphate in stomach and hindgut as a result of drinking in the treated crab. Note trapping of barium sulphate in gills in both crabs. Redrawn from Chung et al. (1999), with permission from the National Academy of Sciences.

In insects, CCAP is not only known to be cardioacceleratory (Tublitz and Evans 1986, Davis et al. 1990, Dulcis et al. 2001), but is also important in a developmental context related to eclosion. Control of blood circulation during wing inflation in *Manduca sexta* (Tublitz and Truman 1985), gut contraction during embryogenesis, and larval wandering (Broadie et al. 1990, Tublitz et al. 1992) is regulated by CCAP, and it is a central component in triggering ecdysis behavior at the larval-pupal molt (Gammie and Truman 1997), among several other biological activities (Dircksen 1998). From targeted ablation of CCAP-containing neurons of *Drosophila*, it is known that knockouts exhibit stage-specific defects in execution and circadian gating of ecdysis behavior (Park et al. 2003).

In crustaceans, analogous actions of CCAP during ecdysis have been investigated in *C. maenas* and *O. limosus*: detailed analyses of behaviors and phenotypes during ecdysis, coupled with measurement of hemolymph CCAP via RIA (Stangier et al. 1988) have shown that CCAP release patterns are tightly correlated with specific events during ecdysis (Phlippen et al. 2000); in particular, a massive release of CCAP, sufficient to deplete stores in the pericardial organ, occurs during ecdysis. Furthermore, the biphasic molting pattern of the isopod *Oniscus asellus* is reflected in changes in CCAP content of the serially iterated CCAP neurons at this time (Johnen et al. 1995). Temporally defined ecdysis behaviors, phenotypes, and corresponding changes in circulating CCAP levels are shown in Fig. 1.6.

The ultimate hormone in arthropod ecdysis has long been known to be bursicon, identified as the hormone involved in cuticle tanning and melanization of the cuticle almost 50 years ago

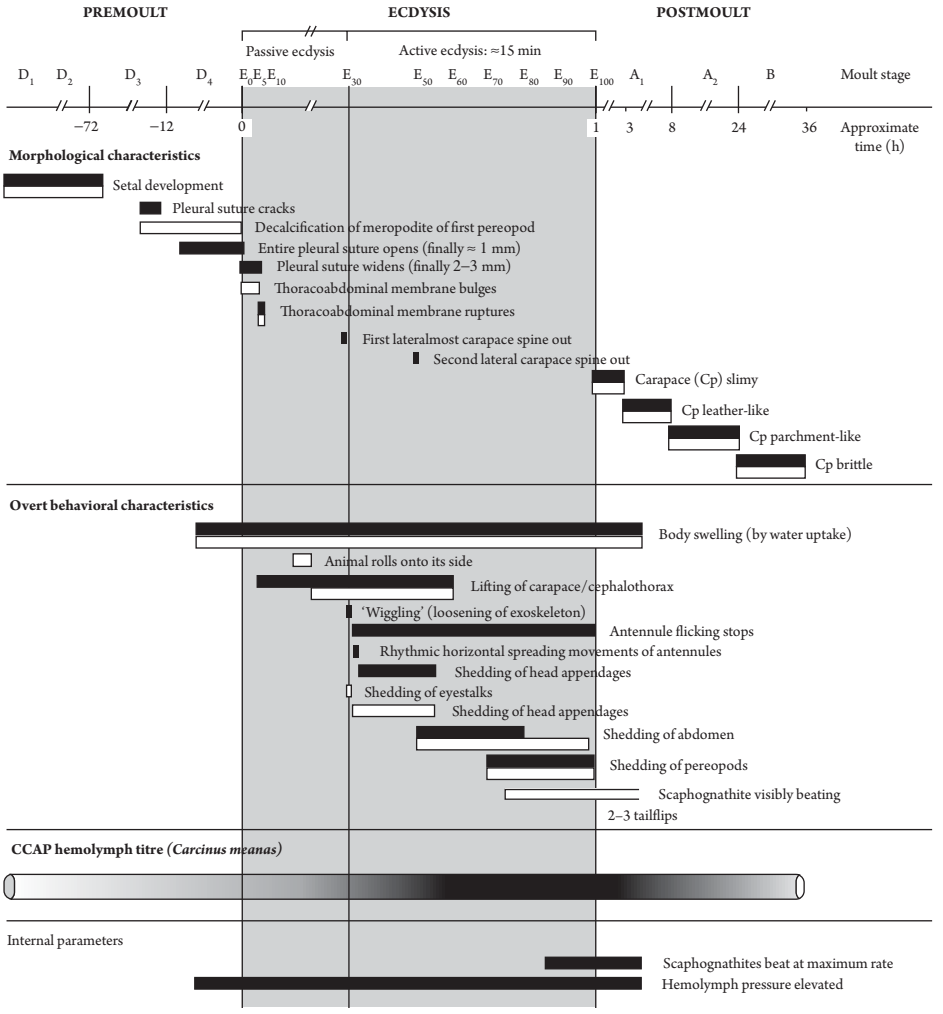


Fig. 1.6.
Morphological and behavioral changes during the molt cycle of crab (*Carcinus maenas*) and crayfish (*Orconectes limosus*) and cartoon of crustacean cardioactive peptide (CCAP) levels in the hemolymph. Open bars, crayfish; filled bars, crab. Molt stages according to Drach and Tchernigovtzeff (1967). From Phlippen et al. (2000), with permission from The Company of Biologists, Ltd.

(Honegger et al. 2008). Following the identification of bursicon in *Drosophila* as a heterodimeric (encoded by *burs* or *bursα*/*pburs* or *bursβ*) cystine knot protein (Luo et al. 2005, Mendive et al. 2005), expressed sequence tag (EST) data from the *D. pulex* transcriptome and EST data from *C. maenas* predicted the presence of burs α (*Daphnia*) and burs β (*Carcinus*), which were subsequently cloned and sequenced in both species (Wilcockson and Webster 2008) and subsequently in *H. gammarus* (Sharp et al. 2010). Contemporary techniques involving *in silico* data mining coupled with MS analysis have confirmed these results in the *D. pulex* transcriptome/genome (Gard et al. 2009, Dircksen et al. 2011) and the neuropeptidome of *C. maenas* (Ma et al. 2009), and similar bursicon molecules have been discovered in *L. vannamei* using this powerful approach (Ma et al. 2010). Patterns of expression of bursicon in the central nervous system (CNS) of *C. maenas* and *H. gammarus* using in situ hybridization and immunohistochemistry have shown that both bursicon and CCAP are always completely colocalized in a set of fully identified neurons in the thoracic and abdominal ganglia (Wilcockson and Webster 2008, Sharp et al. 2010); thus, both hormones should also be co-released from the pericardial organs. Our current studies show that this is indeed the case. Simultaneous measurement of both CCAP (by RIA) and bursicon (by TR-FIA) from the same hemolymph samples at a high temporal resolution have shown that CCAP peaks at exactly 100% ecdysis (1 pmol/mL) as does bursicon (4 pmol/mL); hormone levels of both rapidly decline within 10 min of ecdysis and subsequently return to basal levels within 3 h (Webster, unpublished). Thus, spatial separation of biological actions of both peptides must occur, given co-release. Although salient biological activities of CCAP, such as cardioacceleratory activities, are established in crustaceans, the tacit assumption that bursicon is involved in cuticle hardening in crustaceans cannot be certain as yet. For a detailed discussion of postecdysial changes in cuticle composition, see Chapter 5 in Volume 1.

It is noteworthy that abdominal ganglia extracts of *H. americanus* are active in the *Sarcophaga* cuticle tanning bioassay (Kostron et al. 1995), as is purified *Carcinus* bursicon (Webster, unpublished). Thus, a plausible scenario would be that the action of bursicon in cuticle hardening is somewhat long-lived, compared to the rapid action of CCAP in facilitating emergence behaviors. Injection of bursicon in *Drosophila* followed by gene expression analysis by microarray has shown that at least 13 genes are involved in cuticle sclerotization, and 74 diverse genes apparently unrelated to cuticular hardening are regulated by bursicon (An et al. 2008). It would be interesting to expand these studies to crustaceans, particularly those with well-annotated transcriptomes, to investigate downstream targets of CCAP and bursicon signaling. Current understanding of the release patterns of CHH, CCAP, and bursicon during premolt and ecdysis is diagrammatically summarized in Fig. 1.7.

Methyl Farnesoate

Methyl farnesoate (MF) is the unepoxidated precursor of insect juvenile hormone (JH) III, synthesized and secreted from the mandibular organs (MO) of decapod crustaceans. Since its discovery (Laufer et al. 1987), a large yet somewhat disparate literature has accumulated suggesting that it is a key hormone regulating reproduction in crustaceans. Details of the role of MF in reproduction and regulation of its secretion by neurohormones are outside the scope of this review; comprehensive accounts have been given by Laufer et al. (1993), Homola and Chang (1997), Borst et al. (2001), and Nagaraju (2007). However, it is relevant here to note that MF synthesis is inhibited by type II CHH superfamily hormones (MOIHs) in cancrid crabs (Wainwright et al. 1996, Lu et al. 2000, 2001) and by type I hormones (CHH) in *C. maenas* (Keller et al. 1999) and *L. emarginata* (Liu et al. 1997). By analogy to well-established roles of JHs in insects (Riddiford 1996), there is evidence that MF is involved in maintenance of juvenile morphology, inhibiting gonadal development at this time, and, in the adult, enhancing gonadal maturation.

Regarding the influence of MF on morphogenesis in crustaceans, studies on the spider crab *L. emarginata* have shown that reproductively mature males (characterized by abraded carapaces)

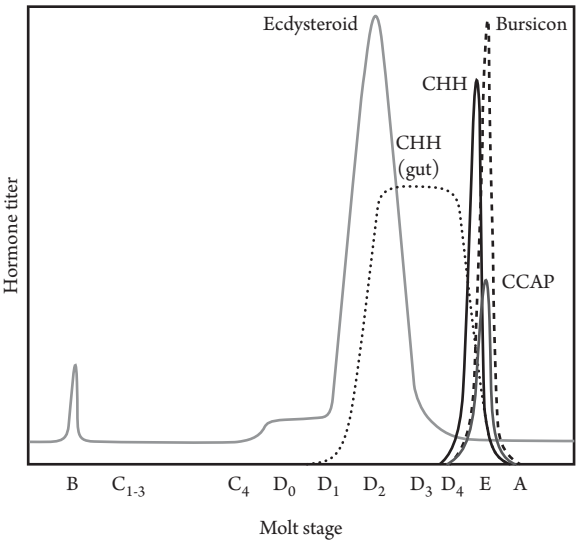


Fig. 1.7.
Schematic of hormone titers during the molt cycle of *Carcinus maenas*. Molt stages and hormone titers not to scale.

possess large chelae and higher levels of MF synthesis and titer than do small-clawed, unabraded, nonreproductive males (Sagi et al. 1993, 1994, Laufer et al. 2002). Additionally, eyestalk ablation in juvenile *Libinia*, which elevates levels of MF, results in maintenance of the juvenile (small chelae) morphotype (Laufer et al. 1997). Similarly, nonreproductive male *P. clarkii* retain an immature morphotype after eyestalk ablation or dietary administration of MF (Laufer et al. 2005).

Juvenilizing effects of MF have been widely reported following exposure of crustacean larvae to MF or dietary administration. Barnacle cyprids (*Balanus amphitrite*) exposed to a high concentration of MF (0.1 μM) in seawater delay metamorphosis (Smith et al. 2000). Similarly, pharmacological doses of synthetic analogues of JH III, hydroprene, and methoprene increase the number of zoeal stages and delay megalopal development in *Rhithropanopeus harrisii* larvae (Christiansen et al. 1997a,b). MF in seawater delays development of *H. americanus* larvae (Borst et al. 1987), and dietary MF retards growth and development of *Macrobrachium rosenbergii* larvae (Abdu et al. 1998).

Exposure of *D. magna* to MF (Olmstead and LeBlanc 2002) and JH mimics (Tatarazako et al. 2003, Olmstead and LeBlanc 2002, Oda et al. 2005) induces parthenogenetically reproducing clones to produce male neonates in a dose-dependent manner. Additionally, it has recently been shown that morphological changes associated with antipredatory responses (elongation of helmet), can be induced in *Daphnia galeata* by administration of MF or the JH mimic fenoxycarb (Oda et al. 2011).

It has long been known that the MOs undergo molt-cycle-related changes in structure, suggesting increased secretory activity, and premolt histological and ultrastructural changes reminiscent of those seen in YOs during premolt (Le Roux 1968, Aoto et al. 1974, Bucholz and Adelung 1980). Injection of MO extracts accelerates molting in heterologous bioassays; for example, *C. sapidus* MO extracts promote molting in *Penaeus setiferus* (Yudin et al. 1980), and *P. clarkii* MO extract accelerates molting in *Caridinia denticulata* (Taketomi et al. 1989). It is notable that MF (1 μM) and co-culture with mandibular organs stimulates ecdysteroid production by YO of *Cancer magister* (Tamone and Chang 1993), and that MF injection can accelerate molting in several crustacean species, for example, *Cherax quadricarinatus* (Abdu et al. 2001), *P. clarkii* (Laufer et al. 2005), and *Oziotelphusa senex senex* (Nagaraju 2003, Reddy et al. 2004). Because MF secretory activity

increases during premolt in several species of crustaceans (Wilder et al. 1995, Ahl and Laufer 1996, Laufer et al. 2005), it is tempting to suggest that MF forms an important part of a feed-forward loop during the phase of rapid increase in circulating ecdysteroid so characteristic of premolt. Further studies to elaborate possible roles of MF in modulating molting and morphogenesis using a species which is well-studied, and, importantly, using contemporary molecular techniques, would clearly be worthwhile.

FUTURE DIRECTIONS

During the past 20 years, there has been a remarkable resurgence of interest in the hormonal control of molting in crustaceans. Many members of the type II CHH hormone superfamily have been identified, and, in particular, research on authentic MIHs has been rewarding. However, in many instances, the biological significance of a number of these peptides remains to be elucidated, particularly in the penaeid shrimps where much overlap in function seems to be common, particularly when the large number of CHH superfamily peptides in any given species of penaeid is taken into account. Comprehensive studies detailing the range of biological activities from the full spectrum of CHH superfamily peptides would do much to reconcile the current situation.

Research on signaling pathways in the YO and control of ecdysteroid synthesis has been significant and has clearly demonstrated that the accepted model of molt control is far from complete; indeed, this may need a major revision. More studies measuring levels of MIH and CHH neuropeptides in the hemolymph at appropriate temporal scales are needed, and the highly sensitive assays needed for such studies are now available for several models. In particular, the determination of the MIH (and other CHH superfamily) hormone receptors is now urgently required, determination of candidate receptor cDNAs expressed in heterologous cells would allow determination of ligand specificity and affinity, and determination of expression of receptors would be invaluable in giving much information on target tissues of respective peptides.

Downstream events related to changes in gene expression in target tissues following administration of hormones would also seem an attractive area of future research. Given current advances in sequencing and gene expression technologies, it is now becoming feasible to investigate global transcriptomic changes, and coupling such studies with proteomic approaches should be an exciting challenge.

Given the recent advances in our knowledge of endocrine cascades during ecdysis in insects, it is now appropriate to investigate these in crustaceans. Genome sequencing, data mining of genomes, transcriptomes, and EST databases are set to revolutionize crustacean neuroendocrinology, as evidenced by novel important studies detailing the peptidome of *D. pulex* (Dircksen et al. 2011, Christie et al. 2011). Given that quite a variety of neuropeptides first identified in insects are involved in the ecdysis cascade, a promising approach to begin corresponding peptide discovery in crustaceans would be to identify possible transcripts and then firmly identify these as translated proteins via proteomics. It should first be reiterated at this point that although there are peptides that seem to be exclusively found in insects, such as PTTH, for which no homologues have yet been identified from the *Daphnia* genome and, conversely, that although MIH (type II) CHH family peptides have not yet been identified in any insect, a rigorously applied concept of "insect" or "crustacean" neuropeptides is widely recognized as being somewhat naïve. Second, until relevant biological actions of such neuropeptides have been confirmed in crustaceans, the insect hormone appellations are, at best, putative. Peptide hormones first identified as important in insect ecdysis and that have been identified using genomic and transcriptomic analyses in crustaceans are briefly reviewed here.

(Arg^7-corazonin), pQTFQYSRGWTNamide (Veenstra 1989) is a key neuropeptide in the insect ecdysis cascade because, in *M. sexta* and *Bombyx mori,* it acts on Inka cells (groups of cells

attached to the tracheae of the prothoracic and abdominal segments of insects to elicit ETH release (Y. J. Kim et al. 2004). This peptide has been predicted from analyses of the *D. pulex* genome (Christie et al. 2011), in EST databases for *Daphnia carinata* (Christie et al. 2010) and *L. vannamei* (Ma et al. 2010), and the peptide has been identified by MS in *D. pulex* (Dircksen et al. 2011). Furthermore, this molecule has been identified by MS of nervous system extracts (brain, thoracic ganglia) of *C. maenas* (Ma et al. 2009) and *L. vannamei* (Ma et al. 2010).

ETH and pre-ETH (PETH) are released from Inka cells to activate specific neuronal circuits involved in regulation of the ecdysis sequence in insects (see Zitnan et al. 2007 for review). A single gene codes for an ETH-like precursor in *D. pulex* (Christie et al. 2011), which encodes two ETHs in tandem. These are DappuETH1: DPSPEPEPFNPNYNRFRQKIPRIamide and DappuETH2: GE GIIAEYMNSESFPHEGSLSNFFLKASKAVPRLamide (which is much longer than insect ETHs); the presence of both of these peptides has been confirmed by MS (Dircksen et al. 2011). As yet, no other ETH-like molecules have been predicted in crustaceans by transcriptome mining.

EH, first isolated in *M. sexta* and *Bombyx mori* (Kono et al. 1987, Katakoa et al. 1987, Marti et al. 1987) and *Drosophila* (Horodyski et al. 1993), acts directly on Inka cells (in *Manduca* and *Bombyx*) to release ETH and increases cGMP in Inka cells, leading to pre-ecdysis and ecdysis burst patterns in the CNS (Žitňan et al. 1996). EH-producing ventromedial (VM) cells in the brain respond to ETH by increased activity and release of hormone (Hewes and Truman 1991, Ewer et al. 1997, Gammie and Truman 1997, 1999), which in turn elicits cGMP production and excitability of 27/704 neurons in the CNS (Ewer et al. 1994, Ewer and Truman 1997, Fuse and Truman 2002). The 27/704 cells in turn produce CCAP and MIPs, which are released at ecdysis (Gammie and Truman 1997, Davis et al. 2003) and are responsible for execution of the ecdysis motor program.

A single gene encoding a putative EH (dappu-*eh*) was identified in the *D. pulex* genome (Christie et al. 2011) using a putative *Penaeus monodon* EH precursor sequence as a query (Christie et al. 2010). This EH has a high sequence similarity with some insect EHs; for example, 71% identity with that of the flour beetle *Tribolium castaneum*. A second gene, encoding a rather longer EH-like molecule with lower sequence similarity to insect EH (Dappu-*ehl*) also occurs, next to *-eh* in a tail-to-tail inverted tandem cluster (Dircksen et al. 2011). In decapods, an EST encoding an EH-like molecule has been identified (*C. sapidus*, Accession No. CV224237), but this has quite a low sequence similarity to insect EHs (Dircksen et al. 2011), and a very similar molecule has been identified by cDNA cloning in *C. maenas* (Webster, unpublished). Additionally, EH-like molecules have been identified by *in silico* data mining in *Triops cancriformis* (FM869498) and *Marsupenaeus japonicus* (CI998674, CI998685, CI999397; Christie et al. 2010). Thus, it seems likely that EH-like molecules are widely distributed in crustaceans, and when the presence of other neuropeptides involved in the ecdysis program are taken into account, it seems likely that this process might be controlled in a similar manner to that seen in insects.

CONCLUSIONS

The hormonal control of molting in crustaceans has been the subject of much interest to comparative endocrinologists since early models of molt control were proposed more than 60 years ago. Although molt cycle-related titers, biosynthesis, and metabolism of molting hormones (ecdysteroids) are broadly similar in all arthropods, in contrast to insects, ecdysteroid synthesis in crustaceans is repressed by MIH. The first structural determinations of these hormones demonstrated that they were members of the ever-expanding CHH family and that, in many instances, gene duplication has resulted in the evolution of specific MIH-type molecules apart from those that exhibit hyperglycemic and molt-inhibiting activity and that can be structurally identified as CHH-type molecules.

In contrast to CHH-type molecules, which are now known to be expressed in several tissues apart from the eyestalk XO-SG system, MIH-type molecules seem to be mainly expressed in these tissues. Furthermore, MIH signal transduction pathways are associated with the only target tissue—the YO—and notable progress has recently been made in elucidating the signal transduction cascade for MIH in crabs. This work suggests that MIH signaling involves a membrane-bound G-protein coupled receptor that signals via a soluble guanylate cyclase and a Ca^{2+}/calmodulin-dependent NO synthase (NOS). However, as yet, the receptors for MIH (and CHH) remain orphans.

The crustacean equivalent of JH (JH-III) is MF, produced by the mandibular organ of decapod crustaceans. The roles of this sequiterpenoid hormone in crustacean development, reproduction, and molting are currently not well understood, yet there are tantalizing glimpses pointing to quite fundamental functions of this hormone, and further studies, using contemporary molecular techniques, are now needed to further define its role in the above-mentioned processes.

It has recently been established that CCAP and bursicon are released during the ecdysis program of decapod crustaceans at precisely timed periods, which mirrors the situation in insect ecdysis. However, the great complexity of the numerous hormones involved in insect ecdysis remains to be explored in crustaceans. With recent advances in transcriptomics, it is expected that several more "insect" neuropeptides will be discovered, and, despite the disadvantage of genetic intractability of crustaceans (in comparison to model insects), it is expected that fundamental progress toward unraveling the undoubted complexity of the hormonal control of molting in crustaceans will be made, and this will no doubt be of interest to arthropod endocrinologists and the aquaculture industry.

ACKNOWLEDGMENTS

The support of the Biotechnology and Biological Sciences Research Council (BBSRC), Natural Environment Research Council (NERC), and Royal Society is gratefully acknowledged. I am particularly indebted to Dr. Sook Chung, Prof. Heiner Dircksen, Prof. Rainer Keller, and Prof. Huw Rees for our combined research, collaboration, and friendship over more than 25 years.

REFERENCES

Abdu, U., P. Takac, H. Laufer, and A. Sagi. 1998. Effect of methyl farnesoate on late larval development and metamorphosis in the prawn *Macrobrachium rosenbergii* (Decapoda, Palaemonidae): a juvenoid-like effect? Biological Bulletin 195:112–119.

Abdu, U., A. Barki, I. Karplus, S. Barel, P. Takac, G. Yehezkel, H. Laufer, and A. Sagi. 2001. Physiological effects of methyl farnesoate and pyroxifen on wintering female crayfish *Cherax quadricarinatus.* Aquaculture 202:163–175.

Ahl, J.S.B., and H. Laufer. 1996. The pubertal molt in crustacean revisited. Invertebrate. Reproduction and Development 30:177–180.

Aiken, D.E. 1973. Proecdysis, setal development and molt prediction in the American lobster (*Homarus americanus*). Journal of the Fisheries Research Board of Canada 30:1337–1344.

An, S., S. Wang, L.I. Gilbert, B. Beerntsen, M. Ellersieck, and Q. Song. 2008. Global identification of bursicon-regulated genes in *Drosophila melanogaster*. BMC Genomics 9:424.

Aoto, T., Y. Kamiguchi, and S. Hisano. 1974. Histological and ultrastructural studies on the Y-organ and mandibular organ of the freshwater prawn *Palaemon paucidens*, with special reference to their relation with the molt cycle. Journal of the Faculty of Science of Hokkaido University 19:295–308.

Arakane, Y., B. Li, S. Muthukrishnan, R.W. Beeman, K.J. Kramer, and Y. Park. 2008. Functional analysis of four neuropeptides, EH, ETH, CCAP and bursicon, and their receptors in adult ecdysis behaviour of the red flour beetle, *Tribolium castaneum*. Mechanisms of Development 125:984–995.

Asazuma, H., S. Nagata, H. Katayama, T. Ohira, and H. Nagasawa. 2005. Characterization of a molt-inhibiting hormone (MIH) receptor in the Y-organ of the kuruma prawn, *Marsupenaeus japonicus*. Annals of the New York Academy of Sciences 1040:215–218.

Asazuma, H., S. Nagata, and H. Nagasawa. 2009. Inhibitory effect of molt-inhibiting hormone on Phantom expression in the Y-organ of the kuruma prawn, *Marsupenaeus japonicus*. Archives of Insect Biochemistry and Physiology 72:220–233.

Baghdassarian, D., N. de Bessé, B. Saïdi, G. Sommé, and F. Lachaise. 1996. Neuropeptide-induced inhibition of steroidogenesis in crab molting glands: involvement of cGMP-dependent protein kinase. General and Comparative Endocrinology 104:41–51.

Baldaia, L., P. Porcheron, J. Coimbra, and P. Cassier. 1984. Ecdysteroids in the shrimp *Palaemon serratus*: relations with molt cycle. General and Comparative Endocrinology 55:437–443.

Blanchet, M.-F., P. Porcheron, and F. Dray. 1976. Etude des variations du taux des ecdysones au cours du cycle d'intermue chez le male d'*Orchestia gammarella* Pallas (Crustacé Amphipode) par dosage radioimmunologique. Comptes Rendus de l'Academie des Sciences, Serie D (Sciences Naturelles) 283:651–654.

Blanchet, M.-F., P. Porcheron, and F. Dray. 1979. Variations du taux ecdystéroides au cours des cycle de mue et de vitellogenèse chez le Crustacé Amphipode, *Orchestia gammarellus*. International Journal of Invertebrate Reproduction 1:133–139.

Basu, A.C., and E.A. Kravitz. 2003. Morphology and monoaminergic modulation of crustacean hyperglycaemic hormone-like immunoreactive neurons in the lobster nervous system. Journal of Neurocytology 32:253–263.

Beyenbach, K.W., H. Skaer, and J.A.T. Dow. 2010. The developmental, molecular and transport biology of Malpighian tubules. Annual Review of Entomology 55:351–374.

Blais, C., M. Sefiani, J.-Y. Toullec, and D. Soyez. 1994. *In vitro* production of ecdysteroids by Y-organs of *Penaeus vannamei* (Crustacea, Decapoda). Correlation with hemolymph titers. Invertebrate Reproduction and Development 26:3–11.

Blais, C., C. Dauphin-Villemant, N. Kovganko, J.P. Girault, C. Descoins, and R. Lafont. 1996. Evidence for the involvement of 3-oxo-Δ4 intermediates in ecdysteroid biosynthesis. Biochemical Journal 320:413–419.

Block, D.S., A.C. Bejarano, and G.T. Chandler. 2003. Ecdysteroid concentrations through various life-stages of the meiobenthic harpactacoid copepod, *Amphiascus tenuiremis* and the benthic estuarine amphipod, *Leptocheirus plumulosus*. General and Comparative Endocrinology 132:151–160.

Böcking, D., C. Dauphin-Villemant, D. Sedlmeier, C. Blais, and R. Lafont. 1993. Ecdysteroid synthesis in moulting glands of the crayfish *Orconectes limosus*: evidence for the synthesis of 3-dehydroecdysone by *in vitro* synthesis and conversion studies. Insect Biochemistry and Molecular Biology 23:57–63.

Böcking, D., C. Dauphin-Villemant, J.-Y. Toullec, C. Blais, and R. Lafont. 1994. Ecdysteroid formation from 25-hydroxycholesterol by arthropod molting glands *in vitro*. Comptes Rendus de l'Academie des Sciences 317:891–898.

Borst, D.W., and J.D. O'Connor. 1972. Arthropod molting hormone: radioimmune assay. Science 178:418–419.

Borst, D.W., and J.D. O'Connor. 1974. Trace analysis of ecdysones by gas-liquid chromatography, radioimmunoassay and bioassay. Steroids 24:637–656.

Borst, D.W., H. Laufer, M. Landau, E.S. Chang, W.A. Hertz, F.C. Baker, and D.A. Schooley. 1987. Methyl farnesoate and its role in crustacean reproduction and development. Insect Biochemistry 17:1123–1127.

Borst, D.W., J. Ogan, B. Tsukimura, T. Claerhout, and K.C. Holford. 2001. Regulation of the crustacean mandibular organ. American Zoologist 41:430–441.

Broadie, K.S., A.W. Sylwester, M. Bate, and N.J. Tublitz. 1990. Immunological, biochemical and physiological analyses of cardioacceleratory peptide 2 (CAP2) activity in the embryo of the tobacco hawkmoth *Manduca sexta*. Development 108:59–71.

Bruce, M.J., and E.S. Chang. 1984. Demonstration of a molt-inhibiting hormone from the sinus gland of the lobster, *Homarus americanus*. Comparative Biochemistry and Physiology 79A:421–424.

Bucholz, C., and D. Adelung. 1980. Ultrastructural basis of steroid production in the Y-organ and mandibular organ of the crabs *Hemigrapsus nudus* Dana. and *Carcinus maenas* L. Cell and Tissue Research 206:83–94.

Bulau, P., A. Okuno, E. Thome, T. Schmitz, J. Peter-Katalinic, and R. Keller. 2005. Characterization of a molt-inhibiting hormone (MIH) of the crayfish, *Orconectes limosus*, by cDNA cloning and mass spectrometric analysis. Peptides 26:2129–2136.

Chan, S.M., and P.L. Gu. 1998. Cloning of a cDNA encoding a putative molt-inhibiting hormone from the eyestalk of the sand shrimp *Metapenaeus ensis*. Molecular Marine Biology and Biotechnology 7:214–220.

Chan, S.M., X.G. Chen, and P.L. Gu. 1998. PCR cloning and expression of the molt-inhibiting hormone gene for the crab *Charybdis feriatus*. Gene 224:23–33.

Chang, E.S., and M.J. Bruce. 1980. Ecdysteroid titers of juvenile lobsters following molt induction. Journal of Experimental Zoology 214:157–160.

Chang, E.S., and D.L. Mykles. 2011. Regulation of crustacean molting: a review and our perspectives. General and Comparative Endocrinology 172:323–330.

Chang, E.S., and J.D. O'Connor. 1977. Secretion of α-ecdysone by crab Y-organs *in vitro*. Proceedings of the National Academy of Sciences of the USA 74:615–618.

Chang, E.S., and J.D. O'Connor. 1978. *In vitro* secretion and hydroxylation of α-ecdysone as a function of the crustacean molt cycle. General and Comparative Endocrinology 36:151–160.

Chang, E.S., B.A. Sage, and J.D. O'Connor. 1976. The qualitative and quantitative determination of ecdysones in tissues of the crab, *Pachygrapsus crassipes,* following molt induction. General and Comparative Endocrinology 30:21–33.

Chang, E.S., G.D. Prestwich, and M.J. Bruce. 1990. Amino acid sequence of a peptide with both molt-inhibiting and hyperglycemic activities in the lobster, *Homarus americanus*. Biochemical and Biophysical Research Communications 171:818–826.

Chang, E.S., S.A. Chang, B.S. Beltz, and E.A. Kravitz. 1999. Crustacean hyperglycaemic hormone in the lobster nervous system: localization and release from cells in the suboesophageal ganglion and thoracic second roots. Journal of Comparative Neurology 414:50–56.

Charmantier, G., and J.-P. Trilles. 1979. La dégénérescence de l'organe Y chez *Sphaeroma serratum* (Fabricius) (Isopoda, Flabellifera) etude ultrastructurale. Crustaceana 36:29–38.

Chen, H.-Y., R.D. Watson, J.-C. Chen, H.-F. Liu, and C.-Y. Lee. 2007. Molecular characterization and gene expression pattern of two putative molt-inhibiting hormones from *Litopenaeus vannamei*. General and Comparative Endocrinology 151:72–81.

Chen, S.H., C.Y. Lin, and C.M. Kuo. 2005. In silico analysis of crustacean hyperglycemic hormone family. Marine Biotechnology (NY) 7:193–206.

Cheng, J.H., and E.S. Chang. 1991. Ecdysteroid treatment delays ecdysis in the lobster, *Homarus americanus*. Biological Bulletin 181:169–174.

Christiansen, M.E., J.D. Costlow Jr., and R.J. Monroe. 1997a. Effects of the juvenile hormone mimic ZR-512 (Altozar) on larval development of the mud-crab *Rhithropanopeus harrisi* at various cyclic temperatures. Marine Biology 39:281–288.

Christiansen, M.E., J.D. Costlow Jr., and R.J. Monroe. 1997b. Effects of the juvenile hormone mimic ZR-515 (altosid) on larval development of the mud crab *Rhithropanopeus harrisii* at various salinities and cyclic temperatures. Marine Biology 39:269–279.

Christie, A.E., C.S. Durkin, N. Hartline, P. Ohno, and P.H. Lenz. 2010. Bioinformatic analyses of the publicly accessible crustacean expressed sequence tags (ESTs) reveal numerous novel neuropeptide-encoding precursor proteins, including ones from members of several little studied taxa. General and Comparative Endocrinology 167:164–178.

Christie, A.E., M.D. Mc Coole, S.M. Harmon, K.N. Baer, and P.H. Lenz. 2011. Genomic analysis of the *Daphnia pulex* peptidome. General and Comparative Endocrinology 171:131–150.

Chung, J.S. 2010. Hemolymph ecdysteroids during the last three molt cycles of the blue crab, *Callinectes sapidus*: quantitative and qualitative analyses and regulation. Archives of Insect Biochemistry and Physiology 73:1–13.

Chung J.S., and S.G. Webster. 2003. Moult cycle related changes in biological activity of moult-inhibiting hormone and crustacean hyperglycaemic hormone (CHH) in the Crab, *Carcinus maenas*. From target to transcript. European Journal of Biochemistry 270:3280–3288.

Chung, J.S., and S.G. Webster. 2004. Expression and release patterns of neuropeptides during embryonic development and hatching of the green shore crab, *Carcinus maenas*. Development 131:4751–4761.

Chung J.S., and S.G. Webster. 2005. Dynamics of in vivo release of molt-inhibiting hormone and crustacean hyperglycaemic hormone (CHH) in the shore crab, *Carcinus maenas*. Endocrinology 146:5545–5551.

Chung, J.S., and N. Zmora. 2008. Functional studies of crustacean hyperglycemic hormones (CHHs) of the blue crab, *Callinectes sapidus* – the expression and release of CHH in eyestalk and pericardial organ in response to environmental stress. FEBS Journal 275:693–704.

Chung, J.S., M.C. Wilkinson, and S.G. Webster. 1996. Determination of the amino acid sequence of the moult-inhibiting hormone from the edible crab, *Cancer pagurus*. Neuropeptides 30:95–101.

Chung, J.S., H. Dircksen, and S.G. Webster. 1999. A remarkable, precisely timed release of hyperglycaemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. Proceedings of the National Academy of Sciences of the USA 96:13103–13107.

Chung, J.S., D.C. Wilcockson, N. Zmora, Y. Zohar, H. Dircksen, and S.G. Webster. 2006. Identification and developmental expression of RNAs encoding crustacean cardioactive peptide in decapod crustaceans. Journal of Experimental Biology 209:3862–3872.

Chung, J.S., N. Zmora, H. Katayama, and N. Tsutsui. 2010. Crustacean hyperglycemic hormone (CHH) neuropeptides family: functions, titers and binding to target tissues. General and Comparative Endocrinology 166:447–454.

Covi, J., S. Gomez, S. Chang, K. Lee, E. Chang, and D. Mykles. 2008. Repression of Y-organ ecdysteroidogenesis by cyclic nucleotides and agonists of NO-sensitive guanylyl cyclase. Pages 37–46 *in* Morris, K.S. and A. Voslom, editors. Fourth meeting of comparative physiologists and biochemists in Africa-Mara 2008 – "Molecules to migration: the pressures of life." Monduzzi Editore International, Bologna, Italy.

Covi, J.A., E.S. Chang, and D.L. Mykles. 2009. Conserved role of cyclic nucleotides in the regulation of ecdysteroidogenesis by the crustacean molting gland. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 152:470–477.

Cuzin-Roudy, J., and C. Tchernigovtzeff. 1985. Chronology of the female molt cycle in *Siriella armata* M. -Edw. (Crustacea: Mysidacea) based on marsupial development. Journal of Crustacean Biology 5:1–14.

Cuzin-Roudy, J., C. Strambi, A. Strambi, and J.-P. Delbeque. 1989. Hemolymph ecdysteroids and molt cycle in males and females of *Siriella armata* M.-Edw. (Crustacea: Mysidacea): possible control by the MI-ME X-organ of the eyestalk. General and Comparative Physiology 74:96–109.

Dauphin-Villemant, C., D. Böcking, and D. Sedlmeier. 1995. Regulation of steroidogenesis in crayfish molting glands: involvement of protein synthesis. Molecular and Cellular Endocrinology 109:97–103.

Dauphin-Villemant, C., D. Böcking, M. Tom, M. Maibeche, and R. Lafont. 1999. Cloning of a novel cytochrome P450 (CYP4C15) differentially expressed in the steroidal glands of an arthropod. Biochemical and Biophysical Research Communications 264:413–418.

Davis, N.T., T.A. Miller, H. Lehman, and J.G. Hildebrand. 1990. Crustacean cardioactive peptide: immunoreactive neurons and physiological actions in *Manduca sexta*. Society for Neuroscience Abstracts 16:856.

Davis, N.T., M.B. Blackburn, E.G. Golubeva, and J.G. Hildebrand. 2003. Localization of myoinhibitory peptide immunoreactivity in *Manduca sexta* and *Bombyx mori*, with implications that the peptide has a role in molting and ecdysis. Journal of Experimental Biology 206:1449–1460.

De Kleijn, D.P.V., A.J.M. Coenen, A.M. Laverdure, C.P. Tensen, and F. Van Herp. 1992. Localization of mRNAs encoding crustacean hyperglycemic hormone (CHH) and gonad-inhibiting hormone (GIH) in the X-organ sinus gland complex of the lobster *Homarus americanus*. Neuroscience 51:121–128.

De Kleijn, D.P.V., F.J.G.T. Sleutels, G.J.M. Matrens, and F. Van Herp. 1994. Cloning and expression of mRNA encoding prepro-gonad-inhibiting hormone (GIH) in the lobster *Homarus americanus*. FEBS Letters 353:253–258.

Dillaman, R.M., R. Roer, T. Shafer, and S. Modla. 2013. The crustacean integument: structure and function. Natural history of the Crustacea, volume 1: functional morphology and diversity. Oxford University Press, New York.

Dircksen, H. 1998. Crustacean cardioactive peptide. Pages 302–333 *in* Coast, G.M., and S.G. Webster, editors. Recent advances in arthropod endocrinology. Society for Experimental Biology seminar series 65. Cambridge University Press.

Dircksen, H., and U. Heyn. 1998. Crustacean hyperglycemic hormone-like peptides in crab and locust peripheral intrinsic neurosecretory cells. Annals of the N.Y. Academy of Sciences 839:392–394.

Dircksen, H., S.G. Webster, and R. Keller. 1988. Immunocytochemical demonstration of the neurosecretory systems containing putative moult-inhibiting hormone and hyperglycemic hormone in the eyestalk of brachyuran crustaceans. Cell and Tissue Research 251:3–12.

Dircksen, H., D. Böcking, U. Heyn, C. Mandel, J.S. Chung, G. Baggerman, P. Verhaert, S. Daufeldt, T. Plösch, P.P. Jaros, E. Waelkens, R. Keller, and S.G. Webster. 2001. Crustacean hyperglycaemic hormone (CHH) like peptides and CHH-precursor-related peptides from pericardial organ neurosecretory cells in the shore crab, *Carcinus maenas*, are putatively spliced and modified products of multiple genes. Biochemical Journal 356:159–170.

Dircksen, H., S. Neupert, R. Predel, P. Verleyen, J. Huybrechts, J. Straus, F. Hauser, E. Stafflinger, M. Schneider, K. Pauwels, L. Schoofs, and C.J.P. Grimmelikhuizen. 2011. Genomics, transcriptomics and peptidomics of *Daphnia pulex* neuropeptides and protein hormones. Journal of Proteome Research 10:4478–4504.

Drach, P., and C. Tchernigovtzeff. 1967. Sur la méthode de determination des stades d'intermue et son application générale aux crustaces. Vie Milieu 18:595–609.

Dulcis, D., N.T. Davis, and J.G. Hildebrand. 2001. Neuronal control of heart reversal in the hawkmoth *Manduca sexta*. Journal of Comparative Physiology A 187:837–849.

Durlait, M., M. Moriniere, and P. Porcheron. 1988. Changes in ecdysteroids in *Astacus leptodactylus* during the molting cycle. Comparative Biochemistry and Physiology 89A:223–229.

Echalier, G. 1959. L'organe Y et le déterminisme de la croissance et de la mue chez *Carcinus maenas* (L.), Crustacé Décapode. Annales des Sciences Naturelles—Zoologie et Biologie Animale 12:1–59.

Edomi, P., E. Azzoni, R. Mettulio, N. Pandolfelli, E.A. Ferrero, and P.G. Giulianini. 2002. Gonad-inhibiting hormone of the Norway lobster (*Nephrops norvegicus*): cDNA cloning, expression, recombinant protein production and immunolocalization. Gene 284:93–102.

Ewer, J. 2007. Neuroendocrinology of eclosion. Pages 555–575 *in* North, G., and R.J. Greenspan, editors. Invertebrate neurobiology. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Ewer, J., and J.W. Truman. 1997. Invariant association of ecdysis with increases in cyclic 3'-5'-guanosine monophosphate immunoreactivity in a small network of peptidergic neurons in the hornworm, *Manduca sexta*. Journal of Comparative Physiology A 181:319–330.

Ewer, J., J. De Vente, and J.W. Truman. 1994. Neuropeptide induction of cGMP increases in the insect CNS: resolution at the level of single identifiable neurons. The Journal of Neuroscience 14:7704–7712.

Ewer, J., S.C. Gammie, and J.W. Truman. 1997. Control of insect ecdysis by a positive-feedback endocrine system: roles of eclosion hormone and ecdysis triggering hormone. Journal of Experimental Biology 200:869–881.

Fuse, M., and J.W. Truman. 2002. Modulation of ecdysis in the moth *Manduca sexta*: the roles of the suboesophageal and thoracic ganglia. Journal of Experimental Biology 205:1047–1058.

Gabe, M. 1953. Sur l'existence, chez quelques Crustacés Malacostracés, d'un organe comparable à la glande de la mue des Insectes. Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences 237:1111–1113.

Gammie, S.C., and J.W. Truman. 1997. Neuropeptide hierarchies and the activation of sequential motor behaviors in the hawkmoth, *Manduca sexta*. The Journal of Neuroscience 17:4389–4397.

Gammie, S.C., and J.W. Truman. 1999. Eclosion hormone provides a link between ecdysis-triggering hormone and crustacean cardioactive peptide in the neuroendocrine cascade that controls ecdysis behaviour. Journal of Experimental Biology 202:343–352.

Gard, A.L., P.H. Lenz, J.R. Shaw, and A.E. Christie. 2009. Identification of putative peptide paracrines/hormones in the water flea *Daphnia pulex* (Crustacea; Branchiopoda; Cladocera) using transcriptomics and immunohistochemistry. General and Comparative Endocrinology 160:271–287.

Gersch, M., K. Richter, and H. Eibisch. 1977. Studies on characterization and action of molt-inhibiting hormone (MIH) of the sinus gland in *Orconectes limosus* Rafinesque (Crustacea-Decapoda). Zoologische Jahrbucher Abteilung fuer Allgemeine Zoologie und Physiologie der Tiere 81:133–152.

Gersch, M., K. Richter, G.-A. Böhm, and H. Eibisch. 1980. Experimentelle Untersuchungen zur saisonalen Abhangigkeit der neurohormonalen Steurung der Gastrolithenbildung und der Ecdysteroidsynthese

des Y-organs von *Orconectes limosus* (Crustacea, Decapoda). Zoologische Jahrbucher Abteilung fuer Allgemeine Zoologie und Physiologie der Tiere 84:164–180.

Gilbert, L.I., and K.F. Rewitz. 2009. The function and evolution of the Halloween genes: the pathway to the arthropod molting hormone. Pages 231–269 *in* Smagghe, G., editor. Ecdysone: structures and function. Springer, The Netherlands.

Gilbert, L.I., R. Rybczynski, and S. Tobe. 1996. Endocrine cascades in insect metamorphosis. Pages 59–107 *in* Gilbert, L.I., J. Tata, and P. Atkinson, editors. Metamorphosis: post-embryonic reprogramming of gene expression in amphibian and insect cells. Academic Press, San Diego, USA.

Gilbert, L.I., R. Rybczynski, and J.T. Warren. 2002. Control and biochemical nature of the ecdysteroidogenic pathway. Annual Review of Entomology 47:883–916.

Girard, P., and R. Maissat. 1983. Variations du taux des ecdystéoides hémolymphatiques chez le male de *Ligia oceanica* (L)., (Crustacea, Isopoda, Oniscoidea) et function du cycle de mue et des modifications structurale de l'organe Y. Canadian Journal of Zoology 61:534–538.

Graf, F., and J.P. Delbeque. 1987. Ecdysteroid titers during the molt cycle of *Orchestia cavimana* (Crustacea, Amphipoda). General and Comparative Endocrinology 65:23–33.

Grève, P., O. Sorokine, T. Berges, C. Lacombe, A. Van Dorsselaer, and G. Martin. 1999. Isolation and amino acid sequence of a peptide with vitellogenesis inhibiting activity from the terrestrial isopod *Armadillidium vulgare* (Crustacea). General and Comparative Endocrinology 115:406–414.

Gu, P.-L., and S.-M. Chan. 1998. Cloning of a cDNA encoding a putative molt-inhibiting hormone from the eyestalk of the sand shrimp *Metapenaeus ensis*. Molecular Marine Biology and Biotechnology 7:14–22.

Gu, P.-L., K.H. Chu, and S.-M. Chan. 2001. Bacterial expression of the shrimp molt-inhibiting hormone (MIH): antibody production, immunocytochemical study and biological assay. Cell and Tissue Research 303:129–136.

Gu, P.-L., S.S. Tobe, B.K.C. Chow, K.H. Chu, J.-G. He, and S.-M. Chan. 2002. Characterization of an additional molt inhibiting hormone-like neuropeptide from the shrimp *Metapenaeus ensis*. Peptides 23:1875–1883.

Gunamali, V., R. Kirubagaran, and T. Subramoniam. 2004. Hormonal coordination of molting and female reproduction by ecdysteroids in the mole crab *Emerita asiatica* (Milne Edwards). General and Comparative Endocrinology 138:128–138.

Han, D.W., N. Patel, and R.D. Watson. 2006. Regulation of protein synthesis in Y-organs of the blue crab (*Callinectes sapidus*): involvement of cyclic AMP. Journal of Experimental Zoology Part A: Comparative Experimental Biology 305:328–334.

Hewes, R.S., and J.W. Truman. 1991. The roles of central and peripheral eclosion hormone release in the control of ecdysis behavior in *Manduca sexta*. Journal of Comparative Physiology A 168:697–708.

Hoarau, F., and M. Hirn. 1978. Evolution du taux des ecdystéroides au cours du cycle de mue chez *Helleria brevicornis* Ebner (Isopode terrestre). Comptes Rendus des Seances de l'Academie des Sciences Serie D, Sciences Naturelles 286:1443–1446.

Homola, E., and E.S. Chang. 1997. Methyl farnesoate: crustacean juvenile hormone in search of functions. Comparative Biochemistry and Physiology 117 B:347–356.

Honegger, H.W., E.M. Dewey, and J. Ewer. 2008. Bursicon, the tanning hormone of insects: recent advances following the discovery of its molecular identity. Journal of Comparative Physiology A 194:989–1005.

Hopkins, P.M. 1982. Effects of neurosecretory factors on in vivo levels of ecdysteroids in hemolymph of the fiddler crab, *Uca pugilator*. American Zoologist 22:938.

Hopkins, P.M. 1983. Patterns of serum ecdysteroids during induced proecdysis in the fiddler crab, *Uca pugilator*. General and Comparative Endocrinology 52:350–356.

Hopkins, P.M. 1986. Ecdysteroid titers and Y-organ activity during late anecdysis and proecdysis in the fiddler crab *Uca pugilator*. General and Comparative Endocrinology 63:362–373.

Hopkins, P.M. 1992. Hormonal control of the molt cycle in the fiddler crab *Uca pugilator*. American Zoologist 32:450–458.

Horn, D.H.S., E.J. Middleton, J.A. Wunderlich, and F. Hampshire. 1966. Identity of the moulting hormones of insects and crustaceans. Chemical Communications 11:339–341.

Horodyski, F.M., J. Ewer, L.M. Riddiford, and J.W. Truman. 1993. Isolation, characterization and expression of the eclosion hormone gene of *Drosophila melanogaster*. European Journal of Biochemistry 215:221–228.

Hsu, Y.W.A., D.I. Messinger, J.S. Chung, S.G. Webster, H.O. de la Inglesia, and A.E. Christie. 2006. Members of the crustacean hyperglycaemic hormone (CHH) peptide family are differentially distributed both between and within neuroendocrine organs of *Cancer* crabs: implications for differential release and pleiotropic function. Journal of Experimental Biology 209:3241–3256.

Ikeda, M., and Y. Naya. 1993. The biotransformation of tritiated 3-dehydroecdysone by crayfish, *Procambarus clarkii*. Experientia 49:1101–1105.

Jegla, T.C., K. Ruland, G. Kegel, and R. Keller. 1983. The role of the Y-organ and cephalic gland in ecdysteroid production and control of molting in the crayfish *Orconectes limosus*. Journal of Comparative Physiology B 152:91–95.

Johnen, C., U. von Gliscynski, and H. Dircksen. 1995. Changes in haemolymph ecdysteroid levels and CNS contents of crustacean cardioactive peptide-immunoreactivity during the moult cycle of the isopod *Oniscus asellus*. Netherlands Journal of Zoology 45:38–40.

Johnson, C.L. 2003. Ecdysteroids in the oceanic copepod *Calanus pacificus*: variation during molt cycle and change associated with diapause. Marine Ecology Progress Series 257:159–165.

Katakoa, H., R.G. Troetschler, S.J. Kramer, B.J. Cesarin, and D.A. Schooley. 1987. Isolation and primary structure of the eclosion hormone of the tobacco hornworm, *Manduca sexta*. *Biochemical and Biophysical Research Communications* 299:924–931.

Katayama, H., and H. Nagasawa. 2004. Effect of a glycine residue insertion into crustacean hyperglycemic hormone on hormonal activity. Zoological Science 21:1121–1124.

Katayama, H., K. Nagata, T. Ohira, F. Yumoto, M. Tanokura, and H. Nagasawa. 2003. The solution structure of molt-inhibiting hormone from the Kuruma prawn *Marsupenaeus japonicus*. The Journal of Biological Chemistry 278:9620–9623.

Keller, R., and E. Schmid. 1979. In vitro secretion of ecdysteroids by Y-organs and lack of secretion by mandibular organs in the crayfish following molt induction. Journal of Comparative Physiology B 130:347–353.

Keller, R., G. Kegel, B. Reichwein, D. Sedlmeier, and D. Soyez. 1999. Biological effects of neurohormones of the CHH/MIH/GIH peptide family in crustaceans. Pages 209–212 *in* Roubos, E.W., S.E. Wendelaar Bonga, H. Vaudry, and A. De Loof, editors. Recent developments in comparative endocrinology and neurobiology. Shaker Publishing, Maastricht, The Netherlands.

Kim, H.W., L.A. Batista, J.L. Hoppes, K.J. Lee, and D.L. Mykles. 2004. A crustacean nitric oxide synthase expressed in nerve ganglia, Y-organ gill and gonad of the tropical land crab, *Gecarcinus lateralis*. Journal of Experimental Biology 207:2845–2857.

Kim, Y.J., I. Spalovska-Valachova, K.H. Cho, I. Žitňanová, Y. Park, M.E. Adams, and D. Žitňan. 2004. Corazonin receptor signalling in ecdysis initiation. Proceedings of the National Academy of Sciences, USA 103:14211–14216.

Kim, Y.J., D. Žitňan, C.G. Galizia, K.H. Cho, and M.E. Adams. 2006. A command chemical triggers an innate behaviour by sequential activation of multiple peptidergic ensembles. Current Biology 16:1395–1407.

Kleijn, J.M., S. Mangerich, D.P.V. De Kleijn, R. Keller, and W.M. Weidemann. 1993. Molecular cloning of crustacean molt-inhibiting hormone (MIH) precursor. FEBS Letters 334:139–142.

Kono, T., H. Nagasawa, A. Isogai, H. Fugo, and A. Suzuki. 1987. Amino acid sequence of eclosion hormone of the silkworm *Bombyx mori*. Agricultural and Biological Chemistry 51:2307–2308.

Kostron, B., K. Marquardt, U. Kaltenhauser, and H. Honegger. 1995. Bursicon, the cuticle sclerotizing hormone-comparison of its molecular mass in different insects. Journal of Insect Physiology 41:1045–1053.

Lachaise, F., and R. Lafont. 1984. Ecdysteroid metabolism in a crab: *Carcinus maenas* L., Steroids 43:243–259.

Lachaise, F., M. Lagueux, R. Feyereisen, and J.A. Hoffmann. 1976. Métabolisme de l'ecdysone au cours du développement de *Carcinus maenas* (Brachyura, Decapoda). Comptes Rendus des Seances de l'Academie des Sciences. Serie D, Sciences Naturelles 283:323–332.

Lachaise, F., M.F. Meister, C. Hetru, and R. Lafont. 1986. Studies on the biosynthesis of ecdysone by the Y-organs of *Carcinus maenas*. Molecular and Cellular Endocrinology 45:253–261.

Lachaise, F., M. Hubert, S.G. Webster, and R. Lafont. 1988. Effect of moult-inhibiting hormone on ketodiol conversion by crab Y-organs. Journal of Insect Physiology 34:557–562.

Lachaise, F., G. Carpentier, G. Sopmme, J. Colardeau, and P. Beydon. 1989. Ecdysteroid synthesis by crab Y-organs. Journal of Experimental Zoology 252:283–292.

Lachaise, F., A. Le Roux, M. Hubert, and R. Lafont. 1993. The molting gland of crustaceans: localization, activity and endocrine control (a review). Journal of Crustacean Biology 13:198–234.

Lachaise, F., G. Sommé, G. Carpentier, E. Granjeon, S. Webster, and D. Bagdhassarian. 1996. A transaldolase: an enzyme implicated in crab steroidogenesis. Endocrine 5:23–32.

Lacombe, C., P. Grève, and G. Martin. 1999. Overview on the sub-grouping of the crustacean hyperglycemic hormone family. Neuropeptides 33:71–80.

Lafont, R., C. Beydon, M. Blais, F. Garcia, F. Lachaise, F. Riera, G. Somme, and J.P. Girault. 1986. Ecdysteroid metabolism-a comparative study. Insect Biochemistry 16:11–16.

Laufer, H., D.W. Borst, F.C. Baker, C. Carrasco, M. Sinkus, C.C. Reuter, L.W. Tsai, and D.A. Schooley. 1987. Identification of a juvenile hormone-like compound in a crustacean. Science 235:202–205.

Laufer, H., J.S.B. Ahl, and A. Sagi. 1993. The role of juvenile hormones in crustacean reproduction. American Zoologist 33:365–374.

Laufer, H., P. Takac, J.S.B. Ahl, and M.R. Laufer. 1997. Methyl farnesoate and the effect of eyestalk ablation on the morphogenesis of the juvenile female spider crab *Libinia emarginata*. International Journal of Invertebrate Reproduction and Development 31:63–68.

Laufer, H., P. Takac, J.S.B. Ahl, G. Rottlant, and B. Baclaski. 2002. Evidence that ecdysteroids and methyl farnesoate control allometric growth and differentiation in a crustacean. Insect Biochemistry and Molecular Biology 32:205–210.

Laufer, H., N. Demir, X. Pan, J.D. Stuart, and J.S.B. Ahl. 2005. Methyl farnesoate controls adult male morphogenesis in the crayfish, *Procambarus clarkii*. Journal of Insect Physiology 51:379–384.

Le Roux, A. 1968. Description d'organes mandibulaires nouveaux chez les Crustacés Décapodes. Comptes Rendus des Seances de l'Academie des Sciences Serie D, Sciences Naturelles 266:1317–1320.

Lee, K.J., and R.D. Watson. 2002. Antipeptide antibodies for detecting crab (*Callinectes sapidus*) molt-inhibiting hormone. Peptides 23:853–862.

Lee, K.J., T.S. Elton, A.-K. Bej, S.A. Watts, and R.D. Watson. 1995. Molecular cloning of a cDNA encoding putative molt-inhibiting hormone from the blue crab, *Callinectes sapidus*. Biochemical and Biophysical Research Communications 209:1126–1131.

Lee, K.J., R.D. Watson, and R.D. Roer. 1998. Molt-inhibiting hormone mRNA levels and ecdysteroid titer during a molt cycle of the blue crab, *Callinectes sapidus*. Biochemical and Biophysical Research Communications 249:624–627.

Lee, K.J., H.-W. Kim, A.M. Gomez, E.S. Chang, J.A. Covi, and D.L. Mykles. 2007. Molt-inhibiting hormone from the tropical land crab, *Gecarcinus lateralis*: cloning, tissue expression, and expression of biologically active recombinant peptide in yeast. General and Comparative Endocrinology 150:505–513.

Lee, S.G., B.D. Bader, E.S. Chang, and D.L. Mykles. 2007a. Effects of elevated ecdysteroid in tissue expression of three guanylyl cyclases in the tropical land crab *Gecarcinus lateralis*: possible roles of neuropeptide signalling in the molting gland. Journal of Experimental Biology 210:3245–3254.

Lee, S.G., H.W. Kim, and D.L. Mykles. 2007b. Guanylyl cyclases in the tropical land crab, *Gecarcinus lateralis*: cloning of soluble (NO-sensitive and-insensitive) and membrane receptor forms. Comparative Biochemistry and Physiology—Part D: Genomics and Proteomics 2:332–344.

Liu, L., H. Laufer, Y. Wang, and T. Hayes. 1997. A neurohormone regulating both methyl farnesoate synthesis and glucose metabolism in a crustacean. Biochemical and Biophysical Research Communications 237:694–701.

Lu, W., G. Wainwright, S.G. Webster, H.H. Rees, and P.C. Turner. 2000. Clustering of mandibular organ-inhibiting hormone and moult-inhibiting hormone genes in the crab, *Cancer pagurus*, and implications for regulation of expression. Gene 253:197–207.

Lu, W., G. Wainwright, L.A. Olohan, S.G. Webster, H.H. Rees, and P.C. Turner. 2001. Characterization of cDNA encoding molt-inhibiting hormone of the crab, *Cancer pagurus*; expression of MIH in non-X-organ tissues. Gene 278:149–159.

Luo, C.W., E.M. Dewey, S. Sudo, J. Ewer, S.Y. Hsu, H.W. Honegger, and A.J. Hsueh. 2005. Bursicon, the insect cuticle hardening hormone is a heterodimeric cystine knot protein that activates G protein-coupled receptor LGR2. Proceedings of the National Academy of Sciences, USA 102:2820–2825.

Ma, M., E.K. Bors, E.S. Dickinson, M.A. Kwiatowski, G.L. Sousa, R.P. Henry, C.M. Smith, D.W. Towle, A.E. Christie, and L. Li. 2009. Characterization of the *Carcinus maenas* neuropeptidome by mass spectrometry and functional genomics. General and Comparative Endocrinology 161:320–334.

Ma, M., A.L. Gard, F. Xiang, J. Wang, N. Davoodian, P.H. Lenz, S.R. Malecha, A.E. Christie, and L. Li. 2010. Combining *in silico* transcriptome mining and biological mass spectrometry for neuropeptide discovery in the Pacific white shrimp *Litopenaeus vannamei*. Peptides 31:27–43.

Marco, H.G., and G. Gäde. 1999. A comparative immunocytochemical study of the hyperglycaemic, moult-inhibiting and vitellogenesis-inhibiting neurohormone family in three species of decapod crustacean. Cell and Tissue Research 295:171–182.

Marti, T., K. Takio, K.A. Walsh, G. Terzi, and J.W. Truman. 1987. Microanalysis of the amino acid sequence of the eclosion hormone from the tobacco hornworm *Manduca sexta*. FEBS Letters 219:415–418.

Martin-Creuzberg, D., S.A. Westerlund, and K.H. Hoffmann. 2007. Ecdysteroid levels in *Daphnia magna* during a molt cycle: determination by radioimmunoassay (RIA) and liquid chromatography-mass spectrometry (LC-MS). General and Comparative Endocrinology 151:66–71.

Mattson, M.P., and E. Spaziani. 1985a. Characterization of molt-inhibiting hormone (MIH) action on crustacean Y-organ segments and dispersed cells in culture and a bioassay for MIH activity. *Journal of Experimental Zoology* 236:93–101.

Mattson, M.P., and E. Spaziani. 1985b. Cyclic AMP mediates the negative regulation of Y-organ ecdysteroid production. Molecular and Cellular Endocrinology 42:185–189.

Mattson, M.P., and E. Spaziani. 1986a. Regulation of Y-organ ecdysteroidogenesis by molt-inhibiting hormone in crabs: involvement of cyclic AMP-mediated protein synthesis. General and Comparative Endocrinology 63:414–423.

Mattson, M.P., and E. Spaziani. 1986b. Regulation of crab Y-organ steroidogenesis *in vitro*: evidence that ecdysteroid production increases through activation of cAMP-phosphodiesterase by calcium-calmodulin. Molecular and Cellular Endocrinology 48:135–151.

Mattson, M.P., and E. Spaziani. 1987. Demonstration of protein kinase C activity in crustacean Y-organs, and partial definition of its role in regulation of ecdysteroidogenesis. Molecular and Cellular Endocrinology 49:159–171.

McCarthy, J.F., and D.M. Skinner. 1977. Proecysial changes in serum ecdysone titers, gastrolith formation, and limb regeneration following molt induction by limb autotomy and/or eyestalk removal in the land crab, *Gecarcinus lateralis*. General and Comparative Endocrinology 33:278–292.

Mendive, F.M., T. Van Loy, S. Claeysen, J. Poels, M. Williamson, F. Hauser, C.J.P. Grimmelikhuizen, G. Vassart, and J. Vanden Broeck. 2005. *Drosophila* molting neurohormone bursicon is a heterodimer and the natural agonist of the orphan receptor DLGR2. FEBS Letters 579:2171–2176.

Montagné, N., Y. Desdevises, D. Soyez, and J.Y. Toullec. 2010. Molecular evolution of the crustacean hyperglycemic hormone family in ecdysozoans. BMC Evolutionary Biology 10:62.

Mykles, D.L. 2011. Ecdysteroid metabolism in crustaceans. The Journal of Steroid Biochemistry and Molecular Biology 127:196–203.

Mykles, D.L., M.E. Adams, G. Gäde, A.B. Lange, H.G. Marco, and I. Orchard. 2010. Neuropeptide action in insects and crustaceans. Physiological and Biochemical Zoology 83:836–846.

Nagaraju, G.P.C. 2003. Mandibular organ; its role in the regulation of reproduction and molting in the crab *Oziotelphusa senex senex*. PhD thesis, Sri Venkateswara University, Tirupati, India.

Nagaraju, G.P.C. 2007. Is methyl farnesoate a crustacean hormone? Aquaculture 272:39–54.

Nagasawa, H., W.-J. Yang, H. Shimizu, K. Aida, H. Tsutsumi, A. Terauchi, and H. Sonobe. 1996. Isolation and amino acid sequence of a molt-inhibiting hormone from the American crayfish, *Procambarus clarkii*. Bioscience, Biotechnology, and Biochemistry 60:554–556.

Nakatsuji, T., and H. Sonobe. 2004. Regulation of ecdysteroid secretion from the Y-organ by molt-inhibiting hormone in the American crayfish, *Procambarus clarkii*. General and Comparative Endocrinology 135:358–364.

Nakatsuji, T., D.-W. Han, M.J. Jablonsky, S.R. Harville, D.D. Muccio, and R.D. Watson. 2006a. Expression of crustacean (*Callinectes sapidus*) molt-inhibiting hormone in *Escherichia coli*: characterization of the recombinant peptide and assessment of its effects on cellular signalling pathways in Y-organs. Molecular and Cellular Endocrinology 253:96–104.

Nakatsuji, T., H. Sonobe, and R.D. Watson. 2006b. Molt-inhibiting hormone-mediated regulation of ecdysteroid synthesis in Y-organs of the crayfish (*Procambarus clarkii*): involvement of cyclic GMP and cyclic nucleotide phosphodiesterase. Molecular and Cellular Endocrinology 253:76–82.

Nakatsuji, T., C.Y. Lee, and R.D. Watson. 2009. Crustacean molt-inhibiting hormone: structure, function and cellular mode of action. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 152:139–148.

Niwa, R., T. Namiki, K. Ito, Y. Shimada-Niwa, M. Kiuchi, S. Kawaoka, T. Kayukawa, Y. Banno, Y. Fujimoto, S. Shigenobu, S. Kobayashi, T. Shimada, S. Katsuma, and T. Shinoda. 2010. *Non-molting glossy/shroud* encodes a short-chain dehydrogenase/reductase that functions in the "Black Box" of the ecdysteroid biosynthesis pathway. Development 137:1991–1999.

Oda, S., N. Tatarazako, H. Watanabe, M. Morita, and T. Iguchi. 2005. Production of male neonates in *Daphnia magna* (Cladocera, Crustacea) exposed to juvenile hormones and their analogs. Chemosphere 61:1168–1174.

Oda, S., Y. Kato, H. Watanabe, N. Tatarazako, and T. Iguchi. 2011. Morphological changes in *Daphnia galeata* induced by a crustacean terpenoid hormone and its analog. Environmental Toxicology & Chemistry 30:232–238.

Ohira, T., T. Watanabe, H. Nagasawa, and K. Aida. 1997. Molecular cloning of a molt-inhibiting hormone cDNA from the kuruma prawn *Penaeus japonicus*. Zoological Science 14:785–789.

Ohira, T., H. Katayama, S. Tominaga, T. Takasuka, T. Nakatsuji, H. Sonobe, K. Aida, and H. Nagasawa. 2005. Cloning and characterization of a molt-inhibiting hormone-like peptide from the prawn *Marsupenaeus japonicus*. Peptides 26:259–268.

Okumura, T., K. Nakamura, K. Aida, and I. Hanyu. 1989. Hemolymph ecdysteroid levels during the molt cycle in the kuruma prawn *Penaeus japonicus*. Nippon Suisan Gakkaishi 55:2091–2098.

Okumura, T., C.H. Han, Y. Suzuki, K. Aida, and I. Hanyu. 1992. Changes in hemolymph vitellogenin and ecdysteroid levels during the reproductive and nonreproductive cycles in the freshwater prawn *Macrobrachium nipponense*. Zoological Science 9:37–45.

Okumura, T., M. Kamba, H. Sonobe, and K. Aida. 2003. *In vitro* secretion of ecdysteroid by Y-organ during molt cycle and evidence for secretion of 3-dehydroecdysone in the giant freshwater prawn, *Macrobrachium rosenbergii* (Crustacea: Decapoda: Caridea). Invertebrate Reproduction and Development 44:1–8.

Ollivaux, C., J. Vinh, D. Soyez, and J.-Y. Toullec. 2006. Crustacean hyperglycemic and vitellogenesis-inhibiting hormones in the lobster *Homarus gammarus*. FEBS Journal 273:2151–2160.

Ollivaux, C., D. Gallois, M. Amiche, M. Boscaméric, and D. Soyez. 2009. Molecular and cellular specificity of post-translational aminoacyl isomerisation in the crustacean hyperglycaemic hormone family. FEBS Journal 276:4790–4802.

Olmstead, A.W., and G.A. LeBlanc. 2002. Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. Journal of Experimental Zoology 293:736–739.

Park, J.H., A.J. Schroeder, C. Helfrich-Förster, F.R. Jackson, and J. Ewer. 2003. Targeted ablation of CCAP neuropeptide containing neurons of *Drosophila* causes specific effects in execution and circadian timing of ecdysis behaviour. Development 130:2645–2656.

Passano, L.M. 1953. Neurosecretory control of molting in crabs by the X-organ sinus gland complex. Physiologia Comparata. et Oecologia 3:155–189.

Phlippen, M.K., S.G. Webster, J.S. Chung, and H. Dircksen. 2000. Ecdysis of decapod crustaceans is associated with a dramatic release of crustacean cardioactive peptide into the haemolymph. Journal of Experimental Biology 203:521–536.

Porcheron, P., J. Foucrier, C. Gros, P. Pradelles, P. Cassier, and F. Dray. 1976. Radioimmunoassay of arthropod moulting hormone: β-ecdysone antibodies production and 125I-iodinated tracer preparation. FEBS Letters 69:159–162.

Porcheron, P., M. Moriniere, J. Grassi, and P. Pradelles. 1989. Development of an enzyme immunoassay for ecdysteroids using acetylcholinesterase as label. Insect Biochemistry 19:117–122.

Qian, Y.Q., L. Dai, J.-S. Yang, D.-F. Chen, Y. Fujiwara, S. Tsuchida, H. Nagasawa, and W.-J. Yang. 2009. CHH family peptides from an 'eyeless' deep-sea hydrothermal vent shrimp, *Rimicaris kairei*: characterization and sequence analysis. Comparative Biochemistry and Physiology B, Biochemistry and Molecular Biology 154:37–47.

Reddy, P.R., G.P.C. Nagaraju, and P.S. Reddy. 2004. Involvement of methyl farnesoate in the regulation of molting and reproduction in the freshwater crab *Oziotelphusa senex senex*. Journal of Crustacean Biology 24:511–515.

Rewitz, K.F., and L.I. Gilbert. 2008. *Daphnia* Halloween genes that encode cytochrome P450s mediating the synthesis of the arthropod molting hormone: evolutionary implications. BMC Evolutionary Biology 8:60.

Rewitz, K., B. Styrishave, and O. Andersen. 2003. CYP330A1 and CYP4C39 enzymes in the shore crab *Carcinus maenas*: sequence and expression regulation by ecdysteroids and xenobiotics. Biochemical and Biophysical Research Communications 310:252–260.

Rewitz, K.F., M.B. O'Connor, and L.I. Gilbert. 2007. Molecular evolution of the insect Halloween family of cytochrome P450s: phylogeny, gene organisation and functional conservation. Insect Biochemistry and Molecular Biology 37:741–753.

Riddiford, L.M. 1989. The epidermis as a model system for ecdysteroid action. Pages 407–413 *in* J. Koolman, editor. Ecdysone: from chemistry to mode of action. Thieme, Stuttgart, Germany.

Riddiford, L.M. 1996. Juvenile hormone: the status of its "status quo" action. Archives of Insect Biochemistry and Physiology 32:271–286.

Rottlant, G., D.P.V. De Kleijn, M. Charmantier-Daures, G. Charmantier, and F. Van Herp. 1993. Localization of crustacean hyperglycemic hormone (CHH) and gonad-inhibiting hormone (GIH) in the eyestalk of *Homarus gammarus* larvae by immunocytochemistry and in situ hybridization. Cell and Tissue Research 271:507–512.

Rudolph, P.H., and E. Spaziani. 1992. Formation of ecdysteroids by Y-organs of the crab, *Menippe mercenaria*. II. Incorporation of cholesterol into 7-dehydrocholesterol and secretion products in vitro. General and Comparative Endocrinology 88:235–242.

Rudolph, P.H., E. Spaziani, and W.L. Wang. 1992. Formation of ecdysteroids by Y-organs of the crab, *Menippe mercenaria*. I. Biosynthesis of 7-dehydrocholesterol in vivo. General and Comparative Endocrinology 88:224–234.

Sagi, A., E. Homola, and H. Laufer. 1993. Distinct reproductive types of male spider crabs *Libinia emarginata* differ in circulating and synthesizing methyl farnesoate. Biological Bulletin 185:168–173.

Sagi, A., J.S.B. Ahl, H. Danaee, and H. Laufer. 1994. Methyl farnesoate levels in male crabs exhibiting active reproductive behavior. Hormones and Behavior 28:261–272.

Saïdi, B., N. De Bessé, S.G. Webster, D. Sedlmeier, and F. Lachaise. 1994. Involvement of cAMP and cGMP in the mode of action of molt-inhibiting hormone (MIH) a neuropeptide which inhibits steroidogenesis in a crab. Molecular and Cellular Endocrinology 102:53–61.

Sedlmeier, D., and R. Fenrich. 1993. Regulation of ecdysteroid biosynthesis in crayfish Y-organs. I. Role of cyclic nucleotides. Journal of Experimental Zoology 265:448–453.

Sefiani, M., J.-P. Le Caer, and D. Soyez. 1996. Characterization of hyperglycemic and molt-inhibiting activity from sinus glands of the penaeid shrimp *Penaeus vannamei*. General and Comparative Endocrinology 103:41–53.

Sharp, J.S., D.C. Wilcockson, and S.G. Webster. 2010. Identification and expression of mRNAs encoding bursicon in the plesiomorphic central nervous system of *Homarus gammarus*. General and Comparative Endocrinology 169:65–74.

Shih, T.-W., Y. Suzuki, H. Nagasawa, and K. Aida. 1998. Immunohistochemical identification of hyperglycemic hormone- and molt-inhibiting hormone-producing cells in the eyestalk of the Kuruma prawn, *Penaeus japonicus*. Zoological Science 15:389–397.

Simione, F.P., and D.L. Hoffman. 1975. Some effects of eyestalk removal on Y-organs of *Cancer irroratus* Say. Biological Bulletin 148:440–447.

Smith, P.A., A.S. Clare, H.H. Rees, M.C. Prescott, G. Wainwright, and M.C. Thorndyke. 2000. Identification of methyl farnesoate in the cypris larva of the barnacle, *Balanus amphitrite*, and its role as a juvenile hormone. Insect Biochemistry and Molecular Biology 30:885–890.

Smith, R.I. 1940. Studies on the effect of eyestalk removal upon young crayfish (*Cambarus clarkii* Girard). Biological Bulletin 79:145–152.

Snyder, M.J., and E.S. Chang. 1991a. Ecdysteroids in relation to the molt cycle of the American lobster, *Homarus americanus*. I. Hemolymph titers and metabolites. General and Comparative Endocrinology 81:133–145.

Snyder, M.J., and E.S. Chang. 1991b. Metabolism and excretion of injected [3H]-ecdysone by female lobsters, *Homarus americanus*. Biological Bulletin 180:475–484.

Snyder, M.J., and E.S. Chang. 1991c. Ecdysteroids in relation to the molt cycle of the American lobster, *Homarus americanus*. II. Excretion of metabolites. General and Comparative Endocrinology 83:118–131.

Snyder, M.J., and E.S. Chang. 1992. Role of the midgut gland in metabolism and excretion of ecdysteroids by lobsters, *Homarus americanus*. General and Comparative Endocrinology 85:286–296.

Sonobe, H., M. Kamba, K. Ohta, M. Ikeda, and Y. Naya. 1991. *In vitro* secretion of ecdysteroids by Y-organs of the crayfish, *Procambarus clarkii*. Experientia 47:948–952.

Soumoff, C., and J.D. O'Connor. 1982. Repression of Y-organ secretory activity by molt-inhibiting hormone in the crab *Pachygrapsus crassipes*. General and Comparative Endocrinology 48:432–439.

Soumoff, C., and D.M. Skinner. 1983. Ecdysteroid titers during the molt cycle of the blue crab resemble those of other crustaceans. Biological Bulletin 165:321–329.

Soyez, D., J.-P. Le Caer, P.Y. Noël, and J. Rossier. 1991. Primary structure of two isoforms of the vitellogenesis-inhibiting hormone from the lobster *Homarus americanus*. Neuropeptides 20:25–32.

Spaziani, E., and S.B. Kater. 1973. Uptake and turnover of cholesterol-14C in Y-organs of the crab *Hemigrapsus* as a function of the molt cycle. General and Comparative Endocrinology 20:534–549.

Spaziani, E., H.H. Rees, W.L. Wang, and R.D. Watson. 1989. Evidence that Y-organs of the crab *Cancer antennarius* secrete 3-dehydroecdysone. Molecular and Cellular Endocrinology 66:17–25.

Spaziani, E., M. Mattson, W.L. Wang, and H.E. McDougall. 1999. Signaling pathways for ecdysteroid hormone synthesis in crustacean Y-organs. American Zoologist 39:496–512.

Spaziani, E., T.C. Jegla, W.L. Wang, J.A. Booth, S.M. Connolly, C.C. Conrad, M.J. Dewall, C.M. Sarno, D.K. Stone, and R. Montgomery. 2001. Further studies on signalling pathways for ecdysteroidogenesis in crustacean Y-organs. American Zoologist 41:418–429.

Stangier, J., C. Hilbich, C.K. Bayreuther, and R. Keller. 1987. Unusual cardioactive peptide (CCAP) from pericardial organs of the shore crab *Carcinus maenas*. Proceedings of the National Academy of Sciences, USA 84:575–579.

Stangier, J., C. Hilbich, H. Dircksen, and R. Keller. 1988. Distribution of a novel cardioactive peptide (CCAP) in the nervous system of the shore crab *Carcinus maenas*. Peptides 9:795–800.

Stevenson, J.R., P.W. Armstrong, E.S. Chang, and J.D. O'Connor. 1979. Ecdysone titers during the molt cycle of the crayfish *Orconectes sanborni*. General and Comparative Endocrinology 39:20–25.

Styrishave, B., T. Lund, and O. Andersen. 2008. Ecdysteroids in female shore crabs *Carcinus maenas* during the moulting cycle and oocyte development. Journal of the Marine Biological Association of the United Kingdom 88:575–581.

Suzuki, S., K. Yamasaki, T. Fujita, Y. Mamiya, and H. Sonobe. 1996. Ovarian and hemolymph ecdysteroids in the terrestrial isopod *Armadillidium vulgare* (Malacostraca, Crustacea). General and Comparative Endocrinology 104:129–138.

Taketomi, T., M. Motono, and M. Miyawaki. 1989. On the function of the mandibular gland of decapod crustacean. Cell Biology International Reports 13:463–469.

Tamone, S.L., and E.S. Chang. 1993. Methyl farnesoate stimulates ecdysteroid secretion from crab Y-organs *in vitro*. General and Comparative Endocrinology 89:425–432.

Tamone, S.L., M.M. Adams, and J.M. Dutton. 2005. Effect of eyestalk-ablation on circulating ecdysteroids in hemolymph of snow crabs, *Chionoecetes opilio*: physiological evidence for a terminal molt. Integrative and Comparative Biology 45:166–171.

Tang, C., W. Lu, G. Wainwright, S.G. Webster, H.H. Rees, and P.C. Turner. 1999. Molecular characterisation and expression of mandibular organ-inhibiting hormone, a recently discovered neuropeptide involved in the regulation of growth and reproduction in the crab *Cancer pagurus*. Biochemistry Journal 343:355–360.

Tatarazako, N., S. Oda, H. Watanabe, M. Morita, and T. Iguchi. 2003. Juvenile hormone agonists affect the occurrence of male *Daphnia*. Chemosphere 53:827–833.

Toullec, J.Y., and C. Dauphin-Villemant. 1994. Dissociated cell-suspensions of *Carcinus maenas* Y-organs as a tool to study ecdysteroid production and its regulation. Experientia 50:153–158.

Treerattrakool, S., A. Udomkit, S.-M. Chan, and S. Panyim. 2008. Molecular characterization of gonad-inhibiting hormone of *Penaeus monodon* and elucidation of its inhibitory role in vitellogenin expression by RNA interference. FEBS Journal 275:970–980.

Truman, J.W. 2005. Hormonal control of insect ecdysis: endocrine cascades for coordinating behavior with physiology. Vitamins and Hormones 73:1–30.

Tublitz, N.J., and P.D. Evans. 1986. Insect cardioactive peptides: cardioacceleratory peptide (CAP) activity is blocked *in vivo* and *in vitro* with a monoclonal antibody. The Journal of Neuroscience 6:2451–2456.

Tublitz, N.J., and J.W. Truman. 1985. Insect cardioactive peptides. II. Neurohormonal control of heart activity by two cardioacceleratory peptides in the tobacco hawkmoth, *Manduca sexta*. Journal of Experimental Biology 114:381–395.

Tublitz, N.J., A.T. Allen, C.C. Cheung, K.K. Edwards, D.P. Kimble, P.K. Loi, and A.W. Sylwester. 1992. Insect cardioactive peptides: regulation of hindgut activity by cardioacceleratory peptide 2 (CAP2) during wandering behaviour in *Manduca sexta* larvae. Journal of Experimental Biology 165:241–264.

Udomkit, A., S. Chooluck, S. Sonthayayon, and S. Panyim. 2000. Molecular cloning of a cDNA encoding a member of the CHH/MIH/GIH family from *Penaeus monodon* and analysis of its gene structure. Journal of Experimental Marine Biology and Ecology 244:145–156.

Umphrey, H.R., K.J. Lee, R.D. Watson, and E. Spaziani. 1998. Molecular cloning of a cDNA encoding molt-inhibiting hormone of the crab, *Cancer magister*. Molecular and Cellular Endocrinology 136:145–149.

Veenstra, J.A. 1989. Isolation and structure of corazonin, a cardioactive peptide from the American cockroach. FEBS Letters 250:231–234.

Wainwright, G., S.G. Webster, M.C. Wilkinson, J.S. Chung, and H.H. Rees. 1996. Structure and significance of mandibular organ-inhibiting hormone in the crab, *Cancer pagurus*; involvement in multihormonal regulation of growth and reproduction. The Journal of Biological Chemistry 271:12749–12754.

Wang, W.L., E. Spaziani, Z.-H. Huang, D.M. Charkowski, Y. Li, and X.M. Liu. 2000. Ecdysteroid hormones and metabolites of the stone crab, *Menippe mercenaria*. Journal of Experimental Zoology 286:725–735.

Wang, Z.Z., C.Z. Jiao, X.J. Zhang, and J.H. Xiang. 2003. Molecular cloning and sequence analysis of full length cDNA encoding molt-inhibiting hormone from *Fenneropenaeus chinensis*. Yi Chuan Xue Bao 30:128–134.

Watson, R.D., and E. Spaziani. 1985. Biosynthesis of ecdysteroids from cholesterol by crab Y-organs, and eyestalk suppression of cholesterol uptake and secretory activity, *in vitro*. General and Comparative Endocrinology 59:140–148.

Webster, S.G. 1986. Neurohormonal control of ecdysteroid synthesis by *Carcinus maenas* Y-organs *in vitro*, and preliminary characterization of the putative molt-inhibiting hormone (MIH). General and Comparative Endocrinology 61:237–247.

Webster, S.G. 1991. Amino acid sequence of putative moult-inhibiting hormone from the crab *Carcinus maenas*. Proceedings of the Royal Society of London B 244:247–252.

Webster, S.G. 1993. High-affinity binding of putative moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) to membrane bound receptors on the Y-organ of the shore crab *Carcinus maenas*. Proceedings of the Royal Society of London B 251:53–59.

Webster, S.G., and H. Dircksen. 1991. Putative molt-inhibiting hormone in larvae of the shore crab *Carcinus maenas* L.: an immunocytochemical approach. Biological Bulletin 180:65–71.

Webster, S.G., and R. Keller. 1986. Purification, characterisation and amino acid composition of the putative moult-inhibiting hormone (MIH) of *Carcinus maenas* (Crustacea, Decapoda). Journal of Comparative Physiology B 156:617–624.

Webster, S.G., H. Dircksen, and J.S. Chung. 2000. Endocrine cells in the gut of the shore crab *Carcinus maenas* immunoreactive to crustacean hyperglycaemic hormone and its precursor-related peptide. Cell and Tissue Research 300:193–205.

Webster, S.G., R. Keller, and H. Dircksen. 2012. The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting and reproduction. General and Comparative Endocrinology 175:217–233.

Wilcockson, D.C., and S.G. Webster. 2008. Identification and developmental expression of mRNAs encoding putative insect cuticle hardening hormone, bursicon in the green shore crab *Carcinus maenas*. General and Comparative Endocrinology 156:113–125.

Wilder, M.N., S. Okada, N. Fusetani, and K. Aida. 1995. Hemolymph profiles of juvenoid substances in the giant freshwater prawn *Macrobrachium rosenbergii* in relation to reproduction and molting. Fisheries Science 61:175–176.

Yang, W.-J., and K.R. Rao. 2001. Cloning of precursors for two MIH/VIH-related peptides in the prawn, *Macrobrachium rosenbergii*. Biochemical and Biophysical Research Communications 289:407–413.

Yodmuang, S., A. Udomkit, S. Treerattrakool, and S. Panyim. 2004. Molecular and biological characterization of molt-inhibiting hormone of *Penaeus monodon*. Journal of Experimental Marine Biology and Ecology 312:101–114.

Yoshiyama, T., T. Namiki, K. Mita, H. Katakoa, and R. Niwa. 2006. Neverland is an evolutionally conserved Rieske-domain protein that is essential for ecdysone synthesis and insect growth. Development 133:2565–2574.

Yudin, A.I., R.A. Diener, W.H. Clark Jr., and E.S. Chang. 1980. Mandibular gland of the blue crab *Callinectes sapidus*. Biological Bulletin 159:760–772.

Zarubin, T.P., E.S. Chang, and D.L. Mykles. 2009. Expression of recombinant eyestalk crustacean hyperglycemic hormone from the tropical land crab, *Gecarcinus lateralis*, that inhibits Y-organ ecdysteroidogenesis in vitro. Molecular Biology Reports 36:1231–1237.

Zeleny, C. 1905. Compensatory regulation. Journal of Experimental Zoology 2:1–102.

Zheng, J., C.-Y. Lee, and R.D. Watson. 2006. Molecular cloning of a putative receptor guanylyl cyclase from Y-organs of the blue crab, *Callinectes sapidus*. General and Comparative Endocrinology 146:329–336.

Zheng, J., T. Nakatsuji, R.D. Roer, and R.D. Watson. 2008. Studies of a receptor guanylyl cyclase cloned from Y-organs of the blue crab (*Callinectes sapidus*), and its possible functional link to ecdysteroidogenesis. General and Comparative Endocrinology 155:780–788.

Žitňan, D., T.G. Kingan, J. Hermesman, and M.E. Adams. 1996. Identification of ecdysis triggering hormone from an epitrachaeal endocrine system. Science 271:88–91.

Žitňan, D., Y.J. Kim, I. Žitňanova, L. Roller, and M.E. Adams. 2007. Complex steroid-peptide-receptor cascade controls insect ecdysis. General and Comparative Endocrinology 153:88–96.

Zmora, N., J. Trant, Y. Zohar, and J.S. Chung. 2009. Molt-inhibiting hormone stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab, *Callinectes sapidus* I: an ovarian stage dependent involvement. Saline Systems 5:7.

2

ENDOCRINOLOGY OF METABOLISM AND WATER BALANCE: CRUSTACEAN HYPERGLYCEMIC HORMONE

Simon G. Webster

Abstract

Fundamentally important regulatory processes in crustaceans, namely energy mobilization and ionic homeostasis, are controlled by crustacean hyperglycemic hormones (CHH), which form part of a large group of structurally related neurohormones that are also involved in control of growth and reproduction. Although it was once thought that these peptides had quite circumscribed roles, principally related to control of hemolymph glucose levels, many more fundamentally important processes are now known to be regulated by these hormones. Recent research, which is highlighted in this chapter, has shown that not only do these peptides perform multifunctional roles, often in the same species, but they are also widely distributed in neural and non-neural tissues in crustaceans. Furthermore, the discovery of related neurohormones—the ion transport peptides (ITP) in insects—point to a long evolutionary history. This is discussed in relation to gene structure and functional diversification within the Arthropoda.

INTRODUCTION

The most widely studied crustacean neurohormone is without doubt crustacean hyperglycemic hormone (CHH), first described as a "diabetogenic factor" in the eyestalks of fiddler crabs, *Uca pugilator*, which dramatically increases blood sugar levels when injected into blue crabs, *Callinectes sapidus* (Abramowitz et al. 1944). This discovery was almost coincident with anatomical studies establishing the sinus gland (SG) as a neurohemal tissue and the neural link to the X-organ (XO; Hanström 1931, 1933, Bliss 1951, Carlisle and Passano 1953). These pioneering

studies (among others) underpinned our early understanding of the eyestalk neurosecretory system as a major player in the endocrine orchestra of crustaceans. CHH is the most abundant peptide in the SG. This fortuitous feature, coupled with the ease of eyestalk extirpation, stimulated early studies concerned with species specificity and purification in particular. Reviews covering the early literature are given by Kleinholz and Keller (1973), Keller et al. (1985), and Keller and Sedlmeier (1988). In this review, recent advances in our knowledge of the structures, functions, and mode of action of CHH are reviewed, highlighting areas where further investigations are now needed.

CHH PEPTIDES, TRANSCRIPTS, AND GENES

Following the first determination by Edman microsequencing of the amino acid sequence of CHH from the SGs of *Carcinus maenas* (Kegel et al. 1989), there has been a tremendous upsurge of interest in determining the structures of CHHs—approximately 80 amino acid sequences of these hormones are now known for about 40 species of decapod crustaceans (Table 2.1), and this number is continually being added to. Additionally, after the identification of the structurally related molt-inhibiting hormone (MIH) in *C. maenas* (Webster 1991), vitellogenesis-inhibiting hormone (VIH) in *Homarus americanus* (Soyez et al. 1991), and that of a functionally defined MIH in the same species, which was structurally a CHH (Chang et al. 1990), an emerging scenario became clear: the CHH neuropeptide family of crustaceans contains many structurally related members that may have several overlapping biological activities. Furthermore, following the discovery of an ion transport peptide (ITP) in the locust *Schistocerca gregaria* corpora cardiaca, which stimulated Cl^- resorption by the ileum (Audsley et al. 1992), it became clear that this hormone was also a member of the CHH peptide family (Audsley et al. 1994, Meredith et al. 1996), thus CHH-like molecules seem to have a wide, if not universal, distribution and a long evolutionary history in arthropods. These concepts are discussed in detail later.

All CHH family members are between 72 and 83 amino acids long and possess (as an invariant structural feature) six cysteyl residues arranged in three disulphide bridges. Additionally, two arginines, one aspartate, and a phenylalanine are in identical positions. For crustaceans, two subfamilies are recognized when amino acid sequences of the mature peptides (and their precursors) are compared. The first, named the CHH subfamily or type I peptides (Lacombe et al. 1999) contain 72 or occasionally 73 amino acids and are, in crabs, crayfish, and lobsters, invariably N-terminally blocked by pyroglutamate and amidated C-terminally (for CHHs produced by the XO-SG). For the penaeid shrimps, sequences are more variable, numerous in single species, and N-termini are often unblocked (Chen et al. 2005). For CHHs determined by *in silico* database mining for the model crustacean *Daphnia pulex* (Gard et al. 2009) and by cDNA cloning in *Daphnia magna* (Montagné et al. 2010), it is notable that these are much more similar in a molecular phylogenetic context to insect ITPs than to their crustacean relatives (Montagné et al. 2010).

The type II peptides (Lacombe et al. 1999) comprise a group of hormones that include members with molt-inhibiting, vitellogenesis-inhibiting, and mandibular organ-inhibiting biological activities named the MIH/VIH group peptides. The distinctness of this hormone group (i.e., the mature peptides are slightly larger than CHHs, lack a precursor-related peptide, have unblocked N- and (commonly) C-termini, have the insertion of a Gly in the N-terminal region, show circumscribed biological activities, and lack hyperglycemic activity) is clear cut. The structures and functions of MIH/VIH peptides are discussed in detail in Chapter 1 (this volume) on molting.

Table 2.1. Crustacean hyperglycemic hormone pre/pro-peptide sequences.

Species	Tissue source	Peptide or conceptual translation	Accession No. (Protein)	Reference
Branchiopoda				
Daphnia magna	Whole animals	ITP ITP-L	ABO43963 ABO43964	Montagné and Toullec 2006 unpub.
Daphnia pulex	*In silico* data mining	ITP ITP-L		Montagné et al. 2010
Malacostraca				
Isopoda				
Armadillidium vulgare	Sinus glands	CHH	P30814	Martin et al. 1993
Eurydice pulchra	Cerebral ganglia	CHH	JF927891	Wilcockson et al. 2011 unpub.
Decapoda				
Astacus astacus	Sinus glands	CHH	P83800	Schmitz et al. 2004 unpub.
Bythograea thermydron	X-organs	CHH	AAK28329	Toullec et al. 2002
Callinectes sapidus	Eyestalk ganglia	CHH	AAS45136	Choi et al. 2006
	Thoracic ganglia	CHH-L	ABC61678	Zheng et al. 2010
	Pericardial organs	CHH-L	DQ667141	Chung and Zmora 2008
Cancer pagurus	Sinus glands	CHH	P81032	Chung et al. 1996
Cancer productus	Eyestalk ganglia	CHH-I*	ABQ41269	Hsu et al. 2008
		CHH-IIa**	ABQ41270	
		CHH-IIb**	ABQ41271	
		CHH-III*	ABQ41272	
Carcinus maenas	Sinus glands, eyestalks	CHH	P14944	Kegel et al. 1989, Weidemann et al. 1989
	Pericardial organs	CHH-L	AAG29432	Dircksen et al. 2001
Cherax destructor	Sinus glands	CHH A	P83485	Bulau et al. 2003
	Sinus glands	CHH B	P83486	
Chionoecetes bairdi	Eyestalk ganglia	CHH	ACG50068	Chung et al. 2009
Discoplax celeste	X-organs	CHH	JF894384	Turner et al. 2011 unpub.
	Pericardial organs	CHH-L	JF894385	
Galathea strigosa	X-organs	CHH	ABS01332	Montagné et al. 2008
Gecarcinus lateralis	Eyestalk ganglia	CHH	ABF48652	Lee et al. 2007
	Hindgut, testis	CHH-L	ABF58091	

(*continued*)

Table 2.1. *(Continued)*

Species	Tissue source	Peptide or conceptual translation	Accession No. (Protein)	Reference
Gecarcoidea natalis	X-organs	CHH	ABL09570	Webster et al. 2006
	Pericardial organs	CHH-L	ABL09571	unpub.
Grapsus tenuicrustatus	X-organs	CHH	JN048801	Webster et al. 2011 unpub.
Homarus americanus	Sinus glands, eyestalks	CHH A	P19806	Chang et al. 1990, De Kleijn et al. 1995
	Eyestalk ganglia	CHH B	Q25154	De Kleijn et al. 1995
Homarus gammarus	X-organs	CHH A	ABA42179	Ollivaux et al. 2006
		CHH B	ABA42180	
Jasus lalandii	Sinus glands	CHH	P56687	Marco et al. 1998
Libinia emarginata	Eyestalk ganglia	CHH (MOIH)	AAD32706	Liu et al. 1997
Litopenaeus schmitti	Sinus glands	CHH	P59685	Huberman et al. 2000
Litopenaeus vannamei	Eyestalk ganglia	CHH	CAA68067	Van Wormhoudt 1996 unpub.
	Eyestalk + gDNA	CHH (MIH 2)	AAR11295	Lago-Lestón et al. 2007
	Eyestalk +gDNA	CHH-L (MIH 1)	AAN86055	
	gDNA	CHH, ITP-L	AAN86055	Tiu et al. 2007
Macrobrachium rosenbergii	Eyestalk ganglia	CHH	AAL40915	Chen et al. 2004
	Several tissues	CHH-L	AAL40916	
Marsupenaeus japonicas	Eyestalk ganglia	CHH 1	O15980	Ohira et al. 1999
		CHH 2	Q9U5D2	unpub.
		CHH 3	BAA13481	Ohira et al. 1997
		CHH 5	O15981	Ohira et al. 1999 unpub.
	Sinus glands	CHH 6	P81700	Yang et al. 1997
	Eyestalk ganglia	CHH 7	O15982	Ohira et al. 1999 unpub.
Metapenaeus ensis	Eyestalk ganglia	CHH A	AAD45233	Gu et al. 2000
		CHH B	AAF63028	
Nephrops norvegicus	Eyestalk ganglia	CHH A*	AY285782	Mettulio et al. 2004
		CHH B*	AY285783	
Orconectes limosus	Eyestalk ganglia	CHH	Q25589	De Kleijn et al. 1994
Pachygrapsus marmoratus	X-organs	CHH	AAO27804	Toullec et al. 2006
	Pericardial organs	CHH-L	AAO27806	
Pagurus bernhardus	X-organs	CHH	ABE02191	Montagné et al. 2008

(*continued*)

Table 2.1. *(Continued)*

Species	Tissue source	Peptide or conceptual translation	Accession No. (Protein)	Reference
Penaeus monodon	Eyestalk ganglia	SGP-II	AAC84143	Davey et al. 1998 unpub.
		CHH 1	O97383	Davey et al. 2000
		CHH 4*	O97386	
		CHH 5	O97387	
		CHH*	AADO3606	Chen et al. 1988 unpub.
Pontastacus leptodactylus	Eyestalk ganglia	CHH	AAX09331	Ollivaux et al. 2004 unpub.
		CHH 1	AAS45406	Mettulio et al. 2004
Portunus trituberculatus	N/A	CHH	EU395808	Zhu et al. 2008 unpub.
Potamon ibericum	X-organs	CHH	ABA70560	Toullec et al. 2006
		CHH-L	ABA70561	
Ptychognathus pusillus	Eyestalk ganglia	CHH	JN048802	Webster et al. 2011 unpub.
Procambarus bouvieri	Sinus glands	CHH	Q10987	Aguilar et al. 1996
Procambarus clarkii	Eyestalk ganglia	CHH	AB027291	Yasuda-Kamatani and Yasuda 2000 unpub.
	Muscle, gDNA	CHH-L	AF474409	Kuepper and Jaros 2002 unpub.
Rimicaris kairei	"Dorsal eyes"/ carapace	CHH	FJ447499	Qian et al. 2009
	"Dorsal eyes"/ carapace	CHH-L	FJ447498	
Scylla olivacea	Eyestalk ganglia	CHH	AY372181	Tsai et al. 2008
	Pericardial organs	CHH-L	EF530127	

Protein accession numbers conceptually determined from various tissue sources by cDNA, gDNA cloning and sequencing, *in silico* data mining, or directly determined by microsequencing (gray highlights indicate mature peptide sequence information only). Asterisks indicate isoforms where mature peptide sequences are identical, but where precursor peptides sequences differ. Abbreviations: MOIH, mandibular organ-inhibiting hormone biological activity; MIH, molt-inhibiting hormone-like structure; ITP, ion transport peptide-like structure; SGP, sinus gland peptide CHH-L; ITP-L denotes corresponding peptides derived from alternative splicing.

A universal feature of all type I CHH prepro-hormones is the presence of a cryptic precursor peptide: CHH precursor-related peptide (CPRP). Unlike the mature CHHs, the size and sequences of CPRPs are not well conserved, ranging from four residues in *Penaeus monodon* (Davey et al. 2000) to 50 in the anomuran *Pagurus bernhardus* (Montagné et al. 2008). Several CPRPs are frequently observed; for example, seven in *Marsupenaeus japonicus* (Yang et al.

1997, Ohira et al. 1997), suggesting that there are many copies of the CHH gene, as is discussed later. Additionally, it has been elegantly shown from nano liquid chromatography-electrospray ionization-quadrupole time of flight mass spectrometry (LC-ESI-QTOF MS/MS) that four complete and 27 truncated CPRPs (including some that are methylated at the C-terminus and that are not extraction artifacts) occur in *Cancer borealis* (Fu et al. 2005). Using matrix-assisted laser desorption ionization-Fourier transform mass spectrometry (MALDI-FTMS), it has recently been shown that individual *Cancer productus* possess different inventories of CPRPs: 61% of the examined crabs express CPRP I and II; 26% CPRP I, II, and, III; and 13% CPRP I, II, and IV (Stemmler et al. 2007). However, to date, no physiological role has been determined for any CPRP. Despite stoichiometric co-release with CHH, CPRP has an extraordinarily long half-life in circulation in *Cancer pagurus*, approximately 60 min, compared with 5–10 min for CHH (Wilcockson et al. 2002), which would perhaps argue against any hormonal function. However, it seems possible that the truncated CPRPs may actually represent the final processed, biologically active peptides (Fu et al. 2005).

Recently, an exception to the rule concerning the presence of CPRP in preprocessed CHH precursors has been observed. cDNA sequences encoding CHH-like peptides that do not contain a CPRP have been found to be expressed in the wall of the spermatophore sac of *Fenneropenaeus chinensis* (Li et al. 2010). Whether these transcripts are translated to peptide and their functionality remain to be determined.

A notable feature of CHHs concerns the diversity of isoforms in individuals. In crab and crayfish SG, both N-terminally blocked and unblocked hormones are found (Chung and Webster 1996, Bulau et al. 2003, 2004). For lobsters and crayfish, further structural diversity is generated by stereo isomerization of the third amino acid, phenylalanine, which is present in L or D forms (Soyez et al. 1994, Soyez et al. 1998, Ollivaux et al. 2009). It is thought that this diversity has biological significance because hyperglycemia induced by injection of the D-Phe^{3} containing hormone in *H. americanus* is prolonged compared to that seen for the corresponding L-Phe^{3} isoform (Soyez et al. 1994), and only the D-Phe^{3} isoform exhibits MIH activity (repression of ecdysteroid synthesis) in *Procambarus clarkii* (Yasuda et al. 1994).

Production of recombinant CHHs for several species of crustaceans has highlighted the importance of the C-terminal amide because unamidated recombinant CHHs (rCHH) (whether expressed in bacteria or yeast) have somewhat limited hyperglycemic activity (Katayama et al. 2002, Trerattrakool et al. 2003), whereas correctly amidated rCHH has good biological activity, at least 10 times that of the unamidated molecule (Mosco et al. 2008, Nagai et al. 2009, Chang et al. 2010). Recombinant CHHs containing point or C-terminal deletions; Mettulio et al. (2004) showed that point mutations that disrupted correct disulphide bridge formation completely removed biological activity, as did C-terminal truncation. Similar results have been found for C-terminal mutational analysis of recombinant ITP expressed in *Drosophila* Kc1 cells and assayed (stimulation of Cl^{-} transport) in the locust ileum (Wang et al. 2000). Clearly, these results underscore the critical importance of C-terminal amidation. Indeed, the lack of hyperglycemic activity observed for the (unamidated) pericardial (PO)-CHH (Dircksen et al. 2001) or biological activity seen for the corresponding splice variant of ITP (ITP-L) in locusts (Wang et al. 2000, Phillips et al. 2001) might also reflect this phenomenon, notwithstanding the rather different C-terminal amino acid sequences in these splice variants, as alluded to later. Interestingly, an rCHH mutant that possessed a Gly^{12} residue, which would mimic MIH/VIH (type II) peptides, has very limited hyperglycemic activity (Katayama and Nagasawa 2004). Additionally, rCHHs expressed in yeast, with a free C-terminus or cMyc/polyhistidine tag, were effective at repressing ecdysteroidogenesis by the Y-organ (YO; albeit at very high doses) but were almost without discernable hyperglycemic activity (Zarubin et al. 2009).

A common feature of many CHH and ITP genes concerns alternative splicing arrangements. It was first shown in *C. maenas* that tissue-specific expression of two different CHHs occurred (Dircksen et al. 2001). The CHH mRNA expressed by XO perikarya (XO-CHH) is encoded for by exons I, II, IV, whereas that of the intrinsic multipolar neurosecretory neurons of the pericardial organ (PO-CHH) is encoded by exons I–IV. This splicing arrangement results in two peptides that differ from amino acid 41 through to the C-terminus (Fig. 2.1). This arrangement of the CHH gene (4 exons) and alternative splicing arrangements seem to be identical for the majority of decapod CHH transcripts and genes thus far identified (e.g., *Macrobrachium rosenbergii*, Chen et al. 2004; *Pachygrapsus marmoratus* and *Potamon ibericum*, Toullec et al. 2006; *Rimicaris kairei*, Qian et al. 2009). However, in the penaeids *Metapenaeus ensis* and *Litopenaeus vannamei*, it has been suggested that 3-exon CHH genes (where exon I is absent) are present (Gu et al. 2000). However, it has recently been shown that for *L. vannamei*, a conventional 4-exon gene encodes CHH, and alternative splicing mechanisms are very similar if not identical to those seen in other crustaceans (Lago-Lestón et al. 2007, Tiu et al. 2007).

Intriguingly, for all the CHH family type II (MIH/VIH) peptides, gene arrangements consist of 3 exons, yet the splice donor/acceptor site between exons II–III corresponds exactly to that seen in all other CHH molecules. This feature clearly points to a long evolutionary history, and scenarios of CHH gene evolution involving either two independent exon duplications or exon duplication and deletion, which would elegantly account for CHH gene structure and diversity across the arthropods, have been recently proposed (Montagné et al. 2010), as shown in Fig. 2.2.

Regarding copy number of CHH genes, genomic Southern blot analysis has indicated at least four CHH genes in *C. maenas* (Dircksen et al. 2001) and at least eight in *M. ensis* (Gu and Chan 1998, Gu et al. 2000). From transcript diversity, six different CHH-like cDNAs have been cloned and sequenced in *P. monodon* (Davey et al. 2000, Udomkit et al. 2000); microheterogeneity is frequently observed in cDNA clones (Dircksen et al. 2001) and can also be seen in individual crabs (Hsu et al. 2008). These results all indicate that multiple copies of CHH genes are present in crustaceans.

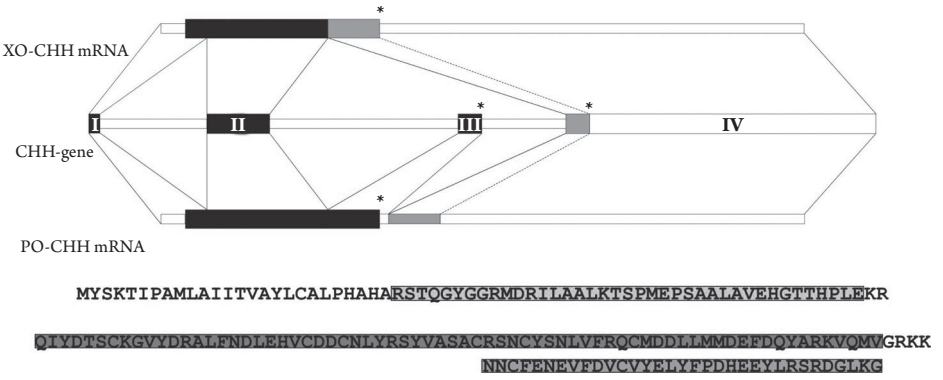


Fig. 2.1.
Schematic representation (not to scale) of crustacean hyperglycemic hormones (CHH) gene, transcript, and peptide structure, illustrated by the *Carcinus maenas* CHH prototype. X-organ (XO)-CHH encoded by exons I, II, IV is expressed by eyestalk neurons and gut paraneurons, whereas pericardial organ (PO)-CHH encoded by exons I–IV is expressed by intrinsic neurons in the POs. Asterisks show positions of stop codons. CHH precursor related peptide (CPRP) is shown outlined in green, XO-CHH in blue, the PO-CHH variant C-terminal (which differs from residues 41–72, and which is unamidated) in yellow. See color version of this figure in the centerfold. Modified from Dircksen et al. (2001), with permission from The Biochemical Society.

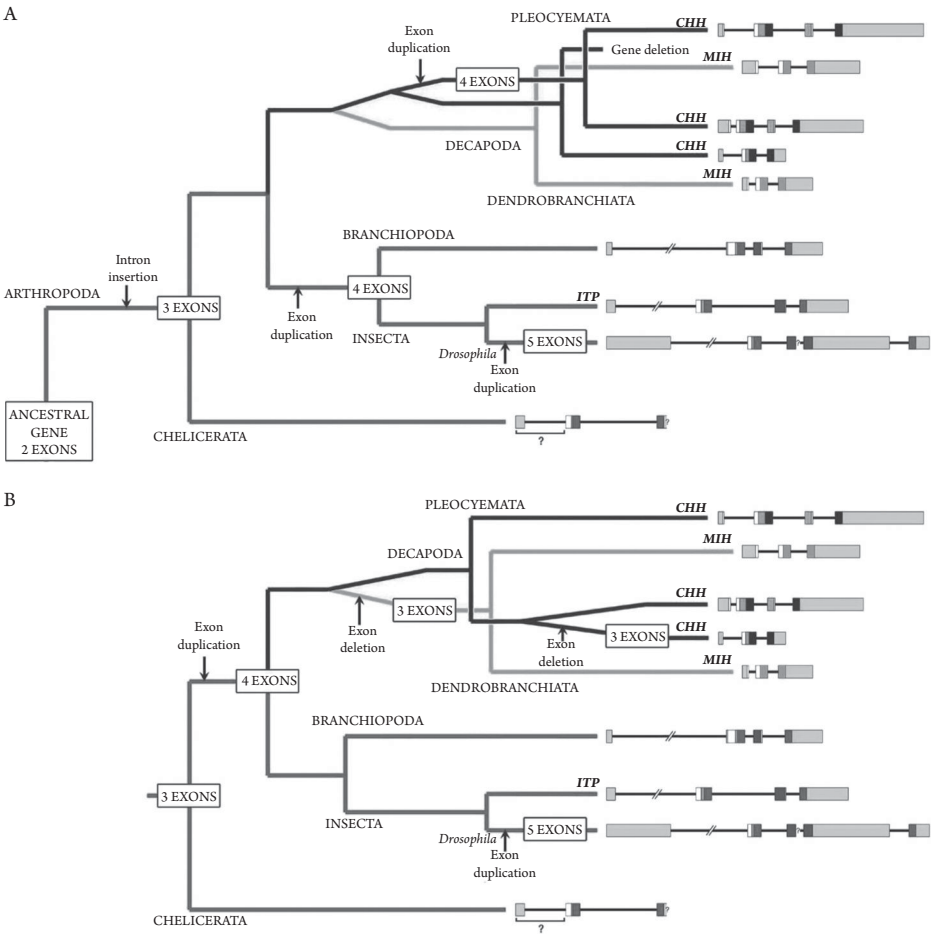


Fig. 2.2.
Evolutionary scenarios of the crustacean hyperglycemic hormones (CHH) family genes in the Arthropoda. (A) An intron insertion in an ancestral 2 exon gene (asterisk) and two independent exon duplications resulted in 4 exons for CHH, and a single-exon duplication gave 3 exon molt-inhibiting hormone (MIH) genes. (B) A single exon duplication accounts for CHH, and exon deletion for MIH gene structure. Note that a further exon duplication can account for the 5 exon ITP gene in *Drosophila.* The scaled schematics show exons represented by boxes, untranslated regions (UTR) (gray), signal peptides (yellow), precursor-related peptides (blue), ITPs (purple), CHHs (red), MIHs (green), alternatively spliced exons encoding ITP-L (violet), and CHH (orange) C-terminus regions. Question marks indicate UTRs whose precise borders are unknown. See color version of this figure in the centerfold. Figure redrawn from Montagné et al. (2010).

BIOLOGICAL ACTIVITIES OF CHH

Well over 60 years have elapsed since the defining role of CHH (and its corresponding appellation) in regulation of carbohydrate metabolism was discovered and named (Chung et al. 2010, Webster et al. 2012). However, in common with many other neurohormones, it is accepted that CHH is truly pleiotropic and influences a variety of central physiologies such as molting, osmoregulation, and reproduction, notwithstanding the influence on some of these processes by other structurally related (type II) peptides in the CHH family, as alluded to earlier.

Regulation of Carbohydrate and Lipid Metabolism

It has long been known that injection of eyestalk, SG extract, or purified CHH leads to rapid and sustained hyperglycemia in crustaceans, almost without exception, in a group- or even species-specific manner (Keller 1969, Leuven et al. 1982). This response is mediated via binding to specific, high-affinity, and saturable CHH receptors (Kummer and Keller 1993, Webster 1993) that are found in many (perhaps all) tissues, as described later. Subsequent increases in cyclic nucleotide levels (cyclic adenosine monophosphate [cAMP] and cyclic guanosine monophosphate [cGMP]) lead to activation of protein kinases and thus to phosphorylase and inhibition of glycogen synthase in a manner reminiscent to that of glucagon action (Sedlmeier and Keller 1981, Sedlmeier 1982, 1985), although, crucially, fundamental aspects such as receptor identification and second-messenger pathways remain to be properly defined. The inevitable result of this cascade is hyperglycemia, yet it is has been suggested that this phenomenon is somewhat artifactual; that is, a result of glucose leakage from the intracellular environment, following glycogen hydrolysis (Santos and Keller 1993a,b).

The physiological significance of hyperglycemia, viz. its essential role as an adaptive hormone, has only relatively recently been defined despite fundamental advances in our knowledge of CHH. Circulating levels of CHH have been determined by radioimmunoassay (RIA; Keller and Orth 1990, Webster 1996, Chung and Zmora 2008) and enzyme-linked immunosorbent assay (ELISA; Chang et al. 1998, Lorenzon et al. 2004). Recently, my laboratory has developed ultrasensitive Eu^{3+}chelate-based time-resolved fluoroimmunoassays (TR-FIA) that are 20–100-fold more sensitive than current immunoassays. These offer the exciting promise of enabling repeated measurements of hormone levels to be made in individuals, thus vividly illustrating remarkably rapid minute-by-minute changes in CHH levels, viz. *Gecarcoidea natalis* (Morris et al. 2010), *Discoplax celeste* (Turner 2010), and *C. maenas* (Webster unpublished).

Changes in CHH titer, which result in sustained increases in glucose 1–2 h after stressful episodes, have been shown repeatedly. Examples for various decapod crustaceans include emersion stress and/or hypoxia (Keller and Orth 1990, Webster 1996, Chang et al. 1998, Chung and Zmora 2008), thermal stress (Zou et al. 2003, Chung and Webster 2005), parasitism (Stentiford et al. 2001), or exposure to heavy metal pollutants (Lorenzon et al. 2004). In particular, forced exercise (by inducing continuous running behavior) seems to be a particularly effective and environmentally relevant stressor. In the Christmas Island red crab, *G. natalis*, this results in rapid hyperlactemia accompanied by immediate release of CHH, which promptly declines after termination of exercise (Morris et al. 2010). Subsequently (within an hour), the animals exhibit hyperglycemia, as summarized in Fig. 2.3. CHH levels appear to be tightly controlled by positive and negative feedback loops. Evidence for both was first provided by Santos and Keller (1993a), who demonstrated that injection of lactate or glucose increased or reduced, respectively, circulating CHH levels in *C. maenas*. Evidence for a negative feedback loop was recently shown in *G. natalis*, where glucose injection could completely inhibit exercise-induced CHH release. This was of particular significance because this negative feedback loop only occurred during the wet season, when crabs were actively migrating, and it is presumably of ecophysiological significance in maximizing energy efficiency during migration. During the dry season, when these crabs are inactive and fossorial, this feedback loop is entirely absent (Morris et al. 2010). The existence of a negative feedback mechanism has been elegantly demonstrated in vitro, in *C. borealis* (Glowik et al. 1997). Dissociated CHH neurons from the XO can be readily cultured in vitro and produce a recognizable phenotype in that they produce lamelliform (veiling) growth cones and contain sufficient CHH for measurement of the peptide content of single cells (Keller et al. 1995). Using single-cell current and voltage clamp techniques, the glucose sensitivity of these cells can be demonstrated, whereby hyperpolarization occurs at concentrations of D-glucose (EC_{50} 0.25 mM) that nicely correspond to levels recorded during hyperglycemia, thus underscoring the physiological relevance of this response (Fig. 2.4).

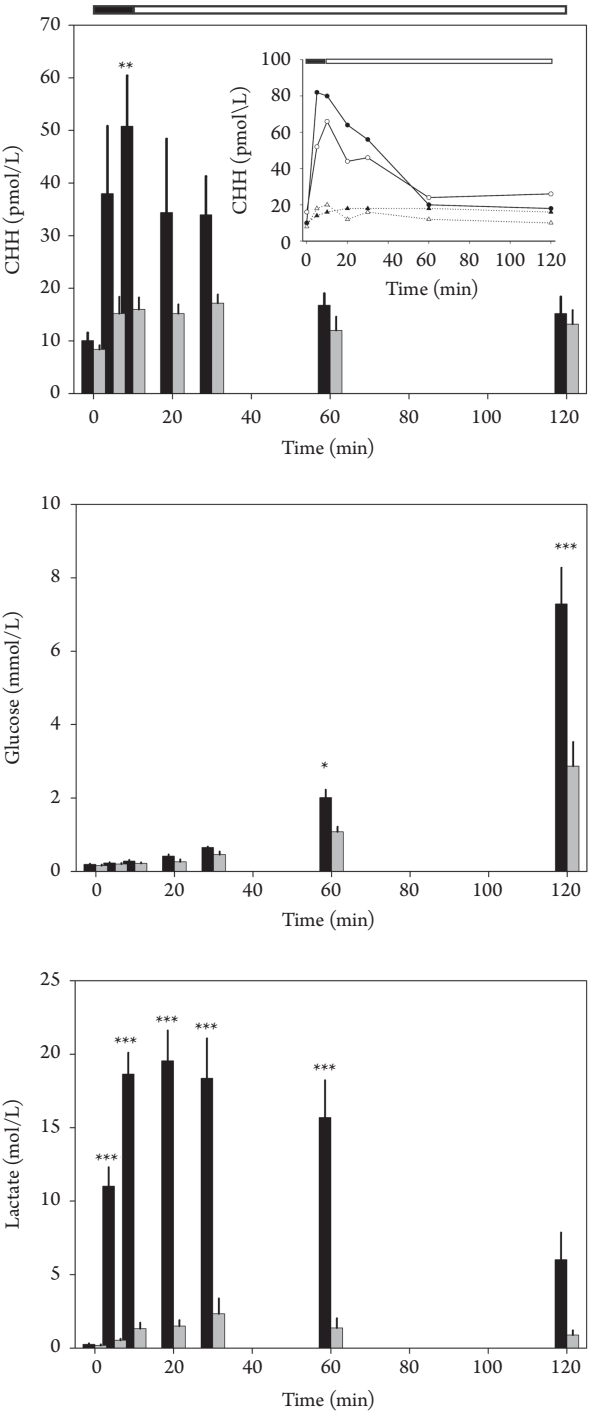


Fig. 2.3.
The effect of exercise on circulating crustacean hyperglycemic hormones (CHH), glucose, and lactate levels in the Christmas Island red crab, *Gecarcoidea natalis*. Crabs were exercised for 10 min (black bar), followed by a 110 min recovery period (white bar). Black columns, exercised animals; gray bars, controls (n = 5 for each group). Error bars= +1 SEM. Inset shows CHH profiles from two exercised (solid lines, circles) and control crabs (dotted lines, triangles). * P<0.05, ** P<0.01, *** P<0.001. From Morris et al. (2010), with permission from The Company of Biologists.

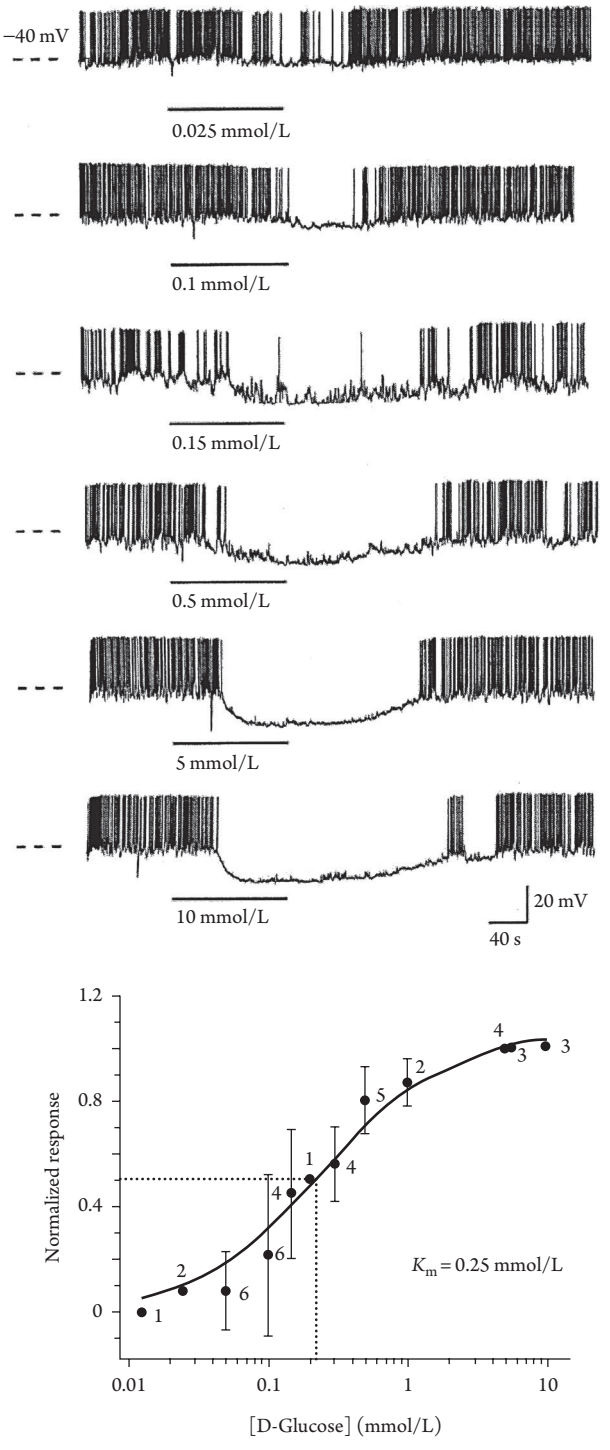


Fig. 2.4.
Glucose dependent responses of isolated crustacean hyperglycemic hormones (CHH) immunoreactive neurons of *Cancer borealis* to 2 min applications of glucose (0.025–10 mM). Graph shows dose–response curve where hyperpolarizations have been normalized to the amplitude of the response at 5 mM. Numbers of cells used are shown for each dose, curve fitted to Michaelis-Menten equation. From Glowik et al. (1997), with permission from The Company of Biologists.

Interestingly, electrical stimulation or K^+ evoked exocytosis of dissected XO-SG of *Cardisoma carnifex* showed that CHH release continued for some time (tens of minutes) after the cessation of stimulus (Keller et al. 1994).

As befits the role of CHH as an adaptive hormone, it is notable that CHH is a secretagogue and stimulates the release of amylase from isolated midgut glands of *C. maenas* and *Orconectes limosus* in a physiologically relevant, dose-dependent manner (Sedlmeier 1988). Additionally, in these species, there is evidence to suggest the involvement of CHH in elevation of free fatty acids and phospholipids in vitro and in vivo (Santos et al. 1997).

It has been proposed, generally on the basis of injection of biogenic amines, peptide neurotransmitters, and simple measurement of hemolymph glucose, that a variety of these may be involved in CHH release. For example, serotonin is well known to cause hyperglycemia in a variety of crustaceans (Lüschen et al. 1993, Lorenzon et al. 2005), and this response can be potentiated by serotonin reuptake inhibitors such as fluoxetine (Santos et al. 2001) and may be mediated via 5-HT_1-like receptors, since antagonists such as cyproheptadine blunt serotonin-induced hyperglycemia (Lorenzon et al. 2004). Nevertheless, these experiments should be interpreted with caution. In some instances, eyestalk removal abolishes serotonin-induced hyperglycemia, whereas in others this neurotransmitter seems to act independently of CHH. The same caveats apply to experiments involving administration of dopamine. This induces hyperglycemia independently of CHH, in that eyestalk-ablated animals show no hyperglycemia following injection (Lüschen et al. 1993, Komali et al. 2005), whereas other studies have shown that dopamine causes hypoglycemia in intact animals (Lorenzon et al. 2004). Interestingly, retinoic acid, which appears to be an authentic signaling molecule in crustaceans (Hopkins 2001), may mediate CHH release (as measured by hyperglycemia) in *Oziotelphusa senex senex*—but only 9-*cis* retinoic acid and not the all-*trans* isomer is active in this respect (Reddy and Sainath 2008).

Clearly, firm conclusions regarding the importance of neurotransmitters can only be reached if CHH levels are measured in conjunction with amine administration, as have been reported (Zou et al. 2003, Lorenzon et al. 2005). These have shown that 5-HT seems to directly stimulate CHH release, in contrast to dopamine. However, the elegant single-cell electrophysiological approach taken by Glowik et al. (1997) shows that 5-HT (and γ-aminobutyric acid [GABA]) hyperpolarize CHH neurons, a result clearly at odds with other observations. Interestingly, in the lobster *H. americanus*, CHH immunopositive somata at the branching point of the second thoracic nerve are in close apposition to neurosecretory terminals of serotonergic and octopaminergic neurons, and, for octopamine, nanomolar levels of bath-applied transmitter excite spontaneous bursting activity of these root neurons, whereas higher concentrations of both transmitters inhibit this activity (Basu and Kravitz 2003).

Regarding the involvement of peptide neurotransmitters such as enkephalins in CHH release, again, few firm conclusions can be drawn. On the one hand, Leu-enkephalin (L-Enk) administration has been reported to lead to hypoglycemia in *U. pugilator, C. maenas,* and *P. clarkii* (Nagabhushanam et al. 1995), Met-enkephalin injection has been reported to cause hyperglycemia in the freshwater crab *O. senex senex* (Reddy 1999). However, more critical and sophisticated recent experiments, whereby CHH release has been measured in isolated eyestalk ganglia of *O. limosus*, coupled with immunohistochemical studies, have shown that the influence of L-Enk is likely inhibitory. Because extensive L-Enk immunoreactive neuropils with dendrites are in close (possibly synaptic) apposition with arborizing dendrites of CHH immunoreactive XO perikarya, enkephalinergic modulation of CHH release (Ollivaux et al. 2002) seems possible. However, this seems likely to be indirect because the isolated CHH neuron preparations of Glowik et al. (1997) are entirely unresponsive to L-Enk. Clearly, the mechanisms of modulation of CHH by relevant neurotransmitters have yet to be satisfactorily resolved and merit further study.

Inhibition of Ecdysteroid and Methyl Farnesoate Synthesis

Clearly identifiable MIHs belonging to the type II CHH family grouping that have been functionally defined by their ability to repress ecdysteroid synthesis in vitro by YO bioassay (Soumoff and O'Connor 1982, Mattson and Spaziani 1985, Webster 1986) seem to be universal in brachyurans (Webster and Keller 1986, Chung and Webster 2003) and relatively common in astacurans (Nagasawa et al. 1996, Nakatsuji and Sonobe 2004) and some penaeid shrimps (Sefiani et al. 1996, Yang et al. 1996). However, in lobsters (Nephropidae), such MIHs have not been identified. Instead, a CHH (type I molecule) acts as a functional MIH (CHH A in *H. americanus*; Chang et al. 1990), whereas in the spiny lobster *Jasus lalandii* (Palinuridae), a distinct, biologically active MIH occurs together with CHH (Marco et al. 1998, 2000).

It is notable that CHHs also exhibit some MIH activity in the YO bioassay, but at much reduced potency (10–20 times less active) compared to MIH (Webster and Keller 1986), and it is possible that both may act synergistically (Webster 1998). However, although high-affinity saturable receptors for both CHH and MIH occur in the YO of *C. maenas* (Webster 1993) and *C. sapidus* (Chung et al. 2010), it is as yet unclear whether CHH can exert a biologically relevant effect in control of molting, in the context of synergistic interaction with MIH, since both peptides are rarely released simultaneously (Chung and Webster 2005), and both have very short half-lives in the hemolymph of between 5 and 10 min (Chung and Webster 2005, 2008). Conversely, it should also be noted that functionally defined type II MIHs have, to date, never been shown to exhibit hyperglycemic activity, again highlighting the distinctiveness of the two peptide families.

Methyl farnesoate, the unepoxidated precursor of insect juvenile hormone III, is synthesized by the mandibular organ (MO) of decapod crustaceans (Borst et al. 1987, Laufer et al. 1987a,b, Tsukimura and Borst 1992). MF synthesis is inhibited by distinct, MIH-like (type II) neuropeptides from the XO in *C. pagurus*: the mandibular organ-inhibiting hormones (MOIH-I, -II; Wainwright et al. 1996). Although similar peptides have been identified in other crabs within the genus (Webster, unpublished), in all other decapod crustaceans so far examined, CHH fulfils the role of a MOIH in that it profoundly inhibits in vitro MF synthesis by MO in *Libinia emarginata* (Liu et al. 1997) and *C. maenas* (Keller et al. 1999). Clearly, further studies are now needed; in an evolutionary context, it seems axiomatic that distinct MOIH molecules are restricted to just one genus of decapod crustaceans.

Inhibition of Ovarian Protein Synthesis and Vitellogenesis

The inhibitory influence of SG peptides on protein and/or vitellin synthesis in decapod crustaceans has long been known (Bomirski et al. 1981, Meusy and Payen 1988, Quackenbush and Keeley 1988). The hormone responsible for this process, vitellogenesis- or gonad-inhibiting hormone (VIH/GIH), first identified in lobsters *H. americanus* (Soyez et al. 1991) and subsequently in woodlice *Armadillidium vulgare* (Grève et al. 1999), is a type II CHH family member. In lobsters, GIH has no hyperglycemic activity (Soyez et al. 1987). However, by measuring semiquantitative CHH and GIH transcript (RNase protection assay) and circulating peptide (EIA) levels, it has been suggested from causality that GIH inhibits the start of vitellogenesis, CHH-A and -B stimulate its onset, and CHH-B stimulates oocyte maturation in the American lobster *H. americanus* (De Kleijn et al. 1998). Nevertheless, for the penaeid shrimps, an emerging scenario is one whereby CHHs possess relevant VIH activity, and where identifiable type II VIH/GIH peptides are noticeably absent in the SG neuropeptide inventory. For example, each of the seven CHH family SG peptides from *M. japonicus* inhibited protein and mRNA synthesis in vitellogenic ovarian explants of *Penaeus semisulcatus* (Khayat et al. 1998), yet a type II molecule (called Pej-SGP-IV), a putative MIH, was entirely without inhibitory activity (Avarre et al. 2001). Subsequently, homologous bioassay via

quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) analysis of vitellogenin (Vtg) mRNA expression in this species showed that a CHH peptide (called Pej-SGP-III) profoundly inhibited Vtg mRNA synthesis and, once again, that the MIH-like SG peptide SGP-IV was ineffective in this respect (Tsutsui et al. 2005). Clearly, further research is now timely regarding the identification of candidate VIH/GIH molecules in brachyurans and the corresponding activities of CHHs in a variety of species. Interestingly, a recent study in which Vtg mRNA levels were measured in hepatopancreas explants of *C. sapidus* has shown that MIH increases Vtg mRNA levels in mid-vitellogenesis (Zmora et al. 2009). Thus, it may be that different crustacean taxa have developed rather divergent strategies in their hormonal control of vitellogenesis, and this is a topic that deserves further study.

Osmo- and Ionoregulation

Early work concerned with the hormonal control of osmotic and ionic regulation in mainly marine crustaceans has been the subject of several comprehensive reviews (Mantel 1985, Muramoto 1988, Kamemoto 1991, Morris 2001). Notwithstanding a considerable volume of literature implicating a

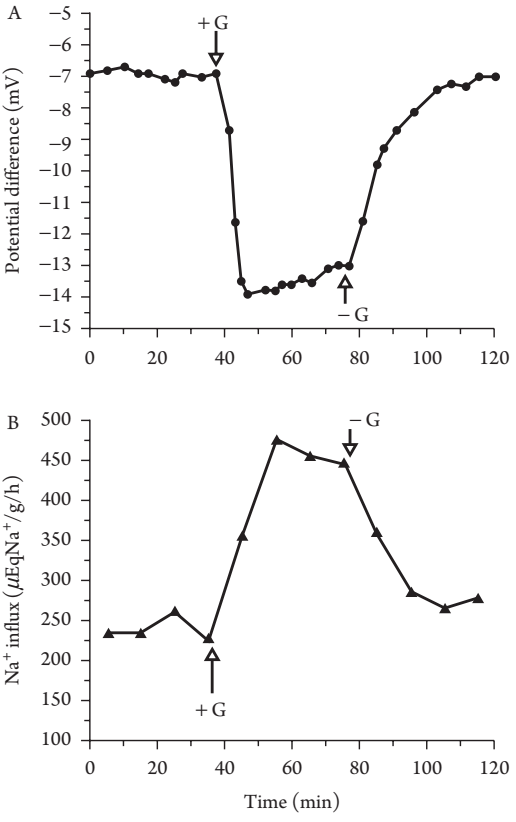


Fig. 2.5.
Perfusion of posterior gills of *Pachygrapsus marmoratus* with high-performance liquid chromatography purified sinus gland extract corresponding to crustacean hyperglycemic hormones (CHH) (four sinus gland equivalents) showing its effect on (A) transepithelial potential difference and (B) Na^+ influx. +G, −G refer to addition and removal of sinus gland extract in gill perfusate. From Spanings-Pierrot et al. (2000) with permission from Elsevier.

variety of neurotransmitters (from almost all of the nervous system) in modulation of ion transport, an emerging scenario from classical ablation/injection-type studies suggested that involvement of hormones from the eyestalk neurosecretory system was relevant in such processes (Kamemoto 1976, Charmantier et al. 1984, Freire and McNamara 1992). In particular, XO-SG peptides were implicated: SG extracts increase hemolymph osmolarity in destalked juvenile lobsters *H. americanus* that were hyperregulating in dilute seawater (Charmantier-Daures et al. 1988), and perfusion of posterior gills of the euryhaline crab *P. marmoratus* with SG extracts stimulated Na^+ influx and transepithelial potential (Pierrot et al. 1994, Eckhardt et al. 1995). Further studies on these species have unequivocally shown CHH to be the candidate peptide involved in stimulation of Na^+ uptake (Charmantier-Daures et al. 1988, Spanings-Pierrot et al. 2000), as shown in Fig. 2.5. In the crayfish *Astacus leptodactylus*, the D-Phe^3 stereoisomer of CHH has been shown to be more effective than the L-Phe^3 stereoisomer in increasing hemolymph osmolarity and Na^+ levels in the hemolymph of destalked animals (Serrano et al. 2003).

In the penaeid shrimp *L. vannamei*, the splice variant of CHH, encoded by exons I–IV and thus equivalent to PO-CHH (see earlier discussion), is widely expressed in several tissues, including the gills (Tiu et al. 2007). Levels of this transcript (quantified by Northern blotting) are highest in the posterior gills, which are considered to be of primary importance in active transport of ions (Mantel and Farmer 1983, Towle and Weihrauch 2001). Stage-specific expression was maximal during late intermolt to early premolt, and highest levels of expression were observed at a salinity of 15 ppt. dsRNA injection gave moderate knockdown of expression (37% after 48 h), and injection of large quantities (0.3 μg) resulted in gill hemorrhage and complete mortality. These results are intriguing because, as alluded to later, the equivalent of CHH in insects (ITP) is involved in chloride resorption in the rectum of orthopteroid insects (Meredith et al. 1996, Phillips et al. 1998a,b). However, it should be noted that only the short form of ITP (equivalent to XO-CHH) is biologically active in insects, and the splice variant expressed in the study by Meredith et al. (1996; ITP-L, which is equivalent to PO-CHH) seems to have no obvious biological activity.

A novel action of CHH concerns that of water uptake during ecdysis. In *C. maenas*, gut paracrine cells in the hindgut and foregut (Fig. 2.6D,G) that are associated with muscle insertions, synthesize XO-CHH only during premolt and release this as a massive but ephemeral surge during the initiation of ecdysis. This release results in a tremendous dipsogenesis, water absorption, and subsequent swelling of the crab, to allow it to reach its subsequent postmolt dimensions (Chung et al. 1999). Interestingly, this hormone surge is almost coincident with that of crustacean cardioactive peptide, which seems to be involved with stereotyped ecdysis behavior, thus allowing the crab to exit from its old exoskeleton well before swelling is complete (Phlippen et al. 2000). Additionally, in this crab, gill plasma membrane preparations show high-affinity saturable binding kinetics with ^{125}I-labeled CHH, indicative of receptor binding, and exposure of crabs to dilute seawater (6 ppt) dramatically increased levels of cGMP in gill tissues (Chung and Webster 2006).

Fig. 2.6 (*Continued*)
branching morphology. (H) CHH immunoreactive structures in the PO and adjacent areas in a *C. maenas* embryo just prior to hatching; arrows point to the PO nerve trunks. Abbreviations: ab, anterior bar; pb, posterior bar. (I) Dual-labeled section of the retina of juvenile crayfish (*Procambarus clarkii*). Red structures (Texas red) show CHH immunoreactive tapetal cells, green structures (FITC) show 5-HT immunopositive retinular cell axons. Upper cell layer shows CHH immunoreactive tapetal cells; lower layer, 5-HT immunopositive retinular cell axons. Scale bars: 50 μm (A, B, F, G, H), 100 μm (C), 200 μm (D) and insert (E), 20 μm (I), 25 μm insert (B). See color version of this figure in the centerfold. Images: (A, B) adapted from Dircksen et al. (1988), with permission from Wiley and Sons, Inc.; (C) courtesy of H. Dircksen; (D, E, F, H) adapted from Chung and Webster (2004), with permission from The Company of Biologists; (G) Webster, unpublished; (I) adapted from Escamilla-Chimal et al. (2001), with permission from The Company of Biologists.

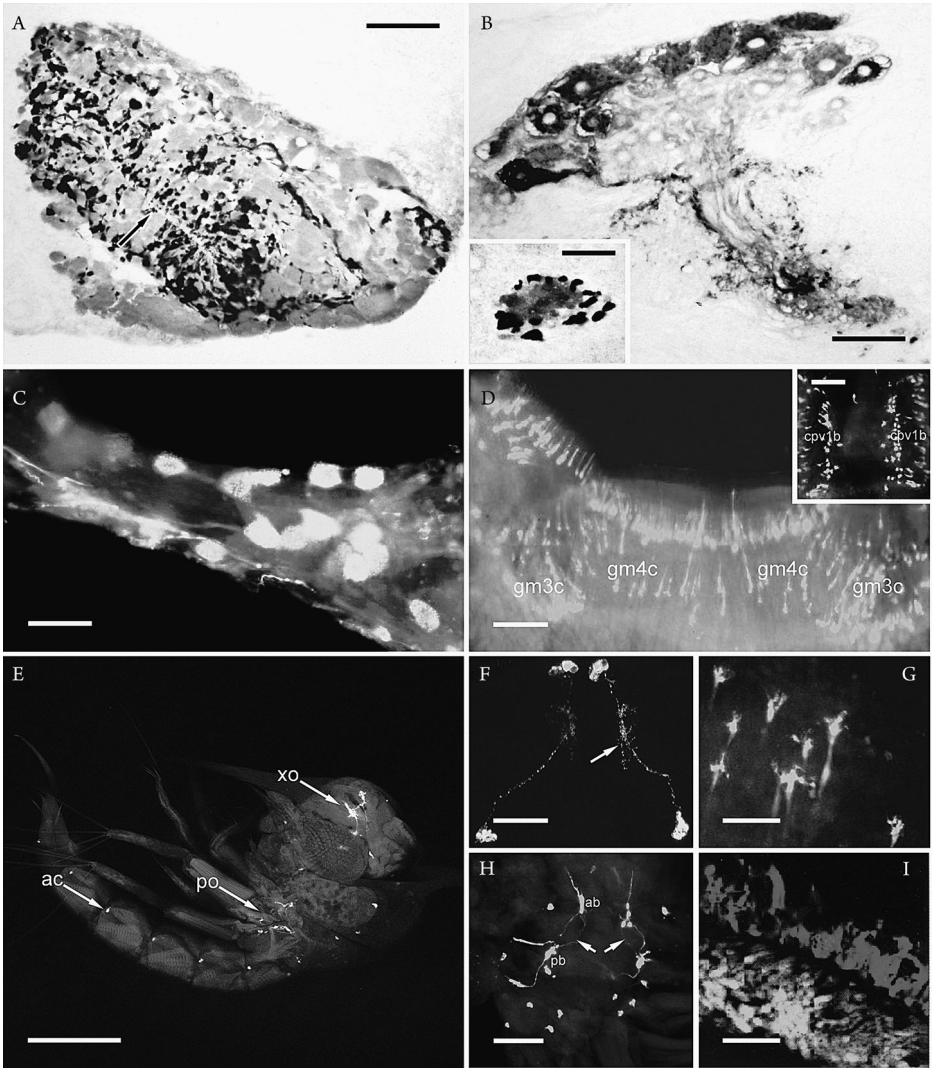


Fig. 2.6.
Immunohistochemical localization of crustacean hyperglycemic hormones (CHH) and molt-inhibiting hormone (MIH) in neural and non-neural tissues of crustaceans. (A) Sinus gland (SG) section of *Carcinus maenas* double immunostained for CHH (brown, peroxidase antiperoxidase/diamainobenzidine) and MIH (black, silver-enhanced immunogold). Arrow points to (collapsed) hemal sinus. (B) Double immunostained (as in A) section of X-organ (XO) of *C. maenas*. Inset shows transverse section of XO-SG tract. (C) Intrinsic CHH neurons in pericardial organ of *C. maenas*. (D) Endocrine cells immunoreactive (whole-mount FITC immunofluorescence) to CHH surrounding the insertions of the gastric and cardiopyloric muscles at the mesocardiac and pterocardiac ossicles of premolt (stage D_2) *C. maenas*, dorsal view. Abbreviations: gm3c, lateral posterior gastric muscles; gm4c, cardiopyloric muscles. Inset shows dorsal view of cells surrounding muscle insertions of the anterior dorsal pyloric dilators (cpv1b) at the posterior mesopyloric and anterior uropyloric ossicles. (E) Structures immunoreactive to CHH (whole-mount immunofluorescence) in a recently hatched *C. maenas* zoea larva. Arrows point to segmentally iterated abdominal cells (ac) close to the insertions of the abdominal flexor muscles, pericardial organs (po) and associated immunopositive cells, and XO-SG neurosecretory system (xo). (F) Double labeled preparation showing localization of two pairs of CHH (red, Cy3) and MIH (green, FITC) immunoreactive neurons in the brain of a *C. maenas* embryo at the mid-eye stage (70–85% development). Arrow indicates extensive arborizing dendrites. Both peptides are not colocalized, but the SG appears yellow due to stacking of the confocal image. (G) CHH immunoreactive cells (whole-mount FITC) in the hindgut of *C. maenas*, imaged from the basal (hemolymph) side, where the cells exhibit a

Clearly, all these results point to a central role of CHH in osmo- and ionoregulation involving not only regulation of the internal milieu during acclimation to diminished salinity, but also regarding one of the most all-pervading processes in crustacean life history: molting and growth. Future directions of research to elucidate the mode of action of CHH in ionoregulation must now concentrate on determining the action of the hormone on specific transport processes. A variety of these Na^+/K^+ ATPase, V-ATPase, NKCC cotransporter, K^+ and Cl^- channels have been identified, cloned, and sequenced in crustacean gills, and models for branchial ionoregulation have been proposed (Freire et al. 2008). Classical Ussing chamber-type studies, together with gene expression profiling and CHH knockdown via RNAi are potentially powerful techniques to further define the roles of CHH in the aforementioned processes.

DISTRIBUTION OF CHH IN THE NERVOUS SYSTEM AND NON-NEURAL TISSUES

Many immunohistochemical studies have localized CHH expression to the XO-SG neurosecretory system in the eyestalk of malacostracan crustaceans. Examples include Brachyura (Dircksen et al. 1988), Astacura (Soyez et al. 1998), Penaeidae (Sithigorngul et al. 2002), Palaemonidae (Sithigorngul et al. 1999), and Isopoda (Martin et al. 1984). In general, these have shown complete localization of CHH in XO perikarya and neurohemal endings in the SG (i.e., type I peptides [CHH] are synthesized in distinct perikarya) and that overlap in cellular expression with type II peptides (MIH, MOIH, VIH) rarely occurs. However, in a few instances, co-expression seems to be observed between CHH and VIH in *Homarus* spp. (De Kleijn et al. 1992, Rottlant et al. 1993) and CHH and MIH in *M. japonicus* (Shih et al. 1998). Fig. 2.6A,B,F shows representative photomicrographs of CHH and MIH immunopositive structures in the XO-SG of *C. maenas*.

For neuropeptides, in which structural diversity due to stereo inversion occurs (Phe^3 for CHH, Trp^4 for VIH) in *Homarus gammarus*, antisera generated against N-terminal sequences that contain the L and D aminoacyl residues of these stereo isomers reveal fascinating and unexpected complexity in expression patterns (Ollivaux et al. 2009). In total, five populations of cells can be recognized (Fig. 2.7), including one that variably co-expresses D isoforms of both CHH and VIH, thus neatly accounting for previously observed co-expression patterns and also raising important questions regarding the physiological significance of this phenomenon with respect to co-release of mixtures of neuropeptides that influence quite disparate physiological processes.

Development of CHH neurosecretory systems during embryogenesis has been studied in *C. maenas* (Chung and Webster 2004). CHH mRNA expression first occurs at about 50% of embryonic development (eye anlagen stage) and subsequently increases dramatically. Whole-mount immunohistochemistry revealed the presence of two pairs of CHH (and MIH) cells in the XO, with projecting axons to the SG (Fig. 2.6F). During larval life, MIH cell number remains invariant (i.e., four cells; Webster and Dircksen 1991), but it is not yet known when further development/recruitment of CHH (or MIH) XO neurons occurs to increase the number to the adult complement of 62–65 in each eyestalk (Dircksen et al. 1988).

Although it was once considered that the XO-SG was the only site of CHH synthesis, it is now known that CHH is expressed in a wide variety of neural and non-neural tissues. More than 25 years ago, CHH was detected by RIA in the PO of *C. maenas* (Keller et al. 1985) and subsequently by EIA in ventral nerve cords of *H. americanus* (Chang et al. 1999). The source of this material has been shown to be from intrinsic multipolar neurons in the PO of *C. maenas* (Dircksen and Heyn 1998) and *C. sapidus* (Chung and Zmora 2008) and in the so-called second thoracic roots of the ventral nerve cord of *H. americanus* (Chang et al. 1999; see Fig. 2.6C). In *C. maenas* embryos, CHH immunopositive neurons are observed in the thoracic ganglion (Fig. 2.6H), which projects axons

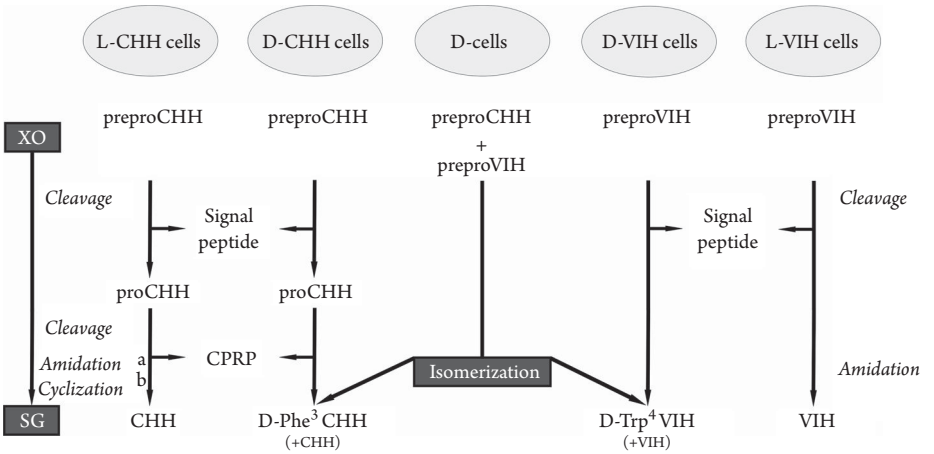


Fig. 2.7.
Diagrammatic representation of cell type specific precursor processing of crustacean hyperglycemic hormones (CHH) and vitellogenesis-inhibiting hormone (VIH) isomers in the X-organ-sinus gland complex. CPRP, CHH precursor-related peptide. Amidation (a) can occur before, during, or after cleavage of CPRP. Cyclization of glutamate to pyroglutamate (b) can only occur after CPRP cleavage. Some cells secrete L-CHH, L-VIH, whereas D-CHH, D-VIH cells, while producing mainly the D-isomers, also secrete variable amounts of L-isomers. However, a small population of cells exclusively secrete the D-isomers of both hormones. See color version of this figure in the centerfold. Adapted from Ollivaux et al. (2009), with permission from John Wiley and Sons, Inc.

along the segmental nerves (Chung and Webster 2004). For crabs, it is known that intrinsic neurons of the PO express the splice variant of CHH encoded by exons I–IV (Dircksen et al. 2001, Chung and Zmora 2008). Other tissues associated with neural structures now known to express CHH include the tapetal cells of the retina in the crayfish *P. clarkii* (Escamilla-Chimal et al. 2001; see Fig. 2.6I). Intriguingly, these cells, together with adjacent retinular cells that express serotonin, undergo daily rhythms of immunoreactive content, as do cells in the XO. This is very reminiscent of observations showing circadian rhythmicity of CHH synthesis in the XO and release from the SG at the beginning of the scotophase and subsequent nocturnal hyperglycemia in *A. leptodactylus* (Gorgels-Kallen and Voorter 1985, Kallen and Abrahamse 1989, Kallen et al. 1990). It has been suggested that modulation of CHH secretion dynamics by release of serotonin from adjacent retinular cells (Escamilla-Chimal et al. 2002) may affect circadian changes in the sensitivity of the eye. These interesting results now need corroboration in other decapod models.

Expression of CHH in non-neural tissues now seems to be a widespread phenomenon in decapod crustaceans. In *C. maenas*, endocrine cells (paraneurons) in the fore- and hindgut express an XO-CHH during premolt, as described earlier (Chung et al. 1999). Detailed analysis of the distribution of these cells demonstrated that they surround the muscle insertions of all extrinsic and intrinsic muscles of the gastric and pyloric stomach, suggesting a mechanoreceptive function. In the hindgut, they are present over the entire longitudinal musculature (Webster et al. 2000; see Fig. 2.6D,G). During ecdysis, complete exocytosis of CHH from these cells occurs, although it is not known whether this is associated with apoptosis. Although correlates have not been observed in embryonic guts, serially iterated peripheral CHH cells are found at the insertions of abdominal flexor muscles during the final stages of embryogenesis in *C. maenas* (Chung and Webster 2004; see Fig. 2.6E). Because the expression and release patterns of these cells are associated with hatching of the prezoea larva, which involves significant water uptake to rupture the eggshell, it is possible that these cells are the functional equivalent of gut paraneurons in the adult.

Transcripts that encode the exon I–IV PO-CHH seem to be present in non-neural tissues including heart, gills, and antennal gland of *M. rosenbergii* (Chen et al. 2004) and epidermis, gill, and gut of *L. vannamei* (Tiu et al. 2007), but, so far, there is no evidence to suggest that these are translated. In a recent study, two rather unusual CHH encoding transcripts, transcribed from three exons that uniquely do not code for a precursor containing a precursor-related peptide (CPRP; see the section "CHH Peptides, Transcripts, and Genes"), were identified in an androgenic gland suppression subtractive hybridization (SSH) library of *F. chinensis* (Li et al. 2010). In situ hybridization studies showed that mRNAs encoding these peptides were restricted to the epithelial cells of the internal wall of the spermatophore sac. This unusual and interesting finding now needs further investigation

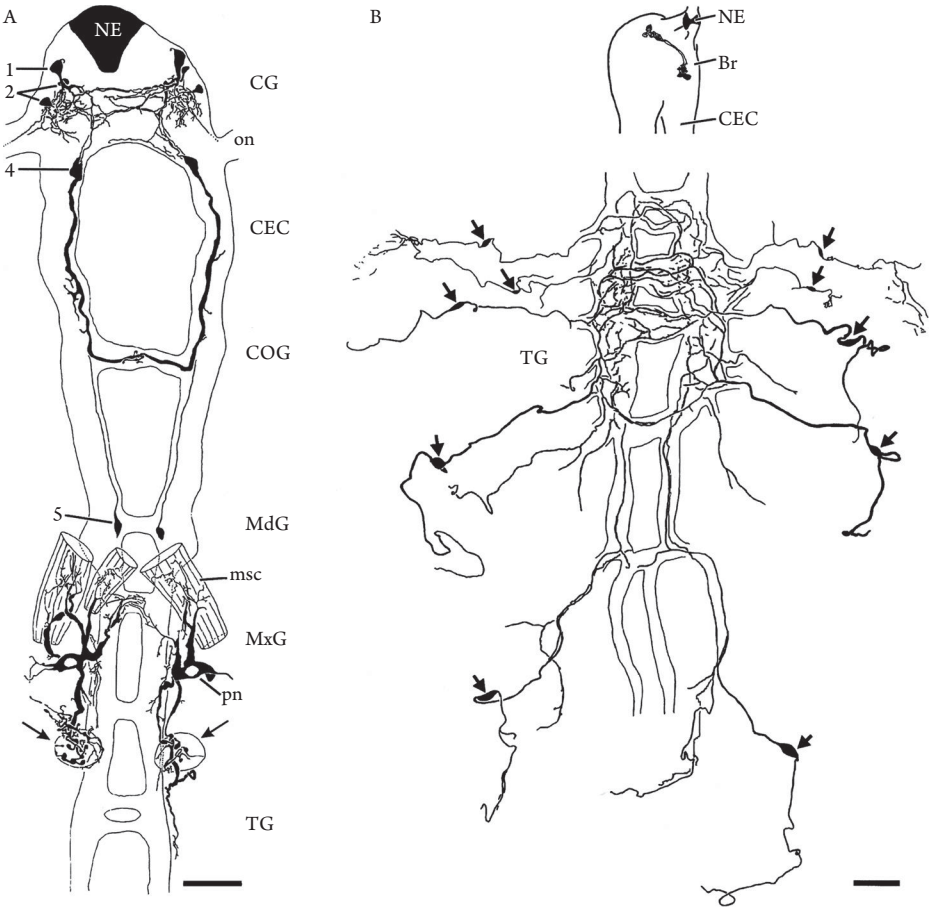


Fig. 2.8.
Camera lucida drawing of CHH immunoreactive neurons in branchiopods. (A) *Artemia salina*. Type 1–4 cells were seen in the brain (type 3 cells not visible in this preparation). Type 5 cells were found in the mandibular ganglion. A strongly stained giant multipolar peripheral neuron (pn) is located on each side of the maxillary segment that innervates unidentified muscles (msc) and bulbous tissues (arrows) in the maxillary segment. (B) *Daphnia magna*. Upper figure shows brain with crustacean hyperglycemic hormones (CHH) immunoreactive neurons. Lower figure shows serially homologous perikarya of bipolar neurons (arrows) within the peripheral nerves of the ventral body. Abbreviations: Br, brain; CEC, circumesophageal connective; CG, cerebral ganglion; COG, connective ganglion; MdG, mandibular ganglion, MxG, maxillary ganglion; NE, nauplius eye; on, optic nerve; TG, thoracic ganglion. Scale bars: 50 μm. Adapted from Zhang et al. (1997), with permission from Springer.

to see whether this is a common occurrence in male decapod crustaceans and to determine tissue distribution and abundance of translated peptide.

Regarding the distribution of CHH-like peptides in nervous systems of nonmalacostracan crustaceans, little is as yet known. The patterns of distribution of CHH immunoreactive neurons have been determined in the branchiopods *D. magna* and *Artemia salina* (Zhang et al. 1997), which are shown as camera lucida reconstructions in Fig. 2.8. Distributions of CHH-ir neurons are rather obviously different from those in the Malacostraca because none is an obvious homologue to those seen in the XO-SG neurosecretory system; of particular interest are the peripheral neurons that project centrally to the ventral nerve cord and to appendage muscles and maxillary somatic muscles. They may be comparable to multipolar cells in the PO of malacostracans, and it has been suggested that they are sensory as well as neurosecretory neurons. *In silico* analysis of the transcriptome of *D. pulex* (Gard et al. 2009) suggests that the CHH-like peptides of *D. pulex* bear a somewhat higher sequence identity to ITP than CHH (see the section "CHH Peptides, Transcripts, and Genes").

CHH-LIKE HORMONES IN OTHER ARTHROPODS: ITP

One of the many physiological adaptations that have been central to the evolutionary success of the insects concerns the homeostatic mechanisms involved in excretion. For insects that live in arid environments, water conservation is of particular importance. More than 30 years ago, using small Ussing chambers to measure short-circuit current (I_{sc}) across ileal preparations of the locust *S. gregaria*, Audsley and Phillips (1990) demonstrated that three factors in the brain, nervous lobe of the corpora cardiaca (NCC), and ventral nerve cord (VNC) stimulated ileal I_{sc} in a dose-dependent manner. Fluid transport across the ileum (Jv) was dependent on Cl^- (Lechleitner et al. 1989a,b) and likewise stimulated by similar nervous system extracts. High-performance liquid chromatography (HPLC) analysis of the bioactive compounds revealed one from the NCC that stimulated ileal Cl^- transport; thus the ITP was partially sequenced (Audsley et al. 1992, 1994), showing it to be very similar to CHH. cDNA library screening identified a full-length sequence of ITP (Meredith et al. 1996), firmly establishing this peptide as a homologous member of the CHH family (see the section "CHH Peptides, Transcripts, and Genes"). As in crustaceans, ITPs from other insects showed a high degree of group or even species specificity in their biological activities, and *C. maenas* CHH was entirely inactive in the ileal bioassay (Meredith et al. 1996). Short and long isoforms of *S. gregaria* ITP (SchgrITP, SchgrITPL) were identified by Meredith et al. (1996) in that species and also in *Locusta migratoria* (Macins et al. 1999). Following the determination of alternative splicing of pre-mRNAs for *C. maenas* CHH (Dircksen et al. 2001), it became obvious that a similar situation occurred in locusts. The structure of *itp* genes and their transcripts are now known for the lepidopterans *Manduca sexta* and *Bombyx mori* (Dai et al. 2007, Drexler et al. 2007) and for the dipterans *Aedes aegypti* and *Drosophila melanogaster* (Dai et al. 2007, Dircksen et al. 2008). Gene, transcript, and peptide structures of CHHs and ITPs have been discussed in more detail (see the section "CHH Peptides, Transcripts, and Genes"), but it is of relevance to note here that the *C. maenas* CHH gene contains 4 exons, *M. sexta* 3, and *D. melanogaster* 5 exons. To date, the biochemical identity (i.e., identification of mature processed peptide) of ITPs has only been unequivocally demonstrated in locusts and fruitflies (Audsley et al. 1992, 2006, Dircksen et al. 2008). In a situation analogous to that of crustaceans, where PO-CHH has no identifiable biological activities to date, there is little information regarding the biological activities or function of the long isoforms of ITP. Certainly, they are inactive in the ileal bioassay, thus leading to the hypothesis that the long isoforms of ITP may antagonize the action of ITP by competing with receptor binding (Phillips et al. 1998b).

Distribution of ITP immunoreactive neurons in the CNS of insects obviously shows that prominent groups of neurosecretory cells in the pars lateralis project axons to the retrocerebral complex,

corpora cardiaca, and corpora allata (where release occurs). Additionally, there are also complex arrangements of ITP-ir interneurons in the brain and subesophageal, thoracic, and abdominal ganglia; in efferent neurons in the abdominal ganglia; and in neurosecretory neurons associated with the neurohemal organs of the peripheral nervous system. For a comprehensive review of the neuroanatomy of ITP immunoreactive structures in insect nervous systems, excellent detailed accounts are given in a recent review (Dircksen 2009), and specific descriptions are available for *M. sexta* (Dai et al. 2007) and *D. melanogaster* (Dircksen et al. 2008). Interestingly, in the flour beetle *Tribolium castaneum*, large numbers of ITP-expressing paraneurons were detected by in situ hybridization in the late larval midgut epithelium (Begum et al. 2009), a situation that is strikingly similar to that seen for CHH in fore- and hindgut tissues of premolt *C. maenas* (Chung et al. 1999).

Regarding other arthropods, CHH-like immunoreactive structures have been observed in neurohemal organs of a myriapod (centipede; *Lithobius forficatus*, Laverdure et al. 1994) and an arachnid (scorpion; *Euscorpius carpathicus*, Stockmann et al. 1997), and *in silico* analysis of expressed sequence tags has shown that transcripts encoding CHH/ITP-like peptides are found in the ixodid ticks *Ixodes scapularis* (Christie 2008), *Amblyomma variegatum*, the mites *Tetranychus urticae* and *Suidasia medanensis*, and the scorpion *Mesobuthus gibbosus* (Christie et al. 2011). Thus, it seems very likely that CHH/ITP-like peptides are widely, perhaps universally, distributed in arthropods.

TARGET TISSUES, BINDING SITES, AND SECOND-MESSENGERS

A long-held view regarding the tissues involved in CHH action was that the midgut gland and abdominal muscle were key targets for CHH action because injection of SG extracts or purified CHH results in increases in cyclic nucleotides followed by a net decrease in glycogen post-hyperglycemia in these tissues (Keller and Sedlmeier 1988). Traditional ^{125}I-CHH based radiolabeled binding experiments have shown that high-affinity, saturable binding is exhibited by plasma membrane preparations of *O. limosus* and *C. maenas* midgut glands (Kummer and Keller 1993) and that, in the latter species, YO membrane preparations showed similar binding characteristics to CHH, together with clearly separate MIH binding activity (Webster 1993). Subsequently, it has been demonstrated in *C. maenas* and *C. sapidus* that a number of other tissue membrane preparations, including those involved in ionoregulation (gills, hindgut) and respiration (heart, scaphognathite) exhibit similar binding characteristics to those of midgut gland and muscle with binding affinities (K_D) 1.2–13 $\times 10^{-10}$ M, maximum number of binding sites (B_{MAX}) 1-5 $\times 10^{-10}$ M/mg protein (Chung and Webster 2006, Katayama and Chung 2009). However, it is not yet known whether these receptors are involved in particular physiological processes, such as ionoregulation, or if their presence is merely correlated with tissue-specific energy homeostasis in highly metabolically active tissues, such as the gills.

Regarding the type of hormone receptor and second-messengers involved, there is some uncertainty. At present, the balance of evidence seems to favor signaling via cGMP. Goy (1990) demonstrated the presence of a membrane-bound guanylate cyclase (MGC) and increases in cGMP, following administration of purified CHH in *H. americanus* tissues. A variety of tissues respond to incubation in CHH (20 nM) with increases of up to 10-fold in cGMP levels within 30 min of hormone administration; incubation with membrane-permeant cGMP analogues such as 8-bromo cGMP increases tissue glucose in *C. maenas* (Chung and Webster 2006). However, for ITP, it seems likely that the transport-stimulating effects of *S. gregaria* ITP in the hindgut are mediated by cAMP—that is, via a G-protein-coupled receptor (GPCR; Phillips and Audsley 1995, Phillips et al. 1998b, Lechleitner et al. 1989a). It is relevant here to mention the proposed signaling pathway of MIH receptors (see Chapter 1 in this volume). The most attractive current model for MIH signaling, based on an elegant fusion of earlier research (review by Chang and Mykles 2011) proposes that

the MIH receptor is a GPCR. Increases in cAMP caused by activation of adenylate cyclase activates protein kinase A (PKA), opening Ca^{2+} channels; increased intracellular Ca^{2+} binds to calmodulin, activating NO synthase (NOS) directly and indirectly via calcineurin. The production of NO activates a NO-sensitive guanylyl cyclase and thus stimulates cGMP production, activating a protein kinase G that, presumably, via numerous phosphorylation steps leads to repression of ecdysteroid synthesis via chronic (translational) and acute (transcriptional) control mechanisms. However, given that MIH most likely evolved from a CHH-like molecule, it seems axiomatic that such closely related peptides might have disparate receptor types and signaling mechanisms.

FUTURE DIRECTIONS

Rapid progress in identification of members of the hyperglycemic hormone family and appreciation that they are not only pleiotropic but of widespread, if not universal occurrence in the arthropods have perhaps been the highlights of crustacean neuroendocrinology over the past 20 years. However, the multiplicity of CHHs in single species, as exemplified by the situation in penaeid shrimps, where there is much confusion regarding the biologically relevant roles of a plethora of very similar CHH-like molecules, begs questions as to their biologically relevant functions. Further research, using either native peptides or (fully) biologically active recombinant molecules is now needed. This is particularly pertinent when ionoregulatory roles are considered. At present, rather more is known about the physiology of ITP-mediated ion and water transport across insect hindguts than the equivalent processes controlled by CHH in crustaceans. Similarly, the recent findings concerning the widespread expression of mRNAs encoding the CHH-L splice variant need urgent attention. Apart from the existence of CHH-L peptide in the intrinsic cells of the POs of crustaceans, it is not known whether tissue-specific expression of mRNA is related to translation of peptide or, indeed, the function of these peptides in many non-neural tissues. A significant gap in our knowledge concerns the nature of the receptors and second-messenger signaling pathways of CHH peptides. Although notable progress has recently been made in identifying signaling pathways for MIH (see Chapter 1 in this volume), almost nothing is known about these processes for CHH. Similarly, for both hormones, receptors remain orphans despite the rapid advances in insect molecular endocrinology, whereby a plethora of neuropeptide receptors are known! Clearly, a contemporary approach to identification of the cognate CHH family receptors will be very informative, particularly regarding identification of new target tissues, and would of course be invaluable in identifying biologically relevant roles for these peptides.

CONCLUSIONS

This chapter has reviewed our current understanding of the nature, sites of synthesis, and biological actions of CHH, with particular emphasis placed on demonstrating the emerging scenario of multifunctionality of these neurohormones in the subphylum. Recent molecular studies have vividly shown the evolutionary history and diversification of both structure and function of hyperglycemic hormones, which have reiterated earlier observations suggesting that a wide range of functions relating to regulation of a variety of quite fundamental physiologies were directly attributable to these hormones.

ACKNOWLEDGMENTS

Research performed in my laboratory has been funded by the Biotechnology and Biological Sciences Research Council (BBSRC).

REFERENCES

Abramowitz, A.A., F.L. Hisaw, and D.N. Papandrea. 1944. The occurrence of a diabetogenic factor in the eyestalk of crustaceans. Biological Bulletin 86:1–5.

Aguilar, M.B., R. Falchetto, J. Shabanowitz, D.F. Hunt, and A. Huberman. 1996. Complete primary structure of the molt-inhibiting hormone (MIH) of the Mexican crayfish *Procambarus bouvieri* (Ortmann). Peptides 17:367–374.

Audsley, N., and J.E. Phillips. 1990. Stimulants of ileal salt transport in neuroendocrine system of the desert locust. General and Comparative Endocrinology 80:127–137.

Audsley, N., C. McIntosh, and J.E. Phillips. 1992. Isolation of a neuropeptide from locust corpus cardiacum which influences ileal transport. Journal of Experimental Biology 173:261–274.

Audsley, N., C. McIntosh, J.E. Phillips, D.A. Schooley, and G.M. Coast. 1994. Neuropeptide regulation of ion and fluid reabsorption in the insect excretory system. Pages 74–80 *in* K.G. Davey, R.E. Peter, and S.S. Tobe, editors. Perspectives of comparative endocrinology. National Research Council of Canada, Ottawa, Canada.

Audsley, N., J. Meredith, and J.E. Phillips. 2006. Haemolymph levels of *Schistocerca gregaria* ion transport peptide and ion transport-like peptide. Physiological Entomology 31:154–163.

Avarre, J.-C., M. Khayat, R. Michelis, H. Nagasawa, A. Tietz, and E. Lubzens. 2001. Inhibition of *de novo* synthesis of a jelly layer precursor protein by crustacean hyperglycemic hormone family peptides and posttranscriptional regulation by sinus gland extracts in *Penaeus semisulcatus* ovaries. General and Comparative Endocrinology 124:257–268.

Basu, A.C., and E.A. Kravitz. 2003. Morphology and monoaminergic modulation of crustacean hyperglycaemic hormone-like immunoreactive neurons in the lobster nervous system. Journal of Neurobiology 32:253–263.

Begum, K., B. Li, R.W. Beeman, and Y. Park. 2009. Functions of ion transport peptide and ion-transport peptide-like in the red flour beetle *Tribolium castaneum*. Insect Biochemistry and Molecular Biology 39:717–725.

Bliss, D.E. 1951. Metabolic effects of sinus gland or eyestalk removal in the land crab *Gecarcinus lateralis.* Anatomical Record 111:502–503.

Bomirski, A., M. Arenarczyk, E. Kawińska, and L.H. Kleinholz. 1981. Partial characterization of crustacean gonad-inhibiting hormone. International Journal of Invertebrate Reproduction 3:213–219.

Borst, D.W., H. Laufer, M. Landau, E.S. Chang, W.A. Hertz, F.C. Baker, and D.A. Schooley. 1987. Methyl farnesoate and its role in crustacean reproduction and development. Insect Biochemistry 17:1123–127.

Bulau, P., I. Meisen, B. Reichwein-Roderburg, J. Peter-Katalinić, and R. Keller. 2003. Two genetic variants of the crustacean hyperglycemic hormone (CHH) from the Australian crayfish, *Cherax destructor*: detection of chiral isoforms due to posttranslational modification. Peptides 24:1871–1879.

Bulau, P., I. Meisen, T. Schmitz, R. Keller, and J. Peter-Katalinić. 2004. Identification of neuropeptides from the sinus gland of the crayfish *Orconectes limosus* using nanoscale on-line liquid chromatography tandem mass spectrometry. Molecular and Cellular Proteomics 3:558–564.

Carlisle, D.B., and L.M. Passano. 1953. The X-organ of Crustacea. Nature 171:1070–1071.

Chang, C.C., K.W. Tsai, N.W. Hsiao, C.-Y. Cheng, C.-L. Lin, R.D. Watson, and C.-Y. Lee. 2010. Structural and functional comparisons and production of recombinant crustacean hyperglycaemic hormone (CHH) and CHH-like peptides from the mud crab *Scylla olivacea.* General and Comparative Endocrinology 167:68–76.

Chang, E.S., and D.L. Mykles. 2011. Regulation of crustacean molting: a review and our perspectives. General and Comparative Endocrinology 172:323–330.

Chang, E.S., G.D. Prestwich, and M.J. Bruce. 1990. Amino acid sequence of a peptide with both molt-inhibiting and hyperglycemic activities in the lobster, *Homarus americanus.* Biochemical and Biophysical Research Communications 171:818–826.

Chang, E.S., R. Keller, and S.A. Chang. 1998. Quantification of crustacean hyperglycaemic hormone by ELISA in hemolymph of the lobster, *Homarus americanus*, following various stresses. General and Comparative Endocrinology 111:359–366.

Chang, E.S., S.A. Chang, B.S. Beltz, and E.A. Kravitz. 1999. Crustacean hyperglycaemic hormone in the lobster nervous system: localization and release from cells in the suboesophageal ganglion and thoracic second roots. Journal of Comparative Neurology 414:50–56.

Charmantier, G., M. Charmantier-Daures, and D.E. Aiken. 1984. Neuroendocrine control of hydromineral regulation in the American lobster *Homarus americanus* H. Milne-Edwards, 1837 (Crustacea Decapoda). 1-Juveniles. General and Comparative Endocrinology 54:8–19.

Charmantier-Daures, M., G. Charmantier, J.E. Van Deijnen, F. Van Herp, P. Thuet, J.-P. Trilles, and D.E. Aiken. 1988. Isolement d'un facteur pédonculaire intervenant dans le contrôle neuroendocrine du métabolisme hydrominérale de *Homarus americanus* (Crustacea, Decapoda). Premiers résultats. Comptes Rendus de L'Académie des Sciences 307:439–444.

Chen, S.H., C.Y. Lin, and C.M. Kuo. 2004. Cloning of two crustacean hyperglycemic hormone isoforms in freshwater giant prawn (*Macrobrachium rosenbergii*): evidence of alternative splicing. Marine Biotechnology 6:83–94.

Chen, S.H., C.Y. Lin, and C.M. Kuo. 2005. *In silico* analysis of crustacean hyperglycemic hormone family. Marine Biotechnology 7:193–206.

Choi, C.Y., J. Zheng, and R.D. Watson. 2006. Molecular cloning of a cDNA encoding a crustacean hyperglycemic hormone from eyestalk ganglia of the blue crab, *Callinectes sapidus.* General and Comparative Endocrinology 148:383–387.

Christie, A.E. 2008. Neuropeptide discovery in Ixodoidea: an *in silico* investigation using publicly accessible expressed sequence tags. General and Comparative Endocrinology 157:174–185.

Christie, A.E., D.H. Nolan, P. Ohno, N. Hartline, and P. Lenz. 2011. Identification of chelicerate neuropeptides using bioinformatics of publicly accessible expressed sequence tags. General and Comparative Endocrinology 170:144–155.

Chung J.S., and S.G. Webster. 1996. Does the N-terminal pyroglutamate residue have any physiological significance for crab hyperglycaemic neuropeptides? European Journal of Biochemistry 240:358–364.

Chung J.S., and S.G. Webster. 2003. Moult cycle-related changes in biological activity of moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) in the crab, *Carcinus maenas*. From target to transcript. European Journal of Biochemistry 270:3280–3288.

Chung, J.S., and S.G. Webster. 2004. Expression and release patterns of neuropeptides during embryonic development and hatching of the green shore crab, *Carcinus maenas.* Development 131:4751–4761.

Chung J.S., and S.G. Webster. 2005. Dynamics of in vivo release of molt-inhibiting hormone and crustacean hyperglycaemic hormone (CHH) in the shore crab, *Carcinus maenas.* Endocrinology 146:5545–5551.

Chung, J.S., and S.G. Webster. 2006. Binding sites of crustacean hyperglycaemic hormone and its second messengers on gills and hindgut of the green shore crab, *Carcinus maenas*: a possible osmoregulatory role. General and Comparative Endocrinology 147:206–213.

Chung, J.S., and S.G. Webster. 2008. Angiotensin-converting enzyme (ACE) like activity in crab gills and its putative role in degradation of crustacean hyperglycaemic hormone (CHH). Archives of Insect Biochemistry and Physiology 68:171–180.

Chung, J.S., M.C. Wilkinson, and S.G. Webster. 1996. Determination of the amino acid sequence of the moult-inhibiting hormone from the edible crab, *Cancer pagurus.* Neuropeptides 30:21–33.

Chung, J.S., and N. Zmora. 2008. Functional studies of crustacean hyperglycaemic hormones (CHHs) of the blue crab, *Callinectes sapidus*: the expression and release of CHH in eyestalk and pericardial organ in response to environmental stress. FEBS Journal 275:693–704.

Chung, J.S., H. Dircksen, and S.G. Webster. 1999. A remarkable, precisely timed release of hyperglycaemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas.* Proceedings of the National Academy of Sciences of the USA 96:13103–13107.

Chung, J.S., S. Bembe, S., S. Tamone, E. Andrews, and H. Thomas. 2009. Molecular cloning of the crustacean hyperglycemic hormone (CHH) precursor from the X-organ and identification of the neuropeptide from sinus gland of the Alaskan Tanner crab, *Chionoecetes bairdi.* General and Comparative Endocrinology 162:129–133.

Chung, J.S., N. Zmora, H. Katayama, and N. Tsutsui. 2010. Crustacean hyperglycemic hormone (CHH) neuropeptides family: functions, titer, and binding to target tissues. General and Comparative Endocrinology 166:447–454.

Dai, L., D Žitňan, and M.E. Davis. 2007. Strategic expression of ion transport peptide gene products in central and peripheral neurons of insects. Journal of Comparative Neurology 500:353–367.

Davey, M.L., M.R. Hall, R.H. Willis, R.W. Oliver, M.J. Thurn, and K.J. Wilson. 2000. Five crustacean hyperglycaemic family hormones of *Penaeus monodon*: complementary DNA sequence and identification in single sinus glands by electrospray ionization-Fourier transform mass spectrometry. Marine Biotechnology 2:80–91.

De Kleijn, D.P.V., A.J.M. Coenen, A.M. Laverdure, C.P. Tensen, and F. Van Herp. 1992. Localization of mRNAs encoding crustacean hyperglycemic hormone (CHH) and gonad-inhibiting hormone (GIH) in the X-organ sinus gland complex of the lobster *Homarus americanus.* Neuroscience 51:121–128.

De Kleijn, D.P.V., K.P.C. Janssen, G.J.M. Martens, and F. Van Herp. 1994. Cloning and expression of two crustacean hyperglycaemic hormone mRNAs in the eyestalk of the crayfish *Orconectes limosus.* European Journal of Biochemistry 224:623–629.

De Kleijn, D.P.V., E.P. de Leeuw, M.C. van den Berg, G.J. Martens, and F. Van Herp. 1995. Cloning and expression of two mRNAs encoding structurally different crustacean hyperglycemic hormone precursors in the lobster *Homarus americanus*. Biochimica et Biophysica Acta 1260:62–66.

De Kleijn, D.P.V., K.P.C. Janssen, S.L. Waddy, R. Hegeman, W.Y. Lai, G.J.M. Martens, and F. Van Herp. 1998. Expression of the crustacean hyperglycaemic hormones and the gonad-inhibiting hormone during the reproductive cycle of the female American lobster *Homarus americanus.* Journal of Endocrinology 156:291–298.

Dircksen, H. 2009. Insect ion transport peptides are derived from alternatively spliced genes and differentially expressed in the central and peripheral nervous system. Journal of Experimental Biology 212:401–412.

Dircksen, H., and U. Heyn. 1998. Crustacean hyperglycemic hormone-like peptides in crab and locust peripheral intrinsic neurosecretory cells. Annals of the New York Academy of Sciences 839:392–394.

Dircksen, H., S.G. Webster, and R. Keller. 1988. Immunocytochemical demonstration of the neurosecretory systems containing putative moult-inhibiting hormone and hyperglycemic hormone in the eyestalk of brachyuran crustaceans. Cell and Tissue Research 251:3–12.

Dircksen, H., D. Böcking, U. Heyn, C Mandel, J.S. Chung, G. Baggerman, P. Verhaert, S. Daufeldt, T. Plösch, P.P. Jaros, E. Waelkens, R. Kellerand, and S.G. Webster. 2001. Crustacean hyperglycaemic hormone (CHH) like peptides and CHH-precursor-related peptides from pericardial organ neurosecretory cells in the shore crab, *Carcinus maenas*, are putatively spliced and modified products of multiple genes. Biochemical Journal 356:159–170.

Dircksen, H., L. Kahsai Tesfai, C. Albus, and D.R. Nässel. 2008. Ion transport peptide splice forms in central and peripheral neurons throughout postembryogenesis of *Drosophila melanogaster.* Journal of Comparative Neurology 509:23–41.

Drexler, A.L., C.C. Harris, M.G. dela Pena, M. Asuncion-Uchi, S. Chung, S. Webster, and M. Fuse. 2007. Molecular characterization and cell-specific expression of an ion transport peptide in the tobacco hornworm, *Manduca sexta.* Cell and Tissue Research 329:391–408.

Eckhardt, E., C. Pierrot, P. Thuet, F. Van Herp, M. Charmantier-Daures, J.-P. Trilles, and G. Charmantier. 1995. Stimulation of osmoregulating processes in the perfused gill of the crab *Pachygrapsus marmoratus* (Crustacea, Decapoda) by a sinus gland peptide. General and Comparative Endocrinology 99:169–177.

Escamilla-Chimal, E.G., F. Van Herp, and M.L. Fanjul-Moles. 2001. Daily variations in crustacean hyperglycaemic hormone and serotonin immunoreactivity during the development of crayfish. Journal of Experimental Biology 204:1073–1081.

Escamilla-Chimal, E.G., M. Hiriart, M.C. Sánchez-Soto, and M.L. Fanjul-Moles. 2002. Serotonin modulation of CHH secretion by isolated cells of the crayfish retina and optic lobe. General and Comparative Endocrinology 125:283–290.

Freire, C., and J.C. McNamara. 1992. Involvement of the central nervous system in neuroendocrine mediation of osmotic and ionic regulation in the freshwater shrimp *Macrobrachium olfersii* (Crustacea, Decapoda). General and Comparative Endocrinology 88:316–327.

Freire, C., H. Onken, and J.C. McNamara. 2008. A structure-function analysis of ion transport in crustacean gills and excretory organs. Comparative Biochemistry and Physiology A 151:272–304.

Fu, Q., A.E. Christie, and L. Li. 2005. Mass spectrometric characterization of crustacean hyperglycemic hormone precursor-related peptides (CPRPs) from the sinus gland of the crab, *Cancer productus.* Peptides 26:2137–2150.

Gard, A.L., P.H. Lenz, J.R. Shaw, and A.E. Christie. 2009. Identification of putative peptide paracrines/hormones in the water flea *Daphnia pulex* (Crustacea; Branchiopoda; Cladocera) using transcriptomics and immunohistochemistry. General and Comparative Endocrinology 160:271–287.

Glowik, R.M., J. Golowasch, R. Keller, and E. Marder. 1997. D-glucose-sensitive neurosecretory cells of the crab *Cancer borealis* and negative feedback regulation of blood glucose level. Journal of Experimental Biology 200:1421–1431.

Gorgels-Kallen, J.L., and C.E.M. Voorter. 1985. The secretory dynamics of the CHH-producing cellgroup of the crayfish *Astacus leptodactylus* in the course of a day/night cycle. Cell and Tissue Research 241:361–366.

Goy, M.F. 1990. Activation of membrane guanylate cyclase by an invertebrate peptide hormone. Journal of Biological Chemistry 265:20220–20227.

Grève, P., O. Sorokine, T. Berges, C. Lacombe, A. Van Dorsselaer, and G. Martin. 1999. Isolation and amino acid sequence of a peptide with vitellogenesis inhibiting activity from the terrestrial isopod *Armadillidium vulgare* (Crustacea). General and Comparative Endocrinology 115:406–414.

Gu, P.L., and S.M. Chan. 1998. The shrimp hyperglycaemic hormone-like neuropeptide is encoded by multiple copies of genes arranged in a cluster. FEBS Letters 441:397–403.

Gu, P.L., K.L. Yu, and S.M. Chan. 2000. Molecular characterization of an additional shrimp hyperglycaemic hormone; cDNA cloning, gene organization, expression and biological assay of recombinant proteins. FEBS Letters 472:122–128.

Hanström, B. 1931. Neue untersuchungen uber Sinnesorgane und Nervensystem der Crustaceen. I. Zeitschrift für Morphologie und Ökologie der Tiere 23:80–236.

Hanström, B. 1933. Neue untersuchungen uber Sinnesorgane und Nervensystem der Crustaceen. II. Zoologische Jahrbücher. Abteilung für Anatomie und Ontogenie der Tiere 56:387–520.

Hopkins, P. 2001. Limb regeneration in the fiddler crab, *Uca pugilator*: hormonal and growth factor control. American Zoologist 41:389–398.

Hsu, Y.W.A., J.R. Weller, A.E. Christie, and H.O. de la Iglesia. 2008. Molecular cloning of four cDNAs encoding prepro-crustacean hyperglycemic hormone (CHH) from the eyestalk of the red rock crab *Cancer productus*: identification of two genetically encoded CHH isoforms and two putative post-translationally derived CHH variants. General and Comparative Endocrinology 155:517–525.

Huberman, A., M.B. Aguilar, I. Navarro-Quiroga, L. Ramos, I. Fernández, F.M. White, and D.F. Hunt. 2000. A hyperglycemic peptide hormone from the Caribbean shrimp *Penaeus (litopenaeus) schmitti.* Peptides 21:331–338.

Kallen, J.L., and S.L. Abrahamse. 1989. Functional aspects of the hyperglycemic hormone producing system of the crayfish *Orconectes limosus* in relation to its day/night rhythm. General and Comparative Endocrinology 74:74.

Kallen, J.L., S.L. Abrahamse, and F. Van Herp. 1990. Circadian rhythmicity of the crustacean hyperglycaemic hormone (CHH) in the hemolymph of crayfish. Biological Bulletin 179:351–357.

Kamemoto, F.I. 1976. Neuroendocrinology of osmoregulation in decapod Crustacea. American Zoologist 16:141–150.

Kamemoto, F.I. 1991. Neuroendocrinology of osmoregulation in crabs. Zoological Science 8:827–833.

Katayama, H., and J.S. Chung. 2009. The specific binding sites of eyestalk- and pericardial organ-crustacean hyperglycaemic hormones (CHHs) in multiple tissues of the blue crab, *Callinectes sapidus*. Journal of Experimental Biology 212:542–549.

Katayama, H., and H. Nagasawa. 2004. Effect of a glycine residue insertion into crustacean hyperglycaemic hormone on hormonal activity. Zoological Science 21:1121–1124.

Katayama, H., T. Ohira, K. Aida, and H. Nagasawa. 2002. Significance of a carboxyl-terminal moiety in the folding and biological activity of crustacean hyperglycaemic hormone. Peptides 23:1537–1546.

Kegel, G., B. Reichwein, S. Weese, G. Gaus, J. Peter-Katalinić, and R. Keller. 1989. Amino acid sequence of the crustacean hyperglycemic hormone (CHH) from the shore crab, *Carcinus maenas.* FEBS Letters 255:10–14.

Keller, R. 1969. Untersuchungen zur Arspezifität eines Crustaceenhormons. Zeitschrift für vergleichende Physiologie 63:137–145.

Keller, R. and H.-P. Orth. 1990. Hyperglycemic neuropeptides in crustaceans. Pages 265–271 *in* A. Epple, C.G. Scanes, and M.H. Stetson, editors. Progress in comparative endocrinology, Wiley-Liss Inc. New York.

Keller, R., and D. Sedlmeier. 1988. A metabolic hormone in crustaceans: The hyperglycaemic neuropeptide. Pages 315–326 *in* H. Laufer, R.G.H. Downer, editors. Endocrinology of selected invertebrates, Vol. 2. Alan R. Liss, New York.

Keller, R., P.P. Jaros, and G. Kegel. 1985. Crustacean hyperglycemic neuropeptides American Zoologist 25:207–221.

Keller, R., B. Haylett, and I.M. Cooke. 1994. Neurosecretion of crustacean hyperglycaemic hormone evoked by axonal stimulation or elevation of saline K+ concentration quantified by a sensitive immunoassay method. Journal of Experimental Biology 188:293–316.

Keller, R., S. Grau, and I.M. Cooke. 1995. Quantitation of peptide hormone in single cultured secretory neurons of the crab, *Cardisoma carnifex*. Cell and Tissue Research 281:525–532.

Keller, R., G. Kegel, B. Reichwein, D. Sedlmeier, and D. Soyez. 1999. Biological effects of neurohormones of the CHH/MIH/GIH peptide family in crustaceans. Pages 209–212 *in* E.W. Roubos, S.E. Wendelaar Bonga, H. Vaudry, and A. De Loof, editors. Recent developments in comparative endocrinology and neurobiology, Shaker Publishing, Maastricht.

Khayat, M., W.-J. Yang, K. Aida, H. Nagasawa, A. Tietz, B. Funkenstein, and E. Lubzens. 1998. Hyperglycaemic hormones inhibit protein and mRNA synthesis in in vitro-incubated ovarian fragments of the marine shrimp *Penaeus semisulcatus.* General and Comparative Endocrinology 110:307–318.

Kleinholz, L.H., and R. Keller. 1973. Comparative studies in crustacean hyperglycemic hormones. I. The initial survey. General and Comparative Endocrinology 21:554–564.

Komali, M., V. Kalarani, C.H. Venkatrayulu, and D.C.S. Reddy. 2005. Hyperglycemic effects of 5-hydroxytryptamine and dopamine in the freshwater prawn *Macrobrachium rosenbergii.* Journal of Experimental Zoology 303:448–455.

Kummer, G., and R. Keller. 1993. High-affinity binding of crustacean hyperglycaemic hormone (CHH) to hepatopancreatic plasma membranes of the crab *Carcinus maenas* and the crayfish *Orconectes limosus*. Peptides 14:103–108.

Lacombe, C., P. Grève, and G. Martin. 1999. Overview on the sub-grouping of the crustacean hyperglycemic hormone family. Neuropeptides 33:71–80.

Lago-Lestón, A.E. Ponce, and M.E. Muñoz. 2007. Cloning and expression of hyperglycemic (CHH) and molt-inhibiting hormones mRNAs from the eyestalk of shrimps of *Litopenaeus vannamei* grown in different temperature and salinity conditions. Aquaculture 270:343–357.

Laufer, H., D. Borst, F.C. Baker, C. Carrasco, M. Sinkus, C.C. Reuter, L.W. Tsai, and D.A. Schooley. 1987a. Identification of a juvenile hormone-like compound in a crustacean. Science 235:202–205.

Laufer, H., M. Landau, E. Homola, and D.W. Borst. 1987b. Methyl farnesoate: its site of synthesis and regulation of secretion in a juvenile crustacean. Insect Biochemistry 17:1129–1131.

Laverdure, A.M., C. Carette-Desmoucelles, M. Breuzet, and M. Descamps. 1994. Neuropeptides and related nucleic acid sequences detected in penaeid shrimps by immunocytochemistry and molecular hybridizations. Neuroscience 60:569–579.

Lechleitner, R.A., N. Audsley, and J.E. Phillips. 1989a. Antidiuretic action of cyclic AMP, corpus cardiacum and ventral ganglia of fluid absorption across locust ileum in vitro. Canadian Journal of Zoology 67:2655–2661.

Lechleitner, R.A., N. Audsley, and J.E. Phillips. 1989b. Composition of fluid transported by locust ileum: influence of natural stimulants and luminal ion ratios. Canadian Journal of Zoology 67:2662–2668.

Lee, K.J., R.M. Doran, and D.L. Mykles. 2007. Crustacean hyperglycaemic hormone from the tropical land crab, *Gecarcinus lateralis*: cloning, isoforms and tissue expression. General and Comparative Endocrinology 154:174–183.

Leuven, R.S.E.W., P.P. Jaros, F. Van Herp, and R. Keller. 1982. Species or group specificity in biological and immunological studies of crustacean hyperglycemic hormone. General and Comparative Endocrinology 46:288–296.

Li, S., F. Li, B. Wang, Y. Xie, R. Wen, and J. Xiang. 2010. Cloning and expression profiles of two isoforms of a CHH-like gene specifically expressed in male Chinese shrimp. *Fenneropenaeus chinensis.* General and Comparative Endocrinology 167:308–316.

Liu, L., H. Laufer, Y. Wang, and T. Hayes. 1997. A neurohormone regulating both methyl farnesoate synthesis and glucose metabolism in a crustacean. Biochemical and Biophysical Research Communications 237:694–701.

Lorenzon, S., P. Edomi, P.G. Giulianini, R. Mettulio, and E.A. Ferrero. 2004. Variation of crustacean hyperglycaemic hormone (cHH) level in the eyestalk and haemolymph of the shrimp *Palaemon elegans* following stress. Journal of Experimental Biology 207:4205–4213.

Lorenzon, S., P. Edomi, P.G. Giulianini, R. Mettulio, and E.A. Ferrero. 2005. Role of biogenic amines in the crustacean hyperglycaemic stress response. Journal of Experimental Biology 208:3341–3347.

Lüschen, W., A. Willig, and P.P. Jaros. 1993. The role of biogenic amines in the control of blood glucose level in the decapod crustacean, *Carcinus maenas* L. Comparative Biochemistry and Physiology C 105:291–296.

Macins, A., J. Meredith, Y. Zhao, H.W. Brock, and J.E. Phillips. 1999. Occurrence of ion transport peptide (ITP) and ion-transport-like peptide (ITP-L) in orthopteroids. Archives of Insect Biochemistry and Physiology 40:107–118.

Mantel, L.H. 1985. Neurohormonal integration of osmotic and ionic regulation. American Zoologist 25:253–263.

Mantel, L.H., and L.L. Farmer. 1983. Osmotic and ionic regulation. Pages 53–61 *in* D.E. Bliss, editor. The biology of crustacea. Vol. 5: internal anatomy and physiological regulation. Academic Press, New York.

Marco, H.G., W. Brandt, and G. Gäde. 1998. Elucidation of the amino acid sequence of a crustacean hyperglycaemic hormone from the spiny lobster, *Jasus lalandii.* Biochemical and Biophysical Research Communications 248:578–583.

Marco, H.G., S. Stoeva, W. Voelter, and G. Gäde. 2000. Characterization and sequence elucidation of a novel peptide with molt-inhibiting activity from the South African spiny lobster, *Jasus lalandii.* Peptides 21:1313–1321.

Martin, G., P.P. Jaros, G. Besse, and R. Keller. 1984. The hyperglycemic neuropeptide of the terrestrial isopod, *Porcellio dilatatus*. II.: immunocytochemical demonstration in neurosecretory structures of the nervous system. General and Comparative Endocrinology 55:217–226.

Martin, G., Sorokine, O., and A. Van Dorsselaer. 1993. Isolation and molecular characterization of a hyperglycemic neuropeptide from the sinus gland of the terrestrial isopod *Armadillidium vulgare* (Crustacea). European Journal of Biochemistry 211:601–607.

Mattson, M.P., and E. Spaziani. 1985. Characterization of molt-inhibiting hormone (MIH) action on crustacean Y-organ segments and dispersed cells in culture and a bioassay for MIH activity. Journal of Experimental Zoology 236:93–101.

Meredith, J., M. Ring, A. Macins, J. Marschall, N.N. Cheng, D. Theilmann, H.W. Brock, and J.E. Phillips. 1996. Locust ion transport peptide (ITP): primary structure, cDNA and expression in a baculovirus system. Journal of Experimental Biology 199:1053–1061.

Mettulio, R., P. Giulianini, E.A. Ferrero, S. Lorenzon, and P. Edomi. 2004. Functional analysis of crustacean hyperglycaemic hormone by in vivo assay with wild type and mutant recombinant proteins. Regulatory Peptides 119:189–197.

Meusy, J.-J., and G.G. Payen. 1988. Female reproduction in malacostracan Crustacea. Zoological Science 5:217–265.

Montagné, N., D. Soyez, D. Gallois, C. Ollivaux, and J.-Y. Toullec. 2008. New insights into evolution of crustacean hyperglycaemic hormone in decapods: first characterization in Anomura. FEBS Journal 275:1039–1052.

Montagné, N., Y. Desdevises, D. Soyez, and J.-Y. Toullec. 2010. Molecular evolution of the crustacean hyperglycaemic hormone family in ecdysozoans. BMC Evolutionary Biology 10:62.

Morris, S. 2001. Neuroendocrine regulation of osmoregulation and the evolution of air-breathing in decapod crustaceans. Journal of Experimental Biology 204:979–989.

Morris, S., U. Postel, Mrinalini, L.M. Turner, J. Palmer, and S.G. Webster. 2010. The adaptive significance of crustacean hyperglycaemic hormone (CHH) in daily and seasonal migratory activities of the Christmas Island red crab *Gecarcoidea natalis*. Journal of Experimental Biology 213:3062–3073.

Mosco, A., P. Edomi, C. Guarnaccia, S. Lorenzon, S. Pongor, E.A. Ferrero, and P.G. Giulianini. 2008. Functional aspects of cHH C-terminal amidation in crayfish species. Regulatory Peptides 147:88–95.

Muramoto, A. 1988. Endocrine control of water balance in decapod crustaceans. Pages 341–356 *in* H. Laufer and R.G.H. Downer, editors. Endocrinology of selected invertebrate types, Vol. II. A.R. Liss, New York.

Nagabhushanam, R., R. Sarojini, P.S. Reddy, M. Devi, and M. Fingerman. 1995. Opioid peptides in invertebrates: localization, distribution and possible functional roles. Current Science 69:659–671.

Nagai, C., H. Asazuma, S. Nagata, S., T. Ohira, and H. Nagasawa. 2009. A convenient method for preparation of biologically active recombinant CHH of the kuruma prawn, *Marsupenaeus japonicus*, using the bacterial expression system. Peptides 30:507–517.

Nagasawa, H., W.-J. Yang, H. Shimizu, K. Aida, H. Tsutsumi, H. Terauchi, and H. Sonobe. 1996. Isolation and amino acid sequence of a molt-inhibiting hormone from the American crayfish, *Procambarus clarkii*. Bioscience Biotechnology and Biochemistry 60:554–556.

Nakatsuji, T., and H. Sonobe. 2004. Regulation of ecdysteroid secretion from the Y-organ by molt-inhibiting hormone in the American crayfish, *Procambarus clarkii.* General and Comparative Endocrinology 135:358–364.

Ohira, T., H. Watanabe, H. Nagasawa, and K. Aida. 1997. Cloning and sequence analysis of a cDNA encoding a crustacean hyperglycemic hormone from the Kuruma prawn, *Penaeus japonicus.* Molecular Marine Biology and Biotechnology 6:59–63.

Ollivaux, C., H. Dircksen, J.-Y. Toullec, and D. Soyez. 2002. Enkephalinergic control of the secretory activity of neurons producing stereoisomers of crustacean hyperglycaemic hormone in the eyestalk of the crayfish *Orconectes limosus.* Journal of Comparative Neurology 444:1–9.

Ollivaux, C., J. Vinh, D. Soyez, and J.-Y. Toullec. 2006. Crustacean hyperglycemic and vitellogenesis-inhibiting hormones in the lobster *Homarus gammarus.* FEBS Journal 273:2151–2160.

Ollivaux, C., D. Gallois, M. Amiche, M. Boscaméric, and D. Soyez. 2009. Molecular and cellular specificity of post-translational aminoacyl isomerisation in the crustacean hyperglycaemic hormone family. FEBS Journal 276:4790–4802.

Phillips, J.E., and N. Audsley. 1995. Neuropeptide control of ion and fluid transport across locust hindgut. American Zoologist 35:503–514.

Phillips, J.E., J. Meredith, N. Audsley, N. Richardson, A. Macins, and M. Ring. 1998a. Locust ion transport peptide (ITP): a putative hormone controlling water and ionic balance in terrestrial insects. American Zoologist 38:461–470.

Phillips, J.E., J. Meredith, N. Audsley, M. Ring, A. Macins, H. Brock, D. Theilmann, and D. Littleford. 1998b. Locust ion transport peptide (ITP): function, structure, cDNA and expression. Pages 210–226 *in* G.M. Coast and S.G. Webster, editors. Recent advances in arthropod endocrinology. Cambridge University Press, Cambridge.

Phillips, J.E., J. Meredith, Y. Wang, Y. Zhao, and H. Brock. 2001. Ion transport peptide (ITP): structure, function, evolution. Pages 745–752 *in* H.J. Goos, R.K. Rastogi, H. Vaudry, and R. Pierantoni, editors. Perspectives in comparative endocrinology: unity and diversity. Medimond, Bologna, Italy.

Phlippen, M.K., S.G. Webster, J.S. Chung, and H. Dircksen. 2000. Ecdysis of decapod crustaceans is associated with a dramatic release of crustacean cardioactive peptide into the haemolymph. Journal of Experimental Biology 203:521–536.

Pierrot, C., E. Eckhardt, F. Van Herp, M. Charmantier-Daures, G. Charmantier, J.-P. Trilles, and P. Thuet. 1994. Effet d'extraits de glandes du sinus sur la physiologie osmorégulatrice de branchies perfusées du crabe *Pachygrapsus marmoratus.* Comptes rendus de l'Académie des Sciences 317:411–418.

Qian, Y.Q., L. Dai, J.S. Yang, F. Yang, D.F. Chen, Y. Fujiwara, S. Tsuchida, H. Nagasawa, and W.J. Yang. 2009. CHH family peptides from an 'eyeless' deep-sea hydrothermal vent shrimp, *Rimicaris kairei*: characterization and sequence analysis. Comparative Biochemistry and Physiology B 154:27–47.

Quackenbush, L.S., and L.L. Keeley. 1988. Regulation of vitellogenesis in the fiddler crab, *Uca pugilator.* Biological Bulletin 175:321–331.

Reddy, P.S. 1999. A neurotransmitter role for methionine-enkephalin in causing hyperglycemia in the freshwater crab *Oziotelphusa senex senex.* Current Science 76:1126–1128.

Reddy P.S., and S.B. Sainath. 2008. Effect of retinoic acid on hemolymph glucose regulation in the fresh water edible crab *Oziotelphusa senex senex.* General and Comparative Endocrinology 155:496–502.

Rottlant, G., D.P.V. De Kleijn, M. Charmantier-Daures, G. Charmantier, and F. Van Herp. 1993. Localization of crustacean hyperglycemic hormone (CHH) and gonad-inhibiting hormone (GIH) in the eyestalk of *Homarus gammarus* larvae by immunocytochemistry and in situ hybridization. Cell and Tissue Research 271:507–512.

Santos, E.A., and R. Keller. 1993a. Regulation of circulating levels of the crustacean hyperglycaemic hormone-evidence of a dual feedback control system. Journal of Comparative Physiology 163A:374–379.

Santos, E.A., and R. Keller. 1993b. Effect of exposure to atmospheric air on blood glucose and lactate in two crustacean species: a role of the crustacean hyperglycemic hormone. Comparative Biochemistry and Physiology A 106:343–347.

Santos, E.A., L.E.M. Nery, R. Keller, and A.A. Gonçalves. 1997. Evidence for the involvement of the crustacean hyperglycemic hormone in the regulation of lipid metabolism. Physiological Zoology 70:415–420.

Santos, E.A., R. Keller, E. Rodriguez, and L. Lopez. 2001. Effects of serotonin and fluoxetine on blood glucose regulation in two decapod species. Brazilian Journal of Medical and Biological Research 34:75–80.

Sedlmeier, D. 1982. The mode of action of the crustacean neurosecretory hyperglycemic hormone (CHH). II. Involvement of glycogen synthase. General and Comparative Endocrinology 47:426–432.

Sedlmeier, D. 1985. Mode of action of the crustacean hyperglycemic hormone. American Zoologist 25:223–232.

Sedlmeier, D. 1988. The crustacean hyperglycemic hormone (CHH) releases amylase from the crayfish midgut gland. Regulatory Peptides 20:91–98.

Sedlmeier, D. and R. Keller. 1981. The mode of action of the crustacean neurosecretory hyperglycemic hormone. I. Involvement of cyclic nucleotides. General and Comparative Endocrinology 45:82–90.

Sefiani, M., J.-P. Le Caer, and D. Soyez. 1996. Characterization of hyperglycaemic and molt-inhibiting activity from sinus glands of the penaeid shrimp *Penaeus vannamei.* General and Comparative Endocrinology 103:41–53.

Serrano, L., G. Blanvillain, D. Soyez, G. Charmantier, E. Grousset, F. Aujoulat, F., and C. Spanings-Pierrot. 2003. Putative involvement of crustacean hyperglycaemic hormone isoforms in the neuroendocrine mediation of osmoregulation in the crayfish *Astacus leptodactylus.* Journal of Experimental Biology 206:979–988.

Shih, T.-W., Y. Suzuki, H. Nagasawa, and K. Aida. 1998. Immunohistochemical identification of hyperglycemic hormone- and molt-inhibiting hormone-producing cells in the eyestalk of the Kuruma prawn, *Penaeus japonicus.* Zoological Science 15:389–397.

Sithigorngul, P., N. Panchan, T. Vilaivan, W. Sithigorngul, and A. Petsom. 1999. Immunochemical analysis and immunocytochemical localization of crustacean hyperglycemic hormone from the eyestalk of *Macrobrachium rosenbergii.* Comparative Biochemistry and Physiology B 124:73–80.

Sithigorngul, P., N. Panchan, P. Chaivisuthangkura, S. Longyant, W. Sithigorngul, and A. Petsom. 2002. Differential expression of CMG peptide and crustacean hyperglycemic hormones (CHHs) in the eyestalk of the giant tiger prawn Penaeus monodon. Peptides 23:1943–1952.

Soumoff, C., and J.D. O'Connor. 1982. Repression of Y-organ secretory activity by molt-inhibiting hormone in the crab *Pachygrapsus crassipes.* General and Comparative Endocrinology 48:432–439.

Soyez, D., J.E. Van Deijnen, and M. Martin. 1987. Isolation and characterization of a vitellogenesis-inhibiting factor from sinus glands of the lobster *Homarus americanus.* Journal of Experimental Zoology 244:479–484.

Soyez, D., J.-P. le Caer, P.Y. Noël, and J. Rossier. 1991. Primary structure of two isoforms of the vitellogenesis-inhibiting hormone from the lobster *Homarus americanus.* Neuropeptides 20:25–32.

Soyez, D., F. Van Herp, J. Rossier, J.-P. Le Caer, C.P. Tensen, and R. Lafont. 1994. Evidence for a conformational polymorphism of invertebrate neurohormones: D-amino acid in crustacean hyperglycaemic peptides. Journal of Biological Chemistry 269:18295–18298.

Soyez, D., A.M. Laverdure, J. Kallen, and F. Van Herp. 1998. Demonstration of a cell-specific isomerisation of invertebrate neuropeptides. Neuroscience 82:935–942.

Spanings-Pierrot, C., D. Soyez, F. Van Herp, M. Gompel, G. Skaret, E. Grousset, and G. Charmantier. 2000. Involvement of crustacean hyperglycemic hormone in the control of gill ion transport in the crab *Pachygrapsus marmoratus.* General and Comparative Endocrinology 119:340–350.

Stemmler, E.A., Y.-W.A. Hsu, C.R. Cashman, D.I. Messinger, H.O. de la Iglesia, P.S. Dickinson, and A.E. Christie. 2007. Direct tissue MALDI-FTMS profiling of individual *Cancer productus* sinus glands reveals that one of three distinct combinations of crustacean hyperglycemic hormone precursor-related peptide (CPRP) isoforms are present in individual crabs. General and Comparative Endocrinology 154:184–192.

Stentiford, G.D., E.S. Chang, S.A. Chang, and D.M. Neil. 2001. Carbohydrate dynamics and the crustacean hyperglycaemic hormone (CHH): effects of parasitic infection in Norway lobsters (*Nephrops norvegicus*). General and Comparative Endocrinology 121:13–22.

Stockmann, R., A.M. Laverdure, and M. Breuzet. 1997. Localization of a crustacean hyperglycaemic hormone-like immunoreactivity in the neuroendocrine system of *Euscorpius carpathicus* (L.) (Scorpionida, Chactidae). General and Comparative Endocrinology 106:320–326.

Tiu, S.H.K., J.G. He, and S.M. Chan. 2007. The LvCHH-ITP gene of the shrimp (*Litopenaeus vannamei*) produces a widely expressed putative ion transport peptide (LvITP) for osmo-regulation. Gene 396:226–235.

Toullec, J.Y., J. Vinh, J.P. le Caer, B. Shillito, and D. Soyez. 2002. Structure and phylogeny of the crustacean hyperglycaemic hormone and its precursor from a hydrothermal vent crustacean: the crab *Bythograea thermydron.* Peptides 23:31–42.

Toullec, J.Y., L. Serrano, P. Lopez, D. Soyez, and C. Spanings-Pierrot. 2006. The crustacean hyperglycemic hormones from an euryhaline crab *Pachygrapsus marmoratus* and a freshwater crab *Potamon ibericum*: eyestalk and pericardial organ isoforms. Peptides 27:1269–1280.

Towle, D.W., and D. Weirauch. 2001. Osmoregulation by gills of euryhaline crabs: molecular analysis of transporters. American Zoologist 41:770–780.

Trerattrakool, S., A. Udomkit, L. Eurwilaichitr, B. Sonthayanon, and S. Panyim. 2003. Expression of biologically active crustacean hyperglycemic hormone (CHH) of *Penaeus monodon* in *Pichia pastoris.* Marine Biotechnology 5:373–379.

Tsai, K.W., S.J. Chang, H.Y. Wu, C.H. Chen, and C.Y. Lee. 2008. Molecular cloning and differential expression pattern of two structural variants of the crustacean hyperglycemic hormone family from the mud crab *Scylla olivacea.* General and Comparative Endocrinology 159:16–25.

Tsukimura, B., and D.W. Borst. 1992. Regulation of methyl farnesoate in the hemolymph and mandibular organ of the lobster, *Homarus americanus.* General and Comparative Endocrinology 86:297–303.

Tsutsui, N., H. Katayama, T. Ohira, H. Nagasawa, M.N. Wilder, and K. Aida. 2005. The effects of crustacean hyperglycemic hormone-family peptides on vitellogenin gene expression in the kuruma prawn, *Marsupenaeus japonicus.* General and Comparative Endocrinology 144:232–239.

Turner, L.M. 2010. A role for crustacean hyperglycaemic hormone (CHH) in the regulation of kidney-like function in freshwater land crabs: a study of the Christmas Island blue crab, *Discoplax hirtipes*. PhD Thesis, University of Bristol, pp. 1–303.

Udomkit, A., S. Chooluck, B. Sonthayanon, and S. Panyim. 2000. Molecular cloning of a cDNA encoding a member of CHH/MIH/GIH family from *Penaeus monodon* and analysis of its gene structure. Journal of Experimental Marine Biology and Ecology 244:145–156.

Wainwright, G., S.G. Webster, M.C. Wilkinson, J.S. Chung, and H.H. Rees. 1996. Structure and significance of mandibular organ-inhibiting hormone in the crab, *Cancer pagurus*; involvement in multihormonal regulation of growth and reproduction. Journal of Biological Chemistry 271:12749–12754.

Wang, Y.-J., Y. Zhao, J. Meredith, J.E. Phillips, D.A. Theilmann, and H.W. Brock. 2000. Mutational analysis of the C-terminus in ion transport peptide (ITP) expressed in *Drosophila* Kc1 cells. Archives of Insect Biochemistry and Physiology 45:129–138.

Webster, S.G. 1986. Neurohormonal control of ecdysteroid synthesis by *Carcinus maenas* Y-organs *in vitro*, and preliminary characterization of the putative molt-inhibiting hormone (MIH). General and Comparative Endocrinology 61:237–247.

Webster, S.G. 1991. Amino acid sequence of putative moult-inhibiting hormone from the crab *Carcinus maenas*. Proceedings of the Royal Society London B 244:247–252.

Webster, S.G. 1993. High-affinity binding of putative moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) to membrane bound receptors on the Y-organ of the shore crab *Carcinus maenas.* Proceedings of the Royal Society London B 251:53–59.

Webster, S.G. 1996. Measurement of crustacean hyperglycaemic hormone levels in the edible crab *Cancer pagurus* during emersion stress. Journal of Experimental Biology 199:1579–1585.

Webster, S.G. 1998. Inhibitory neuropeptides in crustaceans. Pages 33–52 *in* G.M. Coast and S.G. Webster, editors. Recent advances in arthropod endocrinology. Cambridge University Press, Cambridge.

Webster, S.G., and H. Dircksen. 1991. Putative molt-inhibiting hormone in larvae of the shore crab *Carcinus maenas* L.: an immunocytochemical approach. Biological Bulletin 180:65–71.

Webster, S.G., and R. Keller. 1986. Purification, characterisation and amino acid composition of the putative moult-inhibiting hormone (MIH) of *Carcinus maenas* (Crustacea, Decapoda). Journal of Comparative Physiology B 156:617–624.

Webster, S.G., H. Dircksen, and J.S. Chung. 2000. Endocrine cells in the gut of the shore crab *Carcinus maenas* immunoreactive to crustacean hyperglycaemic hormone and its precursor-related peptide. Cell and Tissue Research 300:193–205.

Webster, S.G., R. Keller, and H. Dircksen. 2012. The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting and reproduction. General and Comparative Endocrinology 175:217–233.

Weidemann, W., J. Gromoll, and R. Keller. 1989. Cloning and sequence analysis of cDNA for precursor of a crustacean hyperglycemic hormone. FEBS Letters 257:31–34.

Wilcockson, D.C., J.S. Chung, and S.G. Webster. 2002. Is crustacean hyperglycaemic hormone precursor-related peptide a circulating neurohormone in crabs? Cell and Tissue Research 307:129–138.

Yang, W.J., K. Aida, A. Terauchi, H. Sonobe, and H. Nagasawa. 1996. Amino acid sequence of a peptide with molt-inhibiting activity from the Kuruma prawn *Penaeus japonicus*. Peptides 17:197–202.

Yang, W.J., K. Aida, and H. Nagasawa. 1997. Amino acid sequences and activities of multiple hyperglycemic hormones from the Kuruma prawn, *Penaeus japonicus.* Peptides 18:479–485.

Yasuda, A., Y. Yasuda, T. Fujita, and Y. Naya. 1994. Characterization of crustacean hyperglycemic hormone from the crayfish (*Procambarus clarkii*): multiplicity of molecular forms by stereoinversion and diverse functions. General and Comparative Endocrinology 95:387–398.

Zarubin, T.P., E.S. Chang, and D.L. Mykles. 2009. Expression of recombinant eyestalk crustacean hyperglycemic hormone from the tropical land crab, *Gecarcinus lateralis*, that inhibits Y-organ ecdysteroidogenesis in vitro. Molecular Biology Reports 36:1231–1237.

Zhang, Q., R. Keller, and H. Dircksen. 1997. Crustacean hyperglycaemic hormone in the nervous system of the primitive crustacean species *Daphnia magna* and *Artemia salina* (Crustacea: Branchiopoda). Cell and Tissue Research 287:565–576.

Zheng, J., H.Y. Chen, C.Y. Cheol, C.Y., R.D. Roer, and R.D. Watson. 2010. Molecular cloning of a putative crustacean hyperglycemic hormone isoform from extra-eyestalk tissue of the blue crab (*Callinectes sapidus*) and determination of temporal and spatial patterns of CHH gene expression. General and Comparative Endocrinology 169:174–181.

Zmora, N., J. Trant, Y. Zohar, and J.S. Chung. 2009. Molt-inhibiting hormone stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab, *Callinectes sapidus* I: an ovarian stage dependent involvement. Saline Systems 5:7 doi: 10.1186/1746-1746-1448-5-7.

Zou, H.S., C.C. Juan, S.C. Chen, H.Y. Wang, and C.Y. Lee. 2003. Dopaminergic regulation of crustacean hyperglycaemic hormone and glucose levels in the hemolymph of the crayfish *Procambarus clarkii.* Journal of Experimental Zoology 298 A:44–52.

3

ADAPTIVE COLOR CHANGE AND THE MOLECULAR ENDOCRINOLOGY OF PIGMENT TRANSLOCATION IN CRUSTACEAN CHROMATOPHORES

John Campbell McNamara and
Sarah Ribeiro Milograna

Abstract

This chapter focuses on the cellular underpinnings of rapid adaptive color change in crustaceans. From the standpoint of molecular endocrinology, chromatic adaptation is pertinent to the physiological, cellular, and molecular biological mechanisms that regulate intracellular pigment granule movements. The chapter examines the distribution, organization, and ultrastructure of pigmentary effectors—the epidermal and internal chromatophores and retinal cells—and discusses the original characterization of pigment-aggregating and -dispersing chromatophorotropins that regulate pigment translocation and the sensory mechanisms that lead to their differential release from neurosecretory nuclei. Genes present in various arthropod groups code these peptide neurohormones, and their homology and conservation is discussed in light of evolutionary relationships with the Crustacea. An analysis of putative mechanisms of chromatophorotropin signal transduction is presented, focusing on membrane receptor type and function and subsequent intracellular regulatory cascades. Structural and physiological evidence concerning cytoskeletal molecular motors is investigated. Promising avenues for future investigation are discussed.

AN INTRODUCTION TO COLOR CHANGE IN THE CRUSTACEA

Animal colors and pigments have long fascinated the astute observer, from the dazzling pelt of the golden lion-tamarin and spectacular toco toucan's bill to the shimmering iridescent-blue wings of

the *Morpho* butterfly and the scarlet dye in the cochineal bug. A stunning array of shades, tints, and hues embellishes vertebrates and invertebrates alike, conferring manifest visibility or cryptic concealment as desired.

Crustacean colors are no less spectacular, and many taxa, like the fiddler, mangrove, porcelain, and coral crabs, and the cleaner, pistol, harlequin, hinge-beaked, and hump-backed shrimps, among myriad others, display astonishing color patterns (Bauer 2004). Some color arrays are fixed and are species specific, the product of convergent evolution with a particular substratum, often another organism, whereas others are more labile, the result of rapid color adaptation to changing backgrounds. This chapter focuses on the physiological, cellular, and molecular biological underpinnings of color change in crustaceans, particularly in the Caridea and Brachyura, viewed from the standpoint of molecular endocrinology.

Chromatic adaptation in the Crustacea has long aroused scientific interest, and Kröyer (1842) undertook the first comprehensive study on a broken-back, hippolytid shrimp almost two centuries ago. Adaptive changes in pigmentary patterns may be chromogenic (morphological) in nature, inherent to each species (Fig. 3.1), and changing only very slowly when honed by evolutionary forces or gradually within an individual organism when the number of pigment-bearing cells or chromatophores and their pigments increases or diminishes or when their constituent pigments become altered in color through alterations in metabolic pathways. Such gradual changes also may be associated with a particular developmental or molt stage or may correspond to the specific age of an individual.

However, color changes also may be chromomotor (physiological) in character, brought about by the rapid redistribution of pigment granules within the effector cell cytoplasm, quickly adjusting the individual organism to its current chromatic backdrop, to ambient luminosity and temperature, or to behavioral requirements, including circadian and tidal rhythms and reproductive cycles. It is this process of rapid, hormonally regulated color change that we examine here, beginning with the perception of substratum cues by the eyestalk ommatidia, through to signal transduction and the resulting intracellular signaling cascades in the pigment cells themselves, to the activation of the molecular motors that translocate the individual pigment granules through the cytosol and lead

Fig. 3.1.
The Indo-Pacific harlequin shrimp, *Hymenocera elegans*, exhibits one of the most stunning chromogenic adaptations found among the Crustacea. This gnathophyllid coral reef shrimp displays a fairly fixed, sex- and species-specific coloration pattern that consists of irregular, bluish to purple spots and markings, placed fairly symmetrically on a cream-white background to form a complex pigmentary system. Here, a pair of *H. elegans* (seen in frontal view) explores a prey species, the Indo-Pacific blue star, *Linckia laevigata*. See color version of this figure in the centerfold. With permission from Adriano Morettin, all rights reserved.

to chromatic adaptation. Our focus lies particularly on the molecular mechanisms underlying pigment aggregation in the caridean shrimps, the best-studied models among the decapod Crustacea, although information from brachyuran crabs and insect groups is included for a comparative and evolutionary perspective.

CRUSTACEAN PIGMENTARY EFFECTORS

The Distribution, Location, and Role of Chromatophores in Chromatic Adaptation

Crustacean pigments may be distributed freely within the exoskeleton or contained within pigment-bearing cells or chromatophores (*khrōma*, "color," *phoros*, "bearer"; Bauer 2004). Chromatophores are found in nearly all crustaceans, at all stages of their life cycles, from embryos through larvae, and, particularly, in the benthic, adult forms. The exceptions are perhaps some cave-dwelling and abyssal species, although many of the latter do exhibit bright red pigmentation. These uninuclear cells can contain a single pigment granule type (e.g., a monochromatic yellow chromatophore) or several different pigment granule types (e.g., a polychromatic yellow and blue chromatophore containing separate and distinct yellow and blue pigment granules; see Robison and Charlton 1973, McNamara and Sesso 1983). Chromatophores are often grouped together into multicellular effectors of a dozen or so intimately juxtaposed cells known as a chromatosome (*sōma*, "body"; see Fig. 3.2A,B). These, in turn, may consist of chromatophores of a single color (e.g., a monochromatic red chromatosome) or of several different colors (e.g., a polychromatic yellow and brown chromatosome composed of separate monochromatic yellow and brown chromatophores; see Elofsson and Kauri 1971, McNamara 1981, Bauer 2004; see Fig. 3.2C).

Chromatophores and chromatosomes are found mainly within the integumental epidermis, often protruding into the hemolymph space lined by the basal lamina. They also occur within the fibrous envelopes that cover the internal organs that constitute the reproductive (ovary; see Fig. 3.2B), somatogastric (hepatopancreas, stomach, hindgut; McNamara 1979), and, particularly, the central nervous system (ganglia and ventral nerve cord; McNamara and Sesso 1982, 1983). Highly differentiated pigment cells are also found within the crustacean visual system. Such screening pigments can take the form of nonvisual accessory cells that are associated with the ommatidia. These contain the distal retinal pigments within the elongated distal pigment cells that surround the crystalline cones and ommatidia and the reflecting pigments found in specialized cells that envelope the retinula cells (Fernlund 1976). These pigments exhibit minimal translocation ability and shield the retinula cells from extraneous photons. The proximal screening pigments, however, constitute part of the ommatidia themselves and are located within the retinula cell cytoplasm, through which they migrate to different degrees depending on the species. This visual pigment system is touched on only briefly in the final part of this review.

Chromatosomes, chromatophores, and their pigments constitute the primary means by which crustaceans attune themselves chromatically to their surroundings. This pigmentary system is used in a variety of ways, including species-specific signaling, aposematic signaling, mate attraction, reproductive strategies, protection against ultraviolet (UV) radiation, and thermal regulation, for example. The pigmentary system also responds to circadian and tidal rhythms in species like the fiddler crab (Thurman 1990). A particularly characteristic function salient in the Crustacea—blending into the background or camouflage, be it through mimicry or crypsis—is achieved by the differential distribution of pigment granules within the chromatophore cytoplasm as the individual organism passes from one specific chromatic background to another; that is, rapid or *chromomotor* chromatic adaptation. Thus, when on dark-colored backgrounds, a shrimp's dark pigments will

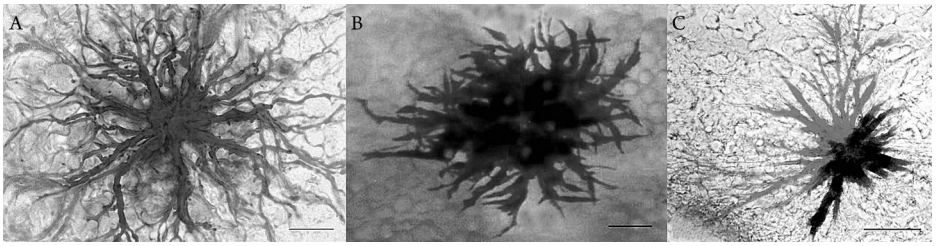


Fig. 3.2.
Chromatosomes consist of multicellular aggregates of highly asymmetrical, single-celled, monochromatic chromatophores. Their intimately apposed, semispherical perikarya contain the pigment granules and form the chromatosome center from which radiate the long chromatophore extensions through which the granules migrate. (A) Monochromatic, red, epidermal chromatosome from the neotropical freshwater shrimp, *Macrobrachium olfersi*. (B) Monochromatic, red chromatosome, showing the nuclei of individual chromatophores (round clear areas within the dark red perikarya) on the dorsal surface of the fibrous capsule containing the ovary of *Macrobrachium olfersi*. These particular pigmentary effectors have been employed with success as models in deciphering the mechanisms of membrane signal transduction, intracellular second-messenger cascades, and regulation of the molecular motors that translocate the pigment granules through the cytosol. (C) Dichromatic, epidermal chromatosome from the New Zealand intertidal shrimp, *Palaemon affinis*, consisting of separate monochromatic yellow (left) and monochromatic red/brown chromatophores (right) containing fully dispersed and partially dispersed pigments, respectively. Scale bars = 50 μm. See color version of this figure in the centerfold.

tend to disperse while its light pigments will tend to aggregate. Conversely, as the animal moves to a lighter background like sand, say, from a darker encrusted rock, its light pigments will disperse while its dark pigments may aggregate (Fig. 3.3). These events can take place within a few minutes and are hormonally mediated because crustacean chromatophores are not innervated.

In contrast, should the individual organism be restricted to a particular background, a slower process of chromatic adjustment ensues (Robison and Charlton 1973). To illustrate, an individual organism's color scheme can exhibit a considerable degree of morphological plasticity, and populations of a single species naturally residing on differently colored substrata may show corresponding chromotypes or different chromotype frequencies (Bauer 1981). Thus, constituent chromatophores may be lost or gained, particularly over the internal organs, whereas epidermal chromatophores may be added during larval ontogeny and on metamorphosis to the postlarval stage, consistent with changes in the chromatic characteristics of the new habitat. Furthermore, the color of the pigments contained within the chromatophores may become altered as a result of catabolic and anabolic metabolic processes, with one pigment type being synthesized while another is degraded. These phenomena, occurring within the individual's lifetime, are known collectively as *chromogenic adaptation* because they are regulated at the level of gene expression.

Chromatophore Organization, Microanatomy, and Ultrastructure

Vertebrate chromatophores originate from the embryonic neural crest (Hall 2008), and "neural crest-like" pigment cell precursors occur in Urochordata and Hemichordata (Jeffery et al. 2004). In contrast, pigment cells are of endomesodermal origin in invertebrates (Hall 2000, Bronner and LeDouarin 2012). Findings refer mainly to the Echinodermata (Oliveri et al. 2008) whose chromatophore structure and physiology are not homologous with the Crustacea. The specific embryonic origin and migratory routes of crustacean chromatophores are yet to be elucidated.

Crustacean chromatophores are highly asymmetrical cells that contain a single nucleus and consist of a spherical perikaryon of approximately 20 μm in diameter from which extend one or two long cell processes of up to 100 μm in length. These subdivide and ramify among the surrounding

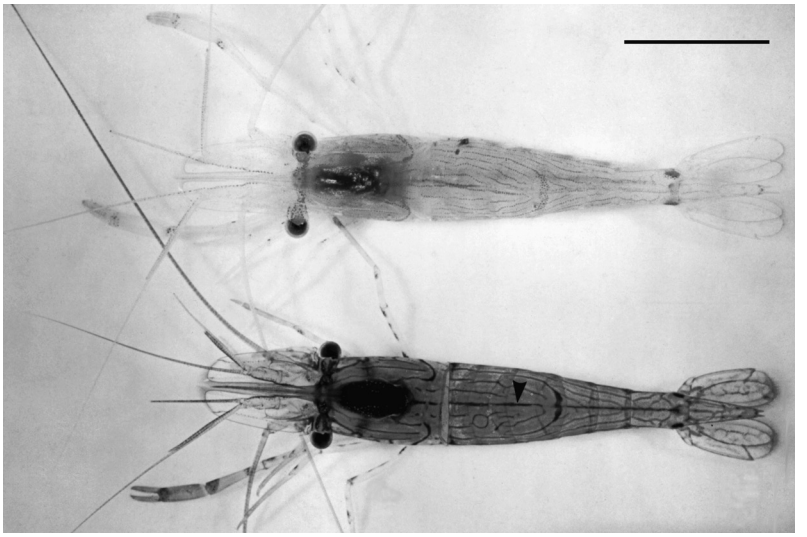


Fig. 3.3.
Chromomotor adaptation in *Palaemon affinis*. The nearly transparent upper shrimp was exposed to an illuminated white background for 2 h, and the brown pigments in its epidermal chromatosomes are fully aggregated; the white somatogastric chromatosome pigments are fully dispersed. The lower shrimp, in which the species' typical chromogenic pattern is clearly visible, was placed on an illuminated black background and displays fully dispersed brown epidermal chromatosome pigments; arrowhead indicates fully dispersed hindgut chromatosomes. Such rapid translocations of the pigment granules through the chromatophore cytosol are regulated by blood-borne neuropeptides called *chromatophorotropins*, such as red pigment concentrating hormone, released from the eyestalk and other neurosecretory centers. Scale bar = 1 cm. See color version of this figure in the centerfold.

epidermal cells (or fibrocytes in the case of internal organ capsules) about 50 μm after leaving the perikaryon. Groups of 10–20 chromatophores are characteristically juxtaposed into multicellular chromatosomes tightly linked by desmosome-like intercellular junctions in the regions of contact between their adjacent perikarya (McNamara 1981). These multicellular pigmentary effectors can attain up to 300 μm in diameter and are easily visible to the naked eye (Fig. 3.2).

In early chromatophores in the process of coalescing into larger chromatosomes, such as those found in the larval stages, numerous pigment granules abound, and both the smooth and rough endoplasmic reticulum are well developed, as are numerous microtubules and occasional mitochondria and polysomes (McNamara 1989). The rough endoplasmic reticulum virtually disappears in fully developed chromatophores, whereas the smooth endoplasmic reticulum (SER) takes the form of a tri-dimensional network that ramifies throughout the cytoplasm, becoming further differentiated into a regular, transversely interconnected, scalariform conformation in the cell extensions, much as seen in the axons of crustacean neurons (McNamara 1981, McNamara and Taylor 1987, McNamara and Ribeiro 1999). The tri-dimensional cisternae are often encountered in structural continuity with the pigment granules in a variety of chromatophore types (McNamara 1980).

In the planar epidermal chromatophores, bundles of 25 nm diameter microtubules abound within the cell extensions to which they lie parallel, often occupying a central, core-like position among the dispersed pigment granules (Elofsson and Kauri 1971, Robison and Charlton 1973, McNamara 1980). Hefty microtubule bundles are also arrayed throughout the perikaryon (see fig. 22 in McNamara and Taylor 1987). This organization of the cytoskeleton appears to be mainly scaffold-like in function since, curiously, microtubule-disrupting agents like colchicine and vinblastine do not affect pigment aggregation at pharmacological concentrations

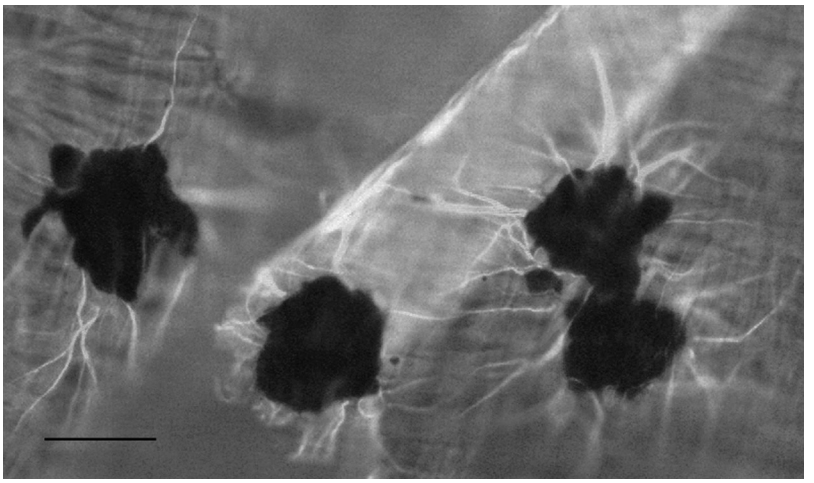


Fig. 3.4.
Epifluorescence microscopy (Leica DM5500B, λ_{ex}=495 nm, λ_{em}=519 nm) showing several red chromatosomes with fully aggregated pigments from the ovary of *Macrobrachium olfersi*. Elongated microtubule bundles (green) lie along the length of the pigment-free cell extensions that project from the perikarya of the individual constituent chromatophores among the surrounding fibroblasts. Monoclonal, anti-β-tubulin primary antibody followed by an Alexa-488 conjugated goat anti-mouse IgG. Scale bar = 100 μm. See color version of this figure in the centerfold.

(Robison and Charlton 1973, Fingerman et al. 1975, Lambert and Fingerman 1976, McNamara 1980). In the chromatophores found within the fibrous capsules positioned around the often-mobile internal organs, microtubules are far fewer and not organized into obvious bundles (McNamara and Ribeiro 1999). However, these longitudinal arrays of tubulin polymers do bind fluorochrome-conjugated antitubulin antibodies, revealing an unequivocal role in supporting and delineating the long cell extensions (Fig. 3.4). In such chromatophores, an actin cytoskeleton sensitive to cytochalasin B predominates throughout the cytoplasm, with the individual actin filaments lying mainly parallel to the cell extensions (Robison and Charlton 1973, McNamara and Ribeiro 1999). The actin cytoskeleton of crustacean chromatophores has been little explored from the structural and functional points of view, although the myosin motors that employ the actin networks to translocate the pigment granules through the cytosol have been partially characterized (Boyle and McNamara 2006).

THE HORMONAL REGULATION OF PIGMENT DISTRIBUTION WITHIN CHROMATOPHORES

The Perception of Environmental Cues

Crustacean chromatophores are not innervated. Rather, they receive neurosecretory hormonal signals derived from the transduction by the nervous system of information predominantly concerning the chromatic and photic environment.

Light reaches the eyestalk ommatidia both directly, impinging on the dorsal surface, and indirectly, reflected from the substratum to the ventral surface. The difference in light intensity, detected by the visual pigments in the retinula cells, constitutes an *albedo ratio*; that is, a measure of the reflectivity of the surface on which the organism is located. An elevated albedo

ratio characterizes a highly reflective, light-colored or whitish substratum, whereas a low albedo ratio signifies little reflection and thus a dark-colored surface. Integration in the optic neuropils and cerebral ganglion thus furnishes the individual crustacean with a notion of overall substratum reflection. This information is passed via afferent innervation to the perikarya of neurosecretory cells that constitute the various neurosecretory nuclei associated with their respective neurohemal organs, the interfaces of distribution between the neurosecretory axon terminals and the hemolymph. The main neurohemal organ linked to color change in the Crustacea is the X-organ/sinus gland (XO-SG) complex located in the eyestalk *medulla terminalis*, although the tritocerebral commissure/postcommissural organ and ventral nerve cord ganglia are also important sources of chromactivating neurosecretions. The axons of adjacent neurosecretory perikarya form short tracts like the XO-SG tract that lead to the capillary spaces on which their axon terminals abut and replete with neurosecretory peptides.

Thus, under appropriate stimuli, originating in the perception of ambient chromatic conditions, reflectivity, and luminosity, the neurosecretory perikarya receive and integrate stimulatory and inhibitory neurotransmissions leading to membrane depolarization, action potential propagation, neurosecretory vesicle fusion with the axon terminal membrane, and the discharge of neurosecretory peptide molecules into the extracellular space near the capillaries. From here, these chromactivating substances diffuse to and are carried via the hemolymph throughout the tissues where they are recognized by specific receptors on the chromatophores. Many neurotransmitters are involved in the further fine regulation of chromactivating neuropeptide release. In the fiddler crab *Uca pugilator* dopamine induces the release of pigment-aggregating hormones and results in aggregation of the red and black epidermal chromatophore pigments (Quackenbush and Fingerman 1984), whereas 5-hydroxytryptamine has the opposite effect (Rao and Fingerman 1983). In contrast, norepinephrine enhances the release of a pigment-dispersing hormone (PDH), causing dispersion of the black chromatophore pigments (Rao and Fingerman 1983, Quackenbush and Fingerman 1984).

Fish and amphibian melanophores can respond directly to UV light by melanosome dispersion (Hunter et al. 1979, Oshima 2001), and such nonhumorally mediated "primary" responses also occur in crustaceans (see Thurman 1988 for review). When exposed to near UVA radiation, pigment granules disperse in black chromatophores of eyestalkless crabs *U. pugilator* (Coohill et al. 1970) and *Neohelice granulata* (Gouveia et al. 2004) in situ. UVA also induces granule dispersion in chromatophores of intact *U. pugilator* (Coohill and Fingerman 1975) and shrimp *Palaemonetes argentinus* (Gouveia et al. 2004). Thus, like retinular cell rhabdomeric membranes (Vargas et al. 2008) and mammalian retinal ganglion cells (Peirson et al. 2009), crustacean chromatophore membranes may contain a light- or UV-transducing opsin.

This primary chromatophore response to light/UV resembles photoreception by the "intrinsically photosensitive retinal ganglion cells" of the mammalian inner retina (Bellingham et al. 2006, 1334), a small subset of which expresses melanopsin (Peirson et al. 2009), an opsin/vitamin A-based photopigment that detects light by photon absorption and triggers a G-protein-mediated phototransduction cascade (Bellingham et al. 2006, Peirson et al. 2009). Photosensitive amphibian melanophores express melanopsin (Provencio et al. 1998), and fish, bird, and lizard genomes contain related sequences (Bellingham et al. 2006, Peirson et al. 2009). Thus, vertebrate melanopsin-based and crustacean primary light/UV photosensitivities may well be evolutionary linked because opsins have been highly conserved during evolution of the Bilateria (Arendt et al. 2004).

Chromatophorotropins: Chromactivating Neurosecretory Peptides

The antagonistic neurosecretory peptides that regulate pigment aggregation and dispersion in the Crustacea are known generically as *chromatophorotropins* and take the form of small peptide

chains of a dozen or so amino acids. The notion that blood-borne chromactivating substances can regulate pigment movement has its origin in pioneering research performed some 100 years ago (Pouchet 1872, Köller 1927, Perkins 1928), investigations that provided the first evidence for the hormonal regulation of physiological processes in crustaceans. Although many different physiological assays for a variety of pigmentary factors located in the neurosecretory cells of the eyestalk medulla, tritocerebral commissure, and ventral nerve cord of many different crustacean species have been developed over the years (Brown 1948, Fingerman 1969, 1970, 1985), the final biochemical characterization and identification of a crustacean chromatophorotropin came only much later.

Red pigment concentrating hormone (RPCH) is an octapeptide originally isolated, identified, and sequenced (pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2) from the eyestalks of the caridean shrimp *Pandalus borealis* (Fernlund and Josefson 1968). It is considered by some to be a universal, plesiomorphic pigment concentrating hormone (PCH) of widespread occurrence throughout the Crustacea because this peptide consistently promotes the aggregation of the red and dark-colored pigments, even in its synthetic form (Josefsson 1983, Rao 1985). Indeed from the 1980s on, few investigators extracted their own chromatophorotropins from neurosecretory tissue—a tedious prerequisite to most studies on signal transduction and crustacean color change—relying instead on synthetic RPCH available from commercial laboratories.

The hormone antagonistic to RPCH, that is, red PDH (RPDH) or PDH, is an octadecapeptide that exists as two isoforms, α-PDH (Asn-Ser-Gly-Met-Ile-Asn-Ser-Ile-Leu-Gly-Ile-Pro-Arg-Val-Met-Thr-Glu-Ala-NH_2; also isolated [Fernlund 1971] and identified and sequenced from *P. borealis* eyestalks [Fernlund 1976, Josefsson 1983]) and β-PDH (Asn-Ser-Glu-Leu-Ile-Asn-Ser-Ile-Leu-Gly-Leu-Pro-Lys-Val-Met-Asn-Asp-Ala-NH_2) isolated from the eyestalks of the fiddler crab *U. pugilator* (Rao et al. 1985), shore crab *Cancer magister* (Kleinholz et al. 1986), and the blue crab *Callinectes sapidus* (Mohrherr et al. 1990). PDH effects are only briefly mentioned here because this review focuses primarily on caridean shrimps in which RPCH evokes pigment aggregation. In *Uca rapax*, both α- and β-PDH induce dose-dependent pigment dispersion, although in the isopod *Ligia exotica* only α-PDH triggers dose-dependent pigment dispersion while β-PDH brings about a partial, dose-independent response. In the freshwater shrimp *Macrobrachium acanthurus* α-PDH causes dose-dependent partial dispersion, but β-PDH is without effect (Tuma et al. 1993). Both α- and β-PDH induce dose-dependent pigment dispersion in *M. potiuna* (Britto et al. 1990) whereas in *M. olfersi*, α-PDH triggers partial pigment dispersion (50%) followed by pigment aggregation (Ribeiro 1998). Curiously, when the eyestalks are removed from otherwise intact shrimps or when caridean chromatophores are isolated from their source of circulating RPCH in physiological preparations in vitro the red and dark chromatophore pigments tend to disperse rapidly and spontaneously (Fingerman et al. 1975, McNamara and Taylor 1987, Tuma et al. 1993); in contrast, in brachyuran crabs treated similarly, the dark pigments aggregate in the absence of PDH (Lambert and Fingerman 1976, Kulkarni and Fingerman 1986). In crustaceans in vivo, the chromatophorotropins RPCH and PDH are secreted to revert pigment distribution from the respective passive states of unstimulated dispersion and aggregation to a hormonally induced, actively aggregated or dispersed condition. This intriguing situation has impacted widely on research in crustacean color change, and few studies, thus, have dealt with pigment dispersion in the Caridea or with pigment aggregation in the Brachyura. In the Caridea, at least, pigment aggregation is experimentally reversible simply by washout of RPCH or by lowering extra- and intracellular calcium (McNamara and Ribeiro 2000; Figs. 3.5 and 3.6), and it is indeed mystifying that pigment dispersion can be brought about experimentally without recourse to a PDH.

Kleinholz et al. (1962) demonstrated that PDH is the same substance as the distal retinal pigment hormone (DRPH) mentioned later, after noting that PDH induced granule migration to the

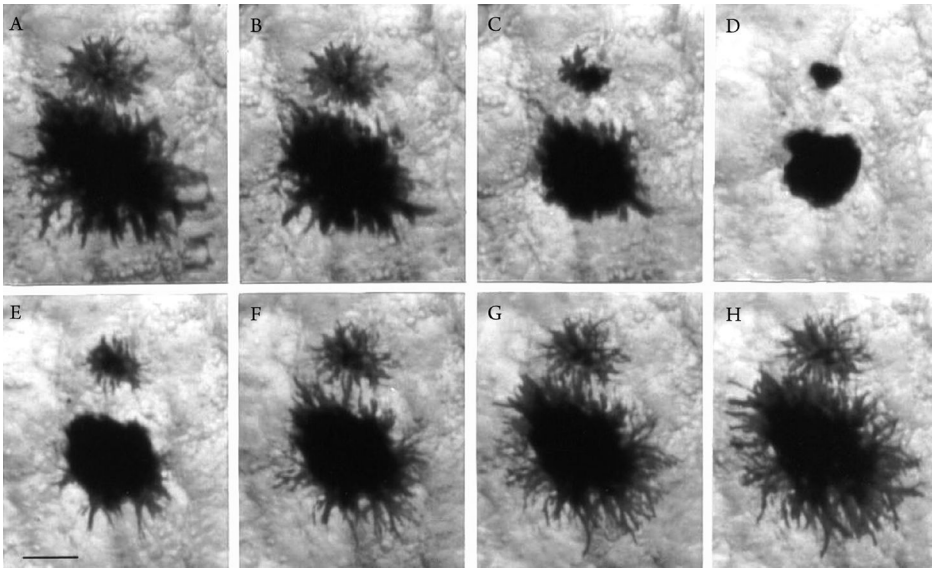


Fig. 3.5.
Sequence of pigment aggregation (A–D) in two red chromatosomes on the ovary of *Macrobrachium olfersi* induced by 30 min perfusion in vitro with 25 μM Ca^{2+}-ionophore A23187 in a 2.5 mM $NaHCO_3$-buffered physiological saline. The pigments were subsequently dispersed (E–H) by 30 min perfusion with a Ca^{2+}-free (10^{-11} M Ca^{2+}), 2 mM EDTA-chelated saline. The pigment translocation rates are not constant (see Fig. 3.6). Scale bar = 50 μm. See color version of this figure in the centerfold.

light-adapted position in the retinal pigment cells of many arthropod species. Thus, PDH is now also known for inducing distal pigment granule movements in the crustacean retina (Kleinholz et al. 1986, Verde et al. 2007).

PDH or factor (PDF) also has been widely implicated in the generation, expression, and synchronization of circadian activity in insects where it plays a role as a neurotransmitter and as a neuromodulator (Verde et al. 2007). The ubiquity of PDF in the insect circadian system strongly suggests a role similar to that of PDH in the Crustacea, where PDH is present both as a neurohormone released from the SG into the circulation and as a neuromodulator and neurotransmitter (Verde et al. 2007).

The crustacean pigment-aggregating chromatophorotropins belong to a larger class of arthropod hormones known as the adipokinetic hormone (AKH)/RPCH family of structurally related, functionally diverse peptides, and RPCH itself is also found in several insect taxa (Gäde et al. 1997, 2003, Rao 2001, Gäde 2009, Zralá et al. 2010). AKHs play an important role in regulating the availability of metabolic substrates in insect flight muscles (Gäde and Marco 2009), a function also attributed to RPCH in isopod muscle glucose metabolism (Zralá et al. 2010). Furthermore, the source of both these neuropeptide hormones in the arthropods (i.e., neurosecretory cells in the *corpora cardiaca* of insects and the eyestalk XO-SG complex in crustaceans) are analogous to the vertebrate hypothalamus/neurohypophysis neurosecretory system (Scharrer and Scharrer 1944). As the resemblance in signaling pathways and intracellular cascades triggered by these peptide neurosecretions is clarified later, it will become apparent that many components of the respective signal transduction mechanisms have been conserved, and the apparent differences between insect and crustacean hormone actions will fade, simply constituting a likely Pan-crustacean (Regier et al. 2010) synapomorphy.

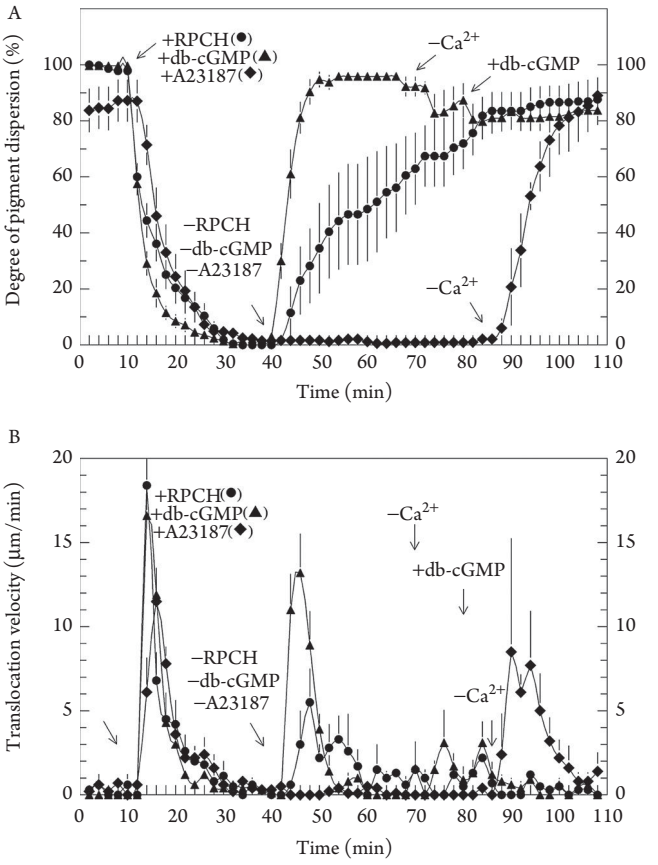


Fig. 3.6.
Remarkable similarity in pigment aggregation profiles triggered by red pigment concentrating hormone (30 nmol/L RPCH, ●), by dibutyryl cyclic guanosine monophosphate (10 μmol/L db-cGMP, ▲) and by the Ca^{2+} ionophore A23187 (25 μmol/L A23187, ♦) in *Macrobrachium olfersi* red ovarian chromatophore preparations perfused in vitro with a Ca^{2+}-containing (5.5 mmol/L) $NaHCO_3$/HEPES-buffered physiological saline. Pigment dispersion was induced by perfusion with saline alone (-RPCH, -cGMP) or with a comparable 2 mmol/L EDTA-chelated/Ca^{2+}-free saline (-Ca^{2+}). (A) Degree of pigment dispersion. (B) Pigment translocation velocities. All effectors produce similar although distinct kinetic phases of pigment aggregation (rapid and slow phases), suggesting crosstalk between the different intracellular effector cascades; the cGMP effect is Ca^{2+}-dependent. Pigment dispersion kinetics on RPCH washout are intermediate between those after cGMP and Ca^{2+} washout. Data are the mean ± SEM, N = 7. Redrawn from McNamara and Ribeiro (2000) and Ribeiro and McNamara (2009).

CHROMATOPHOROTROPINS AND CHROMATOPHOROTROPIN-LIKE HORMONES

Neurosecretory Peptides in Closely Related Groups: Crustaceans and Insects

Interestingly, in addition to AKH, several other arthropod hormones produce chromatophorotropic effects. Crustacean cardioactivating peptide (CCAP) and vertebrate melatonin trigger pigment dispersion in *M. potiuna* red epidermal chromatophores, modulating the response to PDH; in fact, CCAP produces a response threefold stronger than PDH (Nery and Castrucci 1997). Functional substitutions among hormones also suggest their close structural relationships. AKH

shares sequence homology with corazonin (CRZ), a substance to which it is probably ancestrally related (Mercier et al. 2007). CRZ is produced by the lateral brain neurosecretory cells that project into the *corpora cardiaca* of many insects, as well as in a few protocerebral brain neurons (Mercier et al. 2007). CRZ stimulates heart rate and the contraction of hyperneural muscles, acts as a pigmentation-controlling factor in locusts (Hua et al. 2000), and is an initiator of ecdysis behavior (Kim et al. 2004, Žitňan et al. 2007). The multiplicity of neuropeptides found in the invertebrates and the coexistence of diverse structurally and functionally related hormones appears to enable fairly simple nervous systems to perform complex chemical signaling and to enhance their capability to develop and carry out information processing (Gerarts et al. 1992, Nässel 1996).

Other neurohormones, like the crustacean hyperglycemic hormone (CHH) and the molt-inhibiting hormone (MIH) are also produced by the XO neurosecretory perikarya and released by the SG complex. However, these neuropeptides are structurally very distinct from the chromatophorotropins, containing 72–78 amino acids (Mercier et al. 2007). Although they perform very different functions, these hormones induce their intracellular responses through very similar signaling cascades. For example, they effect their cellular responses by increasing cytosolic cyclic guanosine monophosphate (cGMP) concentration (Lee et al. 2007), one of the principal intracellular second-messengers involved in pigment translocation whose role and mode of action is discussed later. Also, in MIH signaling, cGMP is produced via the cytosolic guanylyl cyclase (GC; Lee et al. 2007), rather than by a membrane-linked GC receptor, as is also the case in crustacean chromatophores (Ribeiro and McNamara 2009).

The triggering of very similar intracellular pathways by a diversity of neuropeptide hormones may indicate the prevalence of a single family of receptors present in different physiological systems among the Crustacea and Insecta, thus suggesting the putative evolution of very specific agonist recognition mechanisms and subsequent action through complex processes of signal transduction. Arthropod neuropeptides seem to have co-evolved with their receptors, differently from the situation seen, for example, in the relationship between their biogenic amines and receptors that do not appear to have varied much over the course of evolution in this group (Mercier et al. 2007).

Genetic Coding for the Arthropod AKH/RPCH Family of Peptides

Little is known of the biological function and the regulation of expression of the crustacean chromatophorotropin-encoding genes, as is also true of arthropod neurohormones (Lewis et al. 1997, Rao 2001, Lee and Park 2004). Information on arthropod neurohormone biosynthesis deals mainly with prepro-hormones and their processing (Chin et al. 1990, O'Brien and Taghert 1994). Although the AKH/RPCH peptide family is highly conserved, currently available sequence data are insufficient to disclose clear arthropod evolutionary relationships (Rao 2001). Mature peptide and prepro-hormone sequences are highly conserved not only in arthropods, but also in unrelated species like the nematode *Caenorhabditis elegans* and in soybean and pea seeds. Alignment findings for a prepro-AKH precursor reveal a significantly lower percentage identity, suggesting that, to allow effective function in different organisms, the mature peptide must be highly conserved, whereas the prepro-peptide may be less well conserved to play its role (Taub-Montemayor et al. 1997).

Although numerous cDNAs encoding insect AKH/RPCH precursor peptides are known (e.g., *Locusta migratoria, Schistocerca gregaria, Schistocerca nitans, Blaberus discordalis, Manduca sexta*, and *Drosophila melanogaster*), only three cDNAs have been described from crustaceans: the crabs *Carcinus maenas* and *C. sapidus* and the crayfish *Cherax quadricarinatus* (Martínez-Pérez et al. 2005). The prepro-RPCH from *C. maenas* consists of a 25-amino acid signal peptide, an 8 amino-acid RPCH segment, and a 74 amino-acid RPCH-precursor-related peptide (RPRP; Linck et al. 1993). Compared to the prepro-hormone structure of AKH (O'Shea and Rayne 1992), this RPCH precursor shows a high percentage identity in the region corresponding to the

neuropeptides RPCH and AKH, but a low percentage identity for the precursor-related peptides RPRP and AKH-precursor-related peptide (De Kleijn and Van Herp 1995). The same is also true of other neuropeptides because the cDNA encoding PDH in penaeid shrimp reveals that, although the precursor peptide structures are significantly different, the mature peptides are closely related (Ohira et al. 2006). Thus, elevated percentage identity appears to be restricted to the peptide hormone sequences while the precursor-related peptides are extremely variable, except within groups of very closely related species (Linck et al. 1993). The fact that RPRP is not conserved suggests the lack of an important physiological function (De Kleijn and Van Herp 1995). All AKH and RPCH precursors, however, share a basic architectural plan: the encoding region is subdivided into a signal peptide sequence, the hormone itself, and a precursor-related peptide (Klein et al. 1995).

The gene for the *C. sapidus* RPCH precursor contains an intervening element in the RPCH-PRP coding region, an intron, not identified so far in any other crustacean species. Such introns are present in the genes coding for insect AKH precursors, however. Although the location of this intron is similar to the second intron in the *S. nitans* AKH gene, discrete differences in intron structure between insect (*D. melanogaster* and *S. nitans*) and *C. sapidus* genes are present; the biological relevance of such features is unknown. The *C. sapidus* intron, although exhibiting certain differences with insect AKH encoding genes, shows important similarities with other regions of the RPCH encoding gene. Nevertheless, important differences in the RPCH encoding genes do exist among the arthropods, and the size of the introns suggests the participation of different intronic and exonic elements during splicing to form the precursor mRNA (Martínez-Pérez et al. 2002).

Compared to noncrustacean arthropods then, the estimated amino acid identity of a 590-bp RPCH precursor from *C. quadricarinatus* to insect AKHs (AKH I, II, and III) and hypertrehalosemic hormone (HTH) ranges from 36% to 62% (Martínez-Pérez et al. 2005; see Table 3.1). Within the decapod Crustacea, however, the conceptual translation of a 242-bp cDNA RPCH fragment from *C. quadricarinatus* is 70% identical to that of the crabs *C. sapidus* (Klein et al. 1995) and *C. maenas* (Linck et al. 1993; see Table 3.1).

The isolation and characterization of the *B. discoidalis* HTH gene provided the first description of an AKH/RPCH family prepro-hormone from cockroaches (Lewis et al. 1997). Thus, although cockroaches were distinct from other arthropods by 300 mya (Kukalová-Peck 1990), their HTH precursors have retained the simple organization of the AKH/RPCH prepro-hormones seen in

Table 3.1. Percentage identities of various arthropod AKH/RPCH neuropeptide hormone precursors with the amino acid sequence predicted from a 590-bp cDNA sequence for precursor RPCH from the crab *Cherax quadricarinatus*. See text for references.

Peptide hormone	Species	Identity (%)
RPCH	*Callinectes sapidus*	70
	Carcinus maenas	70
AKH I	*Schistocerca gregaria*	62
	Locusta migratoria	41
AKH II	*Schistocerca nitans*	48
AKH III	*Locusta migratoria*	36
	Drosophila melanogaster	36
HTH	*Blaberus discoidalis*	50

RPCH, crustacean red pigment concentrating hormone; AKH I, II and III, insect adipokinetic hormones I, II and III; HTH, insect hypertrehalosemic hormone.

highly diverged insects and in a distantly related crustacean. Apparently, the tripartite structure of AKH/RPCH family precursors (signal peptide, hormone plus processing site, and C-terminal peptide of variable length and unknown function) arose early in arthropod evolution and has been conserved across broad taxonomic groups (Lewis et al. 1997, Gäde 2004). Although functions have been attributed to signal peptides and hormone segments within AKH/RPCH family precursors, the significance of the C-terminal peptides is obscure. With the exception of crustacean RPCH, C-terminal peptides from various insect species are similar in size but show only 25–35% amino acid positional identity between orders. In comparison, the biologically active peptide segments within the insect prepro-hormones are 44–88% identical, whereas the signal peptides show 16–45% amino acid positional identity (Lewis et al. 1997).

The amino acid sequences of prepro-PDH from the crayfish *Orconectes limosus* (De Kleijn et al. 1993) and from the crabs *C. maenas* (Klein et al. 1992) and *C. sapidus* (Klein et al. 1994) have been deduced from cDNAs obtained employing degenerate primers based on a partial amino acid sequence for *U. pugilator* PDH. The PDH peptides from *O. limosus, C. maenas*, and *C. sapidus* (PDH I) differ by only a single amino acid (aspartic acid for glutamic acid), showing 94% identity, whereas PDH II from *C. sapidus* has up to six different amino acids, corresponding to 64% identity. The preceding RPRPs and signal peptides show 41% and 42% identity, respectively. Given overall prepro-hormone structure in the Crustacea, their prepro-PDHs seem to be well conserved but show no similarity with other known peptides (De Kleijn et al. 1993, De Kleijn and Van Herp 1995).

Like many vertebrate prepro-hormones, such as insulin, post-translational modification of prepro-neuropeptides is common in the Pan-Crustacea and extends to the AKH/RPCH and hyperglycemic (CHH) peptides. In insect AKHs, this usually includes two essential alterations: a Gln to pyro-Glu exchange at the N-terminus and a carboxyamide group at the C-terminus (Gäde et al. 2007). Species-specific modifications include, for example, a conserved Leu2 for Val2 exchange in water scorpion (*Nepa cinerea*) and Ser6 for Thr6 exchange in cricket (*Gryllus bimaculatus*; see Nair et al. 2001, Gäde et al. 2007). In the crayfish *O. limosus*, two distinct genes code for CHH prepro-hormone isoforms that differ slightly in their signal and precursor-related peptides but are identical in CHH coding region (De Kleijn et al. 1994); the two CHH isoforms expressed result from post-translational modifications.

CELL SIGNALING AND PIGMENT MIGRATION IN CHROMATOPHORES

The Chromatophorotropin Receptor

Investigations aiming to identify the RPCH receptor type present in the plasma membrane of crustacean chromatophores have been initiated only very recently. The red ovarian chromatophores of *Macrobrachium olfersi* respond only partially to RPCH when previously perfused in vitro with a generic G-protein antagonist (pGlu-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH_2; Milograna and McNamara 2009a). This constitutes reasonable evidence that RPCH binding to a seven-domain membrane-spanning G-protein-coupled receptor (GPCR) induces pigment aggregation. The AKHs, structurally related to RPCH, also activate intracellular cascades after binding to GPCRs (Van der Horst et al. 1999). AKH receptors cloned from *D. melanogaster* and *Bombyx mori* (Staubli et al. 2002) are structurally related to the GPCR receptors for vertebrate gonadotropin-releasing hormone (Gn-RH). The AKHs that activate glycogen phosphorylase in insect flight muscles act via membrane-located protein G_q and phospholipase C_β, increasing inositol trisphosphate (IP_3) and leading to Ca^{2+} release from IP_3-gated intracellular stores and extracellular influx. In contrast, the lipase-activating AKHs increase protein G_s and adenylyl cyclase (AC) activities, thus elevating intracellular cAMP together with Ca^{2+} release from IP_3-gated stores and extracellular influx (Gäde 2004).

Signal Transduction at the Chromatophore Membrane

Despite notable downstream functional diversity, the initial phase of GPCR signal transduction is fairly uniform: the activated receptor catalyzes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the stimulatory G-protein α subunit ($G_{s\alpha}$), leading to dissociation among the $G_{s\alpha}$-GTP and $G_{\beta\gamma}$ subunits and enabling them to activate or inhibit a diversity of effector proteins ranging from membrane-associated catalytic enzymes and ion channels to soluble second-messenger-producing enzymes (Natochin et al. 2001, Gurevich and Gurevich 2008). The classic enzyme system regulated by GPCRs is the membrane-located AC that catalyzes cAMP production from ATP and its release into the cytosol. Adenylyl cyclase is activated by protein $G_{s\alpha}$ and inhibited by protein $G_{i\alpha}$, whereas the upstream GPCRs respond to diverse external stimuli like light and odorants and to various endogenous cues such as hormones, neurotransmitters, extracellular Ca^{2+}, and enzyme activity (Gurevich and Gurevich 2008).

In addition to the recent investigations on RPCH and AKH action just described (Van der Horst et al. 1999, Staubli et al. 2002, Gäde 2004), other evidence concerning the nature of their signaling cascades suggests that crustacean chromatophorotropins function in a classic manner via G-protein transduction (Milograna and McNamara 2009a). Pigment translocation in crustacean chromatophores can be induced by an increase in cytosolic Ca^{2+}, leading to protein phosphorylation/dephosphorylation owing to downstream activation of Ca^{2+}-regulated specific kinases and/or phosphatases. Ca^{2+} signaling often occurs concomitantly but not necessarily simultaneously with a decrease or increase in cytosolic cAMP or cGMP concentration, brought about by crosstalk between the respective signaling cascades. This phenomenon varies widely among the crustacean orders and depends on chromatophore type; it also varies moderately even at the species level. These signaling cascades are discussed in detail in the following sections. The salient point here is that most chromatophorotropin signaling cascades tend to be characteristic of GPCR signaling.

Chromatophore Membrane Ion Channels and Calcium Movements

In caridean shrimps, RPCH requires extracellular Ca^{2+} (Ca^{2+}_{ext}) to induce and sustain pigment aggregation (Fingerman 1969, McNamara and Taylor 1987, Britto et al. 1990, McNamara and Ribeiro 1999). The Ca^{2+} ionophore, A23187, a Ca^{2+}-specific mobile ion carrier that allows concentration-dependent selective Ca^{2+} movements, induces complete and reversible pigment aggregation with kinetics similar to RPCH (McNamara and Ribeiro 2000) and has been a useful tool to investigate a role for extracellular Ca^{2+} (Figs. 3.5 and 3.6).

During pigment aggregation, Ca^{2+} derives from both extracellular and intracellular sources, following a sequence of intricate and finely regulated events (Ribeiro and McNamara 2007, Milograna et al. 2010). Overall, after RPCH binds to its receptor in the plasma membrane, Ca^{2+} is released from the SER (Ribeiro and McNamara 2007). Subsequent to this initial increase in intracellular Ca^{2+} (Ca^{2+}_{int}) concentration, and perhaps partly as a direct response to it, the plasma membrane resting potential depolarizes from −76 to −34 mV (Milograna et al. 2010), leading to voltage-regulated Ca^{2+} influx from the extracellular fluid and an intracellular Ca^{2+} signaling cascade (Fig. 3.7).

The complete cellular mechanism that leads to membrane depolarization is not yet clear, but Ba^{2+}-sensitive plasma membrane K^+ channels appear to close shortly after RPCH binding, thus restricting K^+ efflux. The resulting accumulation of positive charges below the cytosolic membrane leaflet reduces the potential difference generated naturally in the unstimulated chromatophore and leads to depolarization of the chromatophore membrane in which voltage-sensitive, N- and/or P/Q-type Ca^{2+} channels then open, allowing Ca^{2+}_{ext} influx (Milograna et al. 2010; see Fig. 3.7).

An important issue here concerns the duality of the Ca^{2+} sources, both extra- and intracellular in nature, on which pigment aggregation depends. The velocity of pigment translocation in

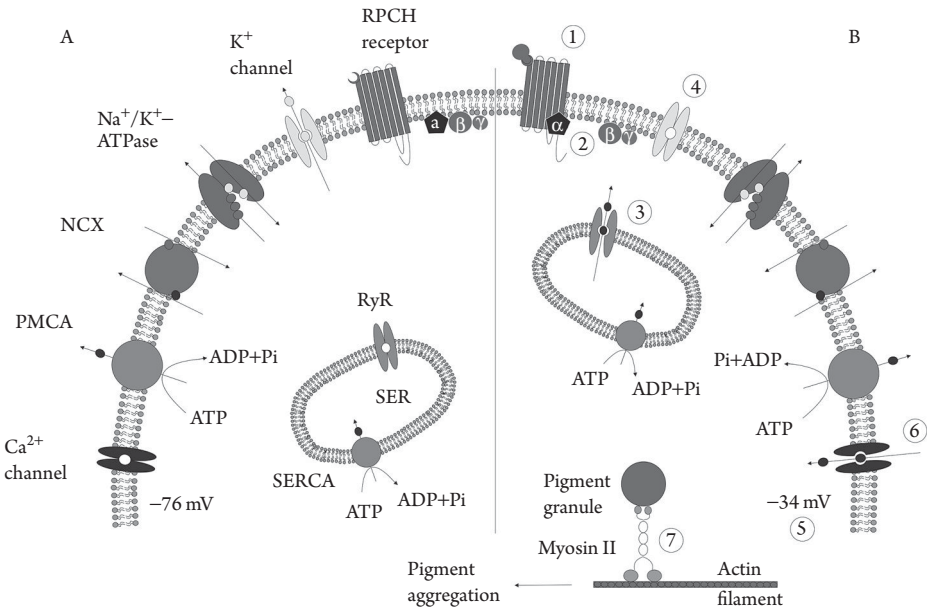


Fig. 3.7.
Model proposed for signal transduction and Ca^{2+}-signaled pigment aggregation after red pigment concentrating hormone (RPCH) binding to its receptor in the chromatophore cell membrane. (A, *left*): Resting state, fully dispersed pigment, RPCH not bound to receptor. Membrane resting voltage (−76 mV) is maintained primarily by the Na^+/K^+-ATPase. K^+ flows outward through open K^+ channels and is recycled by the Na^+/K^+-ATPase that extrudes Na^+. Na^+ influx (Na^+_{ext} = 140 mM) drives Ca^{2+} efflux through a Na^+/Ca^{2+} exchanger (NCX), while plasma membrane (PMCA) and sarco/endoplasmic reticulum (SERCA) Ca^{2+}-ATPases actively transport Ca^{2+} to the extracellular fluid and smooth endoplasmic reticulum (SER) lumen, respectively, maintaining low Ca^{2+}_{int} ($<10^{-9}$ M). The chromatophore membrane is fairly impermeable to Ca^{2+} influx down the Ca^{2+} gradient (Ca^{2+}_{ext} = 5.5 mM) since Ca^{2+} channels are closed. (B, *right*): RPCH bound to putative 7-span G-protein-coupled receptor (GPCR), pigment aggregation initiated. After RPCH binding, the activated GPCR (1) signals a heterotrimeric G-protein, (2) leading to Ca^{2+} release through SER ryanodine receptors (3) via an unknown mechanism. The increased Ca^{2+}_{int} (4) closes membrane K^+ channels, either by a direct Ca^{2+}-dependent effect or through protein kinase C/Ca^{2+}-calmodulin-mediated phosphorylation or by interaction with the G-protein α- or βγ-subunits, thus (5) depolarizing the chromatophore membrane to −34 mV. Voltage-gated N- and/or P/Q-type membrane Ca^{2+} channels then open (6), allowing Ca^{2+} influx from the extracellular fluid down the Ca^{2+} gradient into the cytosol, increasing Ca^{2+}_{int} to $\approx 10^{-3}$ M. The activated intracellular Ca^{2+} and cyclic guanosine monophosphate signaling cascades appear to stimulate Rho protein kinase that then (7) phosphorylates a nonmuscle myosin II molecular motor, resulting in pigment aggregation. From Milograna et al. (2010) with permission from Wiley and Sons, Inc.

palaemonid chromatophores is Ca^{2+} sensitive, or at least increases with increasing Ca^{2+}_{int}, from $<10^{-9}$ M in unstimulated chromatophores with fully dispersed pigments, to $\approx 10^{-3}$ M during pigment aggregation (McNamara and Ribeiro 2000). At 5 mM Ca^{2+}_{ext} (equivalent to the total hemolymph Ca^{2+} concentration), pigment aggregation is almost complete within 5 min of RPCH perfusion in vitro; in ethylene glycol tetraacetic acid-buffered Ca^{2+}-free saline, pigments previously aggregated by RPCH or 1–5 mM Ca^{2+}_{ext} disperse, disclosing a process that responds differentially to variable Ca^{2+} concentrations rather than a simple "on-off" triggering mechanism. Apparently, should the increase in cytosolic Ca^{2+} derive from a single source, the rate of increase in cytosolic Ca^{2+} necessary to trigger the subsequent intracellular Ca^{2+} cascade is not attained as effectively as with a simultaneous or concatenated supply from both sources. Given that Ca^{2+}_{ext} influx obeys a concentration gradient into the cytoplasm, influx capacity may be rate limiting. Furthermore, it may be

energetically expensive for chromatophores to maintain large Ca^{2+} SER stocks, given the paucity of mitochondria and the limited volume of the SER cisternae (McNamara and Ribeiro 1999). Ca^{2+} cycling between the cytosol and the SER thus may be limited, possibly slowing pigment aggregation and dispersion dynamics and affecting the overall efficiency of color change and the camouflage mechanism, for example.

A case in point, like RPCH, during the action of endogenous AHKs employed in both adipokinetic and hyperprolinemic signaling in insects, neither Ca^{2+}_{ext} nor Ca^{2+} from the SER alone is sufficient. The AKH peptide activates phospholipase C_{β}, increasing IP_3 levels and resulting in the depletion of intracellular Ca^{2+} stores, a cue for capacitative Ca^{2+} entry via the plasma membrane (Gäde and Auerswald 2003). Furthermore, extracellular Ca^{2+} is indispensable for AKH activity in many insects and also may be required for hormone receptor binding (Van der Horst et al. 1999).

Caridean chromatophores are endowed with an equally rich Ca^{2+} transport apparatus that enables them to reduce Ca^{2+}_{int} to the low levels attained via the mechanisms just described. This system includes ATP-dependent transport proteins such as the sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) located in the SER membranes, which actively transports free cytosolic Ca^{2+} into the SER lumen. Inhibition of SERCA activity by thapsigargin and cyclopiazonic acid alone, respectively, induces pigment aggregation of between 50% and 100% (Ribeiro and McNamara 2007). The plasma membrane Ca^{2+}-ATPase (PMCA) actively pumps Ca^{2+} up its concentration gradient to the extracellular medium. PMCA inhibition by lanthanum alone induces 12% pigment aggregation (Milograna et al. 2010). This spontaneous pigment aggregation seen on inhibition of the two Ca^{2+}-ATPases results from increased Ca^{2+}_{int}. The plasma membrane Na^+/K^+-ATPase, linked to the Na^+/Ca^{2+}-exchanger driven by the inward Na^+ gradient, is also involved in Ca^{2+} extrusion (Milograna et al. 2010; see Fig. 3.7). These proteins are also crucial to the pigment dispersion mechanism, although whether they can by activated directly by PDHs is not known. They most probably respond constitutively to intracellular Ca^{2+} levels, pumping continuously to hold Ca^{2+}_{int} at the fairly constant low levels typical of pigment dispersion.

Roles for Ca^{2+}-ATPases and Ca^{2+}-exchangers have not been evaluated in crustacean groups other than the palaemonid shrimps, but they likely underlie the pigment-dispersing mechanism in brachyuran crab chromatophores. In these crabs, Ca^{2+}_{int} is related to pigment movement in a manner contrary to that seen in carideans; that is, pigment granules are fully aggregated in unstimulated crab chromatophores (Lambert and Fingerman 1976, Kulkarni and Fingerman 1986), exactly the reverse of their distribution in caridean shrimp chromatophores. In fiddler crab black chromatophores, Ca^{2+}_{int}, increased by the calcium ionophore A23187, induces pigment dispersion (Quackenbush 1981, Rao and Fingerman 1983). This evidence suggests that, in crabs, PDH coupling to the plasma membrane receptor induces increased Ca^{2+}_{int} and dispersion of the initially aggregated pigment granules, possibly through mechanisms of signal transduction similar to that induced by RPCH in shrimps.

Intracellular Second-Messenger Cascades in Chromatophores

In addition to the increase in Ca^{2+}_{int} just described, RPCH binding to a putative GPCR in the plasma membrane also leads to an increase in intracellular cGMP (McNamara and Ribeiro 2000, Ribeiro and McNamara 2007, 2009). Dibutyryl-cGMP (db-cGMP), a lipid-soluble cGMP analogue, likewise induces complete, Ca^{2+}-dependent, reversible pigment aggregation identical to RPCH- and very similar to A23187-triggered aggregation (McNamara and Ribeiro 2000, Ribeiro and McNamara 2009; see Fig. 3.6A,B). The soluble GC activators sodium nitroprusside (SNP) and morpholinosydnonimine (SIN-1) alone induce 40% pigment aggregation with granule transport kinetics similar to that induced by RPCH (Ribeiro and McNamara 2009). Inhibition of the same cytosolic GCs by zinc protoporphyrin IX (ZnPP-IX) and 6-anilino-5,8-quinolinedione (LY83583) reduces RPCH-triggered pigment aggregation by 30% and also reduces the rapid phase of pigment translocation (Ribeiro and

McNamara 2009). However, the type-C plasma membrane receptor GC does not seem to play a role in cGMP synthesis during RPCH action because stimulation with *Escherichia coli* heat-stable enterotoxin 1 (STa-1) does not induce pigment aggregation (Ribeiro and McNamara 2009).

Pigment aggregation in caridean chromatophores also seems to be associated with a decrease in intracellular cyclic adenosine monophosphate (cAMP) concentration (Fingerman 1969, Nery et al. 1997). Furthermore, this cyclic nucleotide second-messenger induces pigment dispersion in *Palaemonetes vulgaris* (Fingerman 1969) and *Macrobrachium potiuna* (Nery et al. 1998) red epidermal chromatophores, although db-cAMP does not cause pigment movement in *M. olfersi* red ovarian chromatophores (Ribeiro and McNamara 1997). The cAMP target, protein kinase A (PKA), also plays a role in pigment dispersion in *M. olfersi* ovarian red chromatophores (Bell et al. 2005). PKA activation leads to a protein phosphorylation cascade that induces pigment dispersion in carideans and brachyurans. This cascade, when deactivated, may lead to pigment aggregation in caridean shrimps (Nery and Castrucci 1997, Nery et al. 1997). Furthermore, a decrease in cytosolic cAMP concentration, when transduced by an inhibitory G-protein (G_i)-coupled receptor, may be an alternative signaling mechanism for pigment aggregation in some crustacean groups (Nery and Castrucci 1997).

Both cGMP and db-cGMP induce dose-dependent pigment dispersion in vitro in the black, white, and red chromatophores of the crab *U. pugilator*, and they inhibit pigment dispersion in red chromatophores but enhance dispersion in black and white chromatophores induced by a partially purified eyestalk hormone in vitro (Rao and Fingerman 1983). cGMP is thus one of the important second-messenger nucleotides that effect pigment translocation in different directions, apparently depending on chromatophore type and taxon. This constitutes further evidence that the pigment granules in crab and shrimp chromatophores migrate in opposite directions in response to the same intracellular stimulus. Thus, the same cGMP signaling pathway may activate different molecular motors that effect opposing pigment granule movements; that is, dispersion in the Brachyura and aggregation in the Caridea (Ribeiro and McNamara 2009).

Nevertheless, pigment aggregation in caridean chromatophores is triggered by db-cGMP only when Ca^{2+} is present in the extracellular medium, clearly revealing that not only does RPCH-triggered aggregation require Ca^{2+}, but also that the cGMP cascade is also Ca^{2+}-dependent (Ribeiro and McNamara 2009; Fig. 3.6A). There thus seem to be one or more points of cross-talk between the Ca^{2+} and cGMP signaling cascades that together induce pigment aggregation, although the Ca^{2+} cascade seems to be fairly independent of the cGMP cascade, at least in vitro. Similarly, in the crabs that rely on different second-messenger cascades like cAMP, cGMP, and Ca^{2+} for black, white, and red pigment dispersion (Quackenbush and Rao 1979, Quackenbush 1981), crosstalk and interdependence seem to prevail. What advantage might reside in employing more than one signaling cascade in a single physiological response? Crosstalk between signal transduction cascades appears to provide cells with complex intracellular systems for the fine tuning of hormone-induced signals (Van der Horst et al. 1999). Thus, transduction systems appear to have evolved with a certain redundancy, thus providing functional security that may enhance the fitness of an individual.

Second-Messenger-Activated Effectors and Kinases

It is not yet clear just how cGMP and Ca^{2+} activate the pigment translocation mechanism in caridean shrimps. Free cytosolic Ca^{2+} can activate many target proteins like Ca^{2+}/calmodulin (Ca^{2+}/CaM), protein kinase C (PKC), and the Ca^{2+}/calmodulin-dependent protein kinase (Ca^{2+}/CaMK), for example, whose activation leads to a wide variety of intracellular responses (Kheifets and Mochly-Rosen 2007). PCH-induced pigment aggregation in *M. potiuna* red chromatophores is dependent on the Ca^{2+}/CaM complex (Nery et al. 1997), and PKC (Bell et al. 2005) and Ca^{2+}/CaM are involved in pigment aggregation in *M. olfersi* red ovarian chromatophores (Milograna et al. 2012), as revealed by N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7)

inhibition. IP_3 and diacylglycerol (DAG) play a role in pigment aggregation in *M. potiuna* red epidermal chromatophores (Nery et al. 1997) but not in *M. olfersi* ovarian chromatophores (Ribeiro 2002), suggesting that different elements of the same signal transduction cascade may vary among different chromatophore types.

Ca^{2+}/CaM can regulate or induce nitric oxide synthase (NOS) activity, a heme-protein cytochrome. This enzyme generates nitric oxide (NO) from L-arginine in a reaction that requires oxygen, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), flavines, and biopterins (Moncada et al. 1991, Murad 1994). A role for NOS and NO during the RPCH signaling cascade leading to pigment aggregation has been demonstrated by Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME) inhibition in perfused shrimp ovarian chromatophores (Milograna et al. 2012). cGMP can be produced by soluble cytosolic GC (GC-S) when activated by NO (Schmidt et al. 1993, Murad 1994, Müller 1997) and a key role for GC-S in red pigment aggregation has been demonstrated in *M. olfersi* ovarian chromatophores (Ribeiro and McNamara 2009; see Fig. 3.8). The cGMP pathway appears to be Ca^{2+}-dependent at several points, and the GC activation step

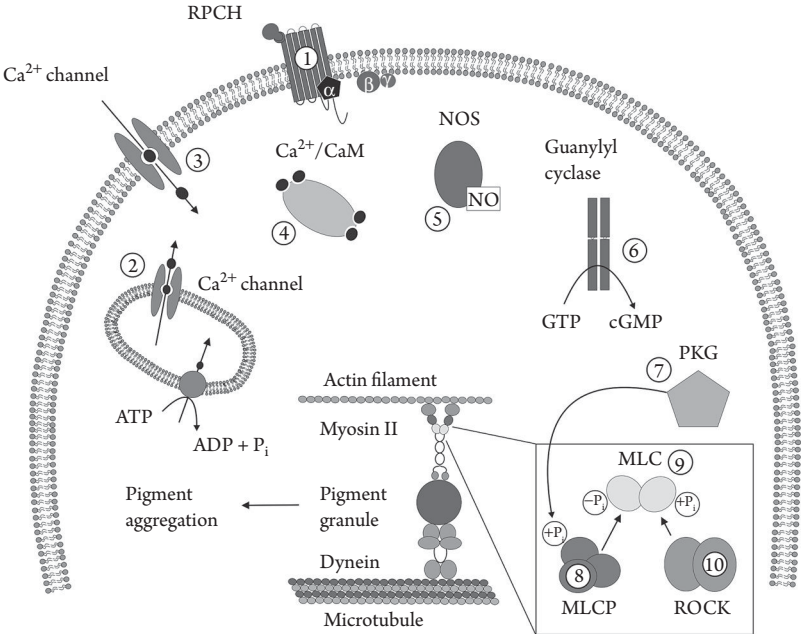


Fig. 3.8.
Model proposed for pigment aggregation signaled by the Ca^{2+}- and cyclic guanosine monophosphate (cGMP) cascades after red pigment concentrating hormone (RPCH) binding to its receptor. Pre-RPCH binding, resting state conditions are provided in Fig. 3.7 (left panel). On RPCH binding, the activated GPCR (1) signals a heterotrimeric G-protein leading to Ca^{2+} release through SER ryanodine receptors (2) and opening of voltage-gated N- and/or P/Q-type membrane Ca^{2+} channels (3), increasing Ca^{2+}_{int} to $\approx 10^{-3}$ M (see Fig. 3.7 for details), a concentration that (4) activates Ca^{2+}/calmodulin (Ca^{2+}/CaM). This intracellular effector stimulates nitric oxide synthase (NOS) (5) to produce nitric oxide (NO) that in turn enables cytosolic guanylyl cyclase (6) to produce cGMP from GTP. cGMP activates protein kinase G (7) that may inhibit myosin light chain phosphatase (MLCP) activity (8), which in turn regulates myosin light chain (MLC) activity (9) through dephosphorylation (inactive state). In contrast, Rho protein kinase (ROCK) (10), possibly activated by Ca^{2+} or by cGMP, phosphorylates the MLC (active state), resulting in pigment aggregation. Thus, MLCP and ROCK together regulate the overall activity of the myosin heavy chain motor domains (myosin II). The microtubule motor, dynein, also may be activated concomitantly by this mechanism and may possibly play a role in rapid-phase aggregation.

may be particularly important in this regard (Ribeiro and McNamara 2009). In crustaceans, components of the NO/cGMP pathway have been identified in many systems that involve sensory perception, such as the neural circuits that control olfaction (Johansson et al. 1996, Johansson and Mellon 1998, Scholz et al. 1998), vision (Lee et al. 2000), rhythmic motor behavior (Scholz et al. 1996, Scholz 2001), escape behavior (Aonuma et al. 2000), and neurosecretion (Lee et al. 2000).

NO induces dose-dependent pigment dispersion in the black epidermal chromatophores of *N. granulata* in vitro (Vargas et al. 2008). UVA/B-induced granule dispersion in crab chromatophores in vivo and in cultured retinal cells also depends on NO (Filgueira et al. 2010), constituting an important protective response against damaging UV effects. Thus, NO may act as a chromatophorotropin agonist in many crustaceans (Vargas et al. 2008), further evidence that the same signaling pathways activate different molecular motors in the Brachyura (dispersion) and Caridea (aggregation) (Ribeiro and McNamara 2009). Apparently, different membrane receptors, such as UV-sensitive opsins and RPCH and PDH receptors, may converge downstream on NOS, allowing perception of diverse environmental cues via primary and humorally mediated responses by the same cells.

In caridean shrimps, in response to RPCH signal transduction, Ca^{2+} apparently occupies its binding sites on the calmodulin molecule, forming the activated Ca^{2+}/CaM complex that in turn activates Ca^{2+}/CaMK, which stimulates NOS to synthesize NO, thus activating GC-S (Ribeiro and McNamara 2009, Milograna et al. 2012). From GTP, this cyclase produces cGMP, which is released into the cytosol and leads to activation of protein kinase G (PKG). In addition to PKG, cGMP can regulate ion channels and phosphodiesterases (Wong and Garbers 1992, Lincoln and Cornwell 1993, Schmidt et al. 1993). PKG plays a crucial role in pigment aggregation in the red ovarian chromatophores of *M. olfersi* (Milograna et al. 2012), as demonstrated by Rp-guanosine cyclic 3′,5′-monophosphate triethylammonium (Rp-cGMP-triethylamine) inhibition. In the caridean shrimps, PKG seems to be the principal kinase responsible for pigment aggregation, although further investigation is necessary to better understand the subsequent steps in this mechanism (Fig. 3.8). Apparently, in crustacean chromatophores, the final distributional state of the pigments at any particular instant reflects the degree of activation of specific effectors like protein kinases G, C, and A and the Ca^{2+}/CaM-dependent kinase at the end points of the signaling cascades and the subsequent phosphorylation of direction-specific molecular motors and their specific kinase-dependent regulators (Ribeiro and McNamara 2009).

Protein kinases are second-messenger-activated enzymes that phosphorylate particular amino acids like serine and threonine at specific sites on diverse effector proteins, to which they transfer a high-energy phosphate group derived from the hydrolysis of ATP to ADP. Once activated, the target protein suffers a conformational change and becomes enabled to perform its functional role. In crustacean chromatophores, protein kinases activated primarily during the second-messenger cascades just described appear to activate other downstream kinases that directly regulate molecular motor activity. For example, actin-myosin-based movements are modulated by kinases and phosphatases that recycle energy in the form of a phosphate group through the regulatory sites of the myosin heavy chains, the so-called *light chains*. The myosin light chain (MLC) is a myosin-associated peptide subunit that regulates contractile and other activity-related movements in nonmuscle cells. Phosphorylation of the MLC serine 19 residue (MLC_{Ser19}) is required for the formation of the actin-myosin complex, thus initiating the myosin motor domain ATPase activity that alters the conformation of the myosin dimer motor domains and leads to progressive myosin movement along the actin filament (Fajmut and Brumen 2008). In *M. olfersi* red ovarian chromatophores, MLC_{Ser19} is apparently phosphorylated by Rho protein kinase (ROCK), a Rho GTPase effector that plays a role in many motor responses involving the cytoskeleton (Riento and Ridley 2003). ROCK blockade by Y-27632 and H-1152 partially inhibits pigment aggregation and accelerates pigment dispersion in *M. olfersi* chromatophores (Milograna et al. 2012; Fig. 3.8).

Table 3.2. Effects of diverse pharmacological and physiological agents on pigment movements in caridean shrimp (*Macrobrachium, Palaemon,* and *Palaemonetes*) and brachyuran crab (*Uca, Neohelice*) chromatophores. These effectors, when perfused in vitro, may induce pigment translocation per se and can inhibit or enhance RPCH-triggered pigment migration, either partially or fully. All abbreviations and references are provided in the text.

Effector	Pharmacological characteristics/activity	Intracellular action	Effect on shrimp chromatophores	Effect on crab chromatophores
RPCH	GPCR agonist	Triggers Ca^{2+} and cGMP signaling cascades	Complete pigment aggregation	Aggregates red pigments
α-, β-PDH	Plasma membrane receptor agonists?	Trigger Ca^{2+} and cAMP signaling cascades	Partial or complete pigment dispersion	Partial or complete pigment dispersion
A23187	Ca^{2+}-specific mobile ion carrier/ionophore	Increases $[Ca^{2+}]_{int}$	Complete pigment aggregation	Complete pigment dispersion
Ca^{2+}	Intracellular second messenger	Activates CaM, Ca^{2+}/CaMK and PKC	Complete pigment aggregation	Complete pigment dispersion
La^{3+}	PMCA inhibitor and Ca^{2+} channel blocker	Increases $[Ca^{2+}]_{int}$; inhibits RPCH-induced Ca^{2+} influx	Partial pigment aggregation; partial inhibition of RPCH-induced aggregation	No data available
Ba^{2+}	K^+ channel blocker	Depolarizes plasma membrane	Partial pigment aggregation	No data available
Thapsigargin	SERCA inhibitor	Increases $[Ca^{2+}]_{int}$	Partial pigment aggregation	No data available
Cyclopiazonic acid	SERCA inhibitor	Increases $[Ca^{2+}]_{int}$	Partial pigment aggregation	No data available
cGMP	Intracellular second messenger	Activates PKG	Complete pigment aggregation	Black, white, red pigment dispersion
db-cGMP	Lipid-soluble cGMP analogue	Activates PKG	Complete pigment aggregation	Black, white, red pigment dispersion
cAMP	Intracellular second messenger	Activates PKA	Pigment dispersion	Pigment dispersion

(*continued*)

Table 3.2. *(Continued)*

Effector	Pharmacological characteristics/activity	Intracellular action	Effect on shrimp chromatophores	Effect on crab chromatophores
IP_3	Intracellular second messenger	Releases Ca^{2+} from REL	Pigment aggregation (*M. potiuna* epidermal red chromatophores)	No data available
DAG	Intracellular second messenger	Activates PKC	Pigment aggregation (*M. potiuna* epidermal red chromatophores)	No data available
STa-1	Plasma membrane receptor guanylyl cyclase activator	Releases NO	No effect	No data available
SIN-1	Soluble guanylyl cyclase activator	Releases NO	Partial pigment aggregation	Black pigment dispersion
SNP	Soluble guanylyl cyclase activator	Releases NO	Partial pigment aggregation	No data available
ZnPP-IX	Soluble guanylyl cyclase inhibitor	Inhibits NO release	Partial inhibition of RPCH-induced aggregation	No data available
UV light	Cell signaling activator	Activates NOS	No data available	Pigment dispersion
L-NAME	NOS inhibitor	Inhibits NO production	Partially inhibits RPCH-induced aggregation	Inhibits black pigment dispersion
W7	Ca^{2+}/CaM inhibitor	Suppresses downstream CaM cascade	Partially inhibits RPCH-induced aggregation	No data available
Rp-cGMP-triethylamine	PKG inhibitor	Suppresses downstream activation of PKG-phosphorylated proteins	Partially inhibits RPCH-induced aggregation	No data available
Y-27632	Rho protein kinase inhibitor	Phosphorylates MLC	Partially inhibits RPCH-induced aggregation; accelerates pigment dispersion	No data available

H-1152	Rho protein kinase inhibitor	Phosphorylates MLC	Partially inhibits RPCH-induced aggregation; accelerates pigment dispersion	No data available
Cantharidin	MLC phosphatase inhibitor	Dephosphorylates MLC	Accelerates pigment aggregation	No data available
BDM	Myosin ATPase inhibitor	Inhibits myosin/actin interaction	Hyperdispersion; reduces pigment aggregation velocity	No data available
Blebbistatin	Non-muscle myosin II inhibitor	Impairs non-muscle myosin II-based transport	Partially inhibits RPCH-induced pigment aggregation	No data available
Pan-myosin antibody	Myosin motor inhibitor	Impairs myosin-based transport	Partially inhibits RPCH-induced pigment aggregation	No data available
Cytochalasin B	Depolymerizes actin filaments	Inhibits actin filament-based transport	Inhibits RPCH-induced pigment aggregation; no effect on A23187-triggered aggregation	Inhibits PDH-triggered dispersion, and aggregation
Colchicine	Depolymerizes microtubules	Impairs microtubule-based transport	Partial pigment aggregation; no effect on RPCH-triggered aggregation	No effect on PDH-triggered dispersion; inhibits aggregation
Taxol	Microtubule stabilizer	Alters cytoskeleton turn-over	Partial pigment aggregation; reduces velocity of RPCH-triggered pigment aggregation	No data available
EHNA	Dynein-ATPase inhibitor	Impairs dynein-based transport	Partially inhibits RPCH-induced pigment aggregation	No data available

MLC dephosphorylation is performed by the MLC phosphatase (MLCP), leading to the uncoupling of the myosin motor domains from the actin filaments or by simply maintaining the crossbridge ligand sites in a dephosphorylated state (Rembold and Murphy 1990). ROCK increases MLC phosphorylation either by direct phosphorylation of the MLC_{Ser19} residue (Amano et al. 1996) or by inhibition of MLCP activity (Feng et al. 1999, Chilcoat et al. 2008; Fig. 3.8). The processes underlying the MLC phosphorylation/dephosphorylation cycle appear to depend on intracellular Ca^{2+} and on the decoding of kinase signaling. When the MLC is held in a phosphorylated state by inhibition of MLCP with cantharidin, RPCH-triggered aggregation velocity in *M. olfersi* chromatophores increases significantly, indicating accelerated myosin head movement along the actin filament (Milograna et al. 2012). This area of investigation requires detailed study because no comparative data on myosin kinases and phosphatases are available in other crustacean pigment cells (Fig. 3.8). Table 3.2 provides summary data for the effects of many different pharmacological and physiological agents on chromatophore pigment movements in the main caridean and brachyuran taxa investigated.

THE MECHANICS OF PIGMENT TRANSLOCATION WITHIN CHROMATOPHORES

Molecular Motors and the Cytoskeleton

The mechano-chemical proteins responsible for intracellular transport are known as "molecular motors" and generally consist of two functional parts: a motor domain that reversibly binds to the cytoskeleton and converts chemical energy into kinetic energy or movement and a tail that interacts with a cargo, either directly or through accessory light chains (Karcher et al. 2002). Knowledge of pigment granule translocation in invertebrate chromatophores is scanty from a biophysical stance (McNamara and Ribeiro 1999). Traditionally, conventional myosins are the molecular motors known to transport cargos along actin filaments, whereas kinesin and dynein interact with microtubules. However, recent studies describe extremely complex interactions among molecular motors, the cytoskeleton, and accessory proteins in cells in general (Gross et al. 2002, Ali et al. 2008), including interactions between many kinds of molecular motors (e.g., an association between kinesin and myosin for transporting cargos along microtubules).

It has long been suggested that more than one molecular motor is involved in pigment movement, even in monochromatic chromatophores (Boyle and McNamara 2006). Pigment aggregation in *M. olfersi* red ovarian chromatophores clearly follows a biphasic kinetic time course: a brief 2 min pulse of rapid-phase activity at around 20 μm/min followed by a 10 min plateau of slow-phase translocation at about 5 μm/min (Fig. 3.6A,B). Also, such chromatophores contain two morphologically distinct red pigment granules that show marked differences in size (1–2 μm versus 80–100 nm diameter) and surface characteristics (membrane-bounded versus carotenoid interface; McNamara and Sesso 1983) and that remain clearly spatially separated in the aggregated state with no physical barrier evident between them (McNamara and Sesso 1982, 1983). This suggests that at least two distinct molecular motors participate in pigment migration and in the spatial separation of the pigment granules (McNamara and Ribeiro 1999).

Pigment Translocation by Actin and/or Tubulin-based Motors

We have demonstrated a prominent role for PKG and myosin in slow-phase pigment aggregation, together with an apparent lack of function for PKG in rapid-phase translocation (Milograna et al. 2012). Slow-phase pigment aggregation velocity can be reduced by 30% by inhibiting myosin ATPase activity with butanedione monoxime and thus appears to be underpinned by actin-myosin-based

granule transport (McNamara and Ribeiro 1999). A myosin motor also actively sustains the aggregated state, regulated by Ca^{2+}-dependent proteins activated by RPCH (McNamara and Ribeiro 1999). Myosin motor inhibition in *M. olfersi* red ovarian chromatophores with dispersed pigments causes pigment hyperdispersion, suggesting that the myosin motor remains activated even in the dispersed pigment state (Boyle and McNamara 2006).

Several different myosin types may effect pigment aggregation in crustacean chromatophores. Myosins are currently classed into more than 20 families, each exhibiting a diversity of structural and functional characteristics. Boyle and McNamara (2006), based on Western blotting in *M. olfersi* ovarian chromatophores, suggest that one of the pigment-aggregating myosin motors might be myosin II or myosin XII. Our recent findings with Alexa-conjugated anti-nonmuscle myosin II antibodies in confocal fluorescence microscopy clearly reveal the broad distribution of nonmuscle myosin II in *M. olfersi* ovarian chromatophores (Fig. 3.9A), as well as the functional relevance of this motor, as demonstrated by pharmacological inhibition of pigment granule aggregation with blebbistatin, a nonmuscle myosin II inhibitor (Milograna and McNamara 2009b). Also, the streptomycin-O mediated introduction of a pan-myosin antibody into ovarian chromatophores causes functional inhibition of RPCH-triggered pigment aggregation (Boyle and McNamara 2006). Tetramethyl-rhodamine-isothiocyanate (TRITC)-labeled pan-myosin antibodies (Boyle and McNamara 2006) and Alexa-conjugated anti-pan myosin antibodies (Fig. 3.9B) also reveal myosin to be intimately associated with the larger pigment granules, suggesting that at least two classes of myosin participate in pigment translocation.

The role of microtubule-based transport in pigment aggregation in crustacean chromatophores is controversial. Disruption by colchicine, vinblastine, and other tubulin depolymerizing agents usually does not affect pigment aggregation at pharmacological concentrations (McNamara 1980, Quackenbush 1981, Tuma et al. 1995). However, microtubule stabilization with Taxol collapses the

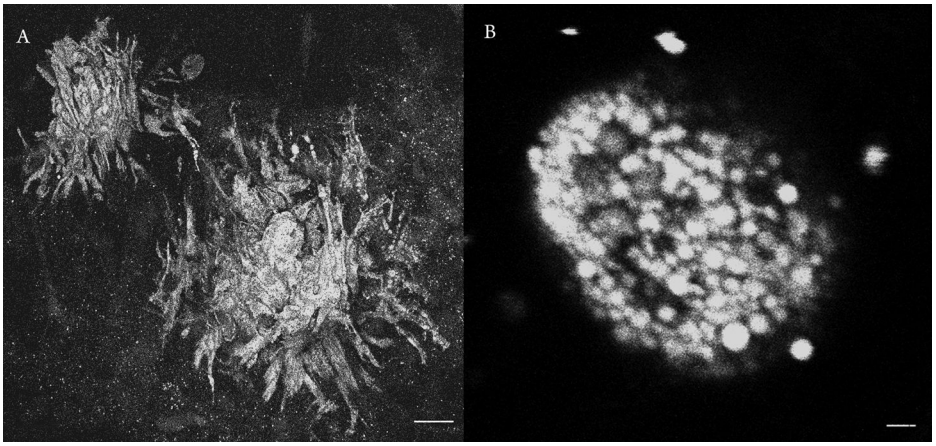


Fig. 3.9.
Myosin molecular motors, intimately associated with the pigment granule membranes, play an important role in granule translocation along the actin cytoskeleton during pigment aggregation and possibly in limiting pigment dispersion brought about by opposing motors. (A) Confocal fluorescence microscopy (Leica TCS SP5, λ_{ex}= 495 nm, λ_{em}= 519 nm) showing the distribution of granule-associated, nonmuscle myosin II throughout the cytoplasm of *M. olfersi* ovarian chromatophores with fully dispersed pigments, revealed using a polyclonal, nonmuscle myosin II primary antibody followed by an Alexa-488 conjugated goat anti-rabbit IgG. (B) Distribution of a skeletal muscle pan-myosin clearly associated with the large membrane-bounded pigment granules in an ovarian chromatophore extension, shown employing a polyclonal, anti-skeletal muscle pan-myosin primary antibody conjugated with an Alexa-488 goat anti-rabbit IgG. Scale bars A = 20 μm, B = 1 μm. See color version of this figure in the centerfold.

chromatophore structure and leads to some degree of pigment aggregation (Bell 2008), perhaps supporting older notions (McNamara 1980, 1981) that microtubule turnover maintains the notable chromatophore asymmetry and, particularly, the integrity of the cell extensions and finer secondary ramifications. Taxol slightly slows RPCH-triggered pigment aggregation in the ovarian chromatophores of *M. olfersi* (Bell 2008) also suggesting that microtubule depolymerization or turnover may be associated with aggregation. However, in the red chromatophores of *M. potiuna*, Taxol accelerates pigment aggregation (Tuma et al. 1995), suggesting that microtubules maintained in a polymerized state somehow sustain granule translocation. Certainly, many polymerized microtubules are present in the cell extensions of caridean epidermal and internal organ chromatophores with aggregated pigments (Robison and Charlton 1973, McNamara and Sesso 1983, McNamara and Taylor 1987; see Fig. 3.4).

The nature of the traditionally microtubule-associated molecular motors has not been well investigated in crustacean chromatophores. Based on TRITC-labeled antikinesin antibody findings, Boyle and McNamara (2006) have demonstrated a strong association between pigment granules and kinesin in *M. olfersi* ovarian chromatophores, suggesting that kinesin may be the molecular motor responsible for pigment dispersion. Both kinesin and dynein, identified by Alexa-conjugated antibodies in confocal fluorescence microscopy, are associated with pigment granules (Fig. 3.10A,B). Physiological inhibition of dynein-ATPase with erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride also partially inhibits pigment aggregation (Ribeiro and McNamara 2004).

Given these findings, pigment aggregation in caridean shrimp chromatophores may involve at least two active motor components. We advance two hypotheses to elucidate the dynamics of pigment aggregation: (i) dynein may function during fast-phase aggregation via the microtubule component of the cytoskeleton associated with the larger cell extensions; nonmuscle myosin II may then attach to the granules, transporting them along the actin filaments in the finer secondary ramifications; or, alternatively, (ii) granule-bound, nonmuscle myosin II may translocate over the actin cytoskeleton during fast-phase aggregation, which would progress slowly by an association

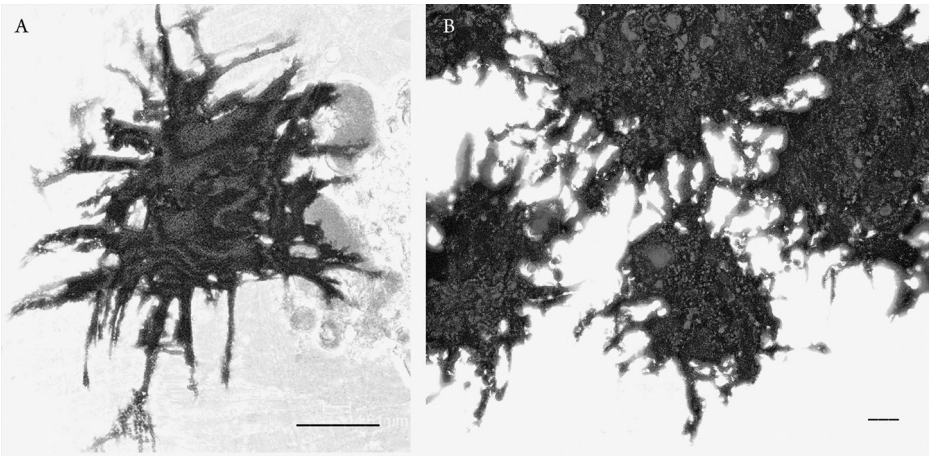


Fig. 3.10.
Molecular motors like kinesin and dynein that respectively transport cargos to the chromatophore periphery or perikaryon along the microtubular component of the cytoskeleton are also associated with the pigment granules. (A) Confocal fluorescence microscopy (Leica TCS SP5, λ_{ex}= 495 nm, λ_{em}= 519 nm) showing the distribution of granule-associated kinesin throughout the cytoplasm of a chromatosome with fully dispersed pigments, revealed using a monoclonal, anti-kinesin primary antibody and an Alexa-488 conjugated goat anti-mouse IgG. (B) Dynein distribution is clearly associated with the large membrane-bound pigment granules in a chromatosome with fully dispersed pigments, shown here employing a monoclonal, anti-dynein primary antibody followed by an Alexa-488 conjugated goat anti-mouse IgG. Bright field and fluorescence images overlaid with background subtraction, scale bars = 20 μm. See color version of this figure in the centerfold.

of dynein and myosin (Fig. 3.8). Kinesin seems to be the molecular motor responsible for pigment dispersion, possibly through an interaction with other motors, although sustaining pharmacological evidence is as yet unavailable. Boyle and McNamara (2008) have proposed a pigment transport model in which kinesin and myosin mechano-chemical motors alternately stretch and compress a structurally unified, elastic pigment matrix over a polymerized tubulin and actin cytoskeleton, producing pigment dispersion and aggregation.

A SPECIAL CASE: THE RETINAL PIGMENTARY SYSTEM

A specialized, labile pigmentary system is also found within the cells that constitute the crustacean compound eye, described briefly here. The photosensitive retinula cells are usually arrayed as groups of eight cells but, depending on the taxon, are also found in groups of from five to seven cells located beneath the cone cells. Usually four cone cells, together with the faceted, transparent, and multilayered cornea, form the dioptric apparatus of the crustacean eye, which is constituted by many similar anatomical units known as *ommatidia*. The retinula cells possess highly folded membrane specializations termed *rhabdomeres* that are the light-receptive elements of the ommatidia and that contain the photosensitive pigments (Porter et al. 2007). Axons from the retinula cells penetrate the basement membrane and terminate in the *lamina ganglionaris*, from where second-order neurons project to other neurons located within the *medulla externa*. A variety of distal and proximal screening pigment cells (Hallberg and Elofsson 1989) completes the basic structure of the crustacean compound eye (Meyer-Rochow 2001). These various eye pigments occupy different positions within the retinula cells, depending on ambient luminosity (Kleinholz 1966). Such pigments act as screening filters, regulating the amount of light reaching the retina (Aréchiga et al. 1993). Photon flux to the receptors is thus a function of the position of the pigment granules located within two distinct sets of cells in the compound eye. In darkness, both sets of pigments are retracted, leaving most of the photoreceptor surface exposed to light. Under illumination, both pigment types become dispersed, depending on light intensity, thus blocking the access of stray light to the photosensitive membranes (rhabdomeres) in the photoreceptors (Garfias et al. 1995). The redistribution of the pigment granules is accompanied by changes in the SER, ranging from numerous small vesicles under conditions of light exposure to fewer large cisternae in the dark (Frixione and Porter 1986). Photomechanically induced changes may thus affect the position and shape of whole cells, the quantity and distribution of organelles, and the chemical composition of membranes and photosensitive pigments, as well as the titers of intracellular messengers (Meyer-Rochow 1999). These alterations are tuned to meet the requirements of the crustacean eye for maximum light sensitivity and acuity while the constituent photoreceptor cells derive maximum protection against damaging radiation (Meyer-Rochow 2001).

The mechanisms by which the conditions evoking light and dark adaptation elicit their corresponding intracellular pigment granule translocations are different. Proximal pigment granule migration is a direct response of the photoreceptors themselves to light and dark (Frixione et al. 1979). The distal pigment cells, however, do not respond directly to light; they are the end effectors of a neuroendocrine reflex. Distal pigment dispersion is triggered by light acting on extraretinal photoreceptors (Aréchiga et al. 1985) and is mediated by the release of a light-adapting hormone (DRPH; Kleinholz 1966). DRPH is released into the SG from neurosecretory axons whose perikarya are located in the protocerebrum (Hernández and Fuentes-Pardo 2001) and in the XO. In response to this neurosecretory peptide, the distal pigment granules then migrate longitudinally, from a distal to a more proximal position in the distal pigment cells, a change that reduces the amount of light entering the retinula cells. The mechanisms by which retraction of the distal retinal pigment granules is induced are yet to be disclosed (Garfias et al. 1995), but Ca^{2+} seems to play a key role in regulating such movements in arthropod retinula cells (Frixione and Aréchiga 1981). In crayfish, the transport mechanism in the retinula cells is controlled by Na^{+}-dependent adjustments

of Ca^{2+} concentration, dependent on uptake by SER cisternae, as is also seen in chromatophores (Frixione and Ruiz 1988). Activation of PKC by DAG released during the phototransduction cascade also may regulate the turnover of phototransductive membrane in arthropods (Blest et al. 1993, Minke and Selinger 1992). In arthropod photoreceptors, DAG may thus constitute one of the light-dependent second-messengers that induce transcription of rhabdomeral membrane precursors (Blest et al. 1993, 1994).

A further modulatory influence on screening pigment migration is exerted by circadian rhythm. At night, the responsiveness of the retinal photoreceptors is much higher than during the day, and both retinal pigments are retracted at night and dispersed during the day. Such rhythmicity persists under conditions of constant illumination (Aréchiga et al. 1993, Garfias et al. 1995) suggesting regulation by a biological clock. The retinula cells also confer polarized vision or sensitivity on some crustacean groups (Kleinlogel and Marshall 2009)—that is, the ability to discriminate between two light sources of the same luminosity but of different e-vector orientation and/or degree of polarization (Kirschfeld 1973)—and some species are also sensitive to UV light (Kleinlogel and Marshall 2006).

FUTURE DIRECTIONS

Although physiological investigations of pigment movements in crustacean chromatophores seem to have reached their zenith toward the end of the past century, many questions still remain that now can be examined employing novel techniques and refined methods. Knowledge of the tissues of origin and the migration pathways of chromatophores during embryogenesis would be very useful, for example. Perhaps the most important underlying issue will be success in culturing crustacean chromatophores and establishing a chromatophore cell line. This will open the way for electrophysiological, biochemical, and molecular biological techniques that require individual cells, pure cell suspensions, or purified chromatophore proteins and membranes. Identification of the membrane receptors involved in chromatophorotropin signal transduction is an essential step in clarifying the early events leading to pigment migration, and an examination of homology with other arthropod hormone receptors requires molecular analysis. The use of well-established patch clamping methodologies also will be important here to reveal voltage-dependent membrane-associated events. Investigation of the cytoskeleton, particularly actin distribution and its associated pigment-translocating molecular motors and their regulation, also require study, given the diverse intracellular signaling cascades now identified. Real-time analyses of calcium movements during pigment translocation using intracellular fluorochromes also would be very useful. Pigment dispersion in the caridean shrimps is still virtually unexplored, and physiological/pharmacological investigations are necessary to reveal the receptor type, signaling cascades, and molecular motors involved. Molecular biological methodologies will be important here to evaluate cytoskeleton and molecular motor turnover through gene expression and to allow a comparative evaluation of how pigment translocation systems have been conserved or have diverged among the different crustacean taxa. Future molecular studies should thus contemplate a wider diversity of crustacean species than investigated to present because most physiological and pharmacological studies have been limited to just a few species of crab, shrimp, and crayfish, within a handful of genera.

CONCLUSIONS

We examined rapid color change in the Caridea and Brachyura from the standpoint of the physiological, cellular, and molecular mechanisms that regulate pigment granule movements within the chromatophores. We also analyzed pigmentary effector organization and revisited the characterization of the pigment-aggregating and -dispersing chromatophorotropins. Genetic coding

for these peptide neurohormones and their homology and conservation were discussed in the light of evolutionary relationships, notably of the Insecta, with the Crustacea. We analyzed putative mechanisms of signal transduction, focusing on receptor type and function, and the cascades that employ both intra- and extracellular calcium and cyclic nucleotide second-messengers to regulate the molecular motor activity that leads to pigment granule translocation. We examined the structural and physiological evidence showing a role for cytoskeletal molecular motors like myosin, kinesin, and dynein, putatively responsible for granule translocation, and their regulation by phosphorylation-dephosphorylation mechanisms brought about by protein kinases and phosphatases.

We conclude that necessary avenues for future investigation should include establishing a chromatophore cell line that will enable the use of electrophysiological, biochemical, and molecular biological techniques with individual cells, pure cell suspensions, or purified chromatophore proteins and membranes. Greater knowledge of the tissues of origin and the migration pathways of chromatophores during embryogenesis is necessary. Identification of the membrane receptors involved in chromatophorotropin signal transduction is essential to clarify early events leading to pigment migration, and examination of homologies with other arthropod hormone receptors requires molecular analysis. Cytoskeleton dynamics, particularly actin distribution, together with its associated pigment-translocating molecular motors, also require study. Calcium movements during pigment translocation using intracellular fluorochromes should be examined. Pigment dispersion in the caridean shrimps is little explored and physiological/pharmacological investigations are necessary to reveal the receptor type, signaling cascades, and molecular motors involved. Molecular and phylogenetic methodologies are necessary to provide a comparative evaluation of how pigment translocation systems have been conserved or have diverged among the different crustacean taxa.

ACKNOWLEDGMENTS

We gratefully acknowledge past and ongoing financial support from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), the Conselho Nacional de Desenvolvimento Tecnológico e Científico (CNPq), and the Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) in the form of research grants and scholarships that have financed this line of investigation over the years. We also thank the Centro de Biologia Marinha (CEBIMAR), Universidade de São Paulo for continued logistical support, and we are most grateful to Prof. Roy Edward Larson and Dr. Munira Muhammad Abdel Baqui (Departamento de Biologia Celular, FMRP, USP) for essential support in preparing material for and performing confocal microscopy.

REFERENCES

Ali, M., H. Lu, C.S. Bookwalter, D.M. Warshaw, and K.M. Trybus. 2008. Myosin V and kinesin act as tethers to enhance each others' processivity. Proceedings of the National Academy of Sciences, USA 105:4691–4696.

Amano, M., M. Ito, K. Kimura, Y. Fukata, K. Chihara, T. Nakano, Y. Matsuura, and K. Kaibuchi. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). Journal of Biological Chemistry 271:20246–20249.

Aonuma, H., T. Nagayama, and M. Takahata. 2000. Modulatory effects of nitric oxide on synaptic depression in the crayfish neuromuscular system. Journal of Experimental Biology 203:3595–3602.

Aréchiga H., J.L. Cortes, U. Garcia, and L. Rodríguez-Sosa. 1985. Neuroendocrine correlates of circadian rhythmicity in crustaceans. American Zoologist 25:265–274.

Aréchiga H., F. Fernandez-Quiroz, M.F. Fernandez, and L. Rodríguez-Sosa. 1993. The circadian system of crustaceans. Chronobiology International 10:101–108.

Arendt D., K. Tessmar-Raible, H. Snyman, A.W. Dorresteijn, and J. Wittbrodt. 2004. Ciliary photoreceptors with a vertebrate-type opsin in an invertebrate brain. Science 306:869–871.

Bauer, R.T. 1981. Grooming behavior and morphology in the decapod Crustacea. Journal of Crustacean Biology 1:153–173.

Bauer, R.T. 2004. Remarkable shrimps: adaptations and natural history of the Carideans. Animal Natural Histories Series, vol. 7. University of Oklahoma Press, Norman, Oklahoma.

Bell, F.T. 2008. Citoesqueleto, motores moleculares e translocação pigmentar em cromatossomos ovarianos do camarão *Macrobrachium olfersii* (Crustacea, Decapoda). M.Sc. Thesis, Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo.

Bell, F.T., M.R. Ribeiro, and J.C. McNamara. 2005. As proteínas quinases A, C e G na translocação de grânulos de pigmento em cromatossomos ovarianos do camarão de água doce *Macrobrachium olfersii* (Crustacea, Decapoda). II Encontro da Biologia Comparada 'Desafios da Biologia Comparada no Conhecimento da Biodiversidade', Ribeirão Preto, Brazil.

Bellingham, J., S.S. Chaurasia, Z. Melyan, C. Liu, M.A. Cameron, E.E. Tarttelin, P.M. Iuvone, M.W. Hankins, G. Tosini, and R.J. Lucas. 2006. Evolution of melanopsin photoreceptors: discovery and characterization of a new melanopsin in nonmammalian vertebrates. Public Library of Science Biology 4:e254.

Blest, A.D., S. Stowe, and A. Delaney. 1993. Diacylglycerols as putative second messengers that regulate phototransductive membrane turnover by arthropods. Journal of Comparative Physiology 173A:57–63.

Blest, A.D., S. Stowe, and A. Delaney. 1994. An inhibitor of diacylglycerol-activated protein kinase Cs blocks the effects of a diacylglycerol lipase inhibitor, U-57908, on the light-dependent renewal of crab rhabdoms in vitro. Journal of Comparative Physiology 175A:611–617.

Boyle, R.T., and J.C. McNamara. 2006. Association of kinesin and myosin with pigment granules in crustacean chromatophores. Pigment Cell Research 19:68–75.

Boyle, R.T., and J.C. McNamara. 2008. A spring-matrix model for pigment translocation in the red ovarian chromatophores of the freshwater shrimp *Macrobrachium olfersi* (Crustacea, Decapoda). Biological Bulletin 214:111–121.

Britto, A.L., A.M. Castrucci, M.A. Visconti, and L. Josefsson. 1990. Quantitative in vitro assay for crustaceans chromatophorotropins and other pigment cell agonists. Pigment Cell Research 3:28–32.

Bronner, M.E., and N.M. LeDouarin. 2012. Development and evolution of the neural crest: an overview. Developmental Biology 366:2–9.

Brown, F.A., Jr. 1948. Hormones in Crustaceans. The Hormones, Chapter V, Academic Press. New York.

Chilcoat, C.D., Y. Sharief, and S.L. Jones. 2008. Tonic protein kinase A activity maintains inactive β2 integrins in unstimulated neutrophils by reducing myosin light-chain phosphorylation: role of myosin light-chain kinase and Rho kinase. Journal of Leukocyte Biology 83:964–971.

Chin, A.C., E.R. Reynolds, and R.H. Scheller. 1990. Organization and expression of the Drosophila FMRFamide-related prohormone gene. DNA and Cell Biology 9:263–271.

Coohill, T.P., and M. Fingerman. 1975. Relative effectiveness of ultraviolet and visible light in eliciting pigment dispersion in melanophores of the fiddler crab, *Uca pugilator*, through the secondary response. Physiological Zoology 48:57–63.

Coohill, T.P., C.K. Bartell, and M. Fingerman. 1970. Relative effectiveness of ultraviolet and visible light in eliciting pigment dispersion directly in melanophores of the fiddler crab, *Uca pugilator*. Physiological Zoology 43:232–239.

De Kleijn, D.P.V., and F. Van Herp. 1995. Molecular biology of neurohormone precursors in the eyestalk of Crustacea. Comparative Biochemistry and Physiology 112B:573–579.

De Kleijn, D.P.V., K.P.C. Janssen, G.J.M. Martens, and F. Van Herp. 1994. Cloning and expression of two crustacean hyperglycemic-hormone mRNAs in the eyestalk of the crayfish *Orconectes limosus*. European Journal of Biochemistry 224:623–629.

De Kleijn, D.P., B. Linck, J.M. Klein, W.M. Weidemann, R. Keller, and F. Van Herp. 1993. Structure and localization of mRNA encoding a pigment dispersing hormone (PDH) in the eyestalk of the crayfish *Orconectes limosus*. FEBS Letters 321:251–255.

Elofsson, R., and T. Kauri. 1971. The ultrastructure of the chromatophores of *Crangon* and *Pandalus* (Crustacea). Journal of Ultrastructure Research 36:263–270.

Fajmut, A., and M. Brumen. 2008. MLC-kinase/phosphatase control of Ca2+ signal transduction in airway smooth muscles. Journal of Theoretical Biology 252:474–481.

Feng, J., M. Ito, K. Ichikawa, N. Isaka, M. Nishikawa, D.J. Hartshorne, and T. Nakano. 1999. Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. Journal of Biological Chemistry 274:37385–37390.

Fernlund, P. 1971. Chromactivating hormones of *Pandalus borealis*: isolation and purification of a light-adapting hormone. Biochimica et Biophysica Acta 237:519–529.

Fernlund, P. 1976. Structure of a light adapting hormone from the shrimp, *Pandalus borealis*. Biochimica et Biophysica Acta 439:17–25.

Fernlund, P., and L. Josefsson. 1968. Chromactivating hormones of *Pandalus borealis*: isolation and purification of the 'red-pigment-concentrating hormone.' Biochemica et Biophysica Acta 158:262–273.

Filgueira, D.M.V.B., L.P. Guterres, A.P.S. Votto, M.A. Vargas, R.T. Boyle, G.S. Trindade, and L.E.M. Nery. 2010. Nitric oxide-dependent pigment migration induced by ultraviolet radiation in retinal pigment cells of the crab *Neohelice granulata*. Photochemistry and Photobiology 86:1278–1284.

Fingerman, M. 1969. Cellular aspects of the control of physiological color changes in crustaceans. American Zoologist 9:443–452.

Fingerman, M. 1970. Comparative physiology: chromatophores. Annual Review of Physiology 32:345–372.

Fingerman, M. 1985. The physiology and pharmacology of crustacean chromatophores. American Zoologist 25:233–252.

Fingerman, M., S.W. Fingerman, and D.T. Lambert. 1975. Colchicine, cytochalasin B and pigment movements in ovarian and integumentary erythrophores of the prawn *Palaemonetes vulgaris*. Biological Bulletin 149:165–177.

Frixione, E., and H. Aréchiga. 1981. Ionic dependence of screening pigment migrations in crayfish retinal photoreceptors. Journal of Comparative Physiology 144A:35–43.

Frixione, E., and R.M. Porter. 1986. Volume and surface changes of smooth endoplasmic reticulum in crayfish retinula cells upon light- and dark-adaptation. Journal of Comparative Physiology 159A:667–674.

Frixione, E., and L. Ruiz. 1988. Calcium uptake by smooth endoplasmic reticulum of peeled retinal photoreceptors of the crayfish. Journal of Comparative Physiology 162A:91–100.

Frixione, E., H. Aréchiga, and V. Tsutsumi. 1979. Photomechanical migrations of pigment granules along the retinula cells of the crayfish. Journal of Neurobiology 10:573–590.

Gäde, G. 2004. Regulation of intermediary metabolism and water balance of insects by neuropeptides. Annual Review of Entomology 49:93–113.

Gäde, G. 2009. Peptides of the adipokinetic hormone/red pigment-concentrating hormone family: a new take on biodiversity. Trends in Comparative Endocrinology and Neurobiology 1163:125–136.

Gäde, G., and L. Auerswald. 2003. Mode of action of neuropeptides from the adipokinetic hormone family. General Comparative Endocrinology 132:10–20.

Gäde, G., and H.G. Marco. 2009. Peptides of the adipokinetic hormone/red pigment-concentrating hormone family with special emphasis on *Caelifera*: primary sequences and functional considerations contrasting grasshoppers and locusts. General and Comparative Endocrinology 162:59–68.

Gäde, G., K.H. Hoffmann, and J.H. Spring. 1997. Hormonal regulation in insects: facts, gaps and future directions. Physiological Reviews 77:963–1032.

Gäde, G., L. Auerswald, P. Simek, H.G. Marco, and D. Kodrík. 2003. Red pigment-concentrating hormone is not limited to crustaceans. Biochemical and Biophysical Research Communications 309:967–973.

Gäde, G., P. Simek, and H.G. Marco. 2007. Water scorpions (Heteroptera, Nepidae) and giant water bugs (Heteroptera, Belostomatidae): sources of new members of the adipokinetic hormone/red pigment-concentrating hormone family. Peptides 28:1359–1367.

Garfias, A., L. Rodríguez-Sosa, and H. Aréchiga. 1995. Modulation of crayfish retinal function by red pigment concentrating hormone. Journal of Experimental Biology 198:1447–1454.

Gerarts, W.P.M., A.B. Smith, K.W. Li, and P.L. Hordijk. 1992. The light green cells of *Lymnae*: a neuroendocrine model system for stimulus-induced expression of multiple peptide genes in a single cell type. Experientia 48:464–473.

Gouveia, G., T.M. Lopes, C.A. Neves, L.E.M. Nery, and G.S. Trindade. 2004. Ultraviolet radiation induces dose-dependent pigment dispersion in crustacean chromatophores. Pigment Cell Research 17:545–548.

Gross, S.P., M.C. Tuma, S.W. Deacon, A.S. Serpinskaya, A.R. Reilein, and V.I. Gelfand. 2002. Interactions and regulation of molecular motors in *Xenopus* melanophores. Journal of Cellular Biology 156:855–865.

Gurevich, V.V., and E.V. Gurevich. 2008. GPCR monomers and oligomers: it takes all kinds. Trend in Neurosciences 31:74–81.

Hall, B.K. 2000. The neural crest as a fourth germ layer and vertebrates as quadroblastic not triploblastic. Evolution & Development 2:3–5.

Hall, B.K. 2008. The neural crest and neural crest cells: discovery and significance for theories of embryonic organization. Journal of Biosciences 33:781–793.

Hallberg, E., and R. Elofsson. 1989. Construction of the pigment shield of the crustacean compound eye: a review. Journal of Crustacean Biology 9:359–372.

Hernández, O.H., and B. Fuentes-Pardo. 2001. Cerebroid ganglion is the presumptive pacemaker of the circadian rhythm of electrical response to light in the crayfish. Biological Rhythm Research 32:125–144.

Hua, Y.-J., J. Ishibashi, H. Saito, A.I. Tawfik, M. Sakakibara, Y. Tanaka, R. Derua, E. Waelkens, G. Baggerman, A.D. Loof, L. Schoofs, and S. Tanaka. 2000. Identification of [Arg7] corazonin in the silkworm, *Bombyx mori* and the cricket, *Gryllus bimaculatus*, as a factor inducing dark color in an albino strain of the locust, *Locusta migratoria*. Journal of Insect Physiology 46:853–860.

Hunter, R.J., J.H. Taylor, and H.G. Moser. 1979. Effects of ultraviolet irradiation on eggs and larvae of the northern anchovy, *Engraulis mordax*, and the Pacific mackerel, *Scomber japonicus*, during embryonic stage. Photochemistry and Photobiology 29:325–338.

Jeffery, W.R., A.G. Strickler, and Y. Yamamoto. 2004. Migratory neural crest-like cells form body pigmentation in a urochordate embryo. Nature 431:696–699.

Johansson, K.U., and D.F. Mellon. 1998. Nitric oxide as a putative messenger molecule in the crayfish olfactory midbrain. Brain Research 807:237–242.

Johansson, K.U., R. Wallen, and E. Hallberg. 1996. Electron microscopic localization and experimental modification of NADPH-diaphorase activity in crustacean sensory axons. Invertebrate Neuroscience 2:167–173.

Josefsson, L. 1983. Chemical properties and physiological actions of crustacean chromatophorotropins. American Zoologist 23:507–515.

Karcher, R.L., S.W. Deacon, and V.I. Gelfand. 2002. Motor–cargo interactions: the key to transport specificity. Trends in Cell Biology 12:21–27.

Kheifets, V., and D. Mochly-Rosen. 2007. Insight into intra- and inter-molecular interactions of PKC: design of specific modulators of kinase function. Pharmacological Research 55:467–476.

Kim, Y.-J., I. Spalovská-Valachová, K.-H. Cho, I. Zitnanova, Y. Park, M.E. Adams, and D. Žitňan. 2004. Corazonin receptor signaling in ecdysis initiation. Proceedings of the National Academy of Sciences, USA 101:6704–6709.

Kirschfeld, K. 1973. Vision of polarised light. Pages 289–296 *in* Symposium Proceedings of the 4th International Biophysics Congress, Moscow.

Klein, J.M., D.P.V. De Kleijn, R. Keller, and W.M. Weidemann. 1992. Molecular cloning of crustacean pigment dispersing hormone precursor. Biochemical and Biophysical Research Communications 189:1509–1514.

Klein, J.M., C.J. Mohrherr, F. Sleutels, J.P. Riehm, and K.R. Rao. 1994. Molecular cloning of two pigment-dispersing hormone (PDH) precursors in the blue crab *Callinectes sapidus* reveals a novel member of the PDH neuropeptide family. Biochemical and Biophysical Research Communications 205:410–416.

Klein, J.M., C. Mohrherr, F. Sleutels, N. Jaenecke, J. Riehm, and K.R. Rao. 1995. A highly conserved red pigment concentrating hormone precursor in the blue crab *Callinectes sapidus*. Biochemical and Biophysical Research Communications 212:151–158.

Kleinholz, L.H. 1966. Separation and purification of crustacean eyestalk hormones. American Zoologist 6:161–167.

Kleinholz, L.H., H. Esper, C. Jonson, and F. Kimball. 1962. Neurosecretion and crustacean retinal pigment hormone: assay and properties of light adapting hormone. Biological Bulletin 123:317–319.

Kleinlogel, S., and N.J. Marshall. 2006. Electrophysiological evidence for linear polarization sensitivity in the compound eyes of the stomatopod crustacean *Gonodactylus chiragra*. Journal of Experimental Biology 209:4262–4272.

Kleinlogel, S., and N.J. Marshall. 2009. Ultraviolet polarisation sensitivity in the stomatopod crustacean *Odontodactylus scyllarus*. Journal of Comparative Physiology 195A:1153–1162.

Kleinholz, L.H., K.R. Rao, J.P. Riehm, G.E. Tarr, L. Johnson, and S. Norton. 1986. Isolation and sequence analysis of a pigment-dispersing hormone from the eyestalks of the crab *Cancer magister*. Biological Bulletin 170:135–143.

Köller, G. 1927. Über Chromatophorensystem, Farbensinn und Farbwechsel bei *Crangon vulgaris*. Zeitschrift für vergleichende Physiologie 5:191–246.

Kröyer, H. 1842. Monographisk fremstilling af slaegten *Hippolyte* nordiske arter. Kongelige Danske Videnskabernes Selskab Skrifter 9:209–361.

Kukalová-Peck, J. 1990. Fossil history and the evolution of hexapod structures. Pages 141–179 *in* T.D. Naumann, editor. The insects of Australia. CSIRO, Melbourne University Press, Melbourne, Australia.

Kulkarni, G.K., and M. Fingerman. 1986. Chromatophorotropic activity of extracts of the brain and nerve chord of the leech, *Macrobdella decora*, in the fiddler crab, *Uca pugilator*: an *in vivo* and *in vitro* study. Comparative Biochemistry and Physiology 84C: 369–372.

Lambert, D.T., and M. Fingerman. 1976. Evidence for a non-microtubular colchicine effect in pigment granule aggregation in melanophores of the fiddler crab, *Uca pugilator*. Comparative Biochemistry and Physiology 53C:25–28.

Lee, C.Y., H.S. Zou, S.M. Yau, Y.R. Ju, and C.S. Liau. 2000. Nitric oxide synthase activity and immunoreactivity in the crayfish *Procambarus clarkii*. Neuroreport 11:1273–1276.

Lee, G., and J.H. Park. 2004. Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone encoding gene in *Drosophila melanogaster*. Genetics 167:311–323.

Lee, S.G., B.D. Bader, E.S. Chang, and D.L. Mykles. 2007. Effects of elevated ecdysteroid on tissue expression of three guanylyl cyclases in the tropical land crab *Gecarcinus lateralis*: possible roles of neuropeptide signaling in the molting gland. Journal of Experimental Biology 210:3245–3254.

Lewis, D.K., M.K. Jezierski, L.L. Keeley, and J.Y. Bradfield. 1997. Hypertrehalosemic hormone in a cockroach: molecular cloning and expression. Molecular and Cellular Endocrinology 20:101–108.

Linck, B., J.M. Klein, S. Mangerich, R. Keller, and W.M. Weidemann. 1993. Molecular cloning of crustacean red pigment concentrating hormone. Biochemical and Biophysical Research Communications 195:807–813.

Lincoln, T.M., and T.L. Cornwell. 1993. Intracellular cyclic GMP receptor proteins. Federation of American Societies for Experimental Biology 7:328–338.

Martínez-Pérez, F., J. Valdés, S. Zinker, and H. Aréchiga. 2002. The genomic organization of the open reading frame of the red pigment concentrating hormone gene in the blue crab *Calinectes sapidus*. Peptides 23:781–786.

Martínez-Pérez, F., S. Zinker, G. Aguilar, J. Valdés, and H. Aréchiga. 2005. Circadian oscillations of RPCH gene expression in the eyestalk of the crayfish *Cherax quadricarinatus*. Peptides 26:2434–2444.

McNamara, J.C. 1979. Ultrastructure of the chromatophores of *Palaemon affinis* Heilprin (Crustacea, Decapoda). Modifications in the shape of hindgut chromatophores associated with pigment movements. Journal of Experimental Marine Biology and Ecology 40:193–199.

McNamara, J.C. 1980. Ultrastructure of the chromatophores of *Palaemon affinis* Heilprin (Crustacea, Decapoda). The structural basis of pigment migration. Journal of Experimental Marine Biology and Ecology 46:219–229.

McNamara, J.C. 1981. Morphological organization of crustacean pigmentary effectors. Biological Bulletin 161:270–280.

McNamara, J.C. 1989. Ultrastructure and development of pigmentary effectors in embryos of the freshwater shrimp *Macrobrachium olfersii* (Wiegmann) (Decapoda, Caridea, Palaemonidae) Crustaceana 57:38–50.

McNamara, J.C., and M.R. Ribeiro. 1999. Kinetic characterization of pigment migration and the role of the cytoskeleton in granule translocation in the red chromatophores of the shrimp *Macrobrachium olfersi* (Crustacea, Decapoda). Journal of Experimental Zoology 283:19–30.

McNamara, J.C., and M.R. Ribeiro. 2000. The calcium dependence of pigment translocation in fresh water shrimp ovarian red chromatophores. Biological Bulletin 198:357–366.

McNamara, J.C., and A. Sesso. 1982. Pigment biogenesis in freshwater shrimp ventral nerve chord chromatophores. Cell Tissue Research 222:167–175.

McNamara, J.C., and A. Sesso. 1983. Freeze fracture study of pigment granule membranes in shrimp ventral nerve chord chromatophores. Journal of Crustacean Biology 3:367–379.

McNamara, J.C., and H.H. Taylor. 1987. Ultrastructural modifications associated with pigment migration in palaemonid shrimp chromatophores (Decapoda, Palaemonidae). Crustaceana 53:113–133.

Mercier, J., D. Doucet, and A. Retnakaran. 2007. Molecular physiology of crustacean and insect neuropeptides. Journal of Pesticide Science 32:345–359.

Meyer-Rochow, V.B. 1999. Compound eye: circadian rhythmicity, illumination, and obscurity. Pages 7–124 *in* Y. Eguchi, editor. Atlas of arthropod sensory receptors. Springer, Tokyo, Japan.

Meyer-Rochow, V.B. 2001. The crustacean eye: dark/light adaptation, polarization sensitivity, flicker fusion frequency, and photoreceptor damage. Zoological Science 18:1175–1197.

Milograna, S.R., and J.C. McNamara. 2009a. Red pigment concentrating hormone receptor type and a role for protein kinase G during pigment aggregation in the red ovarian chromatophores of *Macrobrachium olfersi* (Decapoda). XXIV Reunião Anual da Federação de Sociedades de Biologia Experimental, Águas de Lindóia, São Paulo.

Milograna, S.R., and J.C. McNamara. 2009b. A role for non-myosin II and its light chain phosphatase during pigment aggregation in red ovarian chromatophores of *Macrobrachium olfersi* (Decapoda). XXIV Reunião Anual da Federação de Sociedades de Biologia Experimental, Águas de Lindóia, São Paulo.

Milograna, S.R., F.T. Bell, and J.C. McNamara. 2010. Signal transduction, plasma membrane calcium movements, and pigment translocation in freshwater shrimp chromatophores. Journal of Experimental Zoology 313A:605–617.

Milograna, S.R, F.T. Bell, and J.C. McNamara. 2012. Signaling events during cyclic guanosine monophosphate-regulated pigment aggregation in freshwater shrimp chromatophores. The Biological Bulletin 223:178–191.

Minke, B., and Z. Selinger. 1992. The inositol-lipid pathway is necessary for light excitation in fly photoreceptors. Pages 202–221 *in* D. Corey and S.D. Roper, editors. Sensory transduction. Rockefeller University Press, New York.

Mohrherr, C.J., K.R. Rao, J.P. Riehm, and W.T. Morgan. 1990. Isolation of β-PDH from sinus glands of the blue crab, *Callinectes sapidus*. American Zoologist 30:28A.

Moncada, S., R.M.J. Palmer, and E.A. Higgs. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacological Reviews 43:109–142.

Müller, U. 1997. The nitric oxide system in insects. Progress in Neurobiology 51:363–381.

Murad, F. 1994. Regulation of cytosolic guanylyl cyclase by nitric oxide: the NO-cyclic GMP signal transduction system. Advances in Pharmacology 26:19–33.

Nair, M.M., G.E. Jackson, and G. Gäde. 2001. Conformational study of insect adipokinetic hormones using NMR constrained molecular dynamics. Journal of Computer-Aided Molecular Design 15:259–270.

Nässel, D.R. 1996. Peptidergic neurohormonal control systems in invertebrates. Current Opinion in Neurobiology 6:842–850.

Natochin, M., K.G. Gasimov, and N.O. Artemyev. 2001. Inhibition of GDP/GTP exchange on GR subunits by proteins containing G-protein regulatory motifs. Biochemistry 40:5322–5328.

Nery, L.E., and A.M.L. Castrucci. 1997. Pigment cell signalling for physiological color change. Comparative Biochemistry and Physiology 118A:1135–l144.

Nery, L.E., M.A. Silva, L. Josefsson, and A.M. Castrucci. 1997. Cellular signalling of PCH-induced pigment aggregation in the crustacean *Macrobrachium potiuna* erythrophores. Journal of Comparative Physiology 167B:570–575.

Nery, L.E., M.A. Silva, and A.M.L. Castrucci. 1998. Role of cyclic nucleotides in pigment translocation within the freshwater shrimp, *Macrobrachium potiuna*, erythrophores. Journal of Comparative Physiology 168B:624–630.

O'Brien, M.A., and P.H. Taghert. 1994. The genetic analysis of neuropeptide signaling systems. Zoological Sciences 11:633–645.

Ohira, T., N. Tsutsui, I. Kawasoe, and M.N. Wilder. 2006. Isolation and characterization of two pigment-dispersing hormones from the whiteleg shrimp *Litopenaeus vennamei*. Zoological Sciences 23:601–606.

Oliveri, P., Q. Tu, and E.H. Davidson. 2008. Global regulatory logic for specification of an embryonic cell lineage. Proceedings of the National Academy of Sciences 105:5955–5962.

O'Shea, M., and R.C. Rayne. 1992. Adipokinetic hormones: cell and molecular biology. Cellular and Molecular Sciences 48:430–438.

Oshima, N. 2001. Direct reception of light by chromatophores of lower vertebrates. Pigment Cell Research 14:312–319.

Peirson, S.N., S. Halford, and R.G. Foster. 2009. The evolution of irradiance detection: melanopsin and the non-visual opsins. Philosophical Transactions of the Royal Society B 364: 2849–2865.

Perkins, E.B. 1928. Color changes in crustaceans, especially in *Palaemonetes*. Journal of Experimental Zoology 50:71–105.

Porter, M.L., T.W. Cronin, D.A. McClellan, and K.A. Crandall. 2007. Molecular characterization of crustacean visual pigments and the evolution of pancrustacean opsins. Molecular Biology and Evolution 24:253–268.

Pouchet, G. 1872. Sur les rapides changements de coloration provoqués expérimentalment, chez les Crustacés et sur les colorations bleues des poissons. Journal de l'Anatomie et de la Physiologie 8:401–407.

Provencio, I., G. Jiang, W.J. DeGrip, W.P. Hayes, and M.D. Rollag. 1998. Melanopsin: an opsin in melanophores, brain, and eye. Proceedings of the National Academy of Sciences USA 95:340–345.

Quackenbush, L.S. 1981. Studies on the mechanism of action of a pigment dispersing chromatophorotropin in the fiddler crab, *Uca pugilator*. Comparative Biochemistry and Physiology 68A:597–604.

Quackenbush, L.S., and M. Fingerman. 1984. Regulation of neurohormone release in the fiddler crab, *Uca pugilator*: effects of gamma-aminobutyric acid, octopamine, met-enkephalin, and β-endorphin. Comparative Biochemistry and Physiology 79C:77–84.

Quackenbush, L.S., and K.R. Rao. 1979. Effects of cyclic-nucleotides and A23187 on the chromatophores of the fiddler crab, *Uca pugilator*. American Zoologist 19:917–917.

Rao, K.R. 1985. Pigmentary effectors. Pages 395–462 *in* D.E. Bliss and L.H. Mantel, editors. The biology of Crustacea, Vol. 9. D.E. Bliss, Academic Press, New York.

Rao, K.R. 2001. Crustacean pigmentary-effector hormones: chemistry and functions of RPCH, PDH, and related peptides. American Zoologist 41:364–379.

Rao, K.R., and M. Fingerman. 1983. Regulation of release and mode of action of crustacean chromatophorins. American Zoologist 23:517–527.

Rao, K.R., J.P. Riehm, C.A. Zahnow, Z.H. Kleinholz, G.E. Tarr, L. Johnson, S. Norton, M. Landau, O.J. Semmes, R.M. Sattelberg, W.H. Jorenby, and M.F. Hintz. 1985. Characterization of a pigment dispersing hormone in eyestalks of the fiddler crab *Uca pugilator*. Proceedings of the National Academy of Sciences, USA 82:5319–5322.

Regier, J.C., J.W. Schultz, A. Zwick, A. Hussey, B. Ball, R. Wetzer, J.W. Martin, and C.W. Cunningham. 2010. Arthropod relationships reviewed by phylogenomic analysis of nuclear protein-coding sequences. Nature 463:1079–1083.

Rembold, C.M., and R.A. Murphy. 1990. Latch-bridge model in smooth muscle-[Ca2+]i can quantitatively predict stress. American Journal of Physiology 259:251–257.

Ribeiro, M.R. 1998. Um estudo da cinética da translocação pigmentar nos cromatóforos vermelhos do ovário do camarão de água doce *Macrobrachium olfersii* (Crustacea, Decapoda). M.Sc. Thesis, Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo.

Ribeiro, M.R. 2002. A transdução de sinal e a ativação da translocação pigmentar nos cromatóforos vermelhos do camarão *Macrobrachium olfersii* (Crustacea, Decapoda). Ph.D. Thesis, Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo.

Ribeiro, M.R., and J.C. McNamara. 1997. Possíveis mensageiros intracelulares do RPCH nos cromatóforos vermelhos do ovário do camarão de água doce *Macrobrachium olfersii* (Crustacea, Decapoda). XII Reunião Anual da Federação de Sociedades de Biologia Experimental, Caxambu, Brazil.

Ribeiro, M.R., and J.C. McNamara. 2004. Pigment aggregation in shrimp chromatophores. XXII Congresso da Sociedade Brasileira de Biologia Celular e IX Congresso Iberoamericano de Biologia Celular, Campinas, Brazil. Abstract #P-012.

Ribeiro, M.R., and J.C. McNamara. 2007. Calcium movements during aggregation in freshwater shrimp chromatophores. Pigment Cell Research 20:70–77.

Ribeiro, M.R., and J.C. McNamara. 2009. Cyclic guanosine monophosphate signaling cascade mediates pigment aggregation in freshwater shrimp chromatophores. Biological Bulletin 216:138–148.

Riento, F., and A.J. Ridley. 2003. ROCKS: multifunctional kinases in cell behavior. Nature Reviews/ Molecular Cell Biology 4: 446–456.

Robison, W.G., and J.S. Charlton. 1973. Microtubules, microfilaments and pigment movement in the chromatophores of *Palaemonetes vulgaris* (Crustacea). Journal of Experimental Zoology 186:279–304.

Scharrer, B., and E. Scharrer. 1944. Neurosecretion. IV. Comparison between the intercerebralis–cardiacum–allatum system of the insects and the hypothalamo-hypophyseal system of the vertebrates. Biological Bulletin 87:242–251.

Schmidt, H.H., S.M. Lohmann, and U. Walter. 1993. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. Biochemica et Biophysica Acta 1178:153–175.

Scholz, N.L. 2001. NO/cGMP signaling and the flexible organization of motor behavior in crustaceans. American Zoologist 41:292–303.

Scholz, N.L., M.F. Goy, J.W. Truman, and K. Graubard. 1996. Nitric oxide and peptide neurohormones activate cGMP synthesis in the crab stomatogastric nervous system. Journal of Neuroscience 16:1614–1622.

Scholz, N.L., E.S. Chang, K. Graubard, and J.W. Truman. 1998. The NO/cGMP signaling pathway and the development of neural networks in postembryonic lobsters. Journal of Neurobiology 34:208–226.

Staubli, F., T.J.D. Jorgensen, G. Cazzamali, M. Williamson, and C. Lenz. 2002. Molecular identification of the insect adipokinetic hormone receptors. Proceedings of the National Academy of Sciences, USA 99:3446–3451.

Taub-Montemayor, T.E., K.D. Linse, and M.A. Rankin. 1997. Isolation and characterization of *Melanoplus sanguinipes* adipokinetic hormone: a new member of the AKH/RPCH Family. Biochemical and Biophysical Research Communications 239:763–768.

Thurman, C.L. 1988. Rhythmic physiological color change in Crustacea: a review. Comparative Biochemistry and Physiology 9lC:171–185.

Thurman, C.L. 1990. Adaptative coloration in Texas fiddler crab (*Uca*). Pages 109–125 *in* M. Wicksten, editor. *Adaptative coloration in invertebrates: animal behavior*. Texas A and M University Press, College Station, Texas.

Tuma, M.C.B., A.M.L. Castrucci, and L. Josefsson. 1993. Comparative activities of the chromatophorotropins RPCH, α-PDH, and β-PDH on 3 crustacean species. Physiological Zoology 66:181–192.

Tuma, M.C.B., L. Josefsson, and A.M.L. Castrucci. 1995. Cytoskeleton and PCH-induced pigment aggregation in *Macrobrachium potiuna* erythrophores. Pigment Cell Research 8:215–220.

Van der Horst, D., W.J.A. Van Marrewijk, H.G.B. Vullings, and J.H.B. Diederen. 1999. Metabolic neurohormones: release, signal transduction and physiological responses of adipokinetic hormones in insects. European Journal of Entomology 96:299–308.

Vargas, M.A., B.P. Cruz, F.E. Maciel, M.A. Geihs, J.C.B. Cousin, G.S. Trindade, A.L.M. Baisch, S. Allodi, and L.E.M. Nery. 2008. Participation of nitric oxide in the color change induced by UV radiation in the crab *Chasmagnathus granulatus*. Pigment Cell & Melanoma Research 21:184–191.

Verde, M.A., C. Barriga-Montoya, and B. Fuentes-Pardo. 2007. Pigment dispersing hormone generates a circadian response to light in the crayfish, *Procambarus clarki*. Comparative Biochemistry and Physiology 147A:983–992.

Wong, S.F.-K., and D.L. Garbers. 1992. Receptor guanylyl cyclases. Journal of Clinical Investigation 90:299–305.

Žitňan, D., Y.-J. Kimc, I. Žitňanová, L. Roller, and M.E. Adams. 2007. Complex steroid-peptide-receptor cascade controls insect ecdysis. General and Comparative Endocrinology 153:88–96.

Zralá, J., D. Kodrík, H. Zmahradnícková, R. Zemek, and R. Socha. 2010. A novel function of red pigment-concentrating hormone in crustaceans: *Porcellio scaber* (Isopoda) as a model species. General and Comparative Endocrinology 166:330–336.

4

MUSCLE STRUCTURE, FIBER TYPES, AND PHYSIOLOGY

Scott Medler and Donald L. Mykles

Abstract

Crustacean muscles are striated muscles exhibiting a wide range of structural characteristics and physiological capabilities. Slow fibers possess relatively wide sarcomeres and produce slow sustained contractions used for diverse biological functions. Fast fibers possess narrow sarcomeres and generate the power needed for quick movements and bursts of locomotion. Differences in contractile rates are determined by sarcomere width and by alternate myosin heavy chain (MHC) isoforms. Crustacean fibers exhibit thin-filament regulation of muscle contraction and possess different isoforms of troponins and tropomyosin that influence the kinetics of muscle activation. Crustacean muscles commonly contain fibers that do not fit neatly into one category, but lie along a continuum of fiber types. The sarcolemma of crustacean fibers is often folded into clefts that penetrate deeply into the fiber interior. These clefts are lined with mitochondria and presumably facilitate diffusional exchange across the membrane in large fibers.

INTRODUCTION

Crustacean skeletal muscles have provided a rich area of research for decades, and a number of compelling reasons exist for studying crustacean muscles. For crustacean biologists, skeletal muscles are integral components of many key processes, making their biology relevant to researchers with a wide range of interests. Growth and molting are essential features of crustacean life history, and these complex processes must be coordinated with skeletal muscle atrophy and subsequent growth. Many crustaceans are highly energetic and mobile animals, and any studies of locomotion or other types of performance must incorporate an understanding of muscle function. Crustaceans, principally decapods, are also important components of the aquaculture and commercial seafood

industries that represent hundreds of millions of dollars of economic value annually, so an interest in their skeletal muscles is not completely academic (Oesterling 2012).

For those interested in basic skeletal muscle structure and function, crustacean muscles have provided a number of unique models for comparison with the more intensively studied vertebrate skeletal muscles. Beginning in the mid-1960s, Ashley and colleagues performed a series of experiments in which they injected the calcium-sensitive jellyfish fluorescent protein aqueorin into the giant muscle fibers of barnacles (Ashley and Ridgeway 1970). When the muscles were stimulated to contract, the aqueorin emitted light as calcium was released within the muscle to elicit contraction. These were some of the first experiments demonstrating the inextricable connection between Ca^{2+} ions and muscle contraction. Not quite a decade later, Fred Lang and colleagues embarked on a series of studies focused on the developmental changes in muscle fiber type that occur in juvenile lobster claws (Lang et al. 1977a,b, Govind and Lang 1977). These studies showed that fibers could be completely remodeled from fast to slow (and vice versa) and provided one of the first examples of invertebrate muscle plasticity (see Chapter 5 in this volume). More recently, Steven Kinsey's laboratory has used the exceptionally large muscle fibers of crabs to examine the diffusional limitations in muscle fibers more generally (Kinsey et al. 2007, Hardy et al. 2009, Kinsey et al. 2011; see Chapter 12 in this volume). These studies have provided insights not only into the constraints of cell dimensions on basic physiologic function in crustacean muscles, but also help explain how these limitations have affected the evolution of skeletal muscle design more broadly. Collectively, these studies represent just some of the many insights gained from studying these diverse and fascinating skeletal muscles.

In this chapter, we provide a general overview of crustacean muscle structure and physiological function. We begin by providing some examples of the varied and complex roles crustacean muscles play in an animal's basic biology and life history. Next, we focus on crustacean muscle structure, from the whole-muscle level down to fiber ultrastructure. We then focus on the classification of crustacean muscles into discrete fiber types, with a particular focus on the alternate isoforms of myofibrillar proteins that help define these fiber types. Although the current classification system is limited to just a few species, it is clear that a significant amount of overlap exists even among these well-defined fiber types. Finally, we conclude the chapter with a discussion of crustacean muscle physiology. Throughout the chapter, some natural overlap in subject areas is unavoidable. For example, it is impossible to adequately discuss muscle fiber types without covering the structural feature of sarcomere width or physiological parameters like shortening velocity. Although we have attempted to minimize this type of repetition, some degree of redundancy should be anticipated.

MUSCLE STRUCTURE

Overview

Crustacean muscles come in a variety of sizes, structural organizations, and even subtle colors. Each of these characteristics can be related to the functional roles of specific muscles, and crustacean muscles are highly diverse both in terms of their structural organization and function. Like all skeletal muscles, crustacean muscles are made up of a few to hundreds of individual muscle cells or fibers. These fibers are invariably anchored to the exoskeleton at their origin and are attached to a moveable connection point at their insertion. In certain instances, the insertion point is a well-defined tendon, called the *apodeme*, as is the case in the opener and closer muscles of claws. In others, muscles may insert directly onto a different region of the exoskeleton, as occurs with the extensor and flexor muscles of the tail in lobsters and crayfish. Although many crustacean muscles are organized into bipinnate structures with a central apodeme, the precise architecture of diverse muscles varies considerably.

The wide range in functional organization of crustacean muscles reflects their diverse specialized functional roles. A muscle's functional organization is closely integrated with the exoskeleton to which it is coupled. In some cases, the joints of crustaceans are formed by simple hinges (Warner and Jones 1976, Schenk and Wainwright 2001), whereas others represent highly complex structures with specialized regions of calcification in the exoskeleton that provide essential mechanical properties (Patek et al. 2004, 2007). Several well-studied musculoskeletal systems demonstrate the close coupling between the structure of the exoskeleton and the functional organization of the associated musculature. We discuss three of these systems here, to illustrate the diversity in muscle organization resulting from the functional requirements of the muscles.

The claws of many crustaceans exhibit significant dimorphism in which one claw is more heavily built. The closer muscles of both claws exhibit morphological and cellular adaptations that produce a slow forceful closure of the major claw and a more rapid closure of the minor claw. The major claw, designated the *crusher* in clawed lobsters, has a greater mechanical advantage than its partner, owing to the construction of the dactyl and the insertion point of the closer muscle on the apodeme (Warner and Jones 1976, Costello and Lang 1979, Schenk and Wainwright 2001). In addition to having an architecture that facilitates a forceful claw, all of the fibers that comprise the lobster crusher closer muscle are long-sarcomered slow fibers (Lang et al. 1977a). The slender *cutter* claw in the lobster has a mechanical advantage that favors rapid claw closure, and its closer muscle is built from approximately 65% fast muscle fibers (Lang 1977, see Chapter 5 in this volume).

The first thoracic appendages of mantis shrimp (order Stomatopoda) are specialized into raptorial or hammer-like structures used for predation and defense (Patek et al. 2004, 2007). The velocities generated during the rapid strike from these appendages are among the highest in the animal kingdom and exceed speeds that can be actively generated by contracting skeletal muscles (Burrows 1969, Patek et al. 2004). The function of the appendages has aptly been compared to the operation of a crossbow, in which the energy input to draw back the bow is exceedingly slow in comparison to the rapid release of energy that ensues when the trigger is activated (Patek et al. 2007). In the mantis shrimp, most of the exoskeleton of the merus is very thin and supple, but specialized regions of calcification in the distal portion of the appendage provide structures that function to both store energy and to effectively "cock" the structure (Patek et al. 2007). Although the precise mechanism of energy storage is not well understood, it is clear that a series of calcified ridges in the distal part of the merus are essential to the storage of potential energy generated by the contracture of the extensor muscles (Patek et al. 2007, Zack et al. 2009). The primary muscles used to generate tension for a strike are two large extensors of the carpus that insert onto two calcified sclerites that function as a "click-joint" to lock the carpus in a fully flexed position (Burrows 1969, Burrows and Hoyle 1972, McNeill et al. 1972). Two smaller carpus flexors are used to lock the "cocked" appendage in place for a short period before it is released (Burrows and Hoyle 1972). Each of these muscles is a slow contracting muscle, with ultrastructural and physiological features common to other slow muscles in crustaceans (McNeill et al. 1972). A similar mechanism is used by snapping shrimp (family Alpheidae) to generate their loud snapping sounds (Ritzmann 1974, Versluis et al. 2000; see Chapter 5 in this volume).

In crabs of the family Portunidae, the fifth pereopod has become specialized into a broad paddle that is used as a swimming appendage (Hartnoll 1971, Spirito 1972). These animals are capable of sustained swimming at speeds of up to 1 meter/s and are highly maneuverable (Spirito 1972). The muscles used to generate power for swimming are complex, in terms of both the anatomical arrangement of the muscles and of their fiber composition (White and Spirito 1973, Tse et al. 1983). Swimming is powered by the interaction of four sets of muscles that receive separate innervation and are responsible for distinct physiological activities. White muscles power short-term escape responses, whereas pink-colored muscles drive sustained swimming for prolonged periods. Both types of fibers have structural and physiological features characteristic of fast muscles, but the more

pigmented fibers are subdivided by membrane clefts lined with high concentrations of mitochondria (Tse et al. 1983, Henry et al. 2001). The specialization of these muscles into aerobic engines that power swimming behavior has evolved in several different species of crabs (Hardy et al. 2010). Clearly, these muscles have evolved a phenotype that is well matched to the paddle-like appendage necessary for swimming.

The common theme illustrated by these examples is that crustacean muscles are highly sophisticated organs, precisely matched to the exoskeletal structures with which they are integrated. Some of these systems have evolved for force production, others for explosive speed, and some for sustained power output. They are highly diverse in terms of their structural, metabolic, and physiological properties. Although the physiological systems of invertebrates are sometimes described as being simple, the diversity and complexity of organization are arguably greater in crustacean muscles than in those of vertebrates.

Ultrastructural Organization

Crustacean muscles exhibit a wide variety of structural organization at the cellular level. One of the major differences between crustacean and vertebrate skeletal muscles fibers is that crustacean fibers display a wide range of sarcomere widths (from 3 to 20 μm), while those of vertebrates are uniformly short (~2.5–3 μm; Hoyle 1967, 1983). Sarcomere width has commonly been used to identify different physiological fiber types in crustaceans because long-sarcomered fibers tend to be innervated by slow motor neurons and contract slowly. Fibers with short sarcomeres are frequently innervated by a fast motor neuron and are fast contracting, whereas intermediate fibers exhibit intermediate sarcomere widths and are often innervated by both fast and slow motor neurons (Atwood 1976, Govind and Atwood 1982). Sarcomere width is not simply a descriptive correlate of contractile phenotype but is a direct determinant of contractile strength and speed (Huxley and Niedergerke 1954, Josephson 1975). Short-sarcomered fibers have a proportionately greater number of these contractile units in series and thereby contract with greater speed than the fibers with longer sarcomeres. Long-sarcomered fibers generally have a greater number of myosin cross-bridges available per sarcomere and therefore produce greater forces than do fibers with short sarcomeres. This pattern is consistent with theoretical expectations, and contractile force is directly correlated with sarcomere width in crustacean muscles (Jahromi and Atwood 1969, Taylor 2000).

Sarcomere width in crustacean muscles is also correlated with other common features of myofibrillar organization. Short-sarcomered fibers tend to have straight and well-aligned Z lines, I bands, and A bands. Long-sarcomered fibers often possess Z lines that appear jagged or wavy in longitudinal sections, and the alignment between adjacent myofibrils is often staggered (Jahromi and Atwood 1969, Mykles and Skinner 1981, Mellon and Stephens 1992, West et al. 1992; Fig. 4.1). In many cases, the H zone in the middle of the A band is well-defined in short-sarcomered fibers but is less visible or absent in the fibers with long sarcomeres. In cross-section, short-sarcomered fibers possess thick filaments surrounded by a highly regular array of six thin filaments (Jahromi and Atwood 1969, West et al. 1992). The thick filaments in the long-sarcomered fibers are surrounded by a higher number of thin filaments (~12 or more) that are scattered around the filament without any obvious order (Jahromi and Atwood 1969, 1971, Mykles and Skinner 1981, West et al. 1992). The filaments themselves are narrower in fast muscles with short sarcomeres than in slow muscles. In both muscle types, an organized array of myosin subfilaments is thought to surround a core of paramyosin. Those of slow muscles are composed of a greater number of these myosin subfilaments and have a thicker core of paramyosin than those of fast muscles (Jahromi and Atwood 1969, Chapple 1982). It is generally thought that the combination of wider thick filaments and a higher number of thin filaments associated with slow fibers results in a greater level of generated force than in fast fibers (Mellon and Stephens 1992, Royuela et al. 2000).

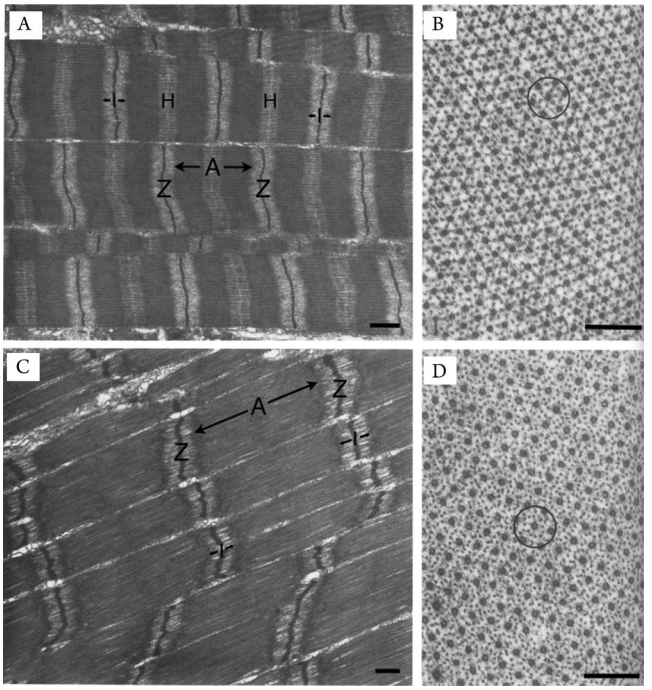


Fig. 4.1.
Structural differences between short- (A, B) and long-sarcomered (C, D) fibers from the claw closer muscles of the Australian yabby, *Cherax destructor*. Longitudinal section of short-sarcomered fiber (A) shows distinct A and I bands, and these regions of adjacent myofibrils line up in register. The H zones and M lines (middle of H zones) in the middle of each A band are distinct. In cross-section (B), each thick filament is surrounded by six thin filaments (enclosed in circle). Longitudinal section of long-sarcomered fiber (C) show that Z lines are less straight, do not line up in register between adjacent myofibrils, and are not always perpendicular to the fiber axis. The H zones and M lines are not readily apparent. In cross-section (D), the long-sarcomered fibers exhibit thick filaments surrounded by an average of 12 thin filaments (enclosed in circle). All figures are transmission electron micrographs (TEM), scale bars = 0.2 μm. From West et al. (1992), with permission from Springer.

Each myofibril within a muscle fiber is surrounded by a collar of membranes formed by the sarcoplasmic reticulum (SR) that is contacted by the network of tubular membranes invaginating from the sarcolemma (Rosenbluth 1969, Franzini-Armstrong et al. 1986, Ushio and Watabe 1993; Fig. 4.2). The extent of the SR varies greatly, depending on the speed and frequency of contractions produced by the muscle. In slow muscles, the SR and corresponding tubular system tend to be less developed than in fast fibers (Ushio and Watabe 1993, Lagersson 2002), although in some cases these differences are minor (Jahromi and Atwood 1969, Jahromi and Atwood 1971). In certain specialized high-frequency muscles, such as the antennal remoter muscle in lobsters, the relative proportion of SR makes up the majority of the fiber volume, and myofibrils only account for about a quarter of the total volume (Rosenbluth 1969).

Crustacean fibers possess a well-developed internal membrane system that functions to carry depolarizations of the sarcolemma into the muscle fiber during excitation-contraction coupling (Peachey 1967, Selverston 1967, Franzini-Armstrong et al. 1986, Ushio and Watabe 1993). In addition to the well-known T tubules that are present in vertebrate skeletal muscles, crustacean fibers variably also possess surface membrane clefts and Z tubules (Peachey 1967, Franzini-Armstrong et al. 1986). The clefts of crustacean fibers are often highly developed and greatly increase the surface area of the sarcolemma. Various tubular systems, running not only transversely, but also in

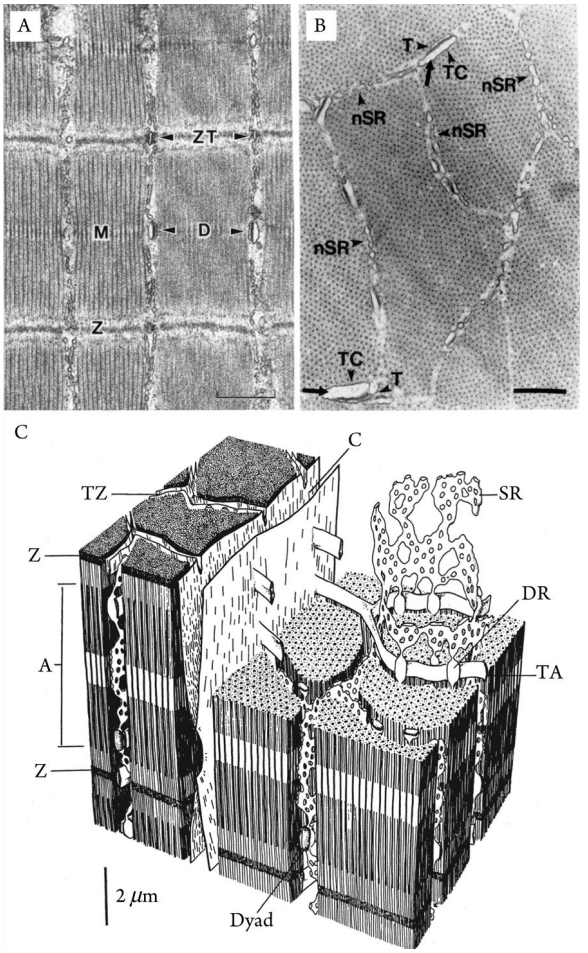


Fig. 4.2.
Membrane systems in crustacean fibers. Longitudinal- (A) and cross-sections (B) of muscle fibers demonstrate the membrane invaginations that form networks around myofibrils (TEM). In both figures, diads are visible where T tubules come into contact with the terminal cisternae of the SR (D in (A); T and arrows in (B)). Other abbreviations: (A) Z tubule, ZT; M line, M; Z line, Z; (B) nonjunctional sarcoplasmic reticulum, nSR; terminal cisternae, TC. Three-dimensional reconstruction (C) of the membrane systems in crustacean fibers demonstrates the relationships among membrane clefts, C, T tubules ("A tubules," TA), SR, and the terminal cisternae ("dilated cisternae," DR). T tubules arise as invaginations of the membrane clefts (C). Other abbreviations in (C): Z tubules, TZ; A band, A; Z line, Z. (A) From Stokes and Josephson (1992), with permission from Springer; (B) from Ushio and Watabe (1993), with permission from Wiley and Sons, Inc.; (C) from Peachey (1967), with permission from Oxford University Press. Scale bars in A and B = 0.5 μm; scale bar in C = 2 μm.

longitudinal and oblique directions, arise from these clefts (Hoyle 1983). Of these, Z tubules penetrate into fibers at the level of the Z line. The T tubules are defined by the fact that they form junctions with the SR and are integral to the process of excitation-contraction coupling, although some junctions with the SR are also formed directly with the clefts themselves. The T tubules form flattened cisternae that are directly opposed to the SR at these positions, where they form dyads and triads (Peachey 1967, Franzini-Armstrong et al. 1986). Fast muscles tend to have a greater density of these connections than slow muscles. In some slow fibers, T tubules regularly penetrate into the fiber at the level of the outer borders of the A bands, but, overall, there is no regular placement of

these tubules. Particularly in fast muscles, the T tubules may penetrate the fiber in different locations (Jahromi and Atwood 1969, Rosenbluth 1969, Franzini-Armstrong et al. 1986, Stokes and Josephson 1992). Franzini-Armstrong et al. (1986) reported that the Z tubules are distinct from the T-tubule system, although continuities between the systems do exist. The Z tubules do not apparently form junctions with the SR, and their function is poorly understood (Jahromi and Atwood 1971, Franzini-Armstrong et al. 1986).

Ultrastructural studies have provided evidence of foot processes that connect the T tubule to the SR at dyadic and triadic junctions that are similar to those observed in vertebrate muscles (Fig. 4.2A,B; see Mellon and Stephens 1992). We now know that these feet are ryanodine receptors (RyR) responsible for coupling depolarization of the T tubule with intracellular Ca^{2+} release by the SR (Franzini-Armstrong and Protasi 1997, Di Biase and Franzini-Armstrong 2005). In vertebrate skeletal muscles, the ryanodine receptors are physically coupled to the dihydropyridine receptors (DHPR) embedded in the T-tubule membranes and function together as Ca^{2+} release units. In the muscles of crustaceans and other invertebrates, the ryanodine receptors serve a similar role in excitation-contraction coupling, but there is not a direct association with the DHPRs (Di Biase and Franzini-Armstrong 2005; and see the section "Excitation-Contraction Coupling").

MUSCLE FIBER TYPES

Overview

The use of specialized muscle cells to generate contraction and movement is a defining characteristic of the Animal Kingdom. All animals, from creeping worms to the fastest vertebrates, rely on the same fundamental processes of muscle contraction to power their movements. Myosin motor proteins are organized into thick filaments that interdigitate with thin filaments of actin and produce force when the myosin motor pulls on the actin filaments. Within this general scheme exists a diverse array of specific levels of muscle organization (Hoyle 1967, Hoyle 1983, Paniagua et al. 1996). In smooth muscles, thick and thin filaments interdigitate with one another, but the spatial organization of the thick and thin filaments within the muscle cell is not well defined. In cross-striated muscles, thick and thin filaments are organized into alternating A bands and I bands along the fiber length, which produces the characteristic repeated banding pattern of these muscles (Fig. 4.1). Obliquely striated muscles are similar to striated muscles in having thick and thin filaments organized into well-defined regions, but the angle of these regions is much less than the right angles observed in striated muscles. Broadly, different animals possess a continuum of muscle fiber types ranging from smooth muscles, to obliquely striated, to striated fibers (Hoyle 1983, Paniagua et al. 1996). In most animal taxa, including the vertebrates and mollusks, multiple types of muscles are present within an organism and even within the same muscle (Paniagua et al. 1996, Royuela et al. 2000). Among the arthropods, all of the muscles are cross-striated, including the muscles of the heart and other visceral organs (Mellon 1992, Paniagua et al. 1996).

Diverse muscles within the Animal Kingdom also differ with respect to their mechanisms of muscle activation (Lehman and Svent-Gyorgyi 1975, Svent-Gyorgyi 1975, Royuela et al. 2000, Hooper et al. 2008). At rest, all muscles possess mechanisms that prohibit the myosin heads of the thick filaments from interacting with the thin filament actins. In the muscles of all animals, an increase in intracellular Ca^{2+} is required to initiate the force-producing interaction between actin and myosin (Szent-Gyorgyi 1975, Hooper et al. 2008). In some muscles, an inhibitory state of the myosin motor must be removed to initiate muscle contraction (*thick filament regulation*). In

molluskan muscles and vertebrate smooth muscles, a light chain of myosin serves as the regulator of muscle contraction (Szent-Gyorgyi 1975, Hooper et al. 2008, Himmel et al. 2009). In others, troponin and tropomyosin proteins associated with the actin filaments effectively inhibit muscle contraction by blocking the myosin binding site on the actin filaments (*thin filament regulation*; Szent-Gyorgyi 1975, Royuela et al. 2000, Hooper et al. 2008). As with the structural organization of muscles, animals broadly possess a whole range of mechanisms used for muscle activation. Many animals possess both thick filament-regulated and thin filament-regulated muscle fiber types (Lehman and Svent-Gyorgyi 1975, Szent-Gyorgyi 1975, Royuela et al. 2000, Hooper et al. 2008). Arthropods, including crustaceans, rely primarily or exclusively on thin filament regulation during muscle activation. However, there is evidence that some crustacean slow muscles possess dually regulated systems (Lehman and Svent-Gyorgyi 1975, Szent-Gyorgyi 1975, Royuela et al. 2000, Hooper et al. 2008). Crustacean muscles are therefore very similar to vertebrate skeletal muscles with respect to their cross-striated organization and in predominantly possessing thin filament regulation of actomyosin activation.

General Classification of Muscle Fiber Types

Striated skeletal muscles are composed of populations of individual cells, or muscle fibers, which represent the cellular basis of muscle contraction. In all animals, distinct populations of muscle fibers are present, providing specialization of contractile function for differing mechanical requirements (Rome et al. 1988, Rome and Lindstedt 1997). Fast-contracting muscles are needed for bursts of power, whereas slower muscles are used for activities that require more prolonged periods of sustained force generation. Metabolic properties of different fiber types are often matched with shortening speed, with faster muscles tending to be less aerobic and mainly relying on glycolysis and intracellular phosphagens to fuel muscle contraction (Rome and Lindstedt 1997). Within these general parameters, specific muscle fiber types from diverse species exhibit a wide range of contractile and metabolic properties (Rome and Lindstedt 1997). Crustacean muscle fibers are as diverse as those of any animal group. Slow fibers often control appendages and body regions where forces need to be maintained over a period of time, but rapid contraction is not a requirement. These include fibers of claw opener and closer muscles and many of the superficial muscles of the abdomen (Jahromi and Atwood 1969, Ogonowski and Lang 1979, Mykles 1988, Fowler and Neil 1992, Neil et al. 1993, Sohn et al. 2000, Medler et al. 2004). Fast fibers are important in muscles that power rapid locomotion, such as the deep abdominal muscles in lobsters and crayfish, as well as in the leg muscles of running crabs (Jahromi and Atwood 1969, Ogonowski and Lang 1979, Mykles 1985a, Li and Mykles 1990, Cotton and Mykles 1993, Medler and Mykles 2003, Perry et al. 2009). Fast fibers are also found in the cutter claws of lobsters and the pincer claws of snapping shrimp (Mellon and Stephens 1978, O'Connor et al. 1982, Govind 1987). Although slow fibers tend to be more aerobic than fast fibers, this is not a strict correlation, and there are many examples of fast aerobic fibers characterized by high mitochondrial densities (see the section "Aerobic Capacity"). Some muscles are specialized to generate not only fast contractions, but have also evolved to produce muscle twitches at high contractile frequencies (Fahrenbach 1963, Rosenbluth 1969, Stokes and Josephson 1992, Josephson and Stokes 1994). These muscles exhibit specializations that include high densities of T-tubule systems and SR necessary to produce rapid increases and decreases in Ca^{2+} concentrations that trigger muscle activation and relaxation, respectively.

Over the years, skeletal muscle biologists have been aware of differences in muscle fiber types and have attempted to classify these fiber types into some logical framework. Initially, muscles were grouped simply into "red" versus "white" fiber types based on their superficial

appearance. In other cases, physiological measurements allowed different fibers to be identified based on their contractile properties as either "fast" or "slow." The development of histochemical assays using frozen muscle sections, particularly myofibrillar ATPase histochemistry, allowed for the identification of several different fiber types (Brooke 1970, Barnard et al. 1971). These techniques led to the classification of mammalian fiber into three fundamental groups: slow (I), fast glycolytic (IIB), and fast oxidative (IIA). Subsequently, the myosin heavy chain (MHC) motors responsible for generating contraction in mammalian muscles were identified (Schiaffino and Reggiani 1996, 2011). These different isoforms are encoded by several distinct genes, and the specific MHC isoform(s) expressed in single fibers has become the standard for classifying muscle fiber types (Schiaffino and Reggiani 1996, 2011, Pette and Staron 2000, 2001). Different MHC isoforms within mammalian fibers are now identified either through labeling muscle sections with monoclonal antibodies directed against specific MHC isoforms or through single-fiber sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) analysis (Booth et al. 2010, Pandorf et al. 2010).

Each of these approaches has been used to distinguish crustacean muscle fiber types. In crustaceans, fast-contracting glycolytic fibers are often large in diameter and appear either translucent or pearly white in coloration. Fast and slow fibers with varying degrees of aerobic capacity range from light brown or tan in coloration, to varying shades of pink or red. Histochemical procedures adapted from those used to identify mammalian muscle fiber types have been used to successfully distinguish crustacean fibers (Ogonowski and Lang 1979, Ogonowski et al. 1980, Silverman and Charlton 1980, Tse et al. 1983, Maier et al. 1984, Rathmayer and Maier 1987, Mykles 1988, Gunzel et al. 1993). These techniques stain crustacean fast muscles dark brown to black but leave slow fibers relatively unstained. Preincubating tissue sections with buffers of different pH reverses the staining reaction and reveals a range of intermediate fiber types. This range of fiber types identified through histochemical methods can be directly correlated with the physiological properties and innervation patterns of single fibers (Rathmayer and Maier 1987). It is reasonable to assume that these staining differences are directly correlated with the expression of distinct MHC isoforms present within different fibers, as is the case for mammalian fiber types (Staron and Pette 1986, Staron and Hikida 1992). This pattern has been confirmed in at least one study of crustacean muscles (Neil et al. 1993). Several different MHC isoforms in a limited number of species have been identified using single-fiber SDS-PAGE analysis (LaFramboise 2000, Medler and Mykles 2003, Medler et al. 2004, Perry et al. 2009). A better understanding of the number and types of crustacean MHC isoforms is needed to objectively classify these fiber types with a classification scheme similar to that used for mammalian fibers. Another common method used to classify crustacean fiber types is sarcomere width, which roughly varies from 2.5 to 20 μm. This range of sarcomere dimensions is starkly different from mammalian fibers, which have evolved to a constant width of ~2.5 μm in fast and slow fiber types alike (Hoyle 1983). Crustacean fast fibers are constructed from narrow sarcomeres (2.5–4 μm) and slow fibers from long sarcomeres (12–20 μm), whereas many fibers possess sarcomeres of intermediate width (Atwood 1976, Govind and Atwood 1982).

A pattern commonly observed in different crustacean muscles is that specialized fiber types are anatomically segregated within the same muscle. As a general rule, the more aerobic and slower fiber types tend to be localized closer to the joint at the most proximal and distal regions of the muscle (Mykles et al. 2002, Medler and Mykles 2003, Perry et al. 2009). In the closer muscle of the cutter claw of lobsters and in the pincer of snapping shrimp, the muscle has a central band of fast muscle fibers surrounded by slow fibers (Ogonowski et al. 1980, Govind 1987). These different fiber types provide for a range of muscle contraction rates for distinct types of claw movements.

Identified Fiber Types in Lobster Muscles

Distinct muscle fiber types identified by specific MHC and other myofibrillar isoforms have revealed a level of diversity and complexity that could not be detected using histochemical techniques alone. The most comprehensive understanding of myofibrillar isoforms in crustacean muscle is from the American lobster, *Homarus americanus*, where alternate isoforms have been identified for MHC, myosin light chains (MLCs), paramyosin, tropomyosin, troponin T, troponin I, troponin C, and actin (Fig. 4.3, Tables 4.1 and 4.2). Here, we discuss the current understanding of these myofibrillar isoforms and what is known from other crustacean species for comparison.

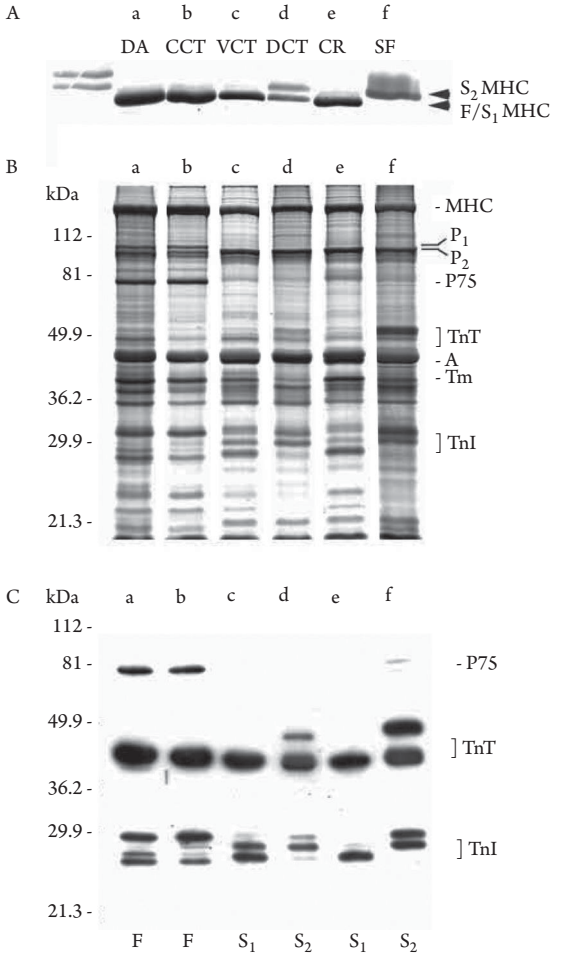


Fig. 4.3.
Myofibrillar protein assemblages in several muscles of the adult lobster *Homarus americanus*. Myosin heavy chain isoforms are shown in (A). Silver-stained gel of multiple myofibrillar proteins are shown in (B). Composite Western blot of P75, TnT isoforms, and TnI isoforms are shown in (C). Multiple isoforms are expressed for many of the myofibrillar proteins, and specific fiber types are characterized by unique combinations of these isoforms. Muscle fibers: deep abdominal (DA), central cutter closer (CCT), ventral cutter closer (VCT), distal cutter closer (DCT), crusher (CR), superficial flexor (SF). These fibers can be classified as fast (F), slow twitch (S_1), or slow tonic (S_2). Abbreviations: myosin heavy chain, MHC; paramyosin, P; 75 kDa protein, P75; troponin T, TnT; actin, A; tropomyosin, Tm; troponin I, TnI. From Medler and Mykles (2003), with permission from The Company of Biologists, Inc.

Table 4.1. Myofibrillar protein isoforms in fiber types of the American lobster *Homarus americanus*. Actin isoforms have been identified from nucleotide sequences alone. Isoforms of MHC, Tm, and TnC have been identified at both the protein and nucleotide levels. Isoforms of paramyosin, P75, TnT, TnI, and MLCs have been identified at the protein level using SDS-PAGE.

Protein	**Fiber Type**		
	Fast	**Slow Twitch (S_1)**	**Slow Tonic (S_2)**
MHC	Fast	S_1	S_2
Paramyosin	P_1>>P_2	P_2	P_2
P75	+	-	-
TnT	T_2	T_3>>T_2	T_1, T_3
Actin	SK4>5>3 (CT) SK8>5>>7 (DA)	SK1>2	
Tm	Fast	S_1	S_2
TnI	I_1>I_2>I_4>I_5>I_3 (CT) I_1,I_5>I_3>I_2 (DA)	I_4>I_2 (CR) I_3>I_2>I_4 (SA)	I_2>I_4 (CR) I_2>I_3 (SA)
TnC	C_3 (CT) C_2 (DA)	C_1, C_3	
MLC (alpha)	LC_2>>LC_1	LC_2>>LC_1>LC_3	LC_2>>LC_1
MLC (beta)	LC_1 (CT) LC_2 (DA)	LC_1	LC_1

Abbreviations: CT, cutter claw closer; CR, crusher claw closer, DA, deep abdominal muscle; SA, superficial abdominal muscle. Table compiled from Mykles 1985a, Mykles 1985b, Mykles 1988, Li and Mykles 1990, Cotton and Mykles 1993, Mykles et al. 1998, Medler and Mykles 2003, Koenders et al. 2004, Kim et al. 2009, Chao et al. 2010. Abbreviations for proteins: myosin heavy chain, MHC; 75 kDa protein, P75; troponin T, TnT; tropomyosin, Tm; troponin I, TnI; troponin C, TnC; myosin light chain, MLC.

MHC exists as at least three isoforms designated fast, slow twitch (S_1), and slow tonic (S_2) in lobster muscles (Li and Mykles 1990, Cotton and Mykles 1993, Medler and Mykles 2003, Medler et al. 2004, 2007). These isoforms have been identified at the protein level using SDS-PAGE analysis (Medler and Mykles 2003, Medler et al. 2004; Fig. 4.3, Table 4.1) and the 3′ terminal sequences that encode the carboxy-terminal rod region of each isoform has been cloned (Cotton and Mykles 1993, Medler and Mykles 2003, Medler et al. 2004; Table 4.2). The overall sequence similarity among the identified sequences is approximately 80% within the open reading frame, and each isoform has a distinct 3′ untranslated region (UTR), which suggests that the alternate isoforms may be encoded by distinct genes. The fast MHC is found within several different muscles, including the closer muscles of the cutter claw and within the deep extensor and flexor muscles of the abdomen. The S_1 MHC is expressed in various slow muscles, including those of the claw openers and within the closer muscles of the crusher claw. This isoform is also expressed to varying levels within the more superficial postural muscles of the abdomen. The S_2 MHC isoform is expressed in muscles that appear to correspond to the physiologically identified slow tonic fibers. These fibers are frequently located within the proximal and distal regions of muscles near joints, and they are likely used to maintain muscle contractions over a period of time. Within the claw closers, S_2 fibers are found in a distal bundle of fibers, and the S_1 isoform is frequently co-expressed to varying degrees within single fibers (Medler and Mykles 2003). A similar pattern of co-expression at both

Table 4.2. Myofibrillar nucleotide sequences of the American lobster *Homarus americanus*. References: [a](Cotton and Mykles 1993), [b](Chao et al. 2010), [c](Garone et al. 1991), [d](Kim et al. 2009), [e](Koenders et al. 2002), [f](Medler and Mykles 2003), [g](Medler et al. 2004), [h](Mykles et al. 1998).

Protein	Isoform	Alternative ID	Sequence Information	GenBank Accession #	Ref
MHC	Fast		Partial cds (1529 bp) C-term	U03091.1	a
	S_1		Partial cds (1795) bp C-term	AY232598.1	f
	S_2		Partial cds (813) bp C-term	AY521626	g
Paramyosin			EST (317 bp)	GO271460.1	
P75			Partial cds (766 bp)	AY302591.1	f
TnT	–	–	–	–	
Actin	SK1		Complete cds (1386 bp)	FJ217207	d
		α actin		AF399872	e
	SK2		Complete cds (1395 bp)	FJ217208	d
	SK3		Complete cds (1224 bp)	FJ217209	d
	SK4		Complete cds (1248 bp)	FJ217210	d
	SK5		Complete cds (1295 bp)	FJ217211	d
	SK6		Complete cds (1243 bp)	FJ217212	d
	SK7		Complete cds (1276 bp)	FJ217213	d
	SK8		Complete cds (1245 bp)		d
Tm	Fast		Complete cds (896 bp)	AF034954.1	h
	S_1		Complete cds (2,223 bp)	AF034953.1	h
	S_2		Complete cds (1526 bp)	AY521627	g
TnI			EST (657 bp)	FD699253.1	
			EST (660 bp)	FD467672.1	
TnC	TnC_1		Complete cds (814 bp)	FJ790218	b
		aa sequence		P29289	c
	TnC_{2a}		Complete cds (639 bp)	FJ790219	b
		aa sequence		P29290	c
	TnC_{2b}		Complete cds (2094 bp)	FJ790220	b
		aa sequence		P29291	c
	TnC_{2b}''		Complete cds (2136 bp)	FJ790221	b
	TnC_3		Complete cds (1046 bp)	HM448422	b
	TnC_4'		Complete cds (1667 bp)	FJ790223	b
	TnC_4''		Complete cds (842 bp)	FJ790222	b
	TnC_4'''		Partial cds (563 bp)	FJ790225	b
	TnC_6		Complete cds (2439 bp)	GQ259153	b
	TnC_{6x}		Complete cds (2171 bp)	GQ259154	b
MLC			EST (695 bp)	FE044128.1	
			EST (194 bp)	GO271581.1	

Abbreviations: aa, amino acid; cds, coding sequence; C-term, C-terminal and 3′ untranslated region; EST, expressed sequence tag; EST identified sequences are only included where published sequences are lacking. Other ESTs encoding *H. americanus* MHC, actin, Tm, TnI, and TnC exist in the GenBank database.

the protein and mRNA levels is observed within the superficial extensor and flexor muscles of the abdomen (Medler et al. 2004). In these single fibers, the S_1 and S_2 isoforms are expressed in varying levels, forming a continuum from the "pure" S_1 and "pure" S_2 fibers. Co-expression of multiple MHCs within single fibers is common in many lobster muscles, even within fibers traditionally classified as either fast or slow.

Six complete MHC sequences have recently been identified from the abdominal muscles of three shrimp species, and these represent the first full-length MHC sequences from crustaceans (Koyama et al. 2012a,b, 2013). Based on sequence comparisons, these represent two different fast MHC isoforms (MHC_1 and MHC_2) expressed in deep abdominal muscles of the shrimp (Koyama et al. 2012a,b, 2013). In addition, several partial sequences from adult pleopod muscles and from developing shrimp muscles have also been identified (Koyama et al. 2013). One of the adult pleopod MHCs exhibits sequence similarities suggesting homology with lobster S_2 MHC (Koyama et al. 2013). In crayfish muscles there are 3–4 different myosin isoforms expressed within different muscles, but their correspondence to the lobster isoforms has not been determined (Sakurai et al. 1996, LaFramboise 2000). Perry et al. (2009) identified three MHC isoforms distributed among distinct fiber types within the carpus extensor and flexor muscle of the ghost crab *Ocypode quadrata*. The 3′ terminal coding sequences and UTRs were also cloned from three distinct MHC isoforms. The similarity among these sequences, and in comparison to those from the lobster, was approximately 80%, but there was no clear correspondence between the crab and lobster MHCs, thus indicating that these may not represent homologous genes. In the large anaerobic fibers from the ghost crab muscles, two MHC isoforms (MHC_1 and MHC_3) were always expressed in approximately 50:50 proportions. More aerobic fibers near the proximal and distal ends of the muscle expressed a distinct isoform (MHC_2), usually as a single isoform but sometimes with one of the isoforms from the fast fibers.

MLCs exist in lobster muscles as 21–23 kDa (α) and 18–18.5 kDa (β) proteins. Three α and two β MLC isoforms are expressed to varying degrees in different lobster fiber types (Table 4.1). A similar pattern occurs in crayfish muscles, where each protein (α and β) is expressed as a fast and slow isoform. In addition, a third 31 kDa MLC is expressed in slow muscles (Sakurai et al. 1996).

Many of the other nonmyosin proteins are also present as multiple isoforms, and their expression largely mirrors that of the MHC isoforms. Many of these isoforms were identified decades ago using SDS-PAGE gels (Costello and Govind 1984, Mykles 1985a,b, 1988). More recently, a significant degree of progress has been made in identifying the gene sequences and tissue-specific expression patterns of these different isoforms. Paramyosin is a large (~105–110 kDa) protein that forms the core of thick filaments in many different invertebrate muscles, and two isoforms of this protein have been identified at the protein level (Table 4.1; Mykles 1985a). In the lobster, fast cutter and deep abdominal muscles preferentially express the larger P_1 isoform, whereas slow crusher claw and superficial abdominal muscles exclusively express the smaller P_2 isoform (Mykles 1985a). Little information is currently available about the gene sequences or mRNA distribution of these paramyosin isoforms, although a putative sequence has been identified as an expressed sequence tag (Table 4.2).

Tropomyosin is formed as a coiled-coil dimeric protein and is coupled to the troponin proteins (TnI, TnT, and TnC) to form the Ca^{2+}-sensitive "switch" in thin-filament regulation of muscle contraction (Hooper and Thuma 2005). In the relaxed state, tropomyosin (Tm) lies in the groove of the actin filament and physically prevents MHC from binding to the actin and generating muscle contraction. When activated, intracellular Ca^{2+} concentrations rise, the Ca^{2+} ions bind to troponin C, and a conformational change takes place in the troponin/tropomyosin complex that moves the tropomyosin away from the myosin binding sites on the actin filament. Therefore, it is anticipated that alternate isoforms of any of these proteins might affect the steepness of the force–Ca^{2+} relationship and thereby influence the sensitivity of muscle activation. Each of the proteins that operate as

a component of this switch (tropomyosin, troponin T, troponin I, and troponin C) exists as multiple isoforms in lobster muscles (Tables 4.1 and 4.2). Tropomyosin exists as three known isoforms, designated as fast, S_1, and S_2, following the MHC nomenclature (Mykles et al. 1998, Medler et al. 2004; Tables 4.1 and 4.2). Each isoform is encoded by a single gene, and specific isoforms are generated through alternative splicing (Mykles et al. 1998, Medler et al. 2004). Three skeletal muscle Tm isoforms are also present in different muscles of the spiny lobster *Panulirus japonicus* and appear to correspond to the isoforms in the American lobster (Ishimoda-Takagi et al. 1997). In addition, an isoform specific to the heart muscle is also present (Ishimoda-Takagi et al. 1997).

Troponin T also exists as three different isoforms, designated simply as TnT_1, TnT_2, and TnT_3 in the order of migration on SDS-PAGE gels ($T_1 < T_2 < T_3$). TnT_2 is preferentially expressed within the fast muscles, TnT_3 in slow twitch (S_1) muscles, and TnT_1 is found specifically within the slow tonic (S_2) fibers (Table 4.1). We do not currently have information about the gene sequences encoding the TnT isoforms in lobster muscles.

Troponin I exists as five different isoforms, with multiple isoforms frequently being expressed within single fibers. During the juvenile stages of muscle differentiation in lobsters, single fibers express several isoforms, but the patterns of expression become more limited as the lobsters reach adulthood (Medler et al. 2007). In fully differentiated fast fibers of the cutter claw, the predominant isoform is TnI_1, whereas in the S_1 fibers of the crusher TnI_4 is the major isoform (Mykles 1985a, Medler et al. 2007). Adult S_1 fibers in the abdominal muscles predominantly express TnI_3 with some levels of TnI_4; S_2 fibers primarily express TnI_2, whereas fast fibers express TnI_1 in combination with other isoforms (Mykles 1985a, Medler et al. 2004). Many fibers exhibiting phenotypes intermediate to the S_1 and S_2 fiber types express varying levels of the TnI isoforms (Medler et al. 2004). Information about the genes encoding the different TnI isoforms is currently lacking, being limited to partial sequences identified as expressed sequence tags (Table 4.2).

Troponin C is the Ca^{2+} binding protein that functions as the Ca^{2+}-sensitive switch in thin filament-regulated muscles. In lobster muscles, three isoforms of TnC have been identified at the protein level from lobster claw and abdominal muscles using SDS-PAGE analysis (Mykles 1985a), and three have been identified through protein purification and amino acid sequencing of isoforms expressed in the abdominal muscles (Garone et al. 1991; Tables 4.1 and 4.2). However, a recent study has revealed a much greater level of complexity than has previously been anticipated. cDNA sequences for 11 different TnC isoforms have now been identified from lobster tissues, with 6–8 of these being predominantly or exclusively expressed within the skeletal muscles (Chao et al. 2010; Table 4.2). The 11 different isoforms are encoded by seven different genes, with several isoforms being generated by alternative splicing of the same gene (Chao et al. 2010). Three of the isoforms identified by their nucleotide sequences identified by Chao et al. (2010) corresponded to those previously identified by Garone et al. (1991).

Until recently, the protein actin that forms the backbone of the thin filament was known to exist as a single isoform in lobster skeletal muscles. However, new data have shown that at least 12 actin isoforms are expressed within various lobster tissues, eight of which are primarily or exclusively expressed within the skeletal muscles (Kim et al. 2009; Table 4.2). These different isoforms are the products of distinct genes, and the expression of specific isoforms is muscle-specific (Kim et al. 2009). In the land crab *Gecarcinus lateralis*, several actin isoforms are present (9–15, encoded by 7–11 genes), but their tissue-specific expression patterns are not known (Varadaraj et al. 1996). In *Artemia*, 8–10 actin genes are present, and four have been cloned (Macias and Sastre 1990). Similar patterns of actin expression are observed in crustaceans from other taxonomic groups, but the functional significance of this diversity is poorly understood (Hooper and Thuma 2005). In *Drosophila* spp., six different actin isoforms are expressed, four being specific to skeletal muscles (Fyrberg et al. 1998, Lovato et al. 2001, Hooper and Thuma 2005). These isoforms are selectively expressed in different muscles (Lovato et al. 2001) and have been shown to possess nonequivalent physiological

functions (Fyrberg et al. 1998). These patterns suggest that different actin isoforms confer subtly different physiological properties to muscles with different functions (Fyrberg et al. 1998, Lovato et al. 2001, Hooper and Thuma 2005).

The multiplicity of myofibrillar isoforms present in crustacean skeletal muscles suggests that the precise contractile properties of the muscle are determined by the specific combination of myofibrillar isoforms within a fiber. In vertebrate muscle fibers, it is well established that muscle shortening velocity is determined directly by the MHC isoform(s) expressed. Alternate isoforms of MHC generally provide for a range of shortening velocities, with the fastest isoforms being roughly 5–10 times greater in their velocities than the slowest (Schiaffino and Reggiani 1996, Reggiani et al. 2000). In lobster muscles, histochemical staining clearly shows that muscles possessing the fast MHC hydrolyze adenosine triphosphate (ATP) at higher rates than the slow S_1 MHC (Ogonowski and Lang 1979, Ogonowski et al. 1980). ATPase activity measured from isolated myofibrillar proteins indicates that the lobster fast MHC hydrolyzes ATP at approximately 2–5 times the rate of the slow S_1 MHC (Mykles 1985a), and ATP hydrolysis rate is directly correlated with the speed of muscle shortening (Schiaffino and Reggiani 1996). Histochemical analysis indicates that the slow S_2 MHC is even slower than the S_1 isoform (Mykles 1988, Fowler and Neil 1992, Neil et al. 1993), and mechanical measurements from the Norway lobster *Nephrops norvegicus* are consistent with this interpretation (Holmes et al. 1999). In many single fibers in lobster muscles, multiple MHC isoforms are expressed (Medler and Mykles 2003, Medler et al. 2004). These fibers are known as "hybrid" fibers and are often interpreted to be transitional fibers, caught in the process of switching from one phenotype to another (Pette and Staron 2000). More recently, it has become clear that hybrid fibers are common components of many normal muscles, in which MHC coexpression is often the rule rather than the exception (Stephenson 2001, Caiozzo et al. 2003). In lobster muscles, a significant level of coexpression of different MHC isoforms is present at both the mRNA and protein levels (Medler and Mykles 2003, Medler et al. 2004, 2007). In the slow superficial muscles of the abdomen, a continuum exists between pure S_1 and S_2 fibers in terms of MHC expression and other myofibrillar isoforms as well (Medler et al. 2004). In the leg muscles of the ghost crab *O. quadrata*, three different MHC isoforms are present, and single anaerobic fast fibers typically express MHC_1 and MHC_3 in approximately 50:50 proportions (Perry et al. 2009). The physiological significance of MHC co-expression is not completely understood, but in mammalian muscles, hybrid fibers possess contractile properties intermediate to the pure fiber types (Reiser et al. 1985, Larsson and Moss 1993, Bottinelli et al. 1996). This suggests that blending of two or more MHCs within single fibers may provide for a continuum of contractile properties.

Alternate isoforms of myofibrillar proteins other than MHC also likely contribute to functional differences among fibers, but their role is even less well understood than that of the MHC isoforms. In principle, alternate isoforms of the thin filament regulatory proteins (tropomyosin, TnI, TnT, and TnC) should affect the sensitivity of muscle activation to Ca^{2+} concentration. Consistent with this expectation, alternate isoforms of TnI in two populations of fast fiber in the yabby, *Cherax destructor*, affect the steepness of the Ca^{2+}–force curve (Koenders et al. 2004). The population of fibers with the greater Ca^{2+}-sensitivity also has slightly shorter sarcomeres, which is also consistent with faster muscle contraction. In running ghost crabs, size-dependent differences exist in the relative proportions of TnI and TnT isoforms that may be related to operational frequency during running (Perry et al. 2009). In dragonfly flight muscles, alternatively spliced variants of TnT significantly influence muscle power output and flight performance (Fitzhugh and Marden 1997, Marden et al. 1999, Marden et al. 2001, Marden and Allen 2002). Collectively, these trends suggest that the relative proportions of the thin filament regulatory proteins may influence the kinetics of muscle activation and deactivation. However, it is possible that some of the diversity in myofibrillar isoforms may simply represent functional redundancies or vestiges of past functional specialization. It is surprising, for example, that so many distinct isoforms of actin are expressed within lobster tissues

(Kim et al. 2009). Actin is generally viewed as being a passive participant in muscle contraction, and a functional role for different actin isoforms would be an unexpected finding. Further studies linking the myofibrillar isoform assemblage with the physiological properties of muscles are needed to reveal how specific isoforms affect muscle function.

Crustacean Muscle Proteins as Allergens

One discipline that has provided unexpected insights into our knowledge of crustacean myofibrillar proteins is the field of seafood allergen research. Crustaceans and mollusks, collectively referred to as shellfish, represent a major proportion of all seafood consumed worldwide (Lehrer et al. 2003, Lopata and Lehrer 2009). A significant number of individuals within the population exhibit allergic reactions to these foods, and, in some cases, the allergic reactions prove to be fatal. Several proteins from crustacean muscles have been identified as allergens, including arginine kinase, SR Ca^{2+} binding protein, and tropomyosin (Lopata and Lehrer 2009). Of these, tropomyosin has by far been the most consistently identified as a major allergen from multiple species of crustaceans, as well as from mollusks (Reese et al. 1999, Ayuso et al. 2002, Lehrer et al. 2003). Tropomyosin is the only major shrimp allergen, and more than 84% of the total IgE antibodies in shrimp-allergic patients are directed against this protein (Lehrer et al. 2003). In addition to allergic reactions caused from consuming or coming in contact with crustacean muscles, tropomyosins from other arthropods (cockroaches and house mites) can cause allergic responses as well (Lehrer et al. 2003). The tropomyosin amino acid sequence is highly conserved among crustaceans, thus providing several common antigenic sites among different tropomyosin isoforms (Motoyama et al. 2007, Suma et al. 2007).

PHYSIOLOGICAL PROPERTIES OF CRUSTACEAN MUSCLES

Nerve–Muscle Interactions

The initial identification of physiological fiber types in crustacean muscles was made independently from the molecular and biochemical determination of muscle fiber types. Several comprehensive reviews of the principles of neuromuscular organization and physiology of crustacean muscles have been published (Atwood 1976, Govind and Atwood 1982, Hoyle 1983, Govind 1987, Govind 1995, Millar and Atwood 2004). These topics are also covered in greater depth in the chapter by Atwood (see Chapter 4 in volume 3), so they will be covered only briefly here. Crustacean muscle innervation patterns significantly complicate the relationship between the physiological properties of different fiber types because a single fiber may be controlled by anywhere from one to five excitatory motor neurons. In addition, many fibers are also affected by an inhibitory motor neuron that can modulate muscle contraction.

Single-fiber analyses of the claw closer muscle in the crab *Eriphia spinifrons* provide an example of the complexities that exist between muscle phenotype and innervation patterns (Rathmayer and Maier 1987; Fig. 4.4). Four different fiber types are present in this muscle, as identified through histochemical, electrophysiological, and enzymatic properties. The fibers are variably innervated by two excitatory motor neurons, one fast and one slow, as well as by a common inhibitory neuron. Type I fibers are classified as slow oxidative. They exhibit low ATPase activities, slow contractions, and are innervated by all three motor neurons. Type II and type III fibers are both classified as fast oxidative glycolytic and exhibit fast contractions and high ATPase activities. The two fiber types also possess moderate to high levels of glycolytic and oxidative enzymes. However, type II fibers receive innervation from all three motor neurons, whereas type III fibers exclusively receive the fast motor neuron. The largest, type IV fibers are fast glycolytic. They exhibit fast contraction, high

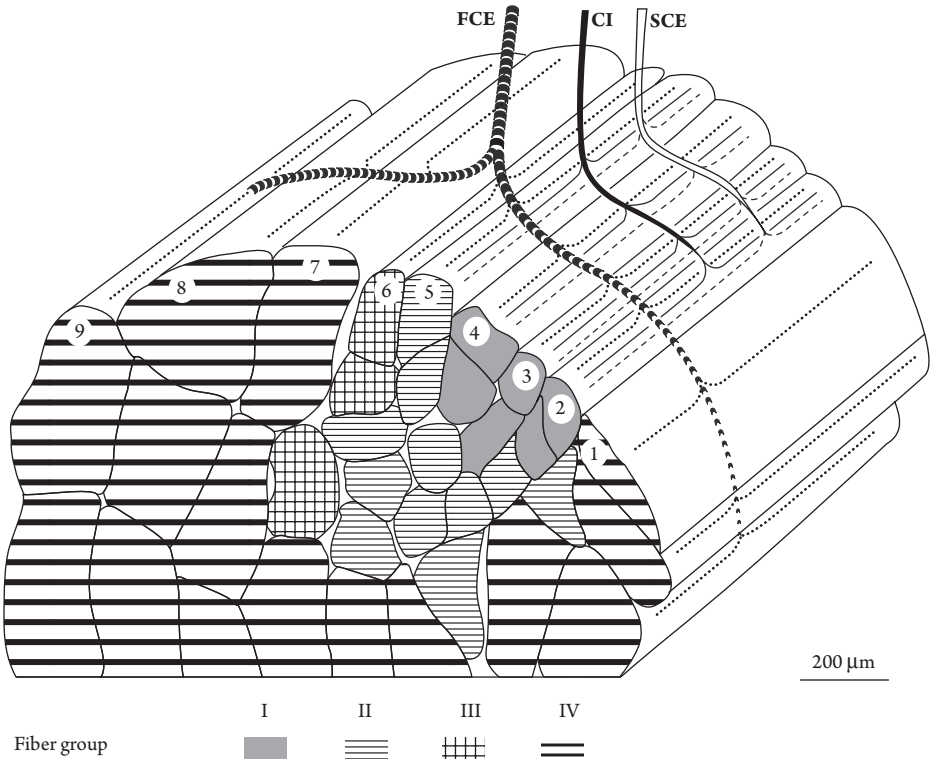


Fig. 4.4.
Schematic diagram of four fiber types present in the leg closer of the crab *Eriphia spinifrons*. Fiber types are identified using combined histochemical, electrophysiological, and biochemical analyses. Three motor neurons variably innervate the different fibers: a fast excitatory motor neuron (FCE), a slow excitatory motor neuron (SCE), and a common inhibitory neuron (CI). Type I fibers are classified as slow oxidative and are controlled by all three neurons. Type II fibers are fast oxidative/glycolytic and are also controlled by all three neurons. Type III fibers are fast oxidative/glycolytic and are controlled only by the FCE. Type IV fibers are fast glycolytic and are also controlled exclusively by the FCE. From Rathmayer and Maier (1987), with permission from Oxford University Press.

ATPase activities, and high levels of glycolytic enzymatic activity, but low oxidative capacity. Like the type III fibers, these fibers are controlled exclusively by the fast motor neuron. These patterns illustrate the principle that crustacean muscle physiology is not determined by fiber type alone, but by potentially complex interactions between the activity of different motor neurons and the cellular and molecular composition of different individual fibers. When contrasted with the organization of mammalian skeletal muscle and motor nerves, some general differences are apparent in crustacean neuromuscular systems. Crustacean muscles tend to have relatively few motor neurons that supply a single muscle, but the number of contacts along a single muscle is greater than in mammalian muscles, which only have a single synapse per fiber (Hoyle 1983, Belanger 2005).

In addition to neurotransmitters released at neuromuscular junctions, crustacean skeletal muscles respond to a number of different neuromodulatory peptides (Kreissl et al. 1999, Mercier et al. 2003, Weiss et al. 2003). These compounds are thought to be released into the circulation from various sources, and they affect different physiological systems including the heart and circulation, digestive system, and skeletal muscles. Their precise role in relation to skeletal muscle function is not completely understood, but we do know that both excitatory and inhibitory peptides exist. Proctolin and FMRFamide-like peptides tend to potentiate muscle contraction, whereas

allatostatins act in an inhibitory capacity (Kreissl et al. 1999, Mercier et al. 2003, Weiss et al. 2003). These effects are exerted through both presynaptic mechanisms and directly on the muscle itself to modulate muscle contractility. There is some evidence that these compounds influence skeletal muscle contractile characteristics through selective phosphorylation of myofibrillar proteins (Brüstle et al. 2001).

Excitation-Contraction Coupling

Overall, the process of excitation-contraction coupling in crustacean muscles appears to be most similar to that in vertebrate cardiac muscles and other invertebrate muscles (Ashley et al. 1993, Palade and Györke 1993, Lea 1996, Quinn et al. 1998, Weiss et al. 2001, Takekura and Franzini-Armstrong 2002). A depolarization of the sarcolemma is carried along the tubular system into the muscle fiber to the dyadic and triadic junctions between the tubule and the enlarged cisternae of the SR. Although the threshold potential needed to initiate contraction is variable among muscles, for most crustacean fibers, the resting potential is more negative than threshold. In muscles that exhibit all-or-none contractions, the threshold is approximately 20–30 mV more positive than the resting potential. In tonic fibers that produce graded contractions, the threshold is closer to the resting potential, and the amount of tension developed is proportional to the level of depolarization (Chapple 1982). Unlike mammalian muscle fibers, the activating depolarization is primarily carried by the inward current of Ca^{2+} ions, rather than by Na^{+} (Ashley et al. 1993, Ushio et al. 1993, Weiss et al. 2001). L-type Ca^{2+} channels within the tubular membranes open, and the inward flux of Ca^{2+} can then activate TnC to initiate contraction, but Ca^{2+} ions also bind to ryanodine (RyR) receptors present in the SR membrane and initiate the release of stored Ca^{2+} (Weiss et al. 2001). This represents a process of Ca^{2+}-induced Ca^{2+} release (CICR), which is the principal mechanism of Ca^{2+} release occurring in the muscles of invertebrates and lower vertebrates (Palade and Györke 1993, Lea 1996, Quinn et al. 1998, Weiss et al. 2001). The threshold pCa for CICR through the RyR in the SR of lobster muscles is approximately 6.0–6.4 (Lea 1996, Quinn et al. 1998). The degree to which Ca^{2+} influx from the extracellular fluid versus that released from the SR initiates muscle contraction probably varies among different muscles, but, in most fibers, CICR appears to play an essential role (Palade and Györke 1993, Ushio et al. 1993, Lea 1996, Quinn et al. 1998, Weiss et al. 2001).

In most vertebrate skeletal muscles, the depolarization of the T tubule triggers a conformational change in the dihydropyridine sensitive L-type Ca^{2+} channels (DHPRs), and a direct mechanical coupling between this protein and the RyR leads to opening of the RyR on the SR membrane (Franzini-Armstrong and Protasi 1997, Endo 2009). In these muscles, the DHPR functions primarily as a voltage sensor rather than a Ca^{2+} channel, and the process is not CICR, being instead a direct coupling between depolarization of the T-tubule membrane and opening of the RyR of the SR (Franzini-Armstrong and Protasi 1997). This is seen as a more advanced form of excitation-contraction coupling and apparently evolved early in the evolution of vertebrates (Di Biase and Franzini-Armstrong 2005). This direct coupling depends, in part, on a physical coupling between the DHPRs within the T-tubule membrane and the RyRs on the SR (Franzini-Armstrong and Protasi 1997, Takekura and Franzini-Armstrong 2002, Di Biase and Franzini-Armstrong 2005). In vertebrates, each RyR is associated with four DHPRs arranged into a square pattern, where each DHPR is attached to one of the four subunits of the RyR (Di Biase and Franzini-Armstrong 2005). Invertebrate muscles, including those of crustaceans, lack the highly ordered arrays of DHPRs, and there is no evidence of a close association between these Ca^{2+} channels and the RyRs (Loesser et al. 1992, Takekura and Franzini-Armstrong 2002, Di Biase and Franzini-Armstrong 2005). This lack of direct coupling between these two molecules supports the view that CICR, rather than direct coupling, provides the mechanism to link depolarization with muscle contraction.

RyRs have been isolated from crustacean muscles and studied in isolated vesicles, as well as in intact myofibrillar bundles (Formelova et al. 1990, Seok et al. 1992, Lea 1996, Quinn et al. 1998, Xiong et al. 1998). Each RyR is composed of four approximately 5,000 amino acid subunits, the same as those found in vertebrate skeletal muscles (Franzini-Armstrong and Protasi 1997, Xiong et al. 1998). Like the RyRs in vertebrates, these channels release Ca^{2+} in response to Ca^{2+} concentrations in the micromolar range, but they are inhibited by Ca^{2+} in the millimollar range (Quinn et al. 1998, Xiong et al. 1998). Two EF-hand domains are present on each RyR subunit, but it is currently unclear whether these Ca^{2+}-binding sites function in channel activation or inhibition (Xiong et al. 1998). The evoked Ca^{2+} currents from lobster RyRs are only about half those of mammalian RyRs (Quinn et al. 1998).

In vertebrates, multiple RyR isoforms are expressed in a tissue-specific manner. In mammals, RyR_1 is the principal isoform expressed in skeletal muscles, RyR_2 in cardiac muscle, and RyR_3 in the brain (Franzini-Armstrong and Protasi 1997). In nonmammalian vertebrates, two isoforms, RyRα and RyRβ, are expressed and are homologous to mammalian isoforms RyR_1 and RyR_3, respectively (Franzini-Armstrong and Protasi 1997). Physiological studies of different muscles in the Australian yabby are consistent with the presence of two different RyR isoforms (Launikonis and Stephenson 2000), but there has yet to be an identification made of multiple crustacean isoforms.

Mechanical Properties

Multiple parameters define the functional performance of skeletal muscles. These include the mechanical properties of muscle stress (force/cross-sectional area) and shortening velocity (muscle lengths per second; L/s). Maximal muscle force is proportional to the physiological cross-sectional area of a muscle, whereas muscle stress is largely determined by sarcomere width or, more precisely, A-band width (Huxley and Niedergerke 1954, Josephson 1975, Taylor 2000). When compared with muscles from a wide range of animals representing different phyla, some crustacean muscles are capable of generating the greatest forces known for any animal (Medler 2002). For example, a variety of muscles from mammals, birds, and other vertebrates generate stresses in the range of 150–200 kN/m^2. By comparison, a number of crustacean muscles produce maximal stresses ranging from 400 to 2,000 kN/m^2 or greater (Taylor 2000, Medler 2002). These trends arise from differences in the anatomical arrangement of the sarcomeres because muscle force is proportional to the sarcomere width whereas shortening velocity is proportional to the number of sarcomeres in series (Huxley and Niedergerke 1954, Josephson 1975, Taylor 2000). This relationship represents a tradeoff between muscle strength and muscle speed, which has resulted in a diverse range of fiber types in crustacean muscles adapted for different uses. In vertebrate muscles, by comparison, the stress generated by different muscles is nearly constant, but muscles' shortening velocities vary over orders of magnitude.

Muscle shortening velocities (typically reported as maximum unloaded shortening velocity or V_{max}) for various crustacean muscles have not been studied as extensively as muscle forces, but the available data suggest that these values are similar to if somewhat slower than those of vertebrate muscles. In the broadest comparison among skeletal muscles representing different phyla, maximal shortening velocity ranges from less than 1 to 25 muscle L/s, which appears to represent an upper limit for shortening velocity (Josephson 1993). Keeping in mind that some level of variability exists among measurement parameters and approaches used in different studies, crustacean muscles exhibit shortening velocities comparable to the muscles of other active animals, including vertebrates. The muscles from the barnacle *Balanus nubilus* have very low rates of contraction (V_{max} = 0.15 L/s), but produce stresses of up to 600 kN/m^2. These fibers possess long sarcomeres (~9 μm) but also have low rates of ATP hydrolysis (Griffiths et al. 1990). Slow fibers in the Norway lobster have maximal shortening velocities of about 0.5 L/s

(Holmes et al. 1999), whereas the flagellum abductor muscle that drives rhythmically active flagella in crabs has a V_{max} of 7.6 L/s (Stokes and Josephson 1994). The extensor and flexor carpus muscles of running ghost crabs were estimated to be capable of maximal shortening velocities of approximately 5–7 L/s (Perry et al. 2009). By comparison, the limb muscles of a comparably sized mouse have shortening velocities that range from 6 L/s (soleus) to 14 L/s (extensor digitorum longus); see Askew and Marsh (1997).

Aerobic Capacity

Crustacean muscles exhibit a range of aerobic capacities, from fibers that possess very few aerobic adaptations to those that are rich in mitochondria and have adaptations that facilitate oxygen exchange (Silverman and Charlton 1980, Tse et al. 1983, Mykles 1988, Stokes and Josephson 1992, Boyle et al. 2003, Johnson et al. 2004, Perry et al. 2009, Hardy et al. 2010). Generally, slow fibers are more aerobic than fast fibers, and slow tonic fibers are more aerobic than slow twitch fibers (Ogonowski and Lang 1979, Lang et al. 1980, Mykles 1988, Fowler and Neil 1992, Neil 1993). However, just as with vertebrate muscles, crustacean fast fibers also exist that possess great aerobic capacities (Silverman and Charlton 1980, Tse et al. 1983, Stokes and Josephson 1992, Hardy et al. 2010). Functionally, aerobic fibers are found in muscles used for slow sustained contractions (Mykles 1988, Fowler and Neil 1992, Neil 1993), those that power swimming and running (Tse et al. 1983, Boyle et al. 2003, Perry et al. 2009, Hardy et al. 2010), and those used for sustained, high-frequency contractions (Silverman and Charlton 1980, Stokes and Josephson 1992). Within single muscles, fibers located near the proximal and distal regions tend to be more aerobic and also tend to be composed of slower fiber types (Lang 1980, Mykles 1988, Mykles et al. 2002, Perry et al. 2009).

A common pattern observed in these crustacean aerobic fibers is one in which the mitochondria are positioned close to the sarcolemma (Fig. 4.5). In many instances, these fibers are highly subdivided by the clefts that penetrate into the fiber from the outer regions, and the subdivisions are lined by high densities of mitochondria. The subsarcolemmal distribution of mitochondria observed in crustacean muscles is distinct from the distribution in mammalian muscle fibers, where the mitochondria are more evenly scattered around the myofibrils (Boyle et al. 2003, Kinsey et al. 2007). The subdivision of individual fibers may be to facilitate exchange of oxygen and nutrients between the hemolymph and the muscle fibers (Hardy et al. 2009). In the aerobic fibers that power swimming in portunid crabs, fibers become more divided by the membrane clefts as they get larger with growth. The result is that the larger fibers in larger crabs are more subdivided, keeping the average width of each subdivision relatively constant (Hardy et al. 2010). We have observed a similar trend in the aerobic fibers of ghost crabs (Medler, unpublished observations). The function of the subsarcolemmal distribution of mitochondria is not completely clear. Although the distribution facilitates the exchange of oxygen between the hemolymph and mitochondria, it requires phosphagens to diffuse across a greater distance from the mitochondria to the fiber interior (Stokes and Josephson 1992, Boyle et al. 2003, Kinsey et al. 2007).

Broadly, crustacean muscle contraction is not only dependent on the contractile properties of the muscle fiber, but also on the motor neuron that controls contraction. Therefore, fatigue resistance in crustacean muscles is not simply a consequence of muscle adaptations, but is also dependent on the motor neuron(s) activating the muscle. Single fibers are often controlled by both a fast motor neuron and a slow motor neuron. The fast motor neurons elicit a greater response from the muscle fiber, but the neurons are quickly fatiguing, whereas the activation from the slow motor neuron generates less force, but the muscles exhibit facilitation over time (Atwood and Cooper 1996, Millar and Atwood 2004).

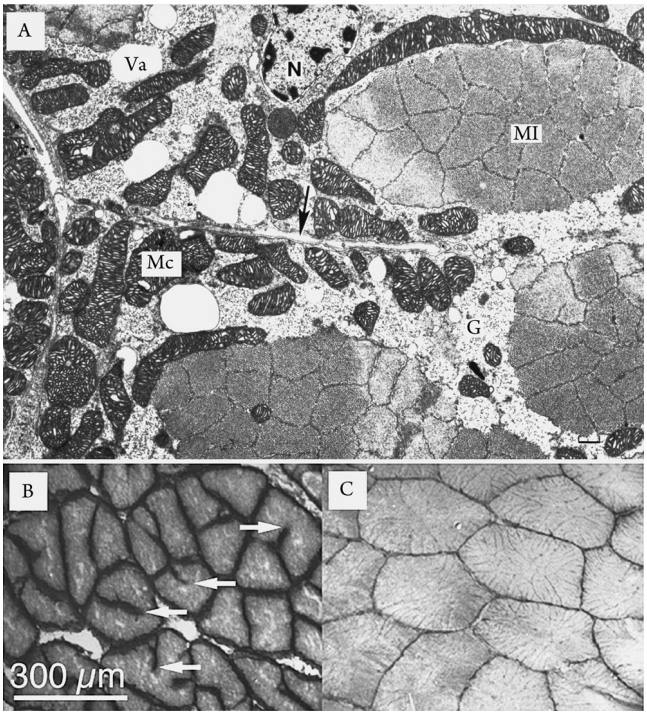


Fig. 4.5.
Structural features associate with aerobic and anaerobic fibers. Aerobic fibers are demonstrated by cross-section of the flagellum abductor (FA) muscle of *Carcinus maenas* (A) and in proximal fibers of the extensor carpus in *Ocypode quadrata* (B). Mid-region fibers of the extensor carpus in *O. quadrata* (C) are not highly aerobic. In *C. maenas*, mitochondria (Mc) are distributed around the fiber periphery in the FA muscle, whereas myofibrils (MI) are located more centrally. A membrane cleft (black arrow) is visible in the figure (TEM). In *O. quadrata*, NADH tetrazolium reductase staining from mitochondrial enzymes reveals a similar distribution in these fibers. In the more proximal fibers (B), high densities of mitochondria are present, and membrane clefts (white arrows) subdivide the fibers. In the mid-region fibers (C), mitochondria are similarly restricted primarily to the subsarcolemmal regions, but with much lower densities. Abbreviations in (A): mitochondria, Mc; glycogen, G; myofibrillar island, MI; nucleus, N; vacuole, Va. (A) is reprinted with permission from Stokes and Josephson (1992), with permission from Springer. (B) and (C) are from Perry et al. (2009), with permission from The Company of Biologists, Inc. The scale bar in A = 0.5 μm.

Physiological Differences Among Identified Fiber Types

Clear physiological differences exist among the S_1, S_2, and fast fiber types identified in the muscles of lobsters and other decapod crustaceans. The fast fibers of the deep abdominal flexors and extensors produce force at greater rates than the slow fibers, but the maximum force is significantly less (Jahromi and Atwood 1969, Ogonowski and Lang 1979). Muscle fibers found in the legs and claws of the lobster show similar differences (Jahromi and Atwood 1971). There are also more subtle differences between the fast muscles of the abdomen and those that compose the fast closer of the cutter claw, although these have not been well studied. The abdominal muscle fibers appear to be a more "pure" fast fiber type, as seen by significant differences in the expression of slow MHC isoforms. The fast muscles of the claw co-express some level of the S_1 MHC isoform, but the expression of that isoform in the abdominal musculature is nearly zero (Medler and Mykles 2003). Additionally, the fast fibers in cutter closer and deep abdominal muscles differ in expression of TnC

isoforms (Chao et al. 2010; Table 4.1). The myosin ATP hydrolysis rate, which is directly correlated with muscle shortening velocity, is also higher in the abdominal musculature when compared to the fast claw fibers (Mykles 1985a). As for the slow fiber types, the S_1 fibers have faster rates of contraction and relaxation than those of the S_2 fibers and also exhibit higher ATPase activity than the S_2 fibers in histochemical staining reactions (Mykles 1988, Galler and Neil 1994, Holmes et al. 1999). By comparison, the S_2 fibers are more sensitive to activation by Ca^{2+}, meaning that they become active at lower Ca^{2+} concentrations (Galler and Neil 1994). The S_2 fibers also exhibit a greater degree of neuromuscular facilitation, providing the capability to maintain force production even after the S_1 fibers have fatigued (Mykles et al. 2002; Fig. 4.6). The classification into these discrete fiber types is really an oversimplification because many fibers possess a phenotype intermediate to these extremes (Costello and Govind 1983, Medler et al. 2004).

In addition to the well-defined fiber types just discussed, there are a number of different crustacean fiber types that do not clearly fit into this system. Physiological studies of several of these different fibers provide further insight into the functionality of different fibers. Using myosin ATPase histochemistry, Rathmayer and colleagues (Rathmayer and Maier 1987, Galler and Rathmayer 1992) identified four different fiber types in the closer muscles of the walking legs of the crab *E. spinifrons* (Fig. 4.4). Mechanical measurements from three of these fiber types showed a gradation in shortening velocities, with the fastest fibers being about 2–3 times faster than the slowest. Ca^{2+} sensitivity also differed among fiber types, but there was no direct correlation with shortening velocity. Sarcomere width was correlated with the different fiber types, with the slowest having the longest sarcomere width (14.6 μm), the fastest having the shortest (9.6 μm), and the intermediate fiber type having an intermediate sarcomere width (12.3 μm). Fast muscles of crayfish abdominal extensors and flexors possess ATPase activities that are approximately 5–7 times higher than the slow muscles of the claw opener (Sakurai et al. 1996). West et al. (1992) found that short-sarcomered (3.25 μm) claw closer fibers in the yabby (*C. destructor*) had slightly higher ATPase activities than those of the long-sarcomered fibers (8.57 μm), but these differences were not significant. Their conclusion

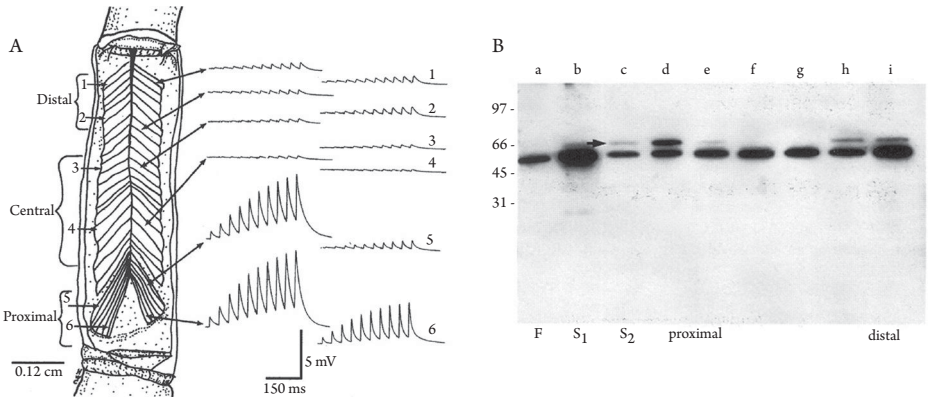


Fig. 4.6.
Myofibrillar protein isoform expression is correlated with synaptic efficacy. Fibers of the crayfish leg opener exhibit regional differences in excitatory postsynaptic potential (EPSP) following stimulation of the excitatory motor neuron (A). Proximal fibers (5 and 6) exhibit the greatest short-term facilitation, the distal fibers (1 and 2) intermediate levels, and fibers of the central region (3 and 4) show the least facilitation. Western blots of TnT isoforms from opener fibers reveal a correlation with these physiological responses (B). The most proximal (d and e) and distal (h and i) fibers express varying levels of TnT_1 (arrow) in combination with TnT_3, identifying them as slow tonic (S_2) fibers. The central fibers (f and g) express only TnT_3 and are slow twitch (S_1). Fibers a–c are controls (F, fast; S_1, slow twitch; S_2, slow tonic). From Mykles et al. (2002), with permission from The Company of Biologists, Inc.

was that the faster contraction of the short-sarcomered fibers was due primarily to the structural arrangement of the sarcomeres, rather than to differences in the myosin cross-bridge kinetics.

Overall, crustacean muscles comprise highly diverse fiber types, as evident from the range of physiological, ultrastructural, and molecular compositions among fibers within and among species. Currently, no unified system exists that can be used to systematically classify crustacean fiber types in an unambiguous way. In extensively studied mammalian muscles, different fibers are classified according to the MHC isoform(s) that they express, and the genes encoding these isoforms have been fully characterized (Schiaffino and Reggiani 1996, Schiaffino and Reggiani 2011). The most precise classification of muscle fiber types in crustaceans is for the American lobster (*H. americanus*) in which fibers are classified as either S_1, S_2, or fast. This system seems to be relevant for crayfish and at least some crabs, but currently there are not enough data for MHC isoforms in different species to know for certain. In ghost crab leg muscles, three MHC isoforms are expressed in different ratios, but their migration pattern on SDS-PAGE gels is different from that of the lobster (Perry et al. 2009). Similarly, several different isoforms have been identified in crayfish muscles, but their migration pattern is different from the lobster (LaFramboise 2000). Sequence comparisons among orthologous decapod MHCs have failed to identify precise fiber type categories among species, and homologies of these different isoforms remain uncertain (Cotton and Mykles 1993, Medler and Mykles 2003, Medler et al. 2004, Perry et al. 2009). Koyama et al. (2012a,b, 2013) have recently presented phylogenetic relationships among available MHC isoforms from several different species, but these are primarily based on the partial sequences available at this time. Partial sequences are also available for the MHC genes of marine isopods (Holmes et al. 2002, Magnay et al. 2003), but these are for the myosin head near the 5′ end of the molecule and offer no comparison with other known sequences. A more comprehensive dataset consisting of both full MHC sequences and corresponding SDS-PAGE migration patterns would be especially helpful in defining crustacean fiber types more objectively. This type of analysis would also be indispensable for an understanding of the evolution of crustacean fiber type diversity. Crustaceans as a group are highly diverse and represent several distinct evolutionary histories. It could be that several different specific fiber type classification schemes are required to accurately classify the number of distinct fiber types and the interrelationships among these.

FUTURE DIRECTIONS

We are currently very limited in our understanding of the relationships among the diverse fiber types that make up crustacean muscles. These limitations exist for several reasons. First, many of the fiber types that have been identified are still only defined by descriptive parameters like fast versus slow, red versus white, or long- versus short-sarcomered fibers. These definitions are useful for broadly grouping fiber types but are imprecise. A second problem is that fibers have been classified using unique systems for different species, and it is unclear how the identified fiber types correspond to one another. Crab fibers defined as types I–IV by Rathmayer and colleagues (Fig. 4.4) likely correspond in some way to the S_1, S_2, and fast scheme of lobster muscles (Fig. 4.3), but data are not currently available to unravel their relationships. Finally, the identification of myofibrillar protein isoforms has been limited to just a few species, and, in most instances, only partial sequences have been identified if molecular data are available at all.

A better understanding of precisely how many different crustacean fiber types exist and how fiber types among different species are related to one another is clearly needed. An unambiguous fiber type scheme would provide a foundation for other studies, such as those focused on nerve–muscle interactions, dynamic muscle function in locomotion, skeletal muscle plasticity, and other areas related to skeletal muscle biology. We advocate using the MHC isoforms expressed in different

fibers as the definitive measure of crustacean fiber type. This has been the standard for mammalian skeletal muscle classification for many years and provides an objective measure of fiber type (Schiaffino and Reggiani 2011). Although the full-length MHC sequences from shrimp (Koyama et al. 2012a,b, 2013) are currently the only complete crustacean MHC sequences available, several partial sequences have been published, and we should work to obtain full sequences whenever possible. When a greater number of full-length sequences are available, we will be able to establish how many different fiber types exist and how they are related to one another.

CONCLUSIONS

Crustacean muscles are highly diverse in both their structure and physiological function. These muscles have evolved into specialized tissues that fulfill a variety of processes, including sustained force generation, very rapid contractions, and sustained power output for long periods of time. Although diverse, crustacean muscles all share several unifying features. All crustacean muscles are striated and rely principally on thin filament regulation of muscle contraction. Fast contracting fibers possess short sarcomeres that are roughly the width of those found in vertebrate muscles (~2.5–4 μm), whereas slow fibers have longer sarcomeres of variable width (5–20 μm). A well-developed tubular network carries membrane depolarization into the fiber interior to come in close contact with the SR where Ca^{2+} is released to trigger contraction. Crustacean fibers use DHPR proteins and RyRs to control Ca^{2+} release from the SR. Unlike the process in mammalian muscles, there is not close coupling between the DHPR and ryanodine receptor, and the process in crustacean muscles is more similar to that in other invertebrates, lower vertebrates, and mammalian cardiac muscles.

Crustacean muscles comprise a number of distinct fiber types. These are most clearly defined in the American lobster, where the types include fast, slow twitch (S_1), and slow tonic (S_2) fibers. Each fiber type can be defined by specific assemblages of myofibrillar protein isoforms. These represent alternate forms of MHC, paramyosin, actin, tropomyosin, troponins, and MLCs. Recent studies have found that the number and expression patterns of these isoforms are much more complex than previously appreciated. This diversity of proteins assembled into different fibers is presumably responsible for the range of physiological properties. Physiological responses are also dependent on the number and pattern of innervation from excitatory and inhibitory motor neurons.

REFERENCES

Ashley, C.C., and E.B. Ridgway. 1970. On relationships between membrane potential, calcium transient and tension in single barnacle muscle fibres. Journal of Physiology, London 209:105–130.

Ashley, C.C., P.J. Griffiths, T.J. Lea, I.P. Mulligan, R.E. Palmer, and S.J. Simnett. 1993. Barnacle muscle: Ca^{2+}, activation and mechanics. Reviews of Physiology, Biochemistry, and Pharmacology 122:149–258.

Askew, G.N., and R.L. Marsh. 1997. The effects of length trajectory on the mechanical power output of mouse skeletal muscle. Journal of Experimental Biology 200:3119–3131.

Atwood, H.L. 1976. Organization and synaptic physiology of crustacean neuromuscular systems. Progress in Neurobiology 7:291–391.

Atwood, H.L., and R.L. Cooper. 1996. Synaptic diversity and differentiation: crustacean neuromuscular junctions. Invertebrate Neuroscience 1:291–307.

Ayuso, R., G. Reese, S. Leong-Kee, M. Plante, and S.B. Lehrer. 2002. Molecular basis of arthropod cross-reactivity: IgE-binding cross-reactive epitopes of shrimp, house dust mite and cockroach tropomyosins. International Archives of Allergy and Immunology 129:38–48.

Barnard, J.R., V.R. Edgerton, T. Furukawa, and J.B. Peter. 1971. Histochemical, biochemical, and contractile properties of red, white, and intermediate fibers. American Journal of Physiology 220:410–414.

Belanger, J.H. 2005. Contrasting tactics in motor control by vertebrates and arthropods. Integrative and Comparative Biology 45:672–678.

Booth, F.W., M.J. Laye, and E.E. Spangenburg. 2010. Gold standards for scientists who are conducting animal-based exercise studies. Journal of Applied Physiology 108:219–221.

Bottinelli, R., M. Canepari, M.A. Pellegrino, and C. Reggiani. 1996. Force-velocity properties of human skeletal muscle fibres: myosin heavy chain isoform and temperature dependence. Journal of Physiology-London 495:573–586.

Boyle, K.L., R.M. Dillaman, and S.T. Kinsey. 2003. Mitochondrial distribution and glycogen dynamics suggest diffusion constraints in muscle fibers of the blue crab, *Callinectes sapidus*. Journal of Experimental Zoology Part A, Comparative Experimental Biology 297A:1–16.

Brooke, M.H., and K.K. Kaiser. 1970. Muscle fiber types: how many and what kind? Archives of Neurology 23:369–379.

Brüstle, B., S. Kreissl, D.L. Mykles, and W. Rathmayer. 2001. The neuropeptide proctolin induces phosphorylation of a 30 kDa protein associated with the thin filament in crustacean muscle. Journal of Experimental Biology 204:2627–2635.

Burrows, M. 1969. The mechanics and neural control of the prey capture strike in the mantis shrimps *Squilla* and *Hemisquilla*. Zeitschrift fur Vergleichende Physiologie 62:361–381.

Burrows, M., and G. Hoyle. 1972. Neuromuscular physiology of the strike mechanism of the mantis shrimp, *Hemisquilla*. Journal of Experimental Zoology 179:379–394.

Caiozzo, V.J., M.J. Baker, K. Huang, H. Chou, Y. Wu, and K. Baldwin. 2003. Single-fiber myosin heavy chain polymorphism: how many patterns and what proportions? American Journal of Physiology, Regulatory Integrative and Comparative Physiology 285:R570–R580.

Chao, E., H.W. Kim, and D.L. Mykles. 2010. Cloning and tissue expression of eleven troponin-C isoforms in the American lobster, *Homarus americanus*. Comparative Biochemistry & Physiology, Part A 157:88–101.

Chapple, W.D. 1982. Muscle. Pages 151–184 *in* H.L. Atwood and D.C. Sandeman, editors. The biology of Crustacea, vol. 3. Academic Press, New York.

Costello, W.J., and C.K. Govind. 1983. Contractile responses of single fibers in lobster claw closer muscles: correlation with structure, histochemistry, and innervation. Journal of Experimental Zoology 227:381–393.

Costello, W.J., and C.K. Govind. 1984. Contractile proteins of fast and slow fibers during differentiation of lobster claw muscle. Developmental Biology 104:434–440.

Costello, W.J., and F. Lang. 1979. Development of the dimorphic claw closer muscles of the loster, *Homarus americanus*: IV. Changes in functional morphology during growth. Biological Bulletin 156:179–195.

Cotton, J.L.S., and D.L. Mykles. 1993. Cloning of a crustacean myosin heavy chain isoform: exclusive expression in fast muscle. Journal of Experimental Zoology 267:578–586.

Di Biase, V., and C. Franzini-Armstrong. 2005. Evolution of skeletal type e-c coupling: a novel means of controlling calcium delivery. Journal of Cell Biology 171:695–704.

Endo, M. 2009. Calcium-induced calcium release in skeletal muscle. Physiological Reviews 89:1153–1176.

Fahrenbach, W.H. 1963. The sarcoplasmic reticulum of striated muscle of a cyclopoid copepod. Journal of Cell Biology 17:629–640.

Fitzhugh, G.H., and J.H. Marden. 1997. Maturational changes in troponin T expression Ca^{2+}-sensitivity and twitch contraction kinetics in dragonfly flight muscle. Journal of Experimental Biology 200:1473–1482.

Formelova, J., O. Hurnak, M. Novotova, and J. Zachar. 1990. Ryanodine receptor purified from crayfish skeletal muscle. General Physiology and Biophysics 9:445–453.

Fowler, W.S., and D.M. Neil. 1992. Histochemical heterogeneity of fibers in the abdominal superficial flexor muscles of the Norway lobster, *Nephrops norvegicus* (L.). Journal of Experimental Zoology 264:406–418.

Franzini-Armstrong, C., and F. Protasi. 1997. Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. Physiological Reviews 77:699–729.

Franzini-Armstrong, C., A.B. Eastwood, and L.D. Peachey. 1986. Shape and disposition of clefts, tubules, and sarcoplasmic reticulum in long and short sarcomere fibers of crab and crayfish. Cell and Tissue Research 244:9–19.

Fyrberg, E.A., C.C. Fyrberg, J.R. Biggs, D. Saville, C.J. Beall, and A. Ketchum. 1998. Functional nonequivalence of *Drosophila* actin isoforms. Biochemical Genetics 36:271–287.

Galler, S., and D.M. Neil. 1994. Calcium-activated and stretch-induced force responses in two biochemically defined muscle fibre types of Norway lobster. Journal of Muscle Research & Cell Motility 15:390–399.

Galler, S., and W. Rathmayer. 1992. Shortening velocity and force Pca relationship in skinned crab muscle fibers of different types. Pflugers Archiv-European Journal of Physiology 420:187–193.

Garone, L., J.L. Theibert, A. Miegel, Y. Maeda, C. Murphy, and J.H. Collins. 1991. Lobster troponin C: amino acid sequences of three isoforms. Archives of Biochemistry & Biophysics 291:89–91.

Govind, C.K. 1987. Muscle and muscle fiber type transformations in clawed crustaceans. American Zoologist 27:1079–1098.

Govind, C.K. 1995. Muscles and their innervation. Pages 291–312 *in* J. R. Factor editor. Biology of the lobster *Homarus americanus*. Academic Press, San Diego.

Govind, C.K., and H.L. Atwood. 1982. Organization of neuromuscular systems. Pages 63–103 *in* H.L. Atwood and D.C. Sandeman, editors. Neurobiology: structure and function, volume 3. Academic Press, New York.

Govind, C.K., and F. Lang. 1977. Development of the dimorphic claw closer muscles of the lobster, *Homarus americanus*: III. Transformation to dimorphic muscles in juveniles. Biological Bulletin 154:55–67.

Griffiths, P.J., J.J. Duchateau, Y. Maeda, J.D. Potter, and C.C. Ashley. 1990. Mechanical characteristics of skinned and intact muscle fibers from the giant barnacle, *Balanus nuciblus*. Pflugers Archiv-European Journal of Physiology 415:554–565.

Gunzel, D., S. Galler, and W. Rathmayer. 1993. Fiber heterogeneity in the closer and opener muscles of crayfish walking legs. Journal of Experimental Biology 175:267–281.

Hardy, K.M., R.M. Dillaman, B.R. Locke, and S.T. Kinsey. 2009. A skeletal muscle model of extreme hypertrophic growth reveals the influence of diffusion on cellular design. American Journal of Physiology, Regulatory Integrative and Comparative Physiology 296:R1855–R1867.

Hardy, K.M., S.C. Lema, and S.T. Kinsey. 2010. The metabolic demands of swimming behavior influence the evolution of skeletal muscle fiber design in the brachyuran crab family Portunidae. Marine Biology 157:221–236.

Hartnoll, R.G. 1971. The occurrence, methods, and significance of swimming in the brachyura. Animal Behaviour 19:34–50.

Henry, R.P., S.M. Bilger, and A.G. Moss. 2001. Carbonic anhydrase isozyme distribution and characterization in metabolic fiber types of the dorsal levator muscle of the blue crab, *Callinectes sapidus*. Journal of Experimental Zoology 290:234–246.

Himmel, D.M., S. Mui, E. O'Neall-Hennessey, A.G. Szent-Gyorgyi, and C. Cohen. 2009. The on-off switch in regulated myosins: different triggers but related mechanisms. Journal of Molecular Biology 394:496–505.

Holmes, J.M., K. Hilber, S. Galler, and D.M. Neil. 1999. Shortening properties of two biochemically defined muscle fibre types of the Norway lobster *Nephrops norvegicus* L. Journal of Muscle Research and Cell Motility 20:265–278.

Holmes, J.M., N.M. Whiteley, J.L. Magnay, and A.J. El Haj. 2002. Comparison of the variable loop regions of myosin heavy chain genes from Antarctic and temperate isopods. Comparative Biochemistry & Physiology Part B, Biochemistry & Molecular Biology 131:349–359.

Hooper, S.L., and J.B. Thuma. 2005. Invertebrate muscles: muscle specific genes and proteins. Physiological Reviews 85:1001–1060.

Hooper, S.L., K.H. Hobbs, and J.B. Thuma. 2008. Invertebrate muscles: thin and thick filament structure; molecular basis of contraction and its regulation, catch and asynchronous muscle. Progress in Neurobiology 86:72–127.

Hoyle, G. 1967. Diversity of striated muscle. American Zoologist 7:435–449.

Hoyle, G. 1983. Muscles and their neural control. Wiley Interscience, New York.

Huxley, A.F., and R. Niedergerke. 1954. Structural changes in muscle during contraction. Nature 173:971–973.

Ishimoda-Takagi, T., M. Itoh, and H. Koyama. 1997. Distribution of tropomyosin isoforms in spiny lobster muscles. Journal of Experimental Zoology 277:87–98.

Jahromi, S.S., and H.L. Atwood. 1969. Correlation of structure, speed of contraction, and total tension in fast and slow abdominal muscle fibers of the lobster. Journal of Experimental Zoology 171:25–38.

Jahromi, S.S., and H.L. Atwood. 1971. Structural and contractile properties of lobster leg-muscle fibers. Journal of Experimental Zoology 176:475–486.

Johnson, L.K., R.M. Dillaman, D.M. Gay, J.E. Blum, and S.T. Kinsey. 2004. Metabolic influences of fiber size in aerobic and anaerobic locomotor muscles of the blue crab, *Callinectes sapidus*. Journal of Experimental Biology 207:4045–4056.

Josephson, R.K. 1975. Extensive and intensive factors determining performance of striated-muscle. Journal of Experimental Zoology 194:135–154.

Josephson, R.K. 1993. Contraction dynamics and power output of skeletal muscle. Annual Review of Physiology 55:527–546.

Josephson, R.K., and D.R. Stokes. 1994. Contractile properties of a high-frequency muscle from a crustacean 3. Mechanical power output. Journal of Experimental Biology 187:295–303.

Kim, B.K., K.S. Kim, C.W. Oh, D.L. Mykles, S.G. Lee, H.J. Kim, and H.W. Kim. 2009. Twelve actin-encoding cDNAs from the American lobster, *Homarus americanus*: cloning and tissue expression of eight skeletal muscle, one heart, and three cytoplasmic isoforms. Comparative Biochemistry and Physiology B, Biochemistry & Molecular Biology 153:178–184.

Kinsey, S.T., K.M. Hardy, and B.R. Locke. 2007. The long and winding road: influences of intracellular metabolite diffusion on cellular organization and metabolism in skeletal muscle. Journal of Experimental Biology 210:3505–3512.

Kinsey, S.T., B.R. Locke, and R.M. Dillaman. 2011. Molecules in motion: influences of diffusion on metabolic structure and function in skeletal muscle. Journal of Experimental Biology 214:263–274.

Koenders, A., X. Yu, E.S. Chang, and D.L. Mykles. 2002. Ubiquitin and actin expression in claw muscles of land crab, *Gecarcinus lateralis*, and American lobster, *Homarus americanus*: differential expression of ubiquitin in two slow muscle fiber types during molt-induced atrophy. Journal of Experimental Zoology 292:618–632.

Koenders, A., T.M. Lamey, S. Medler, J.M. West, and D.L. Mykles. 2004. Two fast-type fibers in claw closer and abdominal deep muscles of the Australian freshwater crustacean, *Cherax destructor*, differ in Ca^{2+} sensitivity and troponin-I isoforms. Journal of Experimental Zoology 301A:588–598.

Koyama, H., D.B. Akolkar, S. Piyapattanakorn, and S. Watabe. 2012a. Cloning, expression, and localization of two types of fast skeletal myosin heavy chain genes from black tiger and pacific white shrimps. Journal of Experimental Zoology 317A:608–621.

Koyama, H., D.B. Akolkar, T. Shiokai, M. Nakaya, S. Piyapattanakorn, and S. Watabe. 2012b. The occurrence of two types of fast skeletal myosin heavy chains from abdominal muscle of kuruma shrimp *Marsupenaeus japonicus* and their different tissue distribution. Journal of Experimental Biology 215:14–21.

Koyama, H., S. Piyapattanakorn, and S. Watabe. 2013. Cloning of skeletal myosin heavy chain gene family from adult pleopod muscle and whole larvae of shrimps. Journal of Experimental Zoology 319A:268–276.

Kreissl, S., T. Weiss, S. Djokaj, O. Balezina, and W. Rathmayer. 1999. Allatostatin modulates skeletal muscle performance in crustaceans through pre- and postsynaptic effects. European Journal of Neuroscience 11:2519–2530.

LaFramboise, W.A., B. Griffis, P. Bonner, W. Warren, D. Scalise, D. Guthrie, and R.L. Cooper. 2000. Muscle type-specific myosin isoforms in crustacean muscles. Journal of Experimental Zoology 286:36–48.

Lagersson, N.C. 2002. The ultrastructure of two types of muscle fibre cells in the cyprid of *Balanus amphitrite* (Crustacea: Cirripedia). Journal of the Marine Biological Association of the United Kingdom 82:573–578.

Lang, F., W.J. Costello, and C.K. Govind. 1977a. Development of the dimorphic claw closer muscles of the lobster *Homarus americanus*: I. Regional distribution of muscle fiber types in adults. Biological Bulletin 152:75–83.

Lang, F., C.K. Govind, and J. She. 1977b. Development of the dimorphic claw closer muscles of the loster, *Homarus americanus*: II. Distribution of muscle fiber types in larval forms. Biological Bulletin 152:382–391.

Lang, F., M.M. Ogonowski, W.J. Costello, R. Hill, B. Roehrig, K. Kent, and J. Sellers. 1980. Neurotrophic influence on lobster skeletal muscle. Science 207:325–327.

Larsson, L., and R.L. Moss. 1993. Maximum velocity of shortening in relation to myosin isoform composition in single fibers from human skeletal-muscles. Journal of Physiology, London 472:595–614.

Launikonis, B.S., and D.G. Stephenson. 2000. Effects of Mg^{2+} on Ca^{2+} release from sarcoplasmic reticulum of skeletal muscle fibres from yabby (crustacean) and rat. Journal of Physiology, London:299–312.

Lea, T.J. 1996. Caffeine and micromolar Ca^{2+} concentrations can release Ca^{2+} from ryanodine-sensitive stores in crab and lobster striated muscle fibres. Journal of Experimental Biology 199, 2419–2428.

Lehman, W., and A.G. Svent-Gyorgyi. 1975. Regulation of muscular contraction. Distribution of actin control and myosin control in the animal kingdom. Journal of General Physiology 66:1–30.

Lehrer, S.B., R. Ayuso, and G. Reese. 2003. Seafood allergy and allergens: a review. Marine Biotechnology 5:339–348.

Li, Y.L., and D.L. Mykles. 1990. Analysis of myosins from lobster muscles—fast and slow isozymes differ in heavy-chain composition. Journal of Experimental Zoology 255:163–170.

Loesser, K.E., L. Castellani, and C. Franzini-Armstrong. 1992. Dispositions of junctional feet in muscles of invertebrates. Journal of Muscle Research & Cell Motility 13:161–173.

Lopata, A.L., and S.B. Lehrer. 2009. New insights into seafood allergy. Current Opinion in Allergy and Clinical Immunology 9:270–277.

Lovato, T.L., S.M. Meadows, P.W. Baker, J.C. Sparrow, and R.M. Cripps. 2001. Characterization of muscle actin genes in *Drosophila virilis* reveals significant molecular complexity in skeletal muscle types. Insect Molecular Biology 10:333–340.

Macias, M.T., and L. Sastre. 1990. Molecular-cloning and expression of 4 actin isoforms during artemia development. Nucleic Acids Research 18:5219–5225.

Magnay, J.L., J.M. Holmes, D.M. Neil, and A.J. El Haj. 2003. Temperature-dependent developmental variation in lobster muscle myosin heavy chain isoforms. Gene 316:119–126.

Maier, L., W. Rathmayer, and D. Pette. 1984. pH lability of myosin ATPase activity permits discrimination of different muscle fibre types in crustaceans. Histochemistry 81:75–77.

Marden, J.H., and L.R. Allen. 2002. Molecules, muscles, and machines: universal performance characteristics of motors. Proceedings of the National Academy of Sciences of the United States of America 99:4161–4166.

Marden, J.H., G.H. Fitzhugh, M.R. Wolf, K.D. Arnold, and B. Rowan. 1999. Alternative splicing, muscle calcium sensitivity, and the modulation of dragonfly flight performance. Proceedings of the National Academy of Sciences of the United States of America 96:15304–15309.

Marden, J.H., G.H. Fitzhugh, M. Girgenrath, M.R. Wolf, and S. Girgenrath. 2001. Alternative splicing, muscle contraction and intraspecific variation: associations between troponin T transcripts, Ca^{2+} sensitivity and the force and power output of dragonfly flight muscles during oscillatory contraction. Journal of Experimental Biology 204:3457–3470.

McNeill, P., M. Burrows, and G. Hoyle. 1972. Fine structure of muscles controlling the strike of the mantis shrimp, *Hemiquilla*. Journal of Experimental Zoology 179:395–416.

Medler, S. 2002. Comparative trends in shortening velocity and force production in skeletal muscles. American Journal of Physiology, Regulatory, Integrative, and Comparative Physiology 283:R368–R378.

Medler, S., D.L. Mykles. 2003. Analysis of myofibrillar proteins and transcripts in adult skeletal muscles of the American lobster *Homarus americanus*: variable expression of myosins, actin and troponins in fast, slow-twitch and slow-tonic fibres. Journal of Experimental Biology 206:3557–3567.

Medler, S., T. Lilley, and D.L. Mykles. 2004. Fiber polymorphism in skeletal muscles of the American lobster, *Homarus americanus*: continuum between slow-twitch (S-1) and slow-tonic (S-2) fibers. Journal of Experimental Biology 207:2755–2767.

Medler, S., T.R. Lilley, J.H. Riehl, E.P. Mulder, E.S. Chang, and D.L. Mykles. 2007. Myofibrillar gene expression in differentiating lobster claw muscles. Journal of Experimental Zoology 307A:281–295.

Mellon, D., and P.J. Stephens. 1978. Limb morphology and function are transformed by contralateral nerve section in snapping shrimps. Nature 272:246–248.

Mellon, D., and P.J. Stephens. 1992. Chapter 4: connective tissue and supporting structures. Pages 77–116 *in* F.W. Harrison, editor. Microscopic anatomy of the invertebrates: Vol. 10: Decapod Crustaceans. Wiley-Liss Inc., New York.

Mercier, A.J., R. Friedrich, and M. Boldt. 2003. Physiological functions of FMRFamide-like peptides (FLPs) in crustaceans. Microscopy Research and Technique 60:313–324.

Millar, A.G., and H.L. Atwood. 2004. Crustacean phasic and tonic motor neurons. Integrative and Comparative Biology 44:4–13.

Motoyama, K., Y. Suma, S. Ishizaki, Y. Nagashima, and K. Shiomi. 2007. Molecular cloning of tropomyosins identified as allergens in six species of crustaceans. Journal of Agricultural and Food Chemistry 55:985–991.

Mykles, D.L. 1985a. Heterogeneity of myofibrillar proteins in lobster fast and slow muscles: variants of troponin, paramyosin, and myosin light chains comprise four distinct protein assemblages. Journal of Experimental Zoology 234:23–32.

Mykles, D.L. 1985b. Multiple variants of myofibrillar proteins in single fibers of lobster claw muscles: evidence for two types of slow fibers in cutter closer muscle. Biological Bulletin 169:476–483.

Mykles, D.L. 1988. Histochemical and biochemical characterization of two slow fiber types in decapod crustacean muscles. Journal of Experimental Zoology 245:232–243.

Mykles, D.L., and D.M. Skinner. 1981. Preferential loss of thin filaments during molt-induced atrophy in crab claw muscle. Journal of Ultrastructure Research 75:314–325.

Mykles, D.L., J.L. Cotton, H. Taniguchi, K. Sano, and Y. Maeda. 1998. Cloning of tropomyosins from lobster (*Homarus americanus*) striated muscles: fast and slow isoforms may be generated from the same transcript. Journal of Muscle Research & Cell Motility 19:105–115.

Mykles, D.L., S. Medler, A. Koenders, and R. Cooper. 2002. Myofibrillar protein isoform expression is correlated with synaptic efficacy in slow fibres of the claw and leg opener muscles of crayfish and lobster. Journal of Experimental Biology 205:513–522.

Neil, D.M., W.S. Fowler, and G. Tobasnick. 1993. Myofibrillar protein composition correlates with histochemistry in fibres of the abdominal flexor muscles of the Norway lobster *Nephrops norvegicus*. Journal of Experimental Biology 183:185–201.

O'Connor, K., P.J. Stephens, and J.M. Leferovich. 1982. Regional distribution of muscle fiber types in asymmetric claws of Californian snapping shrimp. Biological Bulletin 163:329–336.

Oesterling, M.J. 2012. Shellfish—crustaceans. Pages 83–94 *in* L.A. Granata, G.J. Flick, and R.E. Martin, editors. The seafood industry: species, products, processing, and safety. Wiley-Blackwell, Oxford, UK.

Ogonowski, M.M., and F. Lang. 1979. Histochemical evidence for enzyme differences in crustacean fast and slow muscle. Journal of Experimental Zoology 207:143–151.

Ogonowski, M.M., F. Lang, and C.K. Govind. 1980. Histochemistry of lobster claw-closer muscles during development. Journal of Experimental Zoology 213:359–367.

Palade, P., and S. Györke. 1993. Excitation-contraction coupling in Crustacea: do studies on these primitive creatures offer insights about EC coupling more generally? Journal of Muscle Research and Cell Motility 14:283–287.

Pandorf, C.E., V.J. Caiozzo, F. Haddad, and K.M. Baldwin. 2010. A rationale for SDS-PAGE of MHC isoforms as a gold standard for determining contractile phenotype. Journal of Applied Physiology 108:222–222.

Paniagua, R., M. Royuela, R.M. Garcia-Anchuelo, and B. Fraile. 1996. Ultrastructure of invertebrate muscle cell types. Histology and Histopathology 11:181–201.

Patek, S.N., W.L. Korff, and R.L. Caldwell. 2004. Deadly strike mechanism of a mantis shrimp. Nature 428:819–820.

Patek, S.N., B.N. Nowroozi, J.E. Baio, R.L. Caldwell, and A.P. Summers. 2007. Linkage mechanics and power amplification of the mantis shrimp's strike. Journal of Experimental Biology 210:3677–3688.

Peachey, L.D. 1967. Membrane systems of crab fibers. American Zoologist 7:505–513.

Perry, M.J., J. Tait, J. Hu, S.C. White, and S. Medler. 2009. Skeletal muscle fiber types in the ghost crab, *Ocypode quadrata*: implications for running performance. Journal of Experimental Biology 212:673–683.

Pette, D., and R.S. Staron. 2000. Myosin isoforms, muscle fiber types, and transitions. Microscopy Research and Technique 50:500–509.

Pette, D., and R.S. Staron. 2001. Transitions of muscle fiber phenotypic profiles. Histochemistry and Cell Biology 115:359–372.

Quinn, K.E., L. Castellani, K. Ondrias, and B.E. Ehrlich. 1998. Characterization of the ryanodine receptor/channel of invertebrate muscle. American Journal of Physiology, Regulatory Integrative and Comparative Physiology 274:R494–R502.

Rathmayer, W., and L. Maier. 1987. Muscle fiber types in crabs—studies on single identified muscle fibers. American Zoologist 27:1067–1077.

Reese, G., R. Ayuso, and S.B. Lehrer. 1999. Tropomyosin: an invertebrate pan-allergen. International Archives of Allergy and Immunology 119:247–258.

Reggiani, C., R. Bottinelli, and G.J.M. Stienen. 2000. Sarcomeric myosin isoforms: fine tuning of a molecular motor. News in Physiological Sciences 15:26–33.

Reiser, P.J., R.L. Moss, G.G. Giulian, and M.L. Greaser. 1985. Shortening velocity in single fibers from adult-rabbit soleus muscles is correlated with myosin heavy-chain composition. Journal of Biological Chemistry 260:9077–9080.

Ritzmann, R.E. 1974. Mechanisms for the snapping behavior of two Alpheid shrimp, *Alpheus californiensis* and *Alpheus heterochelis*. Journal of Comparative Physiology 95:217–236.

Rome, L.C., and S.L. Lindstedt. 1997. Mechanical and metabolic design of the muscular system in vertebrates. Pages 1587–1651 *in* W. Dantzler, editor. Handbook of Physiology: Comparative Physiology, volume 13. American Physiological Society, Bethesda, Maryland.

Rome, L.C., R.P. Funke, R.M. Alexander, G. Lutz, H. Aldridge, F. Scott, and M. Freadman. 1988. Why animals have different muscle fibre types. Nature 335:824–827.

Rosenbluth, J. 1969. Sarcoplasmic reticulum of an unusually fast-acting crustacean muscle. Journal of Cell Biology 42:534–547.

Royuela, M., B. Fraile, M.I. Arenas, and R. Paniagua. 2000. Characterization of several invertebrate muscle cell types: a comparison with vertebrate muscles. Microscopy Research and Technique 48:107–115.

Sakurai, Y., N. Kanzawa, and K. Maruyama. 1996. Characterization of myosin and paramyosin from crayfish fast and slow muscles. Comparative Biochemistry and Physiology B, Biochemistry & Molecular Biology 113:105–111.

Schenk, S.C., and P.C. Wainwright. 2001. Dimorphism and the functional basis of claw strength in six brachyuran crabs. Journal of Zoology 255:105–119.

Schiaffino, S., and C. Reggiani. 1996. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. Physiological Reviews 76:371–423.

Schiaffino, S., C. Reggiani. 2011. Fiber types in mammalian skeletal muscles. Physiological Reviews 91:1447–1531.

Selverston, A. 1967. Structure and function of the transverse tubular system in crustacean muscle fibers. American Zoologist 7:515–525.

Seok, J.H., L. Xu, N.R. Kramarcy, R. Sealock, and G. Meissner. 1992. The 30 S lobster skeletal muscle Ca^{2+} release channel (ryanodine receptor) has functional properties distinct from the mammalian channel proteins. Journal of Biological Chemistry 267:15893–15901.

Silverman, H., and M.P. Charlton. 1980. A fast-oxidative crustacean muscle: histochemical comparison with other crustacean muscle. Journal of Experimental Zoology 211:267–273.

Sohn, J., D.L. Mykles, and R.L. Cooper. 2000. Characterization of muscles associated with the articular membrane in the dorsal surface of the crayfish abdomen. Journal of Experimental Zoology 287:353–377.

Spirito, C.P. 1972. An analysis of swimming behavior in the portunid crab, *Callinectes sapidus*. Marine Behaviour and Physiology 1:261–276.

Staron, R.S., and R.S. Hikida. 1992. Histochemical, biochemical, and ultrastructural analyses of single human muscle fibers, with special reference to the C-fiber population. Journal of Histochemistry & Cytochemistry 40:563–568.

Staron, R.S., and D. Pette. 1986. Correlation between myofibrillar ATPase activity and myosin heavy chain composition in rabbit muscle fibers. Histochemistry 86:19–23.

Stephenson, G.M.M. 2001. Hybrid skeletal muscle fibres: a rare or common phenomenon? Clinical and Experimental Pharmacology and Physiology 28:692–702.

Stokes, D.R., and R.K. Josephson. 1992. Structural organization of two fast, rhythmically active crustacean muscles. Cell & Tissue Research 267:571–582.

Stokes, D.R., and R.K. Josephson. 1994. Contractile properties of a high frequency muscle from a crustacean 2. contraction kinetics. Journal of Experimental Biology 187:275–293.

Suma, Y., S. Ishizaki, Y. Nagashima, Y. Lu, H. Ushio, and K. Shiomi. 2007. Comparative analysis of barnacle tropomyosin: divergence from decapod tropomyosins and role as a potential allergen. Comparative Biochemistry and Physiology B, Biochemistry & Molecular Biology 147:230–236.

Svent-Gyorgyi, A.G. 1975. Calcium regulation of muscle contraction. Biophysical Journal 15:707–723.

Takekura, H., and C. Franzini-Armstrong. 2002. The structure of Ca^{2+} release units in arthropod body muscle indicates an indirect mechanism for excitation-contraction coupling. Biophysical Journal 83:2742–2753.

Taylor, G.M. 2000. Maximum force production: why are crabs so strong? Proceedings of the Royal Society of London Series B, Biological Sciences 267:1475–1480.

Tse, F.W., C.K. Govind, and H.L. Atwood. 1983. Diverse fiber composition of swimming muscles in the blue crab, *Callinectes sapidus*. Canadian Journal of Zoology 61:52–59.

Ushio, H., and S. Watabe. 1993. Ultrastructural and biochemical analysis of the sarcoplasmic reticulum from crayfish fast and slow striated muscles. Journal of Experimental Zoology 267:9–18.

Ushio, H., S. Watabe, and M. Iino. 1993. Crayfish skeletal muscle requires both influx of external Ca^{2+} and Ca^{2+} release from internal stores for contraction. Journal of Experimental Biology 181:95–105.

Varadaraj, K., S.S. Kumari, and D.M. Skinner. 1996. Actin-encoding cDNAs and gene expression during the intermolt cycle of the Bermuda land crab *Gecarcinus lateralis*. Gene 171:177–184.

Versluis, M., B. Schmitz, A. von der Heydt, and D. Lohse. 2000. How snapping shrimp snap: through cavitating bubbles. Science 289:2114–2117.

Warner, G.F., and A.R. Jones. 1976. Leverage and muscle type in crab chelae (Crustacea: Brachyura). Journal of Zoology (London) 180:57–68.

Weiss, T., C. Erxleben, and W. Rathmayer. 2001. Voltage-clamp analysis of membrane currents and excitation-contraction coupling in a crustacean muscle. Journal of Muscle Research and Cell Motility 22:329–344.

Weiss, T., S. Kreissl, and W. Rathmayer. 2003. Localization of a FMRFamide-related peptide in efferent neurons and analysis of neuromuscular effects of DRNFLRFamide (DF2) in the crustacean *Idotea emarginata*. European Journal of Neuroscience 17:239–248.

West, J.M., D.C. Humphris, and D.G. Stephenson. 1992. Differences in maximal activation properties of skinned short- and long-sarcomere muscle fibres from the claw of the freshwater crustacean *Cherax destructor*. Journal of Muscle Research and Cell Motility 13:668–684.

White, A.Q., and C.P. Spirito. 1973. Anatomy and physiology of the swimming leg musculature in the blue crab, *Callinectes sapidus*. Marine Behaviour and Physiology 2:141–153.

Xiong, H., X.Y. Feng, L. Gao, L. Xu, D.A. Pasek, J.H. Seok, and G. Meissner. 1998. Identification of a two EF-hand Ca^{2+} binding domain in lobster skeletal muscle ryanodine receptor/Ca^{2+} release channel. Biochemistry 37:4804–4814.

Zack, T.I., T. Claverie, and S.N. Patek. 2009. Elastic energy storage in the mantis shrimp's fast predatory strike. Journal of Experimental Biology 212:4002–4009.

5

SKELETAL MUSCLE DIFFERENTIATION, GROWTH, AND PLASTICITY

Donald L. Mykles and Scott Medler

Abstract

During embryogenesis, muscle progenitor cells fuse and form multinucleate myotubes that differentiate into myofibers. Fibers increase in length and diameter as animals move through larval, juvenile, and adult stages. In large fibers, infolding of the cell membrane and the concentration of mitochondria adjacent to the cell membrane reduce diffusional distances for energy metabolism. As fibers grow, nuclei are added and occupy more central locations to maintain a relatively constant myonuclear domain. Contractile properties and size are altered by physiological conditions. Transformation requires coordinated expression of fiber type-specific isoforms of myosin, actin, paramyosin, tropomyosin, and troponin-I and -T. Muscle load and contractile frequency change significantly as crustaceans grow; these size-related differences require cellular remodeling of muscles for locomotion. Proteasome/ubiquitin-dependent and calpain-dependent proteolytic systems degrade myofibrillar proteins and are upregulated in atrophic muscle. Myostatin and mechanistic Target of Rapamycin signaling pathways control the protein turnover required for remodeling fiber myofibril structure.

INTRODUCTION

This chapter reviews the current state of knowledge of skeletal muscle myogenesis, growth, and plasticity. There are few modern studies of muscle specification and differentiation during embryogenesis, and little is known about the genes that control myogenesis. Once established in the larval stages, the muscle fibers grow incrementally in length and width after each molt. Because the number of muscle fibers remains relatively constant, the fibers can achieve large

dimensions in adults. In the American lobster (*Homarus americanus*), for example, fibers in the crusher claw closer muscle can be a centimeter in length and 1–2 mm in diameter. As in other taxa, crustacean skeletal muscles display a remarkable plasticity, and this chapter focuses on the best-known examples. Transformation of fibers from one phenotype to another occurs in the American lobster, snapping shrimp, and Christmas Island red crab. A molt-induced atrophy occurs in the closer muscles of large-clawed decapods, such as American lobster, fiddler crab, crayfish, and blackback land crab. Autotomy of an appendage causes an unweighting atrophy in the corresponding thoracic muscles of green shore crab, fiddler crab, crayfish, and blackback land crab.

Skeletal muscle development begins in the embryo and is completed during the larval stages. The early stages of myogenesis are similar to those in other taxa. Muscle progenitor cells arise in the mesoderm and fuse to form multinucleate myotubes, which differentiate into striated muscle fibers. Some crustaceans develop from a free-swimming nauplius larva, whereas in others the nauplius stage takes place within the egg. In the free-swimming larva, propulsive muscles develop to provide locomotion, and the adult muscles develop later (Kreissl et al. 2008, Hertzler and Freas 2009). In taxa that develop without a free-swimming nauplius, the muscles present in the adult are evident from the earliest stages of development (Harzsch and Kreissl 2010). In the lobster, early muscle fibers are recognized by the appearance of cells containing developing myofilaments and many myonuclei with diffuse chromatin (Kirk and Govind 1992, Govind 1995). Next, thick and thin myofilaments begin to form a regular latticework, but a characteristic sarcomeric banding pattern is not yet recognizable. At this time, signs of innervation, including neuromuscular terminals with clear synaptic vesicles, become evident. Shortly after innervation, distinct myofibrils with recognizable sarcomeric structure become visible (Govind 1995, Lang 1977). Myogenesis in developing crustacean muscles is asynchronous because both long- and short-sarcomered fibers are already present in the larva, whereas undifferentiated myoblasts are present in the same individual (Lang 1977, Jirikowski et al. 2010). The close association between relatively undifferentiated myoblasts and the growing ends of motoneurons suggests that myogenesis and motor neuron growth take place together in newly forming muscles (Harzsch and Kreissl 2010).

Many adult decapods exhibit continual growth throughout their lives. Molting creates additional space for tissue growth (Mykles 1980, Taylor and Kier 2006). Because muscle fibers remain anchored to the new exoskeleton, stretching due to exoskeleton expansion stimulates muscle growth during the postmolt period. This growth can be rapid. For example, in juvenile lobsters, fibers grow to fill most of the available space in the claws by 3 days postecdysis (Medler et al. 2007). In many crustacean species, an individual will increase in mass by several orders of magnitude over its lifetime. This kind of indeterminate growth presents a number of physiological challenges as the animal grows. In terms of skeletal muscles, several different aspects of integrative muscle function are affected by organismal size. One of the most obvious of these is the classical problem of strength-to-weight ratios. As an animal grows in size, its mass increases in proportion to its linear dimensions^3, whereas muscle strength increases in proportion to the cross-sectional area of the muscles (approximately linear dimensions^2; see Schmidt-Nielsen 1984). This reduction in relative muscle strength has varying levels of significance for a species depending on whether it is aquatic or lives primarily a terrestrial existence. Lobsters and king crabs can grow to sizes of several kilograms, but their relatively sedentary existence, where much of their mass is supported by water, largely mitigates the impact of body mass. For a variety of semiterrestrial crabs, increases in body size are expected to have a more significant impact. The largest terrestrial arthropods are members of the infraorder Anomura, and these hermit crabs can attain sizes of up to 3 kg (Greenaway 2003). However, the notion that increases in effective

load constrain the operation of the musculoskeletal systems is not well supported. For example, even large hermit crabs are sufficiently strong relative to their weight that they exhibit effective locomotor abilities, can climb trees, and can even open coconuts (Herreid and Full 1986a, 1986b, Greenaway 2003). Small hermit crabs are able to carry shells equaling their own body mass or more without affecting running velocity (Herreid and Full 1986b). Exoskeletal strains and the safety factor against limb buckling in running ghost crabs are comparable to values for vertebrate bones (Blickhan et al. 1993). Moreover, performance parameters for these crabs (stride frequency, speed at trot-to-gallop transition, muscle shortening velocity) are also similar to comparably sized mammals (Blickhan and Full 1987, Full 1987, Full and Weinstein 1992, Blickhan et al. 1993). A more likely explanation for limitations on crustacean body size stems from limitations in gas exchange rather than the perceived burden of carrying a heavy exoskeleton (Kaiser et al. 2007).

Skeletal muscle can alter its size and fiber type composition in response to a variety of physiological conditions. Heterochely is common in decapod crustaceans (Mellon 1981, Govind 1992, Mariappan et al. 2000), and species with dimorphic claws are well suited for the study of fiber transformation. Some species, such as *Callinectes sapidus, Carcinus maenas*, and *Menippe mercenaria*, exhibit a distinct "handedness," in which the major claw is usually located on one side (Mariappan et al. 2000). In the hermit crab *Pagurus pollicaris*, the crusher claw is always on the right side and the cutter claw is always on the left side (Stephens et al. 1984). Other species, such as *H. americanus, Alpheus heterochaelis*, and *Nephrops norvegicus*, exhibit an equal distribution of left- and right-handed individuals (Mariappan et al. 2000). The claws of larvae are identical in morphology and fiber type composition and then differentiate into major and minor claws during the juvenile stage. Existing fibers of one type transform to a different type, which involves coordinated expression of fiber type-specific genes, as well as remodeling of the contractile apparatus. This is exemplified by *H. americanus*, in which fast fibers transform to slow fibers in the presumptive major (crusher) claw and slow fibers transform to fast in the presumptive minor (cutter) claw (Mykles 1997b). A similar process probably occurs in other species, in which the major and minor claws differ in fiber type composition, such as the fiddler crab *Uca pugilator* and the hermit crab *P. pollicaris* (Stephens et al. 1984, Govind et al. 1986, Ismail and Mykles 1992). Fiber transformation can also occur in adults. Shifts in fiber type occur during claw reversal in snapping shrimp (*Alpheus* sp.), in which the minor claw (pincer) transforms to the major claw (snapper) when the snapper claw is lost. Fast fibers die, and the remaining slow fibers transform to the snapper slow fiber phenotype over several molts. Claw reversal in the blue crab *C. sapidus* does not result in changes in muscle fiber properties (Govind and Blundon 1985). Claw reversal occurs in the stone crab *M. mercenaria*, but fiber transformation was not examined (Simonson 1985). In preparation for long-distance migration, the fibers in the walking legs of adult red crabs (*Gecarcoidea natalis*) transform from a slow-twitch (S_1) phenotype to a more fatigue-resistant slow-tonic (S_2) phenotype.

Species with large claws, such as *H. americanus*, freshwater yabby (*Cherax destructor*), and males of *Gecarcinus lateralis* and *U. pugilator*, have proved to be excellent models for the study of muscle growth and atrophy (Mykles 1997b). Fiber size is determined by the balance between the protein synthetic and degradative rates. When synthesis exceeds degradation, fibers increase in diameter (hypertrophy). Conversely, when degradation exceeds synthesis, fibers decrease in diameter (atrophy). Two types of atrophy occur in crustaceans. Unweighting from claw or leg autotomy causes a "disuse" atrophy of the corresponding thoracic musculature that operates the appendage. A molt-induced atrophy of the claw closer muscle facilitates withdrawal of the claws from the old exoskeleton at ecdysis. The net loss of protein results in a reduction of fiber diameter, whereas an increased protein turnover is associated with remodeling of the contractile apparatus that results from a preferential loss of thin filaments.

MYOGENESIS AND EARLY MUSCLE DEVELOPMENT

Several recent studies of muscle development in crustaceans have shed some light onto these processes, although much remains unknown (Kreissl et al. 2008, Hertzler and Freas 2009, Harzsch and Kreissl 2010, Jirikowski et al. 2010). A common theme among these studies of early myogenesis is that the process appears to follow the establishment of a founding muscle cell that serves as a point of muscle development. These cells, termed *pioneer cells* after the myogenic cells in grasshopper embryos (Ho et al. 1983), apparently migrate to specific anatomical locations to effectively "seed" specific muscles throughout the body (Fig. 5.1). Following the initial muscle establishment by the pioneer cells, some differences in the initial steps of myogenesis have been reported. In isopod muscles, single pioneer cells appear to establish each primordial muscle cell (Kreissl et al. 2008), whereas in lobsters and dendrobranchiate shrimp, muscles are established as a common syncytial muscle precursor that then divides to form several muscles (Harzsch and Kreissl 2010). It is currently unclear whether the founding cells divide on their own to become polynucleate or whether they act to recruit other cells that fuse with the founding cell.

Myogenesis in crustaceans shares a number of similarities with muscle development and differentiation in *Drosophila*, a model organism that has provided one of the most detailed views of muscle development. In *Drosophila*, muscle *founder cells* differentiate from cells within the mesoderm and then migrate to specific locations within the developing embryo. These founder cells then attract and fuse with fusion-competent myoblasts to form the earliest multinucleate

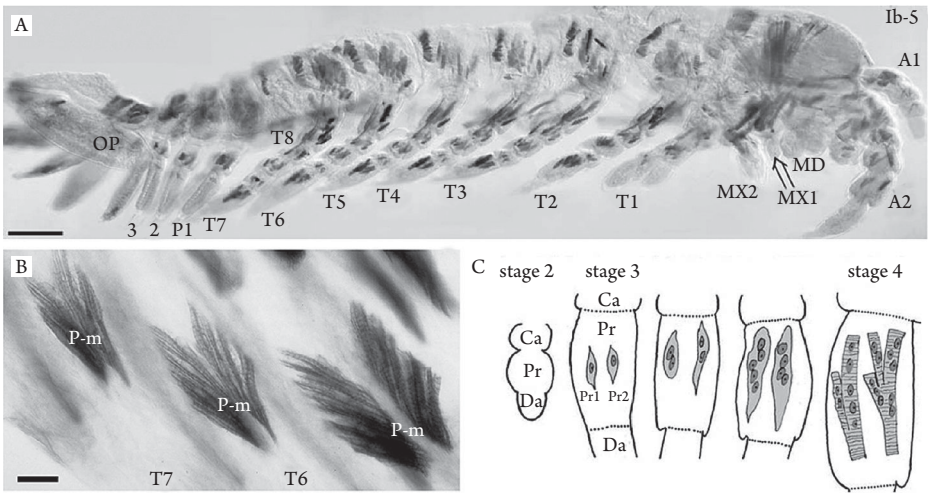


Fig. 5.1.
Myogenesis in crustaceans. (A) Muscle precursor cells in whole mount of a prehatching isopod embryo (*Idotea balthica*). Myogenic cells are labeled (dark) with a monoclonal antibody directed against myosin heavy chain (MHC). Abbreviations: A1, 2, antenna 1 and 2; MD, mandible; MX1, 2, maxilla 1 and 2; OP, operculum; P1-3, pleopods 1 to 3; T1–7, thoracic limbs 1–7 (T1 is a maxilliped); and T8, thoracomere 8. Scale bar = 100 μm. Reprinted from Kreissl et al. (2008), with permission from Springer. (B) Primordial muscles (P-m) in the endopodites of the thoracic limbs of embryonic lobsters (*H. americanus*; embryonic stage E45%). The developing muscles were labeled with a monoclonal antibody against MHC, and these muscle precursors will develop into the closer muscle of these leg segments. Scale bar = 25 μm. Reprinted from Harzsch and Kreissl (2010), with permission from Elsevier. (C) Schematic representation of myogenesis in the propodus of the thoracic limbs of the isopod, *I. balthica*. Muscle founder cells (Pr1 and Pr2) are observed in stage 3 embryos. In subsequent stages, the founder cells enlarge, become multinucleate, and subdivide into distinct subunits. Abbreviations: Ca, carpus; Da, dactylus; and Pr, propodus. From Kreissl et al. (2008), with permission from Springer.

muscle precursor cell. The growing myofiber then elongates toward tendon cells with which they subsequently fuse to form a muscle attachment site (Baylies et al. 1998, Schejter and Baylies 2010). The tendon cells secrete spatial cues that the growing myofibers use to seek out as they elongate toward their future attachment sites (Schejter and Baylies 2010). The founder cell then directs the ongoing differentiation of the developing fiber, with different fiber types being determined by the original founder cell's phenotype. Although our understanding of these events in crustaceans is far from complete, the available data are consistent with a similar developmental process. The common processes between the crustacean muscle development and fly development suggests that arthropod muscles may generally follow a pattern in which founder cells (*pioneer cells* in crustaceans) migrate from undifferentiated mesoderm into a specific anatomical position and then initiate fusion of undifferentiated cells and eventually form a specific muscle fiber type. Further work in this area is clearly needed to understand crustacean muscle differentiation more fully.

Myogenesis in all animals is directed, in part, by groups of transcription factors that orchestrate the processes of muscle differentiation (Baylies et al. 1998, Wigmore and Evans 2002). In vertebrate skeletal muscles, these include the basic helix-loop-helix proteins Myf 5, MyoD, Myf 4, and myogenin, which play complementary roles in directing muscle development (Wigmore and Evans 2002). In *Drosophila*, they include comparable proteins like Twist and Nautilus (Baylies et al. 1998). Although similar myogenic regulatory factors are presumably involved in crustacean myogenesis, the specific proteins have not yet been identified in crustacean muscles.

MUSCLE GROWTH

Once specific muscles have been established during the early stages of development, muscles continue to grow in both length and diameter. The available evidence suggests that increases in overall muscle size in crustaceans are primarily accomplished by increases in the size of individual fibers rather than through increased number of fibers. The hypertrophic growth that characterizes most crustacean muscles means that fiber size may continue to increase over an animal's lifetime. This pattern is fundamentally different from that seen in mammals of vastly different size, in which the size of individual fibers are essentially constant, being on the order of 25–75 μm in diameter (Hoppeler and Fluck 2002, Liu et al. 2009). The mechanism of increasing fiber length is either through the addition of new sarcomeres at the ends of existing muscle fibers, by increasing the length of sarcomeres throughout the fibers, or some combination of both mechanisms (Govind et al. 1974, 1977, Bittner and Traut 1978, El Haj et al. 1984). In fully differentiated lobster muscles, new sarcomeres are added to the ends of existing fibers, but these are the same width as existing sarcomeres (El Haj et al. 1984). The addition of new sarcomeres to the ends of existing fibers is closely linked to the process of molting, when the linear dimensions of the newly formed exoskeleton may increase by approximately 15% (El Haj et al. 1984). Once the old exoskeleton has been shed and the new one has expanded, fiber length increases with the addition of new sarcomeres. It may be that the mechanical stretch provided as the newly formed exoskeleton expands is the physiological cue that initiates fiber elongation (El Haj et al. 1984). In muscles of crayfish, increase in fiber length is accomplished by lengthening of existing sarcomeres. In several different crayfish fibers, sarcomere length increases by more than double in fast, short-sarcomered fibers and up to about fivefold in the fibers of slow muscles (Bittner and Traut 1978). Increasing the length of sarcomeres throughout a fiber would entail an ongoing process of fiber remodeling, whereas the addition of new sarcomeres at the fiber ends would require more restricted remodeling. Altering sarcomere length will also directly impact muscle shortening velocity, with increased sarcomere widths resulting in a slower contracting fiber.

One of the consequences of hypertrophic muscle growth is that diffusion-dependent processes may become limiting (Kinsey et al. 2007, 2011). Large crustacean fibers are often many times larger than mammalian skeletal muscle fibers, and the large size may restrict the diffusion of oxygen, intracellular phosphagens, Ca^{2+}, and other molecules. Kinsey and colleagues have considered this problem in detail and found that, in most cases, diffusion is not limiting to the metabolic processes but may often be on the verge of being diffusion-limited (Kinsey et al. 2011). In the largest anaerobic crustacean muscle fibers, the rate of arginine phosphate resynthesis following exercise is slower than the rate that could become limited by diffusion (Kinsey et al. 2005). However, there are significant structural and physiological adaptations evident in aerobic crustacean fibers that prevent significant diffusion limitation. Aerobic muscle fibers in several crustacean muscles rely on a common mechanism to deal with diffusional limitations associated with hypertrophic growth. In these fibers, the surface membrane of larger fibers becomes invaginated to form clefts that penetrate the fiber center (see Chapter 4 in this volume). In some cases, the fibers become so highly subdivided that it is difficult to determine whether the original fiber is still a single cell or whether it has completely separated into multiple fibers. The mitochondria in these fibers are highly concentrated near the membrane invaginations, so that almost 90% of the mitochondria are found in this location. These aerobic fibers are particularly evident in the muscles used for swimming in portunid crabs (Hardy et al. 2010), as well in the proximal and distal regions of muscle used for running in ghost crabs (Perry et al. 2009).

A related issue is the number and placement of the myonuclei within a muscle fiber. All skeletal muscle fibers are multinucleate, frequently with hundreds of distinct nuclei that originate from multiple myoblasts during development (Baylies and Michelson 2001, Biressi et al. 2007). Each nucleus within a mature muscle fiber is thought to direct the expression of proteins within a limited cytoplasmic space termed the *myonuclear domain* (Allen et al. 1999). In vertebrate muscles, myonuclear domain size is relatively fixed. As vertebrate skeletal muscles grow hypertrophically, nuclei are added to existing fibers to maintain a relatively constant myonuclear domain size (Allen et al. 1999). In mammals ranging over a 100,000-fold difference in body size, the myonuclear domain increases with size but only on the order of three- to fivefold (Liu et al. 2009). In crustaceans, hypertrophic growth of existing muscle fibers also leads to the addition of new nuclei to the fibers to maintain myonuclear domain size (Hardy et al. 2009, Jimenez et al. 2010, Kinsey et al. 2011). In smaller fibers, the nuclei are restricted to the periphery of fibers as they are in mammalian fibers, but, with additional growth, the new nuclei are distributed throughout the fiber, including the fiber interior (Hardy et al. 2009, Kinsey et al. 2011). Overall, the myonuclear domain size in crustacean muscles is comparable to sizes observed in mammalian muscles (~10,000–100,000 μm^3 per nucleus; Liu et al. 2009, Jimenez et al. 2010). In vertebrate muscles, the source of the new nuclei is the population of undifferentiated myoblasts (satellite cells) that lie between the basement membrane and the sarcolemma (Allen et al. 1999, Zammit et al. 2006). In crustacean muscles, putative satellite cells have been identified morphologically, but their role in muscle growth has not been established (Novotová and Uhrík 1992). In snapping shrimp claw muscles undergoing a cycle of degeneration followed by regeneration of a new fiber type, the remnants of the degenerated fibers serve as scaffolding for myoblasts that differentiate into new fibers. The source of these myoblasts was speculated to be either existing satellite cells or from undifferentiated blood cells that transformed into myoblasts (Govind and Pearce 1994).

A different way in which skeletal muscles are impacted by body size is in the frequency of muscle contraction during locomotion. A universal pattern observed among diverse animal taxa is that smaller animals move with higher frequencies in running, flying, and swimming (Hill 1950, Heglund et al. 1974, Full 1997, Medler 2002). As a consequence, the skeletal muscles of smaller animals must have faster intrinsic shortening velocities than in larger animals (Hill 1950, McMahon 1975, Medler and Hulme 2009). The most well-defined example of this pattern is seen in mammalian skeletal

muscles, where homologous isoforms of the myosin heavy chain (MHC) proteins have evolved subtle differences that produce faster muscles in smaller species (Seow and Ford 1991, Reggiani et al. 2000, Pellegrino et al. 2003, Marx et al. 2006). The relative proportion of fast fiber types is greater in the muscles of smaller mammals than in the same muscles of larger animals (Goldspink 1977). In addition to faster muscle shortening, muscles operating at higher contractile frequencies must be able to become activated and inactivated more quickly than in slower contracting muscles (Rome and Lindstedt 1997, Rome 2006). It follows that one mechanism that has evolved to ensure rapid activation and deactivation is a high density of sarcoplasmic reticulum (SR) within muscles used at high frequencies. This ensures that the calcium ions that trigger muscle contraction can be released and then sequestered quickly. The rates of muscle activation, deactivation, and operational frequency are positively correlated with SR density in arthropod muscles, including those of crustaceans (Fahrenbach 1963, Josephson and Young 1987, Stokes and Josephson 1992, Lagersson 2002). In vertebrate muscles, contractile frequency is correlated with concentrations of Ca^{2+}-binding proteins, such as parvalbumin, which buffer cytosolic Ca^{2+} and enhance muscle relaxation (Thys et al. 2001, Rome 2006, Coughlin et al. 2007). Currently, there have not been any parvalbumins or similar proteins identified in crustacean muscles, but it seems likely that they are present. Another mechanism that influences the rate of muscle activation and relaxation is the expression of alternate isoforms of the thin filament proteins that regulate muscle contraction. These could include tropomyosin; troponin (Tn)-I, -T, and -C; or even actin itself. In two fast fiber types in the claws of the freshwater yabby *Cherax destructor*, expression of alternate TnI isoforms is correlated with Ca^{2+}-sensitivity and muscle activation rate (Koenders et al. 2004). In fish muscles, shifts in contractile frequency are correlated with changes in the expression of alternate TnT and TnI isoforms (James et al. 1998, Thys et al. 1998, 2001). In dragonfly flight muscles, the expression of two different TnT splice variants is correlated with contractile frequency and power output (Fitzhugh and Marden 1997, Marden et al. 1999, 2001).

Crustaceans offer compelling examples of size-related changes in muscle function and organization, but it is currently unclear how a systematic shift in operational frequency affects their skeletal muscles. The most complete information comes from studies of semiterrestrial ghost crabs, which are probably the most capable runners of all the crustaceans (Hafemann and Hubbard 1969, Burrows and Hoyle 1973, Full and Weinstein 1992, Perry et al. 2009). These crabs are able to run at top speeds in the range of 1–2 m/sec for short bursts, and the estimated contractile properties are similar to a comparably sized mammal (Hafemann and Hubbard 1969, Burrows and Hoyle 1973, Full and Weinstein 1992, Perry et al. 2009). Stride frequencies during maximal running exhibit a significant correlation with body mass. The smallest ghost crabs reach stride frequencies of approximately 20 Hz, but the larger animals top out at about 4 Hz (Burrows and Hoyle 1973, Blickhan et al. 1993, Perry et al. 2009). Overall, this means that running crabs experience the same scale-dependent shifts in operational frequency as a function of body size known for other kinds of animals. Because these crabs increase in mass by several orders of magnitude over their lifetime, a relevant question is whether the intrinsic properties of the muscles change as the animals grow. A recent study indicates that gradual shifts in the expression of alternate isoforms of MHC, TnT, and TnI is correlated with changes in crab size. Single fibers from the leg extensor and flexor carpopodite muscles express more MHC_2, TnT_1, and TnI_1 in larger crabs than in smaller crabs (Perry et al. 2009). Further work is needed to determine how these differences in myofibrillar protein expression are related to functional differences in the muscles. Over the past two decades, it has become clearer that a significant degree of complexity exists with respect to the number and expression patterns of different myofibrillar isoforms in crustacean muscles (see Chapter 4 in this volume). It is possible that the specific expression of unique combinations of these isoforms provides for a degree of "fine-tuning" in response to the demands placed on different crustacean

skeletal muscles. It remains to be determined what kinds of changes in muscle fiber type accompany continued growth throughout the lifetime of crustaceans.

SKELETAL MUSCLE PLASTICITY

Fiber Transformation in Juvenile Lobsters

The dimorphic claws of the American lobster *H. americanus* differentiate from isomorphic claws during the early juvenile stages (Govind 1984, 1992, Govind et al. 1987, Mykles 1997b). *H. americanus* has three planktonic larval (zoeal) stages separated by two molts. At the third molt, third-stage larvae metamorphose into fourth-stage juveniles. By the fourth molt, fifth-stage juveniles assume a benthic habitat (Herrick 1895). The claws of fourth-stage animals have identical fiber type compositions: there is a central population of fast fibers and dorsal and ventral populations of slow fibers (Lang et al. 1977, Govind and Lang 1978, Ogonowski et al. 1980). At each subsequent juvenile molt, there is an incremental change in the muscle fiber composition and external morphology as the claws differentiate into the cutter and crusher types (Fig. 5.2; Emmel 1908, Govind and Lang 1978). Fibers in the presumptive cutter claw closer muscle transform from the slow to the fast phenotype, whereas fibers in the presumptive crusher claw closer muscle transform from the fast to the slow phenotype (Govind and Lang 1978, Costello and Lang 1979, Ogonowski et al. 1980). There is no evidence of fiber degeneration and replacement during claw differentiation (Lang et al. 1977, Costello and Lang 1979). Fiber transformation is completed in the cutter claw by the ninth stage, whereas transformation in the crusher claw lags behind the cutter claw and it is not completed until the 13th stage or later (Govind and Lang 1978, Lang et al. 1978, Ogonowski et al. 1980, Medler et al. 2007). Differentiation of the claws begins at the fifth stage, but it requires at least 15 molts over several years before the final cutter and crusher claw morphologies of the adult are attained (Costello and Lang 1979, Emmel 1908).

Changes in the innervation pattern and synaptic properties of fast and slow excitatory motor neurons also occur during claw differentiation. In early juveniles (fourth, fifth, and sixth), most fibers in both claws are innervated by both excitatory neurons (Costello et al. 1981). In adults, this pattern is retained in the crusher claw closer muscle but not in the cutter. In the cutter, most of the fast fibers receive only fast excitatory input, and the slow fibers receive either slow excitatory or both slow and fast excitatory input (Lang et al. 1980, Costello et al. 1981, Costello and Govind 1983). In adults, the cutter fast closer excitatory (FCE) motoneuron fires at higher frequencies for shorter bursts than the crusher FCE motoneuron (Lnenicka et al. 1988). Moreover, there are differences in the response of the excitatory motoneuron cell bodies to sensory stimulation; for both the FCE and slow closer excitatory (SCE) motoneurons, the cell bodies on the crusher side in the thoracic ganglion have greater spike frequencies and longer burst periods than those on the cutter side with the same stimulation (Govind and Lang 1981). Changes in the synaptic properties of the FCE motoneurons coincide with the changes in innervation pattern during claw differentiation. Synaptic facilitation is similar in the symmetrical claws of fourth-stage lobsters (Lnenicka et al. 1988). As the animals transition through the fifth, sixth, and seventh stages, facilitation increases in the presumptive cutter claw but remains unchanged in the presumptive crusher claw (Lnenicka et al. 1988). The changes in the pattern and synaptic properties of the FCE motoneurons in the cutter claw contribute to the faster contraction times needed to capture prey.

The determination of claw laterality is random and is restricted to fourth- and fifth-stage lobsters. In wild populations, the numbers of individuals with left-handed and right-handed crusher

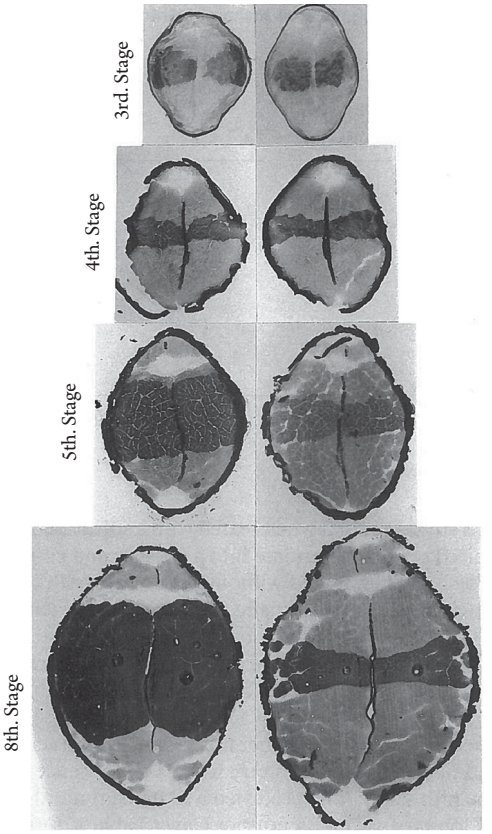


Fig. 5.2.
Fiber transformation during claw differentiation in juvenile American lobster. Transverse sections stained for myofibrillar ATPase activity show developmental changes in the closer muscle as the isomorphic claws of larvae (third and fourth stages) differentiate into the cutter and crusher claws of adults. The central band of dark-staining fast fibers expands in the presumptive cutter claw (left) by transformation of slow fibers to fast fibers. Conversely, the dorsal and ventral bands of slow fibers expand in the presumptive crusher claw (right) by transformation of fast fibers to slow fibers. The fast fibers in the crusher claw closer are completely replaced by slow fibers by the 13th stage. Fiber transformation is restricted to the boundary between fast and slow fiber populations. From Ogonowski et al. (1980), with permission from John Wiley and Sons.

claws are approximately equal (Herrick 1895, Emmel 1908), indicating that claw laterality is not genetically fixed at hatching. Emmel (1908) first demonstrated that autotomy of one of the claws during the fourth or fifth stage induces the remaining claw to differentiate into a crusher. This "forces" the animal to use the intact claw until the next molt, when the contralateral claw regenerate becomes functional (Lang et al. 1978). Once claw laterality is established in an individual, it remains fixed in that individual for the rest of its life (Emmel 1908). Autotomy at larval stages or at sixth and later stages has no effect on claw laterality (Emmel 1908, Lang et al. 1978). Autotomy of both claws during the fourth and fifth stages can delay the critical period for determining claw laterality to the sixth stage (Govind and Pearce 1989).

In an elegant series of experiments, Lang, Govind, and colleagues showed that the establishment of claw laterality requires a minimal reflex activity involving sensory input and neuromuscular output (Govind et al. 1987, Govind 1992). The claw that receives the greater stimulation above a minimum level of activity becomes the crusher claw (Lang et al. 1978, Govind and Kent 1982,

Govind and Pearce 1986, 1992). When juveniles are raised in smooth-bottomed containers without natural substratum or when juveniles receive equal mechanical stimulation through the fourth and fifth stages, most of the animals develop two cutter claws with similar fiber compositions (Lang et al. 1978, Govind and Kent 1982, Govind and Pearce 1986, 1992, Govind et al. 1991). Immobilization has no effect on claw laterality, indicating that a complete reflex arc is needed to determine which claw becomes the crusher (Lang et al. 1978, Govind and Kent 1982). Therefore, the cutter claw is the "default" outcome; a crusher claw only differentiates when one of the claws is used more than the other. The presence of a crusher claw prevents the contralateral claw from differentiating into a crusher. However, "double-crusher" individuals occur rarely in wild populations (Emmel 1908). The fiber compositions differ, even though the claws have the crusher morphology: one of the claws is a "false" crusher because it contains a mixture of fast and slow fibers resembling the composition of the cutter claw; the other claw is a "true" crusher that contains only slow fibers (Govind and Lang 1979). This indicates that fiber transformation can be uncoupled from structural differentiation, presumably from a genetic mutation that prevents repression of the crusher morphology and produces a false crusher.

Changes in muscle protein gene expression occur during claw differentiation. The fast and slow-twitch (S_1) fibers in the differentiated claw of adult *H. americanus* express distinct assemblages of myofibrillar protein isoforms: fast fibers in the cutter claw closer express fast MHC; actinSK3, SK4, and SK5; paramyosin$_1$ (P_1); a 75 kDa protein (P75); troponin-T$_2$ (TnT_2) and troponin-T$_1$ (TnI_1), whereas S_1 fibers in the crusher claw closer express S_1 MHC, actinSK1 and SK2, P_2, TnT_3, and TnI_4 and no P75 (Costello and Govind 1984, Mykles 1985a, 1985b, 1988, Medler and Mykles 2003, Medler et al. 2007, Kim et al. 2009a; see Chapter 4 in this volume). The myofibrillar protein composition of juvenile claw muscle differs from that of differentiated fast and slow fibers in the adult (Fig. 5.3). Claw muscle from fourth-stage animals express P_2, TnT_3, and TnI_4, but not P_1, P75, TnT_2, or TnI_1 (Costello and Govind 1984), which suggests that the fibers are not yet fully differentiated. By the 10th stage, the myofibrillar protein isoform compositions of the cutter and crusher claws resemble those of adults (Costello and Govind 1984), but it may take as long as 2 years for the fast fibers to attain the TnI isoform composition of adults (Fig. 5.3; see Medler et al. 2007). This is supported by analysis of MHC isoform and P75 expression by in situ hybridization and immunocytochemistry, respectively, in juvenile lobsters (Fig. 5.4). In seventh-stage lobsters, P75 and fast and slow MHCs are expressed in all fibers, although P75 and fast MHC are expressed at higher levels in fast fibers and slow MHC is expressed at higher levels in slow fibers (Medler et al. 2007). By the 10th stage, the expression of MHC isoforms and P75 is more discrete between the fast and S_1 fibers (Medler et al. 2007). These data indicate that muscle fibers in juvenile claws are distinct from the fibers in adults and that it may take months or years before the adult fiber phenotypes are achieved.

The mechanism by which the asymmetry in the nervous system directs the orderly transformation of fibers over a period of many molts is poorly understood. Transformation is restricted to the boundary between the fast and slow fiber populations as fibers with intermediate histochemical properties are localized to the boundary zone (Govind et al. 1987). Excitatory motoneuron activity probably plays a role. Chronic electrical stimulation induces changes in fiber properties to a more slow-tonic-like phenotype in crayfish abdominal muscles (Cooper et al. 1998, Gruhn and Rathmayer 2002). In the limb opener muscle, synaptic properties of the excitatory motoneurons may affect the contractile properties of the fibers they innervate; the slow-tonic (S_2) phenotype is correlated with larger excitatory postsynaptic potentials and greater short-term synaptic facilitation (Mykles et al. 2002). However, the innervation pattern and synaptic properties do not strictly correspond with fiber type (Costello et al. 1981, Lnenicka et al. 1988). This suggests that highly localized interactions between fast and slow fibers are needed to restrict transformation to the boundary zone. Further research is needed to determine whether fiber transformation is controlled through direct contacts and/or by paracrine factors.

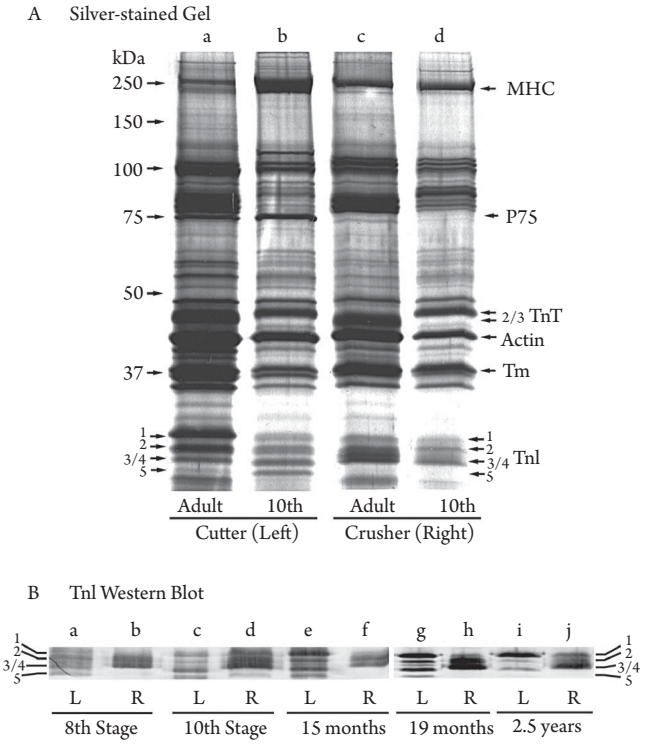


Fig. 5.3.
Differences in myofibrillar proteins between juvenile and adult lobster claw muscles. (A) Myofibrillar protein composition of fibers from cutter and crusher claws from adult and 10th-stage juvenile lobsters (*H. americanus*). Proteins were separated by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and stained with silver. Fast fibers from adult cutter claw express TnI_1 as the predominant isoform. In contrast, fast fibers from the central region of the presumptive cutter claw of 10th-stage juveniles express all five TnI isoforms at comparable levels. Fast fibers in cutter claw from both developmental stages express P75. The slow fibers from the adult crusher claw primarily express the $TnI_{3/4}$ isoforms, whereas the juvenile fibers express less of the $TnI_{3/4}$ isoforms. Both claws in juveniles express a TnT isoform with slower electrophoretic mobility than the TnT_2 and TnT_3 isoforms in adult cutter and crusher claws, respectively. Abbreviations: MHC, myosin heavy chain; Tm, tropomyosin; TnI, troponin-I; and TnT, troponin-T. Positions of molecular mass markers (kDa) indicated at left. (B) Western blot of troponin-I isoforms in dimorphic claws during lobster development. The left claw of fourth-stage larvae was autotomized, which induces the right claw to differentiate into the crusher claw; the left claw regenerates and differentiates into the cutter claw (Govind and Pearce, 1989). Fibers from the central region of the left claw (L; presumptive cutter) and the peripheral region of the right claw (R; presumptive crusher) of juvenile lobsters at eighth stage, 10th stage, 15 months, and 19 months and adult lobster at 2.5 years were analyzed. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with a lobster TnI antibody. Positions of the TnI isoforms indicated at the sides. It may take 2 years for the presumptive cutter claw to achieve the TnI composition of the mature adult. Reprinted with permission from Medler et al. (2007).

Fig. 5.4. (*Continued*)
the seventh molt stage, the developing crusher muscles have a distinct central region of fast fibers and distinct dorsal and ventral regions containing slow fibers (claw from 3 days postmolt animal). (B) Developing seventh-stage cutter claws are primarily composed of fast fibers by this stage (claw from 7 days postmolt animal). The opener muscle and the ventral region of the claw closer are entirely slow. (C, D) 10th-stage claw muscles possess similar staining patterns, but the fast and slow fiber regions are more distinct than the claws from seventh-stage claw muscles (molt stage unknown in 10th-stage claws). This is especially evident in the cutter claw, which completes fiber transformation before the crusher claw. Reprinted with permission from Medler et al. (2007).

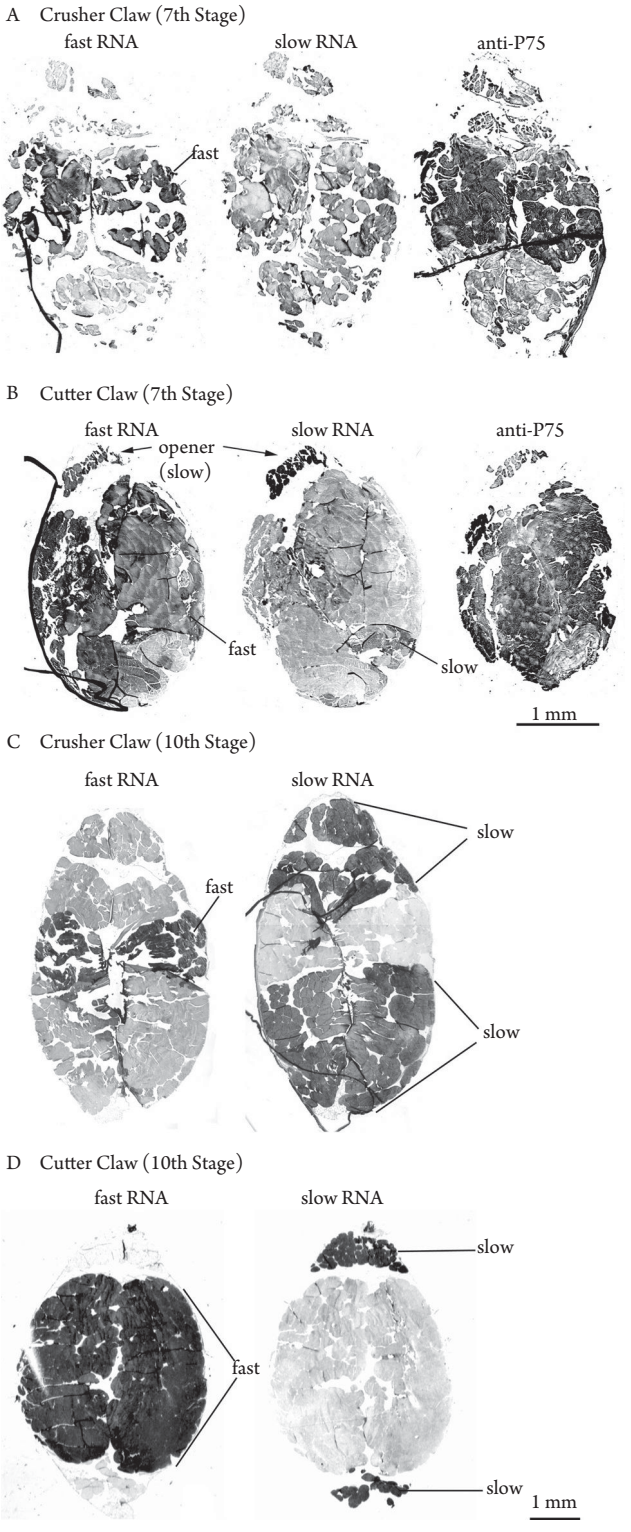


Fig. 5.4.
Differences in myosin heavy chain (MHC) isoform and P75 expression between developing crusher claw muscles and cutter claw muscles in *H. americanus*. Serial cross-sections of muscles were labeled with fast MHC RNA probe (left), S_1 MHC RNA probe (middle), and anti-P75 antibody (seventh stage only) (right). (A) By

Fiber Death and Transformation in Snapping Shrimp

Adult snapping, or pistol shrimps (*Alpheus* and *Synalpheus* spp.) possess dimorphic claws that differ greatly in morphology and function (Mellon 1981, 1999, Govind et al. 1987). The major claw, designated the snapper, has an enlarged dactyl that fits into a corresponding socket in the propodus (Fig. 5.5; Wilson 1903, Darby 1934, Knowlton and Moulton 1963). It is used for prey capture and agonistic behaviors (Darby 1934, Conover and Miller 1978, Knowlton and Keller 1982, Herberholz and Schmitz 1998, Mellon 1999). A rapid release of the dactyl results in a loud popping sound (Ritzmann 1973, Versluis et al. 2000), producing a continuous crackling noise in areas where large populations occur (Johnson et al. 1947, Everest et al. 1948, Knowlton and Moulton 1963, Potter and Chitre 1999). The noise produced by snapping shrimps can serve as a cue for orientation and settlement by invertebrate larvae on coral reefs (Stanley et al. 2010, Vermeij et al. 2010). The minor claw, designated the pincer, is much smaller and is used for feeding and burrowing (Mellon 1999). Right- and left-handed individuals occur in about equal numbers in wild populations (Wilson 1903, Darby 1934). Autotomy of one of the claws in third- or fourth-stage juveniles results in a snapper developing on the contralateral side (Young et al. 1994). These data indicate that claw laterality is established randomly in early juveniles probably by a mechanism similar to that in lobsters.

Unlike lobsters, the laterality of the major and minor claws is not fixed in adults. If the snapper claw is lost, the remaining pincer claw differentiates into a snapper claw over several subsequent

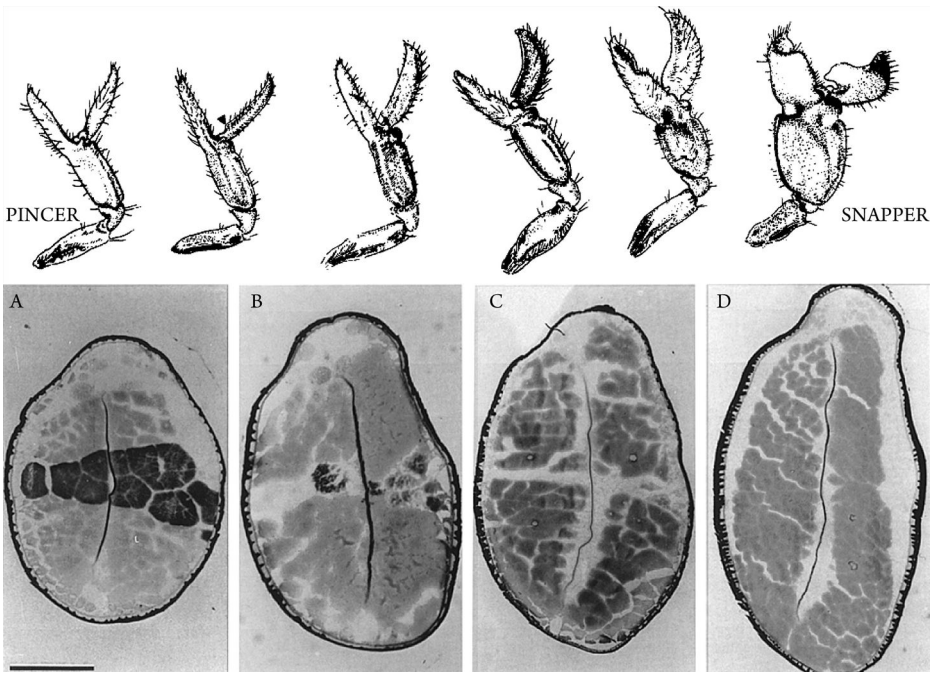


Fig. 5.5.
Claw transformation in snapping shrimp. Autotomy of the snapper claw causes the contralateral pincer claw to differentiate into a snapper over several molts (upper panel), and the pincer regenerates at the location of the original snapper. Myofibrillar ATPase histochemistry shows the dark-staining central band of fast fibers in the pincer (A) degenerating after the first (B) and second (C) molts to the slow fiber composition after the third molt (D). Over the next several molts, the slow fibers transform from the pincer phenotype with intermediate sarcomere lengths (6–8 μm) and a 5–6:1 filament ratio to the snapper phenotype with longer sarcomere lengths (10–15 μm) and a 7:1 filament ratio. Scale bar = 1 mm. Upper panel from Govind et al. (1987), with permission from Oxford University Press; lower panel from Govind and Pearce (1994), with permission from Springer.

molts, while the contralateral claw regenerates into a pincer (Wilson 1903, Mellon and Stephens 1978, Stephens and Mellon 1979). The transforming claw becomes a functional snapper after two molts, but the final snapper morphology is not attained until eight molts after autotomy of the contralateral snapper (Mellon and Stephens 1978, Stephens and Mellon 1979, Mellon and Greer 1987). Claw reversal occurs when only the snapper claw is autotomized, and laterality is maintained when only the pincer claw is autotomized or when both claws are autotomized (Wilson 1903, Darby 1934, Mellon and Stephens 1978). This indicates that there is some "memory" of claw asymmetry but that it can be overcome when the snapper claw is lost or is not functional. Transection of the nerve to the pincer claw prevents or delays reversal (Wilson 1903, Young et al. 1996). Although not as effective as pincer claw autotomy, either transection of the nerve, transection of the apodeme to the closer muscle (tenotomy), or removal of the dactyl (dactylotomy) to the snapper triggers transformation of the contralateral pincer, resulting in animals with two snappers; immobilization of the dactyl has no effect (Mellon and Stephens 1978, Govind et al. 1988, Young et al. 1994, Read and Govind 1997a, 1997b). Maintaining animals in communal tanks increases the incidence of double-snapper individuals (Pearce and Govind 1987). These data indicate that the nervous system transmits the loss of the snapper claw to the contralateral pincer and induces differentiation of the pincer to the snapper.

Differentiation of the pincer to the snapper involves a loss of fast fibers and transformation of slow fibers to a longer sarcomere phenotype. The pincer main closer muscle has a central band of fast fibers flanked dorsally and ventrally by slow fibers (Fig. 5.5), whereas the snapper contains only slow fibers (O'Connor et al. 1982, Mearow and Govind 1986, Quigley and Mellon 1986, Pearce and Govind 1987, Govind et al. 1988, Mellon and Quigley 1988). However, the sarcomere lengths and thin-to-thick filament ratios of the slow fibers in the main closer muscles differ between the two claws: the pincer slow fibers have intermediate sarcomere lengths (6–8 μm) and lower filament ratios (5–6:1) than snapper slow fibers (10–15 μm and 7:1, respectively; Stephens and Mellon 1979, Mellon and Stephens 1980, Stephens et al. 1983, Govind and Pearce 1994). This suggests that the intermediate and long-sarcomere fibers in the main closer muscles of the pincer and snapper claws, respectively, represent the two slow phenotypes. The snapper fibers express a larger TnT isoform that is consistent with the S_2 phenotype (Quigley and Mellon 1984, Govind et al. 1986, Mykles 1988, Ismail and Mykles 1992), but this must be confirmed by a thorough analysis of myofibrillar protein isoforms. The fast fibers are completely gone by the second molt after snapper claw autotomy (Fig. 5.5; Mearow and Govind 1986, Mellon and Quigley 1988, Quigley and Mellon 1986, Govind and Pearce 1994, Young et al. 1996). Following fast fiber degeneration, there is a transformation of the slow fibers in the pincer main closer muscle to the longer sarcomere phenotype in the snapper, which is completed within eight molts after autotomy of the snapper (Stephens and Mellon 1979, Mellon and Stephens 1980, Govind and Pearce 1994). The remodeling of the fibers coincides with the downregulation of pincer myofibrillar protein isoforms (e.g., pincer TnT and fast myosin light chain-2) and the upregulation of snapper myofibrillar proteins (e.g., snapper TnT and slow myosin light chain-2; Quigley and Mellon 1984, Mellon and Quigley 1988). Transection of the nerve to the transforming claw within 2 days after autotomy of the contralateral snapper prevents fast fiber degeneration even though claw morphological changes proceed normally (Mellon and Quigley 1988).

Little is known about the mechanisms controlling the changes in fiber type composition during transformation of the pincer to the snapper. The rapid degeneration of the fast fibers after the first molt is reminiscent of the programmed cell death in the abdominal intersegmental muscles (ISMs) of the hawkmoth *Manduca sexta*. At the end of pupation, contraction of the ISMs splits the pupal case, which allows the adult to escape. After the adult emerges, the ISMs are no longer needed, and the fibers completely degenerate by 30 h after emergence (Schwartz 2008). ISM cell death is triggered by a decline in hemolymph ecdysteroid (molting hormone) titer (Schwartz 2008). Fast fiber degeneration in the pincer claw occurs when ecdysteroid titers are low, which

suggests that a decrease in hemolymph ecdysteroid also triggers programmed cell death in snapping shrimp. Whatever the mechanism, it must explain why fast fibers die while slow fibers do not. The transformation of slow fibers from the pincer phenotype to the snapper phenotype has not been investigated in any detail. Gross motoneuron distribution does not appear to determine the fiber phenotype because the innervation patterns of fast and slow excitatory motoneurons are similar between the pincer and snapper claw closer muscles, and the patterns do not change during transformation in *A. californiensis* (Mellon and Stephens 1979, Stephens et al. 1983). Moreover, synaptic properties are correlated with claw type rather than with fiber type; synapses at fast and intermediate fibers in the pincer are characterized by low quantal neurotransmitter output and minimal facilitation, whereas synapses at long-sarcomere fibers in the snapper are characterized by large quantal output and greater facilitation (Mellon and Stephens 1979, Stephens and Mellon 1979, Stephens et al. 1983). This does not exclude trophic factors or other nerve properties, such as firing patterns and peripheral branching patterns, by which the nervous system may control fiber transformation. The cell bodies of the motoneurons in the thoracic ganglion differ in size, with the cell bodies on the snapper side larger than the cell bodies on the pincer side (Mellon 1981, Mellon et al. 1981). Claw reversal results in the reversal in cell body size after one molt, which is consistent with the primacy of the nervous system in driving fiber transformation (Mellon et al. 1981).

Fiber Transformation Associated with Seasonal Migration of Red Crabs

The red crabs (*G. natalis*) of Christmas Island in the Indian Ocean undergo a seasonal long-distance breeding migration. This species, as well as other members of this family, are obligate air breathers (Adamczewska and Morris 2000). The lining of the branchial chamber is elaborated into a highly vascularized lung-like tissue that allows high aerobic capacity. During the months of the dry season (April–October), animals remain inactive in burrows in the interior of the island to avoid desiccation. At the first monsoon rains, adult males and females begin a migration to the shore that may require walking 1 km/day for 5–6 consecutive days (Adamczewska and Morris 2001a). Upon arrival at the coast, males dig burrows where mating occurs. Males return inland and females remain in the burrows while the eggs develop. After about 2 weeks, females enter the ocean, where contact with seawater stimulates hatching and release of the zoea larvae. Over the course of 3–4 weeks, larvae undergo several molts and metamorphose into juvenile crabs, which come ashore and migrate to interior locations. Thus, there is a single "wave" of coastal migration, followed by three consecutive waves of inland migration: first adult males, then adult females about 2 weeks later, and then juveniles about 4 weeks after that.

The transition of sedentary to migratory phases requires physiological mechanisms for sustained locomotory activity. *G. natalis* has a unique shunt from lungs to the gills, which allows greater oxygenation of the hemolymph during exercise, that is lacking in other land crab species (Adamczewska and Morris 2000, Morris 2002). Even so, dry-season *G. natalis* rely on anaerobic metabolism for sustained moderate exercise. By contrast, wet-season *G. natalis* are completely aerobic and show no metabolic acidosis during migration (Adamczewska and Morris 2001a,b).

Migration causes a change in the fiber-type composition of the muscles in the walking legs to a more fatigue-resistant phenotype. Postel et al. (2010) analyzed 2,118 expressed sequence tags (ESTs) from muscles in the merus of walking legs from wet- and dry-season *G. natalis*. Differences in the expression of several myofibrillar protein isoforms are consistent with a shift from slow-twitch (S_1) to slow-tonic (S_2) fibers in preparation for migration. Histochemical studies show that S_2 fibers have a lower myofibrillar ATPase activity and higher aerobic capacity than S_1 fibers (Mykles 1988). Muscles from dry-season animals express higher levels of an ortholog of lobster actinSK1, which is the dominant actin isoform in S_1 fibers in the lobster crusher claw (Kim et al. 2009a, Medler et al. 2005, Medler and Mykles 2003). Orthologs of lobster slow tropomyosin-1 (Ha-sTm$_1$) and

tropomyosin-2 (Ha-sTm$_2$) are also expressed differentially between dry- and wet-season *G. natalis*. In lobsters, Ha-sTm$_1$ is preferentially expressed in S$_1$ fibers, and Ha-sTm$_2$ is preferentially expressed in S$_2$ fibers (Medler et al. 2004). In *G. natalis*, the S$_2$ tropomyosin ortholog (Gn-TmS2) is expressed at higher levels in migratory animals, whereas there is little or no change in the expression of the S$_1$ ortholog (Gn-TmS1; Postel et al. 2010). There are also changes in 3 TnI and 2 other actin transcripts, but assignment to specific fiber types could not be made (Postel et al. 2010). These data indicate that a shift to slow-tonic fibers in the walking legs contribute to the ability of *G. natalis* to migrate long distances without fatigue.

Transformation of S$_1$ and S$_2$ fibers requires remodeling the myofibrillar structure. In fiddler crabs, S$_1$ fibers have longer sarcomere lengths and higher thin filament-to-thick filament ratios than do S$_2$ fibers (Ismail and Mykles 1992). Consistent with this is the upregulation of two muscle LIM proteins, Mlp (Contig 131) and paxillin (Contig 140), in leg muscles of migrating *G. natalis* (Postel et al. 2010). Orthologs of these genes are implicated in structural remodeling of insect and mammalian skeletal muscles (Postel et al. 2010). This suggests that LIM proteins facilitate the incorporation of newly synthesized myofibrillar protein isoforms into the contractile apparatus so that animals can initiate migration as soon as the monsoon begins.

Temperature Plasticity

Temperature is a significant abiotic factor that affects muscle contraction. Cyprinid fishes exhibit a range of skeletal muscle plasticity in response to temperature changes to maintain muscle performance. In carp, three temperature-specific isoforms of MHC genes are expressed (Watabe 2002). In crustaceans, studies have examined MHC isoform diversity in the context of evolutionary adaptation to environmental temperature and of acclimation to temperature changes. An Antarctic isopod (*Glyptonotus antarcticus*) expresses an MHC isoform that is not present in a temperate isopod species (*Idotea resecata*) or a cold water amphipod species (*Eulimnogammarus verrucosus*; Holmes et al. 2002). This isoform may have evolved to function in the cold Antarctic waters, but further work is needed to demonstrate its functional role. In a subsequent study of seven amphipod species, the expression of four MHC isoforms is correlated with latitudinal distribution (Rock et al. 2009). Although it was concluded that the number of expressed isoforms increased in the more northern populations, one cannot rule out the possibility that these isoforms simply represent differences in common fiber types (i.e., similar to fast, S$_1$, and S$_2$ MHC isoforms). Indeed, two MHC isoforms were later identified as being differentially expressed in fast versus slow muscles (Whiteley et al. 2010). Temperature-specific MHC isoforms have not been identified within any crustacean muscles. Temperature has no effect on the expression of MHC isoforms in the abdominal muscle of lobsters (*H. gammarus*) reared at 10°C, 14°C, and 19°C (Whiteley and El Haj 1997, Magnay et al. 2003). These limited data suggest that crustacean muscles do not possess the level of temperature-dependent plasticity observed in some fish species.

Muscle Atrophy

Disuse Atrophy: Effects of Unweighting, Tenotomy, Denervation, and Immobilization

Crustacean muscles undergo a "disuse" atrophy in response to treatments that reduce the load on the fibers, such as tenotomy and limb autotomy. Tenotomy shortens the fiber resting length 15–35% in the crayfish (*Procambarus clarkii*) claw opener muscle, resulting in a 20% decrease in mean fiber diameter by 15 days (Boone and Bittner 1974). Fiber diameter is reduced about 50% one to two months after tenotomy (Boone and Bittner 1974). Autotomy of a walking leg in *C. maenas* and *U. pugilator* and a claw in crayfish (*Procambarus* sp.) causes an approximately 50% reduction in mass

of the thoracic muscles that operate the appendage, relative to the contralateral muscle (Moffett 1987). Interestingly, the diameter of the fibers in the weighted contralateral anterior levator (AL) muscle is reduced, but not as much as the fibers in the unweighted muscle (Schmiege et al. 1992). Thus, there appears to be an autotomy-induced atrophy in weighted muscles. Innervation remains intact and the muscles continue to function (Boone and Bittner 1974, Velez et al. 1981, Moffett 1987). Atrophy occurs in intermolt animals, indicating that it does not require ecdysteroids. The atrophic changes are reversed when the leg regenerates and becomes functional after the animal molts (Schmiege et al. 1992). Unlike mammalian skeletal muscle, there is no change in fiber phenotype in unweighted *P. clarkii* thoracic muscle (Griffis et al. 2001). Moreover, denervation and immobilization have little to no effect on crayfish claw opener muscle (Boone and Bittner 1974, Velez et al. 1981), which indicates that isometric tension per se is not essential for maintaining muscle mass.

Ultrastructural changes associated with unweighting were examined in *C. maenas* and crayfish (*P. clarkii* and *P. simulans*; Velez et al. 1981, Schmiege et al. 1992). Both autotomy and tenotomy cause disorganization of the sarcomere in *C. maenas* and crayfish, respectively, including fragmentation and loss of the Z line (Velez et al. 1981). Striations are indistinct in tenotomized muscle, probably due to hypercontraction (Velez et al. 1981). The myofibrils have extensive areas of myofilament erosion, and the intermyofibrillar space is increased (Velez et al. 1981, Schmiege et al. 1992). These features are indicative of increased degradation of myofibrillar proteins. There are reductions in mitochondria and nuclei, which are correlated with the presence of multivesicular bodies and lysosomes (Velez et al. 1981, Schmiege et al. 1992).

Molt-induced Atrophy of Claw Closer Muscle

In many decapod crustaceans, the first pair of pereopods develops into powerful claws that have offensive and defensive functions. The penultimate segment (propodus) is enlarged and contains a massive closer muscle that enables the dactyl to close with great force. Although large claws confer a competitive advantage, it creates a mechanical challenge at ecdysis when the claws must be withdrawn through the small basi-ischial joint that connects the claw to the body (Fig. 5.6).

Consequently, the mass of the closer muscle is reduced during the premolt period. The physical problem of withdrawing the claw was recognized by biologists in the 19th century (Salter 1860, Herrick 1895), but it was Couch (1837, 1843) who reported a molt-induced atrophy in *Cancer pagurus* and proposed it functioned in extricating the claws at molt. Skinner (1966) rediscovered the phenomenon in the blackback land crab, *G. lateralis*, in which the claw muscle mass decreases about 40% during the premolt period. Various aspects of this molt-induced muscle atrophy have been reviewed (Mykles and Skinner 1982a, 1985a, 1990a, 1990b, Mykles 1992, 1997b, 1999a, West 1997). The major conclusions are:

1. Atrophy is specific to the claw closer muscle, with fiber types in the claw responding differently to the atrophic signal. Muscles in the walking leg and cephalothorax do not atrophy during the premolt period (Mykles and Skinner 1982a, Schmiege et al. 1992, Griffis et al. 2001). In *G. lateralis*, the claw closer muscle is composed mostly of large-diameter S_1 fibers, with small-diameter S_2 fibers located centrally (Mykles 1988). The major claw of the fiddler crab (*U. pugnax*) contains mostly S_1 fibers with small numbers of S_2 fibers, and the minor claw contains only S_2 fibers (Mykles 1988, Ismail and Mykles 1992). In both species, the S_1 fibers undergo a greater atrophy than the smaller S_2 fibers (Mykles and Skinner 1981, Ismail and Mykles 1992). The claw closer muscle in the yabby *C. destructor* has equal numbers of slow (long sarcomere) and fast (short sarcomere) fibers (West 1997, Koenders et al. 2004). The slow fibers in late premolt animals show morphological changes associated with atrophy, whereas fast fibers do not (West et al. 1995, West 1997). Interestingly, the major claw muscle of

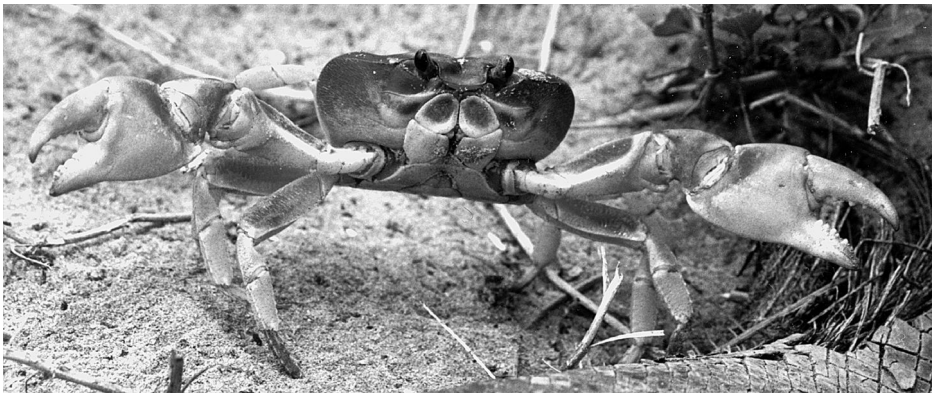


Fig. 5.6.
Dominant male blackback land crab, *G. lateralis*, in threat display. During premolt, the claw closer muscle atrophies. The reduction in muscle mass enables the animal to pull the large claws through the basi-ischial joints at ecdysis. Photograph by D. L. Mykles.

snapping shrimp does not atrophy (Mellon 1999). These animals use a different mechanism to extricate the claw. The exoskeleton splits open at ecdysis, providing a larger opening to remove the appendage (Mellon 1999).

2. The magnitude of claw muscle atrophy is determined by the regeneration load. Growth of limb regenerates occurs during the premolt period, so that a functional claw or leg is restored at ecdysis (Hopkins 2001, Mykles 2001). A large number of limb regenerates can place a significant demand for amino acids needed for tissue growth. In *G. lateralis* and *U. pugnax*, there is about a 40% decrease in mass in animals regenerating no more than one walking leg and about a 78% decrease in mass in animals regenerating 6–8 walking legs (Fig. 5.7; Skinner 1966, Mykles and Skinner 1981, Ismail and Mykles 1992). Thus, muscle protein degradation is accentuated by regeneration of 6–8 walking legs, which would provide amino acids for regenerate growth at a time when animals stop feeding and must rely on endogenous nutrient stores.
3. There is no fiber degeneration. In *G. lateralis*, the twofold reduction in fiber diameter is proportional to the fourfold reduction in myofibrillar cross-sectional area (Mykles and Skinner 1981), indicating that fibers retain the same number of myofibrils. The decrease in myofibrillar diameter results from the removal and degradation of myofilaments, as indicated by areas of myofibrillar erosion and enlarged intermyofibrillar space in atrophic muscle (Fig. 5.7; Mykles and Skinner 1981, Ismail and Mykles 1992, West et al. 1995, West 1997). In *C. destructor*, maximum Ca^{2+}-activated tension decreases about fourfold in both fast and slow fibers in claws of animals immediately before ecdysis (West 1999). There is also a compensatory decrease in the SR, and organelles, such as mitochondria and nuclei, retain their normal appearance (Mykles and Skinner 1981). Secondary lysosomes containing mitochondria and myelin figures indicate increased breakdown of mitochondria and SR membrane as fiber volume decreases (Mykles and Skinner 1982a).
4. There is an extensive remodeling of the sarcomere structure due to a preferential degradation of thin filaments. In *G. lateralis*, 11 thin filaments are removed for each thick filament, resulting in a decrease in the thin-to-thick filament ratio from about 9:1 to 6:1, a 31% decrease in the actin-to-MHC ratio, and a 72% increase in thick filament packing (Fig. 5.8; Mykles and Skinner 1981, 1982b). Similar changes occur in the major claw of male fiddler crabs *U. pugnax* (Ismail and Mykles 1992). In the S_1 fibers, the thin-to-thick filament ratio decreases from about 9:1

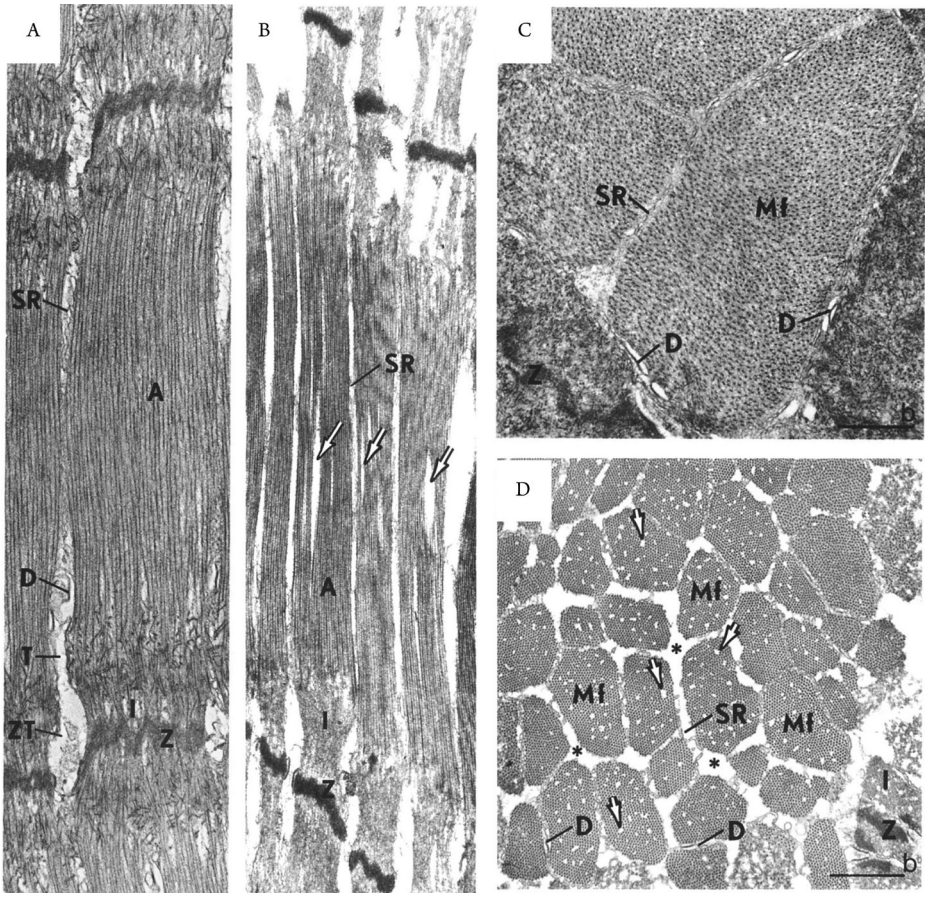


Fig. 5.7.
Molt-induced atrophy in the claw closer muscle of blackback land crab. Electron micrographs show the ultrastructure of the myofibrils in fibers from intermolt (A, C) and late premolt (B, D) animals in longitudinal (A, B) and transverse (C, D) sections. Myofibrillar cross-sectional area is reduced in late premolt fibers while the structure of the sarcomere is retained. Arrows indicate areas of erosion within the myofibrils, and asterisks (*) indicate enlarged interfibrillar space in the premolt fibers. Abbreviations: A, A band; D, dyad; I, I band; Mf, myofibril; SR, sarcoplasmic reticulum; T, transverse tubule; Z, Z-line; and ZT, Z tubule. Scale bar (C, D) = 1 μm. From Mykles and Skinner (1981), with permission from Elsevier.

to 6:1, the actin-to-MHC ratio decreases 74%, and the thick filament packing increases 51% (Ismail and Mykles 1992).

Molecular Mechanisms Regulating Protein Turnover in Crustacean Muscle

In mammalian muscle, atrophy results from a net increase in protein degradation, either from increased proteolysis and no change in protein synthesis, decreased synthesis and no change in proteolysis, or increased proteolysis and decreased synthesis (Schakman et al. 2009, McCarthy and Esser 2010, Goodman et al. 2011). Molt-induced claw muscle atrophy is atypical because protein synthesis is increased as much as 13-fold in vivo and in vitro in claw muscle from late premolt animals (Skinner 1965, El Haj et al. 1996, Covi et al. 2010). The large increase in protein synthesis

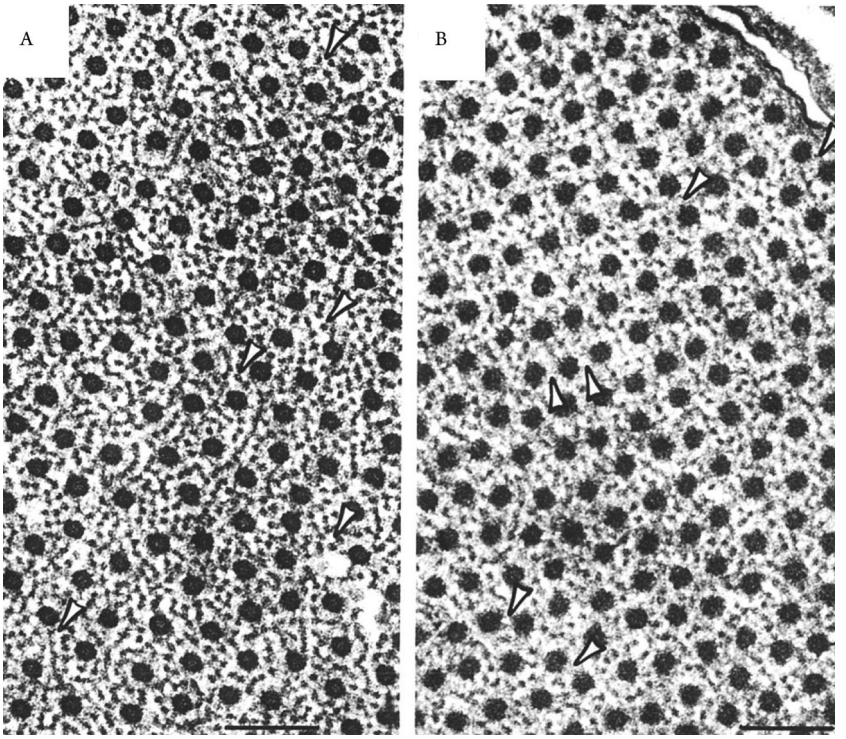


Fig. 5.8.
Molt-induced claw muscle atrophy causes changes in myofilament ratios and packing. (A) Transverse section of myofibril from an intermolt animal showing thick filaments surrounded by 10–15 thin filaments; the thin-to-thick filament ratio is about 9:1. Arrowheads indicate areas where thick filaments are separated by two rows of thin filaments. The average spacing of thick filaments is 51 nm. (B) Transverse section of myofibril from a late premolt animal showing thick filaments surrounded by 7–11 thin filaments; the thin-to-thick filament ratio is about 6:1. Arrowheads indicate areas lacking a row of thin filaments between thick filaments. The average spacing of thick filaments is 45 nm. Scale bars = 0.1 μm. From Mykles and Skinner (1981), with permission from Elsevier.

seems counterproductive because it necessitates an even greater increase in protein degradation to produce a reduction in mass. However, an accelerated protein turnover is necessary for the extensive remodeling of the fibers that occurs during premolt, as described earlier. Increased protein turnover is associated with remodeling in mammalian muscle (Bassel-Duby and Olson 2006). Any modification in the filament lattice requires the exchange of proteins in the myofibril with newly synthesized proteins in the cytoplasm (Russell et al. 2000). For example, the soluble myofilament pool increases under catabolic conditions (Dahlmann et al. 1986). Thus, the restructuring of the sarcomere depends on the flux, or turnover, of protein between the soluble and myofibrillar protein pools.

Protein degradation is mediated by calpain and ubiquitin (Ub)/proteasome proteolytic systems. Their role in molt-induced atrophy is briefly summarized here and emphasizes recent research. The reader is referred to reviews for further details on their biochemical properties (Mykles and Skinner 1985b, 1990a, 1990b, Beyette and Mykles 1999, Mykles 1992, 1993, 1997a, 1997b, 1998, 1999a, 1999b, 2000). Calpains are cytoplasmic Ca^{2+}-dependent proteinases (CDPs) that completely degrade myofibrillar proteins in vitro and in situ (Mykles and Skinner 1982b, 1983, 1986, Mattson and Mykles 1993, Mykles 1990). Four calpain activities, designated CDP I, IIa, IIb, and III, occur in crustacean

muscle, and cDNAs encoding three calpains, designated CalpB, CalpM, and CalpT, have been characterized (Table 5.1; Mykles 2000). Total calpain activity increases twofold in atrophic claw muscle (Mykles and Skinner 1982b).

The Ub/proteasome proteolytic system is stimulated in atrophic claw muscle. Ub is a highly conserved protein that, when conjugated to a protein, targets that protein for degradation by the proteasome (Mykles 1998, Glass 2010). Ubiquitin mRNA, Ub-protein conjugates, and proteasome subunits increase five-, eight-, and twofold, respectively, in atrophic claw muscle of *G. lateralis*, with greater ubiquitin expression in S_1 fibers (Shean and Mykles 1995, Koenders et al. 2002). In *H. americanus*, Ub expression is upregulated in claw muscle during premolt (Koenders et al. 2002). During larval development of the European lobster *H. gammarus*, proteasome activity in the claw muscle is higher during the pre- and postmolt stages (Götze and Saborowski 2011). The proteasome has limited ability to degrade myofibrillar proteins (Mykles 1989, Mykles and Haire 1991, 1995). Its precise role in muscle atrophy has yet to be determined, but its function appears secondary to calpains. As calpains preferentially degrade the Z line (Mykles 1990), calpains initiate protein degradation by releasing filaments from the myofibril; filaments are subsequently degraded by calpain and Ub/proteasome systems (Mykles 1997b).

Ecdysteroids control protein metabolism in the claw closer muscle. Protein synthesis increases during premolt, with maximum rates at late premolt when hemolymph ecdysteroid titers are at their peak (Covi et al. 2010, Mykles, 2011). Coincident with the increase in protein synthesis, there is a decrease in myostatin (Mstn) expression. Mstn, a member of the transforming growth factor-β (TGFβ) family, is an autocrine factor that inhibits protein synthesis and stimulates protein degradation in mammalian muscle (Schakman et al. 2009, McCarthy

Table 5.1. Comparison of masses of deduced sequences from crustacean calpain cDNAs with masses of lobster Ca^{2+}-dependent proteinases (CDPs) characterized biochemically.

Type	Calculated mass (cDNA)	Accession Number	CDP activity (subunit mass)	Reference
B Calpain:				
Gl-CalpB	88.9 kDa	AY639153	CDP IIb (95 kDa[b])	(Kim et al. 2005*a*)
Lv-CalpB	Partial cDNA	GQ179742		unpublished
M Calpain:				
Gl-CalpM	65.2 kDa	AY639152	CDP III (59 kDa[a];	(Kim et al. 2005*a*)
Ha-CalpM	66.3 kDa	AY124009	62 and 68 kDa[b])	(Yu and Mykles 2003)
Nn-CalpM	65.9 kDa	FJ666100		(Gornik et al. 2010)
T Calpain:				
Gl-CalpT	74.6 kDa	AY639154	CDP I or IIa (60 kDa[b])	(Kim et al. 2005*a*)

Abbreviations: Gl, *Gecarcinus lateralis*; Ha, *Homarus americanus*; Lv, *Litopenaeus vannamei*; Nn, *Nephrops norvegicus*.
Native masses of lobster CDPs I, IIa, IIb, and III are 310 kDa, 125 kDa, 195 kDa, and 59 kDa, respectively (Mykles and Skinner 1986). The putative identities of CalpB with CDP IIb and CapT with CDP I or IIa have not been established. The subunit composition of CDP I is not known.
[a] Mass estimated by gel filtration column chromatography (Mykles and Skinner 1986).
[b] Mass estimated by SDS-polyacrylamide gel electrophoresis (Beyette et al. 1993, 1997, Beyette and Mykles 1997, Yu and Mykles 2003). A 62 kDa isoform of Ha-CalpM is expressed in claw muscle, and a 68 kDa isoform is expressed in abdominal muscle (Yu and Mykles 2003).
Modified from Kim et al. (2005*a*).

and Esser 2010). cDNAs encoding the crustacean ortholog have been cloned from *G. lateralis, H. americanus, C. maenas, Eriocheir sinensis, Litopenaeus vannamei, Penaeus monodon*, and *Pandalopsis japonica* (Covi et al. 2008, Cho et al. 2009, Kim et al. 2009b, 2010, MacLea et al. 2010, De Santis et al. 2011, Qian et al. 2013). In *G. lateralis*, Gl-Mstn mRNA is reduced in both the claw closer and thoracic muscles during premolt (Covi et al. 2010). However, the effect of molting is greater in the claw muscle. By late premolt, Gl-Mstn mRNA in the claw muscle decreases by 81% (approximately fivefold) and 94% (approximately 17-fold) in animals induced by eyestalk ablation (ESA) and by multiple leg autotomy (MLA), respectively, and is negatively correlated with ecdysteroids (Covi et al. 2010). Gl-Mstn mRNA in thoracic muscle decreases by 68% (approximately threefold) and 82% (approximately fivefold) in ESA and MLA animals, respectively, and is only weakly correlated with ecdysteroid (Covi et al. 2010). In *L. vannamei*, the effects of 20-hydroxyecdysone (20E) injection on *Lv-Mstn* expression differed between muscles: 20E decreased mRNA levels in abdominal and pleopod muscles, increased mRNA levels in pereopod muscle, and had no effect on mRNA levels in thoracic muscle (Qian et al. 2013). However, because the control injections consisted of an equivalent volume of saline rather than the ethanol vehicle, it is possible that the changes in *Lv-Mstn* mRNA were not strictly in response to 20E (Qian et al. 2013). In *H. americanus*, spontaneous molting results in a larger decrease (82%) in Ha-Mstn expression in crusher claw muscle than in cutter claw (51%) or deep abdominal (69%) muscles (MacLea et al. 2010). However, an acute increase in ecdysteroids caused by ESA has no effect on Ha-Mstn mRNA levels in the three muscles (MacLea et al. 2010). These data suggest that ecdysteroids stimulate protein synthesis by downregulating Mstn. The differential response of the claw and thoracic muscles to ecdysteroid may be due to differences in the expression of the ecdysteroid receptor (Gl-EcR and Gl-RXR isoforms; Kim et al. 2005a,b, Covi et al. 2010).

Ecdysteroids appear to activate mechanistic target of rapamycin (mTOR)-dependent protein synthesis. mTOR is a highly conserved protein kinase that stimulates translation by phosphorylating ribosomal S6 kinase (S6K) and 4EF-binding protein-1 (Schakman et al. 2009, McCarthy and Esser 2010, Goodman et al. 2011, Frost and Lang 2012). mTOR functions as a sensor that controls cellular growth. Its activity is regulated by nutrients, intracellular energy levels, hypoxia, stress, and growth factors (Laplante and Sabatini 2012). The stimulation of muscle protein synthesis by ecdysteroids is primarily at the translational level, as indicated by increases in ribosomal activity and global protein synthesis during premolt (Skinner 1968, El Haj et al. 1996, Covi et al. 2010). Elevated ecdysteroids have little effect on MHC and actin mRNA levels (Whiteley and El Haj 1997, El Haj 1999, Medler et al. 2005). Expression of Rheb (*R*as *h*omolog *e*nriched in *b*rain), an activator of mTOR, is increased nearly fourfold in claw muscles from premolt *G. lateralis*, and Gl-Rheb mRNA levels are positively correlated with hemolymph ecdysteroid levels (MacLea et al. 2012). The mRNA levels of other mTOR signaling components (mTOR, Akt, and S6K) are not correlated with hemolymph ecdysteroid titers (MacLea et al. 2012). These data indicate that mTOR is activated by Rheb, resulting in increased synthesis of cytosolic and myofibrillar proteins (Covi et al. 2010). Thus, Rheb expression may serve as a molecular marker for tissue growth in crustaceans.

Mstn may have pleiotropic functions in crustaceans. Mstn is expressed in a wide variety of tissues, which suggests its function is not restricted to controlling muscle growth (Covi et al. 2008, Kim et al. 2009b, 2010, MacLea et al. 2010, De Santis et al. 2011, Qian et al. 2013). Studies on shrimp (*P. monodon* and *L. vannamei*) illustrate the complex and potentially multimodal actions of Mstn in crustaceans (De Santis et al. 2011, Qian et al. 2013). The expression of Pm-Mstn in abdominal muscle varies during the molting cycle, although there is not a clear relationship between Pm-Mstn mRNA levels and periods of muscle growth. Pm-Mstn mRNA level is elevated immediately after ecdysis (postmolt stage A), when muscles grow in

response to stretching from the expansion of the new exoskeleton. Pm-Mstn expression is low at intermolt stage when abdominal muscle growth is complete. Expression of *Lv-Mstn* in "mixed muscle" samples exhibits the same pattern (Qian et al. 2013). This suggests that Mstn is a myotropic factor that stimulates muscle protein synthesis. An apparent contradiction is that Pm-Mstn mRNA levels are also elevated at premolt stages, when the abdominal muscle is not growing (De Santis et al. 2011). Moreover, abdominal muscles in decapods with elongated body plans (e.g., shrimp, lobsters, and crayfish) do not atrophy because the abdomen is easily withdrawn through the large opening created at the junction between the cephalothorax and abdomen at ecdysis (Chang and Mykles 2011). However, ribosomal activity and protein synthesis are increased in lobster abdominal muscles during premolt (Whiteley and El Haj 1997). If protein synthesis is similarly elevated in premolt shrimp abdominal muscle, the Pm-Mstn expression pattern over the molting cycle is consistent with its function as a stimulatory growth factor. This is supported by knockdown experiments, in which injections of Pm-Mstn ds-RNA into the abdominal muscle significantly reduced shrimp growth over a 45-day period (De Santis et al. 2011). Interpretation of these results should be tempered by possible systemic effects because the Pm-Mstn knockdown was not restricted to the abdominal muscle. Pleopod muscle harvested at 7 days had a similar decrease (~40%) in Pm-Mstn mRNA as abdominal muscle harvested at 45 days, indicating the effect had spread to other regions of the abdomen. Other tissues with high levels of Pm-Mstn expression, such as heart, gill, eyestalk, and stomach, were not included in the qPCR analysis (De Santis et al. 2011). An alternative explanation is that Pm-Mstn knockdown inhibited molting by decreasing the ecdysteroidogenic activity of the molting glands (Y-organs) because Gl-Mstn is highly expressed in the Y-organ (Mudron and Mykles, data not shown). A reduction in molting frequency would explain the lower growth rate of Pm-Mstn ds-RNA-injected animals. Unfortunately, the molting data were not reported, thus leaving the matter unresolved (De Santis et al. 2011).

Little is known about how ecdysteroids regulate protein degradation in crustacean muscles. In *G. lateralis*, ESA causes a transient (1–3 days) increase in Gl-CalpT and Gl-EcR mRNAs in claw muscle, but not in thoracic muscle; ESA has no effect on Gl-CalpB and Gl-CalpM expression (Kim et al. 2005a). Interestingly, the expression of Gl-CalpT and Gl-EcR is correlated in both claw and thoracic muscles, suggesting the two genes are co-regulated (Kim et al. 2005a). However, the expression of ecdysteroid receptor genes (Gl-EcR and Gl-RXR) and Gl-CalpT is not correlated with ecdysteroid (Covi et al. 2010). In *H. americanus*, Ha-CalpM expression in the crusher claw is not affected by molt stage (Yu and Mykles 2003). These data suggest that sustained activation of calpains in atrophic muscle involves post-transcriptional mechanisms.

Atrophy in response to unweighting has a different effect on the expression of genes involved in protein synthesis and degradation. By contrast to the effects of molt induction in *G. lateralis*, Gl-Mstn mRNA increases threefold and Gl-CalpT mRNA decreases 40% in unweighted muscles with respect to weighted contralateral muscle (Covi et al. 2010). In premolt animals, the decrease in Gl-Mstn mRNA in unweighted muscle parallels the decrease in Gl-Mstn mRNA in weighted muscle, indicating that increased ecdysteroids supersede any effect that unloading has on Gl-Mstn expression. In other words, low ecdysteroids permit the upregulation of Mstn in unweighted thoracic muscle. Gl-Rheb and Gl-S6K are increased 2.2-fold and 1.3-fold, respectively, in unweighted thoracic muscle, indicating that mTOR-dependent protein synthesis is stimulated (MacLea et al. 2012). Unweighting has no effect on Gl-Akt, Gl-mTOR, Gl-EcR, and Gl-RXR mRNA levels (Covi et al. 2010, MacLea et al. 2012). These data, which are consistent with the positive relationship between Gl-Rheb and Gl-Mstn expression in weighted thoracic muscle (MacLea et al. 2012), indicate that Mstn stimulates protein synthesis in thoracic muscle.

FUTURE DIRECTIONS

Research should now turn toward elucidating the molecular control of fiber differentiation, phenotype, and size by neural activity and endocrine and paracrine factors. We know little about the molecular mechanisms regulating muscle differentiation during development, in particular the roles of transcription factors that specify skeletal muscle in other organisms (e.g., Mef2, Myf4, Myf5, MyoD, and myogenin in vertebrates) and how neuronal activity alters myofibrillar gene expression. The control of protein turnover is central to muscle plasticity. Fiber transformation requires the coordinated expression of fiber type-specific myofibrillar protein isoforms, which must be assembled and incorporated into the myofibrils while myofilaments containing protein isoforms of the former fiber type are removed and degraded. The relative rates of protein synthesis and degradation determine whether a muscle grows or atrophies. mTOR probably plays a critical role because increased protein synthesis is associated with skeletal muscle growth and remodeling in diverse organisms. In mammals, Mstn controls muscle growth by suppressing mTOR activity and activating Ub/proteasome-dependent degradation (Schakman et al. 2009, McCarthy and Esser 2010, Goodman et al. 2011, Frost and Lang 2012). The function of Mstn in crustacean muscle is not fully understood. Mstn appears to be a stimulator of protein synthesis in abdominal muscles in *P. monodon* and unweighted thoracic muscle in *G. lateralis* (Covi et al. 2010, De Santis et al. 2011, MacLea et al. 2012). In *G. lateralis* claw muscles, the downregulation of Gl-Mstn and the upregulation of Gl-Rheb indicate that Mstn inhibits mTOR-mediated protein synthesis (Covi et al. 2010, MacLea et al. 2012). Thus, muscles in the same species differ in the relationship between Gl-Mstn and Gl-Rheb expression. In claw muscle, Gl-Rheb and Gl-Mstn mRNAs are negatively correlated in animals induced to molt by ESA but not in animals induced to molt by MLA (MacLea et al. 2012). By contrast, Gl-Rheb and Gl-Mstn mRNAs are positively correlated in weighted thoracic muscle from both ESA and MLA animals (MacLea et al. 2012). The differences in the relationship between Gl-Mstn and Gl-Rheb expression may be due to differences in the responses of the two muscles to ecdysteroids (Covi et al. 2010). Gl-Mstn and Gl-Rheb mRNA levels in claw muscles are significantly correlated with hemolymph ecdysteroid titers, whereas there is no correlation between Gl-Mstn and Gl-Rheb mRNA levels and hemolymph ecdysteroid titers in thoracic muscles (MacLea et al. 2012). The next step is to gain a mechanistic understanding of the complex interactions between endocrine/paracrine factors and signaling pathways controlling protein turnover.

CONCLUSIONS

Relatively little is known about the early stages of crustacean myogenesis, but the available data suggest a process similar to that in insects. Muscle precursor cells migrate to specific locations in the embryo and then differentiate into fibers. Muscle fibers with motor nerves are in place at relatively early stages of development, and subsequent growth over several molt cycles produces adult muscles. The genes that control muscle differentiation remain to be studied. Molting stimulates the growth of muscle fibers, which increase in length by the addition of sarcomeres and/or lengthening of existing sarcomeres and in diameter by sarcomere splitting and/or increases in myofibril cross-sectional area. As in mammals, crustacean skeletal muscle is a dynamic tissue that can undergo dramatic changes in mass and contractile properties. Molecular mechanisms controlling the changes in fiber composition by cell death and fiber transformation are complex and probably involve a combination of neurotrophic, endocrine, and paracrine factors, which have yet to be identified. Changes in fiber composition are coordinated with changes in claw morphology, but they can, on rare occasions, be unlinked. In "double-crusher" lobsters, the closer muscle in the "false" crusher has the cutter claw fiber composition. Also, fiber transformation is completed before the

adult claw morphology is attained. Because both fiber transformation and molt-induced atrophy involve extensive remodeling of the sarcomeric structure, it is likely that both processes require an increase in protein turnover. The main difference, however, is that there is a net loss of protein in muscle atrophy, whereas there is no net loss or net gain in protein in transforming fibers. LIM proteins may play a role in both processes (Postel et al. 2010). Calpains degrade myofibrillar proteins, but we know little about how their activities are regulated by ecdysteroids. Atrophy of unweighted thoracic muscle is ecdysteroid-independent and is associated with modest increases in the expression of Gl-Mstn, Gl-Rheb, and Gl-S6K and decreases in the expression of Gl-CalT (Covi et al. 2010, MacLea et al. 2012). The stimulation of muscle protein synthesis by ecdysteroids in molt-induced claw muscle atrophy involves both Mstn and mTOR signaling pathways (Covi et al. 2010, MacLea et al. 2012).

ACKNOWLEDGMENTS

The authors thank the many students, postdoctoral fellows, and collaborators for their contributions. The authors recognize the support of grants from the National Science Foundation and the National Institutes of Health.

REFERENCES

Adamczewska, A.M., and S. Morris. 2000. Locomotion, respiratory physiology, and energetics of amphibious and terrestrial crabs. Physiological Zoology 73:706–725.

Adamczewska, A.M., and S. Morris. 2001a. Ecology and behavior of *Gecarcoidea natalis*, the Christmas Island red crab, during the annual breeding migration. Biological Bulletin 200:305–320.

Adamczewska, A.M., and S. Morris. 2001b. Metabolic status and respiratory physiology of *Gecarcoidea natalis*, the Christmas Island red crab, during the annual breeding migration. Biological Bulletin 200:321–335.

Allen, D.L., R.R. Roy, and V.R. Edgerton. 1999. Myonuclear domains in muscle adaptation and disease. Muscle & Nerve 22:1350–1360.

Bassel-Duby, R., and E.N. Olson. 2006. Signaling pathways in skeletal muscle remodeling. Annual Review of Biochemistry 75:19–37.

Baylies, M.K., M. Bate, and M.R. Gomez. 1998. Myogenesis: a view from *Drosophila*. Cell 93:921–927.

Baylies, M.K., and A.M. Michelson. 2001. Invertebrate myogenesis: looking back to the future of muscle development. Current Opinion in Genetics and Development 11:431–439.

Beyette, J.R., and D.L. Mykles. 1997. Autolysis and biochemical properties of a lobster muscle calpain-like proteinase. Journal of Experimental Zoology 277:106–119.

Beyette, J.R., and D.L. Mykles. 1999. Crustacean calcium-dependent proteinases. Pages 409–427 *in* K.K.W. Wang, and P.-W. Yuen, editors. The Pharmacology of Calpain. Taylor & Francis, Washington, DC.

Beyette, J.R., J.S. Ma, and D.L. Mykles. 1993. Purification and autolytic degradation of a calpain-like calcium-dependent proteinase from lobster (*Homarus americanus*) striated muscle. Comparative Biochemistry and Physiology 104B:95–99.

Beyette, J.R., Y. Emori, and D.L. Mykles. 1997. Immunological analysis of two calpain-like Ca^{2+}-dependent proteinases from lobster striated muscles: relationship to mammalian and *Drosophila* calpains. Archives of Biochemistry and Biophysics 337:232–238.

Biressi, S., M. Molinaro, and G. Cossu. 2007. Cellular heterogeneity during vertebrate skeletal muscle development. Developmental Biology 308:281–293.

Bittner, G.D., and D.L. Traut. 1978. Growth of crustacean muscles and muscle fibers. Journal of Comparative Physiology 124:277–285.

Blickhan, R., and R.J. Full. 1987. Locomotion energetics of the ghost crab. 2. Mechanics of the center of mass during walking and running. The Journal of Experimental Biology 130:155–174.

Blickhan, R., R.J. Full, and L. Ting. 1993. Exoskeletal strain: evidence for a trot-gallop transition in rapidly running ghost crabs. The Journal of Experimental Biology 179:301–321.

Boone, L.P., and G.D. Bittner. 1974. Morphological and physiological measures of trophic dependence in a crustacean muscle. Journal of Comparative Physiology 89:123–144.

Burrows, M., and G. Hoyle. 1973. Mechanism of rapid running in ghost crab *Ocypode ceratophthalma*. The Journal of Experimental Biology 58:327–349.

Chang, E.S. and D.L. Mykles. 2011. Regulation of crustacean molting: a review and our perspectives. General and Comparative Endocrinology 172:323–330.

Cho, I., J.A. Covi, B.D. Bader, and D.L. Mykles. 2009. Expression of a myostatin transcript in *Carcinus maenas*: response to ecdysteroid levels. Integrative and Comparative Biology 49:E212.

Conover, M.R., and D.E. Miller. 1978. Importance of the large chela in the territorial behavior and pairing behaviour of the snapping shrimp, *Alpheus heterochealis*. Marine Behavior and Physiology 5:185–192.

Cooper, R.L., W.M. Warren, and H.E. Ashby. 1998. Activity of phasic motor neurons partially transforms the neuronal and muscle phenotype to a tonic-like state. Muscle and Nerve 21:921–931.

Costello, W.J., and C.K. Govind. 1983. Contractile responses of single fibers in lobster claw closer muscles: correlation with structure, histochemistry, and innervation. Journal of Experimental Zoology 227:381–393.

Costello, W.J., and C.K. Govind. 1984. Contractile proteins of fast and slow fibers during differentiation of lobster claw muscle. Developmental Biology 104:434–440.

Costello, W.J., and F. Lang. 1979. Development of the dimorphic claw closer muscles of the lobster *Homarus americanus*. IV. Changes in functional morphology during growth. Biological Bulletin 156:179–195.

Costello, W.J., R. Hill, and F. Lang. 1981. Innervation patterns of fast and slow motor neurons during development of a lobster neuromuscular system. The Journal of Experimental Biology 91:271–284.

Couch, J. 1837. Observations on the process of exuviation in the common crab (*Cancer pagurus*, Linn.). Magazine of Zoology and Botany 1:341–344.

Couch, J. 1843. On the process of exuviation and growth in crabs and lobsters, and other British species of stalk-eyed crustacean animals. Annual Report of the Royal Cornwall Polytechnic Society 11:1–15.

Coughlin, D.J., S. Solomon, and J.L. Wilwert. 2007. Parvalbumin expression in trout swimming muscle correlates with relaxation rate. Comparative Biochemistry and Physiology 147A:1074–1082.

Covi, J.A., H.W. Kim, and D.L. Mykles. 2008. Expression of alternatively spliced transcripts for a myostatin-like protein in the blackback land crab, *Gecarcinus lateralis*. Comparative Biochemistry and Physiology 150A:423–430.

Covi, J.A., B.D. Bader, E.S. Chang, and D.L. Mykles. 2010. Molt cycle regulation of protein synthesis in skeletal muscle of the blackback land crab, *Gecarcinus lateralis*, and the differential expression of a myostatin-like factor during atrophy induced by molting or unweighting. The Journal of Experimental Biology 213:172–183.

Dahlmann, B., M. Rutschmann, and H. Reinauer. 1986. Effect of starvation or treatment with corticosterone on the amount of easily releaseable myofilaments in rat skeletal muscles. Biochemical Journal 234:659–664.

Darby, H.H. 1934. The mechanism of asymmetry in the Alpheidae. Publications from the Carnegie Institution of Washington, D.C. 435:347–361.

De Santis, C., N.M. Wade, D.R. Jerry, N.P. Preston, B.D. Glencross, and M.J. Sellars. 2011. Growing backwards: an inverted role for the shrimp ortholog of vertebrate myostatin and GDF11. The Journal of Experimental Biology 214:2671–2677.

El Haj, A.J. 1999. Regulation of muscle growth and sarcomeric protein gene expression over the intermolt cycle. American Zoologist 39:570–579.

El Haj, A.J., C.K. Govind, and D.F. Houlihan. 1984. Growth of lobster leg muscle fibers over intermolt and molt. Journal of Crustacean Biology 4:536–545.

El Haj, A.J., S.R. Clarke, P. Harrison, and E.S. Chang. 1996. *In vivo* muscle protein synthesis rates in the American lobster *Homarus americanus* during the moult cycle and in response to 20-hydroxyecdysone. The Journal of Experimental Biology 199:579–585.

Emmel, V.E. 1908. The experimental control of asymmetry at different stages in the development of the lobster *Homarus americanus*. Journal of Experimental Zoology 231:167–175.

Everest, F.A., R.W. Young, and M.W. Johnson. 1948. Acoustical characteristics of noise produced by snapping shrimp. Journal of the Acoustical Society of America 20:137–142.

Fahrenbach, W.H. 1963. Sarcoplasmic reticulum of striated muscle of a cyclopoid copepod. Journal of Cell Biology 17:629–640.

Fitzhugh, G.H., and J.H. Marden. 1997. Maturational changes in troponin T expression Ca^{2+}-sensitivity and twitch contraction kinetics in dragonfly flight muscle. The Journal of Experimental Biology 200:1473–1482.

Frost, R.A. and C.H. Lang. 2012. Multifaceted role of insulin-like growth factors and mammalian Target of Rapamycin in skeletal muscle. Endocrinology and Metabolism Clinics of North America 41:297–322.

Full, R.J. 1987. Locomotion energetics of the ghost crab. 1. Metabolic cost and endurance. The Journal of Experimental Biology 130:137–153.

Full, R.J. 1997. Invertebrate locomotor systems. Pages 853–930 *in* W. Dantzler, editor. Handbook of Physiology: Comparative Physiology. American Physiological Society, Bethesda, Maryland.

Full, R.J., and R.B. Weinstein. 1992. Integrating the physiology, mechanics and behavior of rapid running ghost crabs: slow and steady doesn't always win the race. American Zoologist 32:382–395.

Glass, D.J. 2010. Signaling pathways perturbing muscle mass. Current Opinion in Clinical Nutrition and Metabolic Care 13:225–229.

Goldspink, G. 1977. Mechanics and energetics of muscle in animals of different sizes, with particular reference to the muscle fibre composition of vertebrate muscle. Pages 37–55 *in* T.J. Pedley, editor. Scale Effects in Animal Locomotion. Academic Press, London.

Goodman, C.A., D.L. Mayhew, and T.A. Hornberger. 2011. Recent progress toward understanding the molecular mechanisms that regulate skeletal muscle mass. Cellular Signaling 23:1896–1906.

Gornik, S.G., G.D. Westrop, G.H. Coombs, and D.M. Neil. 2010. Molecular cloning and localization of a calpain-like protease from the abdominal muscle of Norway lobster *Nephrops norvegicus*. Molecular Biology Reports 37:2009–2019.

Götze, S., and R. Saborowski. 2011. Proteasomal activities in the claw muscle tissue of European lobster, *Homarus gammarus*, during larval development. Journal of Comparative Physiology 181:861–871.

Govind, C.K. 1984. Development of asymmetry in the neuromuscular system of lobster claws. Biological Bulletin 167:94–119.

Govind, C.K. 1992. Claw asymmetry in lobsters: case study in developmental neuroethology. Journal of Neurobiology 23:1423–1445.

Govind, C.K. 1995. Muscles and their innervation. Pages 291–312 *in* J.R. Factor, editor. Biology of the Lobster *Homarus americanus*, Academic Press, San Diego.

Govind, C.K., and J.A. Blundon. 1985. Form and function of the asymmetric chelae in blue crabs with normal and reversed handedness. Biological Bulletin 168:321–331.

Govind, C.K., and K.S. Kent. 1982. Transformation of fast fibres to slow prevented by lack of activity in developing lobster muscle. Nature 298:755–757.

Govind, C.K., and F. Lang. 1978. Development of the dimorphic claw closer muscles of the lobster *Homarus americanus*. III. Transformation to dimorphic muscles in juveniles. Biological Bulletin 154:55–67.

Govind, C.K., and F. Lang. 1979. Physiological asymmetry in the bilateral crusher claws of a lobster. Journal of Experimental Zoology 207:27–32.

Govind, C.K., and F. Lang. 1981. Physiological identification and asymmetry of lobster claw closer motoneurons. The Journal of Experimental Biology 94:329–339.

Govind, C.K., and J. Pearce. 1986. Differential reflex activity determines claw and closer muscle asymmetry in developing lobsters. Science 233:354–356.

Govind, C.K., and J. Pearce. 1989. Delayed determination of claw laterality in lobsters following loss of target. Development 107:547–551.

Govind, C.K., and J. Pearce. 1992. Mechanoreceptors and minimal reflex activity determining claw laterality in developing lobsters. The Journal of Experimental Biology 171:149–162.

Govind, C.K., and J. Pearce. 1994. Muscle remodeling in adult snapping shrimps via fast-fiber degeneration and slow-fiber genesis and transformation. Cell and Tissue Research 276:445–454.

Govind, C.K., H.L. Atwood, and F. Lang. 1974. Sarcomere length increases in developing crustacean muscle. Journal of Experimental Zoology 189:395–400.

Govind, C.K., J. She, and F. Lang. 1977. Lengthening of lobster muscle fibres by two age-dependent mechanisms. Experientia 33:35–36.

Govind, C.K., M.M. Quigley, and K.M. Mearow. 1986. The closer muscle in the dimorphic claws of male fiddler crabs. Biological Bulletin 170:481–493.
Govind, C.K., D. Mellon, Jr., and M.M. Quigley. 1987. Muscle and muscle fiber type transformation in clawed crustaceans. American Zoologist 27:1079–1098.
Govind, C.K., A. Wong, and J. Pearce. 1988. Experimental induction of claw transformation in snapping shrimps. Journal of Experimental Zoology 248:371–375.
Govind, C.K., C. Gee, and J. Pearce. 1991. Retarded and mosaic phenotype in regenerated claw closer muscles of juvenile lobsters. Biological Bulletin 180:28–33.
Greenaway, P. 2003. Terrestrial adaptations in the Anomura (Crustacea: Decapoda). Memoirs of Museum Victoria 60:13–26.
Griffis, B., S.B. Moffett, and R.L. Cooper. 2001. Muscle phenotype remains unaltered after limb autotomy and unloading. Journal of Experimental Zoology 289:10–22.
Gruhn, M., and W. Rathmayer. 2002. Phenotype plasticity in postural muscles of the crayfish *Orconectes limosus* Raf.: correlation of myofibrillar ATPase-based fiber typing with electrophysiological fiber properties and the effect of chronic nerve stimulation. Journal of Experimental Zoology 293:127–140.
Hafemann, D.R., and J.I. Hubbard. 1969. On rapid running of ghost crabs (*Ocypode ceratophthalma*). Journal of Experimental Zoology 170:25–32.
Hardy, K.M., R.M. Dillaman, B.R. Locke, and S.T. Kinsey. 2009. A skeletal muscle model of extreme hypertrophic growth reveals the influence of diffusion on cellular design. American Journal of Physiology 296:R1855–R1867.
Hardy, K.M., S.C. Lema, and S.T. Kinsey. 2010. The metabolic demands of swimming behavior influence the evolution of skeletal muscle fiber design in the brachyuran crab family Portunidae. Marine Biology 157:221–236.
Harzsch, S., and S. Kreissl. 2010. Myogenesis in the thoracic limbs of the American lobster. Arthropod Structure and Development 39:423–435.
Heglund, N.C., C.R. Taylor, and T.A. McMahon. 1974. Scaling stride frequency and gait to animal size—mice to horses. Science 186:1112–1113.
Herberholz, J., and B. Schmitz. 1998. Role of mechanosensory stimuli in intraspecific agonistic encounters of the snapping shrimp (*Alpheus heterochaelis*). Biological Bulletin 195:156–167.
Herreid, C.F., and R.J. Full. 1986a. Energetics of hermit crabs during locomotion: the cost of carrying a shell. The Journal of Experimental Biology 120:297–308.
Herreid, C.F., and R.J. Full. 1986b. Locomotion of hermit crabs (*Coenobita compressa*) on beach and treadmill. The Journal of Experimental Biology 120:283–296.
Herrick, F.H. 1895. The American lobster. A study of its habits and development. Bulletin of the United States Fisheries Commission 15:1–252.
Hertzler, P.L., and W.R. Freas. 2009. Pleonal muscle development in the shrimp *Penaeus* (*Litopenaeus*) *vannamei* (Crustacea: Malacostraca: Decapoda: Dendrobranchiata). Arthropod Structure and Development 38:235–246.
Hill, A.V. 1950. The dimensions of animals and their muscular dynamics. Science Progress 38:209–230.
Ho, R.K., E.E. Ball, and C.S. Goodman. 1983. Muscle pioneers: large mesodermal cells that erect a scaffold for developing muscles and motoneurons in grasshopper embryos. Nature 301:66–69.
Holmes, J.M., N.M. Whiteley, J.L. Magnay, and A.J. El Haj. 2002. Comparison of the variable loop regions of myosin heavy chain genes from Antarctic and temperate isopods. Comparative Biochemistry and Physiology 131B:349–359.
Hopkins, P.M. 2001. Limb regeneration in the fiddler crab, *Uca pugilator*: hormonal and growth factor control. American Zoologist 41:389–398.
Hoppeler, H., and M. Fluck. 2002. Normal mammalian skeletal muscle and its phenotypic plasticity. The Journal of Experimental Biology 205:2143–2152.
Ismail, S.Z.M., and D.L. Mykles. 1992. Differential molt-induced atrophy in the dimorphic claws of male fiddler crabs, *Uca pugnax*. Journal of Experimental Zoology 263:18–31.
James, R.S., N.J. Cole, M.L.F. Davies, and I.A. Johnston. 1998. Scaling of intrinsic contractile properties and myofibrillar protein composition of fast muscle in the fish *Myoxocephalus scorpius* L. The Journal of Experimental Biology 201:901–912.

Jimenez, A.G., S.T. Kinsey., R.M. Dillaman, and D.F. Kapraun. 2010. Nuclear DNA content variation associated with muscle fiber hypertrophic growth in decapod crustaceans. Genome 53:161–171.

Jirikowski, G., S. Kreissl, S. Richter, and C. Wolff. 2010. Muscle development in the marbled crayfish-insights from an emerging model organism (Crustacea, Malacostraca, Decapoda). Development Genes and Evolution 220:89–105.

Johnson, M.W., F.A. Everest, and R.W. Young. 1947. The role of snapping shrimp (*Crangon* and *Synalpheus*) in the production of underwater noise in the sea. Biological Bulletin 93:122–138.

Josephson, R.K., and D. Young. 1987. Fiber ultrastructure and contraction kinetics in insect fast muscles. American Zoologist 27:991–1000.

Kaiser, A., C.J. Klok, J.J. Socha, W.K. Lee, M.C. Quinlan, and J.F. Harrison. 2007. Increase in tracheal investment with beetle size supports hypothesis of oxygen limitation on insect gigantism. Proceedings of the National Academy of Sciences USA 104:13198–13203.

Kim, B.K., K.S. Kim, C.W. Oh, D.L. Mykles., S.G. Lee, H.J. Kim, and H.W. Kim. 2009a. Twelve actin-encoding cDNAs from the American lobster, *Homarus americanus*: cloning and tissue expression of eight skeletal muscle, one heart, and three cytoplasmic isoforms. Comparative Biochemistry and Physiology 153B:178–184.

Kim, H.W., E.S. Chang, and D.L. Mykles. 2005a. Three calpains and ecdysone receptor in the land crab, *Gecarcinus lateralis*: sequences, expression, and effects of elevated ecdysteroid induced by eyestalk ablation. The Journal of Experimental Biology 208:3177–3197.

Kim, H.W., S.G. Lee, and D.L. Mykles. 2005b. Ecdysteroid-responsive genes, RXR and E75, in the tropical land crab, *Gecarcinus lateralis*: differential tissue expression of multiple RXR isoforms generated at three alternative splicing sites in the hinge and ligand-binding domains. Molecular and Cellular Endocrinology 242:80–95.

Kim, K.S., J.M. Jeon, and H.W. Kim. 2009b. A myostatin-like gene expressed highly in the muscle tissue of Chinese mitten crab, *Eriocheir sinensis*. Fisheries and Aquatic Sciences 12:185–193.

Kim, K.S., Y.J. Kim, J.M. Jeon, Y.S. Kang, C.W. Oh, and H.W. Kim. 2010. Molecular characterization of myostatin-like genes expressed highly in the muscle tissue from Morotoge shrimp, *Pandalopsis japonica*. Aquaculture Research 41:e862–e871.

Kinsey, S.T., P. Pathi, K.M. Hardy, A. Jordan, and B.R. Locke. 2005. Does intracellular metabolite diffusion limit post-contractile recovery in burst locomotor muscle? The Journal of Experimental Biology 208:2641–2652.

Kinsey, S.T., K.M. Hardy, and B.R. Locke. 2007. The long and winding road: influences of intracellular metabolite diffusion on cellular organization and metabolism in skeletal muscle. The Journal of Experimental Biology 210:3505–3512.

Kinsey, S.T., B.R. Locke, and R.M. Dillaman. 2011. Molecules in motion: influences of diffusion on metabolic structure and function in skeletal muscle. The Journal of Experimental Biology 214:263–274.

Kirk, M.D., and C.K. Govind. 1992. Early innervation of abdominal swimmeret muscles in developing lobsters. Journal of Experimental Zoology 261:298–309.

Knowlton, N., and B.D. Keller. 1982. Symmetric fights as a measure of escalation potential in a symbiotic, territorial snapping shrimp. Behavioral Ecology and Sociobiology 10:289–292.

Knowlton, R.E., and J.M. Moulton. 1963. Sound production in snapping shrimps *Alpheus* (*Crangon*) and *Synalpheus*. Biological Bulletin 125:311–331.

Koenders, A., T.M. Lamey, S. Medler, J.M. West, and D.L. Mykles. 2004. Two fast-type fibers in claw closer and abdominal deep muscles of the Australian freshwater crustacean, *Cherax destructor*, differ in Ca^{2+} sensitivity and troponin-I isoforms. Journal of Experimental Zoology 301A:588–598.

Koenders, A., X.L. Yu, E.S. Chang, and D.L. Mykles. 2002. Ubiquitin and actin expression in claw muscles of land crab, *Gecarcinus lateralis*, and American lobster, *Homarus americanus*: differential expression of ubiquitin in two slow muscle fiber types during molt-induced atrophy. Journal of Experimental Zoology 292:618–632.

Kreissl, S., A. Uber, and S. Harzsch. 2008. Muscle precursor cells in the developing limbs of two isopods (Crustacea, Peracarida): an immunohistochemical study using a novel monoclonal antibody against myosin heavy chain. Development Genes and Evolution 218:253–265.

Lagersson, N.C. 2002. The ultrastructure of two types of muscle fibre cells in the cyprid of *Balanus amphitrite* (Crustacea: Cirripedia). Journal of the Marine Biological Association of the U.K. 82:573–578.

Lang, F. 1977. Synaptic and septate neuromuscular junctions in embryonic lobster muscle. Nature 268: 458–460.

Lang, F., C.K. Govind, and J. She. 1977. Development of dimorphic claw closer muscles of lobster, *Homarus americanus*: II. Distribution of muscle fiber types in larval forms. Biological Bulletin 152: 382–391.

Lang, F., C.K. Govind, and W.J. Costello. 1978. Experimental transformation of muscle fiber properties in lobster. Science 201: 1037–1039.

Lang, F., M.M. Ogonowski, W.J. Costello, R. Hill, B. Roehrig, and K. Kent. 1980. Neurotrophic influence on lobster skeletal muscle. Science 207: 325–327.

Laplante, M., and D.M. Sabatini. 2012. mTOR signaling in growth control and disease. Cell 149: 274–293.

Liu, J.X., A.S. Hoglund, P. Karlsson, J. Lindblad, R. Qaisar, S. Aare, E. Bengtsson, and L. Larsson. 2009. Myonuclear domain size and myosin isoform expression in muscle fibres from mammals representing a 100 000-fold difference in body size. Experimental Physiology 94:117–129.

Lnenicka, G.A., J.A. Blundon, and C.K. Govind. 1988. Early experience influences the development of bilateral asymmetry in a lobster motoneuron. Developmental Biology 129:84–90.

MacLea, K.S., J.A. Covi, H.W. Kim, E. Chao, S. Medler, E.S. Chang, and D.L. Mykles. 2010. Myostatin from the American lobster, *Homarus americanus*: cloning and effects of molting on expression in skeletal muscles. Comparative Biochemistry and Physiology 157A:328–337.

MacLea, K.S., A.M. Abuhagr, N.L. Pitts, J.A. Covi, B.D. Bader, E.S. Chang, and D.L. Mykles. 2012. Rheb, an activator of target of rapamycin, in the blackback land crab, *Gecarcinus lateralis*: cloning and effects of molting and unweighting on expression in skeletal muscle. The Journal of Experimental Biology 215:590–604.

Magnay, J.L., J.M. Holmes, D.M. Neil, and A.J. El Haj. 2003. Temperature-dependent developmental variation in lobster muscle myosin heavy chain isoforms. Gene 316:119–126.

Marden, J.H., G.H. Fitzhugh, M.R. Wolf, K.D. Arnold, and B. Rowan. 1999. Alternative splicing, muscle calcium sensitivity, and the modulation of dragonfly flight performance. Proceedings of the National Academy of Sciences USA 96:15304–15309.

Marden, J.H., G.H. Fitzhugh, M. Girgenrath, M.R. Wolf, and S. Girgenrath. 2001. Alternative splicing, muscle contraction and intraspecific variation: associations between troponin T transcripts, Ca^{2+} sensitivity and the force and power output of dragonfly flight muscles during oscillatory contraction. Journal of Experimental Biology 204:3457–3470.

Mariappan, P., C. Balasundaram, and B. Schmitz. 2000. Decapod crustacean chelipeds: an overview. Journal of Bioscience 25:301–313.

Marx, J.O., M.C. Olsson, and L. Larsson. 2006. Scaling of skeletal muscle shortening velocity in mammals representing a 100,000-fold difference in body size. Pflugers Archiv-European Journal of Physiology 452:222–230.

Mattson, J.M., and D.L. Mykles. 1993. Differential degradation of myofibrillar proteins by four calcium-dependent proteinase activities from lobster muscle. Journal of Experimental Zoology 265:97–106.

McCarthy, J.J., and K.A. Esser. 2010. Anabolic and catabolic pathways regulating skeletal muscle mass. Current Opinion in Clinical Nutrition and Metabolic Care 13:230–235.

McMahon, T.A. 1975. Using body size to understand structural design of animals—quadrupedal locomotion. Journal of Applied Physiology 39:619–627.

Mearow, K.M., and C.K. Govind. 1986. Selective degeneration of fast muscle during claw transformation in snapping shrimps. Developmental Biology 118:314–318.

Medler, S. 2002. Comparative trends in shortening velocity and force production in skeletal muscles. American Journal of Physiology 283:R368–R378.

Medler, S., and K. Hulme. 2009. Frequency-dependent power output and skeletal muscle design. Comparative Biochemistry and Physiology 152A:407–417.

Medler, S., and D.L. Mykles. 2003. Analysis of myofibrillar proteins and transcripts in adult skeletal muscles of the American lobster *Homarus americanus*: variable expression of myosins, actin and troponins in fast, slow-twitch and slow-tonic fibres. The Journal of Experimental Biology 206:3557–3567.

Medler, S., T. Lilley, and D.L. Mykles. 2004. Fiber polymorphism in skeletal muscles of the American lobster, *Homarus americanus*: continuum between slow-twitch (S_1) and slow-tonic (S_2) fibers. The Journal of Experimental Biology 207:2755–2767.

Medler, S., K.J. Brown, E.S. Chang, and D.L. Mykles. 2005. Eyestalk ablation has little effect on actin and myosin heavy chain gene expression in adult lobster skeletal muscles. Biological Bulletin 208:127–137.

Medler, S., T.R. Lilley, J.H. Riehl, E.P. Mulder, E.S. Chang, and D.L. Mykles. 2007. Myofibrillar gene expression in differentiating lobster claw muscles. Journal of Experimental Zoology 307A:281–295.

Mellon, D., Jr. 1981. Nerves and the transformation of claw type in snapping shrimps. Trends in Neuroscience 4:245–248.

Mellon, D., Jr. 1999. Muscle restructuring in crustaceans: myofiber death, transfiguration and rebirth. American Zoologist 39:527–540.

Mellon, D., Jr., and E. Greer. 1987. Induction of precocious molting and claw transformation in alpheid shrimps by exogenous 20-hydoxyecdysone. Biological Bulletin 172:350–356.

Mellon, D., Jr., and M.M. Quigley. 1988. Disruption of muscle reorganization by lesions of the peripheral nerve in transforming claws of snapping shrimps. Journal of Neurobiology 19:532–551.

Mellon, D., Jr., and P.J. Stephens. 1978. Limb morphology and function are transformed by contralateral nerve section in snapping shrimps. Nature 272:246–248.

Mellon, D., Jr., and P.J. Stephens. 1979. The motor organization of claw closer muscles in snapping shrimp. Journal of Comparative Physiology 132:109–115.

Mellon, D., Jr., and P.J. Stephens. 1980. Modifications in the arrangement of thick and thin filaments in transforming shrimp muscle. Journal of Experimental Zoology 213:173–179.

Mellon, D., Jr., J.A. Wilson, and C.E. Phillips. 1981. Modification of motor neuron size and position in the central nervous system of adult snapping shrimps. Brain Research Bulletin 223:134–140.

Moffett, S. 1987. Muscles proximal to the fracture plane atrophy after limb autotomy in decapod crustaceans. Journal of Experimental Zoology 244:485–490.

Morris, S. 2002. The ecophysiology of air-breathing in crabs with special reference to *Gecarcoidea natalis*. Comparative Biochemistry and Physiology 131B:559–570.

Mykles, D.L. 1980. The mechanism of fluid absorption at ecdysis in the American lobster, *Homarus americanus*. The Journal of Experimental Biology 84:89–101.

Mykles, D.L. 1985a. Heterogeneity of myofibrillar proteins in lobster fast and slow muscles: variants of troponin, paramyosin, and myosin light chains comprise four distinct protein assemblages. Journal of Experimental Zoology 234:23–32.

Mykles, D.L. 1985b. Multiple variants of myofibrillar proteins in single fibers of lobster claw muscles: evidence for two types of slow fibers in the cutter closer muscle. Biological Bulletin 169:476–483.

Mykles, D.L. 1988. Histochemical and biochemical characterization of two slow fiber types in decapod crustacean muscles. Journal of Experimental Zoology 245:232–243.

Mykles, D.L. 1989. High-molecular-weight serine proteinase from lobster muscle that degrades myofibrillar proteins. Journal of Experimental Zoology 250:244–252.

Mykles, D.L. 1990. Calcium-dependent proteolysis in crustacean claw closer muscle maintained *in vitro*. Journal of Experimental Zoology 256:16–30.

Mykles, D.L. 1992. Getting out of a tight squeeze—Enzymatic regulation of claw muscle atrophy in molting. American Zoologist 32:485–494.

Mykles, D.L. 1993. Lobster muscle proteasome and the degradation of myofibrillar proteins. Enzyme and Protein 47:220–231.

Mykles, D.L. 1997a. Biochemical properties of insect and crustacean proteasomes. Molecular Biology Reports 24:133–138.

Mykles, D.L. 1997b. Crustacean muscle plasticity: molecular mechanisms determining mass and contractile properties. Comparative Biochemistry and Physiology 117B:367–378.

Mykles, D.L. 1998. Intracellular proteinases of invertebrates: calcium-dependent and proteasome/ubiquitin-dependent systems. International Review of Cytology 184:157–289.

Mykles, D.L. 1999a. Proteolytic processes underlying molt-induced claw muscle atrophy in decapod crustaceans. American Zoologist 39:541–551.

Mykles, D.L. 1999b. Structure and functions of arthropod proteasomes. Molecular Biology Reports 26:103–111.

Mykles, D.L. 2000. Purification and characterization of crustacean calpain-like proteinases. Pages 55–66 *in* J.S. Elce, editor. Calpain Methods and Protocols, Human Press, Totowa.

Mykles, D.L. 2001. Interactions between limb regeneration and molting in decapod crustaceans. American Zoologist 41:399–406.

Mykles, D.L. 2011. Ecdysteroid metabolism in crustaceans. Journal of Steroid Biochemistry and Molecular Biology 127:196–203.

Mykles, D.L., and M.F. Haire. 1991. Sodium dodecyl sulfate and heat induce two distinct forms of lobster muscle multicatalytic proteinase: the heat-activated form degrades myofibrillar proteins. Archives of Biochemistry and Biophysics 288:543–551.

Mykles, D.L., and M.F. Haire. 1995. Branched-chain-amino-acid-preferring peptidase activity of the lobster multicatalytic proteinase (proteasome) and the degradation of myofibrillar proteins. Biochemical Journal 306:285–291.

Mykles, D.L., and D.M. Skinner. 1981. Preferential loss of thin filaments during molt-induced atrophy in crab claw muscle. Journal of Ultrastructure Research 75:314–325.

Mykles, D.L., and D.M. Skinner. 1982a. Crustacean muscles: atrophy and regeneration during molting. Pages 337–357 *in* B.M. Twarog, R.J.C. Levine, and M.M. Dewey, editors. Basic Biology of Muscles: A Comparative Approach, Raven Press, New York.

Mykles, D.L., and D.M. Skinner. 1982b. Molt cycle-associated changes in calcium-dependent proteinase activity that degrades actin and myosin in crustacean muscle. Developmental Biology 92:386–397.

Mykles, D.L., and D.M. Skinner. 1983. Ca^{2+}-dependent proteolytic activity in crab claw muscle. Effects of inhibitors and specificity for myofibrillar proteins. Journal of Biological Chemistry 258:10474–10480.

Mykles, D.L., and D.M. Skinner. 1985a. Muscle atrophy and restoration during molting. Pages 31–46 *in* A.M. Wenner, editor. Crustacean Issues 3. Factors in Adult Growth, A.A. Balkema, Rotterdam.

Mykles, D.L., and D.M. Skinner. 1985b. The role of calcium-dependent proteinase in molt-induced claw muscle atrophy. Pages 141–150 *in* E.A. Khairallah, J.S. Bond, and J.W.C. Bird, editors. Intracellular Protein Catabolism, Alan R. Liss, New York.

Mykles, D.L., and D.M. Skinner. 1986. Four Ca^{2+}-dependent proteinase activities isolated from crustacean muscle differ in size, net charge, and sensitivity to Ca^{2+} and inhibitors. Journal of Biological Chemistry 261:9865–9871.

Mykles, D.L., and D.M. Skinner. 1990a. Atrophy of crustacean somatic muscle and the proteinases that do the job—a review. Journal of Crustacean Biology 10:577–594.

Mykles, D.L., and D.M. Skinner. 1990b. Calcium-dependent proteinases in crustaceans. Pages 139–154 *in* R.L. Mellgren, and T. Murachi, editors. Intracellular Calcium-Dependent Proteolysis, CRC Press, Boca Raton.

Mykles, D.L., S. Medler, A. Koenders, and R. Cooper. 2002. Myofibrillar protein isoform expression is correlated with synaptic efficacy in slow fibres of the claw and leg opener muscles of crayfish and lobster. The Journal of Experimental Biology 205:513–522.

Novotová, M., and B. Uhrík. 1992. Structural characteristics and distribution of satellite cells along crayfish muscle fibers. Experientia 48:593–596.

O'Connor, K., P.J. Stephens, and J.M. Leferovich. 1982. Regional distribution of muscle fiber types in the asymmetric claws of Californian snapping shrimp. Biological Bulletin 163:329–336.

Ogonowski, M.M., F. Lang, and C.K. Govind. 1980. Histochemistry of lobster claw-closer muscles during development. Journal of Experimental Zoology 213:359–367.

Pearce, J., and C.K. Govind. 1987. Spontaneous generation of bilateral symmetry in the paired claws and closer muscles of adult snapping shrimps. Development 100:57–63.

Pellegrino, M.A., M. Canepari, R. Rossi, G. D'Antona, C. Reggiani, and R. Bottinelli. 2003. Orthologous myosin isoforms and scaling of shortening velocity with body size in mouse, rat, rabbit and human muscles. Journal of Physiology London 546:677–689.

Perry, M.J., J. Tait, J. Hu, S.C. White, and S. Medler. 2009. Skeletal muscle fiber types in the ghost crab, *Ocypode quadrata*: implications for running performance. Journal of Experimental Biology 212:673–683.

Postel, U., F. Thompson, G. Barker, M. Viney, and S. Morris. 2010. Migration-related changes in gene expression in leg muscle of the Christmas Island red crab *Gecarcoidea natalis*: seasonal preparation for long-distance walking. The Journal of Experimental Biology 213:1740–1750.

Potter, J.R., and M. Chitre. 1999. Ambient noise imaging in warm shallow seas; second-order moment and model-based imaging algorithms. Journal of the Acoustical Society of America 106:3201–3210.

Qian, Z.Y., X. Mi, X.Z. Wang, S.L. He, Y.J. Liu, F.J. Hou, Q. Liu, and X.L. Liu. 2013. cDNA cloning and expression analysis of myostatin/GDF11 in shrimp, *Litopenaeus vannamei*. Comparative Biochemistry and Physiology 165A:30–39.

Quigley, M.M., and D. Mellon, Jr. 1984. Changes in myofibrillar gene expression during fiber-type transformation in the claw closer muscles of the snapping shrimp, *Alpheus heterochelis* Developmental Biology 106:262–265.

Quigley, M.M., and D. Mellon, Jr. 1986. Myofiber death plays a role in determining fiber type composition in the claw closer muscles of the snapping shrimp, *Alpheus heterochelis*. Journal of Experimental Zoology 239:299–305.

Read, A.T., and C.K. Govind. 1997a. Claw transformation and regeneration in adult snapping shrimp: test of the inhibition hypothesis for maintaining bilateral asymmetry. Biological Bulletin 193:401–409.

Read, A.T., and C.K. Govind. 1997b. Regeneration and sex-biased transformation of the sexually dimorphic pincer claw in adult snapping shrimps. Journal of Experimental Zoology 279:356–366.

Reggiani, C., R. Bottinelli, and G.J.M. Stienen. 2000. Sarcomeric myosin isoforms: fine tuning of a molecular motor. News in Physiological Science 15:26–33.

Ritzmann, R. 1973. Snapping behavior of shrimp *Alpheus californiensis*. Science 181:459–460.

Rock, J., J.L. Magnay, S. Beech, A.J. El Haj, G. Goldspink, D.H. Lunt, and N.M. Whiteley. 2009. Linking functional molecular variation with environmental gradients: myosin gene diversity in a crustacean broadly distributed across variable thermal environments. Gene 437:60–70.

Rome, L.C. 2006. Design and function of superfast muscles: new insights into the physiology of skeletal muscle. Annual Review of Physiology 68:193–221.

Rome, L.C., and S.L. Lindstedt. 1997. Mechanical and metabolic design of the muscular system in vertebrates. Pages 1587–1651 *in* W. Dantzler, editor. Handbook of Physiology: Comparative Physiology. American Physiological Society, Bethesda, Maryland.

Russell, B., D. Motlagh, and W.W. Ashley. 2000. Form follows function: how muscle shape is regulated by work. Journal of Applied Physiology 88: 1127–1132.

Salter, S.J.A. 1860. On the moulting of the common lobster (*Homarus vulgaris*) and the shore crab (*Carcinus maenas*). Journal of the Proceedings of the Linnean Society of London 4:30–35.

Schakman, O., H. Gilson, S. Kalista, and J.P. Thissen. 2009. Mechanisms of muscle atrophy induced by glucocorticoids. Hormone Research 72:36–41.

Schejter, E.D., and M.K. Baylies. 2010. Born to run: creating the muscle fiber. Current Opinion in Cell Biology 22:566–574.

Schmidt-Nielsen, K. 1984. Scaling: why is animal size so important? Cambridge University Press, Cambridge.

Schmiege, D.L., R.L. Ridgway, and S.B. Moffett. 1992. Ultrastructure of autotomy-induced atrophy of muscles in the crab *Carcinus maenas*. Canadian Journal of Zoology 70:841–851.

Schwartz, L.M. 2008. Atrophy and programmed cell death of skeletal muscle. Cell Death and Differentiation 15:1163–1169.

Seow, C.Y., and L.E. Ford. 1991. Shortening velocity and power output of skinned muscle fibers from mammals having a 25,000-fold range of body mass. Journal of General Physiology 97:541–560.

Shean, B.S., and D.L. Mykles. 1995. Polyubiquitin in crustacean striated muscle: increased expression and conjugation during molt-induced claw muscle atrophy. Biochimica et Biophysica Acta 1264:312–322.

Simonson, J.L. 1985. Reversal of handedness, growth, and claw stridulatory patterns in the stone crab *Menippe mercenaria* (Say) (Crustacea: Xanthidae). Journal of Crustacean Biology 5:281–293.

Skinner, D.M. 1965. Amino acid incorporation into protein during the molt cycle of the land crab, *Gecarcinus lateralis*. Journal of Experimental Zoology 160:225–234.

Skinner, D.M. 1966. Breakdown and reformation of somatic muscle during the molt cycle of the land crab, *Gecarcinus lateralis*. Journal of Experimental Zoology 163:115–124.

Skinner, D.M. 1968. Isolation and characterization of ribosomal ribonucleic acid from crustacean, *Gecarcinus lateralis*. Journal of Experimental Zoology 169:347–356.

Stanley, J.A., C.A. Radford, and A.G. Jeffs. 2010. Induction of settlement in crab megalopae by ambient underwater reef sound. Behavioral Ecology 21:113–120.

Stephens, P.J., and D. Mellon, Jr. 1979. Modification of structure and synaptic physiology in transformed shrimp muscle. Journal of Comparative Physiology 132:97–108.

Stephens, P.J., K. O'Connor, and J.M. Leferovich. 1983. Neuromuscular relationships in the asymmetric claws of California snapping shrimp. Journal of Experimental Zoology 225:53–61.

Stephens, P.J., L.M. Lofton, and P. Klainer. 1984. The dimorphic claws of the hermit crab, *Pagurus pollicaris*: properties of the closer muscle. Biological Bulletin 167:713–721.

Stokes, D.R., and R.K. Josephson. 1992. Structural organization of two fast, rhythmically active crustacean muscles. Cell and Tissue Research 267:571–582.

Taylor, J.R.A., and W.M. Kier. 2006. A pneumo-hydrostatic skeleton in land crabs—A sophisticated dual support system enables a crab to stay mobile immediately after moulting. Nature 440:1005.

Thys, T.M., J.M. Blank, and F.H. Schachat. 1998. Rostral-caudal variation in troponin T and parvalbumin correlates with differences in relaxation rates of cod axial muscle. Journal of Experimental Biology 201:2993–3001.

Thys, T.M., J.M. Blank, D.J. Coughlin, and F.H. Schachat. 2001. Longitudinal variation in muscle protein expression and contraction kinetics of largemouth bass axial muscle. Journal of Experimental Biology 204:4249–4257.

Velez, S.J., G.D. Bittner, H.L. Atwood, and C.K. Govind. 1981. Trophic reactions of crayfish muscle fibers and neuromuscular synapses after denervation, tenotomy, and immobilization. Experimental Neurology 71:307–325.

Vermeij, M.J.A., K.L Marhaver, C.M. Huijbers, I. Nagelkerken, and S.D. Simpson. 2010. Coral larvae move toward reef sounds. Plos One 5.

Versluis, M., B. Schmitz, A. von der Heydt, and D. Lohse. 2000. How snapping shrimp snap: through cavitating bubbles. Science 289:2114–2117.

Watabe, S. 2002. Temperature plasticity of contractile proteins in fish muscle. Journal of Experimental Biology 205:2231–2236.

West, J.M. 1997. Ultrastructural and contractile activation properties of crustacean muscle fibres over the moult cycle. Comparative Biochemistry and Physiology 117B:333–345.

West, J.M. 1999. Ca^{2+}-activated force production and calcium handling by the sarcoplasmic reticulum of crustacean muscles during molt-induced atrophy. American Zoologist 39:552–569.

West, J.M., D.C. Humphris, and D.G. Stephenson. 1995. Characterization of ultrastructural and contractile activation properties of crustacean (*Cherax destructor*) muscle fibres during claw regeneration and moulting. Journal of Muscle Research and Cell Motility 16:267–284.

Whiteley, N.M., and A.J. El Haj. 1997. Regulation of muscle gene expression over the moult in Crustacea. Comparative Biochemistry and Physiology 117B:323–331.

Whiteley, N.M., J.L. Magnay, S.J. McCleary, S.K. Nia, A.J. El Haj, and J. Rock. 2010. Characterisation of myosin heavy chain gene variants in the fast and slow muscle fibres of gammarid amphipods. Comparative Biochemistry and Physiology 157A:116–122.

Wigmore, P.M., and D.J.R. Evans. 2002. Molecular and cellular mechanisms involved in the generation of fiber diversity during myogenesis. International Review of Cytology 216:175–232.

Wilson, E.B. 1903. Notes on the reversal of asymmetry in the regeneration of the chelae in *Alpheus heterochelis*. Biological Bulletin 4:197–210.

Young, R.E., J. Pearce, and C.K. Govind. 1994. Establishment and maintenance of claw bilateral asymmetry in snapping shrimps. Journal of Experimental Zoology 269:319–326.

Young, R.E., A. Wong, J. Pearce, and C.K. Govind. 1996. Neural factors influence the degeneration of muscle fibers in the chelae of snapping shrimps. Molecular and Chemical Neuropathology 28:295–300.

Yu, X.L., and D.L. Mykles. 2003. Cloning of a muscle-specific calpain from the American lobster (*Homarus americanus*): expression associated with muscle atrophy and restoration during moulting. The Journal of Experimental Biology 206:561–575.

Zammit, P.S., T.A. Partridge, and Z. Yablonka-Reuveni. 2006. The skeletal muscle satellite cell: the stem cell that came in from the cold. Journal of Histochemistry and Cytochemistry 54:1177–1191.

6

REGENERATION IN CRUSTACEANS

Penny M. Hopkins and Sunetra Das

Abstract

The ability to regenerate missing appendages is highly developed in many crustaceans. Regeneration is often coupled with the ability to lose a limb at a preformed fracture/autotomy plane that is part of an adaptive response to predation. Regeneration of a lost limb commences with wound healing and blastema formation followed by development of a papilla/basal bud. Regeneration is controlled to some extent by hormones: receptors for ecdysteroids are present in the limb bud tissues at all stages of regeneration. Regeneration itself has profound effects on the molt cycle, and the presence of regenerating limb buds can affect the entire cycle. Genes associated with regeneration in insect legs and imaginal discs have been identified and may play a role in crustacean limb regeneration as well.

INTRODUCTION

Regeneration is the ability to regrow lost or damaged body parts. Frequently, regeneration consists simply of the replacement of lost tissues by mitotic events. This basic regeneration is called *compensatory hyperplasia* and it follows injury in many tissues (e.g., the mammalian liver). A second more complex type of regeneration—called *epimorphic regeneration*—occurs in many organisms. An animal that is damaged by prey or in a conspecific fight can avoid a potentially lethal event by surrendering part of its body—usually a limb—followed by regeneration of the lost component.

The loss of a limb—especially if it is a locomotor appendage—can present serious hardships because such appendages are important in foraging, mating, and predator evasion. Moreover, if a lost limb contains specialized sensory structures, there can also be a reduction in sensory input. Epimorphic regeneration of lost appendages is also metabolically costly to the organism.

Regeneration of lost tissues requires reallocation of body resources, which places the organism at a disadvantage in terms of metabolic reserves necessary for routine growth and reproduction. Regenerated structures may also be smaller or less ornamented than the lost structure, which in turn can interfere with social and food-seeking activities (Hopkins 1985, Uetz et al. 1996).

Evolution seems to have favored a higher degree of specialized function over regenerative capacity. Thus, regeneration in its most spectacular manifestations is found more often in the "less derived" more "basal" animals. In many of these animals, regeneration involves the formation of a blastema with some degree of tissue redifferentiation linked to tissue hyperplasia. Epimorphic regeneration occurs to variable degrees in vertebrates such as bony fish, amphibians, and reptiles but is seen most dramatically in invertebrates such as annelids, echinoderms, mollusks, and arthropods (Maginnis 2006).

A group of invertebrates in which epimorphic regeneration has been widely studied is the phylum Arthropoda. Because overall body growth in the arthropods is episodic (due to the rigid exoskeleton), arthropod regeneration is substantially different from regeneration in other groups of animals. Among the arthropods, regeneration has been studied most widely in the insects and crustaceans and has captured the interest and imagination of many scientists, beginning with Aristotle and including such luminaries as Charles Darwin, H. Przibram, T.H. Morgan, and T.H. Huxley.

In both insects and crustaceans, molting and growth are controlled by a number of chemical factors. A complement of neurosecretory and steroid hormones coordinate molting and growth (as well as reproduction) in these arthropods. The arthropod steroid hormones are called *ecdysteroids* and are found almost exclusively in arthropods. These multifunctional steroid hormones are able to stimulate the physiological events leading to the shedding of the exoskeleton and thereby they stimulate growth as well as reproductive development, especially in female arthropods. Moreover, the same steroid hormones are also involved in the control of regeneration in both insects and crustaceans (Bodenstein 1955, Halme et al. 2010).

This review looks at the temporal organization of crustacean regeneration from autotomy to wound healing, to blastema formation, and finally to hypertrophic growth. It also addresses the roles of blood-borne factors in controlling regeneration. We do not have a complete genome for any of the malacostracan crustaceans from whom most of the regeneration data have been accumulated, so we know very little about the genes that are controlled by the factors that control regeneration. We do, however, have the complete genome for some insects. We will, therefore, look at a few specific genes involved in insect limb development and regeneration with the hope that they will help us gain insight into the control of crustacean limb regeneration.

REGENERATION IN THE PHYLUM ARTHROPODA

Regeneration in the two major groups of arthropods, the insects and the crustaceans, is somewhat different since many adult insects do not molt, and regeneration in arthropods is linked, in part, to the molt cycle. Nevertheless, within the insects, at least 38 genera and 43 species have been shown to undergo some degree of regeneration. Regeneration occurs in at least three of the six classes of Crustacea, with at least 35 crustacean genera (and 45 species) able to regenerate limbs (Maginnis 2006).

Holo- and hemimetabolous insects have life cycle stages that include egg, immature stages (such as larva or nymphs), pupal, and adult stages. These insects molt and grow only during the immature stages. After these insects metamorphose into adults, they stop growing in order to put their metabolic energies into reproductive activity and many die soon after reproducing. Regeneration in these insects is limited to those times during which molting and growth occur (i.e., larval or nymphal stages). On the other hand, the less derived apterygote insect orders (Archaeognatha and

Thysanura) do not go through metamorphosis. These insects molt, grow, and regenerate throughout their entire life cycle (Bodenstein 1955).

Regeneration of limbs in the less derived insects such as cockroaches, crickets, and beetles does occur, but, for the most part, in these insects only immature tissues regenerate. Regeneration of nymph legs, antennae, and cerci has been studied in six different species of cockroaches (order Blattodea). Regeneration of limbs has also been studied in crickets, walking sticks, and firebrats (orders Orthoptera, Phasmatodea, and Thysanura, respectively), as well as in true bugs. In holometabolous insects, primordia of adult structures such as legs and wings are retained internally during the larval and pupal stages. These primordia (called *imaginal discs*) are invaginations of the larval epidermis that eventually give rise to the external adult structures of the head and thorax at metamorphosis. Whereas the adult structures do not normally regenerate, these dedicated (but undifferentiated) imaginal discs are capable of regeneration. Imaginal disc regeneration has been described in the fruitfly *Drosophila melanogaster*, the moth *Galleria mellonella*, and the mealmoth *Ephestia kuhniella* (Bergantinos et al. 2010).

Like the more basal insects, the crustaceans continue to molt, grow, and reproduce as adults. Once these arthropods reach the adult stage, however, they must balance the physiological activities associated with growth (and regeneration) with the physiological demands of reproduction. In the Branchiopoda, the cladocerans *Simorephalits gibbosus* and *Daphnia carcinata* (and *Daphnia magna*) can regenerate portions of the carapace and setae (Agar 1930, Anderson 1933). In the Maxillopoda, the cirripedian *Balanus balanoides* can regenerate portions of shell and mantle (Tighe-Ford and Vaile 1974). In the Malacostraca, at least four orders have been shown to regenerate: the isopods, the amphipods, the stomatopods, and the decapods. The isopods *Asellus aquaticus* and *Idotea baltica* can regenerate thoracic legs and antennae (Needham 1945, 1965, Varese 1960, Giordano 1961). The isopods *Porcellio dilatatus* and *Helleria brevicornis* have been shown to regenerate legs (Hoarau 1973, Noulin and Maissiat 1974). The amphipod *Gammarus chevreuxi* can regenerate a first or second antennae (Dixey 1938), whereas *Orchestia gammarella* regenerates gnathopods (Charniaux-Cotton 1957). The stomatopod *Squilla* sp. has been reported to regenerate antennae (Giesbrecht 1910). In some decapods, the ability to regenerate is intimately linked to the ability to undergo autotomy of a damaged limb. Autotomy of a limb, however, does not always signify that the organism can regenerate the structure that is lost.

Autotomy in the Arthropoda

Autotomy is a reflexive response to injury that results in the casting off of an injured limb at a predetermined point proximal to the injury. The advantage to an animal of an autotomy reflex is that it allows the sacrifice of a limb at a preformed breakage site that is designed to keep blood loss and trauma to a minimum. Injury-induced autotomy is well-documented in insects and crustaceans. Injury can be inflicted during conspecific fights for territory or mates, as well as during predator–prey interaction (McPeek 1997). Most "intraspecific competitive interactions" in many crustaceans, however, are only ritualistic and do not result in autotomy (Juanes and Smith 1995).

The release of a limb via autotomy is a distraction to a predator. The crab *Uca pugilator* scavenges in "herds" for food on exposed intertidal flats during low tide. Shore birds swoop down to feast on the vulnerable foragers. When a bird seizes a crab by the leg, the crab autotomizes the leg, leaving the predator with only a small morsel to eat. This strategy enables the crab to escape. Autotomy of limbs also can occur when an arthropod is shedding its rigid exoskeleton: damage to the exoskeleton during molt or physical problems in climbing out of the old exoskeleton can cause an arthropod to shed a trapped appendage. Limbs caught in the filaments of a fishing line but otherwise uninjured will also be autotomized for escape and survival

(Juanes and Smith 1995, Maginnis 2006). Autotomy is an exquisite escape mechanism that has evolved in many groups of animals.

The term *autotomy* (or "self-cutting") has recently and extensively been used to describe any act that results in the loss of a body part irrespective of the mechanism (Maginnis 2006). In this review, the term "autotomy" will be reserved to meet two major criteria: the loss of an appendage is a true "autotomy event" if (i) the event occurs at a predetermined "fracture plane" and involves intrinsic neural and muscular responses (McVean 1975), and, (ii) the event serves primarily as a defensive function and improves the animal's survival in an escape encounter (Wasson et al. 2002, Fleming et al. 2007).

Extent of Autotomy in Arthropods

Examples of autotomy have been reported among many vertebrates and invertebrates. Autotomy that meets the criteria listed here, however, is limited almost exclusively to invertebrates. Loss of appendages in response to predation has been reported in many invertebrate phyla such as Cnidaria, Annelida, Mollusca, and Echinodermata. But the phylum in which autotomy has been more extensively studied is the Arthropoda. In addition to jettisoning walking appendages, some arthropods are capable of autotomizing feeding structures, antennae, antennules, and stingers. Examples of autotomy are found in all of the subphyla of the Arthropoda. Spiders and centipedes lose walking legs in response to predation, but by far the best studied examples of autotomy are in the subphyla Hexapoda (insects) and Crustacea (Maginnis 2006, Fleming et al. 2007).

Among the crustaceans, the autotomy reflex is limited to the class Malacostraca and most commonly to the brachyuran crabs. No autotomy has been reported in the classes Maxillopoda, Ostracoda, Branchiopoda, Cephalocarida, or Remipedia (although Needham stated in 1965 that the autotomy mechanism was found in members of all classes of arthropods). Among the decapods, the ability to autotomize limbs is found in both natantians and reptantians. Some natantians have a true autotomy response, whereas other species possess a fracture plane but lack the musculature for the reflex (Bliss 1956). Of the peracaridans, the amphipods and at least one species of isopods can autotomize limbs. Some malacostracans possess a true autotomy reflex, whereas others possess either *autospasy* (the pulling off of a limb at a predetermined locus of weakness) or *autotilly* (the detachment of an injured limb at a predetermined point using mouth parts or other limbs). In the Malacostraca, the macrurans and anomurans have limited autotomy responses. Some reptantians possess the autotomy reflex in some limbs and autospasy or autotilly in remaining limbs (Wood and Wood 1932). The degree of autotomy in a variety of arthropod populations as measured by the number of individuals with missing or regenerating limbs is variable and can range from 10% to 90% of the population (Fleming et al. 2007).

Some insects and crustaceans, however, can regenerate without the facilitation of autotomy, and the ability to undergo autotomy does not always result in the ability to regenerate.

Process of Autotomy in Crustaceans

Autotomy in brachyuran crustaceans (the true crabs) has been described in detail (McVean and Findlay 1976, McVean 1984). It is a reflexive behavior involving specialized autotomy muscles. Specialized levator muscles insert on the proximal edge of the basioischiopodite segment of the walking leg. The most crucial muscle to the autotomy reflex is a rotating levator muscle (rplm in Fig. 6.1) which, when stimulated, switches the tension exerted by the remaining levators (alm1 in Fig. 6.1) so that a crucial cuticular connection between the basioischium and coxa (cp in

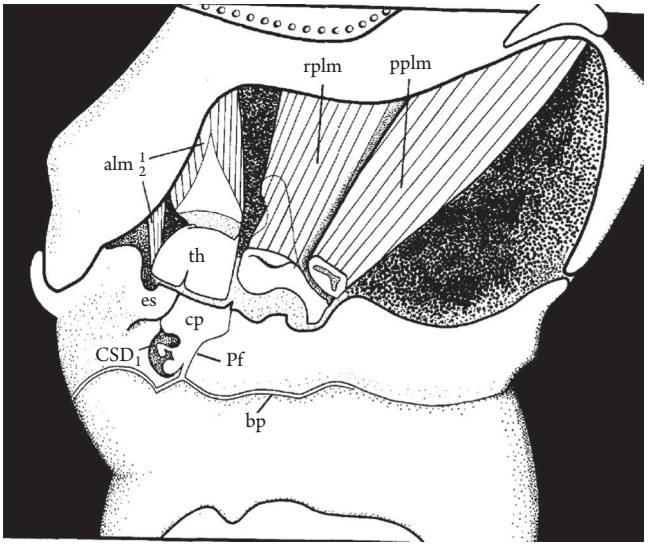


Fig. 6.1.
The levator muscles in the baschioischium leg segment of *Carcinus maenas.* In the walking legs, the anterior levator muscle (alm) is subdivided into two parts, as is the posterior levator group (plm). The tendon head (th) of the anterior levator muscle 1 (alm1) makes contact with the dorsal rim of the basioischium at two locations—at a cuticular projection (es) and onto a cuticular plug (cp) that forms, at its distal point, the intact bridge across the breakage plane (bp). This cp is bounded anteriorly by the membrane overlying the cuticular stress detector 1 (CSD1) and posteriorly by a furrow (Pf). The posterior levator muscle (pplm) helps to keep the faces of the abutting heads closed. The main elements in the walking limbs of *Carcinus* that contribute to limb autotomy are the main levator muscle alm 1, which connects to the es. The es is capable of acting as an energy store. Functional connection is maintained to the energy store, which is tolerant of considerable distortion as long as the catch between the abutting heads is engaged. When the catch is disconnected by rotation of the rotary posterior levator muscle (rplm) tendon, tension accumulated in alm 1 is transferred so that it impinges directly onto the cuticular plug (cp), which causes the breakage plane to snap open, and the basioischium is released from the underlying coxal rim. From McVean and Findlay (1976), with permission from Springer.

Fig. 6.1) is broken, thus permitting a preformed breakage plane (bp in Fig. 6.1) to separate and releasing the basioischiopodite from the coxal stump.

The breakage plane in both insects and crustaceans is usually an area of the cuticle with reduced thickness that allows for easy fracture (Findlay and McVean 1977). There are species differences in the severity of the injury stimulus needed to induce autotomy in crustaceans and insects (Maginnis 2006). Regeneration in crustaceans is always most efficient when amputation of a limb occurs near or at the plane of autotomy (Needham 1965). In some insects, there may be more than one plane of autotomy on each appendage (Woodruff 1937), and there is a gradient of regenerative potential along the length of the limb. The strength of the wound stimulus to induce autotomy must increase if other limbs are missing, and there is also some evidence of a proximo-distal sensitivity gradient along the length of the limb: some crustaceans will not autotomize a limb if the injury is only to the dactyl but will autotomize with increasing readiness as the injury approaches the body (Wood and Wood 1932).

The advantage of autotomy to an animal is because the preformed "plane of autotomy" contains a suite of structures that ensure that blood loss and injury are kept to a minimum. Immediately proximal to the preformed fracture plane in brachyurans is a connective tissue septum called the *autotomy membrane* (AM). The AM is a double membrane that extends across the entire limb base in such a way that it divides the hemocoelic cavity (open circulatory blood space) in the coxal

segment from the cavity of the basioischiopodite (Fig. 6.2A). Following autotomy, the distal part of the AM acts as a valve: blood pressure in the hemocoel immediately inflates the distal AM so that it balloons into the gap left by the autotomized limb, immediately closing the hole (Hopkins 2001). This "immediate closure" assures that there is very little blood loss and minimal bacterial invasion. No muscle tissues cross the crustacean AM, therefore, at autotomy, the only tissue damage is to the pedal nerve and two blood vessels that pass through a hole in the center of the AM. In insects, some coxal muscles cross the autotomy plane and are severed at autotomy. These severed muscles, which serve to move the femur–tibia joint, are phagocytized by blood cells within 30 h following autotomy (Bodenstein 1957). In crustaceans, on the other hand, since there is so little tissue damage during autotomy, there is very little phagocytotic activity by crustacean blood cells.

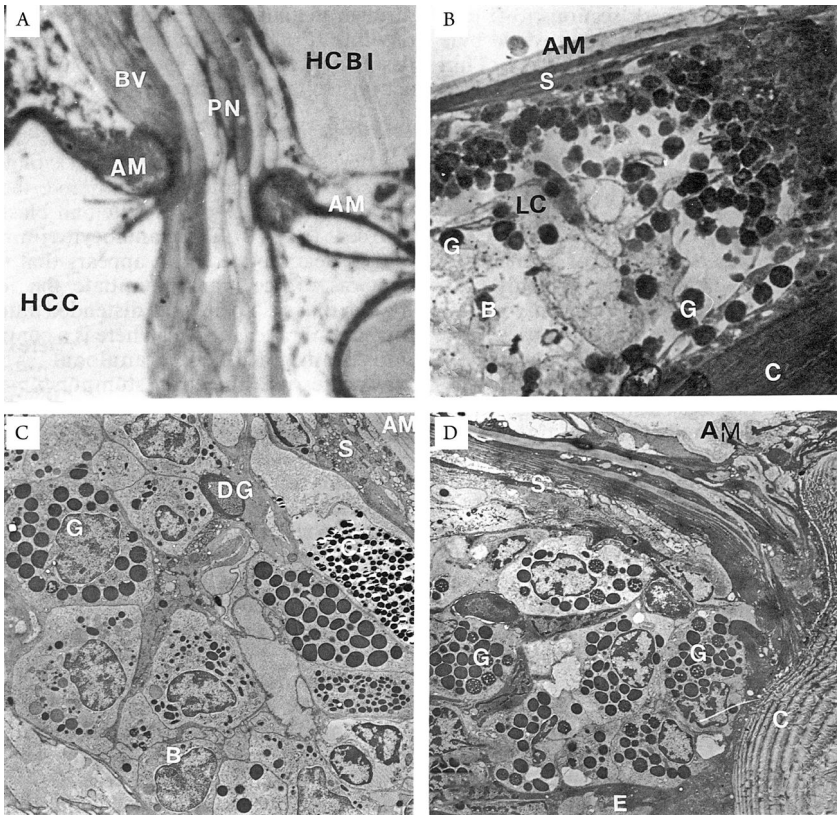


Fig. 6.2.
(A) Autotomy membrane, AM, in an intact limb from the crab, *Uca pugilator* (tissues prepared and processed as described in Hopkins [1993] and Hopkins and Durica [1995]). The pedal nerve, PN, and blood vessels, BV, pass through the AM as a bundle. The thickened area of the AM is the dark area in direct contact with nerve sheath. HCC, the hemocoelic space of the coxa; HCBI, hemocoelic space of the baschioischium. (B) Light micrograph of the autotomy membrane, AM, 4 h after autotomy. The forming scab, S, can be seen immediately below the AM and overlying the limb bud chamber, LC. Granulocytes, G, and hyalinocytes, B, have accumulated below the scab. The coxal wall, C, can be seen to the side. (C) An electron micrograph of the same area as shown in B. The AM and S are seen to the right, and below them are the accumulated granulocytes, G, and hyalanocytes, as well as a degranulated granulocyte, DG. (D) An electron micrograph of the area at the coxal wall where the AM is attached. Granulocytes, G, are evident, and a detached epidermal cell is seen at the bottom, E, getting ready to migrate out under the scab, S. From Hopkins (1993), with permission from Oxford University Press.

Following autotomy or limb loss in insects and crustaceans, the process of regeneration consists of several distinct phases: (i) wound repair, (ii) local activation and proliferation (blastema formation), (iii) pattern formation with differentiation (cell fate determination), and (iv) hypertrophic growth of the regenerate. The similarities of leg regeneration to leg development in insects are quite striking and have been looked at most carefully in those insects where there is genomic information available. A closer look at developmental and regeneration cascades in insects can help us better understand limb regeneration in crustaceans.

Wound Repair

In many arthropods, when a wound occurs to the body or walking limbs, it triggers a series of events that eventually lead to healing and in some cases regeneration. The wound initiates a signaling cascade that activates specific wound-response genes, facilitates wound repair, and recruits blood cells (Moussian and Uv 2005). Repair of wounds by itself without subsequent regeneration involves replacement of only small amounts of tissue and does not usually require the extensive tissue redifferentiation seen in epimorphic regeneration. Wound repair is extremely important in all animals, but especially in the arthropods that have an open circulatory system. In nature, wounds are frequently acquired during bouts of fighting with predators or with conspecifics and occur most often in newly molted arthropods.

Wound Healing

Wound healing occurs following various induced injuries in many insects and crustaceans. Moreover, wound healing is the first step in any regeneration event. Wound healing includes wound closure and epidermal repair. If the cuticle or carapace is damaged, there is also secretion of new cuticle/exoskeleton with production of exoskeleton sculptures such as setae and spines. Although epimorphic regeneration in insects for the most part is limited to immature forms, wound healing has been documented in life stages that normally do not regenerate limbs. The events that culminate in wound healing in both crustaceans and insects are somewhat similar (Wu et al. 2004, Baek et al. 2010, Kwon et al. 2010). Because a great deal more information is available on insect would healing, it is discussed here in some detail with the underlying assumption that a number of the molecular events involved in insect wound healing may soon be shown to be important in crustacean wound healing.

Following wounding in both crustaceans and insects, the first response is an influx of blood cells to the wound site that is thought to be in response to chemotactic signals that attract circulating blood cells necessary for wound closure (Vafopoulou et al. 2007). Closure of wounds by blood cell invasion is subsequently followed by rapid scab formation and migration of adjacent epidermal cells.

The nucleated blood cells of crustaceans that aggregate at wound sites in response to chemical signals are called *hemocytes* (Fig. 6.2B,C,D). It is generally (but not universally) agreed that there are three types of circulating hemocytes in crustaceans: large and small granulocytes and a type of plasmatocyte called a hyalinocyte. The different morphologies of these hemocytes are thought to reflect different functions. The relative abundance of these cell types varies from species to species, and fluctuating abundances may reflect differences in molt cycle, nutritional status, or disease state (Matozzo and Marin 2010). Hemocytes function to (i) release clotting and melanization enzymes, (ii) join with clotted blood to form a plug that further closes injury-induced breaches in the body cavity, (iii) encapsulate invading bacteria, (iv) phagocytize necrotic tissues, and (v) possibly release growth factors and cytokines that may help promote healing (Needham 1965).

Clotting in arthropods is unique and has very little similarity to clotting in other groups of animals. Clotting in crustaceans is part of a well-described innate immunity system (Theopold et al. 2004). Most of the components of the crustacean clotting mechanisms are stored in the hemocyte granules and released upon activation by the lipopolysaccharide coat of invading bacteria or by putative chemical signals released from damaged/wounded cells. In *Drosophila*, one chemotactic wound signal has been identified. It is a peptide that is released by blood cells and/or by damaged epidermis surrounding the wound (Nakatogawa et al. 2009). The extensive clotting that occurs upon wounding is accompanied by another chemical reaction called *melanization*.

The two major clotting cascades in crustaceans are (i) the prophenoloxidase activating system and (ii) the transglutaminase system (Theopold et al. 2004). The prophenoloxidase cascade results in production of melanin (melanization) and other active compounds that stimulate scab formation and increase other immune responses to kill invading bacteria. The second cascade is the result of a transglutaminase released from granulocytes that crosslinks large blood proteins into solid clots that are further crosslinked by the phenoloxidase products (Jiravanichpaisal et al. 2007). Both clotting and sclerotization reactions are essential for wound healing. Adult mutant *Drosophila* lacking hemolymphatic melanization enzymes had 50% mortality when wounded, and the flies that lived had a poor recovery from the injury (Ramet et al. 2002). Within 30 min of wounding, two important melanization enzyme transcripts accumulate in the epidermal cells nearest to the wound. Dopadecarboxylase (*ddc*) and tyrosine hydroxylase (*ple*) catalyze the production of catecholamines that are further converted to quinones by phenol oxidase. Quinones play an important role in clot formation and melanization of a scab (and later in cuticular protein crosslinking). It is thought that these genes are direct targets of a wound-induced factor that appears to signal via an extracellular signal-responsive kinase (ERK) and the transcription factor grainy head in *Drosophila* (Mace et al. 2005).

In decapod crustaceans, autotomy leads to minimal wound trauma. There is immediate wound closure by the connective tissue AM, which spans the base of all appendages that undergo autotomy. As described earlier, the AM is designed to close the breach left by the removal of the limb attached to that coxa. The AM is a complex structure with a unique double membrane that is penetrated by the large pedal nerves and two blood vessels (Fig. 6.2A). These structures pass through the AM together as a bundle through a small hole in the center of the AM. There is an enlarged space between the double membranes at the point through which the bundle of blood vessels and pedal nerve pass. A large number of blood cells are stored in this space, which is in direct contact with the nerve and vessels. In the crustacean coxa, the double AM separates upon autotomy and releases the stored granulated hemocytes. The granulocytes immediately degranulate and form a clot to seal the small hole left by the nerve bundle in the more distal AM. Within 30 min after autotomy, the hemocoelic space immediately below the plane of autotomy is filled with granulocyte cells in both insects and crustaceans (Bodenstein 1957, Adiyodi 1972). Some of these blood cells are from the store located in the double AM membrane, but many of them are recruited from the general circulation.

While the most distal portion of the crustacean AM balloons outward to seal the breach in the coxa, the more proximal portion of the AM remains attached to the sheath of the pedal nerve (Hopkins et al. 1999). This nerve is severed during autotomy at the preformed breakage plane, and the nerve stub retracts back into the coxal stump but is held in place by the attachment of the more proximal AM. The end of the severed nerve is thus kept in close proximity to the point at which it was located prior to autotomy. The major site of blood loss following autotomy is the small hole in the middle of the distal AM through which the pedal nerve and vessels pass. A clot forms very quickly at this hole (as described earlier). Further clotting and melanization occurs along the span of the AM. The AM together with the degranulated blood cells are incorporated into a black "scab"

that gains its color from the chemical sclerotization described earlier. This scab seals the autotomy plane and is visible on the outside of the coxa.

Insects have no comparable structure to the AM of crustaceans. Wounds to soft-bodied insect larvae and the pliable larval wing discs are closed by a contraction of the wound edges. Larval imaginal discs are "set aside" tissues in holometabolous insect larva that will become appendages in the adult insect at metamorphosis. The pairs of discs within the body of the larva can become wings, legs, genitalia, antennae, and eyes in the adult. When these discs are injured, they can regenerate. Initial wound closure in a wounded disc is a reorganization of actin at the edges of epidermal cells that line the injury. This actin forms a cable-like structure that is also seen in developing insect embryos. Contraction of this cable helps to close the wound in a "purse-string" mechanism. The wound edge contracts due to the actin purse-string, and, as the edges approach each other, the epidermal cells join to anchor the two sides together and close the wound (Bosch et al. 2005).

In crustaceans, it is possible to damage only the calcified outer cuticular layers of the exoskeleton without injury to the underlying epidermis. Even if the epidermis is visually unharmed, hemocytes will invade the area under the damaged exoskeleton. The epidermal cells that lie beneath a damaged exoskeleton become activated in the crab *Carcinus maenas* and are able to recruit hemocytes (Dillaman and Roer 1980). In the crayfish *Procambarus clarkii*, wounding of the carapace plus the underlying epidermis results in the onset of a molt cycle (Vafopoulou et al. 2007). The induced cycle is different in its time frame from a cycle induced by other means but, nevertheless, wounding results in an early onset of molt compared to the controls. This suggests that the chemical signals released at the wound site not only attract hemocytes but may also interact with the endocrine system of the crustacean in a way that ensures that the wound will be further repaired by means of a hastened and subsequent molt.

Epidermal Repair

When a wound breaches the cuticle and damages the underlying epidermis, the closure of the injury gap by a blood clot and scab is followed within 6–8 hours by further activation of the epidermal tissues adjacent to the wound. In crustacean penaeid shrimp, epidermal cells surrounding a wound transform from a squamous to a columnar shape, begin to enlarge, and start to migrate across the damaged surface below the wound (Fontaine and Lightner 1973). This same response of the epidermal cells lining the plane of autotomy is seen following autotomy (Hopkins 1993). The epidermal cells that line the coax—which is the most proximal segment of the lost leg that remains attached to the animal's body—are activated by the autotomy of the limb. At this time, these cells detach from the coxal cuticle and change shape (Fig. 6.2C).

In insects, epidermal cells of regenerating imaginal discs have lamellipodia or filipodia that reach toward the cells on the opposite side of the wound. They appear to migrate under the melanized clot but are pulled together by an actin "purse string" mechanism (as described earlier). As the filipodia from each group of cells make contact with filipodia cells from the opposite side of the wound, they form a suture. This closure of the insect epithelium occurs within 18 hours after injury, and the cells return to their normal shape (Ramet et al. 2002).

The initial re-epithelization of a wound in crustaceans involves only hypertrophy and migration of existing cells with no mitotic figures evident (Fontaine and Lightner 1973). The migration of epidermal cells across the plane of autotomy is accompanied by cellular changes that include increases in chromophilia of nuclei and enlarged nucleoli. These changes suggested to Adiyodi (1972) some degree of cellular de-differentiation in the crab *Parathelphusa hydrodromus*, although Needham (1965) reported that de-differentiation was not extensive following autotomy in most arthropods. In crustaceans, healing continues with further hypertrophy and migration of

epidermal cells under the scab. In addition to migration of epidermal cells following autotomy, there is a marked influx of other nonepidermal, fibroblast-like cells from the coxa, the origins of which are unclear (Needham 1965).

In *Drosophilia*, the JUN N-terminal kinase (JNK) signaling pathway plays a crucial role in re-epithelization of wounds in embryos, juveniles, and adults. Re-epithelialization of the wound site probably does not require signals from the invading hemocytes because in *Drosophila* wing discs that are injured there is no hemocyte mediation of wound healing (Wang and Xia 2010). The upstream activating signal for JNK may be an insulin-like compound released by developing and injured wing discs (Colombani et al. 2012, Garelli et al. 2012). The JNK pathway contains a number of key gene products that stimulate the expression of other important genes like *puckered (puc), hemipterous (hep), basket (bsk)*, and *kayak (Dfos* or *kay)* that are upregulated in wound epithelia and thus implicated in the closure and healing of wounds (Ramet et al. 2002, Xia and Karin 2004, Bosch et al. 2005). Because *puc* expression is stimulated in tissues surrounding necrotic regions, it has been suggested that there is some signal from necrotic cells that stimulates the onset of the JNK signaling pathway in the adjacent uninjured epithelia (Mace et al. 2005, Wang and Xia 2010). The activation of this cascade in the epidermal cells adjacent to the wound leads to the movement and fusion of the cells during wound healing in both *Drosophila* adult exoskeleton and imaginal discs (Ramet et al. 2002, Bosch et al. 2005). In the embryo of *Drosophila*, the wound epidermis also expresses α-catenin and E-cadherin (Garcia-Fernandez et al. 2009).

Blastema Formation

In the epimorphic regeneration that follows autotomy and wound repair, a blastema is formed. A blastema is a mass of undifferentiated cells from which an organ or part can grow (Slack 2003). During wound healing, the major cellular response is epidermal activation and mitosis, but during blastema formation, in addition to epidermal activation, there is extensive cellular proliferation and respecification. Many arthropods that lack the autotomy response are capable of blastema formation and regeneration of complete limbs. In the case of limb loss by means other than autotomy, however, wound closure events take a considerably longer time, and these animals have longer latency periods between limb loss and limb regeneration. In nonautomized wounds, there is tissue damage, blood loss, and bacterial invasion. In the crab *U. pugilator*, a limb can be amputated distal to the preformed breakage plane very soon after molt when the animal is still soft and unable to contract its autotomy muscles. (If the limb were fully hardened, any injury along the length of the limb would force autotomy at the plane of autotomy.) Wound healing, blastema formation, and regeneration can occur in these soft-shelled crabs, and only the amputated portion is regenerated (Wu et al. 2004). Thus, positional information is found along the entire length of the limb, appears to be inherent in the blastema itself, and does not rely on information restricted to the plane of autotomy. The plane of autotomy, with its built-in safety valve, is the most dependable place for limb loss to occur, but the entire length of the limb is capable of organizing a blastema and regenerating the missing parts of the lost limb.

Cuticular Secretion

In crustaceans, following the initial wound closure after autotomy, the epidermal cells that have migrated across the wound surface and under the scab begin to secrete a cuticle. These activated epidermal cells secrete a thin, cuticle-like layer that helps strengthen the wound closure and further seals the coxal gap (Needham 1965, Hopkins 2001). Seven to ten days after autotomy, the scab drops off and the cuticular seal of the coxal surface is visible. This cuticle is quite flexible

and forms a crenulated sac that expands as the growth of the regenerating limb bud grows. This cuticular sac is distinct and separate from the cuticle that is secreted by the developing epidermal shell of the regenerating segments. The cuticular sac is unique to crustaceans and has not been reported for the insects. The cuticular sac protects the growing, regenerating crustacean limb bud. It is shed at the time of molt when the newly regenerated but still folded limb is pulled out of the sac and expanded with blood.

Regeneration of Limbs in Insects

As mentioned earlier, some insects can heal wounds as adults, but regeneration in the more derived insects is limited to juvenile forms and immature tissues. The legs of hemimetabolous insect nymphs and naiads regenerate readily, as do the imaginal discs in holometabolous insects (e.g., *Drosophila* and *Manduca*). In the nymph of the hemimetabolous cockroach *Periplaneta americana*, amputation of a leg results in regeneration of a new leg. Adult cockroaches that do not normally regenerate lost limbs will regenerate limbs if parabiosed to a nymph, suggesting that some blood-borne factor is necessary for regeneration competence (Bodenstein 1955). Following amputation in hemimetabolous and immature insects, (i) a new leg forms in the most proximal coxal segment, (ii) degeneration of coxal muscles creates a space in which the regenerate develops, and (iii) as regeneration progresses, more muscles degenerate and pull away from the old exoskeleton to provide more room for the developing regenerate. The severed leg nerve regenerates into the newly forming limb.

Insect Leg Development Compared to Leg Regeneration

The cricket leg develops from a limb bud, and adult *Drosophilia* legs develop from imaginal discs. The molecular cascade that controls development in these structures is thought to be duplicated during regeneration of these structures. It has been suggested that the regenerating leg uses the same "molecular boundary" model for signaling during regeneration as is used during development (Meinhardt 1983). The same signaling pathway mentioned earlier (JNK) that triggers wound healing also stimulates epithelial movement and morphogenetic early genes involved in both leg development and regeneration in insects. The first step in pattern formation in both development and regeneration involves the establishment of a posterior/anterior compartmentalization. Activation of JNK in blastema cells of the imaginal disc is one of the first responses to injury and is necessary for subsequent wound healing and regeneration. Gene regulatory networks that function in developing limbs also regulate regeneration in imaginal discs. Using RNAi, it was shown that, in the regenerating leg of the cricket *Gryllus bimaculatus*, the expression of *hedgehog* (*hh*), *wingless* (*wg*), and *decapentaplegic* (*dpp*) genes along with epidermal growth factor-related ligands set the boundaries for posterior and anterior compartments. These compartments will eventually form the segments of a new regenerated limb (Bergantinos et al. 2010).

In insect leg development, leg segments are sequentially formed by intercalation—starting with the coxa/pretarsus boundary. The segments are laid down with a proximal segment intercalated between the most proximal and most distal structures. This same pattern of proximo-distal intercalated segmentation is seen during limb regeneration in insects, and specific limb segments can be identified by segment-specific gene regulatory networks. The patterns of gene expression and signaling molecules can be explained by the molecular boundary model mentioned earlier (Nakamura et al. 2008). RNAi knockdown of specific regulatory genes—such as *armadillo, hedgehog, engrailed, distaless, efgr, fat*, and *dachshund*—results in defective development and/or leg regeneration due to lack of positional information with the lack of distal-to-proximal respecification of the regenerate.

The ability of holometabolous insect imaginal discs to regenerate has been known for some time (Hadorn and Buck 1962, Madhavan and Schneiderman 1969, 1977, Schubiger 1971), and access

to the *Drosophila* genome makes these tissues ideal to study the molecular control of regeneration (Bergantinos et al. 2010). Damage to *Drosophila* wing discs results in the upregulation of several genes, the two most important being *wingless* (*wg*) and *myc* (Smith-Bolton et al. 2009). Injured discs also appear to release an insulin-like peptide that is capable of retarding growth in the rest of the animal until disc regeneration is complete (Colombani et al. 2012, Garelli et al. 2012). Wing discs, however, lose their regenerative capacity during the middle of the third instar (Smith-Bolton et al. 2009). The imaginal discs of some insects can actually change fates. Antenna discs that are injured in vivo by ultraviolet radiation can be transformed so that they will grow into wing discs (McClure and Schubiger 2007). This change in tissue identity during regeneration is called *transdetermination*. Transdetermination may be a widely conserved feature in insect and crustacean regeneration and is thought to be a result of downregulation of the gene *distal-less* (*dll*), which is an important development gene found in all arthropod limbs (Panganiban et al. 1994, Suzuki et al. 2009).

Regeneration of Limbs in Crustaceans

By 2–4 days after autotomy, most of the granulocyte cells in the area between the two AMs disappear. The hypertrophied immigrant epidermal cells (the wound epidermis) have moved from the coxal walls and completely underlie the scab in the crabs *Parathelphusa* and *Uca* (Adiyodi 1972, Hopkins 1993). The highly activated wound epidermis of the crab secretes a thin cuticle between the scab and itself and begins mitotic activity. Mitosis in crustacean blastemas (as measured by tritiated thymidine incorporation) increases 4 days after autotomy (Hopkins 1989). This first peak of mitotic activity corresponds to the initial invagination of the epidermis to form the walls of developing segments in the crab. The formation of these epidermal foldings is the first sign of segmentation and differentiation in the nascent limb bud. By 6–7 days after autotomy, the blastema has proliferated so much that it pushes aside the scab from the face of the coxa. At this point in development, the regenerating structure is called a *papilla*. The papilla is a small structure that consists of a single segment with no internal substructure.

A second peak of mitotic activity accompanies the emergence of the papilla. This second, large peak of mitotic activity corresponds to the formation of further limb segments and within 1–2 days transforms the papilla into a small bud (a basal bud). Mitotic figures are first seen in the crustacean epidermis, then in the more internal cells. The mitotic growth of the bud continues for a few more days and then ceases (Bliss 1956; Fig. 6.3). The differentiation of the segmented exoskeleton appears to occur solely during this early basal growth phase and is complete by the end the first 2 weeks of basal growth.

In insects, regenerating leg segments are formed by intercalation, but differentiation of crustacean segments occurs sequentially. The direction of differentiation in crustacean segments appears to vary from group to group. In some crustaceans, regeneration of segments lacks any orientation or they have opposite orientations, but in *Uca* the segments form in a proximo-distal sequence beginning with the formation of the merus segment with further addition of more distal segments.

As the papilla emerges from the coxa and it begins to differentiate into a basal bud, there is asymmetrical growth: one side of the developing limb bud grows faster (i.e., has more mitotic figures) than the other (Adiyodi 1972). Asymmetrical growth causes the small basal bud to bend in upon itself so that, as new segments are added to the regenerate, they are folded upon one another. The basal bud is a folded structure enclosed inside the cuticular sac secreted earlier (Needham 1965; Fig. 6.4). A comparably folded regenerating limb is also seen in a few insects. In some crustaceans such as the macrurans, the limb buds are more elongated and less folded. There is some bending of the regenerating macruran limb toward the body, but the regenerate is not tightly folded within a sac as it is in the brachyurans. The crayfish *Orconectes obscurus* retains the fully extended regenerating limb bud within the coxa of the old limb and liberates the fully formed limb only upon molt.

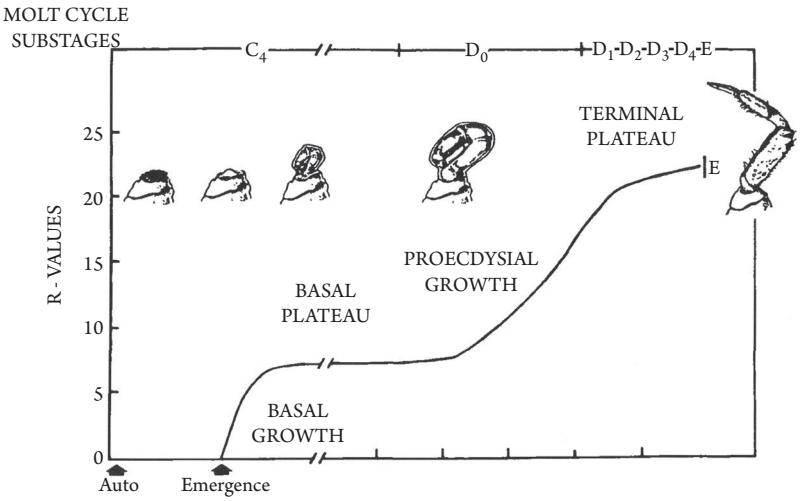


Fig. 6.3.
Diagrammatic representation of the growth curve from a single walking leg from the crab *Uca pugilator*. The large arrow at bottom left represents the point at which autotomy (Auto) occurred. The second large arrow to the right is the point at which the regenerating bud emerged from the coxa as a papilla (Emergence). At the top are Drach's (1939) stages of proecdysis, with (Travis 1960) additional D_o stage, which is characteristically found in crabs undergoing regeneration. In the center are four drawings of the limb bud at the various stages. From left to right, the pictures depict the scab covering the coxa, a papilla emerging from the coxa, a basal bud, and a PE bud. To the far fight is a complete walking leg. R-values are measurements of a regenerating right third (R_3) walking leg (calculated by measuring the length of the bud and dividing it by the width of the carapace then multiplying that by 100). The solid line is a representative plot of the R_3 values (y-axis) as a function of time (x-axis) after autotomy. From Hopkins (1993), with permission from Oxford University Press.

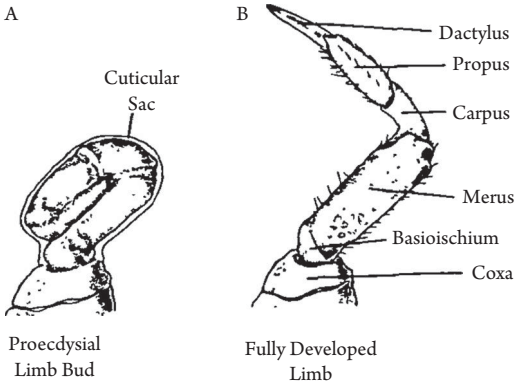


Fig. 6.4.
(A) Drawing of regenerating proecdysis limb bud from the crab *Uca pugilator*, folded and surrounded by a cuticular sac. (B) The same newly regenerated limb after ecdysis. Individual segments are labeled. Redrawn from Hopkins (1993), with permission from Oxford University Press.

Muscle Regeneration

The origin of new muscle tissue in the crustacean regenerating limb is unclear: the immigrant epidermal cells appear to be the main source of epidermal cells in the regenerated limb bud, but there is some question as to the origin of the other blastemal tissues (Bodenstein 1955, Skinner 1985). There is some evidence that muscle tissues regenerate metaplastically from local epidermis, but other reports suggest that muscle tissues arise from immigrant cells moving up from the base of the coxa—which is the site of the large muscles that control the walking legs. Myobundles form from cells that may be de-differentiated cells from a variety of sources, but they may also be reserve cells located at distant points. Whatever the source, the regeneration of muscle tissue lags behind that of segmentation by 1–2 days (Adiyodi 1972), so that the first appearance of the blastema is one of an epidermal shell that later fills with immigrant cells that organize into myobundles (Needham 1965, Hopkins and Durica 1995). Some of the cuticular in-growths in the crustacean blastema arising during the second peak of mitotic activity form the cuticular attachment sites (apodemes) for developing tendons of flexors and extensor muscles (Hopkins 2001). There is also a nonuniform development of muscle fibers that is thought to be a function of variable innervation due to a variable branching pattern of regenerating nerves (Lang et al. 1980).

Nerve Regeneration

As the segments develop in crustaceans, regeneration of the severed pedal nerve begins. The cut surface of the pedal nerve is held in close proximity to the developing blastema by the attachments of the proximal AM to the pedal nerve sheath. The regenerating nerve grows as an elongating cone into the developing segments as they sequentially form (Hopkins and Durica 1995). It is assumed that the pedal nerve regenerates to form the motor input to the new limb, but the source of sensory neurons is unclear. It has been suggested that new sensory neurons differentiate from the immigrant hypodermal cells (Skinner 1985), but the exact source of sensory nerve cells (as well as the chromatophore cells) is unknown.

Differentiation of the Crustacean Limb Bud

When the limb blastema emerges from the surface of the fracture plane as a papilla, it is poorly differentiated and consists of a single segment. The length of time between autotomy and papilla emergence depends on the same variables that determine intermolt duration. Papilla emergence can occur at any time during the intermolt stage but is inhibited if the animal has begun preparations for molt or if exogenous molting hormones (the ecdysteroids) are administered during this time. After papilla emergence, segmentation of the forming limb bud is evident, and the papilla becomes a basal bud (Fig. 6.3). In the basal bud, the cuticularized segments become quite apparent. Some organized tissues are evident within the cuticular shells, but the segments are primarily filled with masses of what appear to be undifferentiated cells and early myocytes (Adiyodi 1972, Hopkins et al. 1999).

During the "basal bud growth" period, organization of the tissues continues with differentiation of specialized chromatophore cells, cuticular elaborations, and further muscle organization. Proliferation (or DNA replication) also continues during basal growth, but mitosis slows down by about 12 days after autotomy (Hopkins et al. 1999). Differentiation of the segments is pretty much completed during the basal growth period, and the bud is encased inside the flexible cuticular sac that expands as the bud grows. Basal growth ceases about 10–14 days after emergence and, thereafter, the small basal bud stays the same small size. This period when basal growth ceases is called *basal plateau* (Fig. 6.3; Bliss 1956, Adiyodi 1972). During basal plateau, proliferation stops, epidermal

cells secrete the secondary cuticle, and myofibrils appear within the relatively empty center of the forming segments. Myofibrils occur in clusters attached to apodemes and are surrounded by granular sarcoplasm. Functional synapses are present in regenerating basal buds of the crab *Grapsus grapsus* following basal growth, and a new AM forms. Basal buds of the crab *Gecarcinus lateralis* are capable of an autotomy response (Skinner 1985). The basal bud remains small even after attaining some degree of differentiation. This small basal bud remains folded and sequestered between the coxa of the other, nonregenerating limbs so that the crab can participate in the foraging and reproductive activities characteristic of the nonmolting periods.

Hypertrophic Growth of the Crustacean Limb Bud

As the crab that is regenerating a new limb enters preparations for molting, the small basal limb bud begins a secondary spurt of growth that occurs only during the period just before molt called *proecdysis* (PE or premolt). This growth spurt is called *proecdysial growth* (Fig. 6.3; Bliss 1956). This growth is almost entirely hypertrophic, with the size of the bud reaching as much as a threefold increase in size due primarily to muscle protein synthesis and water uptake (Hopkins et al. 1999). During the last few days prior to ecdysis/shedding, the regenerated bud does not grow at all. This cessation of growth is called *terminal plateau of regeneration* (Fig. 6.3; Bliss 1956).

Crustacean Leg Development

Hox gene expression and its correlation with appendage morphology have been well studied in arthropods (Averof and Patel 1997, Shiga et al. 2006). The *hox* genes, first discovered in *Drosophila*, specify segment identities along the anteroposterior axis of both vertebrates and invertebrates. Crustaceans have diverse body plans, with limbs developing in abdominal, thoracic, and head segments, whereas in insects, limb development is restricted to the thoracic and head segments. In *Drosophila*, one of the earliest genes to be activated in the limb primordia is *dll* (Cohen et al. 1989). This gene specifies the distal region of the limbs (Panganiban et al. 1994), and the distal structures are not formed in absence of this gene (Cohen and Jurgen 1989). It has been proposed that the variation in arthropod limbs may result from variation in *dll* regulation (Panganiban et al. 1995).

Homeotic gene products like *ubx* (*ultrabithorax*) and *abdA* (*abdominal A*) regulate limb number. There is a significant difference in expression of *ubx, abdA,* and *dll* between insects and crustaceans. In crustaceans like *Artemia*, the genes *ubx/abdA* and *dll* are co-expressed in thoracic limb primordial, whereas in insects, *ubx/abdA* represses *dll* in abdominal segments, which in turn inhibit limb development in that region (Pavlopoulos and Averof 2002). Studies conducted in various crustaceans (Branchiopoda, Maxillopoda, and Malacostraca) have shown that the *hox* gene *ubx* is usually expressed in the thoracic segments that are associated with locomotory appendages and excluded from thoracic segments that bear maxillipeds (Averof and Patel 1997). In *Parhyale hawaiensis*, a malacostracan amphipod, RNAi-mediated knockdown of *ubx* results in transformation of multiple walking legs into maxilliped-like appendages (Liubicich et al. 2009). This suggests that *ubx* plays a role in distinguishing posterior thoracic appendages from the more anterior maxillipeds.

Further studies to decipher the function of *hox* genes in crustaceans during early limb development will lead to identification of downstream genes necessary for this process. These genes in turn might be activated during regeneration of limbs in adult crustaceans. The crustacean genes mentioned here have been found in a wide variety of developing limbs from vertebrates to invertebrates, and the argument has been made for an "evolutionarily conserved mechanism of leg pattern formation from protostomes to deuterostomes" (Nakamura et al. 2008, Suzuki, et al. 2009).

CONTROL OF REGENERATION IN CRUSTACEANS

Many factors have been reported to either hasten or inhibit regeneration in crustaceans and insects: (i) wound factors, (ii) local tissues and nerve supply, plus (iii) environmental conditions all exert some influence upon the extent and rate of regeneration. And, as mentioned earlier, (iv) circulating ecdysteroid hormones can exert profound effects on the rate and nature of regeneration.

Wound Factors

In crustaceans, there is little evidence to indicate what (if any) factor(s) stimulate the recruitment of blood cells into a damaged area or the activation and migration of the epidermal cells immediately following injury. A cytokine has been identified in insects that is released by wounded epidermal and hemocyte cells and acts as a chemotactic agent to attract hemocytes to the wound in the moth *Pseudaleta separata* (Nakatogawa et al. 2009). In crustaceans, however, there is probably more than one message released from damaged cells because (i) hemocyte recruitment processes are similar following limb amputation (with massive tissue damage) and autotomy (with minimal tissue damage) and (ii) there is epidermal activation and massive influx of hemocytes into a region where only the exoskeleton is damaged with no disruption or injury to the underlying hypodermal cells (Vafopoulou et al. 2007). Damaged imaginal discs from the fruitfly *Drosophila* may release a retinoid message into the blood that helps modulate ecdysteroid levels in the blood (Halme et al. 2010). Such a blood-borne signal could also serve as an in situ regeneration factor, as has been recently shown in the developing chick limb bud where retinoic acid can serve as a diffusible developmental signal for proximodistal organization (Rosello-Diez et al. 2011). Damaged tissues may produce a signal that elevates juvenile hormones (JH) levels (or JH sensitivity) in some insects (Krishnakumaran 1972). In the flour beetle, *Tribolium castaneum*, RNAi knockdown of the *dll* gene caused sufficient damage to developing limbs to produce extra larval molts, which the authors suggest was due to elevated JH levels because the phenotype could be rescued with RNAi to an enzyme that produces JH (JH methyl transferase) and a putative JH receptor (methoprene tolerant [met]; Suzuki et al. 2009). As mentioned earlier, chemical signals released at a wound site in the crayfish *P. clarkii* may serve to recruit hemocytes and may interact with the endocrine system to influence ecdysteroid levels (Vafopoulou et al. 2007).

Local Tissue Effects

Another important control of the regeneration process is that of local tissues and positional information. Regeneration of transplanted homoeotic legs in the crayfish *P. clarkii* is affected by the host site. Cells adjacent to a wound or regeneration site retain positional information (Mittenthal et al. 1980). Regeneration of the uropod in the isopod *A. aquaticus* can be blocked by excising the base of the limb proximal to the autotomy plane (Needham 1965). Regeneration in legs from the crayfish *P. clarkii* restores the continuity of that positional information (Mittenthal and Trevarrow 1983). Claw buds transplanted from male to female *Uca pugnax* crabs acquire after three molts some morphological characteristics (shape and sarcomere length) intermediate to both donor and recipient. Also, claws regenerated from a coxa onto which a transplanted claw had been previously attached and then removed prior to the regeneration showed some donor-like characteristics (Trinkaus-Randall 1982). Such an effect can be explained by a transfer of donor tissue with the first transplant. Thus, when the new regenerate grows, the transferred donor tissue exerts some effect on the morphological determination of that new regenerate. It must be stressed again, however, that positional information is found along the entire length of the crustacean limb and is not unique to the plane of autotomy (Wu et al. 2004).

Local Nerve Supply

Epimorphic regeneration in all animals that produce blastemas is nerve dependent (Singer 1952, Kumar et al. 2007). Needham (1945) demonstrated in the isopod *A. aquaticus* that the presence of some intact nerve is necessary for early stages of regeneration. Bodenstein (1955) was unclear, however, as to the importance of intact nerve input into regeneration in the cockroach. In the lobster *Homarus americanus*, there is some indication that developing claw muscle fiber properties are determined by innervation (Lang et al. 1980). The shrimps *Alpheus heterochelis* and *A. armillatus* have two asymmetrical claws. One is a large snapper, and the other is a smaller pincer. If the snapper is removed, a nonsnapper (pincer) will regenerate in its place, and the contralateral pincer will transform into a snapper within two molts (Mellon and Stephens 1978). Severing of the snapper claw nerves will accomplish the same transformation. The two claws also differ in motor nerve size as well as muscle size. The switch in development is mediated by elements within the limb nerves (Mellon 1981). The pesticide dichlorodiphenyltrichloroethane (DDT) causes repetitive neuronal discharges in crustacean nerves (Gordon and Welsh 1948). When DDT is placed in the holding water of the crabs *U. pugilator* and *U. pugnax*, the growth rate of regenerating limbs is hastened (Weis and Mantel 1976). It has been suggested in the crab *U. pugilator* that the pedal nerve that is severed at autotomy releases growth factors that initiate early events in limb blastema organization (Hopkins 2001).

Environmental Effects on Regeneration

In many autotomy-induced regenerations, environmental factors have been shown to exert an influence. Light conditions were shown to affect PE regeneration in the crab *G. lateralis* (Bliss 1956, Bliss and Boyer 1964) and the crayfish *Orconectes virilis* and *Procambarus clarkii* (Stephens 1955, Aiken 1969). Temperature also exerts an effect on PE regeneration in the crabs *Gecarcinus, Uca*, and *Pachygrapsus* (Passano 1960, Rouquette and Vernet-Cornubert 1964, Bliss and Boyer 1964), and the crayfish *Faxonella clypeata* (Mobberly 1963). Lack of privacy or crowding has an inhibitory effect on regeneration rates in the crabs *Ocypode macrocera, G. lateralis*, and *U. pugilator* (Rao 1965, Skinner and Graham 1972, Weis 1976). Low temperature slows basal growth in the crabs *U. pugnax* and *O. macrocera* (Passano 1960, Rao 1965). Starvation does not slow regeneration in the crabs *O. macrocera* and *U. pugilator* (Rao 1965, Weis 1976) or in the cladoceran *D. carcinata* (Agar 1930). Addition of inorganic phosphate to the external medium of the isopod *A. aquaticus*, however, results in larger postmolt regenerates (Needham 1947). It is believed that most of these environmental effects are mediated by the central nervous system and effected by the endocrine system.

Hormonal Control of Regeneration

Ecdysteroid hormones—20-hydroxyecdysone (20E) and ponasterone A (PA)—are the main effectors of molt in arthropods. As early as 1955, Bodenstein concluded that the prothoracic glands (PGs) of insects that produce ecdysteroids and control molting also control regeneration. Early experiments with parabiosed nymphs and adults and with reimplantation of PGs into adult cockroaches (*P. americana*) showed that the ability to regenerate (and to molt) could be reinstated by a hormonal signal from the PGs, and the receptors for ecdysteroid hormones are present in regenerating tissue throughout limb regeneration in the crab *U. pugilator* (Hopkins et al. 1999). The putative source of ecdysteroids in crustaceans are two epidermally derived structures called the Y-organs (YOs) that are located in the anterior cephalothorax (Lachaise et al. 1993).

Exogenously administered ecdysteroids can (under certain circumstances) induce proecdysis and ecdysis in some crustaceans, but the effects of exogenous ecdysteroids on limb regeneration

are quite variable (Krishnakumaran and Schneiderman 1969, 1970, Lowe et al. 1968, Flint 1972, Charmantier-Daures 1976, Bazin 1977, Gilgan et al. 1977). The effect of ecdysone and 20E on early stages of regeneration (blastema formation through papilla emergence) is inhibitory in the crabs *U. pugilator* and *G. lateralis* (Rao 1978, Hopkins et al. 1979). Ecdysteroids are also inhibitory to PE limb-bud regeneration in crabs *C. maenas, Pachygrapsus marmoratus, Rhithropanopeus harrisii* megalopa; in the crayfish *Procambarus* sp.; in the lobster *H. americanus;* and in the isopod *P. dilatus.* PE growth in the crab *U. pugilator* and basal growth in the isopod *H. brevicornis* are accelerated by exogenous ecdysteroids (Krishnakumaran and Schneiderman 1970, Flint 1972, Hoarau 1973, Noulin and Maissaiat 1974, Charmantier-Daures 1976, Bazin 1977, Rao 1978, McConaugha and Costlow 1980). Furthermore, in the crab *G. lateralis*, PE limb-bud growth was unaffected by exogenous ecdysteroids (Skinner and Graham 1972, Hopkins et al. 1979). Injected, labeled ecdysteroids are cleared from crustacean hemolymph very quickly (McCarthy 1982). In the crab *C. maenas*, 80% of labeled injected hormone was eliminated from the circulation 4 h after injection. By 24 h, however, circulating levels of unlabeled steroid rose, suggesting that the crabs own source of ecdysteroids was stimulated by the injection (Adelung 1964). Injected ecdysteroids may be quickly eliminated and may exert their effects indirectly through feedback at the YOs. The extremely variable results of exogenous ecdysteroid treatment may reflect variable sensitivity of the YOs and target tissues to stimulation and/or variable rates of elimination.

In crustaceans, the last phase of regeneration (PE growth) occurs only in animals that are getting ready to molt, which suggests a close relationship between the control of molting and the control of regeneration. Bliss (1956) pointed out that the rapidly growing crustacean limb bud was an excellent external signal for the beginning of the PE stage of the molt cycle. The earliest stage of proecdysis (stage D_o in Fig. 6.3) coincides with the onset of the PE growth phase of crustacean limb regeneration. In the crabs *C. maenas* and *U. pugilator*, the transition from basal to PE growth coincides with a small, transient peak of circulating ecdysteroids (Adelung 1971, Hopkins 1983). This small peak marks the transition from molt stage C_4 to stage D_o. This temporary peak is followed by an obligatory drop in circulating ecdysteroids. If the levels of circulating ecdysteroid does not drop, PE growth of the bud does not occur (Hopkins 1983). It is thought that the transient rise is the signal that the animal is getting ready to molt, but the presence of regenerating limb buds forces a temporary reversal of that increase to allow the limb buds to complete the PE phase of regeneration.

PE growth of limb buds is rapid and can occur within 7–10 days. During PE growth, the size of the limb bud can increase threefold. PE growth of the bud terminates with the rise in ecdysteroids that is necessary for apolysis of the carapace and the actual molt—during stages D_{1-4}. The rise in circulating ecdysteroids that is necessary to coordinate the molt event is inhibitory to further growth of the limb bud. Bodenstein (1955) suggested that a molting hormone titer high enough to inaugurate molting is too high to support regeneration. All phases of regeneration in crustaceans—blastema formation, differentiation, and early and late growth—are inhibited by elevated levels of 20E (Hopkins 1983, Hopkins et al. 1979). The presence of regenerating tissue actually feeds back to inhibit increases in circulating ecdysteroids and to maintain the low ecdysteroid levels necessary for regeneration of limbs (Skinner 1985, Yu et al. 2002).

Wounding of the carapace of the crayfish *P. clarkii* results in the onset of a molt much like the loss of a limb (Vafopoulou et al. 2007). When the carapace is wounded, there is the induction of a new molt cycle that is similar to the cycle induced by eyestalk removal. Both groups of animals molt by 55 and 50 days, respectively. The increase in ecdysteroids in the hemolymph in wounded animals, however, is quite different in the wounded versus the eyestalkless groups of animals. Eyestalk removal results in a steady increase in circulating ecdysteroids until PE peak approximately 2 weeks prior to the actual molt. In wounded animals, the final PE peak occurs only a week prior to ecdysis. The difference in circulating ecdysteroid levels in a wounded animal is a delay in the rapid increase of ecdysteroids. Following wounding, the circulating ecdysteroids rise, but only to basal levels, and

then stay at those basal levels for 10–12 days. After the wound is healed, the ecdysteroid levels begin to rise to PE levels (as in the eyestalkless animals), and the wounded animals almost catch up with the cycles of the eyestalkless animals. This suggests that there are blood-borne signals from any wounded epithelium that may have effects on the molting hormone production in crustaceans. The signals allow for basal levels of ecdysteroid production that are necessary for wound healing (and basal growth of regenerating limbs) but inhibit the surge of ecdysteroids that are necessary for the actual ecdysis.

In crustaceans with intact eyestalks, there is a rise in circulating ecdysteroids shortly before ecdysis (during PE): about a week prior to ecdysis, a series of large peaks of circulating ecdysteroids are seen (Hopkins 1983). The large peaks at the end of PE occur at (and probably induce) the terminal plateau (or the cessation of regenerating limb-bud growth). Thus, in intact crabs (specifically, those retaining both eyestalks and missing only one walking leg), the periods of active basal and PE limb-bud growth are correlated with periods of low circulating ecdysteroids during which the predominant ecdysteroid varies (Hopkins 1992). The bulk of the information in the literature suggests that injected (or infused) ecdysteroids are mostly inhibitory to all stages of regeneration in crustaceans despite the fact that ecdysteroid receptors are present throughout these stages. This underscores the importance of the fine control we see in the levels of ecdysteroids during regeneration in both insects and crustaceans.

Insect limb regeneration is also linked to molting and ecdysteroid levels. Most adult insects, however, do not molt because the PGs in many adult insects degenerate after the last juvenile molt. Adult insects that do not molt may autotomize a limb for defense but very rarely regenerate the lost structure. When adult cockroaches (*Leucophaea maderae* and *P. americanus*) that normally do not regenerate are attached by parabiosis to juvenile nymphs and an adult appendage is removed, the adult will regenerate the limb, but after a much longer period of time. Imaginal discs from *Drosophila* will also regenerate after damage (by cutting or irradiation) but lose their ability to regenerate during the last part of the third instar when ecdysteroid levels are rising as the larva prepares for pupariation. The *wg* gene, which is necessary for disc regeneration, is repressed by ecdysteroids (Mitchell et al. 2008, Smith-Bolton et al. 2009). Regeneration of imaginal discs in *Drosophila* also inhibits the production of ecdysteroids by reducing the expression of the *ptth* gene that is responsible for the activation of ecdysteroidogenesis by the PGs (Halme et al. 2010). This reduction of ecdysteroid levels delays the onset of pupariation and allows for full regeneration of the discs.

Similarly, development of imaginal discs is sensitive to ecdysteroid levels. Discs will only proliferate when implanted into adult abdomens, but will differentiate, metamorphose, and proliferate when implanted into third-instar larvae body cavities. These experiments suggest that there is a blood-borne factor that allows competency and is found only in larval insects. A candidate for such a factor may be the terpenoid JH that is the epoxidated form of retinoic acid. JH has been reported to suppress morphogenetic growth in imaginal discs (Truman and Riddiford 1999), and disc sensitivity to JH appears to be lost during larval development (Madhavan and Schneiderman 1977). In basal insects, JH is not present when appendages begin to grow but reappears as nymphal differentiation occurs. In the flour beetle, *T. castaneum*, RNAi knockdown of the leg patterning genes results in loss of larval appendages and the prevention of the onset of metamorphosis (Suzuki et al. 2009). Partial de-differentiation of appendages in insect larvae maintains high JH levels to prevent metamorphosis. In vitro evidence with eye imaginal discs from the moth *Manduca sexta* showed that the cell fate of these discs requires a "tonic presence" of ecdysteroids but at levels too low to activate the ecdysteroid cascade that leads to metamorphosis/molt (Champlin and Truman 1998). Transplanted *Drosophila* imaginal discs grow more robustly in the abdomen of a female adult than in a male abdomen. Ecdysteroid levels in adult females are low but are not detectable in adult males (Madhavan and Schneiderman 1969). Growth in the male abdomen is enhanced with co-transplantation of a ring gland (containing the PG) or injection of ecdysteroids. In vitro

imaginal discs from the fleshfly, *Sarcophaga peregrina*, in the presence of 2.5×10^{-8} M 20E discs, will form a blastema, yet in the presence of a higher dose (1×10^{-6} M 20E) these same discs will begin to differentiate and elongate. The concentration of ecdysteroid was critical for the correct developmental response of this tissue (Kunieda et al. 1997). Wing discs in *Drosophila* that require ecdysteroid for cell determination will develop normally in the absence of steroid if the Ultraspiracle (USP) partner for ecdysteroid receptor (EcR) is missing (Schubiger and Truman 2000). This suggests that during normal larval development, the USP/EcR complex might inhibit development so that the presence of ecdysteroids releases the suppression, and/or a ligand for USP may function to modulate the activity of EcR. Regeneration and development require low levels of ecdysteroids (as seen in the crustaceans), and the low levels of ecdysteroids that support regeneration are insufficient to trigger ecdysis. These data suggest that ecdysteroids subserve different functions at different concentrations during development. Low levels of ecdysteroid are critical for differentiation, whereas higher levels of the same ecdysteroid are necessary for growth.

The effects of ecdysteroids are mediated by a gene activation cascade that has been known for some time (Clever and Karlson 1960, Ashburner 1973). The genes of the ecdysteroid cascade are ideal candidates for coordinating downstream processes of tissue patterning and morphogenesis (Truman and Riddiford 2002). Ecdysteroids exert their effects by interacting with specific nuclear receptors. The functional receptor for ecdysteroid hormones has been isolated and cloned for both insects and crustaceans (Koelle et al. 1991, Guo et al. 1998, Chung et al. 1998) and is found in abundance in limb bud tissues during regeneration in the crab *U. pugilator* (Chung et al. 1998). mRNA and protein for the functional receptor are expressed in regenerating limb buds during early blastema formation and during the PE stages (Fig. 6.5; Chung et al. 1998, Hopkins et al. 1999, Wu et al. 2004). The dimerization partner of the EcR is the retinoid X receptor (RXR), which is the homologue of USP in insects. There is an 88% deduced amino acid identity (>97% similarity) between the insect EcR and the crustacean EcR. High levels of crustacean EcR mRNA and RXR are found in limb buds when circulating levels of ecdysteroids are lowest. Knocking down the EcR heterodimer complex during basal growth phase in the crab *U. pugilator* disrupts formation and growth of the limb buds. Simultaneous injection of EcR and RXR RNAi into early limb blastemas does not affect migration of epidermal cells into the area but does block the proliferation of the blastema. Taken together, these data suggest that ecdysteroid signaling plays a role in maintaining growth and subsequent differentiation of regenerating limbs in this brachyuran species (Das and Durica 2013) but only at relatively low concentrations.

The in vitro affinity for ecdysteroids of the *Uca* EcR/RXR complex is affected when putative ligands for RXR are present (Hopkins et al. 2008). One such ligand is 9-cis-retinoic acid (9cRA), which is an endogenous compound found in the developing blastema of *Uca* (Hopkins et al. 2008) and has been shown to disrupt blastema organization/development in *Uca*. The presence of retinoids in the developing blastema may be multifold in (i) controlling tissue respecification (Hopkins and Durica 1995); (ii) "fine-tuning" receptor affinity so that the tissues can respond to the obligatory low levels of circulating ecdysteroids, with lower affinity receptor conformations set aside to control physiological responses related to molting of the carapace when circulating ecdysteroid levels are highest (Hopkins et al. 2008); and (iii) acting as a blood-borne signal for controlling other hormone levels that in turn control regeneration (Halme et al. 2010).

The Role of the YO

Ecdysteroids are thought to be produced solely in the YOs of crustaceans (see Lachaise et al. 1993). In the isopod *P. dilatatus*, the YOs are necessary for both basal and PE growth (Noulin and Maissiat 1974). Likewise, it was reported for the crab *C. maenas* that intact YOs were necessary for both basal and PE growth (Echalier 1956). Other work on this same crab suggests that basal growth can occur

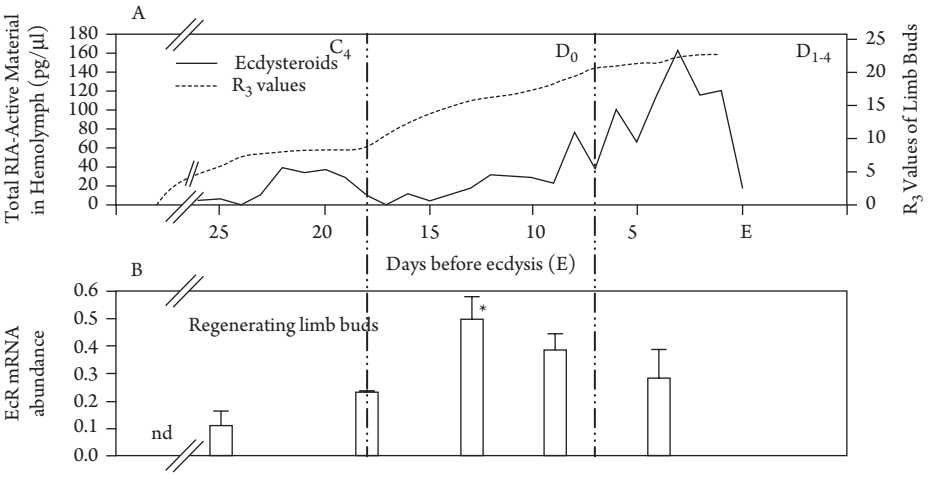


Fig. 6.5.
Distribution of *UpEcR* mRNA in limb buds from the crab *Uca pugilator* during late anecdysis (stage C_4) and proecdysis (stages D_o and D_{1-4}). The solid line in the upper panel (A) represents levels of ecdysteroid (RIA-active material) in hemolymph during the molt cycle, matched backward from ecdysis. The dotted line in (A) represents the same growth curve of an R_3 walking leg as shown in Fig. 6.3. The bottom panel (B) represents the amounts of mRNA extracted from pooled limb buds corresponding to points in the top panel (A). From Chung et al. (1998), with permission from Elsevier.

in the absence of YOs, but PE growth cannot (Bazin and Demeusy 1972, Demeusy 1973). The crab *Sesarma reticulatum* can complete basal growth without YOs but cannot proceed into PE (Jyssum and Passano 1957, Passano and Jyssum 1963). The crabs *P. marmoratus, Pilumnus hirtellus*, and *G. lateralis* can complete both basal growth and PE growth without intact YOs (Charmantier-Daures and Vernet 1974, Charmantier-Daures 1975, Demeusy 1982, Skinner 1985). In some YO-less crabs, the titers of ecdysteroids rise during PE (Charmantier-Daures and DiReggi 1980). Thus, the YOs are not indispensable to the production of ecdysteroids.

EFFECTS OF REGENERATION ON THE CRUSTACEAN MOLT CYCLE AND GROWTH

Regeneration in larval insects delays the onset of a subsequent larval molt (O'Farrell and Stock 1953) and can accelerate subsequent molts in adult firebrats (Buck and Edwards 1990). It was suggested that regenerating tissues were capable of reducing the concentration of molting hormone, and it has recently been shown that regenerating imaginal discs in *Drosophila* can inhibit the release of a prothoracicotropic hormone from the brain and thereby lower ecdysteroid production (Halme et al. 2010). Yet full regeneration requires at least a basal level of ecdysteroids (Madhaven and Schneiderman 1969).

Animals undergoing regeneration of many limbs have smaller postecdysial carapace dimensions (Bennett 1973, Kuris and Mager 1975, Hopkins 1982, Barría and González 2008) and smaller postecdysial limbs (Skinner and Graham 1972, Fingerman and Fingerman 1974, Hopkins 1982). The increase in size of the postecdysial carapace is due to (i) inflation and stretching of the new cells of the hypodermis (Bliss et al. 1966) and (ii) increases in size and number of hypodermal cells. Part of the increase in postecdysial carapace width is due to water uptake at molt (Bliss and Boyer l964). One effect of regeneration on postmolt carapace size is mediated through the mechanical distribution of this water. There is a hormonally fixed amount of water that is taken up at molt.

At molt, newly regenerated limbs that have remained folded throughout PE unfold and become inflated. If the amount of water taken up at molt is limited, then the added volume of many legs to be inflated will decrease the amount of water available to inflate the carapace itself. However, Kuris and Mager (1975) were unable to demonstrate an increase in size in crabs *Hemigrapsus oregonensis* or *Pachygrapsus crassipes* when large limb buds were removed immediately prior to ecdysis. Eyestalkless *Uca* increase in carapace width at molt much more than intact crabs (Abramowitz and Abramowitz 1940). This effect may be due to the loss of eyestalk hormones that restrict the amount of water taken up at molt. Regeneration of many limbs decreases this carapace growth in eyestalkless crabs by the same proportions that it reduces it in intact *U. pugilator* (Hopkins 1982), thus suggesting that whatever the volume of water taken up at molt, the requirement of inflating additional limbs reduces the mechanical aspects of growth at molt.

Regenerated limbs themselves are also smaller than unregenerated limbs from the same animals. Tchernigovtzeff (1965) reported that in the shrimp *Palaemon* and crab *Carcinus* there is a large increase in mitotic figures in the carapace during PE. However, in the crab *G. lateralis*, there is no evidence of mitotic activity during PE in integument from the branchiostegites (Skinner 1962). In *G. lateralis*, a single regenerate is 75% of the size of a nonregenerated limb, whereas when several limbs are autotomized, the postecdysial limbs are 50% of controls (Holland and Skinner 1976). Regenerated limbs in the spider crab *Chionoecetes opilio* are 48% of their former size (Miller and Watson 1976), and regenerated limbs of the large crab *Paralithodes camtschatica* are 35% (for juveniles) and 29% (for adults) of nonregenerated legs (Niwa and Kurata 1964). A second restriction on limb growth may be stored metabolic resources.

In *Uca*, regenerated walking legs from crabs missing eight legs are 68% the size of controls (Hopkins 1982). Postecdysial regenerated limbs also contain only 38% of the protein of control limbs. *Gecarcinus* and *Uca* can regenerate only a fixed amount of protein per molt cycle (Skinner and Graham 1972, Hopkins 1982). This protein is shared by all regenerating limbs. The more limbs that are missing, the smaller the proportion of protein per limb. The amount of protein in a newly regenerated limb is reduced even further if autotomy of many limbs is coupled with eyestalk ablation (Hopkins 1982). When several limbs are autotomized during PE induced by eyestalk removal, there is a prolongation of PE to allow for the organization of the limb bud and subsequent growth prior to ecdysis. There is an incomplete growth of these buds. They grow less during PE, and the molted limbs are smaller with much less protein.

The crab *U. pugilator* not only has a low threshold for molt induction, but there is also a graded effect: the more limbs that are lost, the sooner the onset of PE (Fingerman and Fingerman 1974). Once PE begins in *Uca*, it is of the same duration in crabs missing two as it is in crabs missing eight limbs (Hopkins 1982). The effect of autotomy in *Uca* is then to hasten the onset of PE but not the duration of PE itself. In the crab *P. marmoratus*, loss of eight walking legs reduces the actual length of PE from 40 to 34 days. If limb losses occur during PE, there is an increase in the duration of PE. This effect is seen whether limb loss occurs during a normal PE or during an induced PE (Fingerman and Fingerman 1974).

In many species of brachyurans, loss of multiple limbs during intermolt hastens the onset of PE in order that the missing limbs can be replaced as soon as possible. It was first shown in the crab *G. lateralis* and later in the crab *P. marmoratus* that removal of several walking legs is sufficient to induce PE in intermolt animals (Bliss et al. 1966, Vernet-Cornubert 1961). The sensitivity of animals to autotomy-induced PE varies from group to group. In the xanthid crab *P. hirtellus*, loss of a single limb is sufficient to hasten PE, whereas in *U. pugilator*, loss of at least two walking legs is necessary (Fingerman and Fingerman 1974, Demeusy 1982, Hopkins 1982). In *U. pugilator*, loss of the large male chelae is more effective than loss of a single walking leg in inducing the onset of PE (Hopkins 1982). In *P. marmoratus*, the minimum number of limbs that must be autotomized before PE will begin is four, and in *G. lateralis* five to six legs must be removed to induce PE (Bliss 1956, Rouquette and

Vernet-Cornubert 1964). In *Libinia emarginata*, autotomy of as many as eight walking legs will not induce PE (Skinner and Graham 1972). Moreover, loss of multiple limbs after the beginning of the preparation for molt will halt the process by causing a reduction in circulating ecdysteroids (Holland and Skinner 1976). This reduction is necessary for the early blastema to organize and grow.

The apparently contradictory effects of autotomy and regeneration on the onset and duration of PE in crustaceans can be explained by a single mechanism, if it is assumed that autotomy and regeneration are able to reset the animal and its YO to the substage D_o of PE. Drach (1939) developed a series of stages for the molt cycle of crustaceans. Stage D was reserved for PE, the time during which the animal prepares for molt. Travis (1960) modified Drach's stages to include an additional D_o substage that encompasses the earliest stage of PE (Fig. 6.3). Substage D_o is the period during which PE growth occurs in regenerating limbs and is a time of obligatory low levels of circulating ecdysteroids. If autotomy and regeneration result in physiological conditions that usually occur only at substage D_o, then autotomy performed in intermolt will appear to hasten the onset of PE. Autotomy during D_o will inhibit PE by resetting it back to the beginning of D_o (Skinner and Graham 1972, Hopkins 1982). Both of these contradictory effects suggest that there is some crosstalk between regenerating tissue and the YOs. The feedback ensures optimal levels of ecdysteroid for full regeneration. In *P. marmoratus*, multiple limb loss (and "intensive regenerations") in YO-less crabs causes a shorter PE period when compared to YO-less controls. Both groups have levels of circulating ecdysteroids comparable to intact controls (except during late PE). Thus, in *Pachygrapsus*, autotomy is capable of affecting the duration of PE without affecting the YOs. Other work by these same authors shows that, in *Pachygrapsus* at least, loss of many limbs in YO-intact animals results in elevated levels of circulating ecdysteroids during PE. At this time, it appears that autotomy in crabs stimulates the YOs but also has a stimulatory effect on the onset and duration of PE that is independent of the YOs.

What, then, might be the mechanism responsible for the autotomy-induced resetting of early PE? A putative factor (limb autotomy factor) has been identified in extracts of limb buds from the crab *G. lateralis*. The extracts inhibit PE growth and delay molting, but the exact nature of the putative factor, its source, and its chemical identity remain unknown (Yu et al. 2002). Imaginal discs from *Drosophila* autonomously release an insulin-like peptide that can modulate normal growth of the entire animal to ensure that disc maturation coincides with growth events in the entire animal (Colombani et al. 2012, Garelli et al. 2012). Perhaps such a molecule is produced and released by the developing limb buds of crustaceans. Moreover, regenerating wing discs in *Drosophila* signal the central nervous system via a retinoid intermediate (Halme et al. 2009) to keep ecdysteroid levels low enough to allow for full regeneration. Because retinoids have been identified in the blastemas of a regenerating crustacean, retinoids may play a role in resetting PE in crustaceans (Hopkins et al. 2008).

FUTURE DIRECTIONS

The field of crustacean regeneration control has many unanswered questions and is suitable for further investigation. What is most needed at this point in crustacean regeneration studies (and crustacean endocrinology as well) is a genome of at least one decapod crustacean. Most of the research on crustacean regeneration control has been done on decapods, and these are the animals in which further molecular biological investigation is absolutely essential. There are numerous expressed sequence tag libraries for decapods, but these have limited utility in working out hormone relationships and particularly in examination of control sites in hormonally triggered cascades.

One of the most important unanswered questions is to determine the factors involved in the "resetting" effects of regeneration on the molt cycle. What does it mean exactly to reset the

animal to stage D_o? What compounds are released from the regenerating tissues, and what is their effect on the YO? It appears that the control of the YO production of ecdysteroids is much more complicated than a mere on/off switch mediated by the molt-inhibiting hormone. Is there a positive control of ecdysteroidogenesis from a source other than the eyestalk? Another area that is ready for further study is the actual de-differentiation/respecification of the tissues of the regenerating limb bud. Where do the new tissues arise, and what kinds of growth factors may be involved? It is still unknown as to what sequence the new segments are synthesized and differentiated. Moreover, the specific genes and gene cascades that control early respecification, as well as later hypertrophic growth, are unknown in crustaceans and need to be investigated. There is a wealth of information yet to be mined from the crustacean limb regenerating system, and it may still provide us with new and important information about regenerative mechanisms with wide applications and utilities.

CONCLUSIONS

This review focuses on histological, cellular, and morphological changes during limb regeneration in crustaceans. The regulation of these phenomena and the similarity to leg development and imaginal disc regeneration in insects are discussed. Studies on crustacean limb regeneration have focused extensively on defining two distinct phases: basal growth (which includes wound healing and blastema formation) and PE growth (which includes increases in tissue mass). The regulation of these phases of regeneration in crustaceans appears to vary with the phase. In general, crustacean regeneration is thought to be under the control of both growth factors and hormones, and one of the important aspects of regeneration is how such factors will exert their influence at the cellular and molecular levels. The sources of cells giving rise to different regenerating tissues within the crustacean limb buds are not fully determined. In addition, the molecular networks that produce functional limbs from blastemas are still not completely ascertained. Limb regeneration in crustaceans is coupled with molting and regulated by several blood-borne factors that culminate in changes in gene expression. Because ecdysteroid receptors are expressed in regenerating crustacean limbs throughout the regeneration and molt cycles, it is assumed that ecdysteroids play a role in the control of downstream genes during regeneration. The gene networks that are controlled by ecdysteroids during regeneration are, however, still unknown. These uncertainties are dictating the direction of future limb regeneration studies in crustaceans.

Limb regeneration studies in vertebrates (especially in the salamander *Ambystoma mexicanum*) have revealed that the cell potential of the cells within the blastema is restricted, and this suggests that complete de-differentiation of blastema is not required during vertebrate limb regeneration. In crustaceans, the degree of de-differentiation of epidermal cells in the blastema is still unknown. The tissues arising from the blastema are quite diverse: for example, the de-differentiated epidermal cells of the blastema give rise to muscle, cuticle, nerve, chromatophores, and more. This review also discusses the molecular networks and genes associated with regenerating imaginal discs in *Drosophila* and the parallel networks that may also be involved in differentiation process in crustaceans. In *Drosophila*, during regeneration of damaged imaginal discs, the growth of the remaining intact disc is held up. This retardation appears to be due to release of growth-controlling factors from the injured disc tissue. Similarly in crustaceans, the autotomy of a limb can retard the regenerating process of previously autotomized limbs that are in basal growth, but the growth-controlling compounds in crustaceans are still under investigation. Leg developmental processes are compared to leg regenerative processes in both insects and crustaceans, and it is generally supposed that the gene cascades and the control mechanisms in both development and regeneration are closely related.

REFERENCES

Abramowitz, R.K., and A. Abramowitz. 1940. Moulting, growth, and survival after eyestalk removal in *Uca pugilator*. Biological Bulletin 78:179–188.

Adelung, D. 1964. Der EinfluB der Umweltfaktoren und der innersekretorischen Organe den autungszyklus. Zoologische Anzeiger 30:264–272.

Adelung, D. 1971. Untersuchungen zur Häutungsphysiologie der dekapoden Krebse am Beispiel der Strandkrabbe *Carcinus maenas*. Helgoliinder wiss. Meeresunters 22:66–119.

Adiyodi, R. 1972. Wound healing and regeneration in the crab, *Parathelphusa hydrodromus*. International Review of Cytology 30:257–290.

Agar, W.E. 1930. A statistical study of regeneration in two species of Crustacea. Journal of Experimental Biology 7:349–369.

Aiken, D.E. 1969. Photoperiod, endocrinology, and the crustacean molt cycle. Science 164:149–155.

Anderson, B.G. 1933. Regeneration in the carapace of *Daphnia magna.* I. The relation between the amount of regeneration and the area of the wound during single adult instars. Biological Bulletin 64:70–85.

Ashburner, M. 1973. Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster.* I. Dependance upon ecdysone concentration. Developmental Biology 35:47–61.

Averof, M., and N.H. Patel. 1997. Crustacean appendage evolution associated with changes in *Hox* gene expression. Nature 388:682–686.

Baek, S.H., Y-C. Kwon, H. Lee and K-M Cho. 2010. Rho-family small GTPases are required for cell polarization and directional sensing in *Drosophila* wound healing. Biochemical and Biophysical Research Communications 394:488–492.

Barría, E.M., and M.I. González. 2008. Effect of autotomy and regeneration of the chelipeds on growth and development in *Petrolisthes laevigatus* (Guerin, 1835) (Decapoda, Anomura, Porcellanidae). Crustaceana 81:641–652.

Bazin, F. 1977. Effets d'injections d'ecdysterone sur la mue et la regeneration chez le crabe *Carcinus maenas*. Comptes Rendus de l Académie des Sciences 284:765–768.

Bazin, F. and N. Demeusy. 1972. Processus de cicatrisation consecutif a l'autotomie d'un pereiopode chez le crabe *Carcinus maenas*(L.). Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences, Serie D 274:2603–2605.

Bergantinos, C., X. Vilana, M. Corominas, and F. Serras. 2010. Imaginal discs: Renaissance of a model for regenerative biology. BioEssays 32:207–217.

Bennett, D.B. 1973. The effect of limb loss and regeneration on the growth of the edible crab, *Cancer pagurus* L. Journal of Experimental Marine Biology and Ecology 13:45–53.

Bliss, D.E. 1956. Neurosecretion and the control of growth in a decapod crustacean. Pages 56–75 *in* K.G. Wingstrand, editor. Betril Hanstrom, Zoological Papers in Honour of His Sixty-fifth Birthday. Lund, Sweden: Zoological Institute.

Bliss, D.E., and J.R. Boyer. 1964. Environmental regulation of growth in the decapod crustacean *Gecarcinus lateralis*. General and Comparative Endocrinology 4:15–41.

Bliss, D.E., S.M.E. Wang, and E.A. Martinez. 1966. Water balance in the land crab, *Gecarcinus lateralis*, during the intermolt cycle. American Zoologist 6:197–212.

Bodenstein, D. 1955. Contributions to the problem of regeneration in insects. Journal of Experimental Zoology 129:209–224.

Bodenstein, D. 1957. Studies on nerve regeneration in *Periplaneta americana*. Journal of Experimental Zoology 136:89–116.

Bosch M., F. Serras, E. Martin-Blanco, and J. Baguna. 2005. JNK signaling pathway required for wound healing in regenerating *Drosophila* wing imaginal discs. Developmental Biology 280:73–86.

Buck, C., and J.S. Edwards. 1990. The effect of appendage and scale loss on instar duration in adult firebrats, *Thermobia domestica* (Thysanura). Journal of Experimental Biology 151:341–347.

Champlin, D.T., and J.W. Truman. 1998. Ecdysteroids govern two phases of eye development during metamorphosis of the moth, *Manduca sexta*. Development 125:2009–2018.

Charmantier-Daures, M. 1975. Relations entre l'organe Y, la mue et la regeneration chez *Pachygrapsus marmoratus* (Crustace, Decapode). Archives de Zoologie Experimentale et Generale 116:481–495.

Charmantier-Daures, M. 1976. Action de la regeneration intensive et de l'ecdysterone sur la mue de *Pachygrapsus marmoratus* (Decapode, Brachyoure). Archives de Zoologie Experimentale et Generale 115:395–410.

Charmantier-Daures, M., and M. De Reggi. 1980. Aspects preliminaires des variations hemolymphatiques du taux d'ecdysteroides chez *Pachygrapsus marmoratus* (Crustace, Decapode): influence de la regeneration intensive et de l'ablation des organes Y. Bulletin de la Société Zoologique de France 105:81–86.

Charmantier-Daures, M., and G. Vernet. 1974. Nouvelles données sur le rôle de l'organe Y dans le déroulement de la mue chez *Pachygrapsus marmoratus* (Décapode, Grapsidé). Influence de la régénération intensive. Comptes Rendus de l Académie des Sciences 278:3367–3370.

Charniaux-Cotton, H. 1957. Croissance, regeneration et determinisme endocrinien des caracteres sexuels *d'Orchestia gammarella* Pallas (Crustace Amphipode). Annales Des Sciences Naturelles 19:411–560.

Chung, A.C., D.S. Durica, and P.M. Hopkins. 1998. Tissue-specific patterns and steady-state concentrations of ecdysteroid receptor and retinoid-x-receptor mRNA during the molt cycle of the fiddler crab, *Uca pugilator*. General and Comparative Endocrinology 109:375–389.

Clever, U., and P. Karlson. 1960. Induktion von Puff-Verainderungen in den Speicheldrusenchromosomen von *Choronomus tentans* durch Ecdyson. Experimental Cell Research 20:623–626.

Cohen, S.M., and G. Jürgens. 1989. Proximal-distal pattern formation in *Drosophila*: cell autonomous requirement for *Distal-less* gene activity in limb development. European Molecular Biology Organization Journal 8:2045–2055.

Cohen, S.M., G. Bronner, F. Kuttner, G. Jürgens, and H. Jackie. 1989. Distalless encodes a homeodomain protein required for limb development in *Drosophila*. Nature 338:432–434.

Colombani, J., D. Andersen, and P. Leopold. 2012. Secreted peptide Dilp8 coordinates Drosophila tissue growth with developmental timing. Science 336:582–585.

Das, S. and D.S. Durica. 2013. Ecdysteroid receptor signaling disruption obstructs blastemal cell proliferation during limb regeneration in the fiddler crab, *Uca pugilator*. Molecular and Cellular Endocrinology 365:249–259.

Demeusy, N. 1973. Libération d'appendices thoraciques au cours de leur régénération chez le crabe *Carcinus maenas* L. et poursuite de leur evolution. Cahiers de Biologie Marine 14:189–204.

Demeusy, N. 1982. Mue, glande de mue et régénération chez le Xanthidae *Pilumnus hirlellus* (Crust. Décapodes Brachyoures). Comptes Rendus de l'Académie des Sciences 294:823–826.

Dillaman, R.M. and R.D. Roer. 1980. Carapace repair in the green crab, *Carcinus maenas* (L.). Journal of Morphology 163:135–155.

Dixey, L.R. 1938. Effects of repeated regeneration of the antenna in *Gammarus chevreuxi*. Proceedings of the Zoological Society London A108:289–296.

Drach, P. 1939. Mue et cycle d'intermue chez les Crustaces Décapodes. Annales de l'Institut Oceanographique, Paris 19:103–391.

Echalier,G. 1956. Influence de l'organe Y sur la régénération des pattes, chez *Carcinides maenas* L. (Crustacé Décapode), Comptes Rendus de l'Académie des Sciences 242:2179–2180.

Findlay, I., and A.R. McVean. 1977. The nervous control of limb autotomy in the hermit crab *Pagurus bernhardus* (L.) and the role of the cuticular stress detector, CSD1. Journal of Experimental Biology 70:93–104.

Fingerman, M., and S.W. Fingerman. 1974. The effects of limb removal on the rates of ecdysis of eyed and eyestalkless fiddler crabs, *Uca pugilator*. Zoologische Jahrbucher-Abteilung fur Allgemeine Zoologie und Physiologie der Tiere 78:301–309.

Fleming, P.A., D. Muller, and P.W. Bateman. 2007. Leave it all behind: a taxonomic perspective of autotomy in invertebrates. Biological Reviews 82:481–510.

Flint, R.W. 1972. Effects of eyestalk removal and ecdysterone infusion on moulting in *Homarus americanus*. Journal of the Fisheries Research Board of Canada 20:1229–1233.

Fontaine, C.T., and D.V. Lightner. 1973. Observations on the process of wound repair in penaeid shrimp. Journal of Invertebrate Pathology 2:23–33.

Garcia-Fernandez, B., I. Campos, J. Geiger, and A.C. Santos. 2009. Epithelial resealing. International Journal of Developmental Biology 53:1549–1556.

Garelli, A., A. Gontijo, V. Miguela, E. Caparros, and M. Dominquez. 2012. Imaginal discs secrete insulin-like peptide 8 to mediate plasticity of growth and maturation. Science 336: 579–582.

Giesbrecht, W. 1910. Stomatopoden. Fauna und Flora des Golfs von Neapel 33:1–11.
Gilgan, M.W., T.E. Farquharson, and B.G. Burns. 1977. The effect of α-ecdysone, ecdysterone and inokosterone treatment, separately or in combinations, on proecdysial development and molting in adult male lobsters (*Homarus americanus*). Comparative Biochemistry and Physiology 56A:43–49.
Giordano, A. 1961. Sur la regeneration des pereiopodes de Isopode *Idotea baltica* (Aud.). Comptes Rendus de l'Académie des Sciences 253:554–555.
Gordon, H.T., and J.H. Welsh. 1948. The role of ions in axon surface reactions to toxic organic compounds. Journal of Cellular and Comparative Physiology 31:395–419.
Guo, X., Q. Xu, M.A. Harmon, X. Jin, V. Laudet, D.J. Mangelsdorf, and M.J. Palmer. 1998. Isolation of two functional retinoid X receptor subtypes from the Ixodid tick, *Amblyomma americanum* (L.). Molecular and Cellular Endocrinology 139:45–60.
Hadorn, E., and D. Buck. 1962. Uber entwicklungsleistungen transplantierter teilstucke von flugel-imaginalscheiben von *Drosophila melanogaster*. Revue Suisse de Zoologie 69:302–310.
Halme, A., M. Cheng, and I.K. Hariharan. 2010. Retinoids regulate a developmental checkpoint for tissue regeneration in *Drosophila*. Current Biology 20:458–463.
Hoarau, F. 1973. Comportement de l'hypoderme et progression de la différenciation au cours de la régénération d'un péréiopode chez l'Isopode terrestre *Helleria brevicornis* Ebner. Annals of Embryology and Morphology 6:125–135.
Holland, C.A., and D.M. Skinner. 1976. Interactions between molting and regeneration in the land crab. Biological Bulletin 150:222–240.
Hopkins, P.M. 1982. Growth and regeneration patterns in the fiddler crab, *Uca pugilator*. Biological Bulletin 163:301–319.
Hopkins, P.M. 1983. Patterns of serum ecdysteroids during induced and uninduced proecdysis in the fiddler crab, *Uca pugilator*. General and Comparative Endocrinology 52:350–356.
Hopkins, P.M. 1985. Regeneration and relative growth in the fiddler crab. Pages 265–275 *in* A.M. Wenner, editor. Crustacean. Issues 3. Factors in Adult Growth. A.A. Balkema Rotterdam.
Hopkins, P.M. 1989. Ecdysteroids and regeneration in fiddler crabs, *Uca pugilator*. Journal of Experimental Zoology 252:293–299.
Hopkins, P.M. 1992. Hormonal control of the molt cycle in the fiddler crab, *Uca pugilator*. American Zoologist 32:450–458.
Hopkins, P.M. 1993. Regeneration of walking legs in the fiddler crab, *Uca pugilator*. American Zoologist 33:348–356.
Hopkins, P.M. 2001. Limb regeneration in the fiddler crab, *Uca pugilator*: hormonal and growth factor control. American Zoologist 41:389–398.
Hopkins, P.M. and D.S. Durica. 1995. Effects of all-trans retinoic acid on regenerating limbs of the fiddler crab, *Uca pugilator*. Journal of Experimental Zoology 272:455–63.
Hopkins, P.M., D.E. Bliss, S.W. Sheehan, and J.R. Boyer. 1979. Limb growth-controlling factors in the crab *Gecarcinus lateralis*, with special reference to the limb growth-inhibiting factor. General and Comparative Endocrinology 39:192–207.
Hopkins P.M., A.C-K. Chung, and D.S. Durica. 1999. Limb regeneration in fiddler crab, *Uca pugilator*: histological, physiological and molecular considerations. American Zoologist 39:513–526.
Hopkins, P.M., D.S. Durica, and T. Washington. 2008. RXR isoforms and endogenous retinoids in the fiddler crab, *Uca pugilator*. Comparative Biochemistry Physiology A 151:602–614.
Jiravanichpaisal, P., S.Y. Lee, Y.A. Kim, T. Andren, and I. Söderhäll. 2007. Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: characterization and expression pattern. Developmental and Comparative Immunology 31:441–455.
Juanes, F., and L.D. Smith. 1995. The ecological consequences of limb damage and loss in decapod crustaceans: a review and prospectus. Journal of Experimental Marine Biology and Ecology 193:197–223.
Jyssum, S., and L.M. Passano. 1957. Endocrine regulation of preliminary limb regeneration and molting in the crab *Sesarma*. Anatomical Record 128:571–572.
Koelle, M.R., W.S. Talbot, W.A. Segraves, M.T. Bender, P. Cherbas, and D.S. Hogness. 1991. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell 67:59–77.

Krishnakumaran, A. 1972. Injury induced molting in *Galleria mellonella* larvae. Biological Bulletin 142:281–292.
Krishnakumaran, A., and H.A. Schneiderman. 1969. Induction of molting in Crustacea by an insect molting hormone. General and Comparative Endocrinology 12:515–518.
Krishnakumaran, A., and H.A. Schneiderman. 1970. Control of molting in mandibulate and chelicerate arthropods by ecdysones. Biological Bulletin 139:520–528.
Kumar, A., J.W. Godwin, P.B. Gates, A.A. Garza-Garcia, and J.P. Brockes. 2007. Molecular basis for the nerve dependence of limb regeneration in an adult vertebrate. Science 318:772–777.
Kunieda, T., S. Kurata, and S. Natori. 1997. Regeneration of Sarcophaga imaginal discs in vitro: implication of 20-hydroxyecdysone. Developmental Biology 183:86–94.
Kuris, A.M., and M. Mager. 1975. Effect of limb regeneration on size increase at molt of the shore crabs *Hemigrapsus oregonensis* and *Pachygrapsus crassipes*. Journal of Experimental Zoology 193:353–360.
Kwon, Y-C., S.H. Baek, H. Lee, and K-M. Choe. 2010. Nonmuscle myosin II localization is regulated by JNK during *Drosophila* larval wound healing. Biochemical and Biophysical Research Communications 393:656–661.
Lachaise F., A. Le Roux, M. Hubert, and R. Lafont. 1993. The molting gland of crustaceans: localization, activity, and endocrine control (a review). Journal of Crustacean Biology 13:198–234.
Lang, F., M. Oganowski, R. Hill, B. Roehrig, K. Kent, and J. Sellers. 1980. Neurotrophic influence on lobster skeletal muscle. Science 207:325–327.
Liubicich, D.M., J.M. Serano, A. Pavlopoulos, Z. Kontarkis, M. Protas, E. Kwan, S. Chatterjee, K. Tran, M. Averof, and N.H. Patel. 2009. Knockdown of *Parhyale* Ultrabithorax recapitulates evolutionary changes in crustacean appendage morphology. Proceedings of the National Academy of Sciences 106:13892–13896.
Lowe, M.E., D.H.S. Horn, and M.N. Galbraith. 1968. The role of crustecdysone in the moulting crayfish. Experientia 24:518–519.
Mace, K.A., J.C. Pearson, and W. McGinnis. 2005. An epidermal barrier wound repair pathway in *Drosophila* is mediated by *grainy head*. Science 308:381–385.
Madhavan K., and H.A. Schneiderman. 1969. Hormonal control of imaginal regeneration in *Galleria mellonella* (Lepidoptera). Biological Bulletin 137:321–331.
Madhavan, K., and H.A. Schneiderman. 1977. Histological analysis of the dynamics of growth of imaginal discs and histoblast nests during larval development of *Drosophila melanogaster*. Roux's Archives of Developmental Biology 183:269–305.
Maginnis, T.L. 2006. The costs of autotomy and regeneration in animals: a review and framework for future research. Behavioral Ecology 17:857–872.
Matozzo, V., and M.G. Marin. 2010. The role of haemocytes from the crab *Carcinus aestuarii* (Crustacea, Decapoda) in immune responses: a first survey. Fish Shellfish Immunology 28:534–541.
McCarthy, J.F. 1982. Ecdysone metabolism in proecdysial land crabs (*Gecarcinus lateralis*). General and Comparative Endocrinology 47:323–332.
McClure, K.D., and G. Schubiger. 2007. Transdetermination: *Drosophila* imaginal disc cells exhibit stem cell-like potency. International Journal of Biochemical and Cellular Biology 39:1105–1118.
McConaugha, J.R., and J.D. Costlow. 1980. Regeneration in larvae and juveniles of the mud crab, *Rhithropanopeus harrisii*. Journal of Experimental Zoology 213:247–256.
McPeek, M.A. 1997. Measuring phenotypic selection on an adaptation: lamellae of damselflies experiencing dragonfly predation. Evolution 51:459–466.
McVean, A.R. 1975. Autotomy. Comparative Biochemistry and Physiology A. 51:497–505.
McVean, A.R. 1984. Autotomy. Pages 107–132 *in* D.E. Bliss, editor. The biology of Crustacea. Academic Press, New York.
McVean, A.R., and I. Findlay. 1976. Autotomy in *Carcinus maenas*: the role of the basi-ischiopodite posterior levator muscle. Journal of Comparative Physiology 110:367–381.
Meinhardt, H. 1983. A boundary model for pattern formation in vertebrate limbs. Journal of Embryology and Experimental Morphology 76:115–137.
Mellon, DeF. 1981. Nerves and the transformation of claw type in snapping shrimps. Trends in Neuroscience 4:245–248.

Mellon, DeF., and P.J. Stephens. 1978. Limb morphology and function are transformed by contra lateral section in snapping shrimps. Nature 272:246–248.

Miller, R.J., and J. Watson. 1976. Growth per molt and limb regeneration in the spider crab, *Chionoecetes opilio*. Journal of the Fisheries Research Board of Canada 33:1644–1649.

Mitchell, N., N. Cranna, H. Richardson, and L. Quinn. 2008. The Ecdysone inducible zinc-finger transcription factor Crol regulates Wg transcription and cell cycle progression in *Drosophila*. Development 135:2707–2716.

Mittenthal, J.E., and W.W. Trevarrow. 1983. Intercalary regeneration in legs of crayfish: central segments. Journal of Experimental Zoology 225:15–31.

Mittenthal, J.E., M.C. Olson, and G.D. Cummings. 1980. Morphology of the closer muscles in normal and homoeotic legs of crayfish. Biological Bulletin 159:714–727.

Mobberly, W.C. Jr. 1963. Hormonal and environmental regulation of the molting cycle in the crayfish *Faxonella clypeata*. Tulane Studies in Zoology 11:79–96.

Moussian, B., and A.E. Uv. 2005. An ancient control of epithelial barrier formation and wound healing. BioEssays 27:987–990.

Nakamura, T., T. Mito, T. Bando, H. Ohuchi, and S. Noji. 2008. Dissecting leg regeneration through RNA interference. Cellular and Molecular Life Sciences 65:64–72.

Nakatogawa, S., Y. Oda, M. Kamiya, T. Kamijima, T. Aizawa, K.D. Clark, M. Demura, K. Kawano, M.R. Strand, and Y. Hayakawa. 2009. A novel peptide mediates aggregation and migration of hemocytes from an insect. Current Biology 19:779–785.

Needham, A.E. 1945. Peripheral nerve and regeneration in Crustacea. Journal of Experimental Biology 21:144–146.

Needham, A.E. 1947. Local factors and regeneration in Crustacea. Journal of Experimental Biology 24:220–226.

Needham, A.E. 1965. Regeneration in the Arthropoda and its endocrine control. Pages 283–323 *in* Kiortsis, V., and H.A.L. Trampusch, editors. Regeneration in animals and related problems. International Symposium 1964. North Hall Publishing Company, Amsterdam.

Niwa, K., and Kurata, H. 1964. Limb loss and regeneration in the adult king crab *Paralithodes camtschatica*. Bulletin of the Hokkaido Region Fisheries Research Lab 28:51–55.

Noulin, G., and J. Maissiat. 1974. Etude du role de l'organe Y et de l'effet de l'ecdysterone dans la regeneration d'un appendice chez l'oniscoi'de *Porcellio dialatatus*. Journal of Insect Physiology 20:1963–1974.

O'Farrell, A.F., and A. Stock. 1953. Regeneration and the moulting cycle in *Blattella germanica*: single regeneration initiated during the first instar. Australian Journal of Biological Sciences 6:485–500.

Panganiban, G., L.M. Nagy, and S.B. Carroll. 1994. The role of the *distal-less* gene in the development and evolution of insect limbs. Current Biology 4:671–675.

Panganiban, G., A. Sebring, L.M. Nagy, and S.B. Carroll. 1995. The development of crustacean limbs and the evolution of arthropods. Science 270:1362–1366.

Passano, L.M. 1960. Molting and its control. Pages 473–536 *in* T.H. Waterman, editor. The physiology of Crustacea. New York and London, Academic Press, Inc.

Passano, L.M., and S. Jyssum. 1963. The role of the Y-organ in crab proecdysis and limb regeneration. Comparative Biochemistry and Physiology 9:195–213.

Pavlopoulos, A., and M. Averof. 2002. Developmental evolution: Hox protein ring the changes. Current Biology 12:291–293.

Ramet, M., R. Lanot, D. Zachary, and P. Manfruelli. 2002. JNK signaling pathway is required for efficient wound healing in *Drosophila*. Developmental Biology 241:145–156.

Rao, K.R. 1965. Studies on the influence of environmental factors on growth in the crab *Ocypode macrocera*. H. Milne Edwards. Crustaceana 11:257–276.

Rao, K.R. 1978. Effects of ecdysterone, inokosterone and eyestalk ablation on limb regeneration in the fiddler crab, *Uca pugilator*. Journal of Experimental Zoology 203:257–269.

Rosello-Diez, A., M.A. Ros, and M. Torres. 2011. Diffusible signals, not autonomous mechanisms, determine the main prosimodistal limb subdivisions. Science 332:1086–1088.

Rouquette, M., and G. Vernet-Cornubert. 1964. Influence de la temperature sur la mue et la régénération chez le crabe *Pachygrapsus marmoratus*. Fabricius—Archives de Zoologie Expérimentale et Générale, et Notes et Revues 2:104–126.

Schubiger, G. 1971. Regeneration, duplication and transdetermination in fragments of the leg disc of *Drosophila*. Developmental Biology 26:277–295.

Schubiger, M., and J.W. Truman. 2000. The RXR-ortholog USP suppresses early metamorphic processes in the absence of ecdysteroids. Development 127:1151–1159.

Shiga, Y., K. Sagawa, R. Takai, H. Sakaguchi, and H. Yamagata. 2006. Transcriptional read through of *Hox* genes *Ubx* and *Antp* and their divergent post-transcriptional control during crustacean evolution. Evolution and Development 8:407–414.

Singer, M. 1952. The influence of the nerve in regeneration of the amphibian extremity. Quarterly Review of Biology 27:169–200.

Skinner, D.M. 1962. The structure and metabolism of a crustacean integumentary tissue during a molt cycle. Biological Bulletin 123:635–647.

Skinner, D.M. 1985. Molting and regeneration. Pages: 43–146 *in* Bliss and L.H. Mantel, editors. The biology of Crustacea. D.E. New York: Academic Press.

Skinner, D.M., and D.E. Graham. 1972. Loss of limbs as a stimulus to ecdysis in brachyura (true crabs). Biological Bulletin 143:222–233.

Slack, J.M. 2003. Regeneration research today. Developmental Dynamics 226:162–166.

Smith-Bolton, R.K., M.I. Worley, H. Kanda, and I.K. Hariharan. 2009. Regenerative growth in *Drosophila* imaginal discs is regulated by Wingless and Myc. Developmental Cell 16: 797–809.

Stephens, G.C. 1955. Induction of molting in the crayfish, *Cambarus*, by modification of daily photoperiod. Biological Bulletin 108:235–241.

Suzuki, Y., D.C. Squires, and L.M. Riddiford. 2009. Larval leg integrity is maintained by *Distal-less* and is required for proper timing of metamorphosis in the flour beetle, *Tribolium castaneum*. Developmental Biology 326:60–67.

Tchernigovtzeff, C. 1965. Multiplication cellulaire et régénération au cours du cycle d'intermue des crustacés décapodes. Archieve de Zoologie Experimentale et Generale 106:377–497.

Theopold, U., O. Schmidt, K. Söderhäll, and M.S. Dushay. 2004. Coagulation in arthropods: defence, wound closure and healing. Trends in Immunology 25:289–294.

Tighe-Ford, D.J., and D.C. Vaile. 1974. Wound-stimulated moulting and calcification responses in the adult cirripede *Balanus balanofdes* (L.). Journal of Experimental Marine Biology and Ecology 14:295–306.

Travis, D. 1960. The deposition of skeletal structures in the Crustacea. I. The histology of the gastrolith skeletal tissue complex in the crayfish *Orconectes (Cambarus) virilis* Hagen—Decapoda. Biological Bulletin 118:137–149.

Trinkaus-Randall, V. 1982. Regeneration of transplanted chelae in two species of fiddler crabs Uca *pugilator* and *Uca pugnax*. The Journal of Experimental Zoology 224:13–24.

Truman, J.W., and L.M. Riddiford. 1999. The origins of insect metamorphosis. Nature 410:447–452.

Truman, J.W., and L.M. Riddiford. 2002. Endocrine insights into the evolution of metamorphosis in insects. Annual Review of Entomology 47:467–500.

Uetz, G.W., W.J. McClintock, D. Miller, E.I. Smith, and K.K. Cook. 1996. Limb regeneration and subsequent asymmetry in a male secondary sexual character influences sexual selection in wolf spiders. Behavioral Ecology and Sociobiology 38:253–257.

Vafopoulou, X., H. Laufer, and C.G.H. Steel. 2007. Spatial and temporal distribution of the ecdysteroid receptor (EcR) in haemocytes and epidermal cells during wound healing in the crayfish, *Procambarus clarkii*. General and Comparative Endocrinology 152:359–370.

Varese, J. 1960. Sur la regeneration des antennes de l'isopode *Idotea baltica* (Aud.). Comptes Rendus de l'Académie des Sciences Paris 250:3399–3400.

Vernet-Cornubert, G. 1961. Connaissances actuelles sur le determinisme hormonal de la mue chez les Decapodes et etude de quelques phenomenes qui lui sont lies. Archives de Zoologie Experimentale et Generale 99:57–76.

Wang, J., and Y. Xia. 2010. Construction and preliminary analysis of a normalized cDNA library from *Locusta migratoria manilensis* topically infected with *Metarhizium anisopliae* var. *acridum*. Journal of Insect Physiology 56:998–1002.

Wasson, K., B.E. Lyon, and M. Knope. 2002. Hair-trigger autotomy in porcelain crabs is a highly effective escape strategy. Behavioral Ecology 13:481–486.

Weis, J.S. 1976. Effects of environmental factors on regeneration and molting in the fiddler crabs. Biological Bulletin 150:152–162.

Weis, J.S., and L.H. Mantel. 1976. DDT as an accelerator of limb regeneration and molting in fiddler crabs. Estuary and Coastal Marine Science 4:461–466.

Wood, F.D., and H.E. Wood. 1932. Autotomy in decapod Crustacea. Journal of Experimental Zoology 62:1–49.

Woodruff, L.C. 1937. Autospasy and regeneration in the roach *Blattella germanica* (Linnaeus). Journal of the Kansas Entomological Society 10:1–9.

Wu, X., P.M. Hopkins, and D.S. Durica. 2004. Observations of autotomy-independent limb regeneration in the fiddler crab, *Uca pugilator*. SAAS Bull. Biochemistry and Biotechnology 17:9–19.

Xia, Y., and M. Karin. 2004. The control of cell motility and epithelial morphogenesis by Jun kinases. Trends in Cell Biology 14:94–101.

Yu, X., E.S. Chang, and D.L. Mykles. 2002. Characterization of limb autotomy factor-proecdysis isolated from limb regenerates that suspends molting in the land crab, *Gecarcinus lateralis*. Biological Bulletin 202:204–212.

7

CIRCULATORY PHYSIOLOGY

Iain J. McGaw and Carl L. Reiber

Abstract

The circulatory system of the subphylum Crustacea varies significantly in both anatomical and physiological complexity. The most highly developed system occurs in decapods, where precise physiological control mechanisms rival some simple vertebrate closed systems. Changes in heart rate have been used as an important indicator of stress and energy expenditure, and recent technological advances allow measurement of stroke volume, cardiac output, and hemolymph redistribution in intact animals. Decapods regulate the amount of hemolymph delivered through each arterial system via the muscular cardioarterial valves or by changing downstream resistance in the vessels. A number of naturally occurring peptide and amine neurohormones modulate cardiac activity and differential hemolymph flow by acting directly on the heart and cardiac nerves, or cardioarterial valves and artery walls. This chapter covers the basic functional anatomy of the heart and vessels; developmental, neurological, and hormonal control mechanisms; and cardiovascular responses to environmental and biotic perturbations in decapod crustaceans.

INTRODUCTION

The cardiovascular system of the subphylum Crustacea varies significantly in both anatomical and physiological complexity. The circulatory system is rudimentary in the class Cirripedia and consists of a series of ill-defined sinuses. There is no heart associated with these sinuses; instead, the hemolymph is circulated by way of contraction of the cirri and gut muscles. In the classes Branchiopoda and Ostracoda, a single-chambered heart is evident, which may be globular or tubular in shape. Most of the crustaceans within these classes lack blood vessels; hemolymph circulates directly from

the heart into the hemocoel of the body before emptying back into the heart via paired openings or ostia (Maynard 1960, McLaughlin 1983). The circulatory system increases in complexity in the orders Isopoda, Peracarida, and Mysidacea, consisting in these of a segmental heart and associated vessels (Wirkner and Richter 2003, 2007a,b, 2008). The most highly developed circulatory system is seen in the order Decapoda. A single-chambered heart pumps hemolymph into a well-defined series of arteries that split into capillary-like vessels that ramify among the tissues. Some of the areas of the system, as well as its physiological control mechanisms, are highly developed (Reiber and McGaw 2009).

A number of the most highly cited articles on crustacean cardiovascular anatomy and physiology are published in the extensive series *The Biology of Crustacea* (Bliss and Mantel 1983). These have remained the standard texts for crustacean cardiovascular anatomy and physiology for nearly three decades. McLaughlin's (1983) chapter on internal anatomy includes reference to fine historical articles spanning more than a century and a half. However, most of these articles were written at the beginning of the past century or the end of the 19th century, before the advent of modern imaging techniques allowed the detailed analysis of the circulatory system to be resolved (Haeckel 1857, Bouvier 1891, Pearson 1908, Brody and Perkins 1930). Although a few review articles and specialized book chapters on the physiology of the cardiovascular system have been written within the past two decades, McMahon and Wilkens (1983) chapter on ventilation, perfusion, and oxygen (O_2) uptake has remained the gold standard for crustacean cardiorespiratory physiology. Nonetheless, the crustacean circulatory system has received extensive attention since this work was published. The system is now recognized as being relatively complex, with precise physiological control mechanisms to shunt blood through specific arterial systems and pressures that rival some of the simple vertebrate closed systems (McMahon and Burnett 1990, Wilkens 1999a, McMahon 2001, Reiber and McGaw 2009). The circulatory system of decapod crustaceans may now (at least in physiologically terms) be classified as one that is partially closed, rather than open (Reiber and McGaw 2009).

In addition to anatomical and morphological adaptations, the cardiovascular physiology of crustaceans has also been studied extensively. For more than half a century, changes in heart rate have been used consistently as an important measure of stress and energy expenditure in crustaceans (deFur and Mangum 1979, Handy and Depledge 1999, Brown et al. 2004). However, heart rate alone is not an accurate means of assessing the total amount of hemolymph (cardiac output) delivered to the system. The cardiac output is also dependent on stroke volume of the heart, which can vary independently of heart rate (McGaw and McMahon 1996). Although various methods, such as the Fick principle and thermodilution (Johansen et al. 1970, Burnett 1979, McMahon et al. 1979, Burnett and Bridges 1981, Bradford and Taylor 1982, Wilkes and McMahon 1982), were used to calculate cardiac output and stroke volume of the heart, they had their limitations (Airriess and McMahon 1994). More recently, the development of less invasive techniques such as the pulsed-Doppler flowmeter, sonomicrometry, and digital imaging techniques have allowed greater detail to be resolved in a wider array and size of species (Airriess et al. 1994, Paul et al. 1997, Reiber et al. 1997, Bock et al. 2001, Guadagnoli and Reiber 2005).

Decapod crustaceans are able to regulate the amount of hemolymph delivered through each arterial system via the muscular cardioarterial valves at the entrance of each major artery or by changing the downstream resistance in the vessels (Wilkens 1997, 1999a, McGaw and Reiber 2002). This is not only important for efficient delivery of nutrients and gases, but the diversion of flow to metabolically active tissues also may enhance the ability of animals to cope with environmental changes. Cardiac parameters and hemolymph flow rates have been found to change in response to exercise (DeWachter and McMahon 1996a, McGaw and McMahon 1998), feeding (McGaw and Reiber 2000, McGaw 2006a), hypoxia (Airriess and McMahon 1994, Reiber 1995b,

Reiber and McMahon 1998), emersion (Airriess and McMahon 1996), low salinity (McGaw and McMahon 1996, McGaw and Reiber 1998, McGaw 2006b), and temperature (DeWachter and McMahon 1996b).

Cardiac function and blood flow are controlled by both neural and hormonal control mechanisms. Neural control can occur via the action of the cardioregulatory nerves (inhibitory and acceleratory fibers) that influence both the rate and force of contraction of the heart by acting on the cardiac ganglion. Additionally, the tonus of the cardioarterial valves can be modulated through neuronal stimulation, which regulates hemolymph flow (Kuramoto and Kuwasawa 1980, Kuramoto and Ebara 1984). A number of naturally occurring peptide and amine neurohormones modulate cardiac activity and differential hemolymph flow in crustaceans (Saver and Wilkens 1998, Wilkens and Kuramoto 1998, Wilkens 1995, 1999a). These hormones act on the cardioarterial valves, the artery walls, central nervous system (CNS), or the cardiac muscle itself (Wilkens et al. 1996, Saver et al. 1998, Wilkens 1997, 1999a,b, McMahon 2001, Stevens et al. 2009).

This chapter focuses primarily on articles published since the last extensive review of the topic in *The Biology of Crustacea* (Bliss and Mantel 1983). Because the Malacostraca represent the bulk of the crustaceans studied and include species with complex anatomical and physiological systems, the physiological responses of these are covered in detail and relevant comparisons made with other families. This chapter covers the basic anatomy and functional morphology of the heart and vessels and their developmental, neurological, and hormonal control mechanisms, as well as cardiovascular responses to environmental perturbations.

FUNCTIONAL ANATOMY

The Heart

The anatomy of the crustacean heart is as diverse as the taxa and ranges from a simple tubal heart to the complex globular hearts seen in decapods. The generalities used here illustrate primary patterns observed within the decapod crustaceans, and, therefore, the cardiac anatomy outlined may be used as a general model with some deviations between genera and species (Fig. 7.1).

The heart of the decapod crustaceans is a single-chambered box-like structure (the ventricle) with walls consisting of bands of cardiac muscle arranged in a three-dimensional network to maximize the ability to eject hemolymph upon contraction. This trabecular network of branching and anastomosing myocardial cells forms an avascular myocardium that acts as a functional syncytia to facilitate coordinated contraction (Howse et al. 1971). The ventricle is suspended within the pericardial cavity by elastic alary ligaments (Maynard 1960). The myocardium, alary ligaments, and pericardial cavity together make up a functional hemolymph pump. Ventricular contraction results in hemolymph ejection into the arterial system. Contraction is stimulated by nerves to the myocardium from cardiac pacemaker cells found in the cardiac ganglion. The cardiac ganglion is located along the dorsal surface of the heart and regulates heart rate and muscle contractile force (Wiersma and Novitiski 1942, Maynard 1960, McMahon and Wilkens 1983). Some of the energy of contraction is stored in the elastic alary ligaments attached to the surrounding tissues. A passive expansion of the ventricle results from this attachment, and diastolic filling begins. Venous pressure (filling pressure) is aided by this passive expansion and allows hemolymph to fill the heart through three paired ostia located dorsally, laterally, and ventrally on the heart. Once diastolic filling is complete, systolic contraction begins. Initial cardiac myocardial contraction places tension on the edges of the ostia, resulting in their closure and preventing backflow of hemolymph into the pericardial sinus (Maynard 1960).

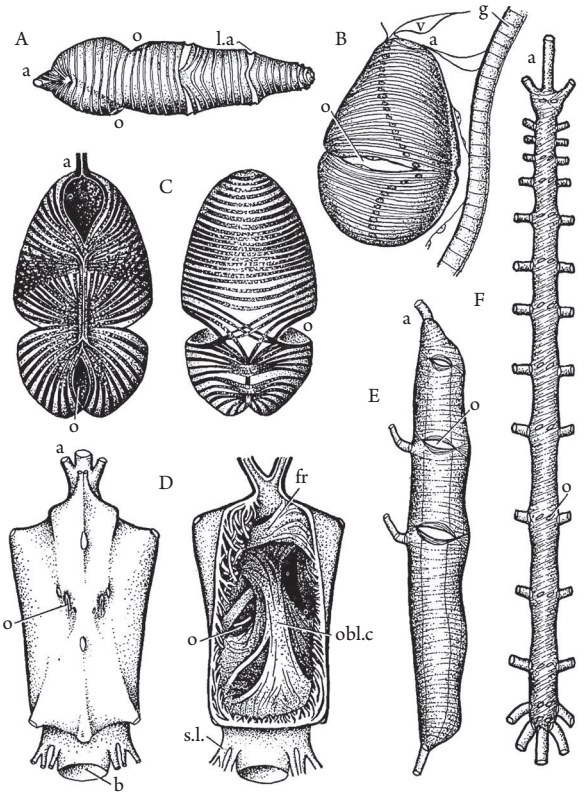


Fig. 7.1.
Types of crustacean hearts. (A) *Diastylis rathkei* (Malacostraca, Cumacea), dorsal view, length is approximately 3 mm. (B) *Daphnia magna* (Branchiopoda, Cladocera) lateral view, length is approximately 200–300 μm. (C) *Calanus finmarchicus* (Copepoda, Calanoida), ventral (left) and dorsal views, length is approximately 330 μm. (D) *Astacus astacus* (Malacostraca, Decapoda), dorsal view (left) and dorsal view with dorsal heart wall removed, length is approximately 1 cm. (E) *Phronima sedentaria* (Malacostraca, Amphipoda), lateral view, length is approximately 2.5 cm. (F) *Squilla mantis* (Malacostraca, Stomatopoda), dorsal view, length is approximately 8 cm. Abbreviations: a, anterior aorta or opening from the heart into the anterior aorta; b, bulbus arteriosus; fr, musculus frontalis; g, wall of the intestine; l.a., opening from the heart into the lateral artery; o, ostium; obl.c., musculus obliquus cordis; s.l., suspensory ligaments; v, cardioarterial valve. From Maynard (1960), with permission from Elsevier.

The Vessels

The crustaceans comprise a group of morphologically diverse organisms making it somewhat difficult to present a general description of the circulatory system (Maynard 1960, McMahon and Burnett 1990, McMahon 2001). Recently, a number of papers and a comprehensive review have addressed the comparative anatomy and evolutionary relationships of the circulatory and respiratory systems (see Wirkner 2009, Wirkner and Richter 2013). Briefly, a wide array of systems exist, ranging from the simple circulatory system of the cirripeds, which have no heart and simple open sinuses, through crustaceans such as the copepods and anostracans, which possess a simple tubular heart, but no vessels, to the most complex distribution systems seen in adult decapods (McMahon 2001). It is assumed that these variations in morphology of the circulatory system are evolutionary, and a complex segmental arterial system may have allowed animals to become large and active (Wilkens 1999b). In particular, the ability of the malacostracan crustaceans to control differential

blood flow through the tissues may have been important in their adaptive radiation and their ability to colonize physiologically diverse environments (Wilkens 1999b).

At the top of the evolutionary chain, the circulatory system of adult decapods is the most complex (Fig. 7.2), so much so that it may now be considered incompletely closed, rather than open (Reiber and McGaw 2009); this section concentrates on this system. Hemolymph is pumped from

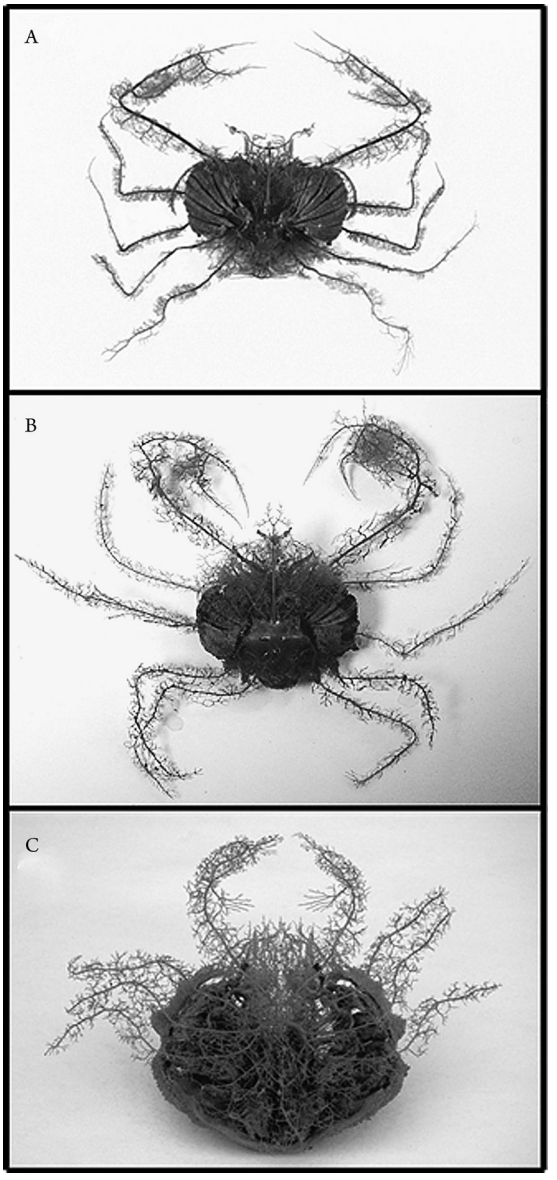


Fig. 7.2.
Corrosion casts showing the complexity of the circulatory system of decapod crustaceans. (A) Dorsal view of the blue crab *Callinectes sapidus*, adapted from McGaw and Reiber (2002), with permission from Wiley and Sons, Inc. (B) Dorsal view of the kelp crab *Pugettia producta*, adapted from McGaw and Stillman (2010), with permission from Elsevier. (C) Ventral view of the Puget Sound king crab, adapted from McGaw and Duff (2008), with permission from Wiley-Liss, Inc.

the single-chambered ventricle into seven arteries (five arterial systems); the small anterior aorta and the paired anterolateral arteries and hepatic arteries flow anteriorly, the large sternal artery exits the heart ventrally, and the posterior aorta complex exits from the posterior aspect of the heart. The anatomical arrangement of the vessels is covered in detail elsewhere (McGaw and Reiber 2002, McGaw 2005a, Wirkner and Richter 2013). The arteries subsequently branch into smaller arteries and fine capillary-like vessels that ramify within the tissues. Decapod crustaceans lack a complete venous system; instead, hemolymph drains into small irregular spaces between the tissues, the interstitial lacunae, and then into sinuses, which are large irregular spaces between the tissues (Maynard 1960). Convention states that arterial hemolymph perfuses the tissues at the level of the lacunae where gas, nutrient, and waste exchange takes place. However, recent work suggests that the hemolymph may remain contained within an endothelial layer down to, or even to the level of the lacunae, which would make lacunar exchange somewhat analogous to capillary beds of vertebrate systems (Wilkens 1997, 1999a,b, Reiber and McGaw 2009). Spent hemolymph (that has perfused the tissues) subsequently collects in sinuses before flowing over the gills and back into the heart. It was once thought that sinuses were so ill defined that they almost defied successful demonstration (Pyle and Cronin 1950). However, recent work shows the sinuses to be distinct structures associated with a fine latticework of lacunae, bordered by fibrous connective tissue (McGaw and Duff 2008, McGaw and Stillman 2010). Haeckel (1857) suggested that there were no unbound sinuses within the decapod system. The difference between sinus and capillary therefore becomes less apparent, suggesting a primarily histological term rather than a physiological one (Reiber and McGaw 2009). The individual sinuses eventually drain into the large ventral thoracic sinus; from here, hemolymph collects in the infrabranchial sinuses before flowing through the gills where it is reoxygenated. By the time the hemolymph has reached the gill vessels, the pressures are only a few centimeters of water (Blatchford 1971, McMahon and Wilkens 1983). The gill lamellae have a low branchial resistance, and these thin-walled vessels are able to distend in response to pressure pulses (Taylor 1989). This means that flow through the small vessels of the gill lamellae might be concerned with the redistribution of lamellar flow, thus maximizing O_2 uptake (Taylor 1989). After passing through the lamellae, hemolymph drains into the pericardial sinus and back into the heart through the ostia.

Although the gills are presumed to be the major return route for venous blood, an alternative return route has been proposed. The branchiostegal sinus is a network of lacunae and veins that perfuses the dorsal surface of the branchial chamber and eventually drains back into the pericardial sinus (Taylor and Taylor 1992). Although the branchiostegal network can bypass the gills, its exact function and the partitioning of venous flow between the branchial and branchiostegal sinuses is not fully understood (Taylor and Greenaway 1984, Greenaway and Farrelly 1990, Taylor and Taylor 1992). The branchiostegal circulation is well developed in terrestrial and amphibious crabs; in these animals, it is used for aerial gas exchange (Taylor and Greenaway 1979, Greenaway and Farrelly 1990, Greenaway et al. 1996). However, the branchiostegal network is also well developed in many subtidal species (McGaw and Duff 2008, McGaw and Stillman 2010). The exact function of ventilatory reversals has long been surmised, but they are thought to aid ventilation of dead spaces in the branchial chamber (Hughes et al. 1969, Davidson and Taylor 1995). There is nothing to suggest that additional O_2 could not be extracted from the water in the branchial chambers via the branchiostegal sinus. If not, there would be a substantial mix of deoxygenated and oxygenated blood returning to the heart, and mixing appears to be minimal (McMahon 2001; see Fig. 7.3).

Unlike mammalian systems, the majority of crustaceans do not have layers of smooth muscle in their arterial walls; instead, the vessel walls consist of an adventitial layer of collagen fibers surrounding a concentric network of fibrous elastic tissue (Shadwick et al. 1990). However, at the base of each artery exiting the heart is a set of muscular cardioarterial valves (Alexandrowicz 1932). The valves are innervated (Kuramoto and Ebara 1984, Kihara and Kuwasawa 1984, Tsukamoto et al. 1992), and neural or neurohormonal stimulation of the valves causes them to contract, progressively

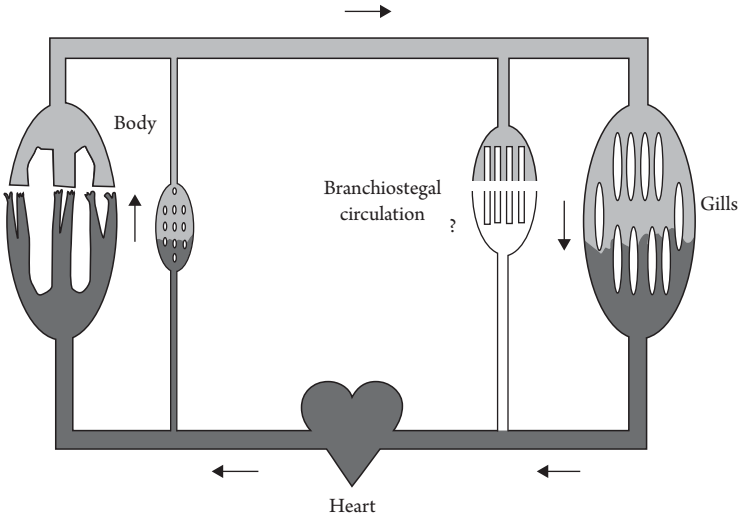


Fig. 7.3.
Pictograph to show the flow of hemolymph in decapod crustaceans. Hemolymph is pumped from the single-chambered ventricle into well-defined arteries. Most of these arteries split into smaller lacunae, which possess a well-defined structure, before emptying into sinuses or veins. In certain areas (e.g., brain, antennal gland), the hemolymph may flow through capillaries with a closed-loop system. Spent hemolymph collects in sinuses before it flows through well-defined gill lamellae where it is oxygenated before returning to the heart. An alternative route through the branchiostegal circulation has also been proposed. The oxygenation status is intentionally left blank because, at this stage, the partitioning of the flow between the gills and branchiostegal circulation, and whether the hemolymph gains oxygen or not when passing through the branchiostegites, is still unknown.

restricting hemolymph flow into the artery. Blocks of striated muscle have been reported in the abdominal arteries of a number of crustaceans (Shadwick et al. 1990). Because the abdominal artery is usually the only artery that contains muscle tissue, it is postulated that this may actually be the evolutionary remnant of a tubular heart (Wilkens 1995, Wilkens et al. 2008). The exact function of these striated muscle blocks is unclear; however, a number of neurohormones have been shown to cause their contraction. Whether these actually control regional blood flow as occurs in closed systems is unclear (Martin et al. 1989, Wilkens 1997). Recent evidence suggests that the cellular make-up of the vessel walls themselves may be important. Lobster arteries are composed of three layers. The inner layer is an elastic connective tissue, and the outer layer is primarily composed of collagen; the middle layer of the artery is composed of cells containing microfilaments. These cells contain actin, myosin, and tropomyosin (Cavey and Wilkens 2000, Cavey et al. 2008, Wilkens et al. 2008). Proctolin and glutamate elicit contraction of rings of arterial tissue, possibly acting on the actin subunits in the walls (Wilkens et al. 2008). These contractions tend to be slow (>20 min to reach maximum) with a very slow recovery time (>2 h). However, even slight arterial contraction will have profound effects on resistance to blood flow and may be an important component of the control mechanisms regulating blood distribution (Wilkens et al. 2008).

Calculations of the hemolymph capacity of each arterial system have been made from corrosion casts (McGaw and Reiber 2002, McGaw and Duff 2008, McGaw and Stillman 2010). In typical brachyuran crabs about 40–45% of hemolymph is contained in the arteries and capillaries; as in vertebrates, the venous system (sinuses, veins, heart, and gills) acts as a reservoir holding 50–65% of the hemolymph at any one time (McGaw and Reiber 2002). A series of papers using pulsed-Doppler, microsphere perfusion, and dye dilution techniques (Airriess et al. 1994, Guadagnoli and Reiber 2005) have shown that decapod crustaceans can partition hemolymph flow through the different

arterial systems, sending blood to more metabolically active tissues. Depending on the species and the stress encountered, the sternal artery and associated branches receive most of the hemolymph, at between 40% and 85%; the paired hepatic arteries receive between 8% and 25%, the paired anterolateral arteries between 8% and 40%, the anterior aorta between 0.05% and 6%, and the posterior aorta complex receives between 0.05% and 8% of the hemolymph pumped by the heart (McGaw et al. 1994a,b, McGaw and McMahon 1995, Reiber et al. 1997).

PHYSIOLOGICAL CONTROL

Developmental Aspects of Cardiac Function

The primary focus of research in crustacean cardiac physiology has been to gain an understanding of the function and regulation of adult systems. This has led to major revisions in our understanding of the complexity of the cardiovascular system's regulatory capabilities in this group of animals (McMahon and Burnett 1990, Airriess and McMahon 1992, 1994, Hill and Kuwasawa 1992, Reiber et al. 1992, Reiber et al. 1997, Romney and Reiber 2013). Investigations by Yamagishi (1990) and Yamagishi and Hirose (1992, 1997) have focused on the neural coupling of the CNS to the cardiorespiratory systems in the direct developing semiterrestrial isopod (*Ligia exotica*) and the tadpole shrimp (*Triops longicaudatus*; Yamagishi et al. 1997). The heart of the isopod was found to be myogenic (intrinsic cardiac pacemaker) during embryonic and juvenile stages. As cardiac development proceeded, the more typical crustacean neurogenic pattern developed. A similar pattern of cardiac regulation has been suggested for the chelicerate, the horseshoe crab (*Limulus*; Carlson and Meek 1908, Crozier and Stier 1927). The heart of both juvenile (larval) and adult tadpole shrimp was found to be myogenic and without direct nervous innervation (Yamagishi et al. 1997). At this time, due to the limited data available, we cannot determine if the *Triops* (myogenic) pattern is unique or a more typical pattern for Crustacea.

The development of cardiac physiological function (heart rate) in the brine shrimp (*Artemia franciscana*) has been shown to be dependent on cardiac differentiation early in development, with heart rate increasing with body mass (Spicer 1994, Spicer and Morritt 1996). This pattern is followed by a dependence on cardiac elongation with heart rate decreasing with body mass. This relationship between differentiation and elongation is significant regarding cardiac function and its role in developmental processes. Early cardiac activity in *Artemia* and in the copepod *Argulus americanus* (Wilson 1904) depends more on morphological than physiological development. It is hypothesized that there is a transition from a diffusion dependent system during early development to an increased dependence on convective processes facilitated by the heart and gills (Spicer 1994, Spicer and Morritt 1996). Supporting this hypothesis is the observation that the beat frequency of natatory limbs (second antennae, used as accessory respiratory structures) decreases with development, resulting in a decrease in gas exchange. The heart begins to take on a physiologically significant role as natatory limb beat frequency declines. Increased cardiac and ventilatory activity compensates for the resulting loss of respiratory function (Barlow and Sleigh 1980). This again leads to the question of how the onset of cardiac function can be influenced by limitations in gas exchange (environmental hypoxia).

Cardiac physiology has been investigated during development from embryonic through larval and adult stages in a number of decapod crustaceans (*Procambarus clarkii*; Reiber 1997, Reiber and Harper 2001, Harper and Reiber 2004). The embryonic crayfish tubal heart showed early signs of contraction (although unorganized) by naupliar stage 4 (incubation day 12 at 25°C). Heart rate increased until naupliar stage 6 then showed a progressive decrease until hatching and larval

development was initiated. They also showed a parallel decrease in hypoxic sensitivity (Reiber 1997). Post-hatching heart rate increased through larval stages 1–3, followed by a decline as the animal gained mass (an allometric relationship; Fig. 7.4). The decrease in heart rate and hypoxic sensitivity during later embryonic stages indicated a potentially diffusion-limited system as embryonic metabolic mass increased beyond the exchange capabilities of the egg membrane (Reiber 1997). It has also been found that embryonic heart rate increases as the partial pressure of oxygen (P_{O_2}) in water is raised above 200 mm Hg, which also suggests the animals are O_2 limited. Embryonic and larval cardiac stroke volume was monitored during hypoxic exposure. Embryonic stroke volume and cardiac output decreased in parallel with heart rate. After hatching, larval stroke volume increased dramatically, compensating for the lower heart rate and resulting in an increase in cardiac output (Reiber 1995b, 1996, Harper and Reiber 2001, Harper and Reiber 2006b). It was also found that if 10-day-old embryos were incubated under hypoxic conditions (P_{O_2} = 75 mm Hg), hatching occurred 4 days earlier than in control animals that were maintained under normoxic conditions throughout development.

This abrupt termination of embryonic development was not a simple time compression of developmental processes (as seen when incubation temperature is increased) but an abbreviation of the ontogenic pattern. These experiments point toward a diffusion-limited embryo that can alter its developmental pattern depending on available O_2 (Reiber 1995a, 1996). If embryonic metabolism cannot be maintained, the animal will hatch, bringing online respiratory mechanisms into play to facilitate gas exchange. This work complements the investigations on brine shrimp. The ontogeny of crayfish cardiac neurohormonal regulation appears to be quite complex, with a defined sequence of neurohormonal sensitivity developing as the animal progresses from stage to stage. It has yet to be established if these ontogenetic changes in myocardial sensitivity to neurohormones are due to a temporal sequence of receptor and/or ligand production or to the development of intracellular pathways.

Injections of octopamine, serotonin, γ-aminobutyric acid (GABA), and dopamine into the pericardial sinus of embryos and larvae did not elicit adult-like cardiac responses in terms of heart rate, stroke volume, or cardiac output until late larval or early juvenile stages. Stage-3 larvae showed a minimal response to injections; it was not until the animals reached juvenile stages (weight of 25 mg or greater) that adult-like responses were observed. Of particular interest was the embryonic response toward GABA injection, which caused an increase in cardiac parameters, a paradoxical response. An adult-like response was not observed until late larval stages had been reached (Kuramoto and Ebara 1984, McMahon 1992, Wilkens 1995, Chapman and Reiber 1998, Reiber and McMahon 1998, Harper and Reiber 2000). The response to GABA injection is obviously not a simple one and may involve changes in receptor populations. More research is clearly needed to understand this system. This insensitivity to injection of cardioactive drugs during early larval stages would indicate that cardiac regulatory mechanisms do not mature until later in development (Reiber 1996, Chapman and Reiber 1998, Harper and Reiber 2000).

Histological evidence supports these conclusions. Embryos stained for nerves were not found to contain nervous innervation of the heart. The cardiac ganglion was not observed to invade the heart until the second larval stage, which corresponds with initial sensitivity to cardioactive drug injections. Using techniques similar to those described for crayfish, cardiovascular development was investigated in the shrimp *Metapenaeus ensis* (McMahon and Chu 1994, Chu et al. 1995). In *M. ensis*, the heart does not start to beat until late naupliar developmental stages. Heart rate increased through the third protozoeal stage then fell as mysid and pelagic stages were reached (McMahon et al. 1997). The changes in heart rate were not due to cardiac elongation, as is evident in *Artemia*, but were attributed to changes in regulatory pathways. It is interesting to note that the heart begins to beat in directly developing shrimp when comparing developmental stages (early naupliar stages in the crayfish vs. late naupliar stages in the shrimp). The advent of internal convective processes

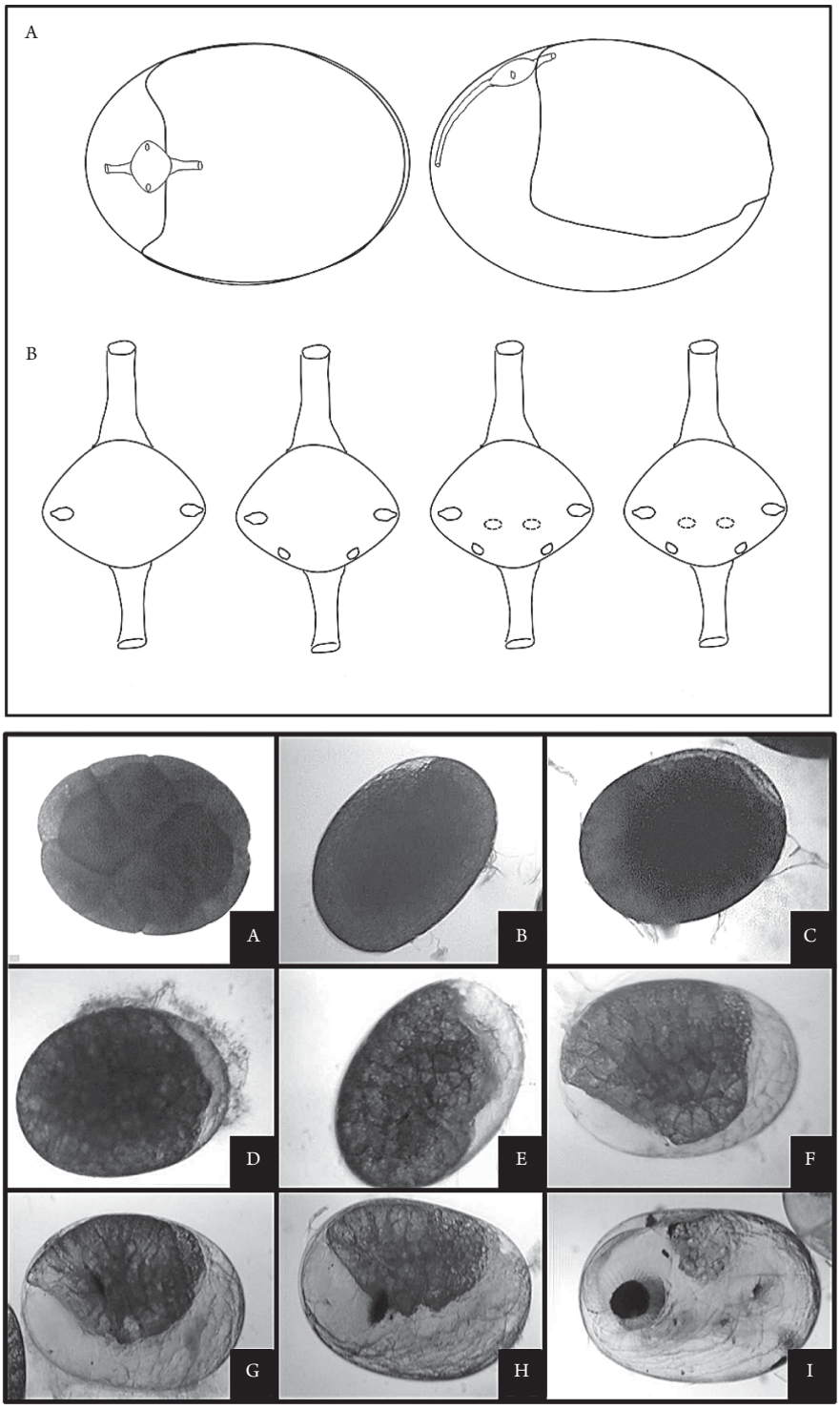


Fig. 7.4.
(Top) Illustrations of the dorsal view of the ventricle during embryonic stages of *Palaemonetes pugio* (Holthuis 1949). Dimensional arrangement of the ventricle for analysis. The Y-axis runs the length of the long axis of the

early in development increases O_2 transport rates, thus allowing the crayfish to partially compensate for the limitations in the diffusional surface area of the egg membrane. This compensatory strategy may allow directly developing species to remain in embryonic stages longer and thus have more time to develop morphologically and hatch as a well-developed larvae or juvenile.

Much of what we know regarding crustacean cardiovascular development comes from anatomical observations or from a few early studies on a limited number of species (McMahon and Chu 1994, Chu et al. 1995, McMahon and Doyle 1995, Reiber 1995b, 1996, 1997). This has resulted in large gaps in our understanding of cardiovascular and respiratory physiological development in Crustacea that would alone justify investigation. Added to this is the unique opportunity crustacean cardiovascular development offers for the investigation of cardiac malleability and physiological trajectories.

Neural Control of Cardiac Function and Blood Flow

Control of Cardiac Function

The neurogenic myocardium of the crustacean heart is stimulated to contract by the nerves from the cardiac ganglion. Overlying and embedded within the cardiac muscle, the cardiac ganglion serves as pacemaker and regulator of muscle contractile force (Maynard 1960, Taylor 1982, McMahon and Burnett 1990). The cardiac ganglion is composed of only a small number of neurons of two cell types, with distinct cell sizes (McMahon and Wilkens 1983, McMahon et al. 1989, Kuramoto and Yamagishi 1990). In the crayfish, the cardiac ganglion is approximately 5 mm in length and contains 16 neurons: eight small and eight large. The two morphologically distinct cell types have functional differences. Normally, the smaller neurons act as a pacemaker by establishing burst frequency. The smaller cells, through chemical synapses, drive the larger neurons, which act as follower cells and innervate the myocardium directly. Therefore, the rate of heart contraction is determined by the burst frequency of the small cells of the cardiac ganglion. The force of the contraction is dependent on the spike frequency within each burst.

The larger cells of the cardiac ganglion innervate the myocardium directly and show mechanoreceptor-like sensitivity (Maynard 1960). Distention of the lobster cardiac ganglia occurs when the heart is exposed to high filling pressure; heart frequency is then determined by the bursting pattern of the large nerves (Kuramoto and Kuwasawa 1980, Kuramoto and Ebara 1985, Cooke 1988). When the heart is exposed to neurohormones such as serotonin, heart frequency is set by the smaller neurons. The system has two layers, with the smaller cells acting as primary pacemaker driving the larger cells; however, as just demonstrated, modulation of heart frequency can depend on both cell types.

The neurons of the cardiac ganglion are influenced by the cardioregulatory nerves, one pair of inhibitory fibers (SN 11) and two pairs of acceleratory fibers (SN 111; see Wiersma and Novitiski

Fig. 7.4 (*Continued*)

(A) Dorsal and lateral position of the heart in reference to the embryonic shrimp. (B) Development of the ventricle during stages of embryonic development. Functional ostial pairs can be seen developing through the stages. Solid lines signify a structure seen on the dorsal surface of the heart, and dashed lines signify a structure seen through the heart on the ventral surface. From Romney and Reiber (2013), with permission from Brill, Inc. (Bottom) Images of embryonic stages of development of *Palaemonetes pugio* (Holthuis 1949), taken under a stereoscope. Stage I and stage VIb are omitted for irrelevance of a single-shot image properly representing characteristics for those articular stages. Stages pictured include (A) stage II, cleavage; (B) stage IIIa, gastrulation with blastopore; (C) stage IIIb, gastrulation with V-depression; (D) stage IV, early nauplius; (E) stage V, mid-nauplius; (F) stage VIa, late nauplius with posterior segmentation; (G) stage VIIa, post-nauplius with eye pigmentation; (H) stage VIIb, post-nauplius with eye condensation; (I) stage VIII; pre-hatch embryo. Images of animals shown here are not processed together to scale.

1942, Maynard 1960, McMahon and Wilkens 1983). Their origins are in the anterior ventral nerve cord and the subesophageal ganglion. The three nerves come together at the lateral pericardial plexus and enter the pericardial sinus. Here, they separate and terminate in a variety of regions. SN III sends out axons that innervate the pericardial organ, the cardiac ganglion, the dorsal muscles of the myocardium, and the alary ligaments. Cardioaccelerator and inhibitor fibers have been found to fire tonically, with intermittent bursts of higher frequency spikes (Wiersma and Novitiski 1942, Wilkens et al. 1974, Taylor 1982, McMahon and Wilkens 1983). If a similar burst pattern is artificially induced electrically in the cardiac ganglion, alteration of heart rate results.

Central neural integration of heart function originates in the command interneurons found in the circumesophageal ganglion. Interneurons have been identified that cause tachycardia, bradycardia, or cardiac arrest (McMahon and Wilkens 1983). The impulse frequency of cardioinhibitory fibers has been found to vary linearly with stimulation of specific interneurons. It is interesting to note that when these interneurons are stimulated or sensory stimulus is given, the cardioaccelerator nerve and inhibitor nerve act in reciprocal fashion, such that when one increases firing frequency, the other decreases its rate.

Additional nerves act on the cardiovascular system to influence cardiac output and arterial perfusion. The regulation of aperture size in the cardioarterial valves could be a prime means by which decapods regulate distribution of the cardiac output (Kuramoto and Kuwasawa 1980, Kuramoto and Ebara 1984a,b, Kuramoto and Ebara 1988). The cardioarterial valves found at the origin of each of the major vessels as they leave the heart prevent backflow of hemolymph during diastole, as well as regulate flow to their respective artery. This is accomplished through excitation of the two striated muscle flaps that make up the valve. When the valve is stimulated, the free edges of the flaps contract, resulting in reduced systolic pressure. The flap muscles contract in response to direct stimulation by an axon of a segmental nerve from the ventral ganglion. Contraction may also result from actions of neurohormones such as proctolin, octopamine, dopamine, noradrenalin, acetylcholine, and serotonin (Maynard 1960, Kuramoto and Ebara 1984, 1989).

The alary ligaments that support the heart within the pericardium are also innervated. These ligaments are made up of elastic tissue and striated muscle. The significance of their innervation is not known (Maynard 1960). It has been suggested that the passive expansion of the heart during diastole may be aided by the contraction of these ligaments (McMahon and Burnett 1990). A control of cardiac volume has also been proposed as a function for the active contraction of the alary ligaments. This may come about through a distention of the heart by the ligaments, resulting in a greater end diastolic volume that may increase stroke volume in a manner similar to the Frank-Starling mechanism seen in mammalian systems. In the myogenic heart of mammals, one of the important regulatory factors is the Starling relation (McMahon and Wilkens 1983, Cooke 1988, Burggren et al. 1990, McMahon and Burnett 1990). When intracardiac pressure is increased, greater stretching of the cardiac muscle occurs, resulting in greater contraction and an increased stroke volume. In the neurogenic heart of the crustaceans, it is thought that increased cardiac distention and/or increased intracardiac pressure will result in a distension of the cardiac muscle, which distorts the cardiac ganglion. This distortion will cause an increased burst frequency, which results in an increased heart rate and greater cardiac ejection (Kuramoto and Ebara 1984a,b).

Cardiac performance in invertebrates may be influenced by the actions of neurohormones and neuromodulators (Kuramoto and Ebara 1984a, Zatta 1987, Barthe et al. 1989, Freschi 1989, Freschi and Livengood 1989, Preiffer-Linn and Glantz 1989). Neuromodulation of the cardioarterial valves of decapods (already discussed) plays a key role in redistribution of the cardiac output. Neuromodulation of the heart also plays a significant role in control and regulation of heart rate and stroke volume. The action of these cardioactive chemicals can result in changes in the output of the cells of the cardiac ganglion and the contractile force of the myocardium itself.

Crustacean heart rate and contraction force are determined by the summation of information coming to the cardiac ganglion (McMahon and Wilkens 1983). Stroke volume and distribution of flow are more complex than previously thought and involve a number of possible interactions, the result of which alters cardiac output. Modulation of cardiac performance results from numerous factors, including internal interactions such as neural modulation, increase hemolymph pressure, metabolic effects, sensory modulation, circadian rhythms, and animal movement (Larimer 1962, 1964, Larimer and Tindel 1966, Freadman and Watson 1989). External factors may also play a role in altering cardiac performance. Sensory stimuli, such as visual and mechanical, will alter heart rate. Local environmental conditions such as water O_2 and carbon dioxide (CO_2) levels, osmotic regulation, and temperature will all alter cardiovascular functions (Patterson and deFur 1988, Wells 1988, Wheatly 1989, Wheatly and Toop 1989).

Control of Vascular Flow

There are four broad mechanisms by which decapods can regulate vascular hemolymph flow: cardioarterial valves, peripheral valves, skeletal muscle contractions, and vascular contraction, with the last being speculative at this time. As previously discussed in this chapter, at the interface between the heart and each arterial system is a muscular cardioarterial valve that is under neuronal and neurohormonal control (Kuramoto and Kuwasawa 1980, Kuramoto and Ebara 1984a,b, Kuramoto and Ebara 1988, Kuramoto et al. 1995, Okada et al. 1997). As the tonus of these valves changes, flow (resistance) to each peripheral arterial system can be modulated to control bulk arterial flow. In two species of lobsters, peripheral valves located downstream from the cardioarterial valves have been shown to have the active ability to regulate resistance and thus hemolymph flow to individual vessels within a system (Wilkens et al. 1997, Davidson et al. 1998, Wilkens and Taylor 2003). These two regulatory systems provide the ability to not only regulate bulk arterial flow, but to also provide a fine level of hemolymph flow regulation to specific vascular systems within the animal. Furthermore, fine regulatory capability may come from observations in lobster arteries of contractility and elasticity along with the identification of both actin and myosin, which may provide fine vascular regulation of hemolymph flow (Cavey et al. 2008, Wilkens et al. 2008). Specific and refined vascular hemolymph flow regulation in decapods accounts for the system-specific metabolic function observed in these complex animals.

The last mechanism by which decapods, specifically macrurans, modulate arterial resistance is through contraction of major skeletal muscle groups. Although this mechanism is not inherent to the vascular system itself, major changes in resistance and flow have been demonstrated during activity (Reiber et al. 1997). Flow was altered substantially as a result of tail flexion in both lobsters and crayfish. Contraction of the abdominal muscles during tail flexion increased stroke volume substantially and caused a redistribution of cardiac output but had no effect on hart rate. Cardiac output was redistributed during tail flexion, increasing the blood supply to the abdominal muscles and thus serving the increased metabolic demand during activity. Blood supply also increased to the limbs, the mouthparts, and the muscles supplying the scaphognathites. A similar shunting of flow toward active tissues was noted during struggling in the crab *Cancer magister* by Bourne and McMahon (1989).

Hormonal Control of Cardiac Function and Blood Flow

A number of neurohormones have been implicated in controlling cardiac function, primarily heart rate, and to a lesser degree stroke volume, cardiac output, and hemolymph perfusion through individual blood vessels. The actions of these neurohormones can vary among different species or even within one species (McGaw et al. 1995). A number of comprehensive reviews on crustacean

neurohormones, including their molecular structure, control pathways, and physiological actions, have recently appeared (Christie et al. 2010a, Christie 2011). In addition, aspects of crustacean endocrinology are covered in Chapters 1 to 3 of this volume. This section provides a brief review of some of the more important neurohormones and how they affect cardiac function and regional blood flow. The hormones may be produced in and released from the CNS and associated ganglia, or specific neuroendocrine organs such as the pericardial organs of the heart and the X-organ complex of the sinus gland (Cooke and Sullivan 1982, Christie 2011). They are released into the circulation, bathing all tissues and bringing about their effects simultaneously at multiple sites (Christie et al. 2010a). In crustaceans, they may act on the cardioregulatory nerves, cardiac ganglion, cardiac muscle, muscle valves, or on the walls of the arteries themselves.

Crustacean Cardioactive Peptide (CCAP)

CCAP, originally isolated from the pericardial organs of the green crab *Carcinus maenas*, is named for its cardioactive effects (Stangier et al. 1987). The primary site of action of CCAP is the cardiac ganglion, where it acts to increase burst frequency and amplitude (Saver et al. 1999, Cruz-Bermudez and Marder 2007). It is known to exert positive chronotropic and inotropic effects in a number of crustaceans. Infusion of 10^{-9} M CCAP onto isolated heart preparations of *C. maenas* produces a pronounced chronotropic and a lesser inotropic effect (Wilkens and Mercier 1993, Saver and Wilkens 1998). CCAP exerts both chronotropic and inotropic actions on the heart of the crayfish *Orconectes limosus* at concentrations above 10^{-8} M (Stangier 1991). In the blue crab *Callinectes sapidus*, it causes a dose-dependent increase in heart rate and stroke volume (Fort et al. 2007a). These chronotropic and inotropic effects appear to be disconnected because the response level for changes in contraction occur between 10^{-10} and 10^{-9} M, whereas modulation of heart rate occurs at higher concentrations of 10^{-8} to 10^{-7} M and typically lasts for less time (Fort et al. 2007a). CCAP also produces smaller chronotropic and inotropic increases in the giant hermit crab *Aniculus aniculus* at concentrations of 10^{-6} to 10^{-5} M (Yazawa and Kuwasawa 1992).

CCAP does not appear to affect the heart rate of *Cancer pagurus, C. magister*, or *Homarus americanus* (Stangier and Keller 1990, Stangier 1991, McGaw et al. 1994a, Wilkens et al. 1996). Although CCAP had no effect on heart rate of *C. magister*, perfusion of 10^{-8} M CCAP causes an increase in stroke volume. The ensuing increase in cardiac output results in an increase in hemolymph flow delivered through the anterolateral arteries (McGaw et al. 1994a). This suggests that CCAP is either acting on the nerves of the cardioarterial valves or directly on the valve muscle itself (McGaw et al. 1994a). CCAP is also implicated in blood flow control in the smaller arteries and arterioles: it causes an increase in vascular resistance of lobster arteries and may be used for regulation of blood flow in the peripheral circulation (Wilkens et al. 1997, Wilkens and Taylor 2003; Fig. 7.5).

Proctolin

Proctolin was first isolated and sequenced from the hindgut of the cockroach (Starratt and Brown 1975). It has a wide array of effects in crustaceans: its primary cardiovascular effects tend to be excitatory, increasing rate and force of contraction of the heart (Keller 1992). Proctolin has multiple sites of action including the cardiac ganglion, heart muscle, CNS, the heart valves, and arteries of the peripheral circulation (Sullivan and Miller 1984, Saver and Wilkens 1998, Saver et al. 1998, Wilkens et al. 2005, 2008).

This peptide causes an increased heart rate in the lobster *H. americanus* (Wilkens et al. 1996, Wilkens and Kuramoto 1998) and the shore crab *C. maenas*, with threshold effects observed at 10^{-8} M for both species (Wilkens et al. 1985, Wilkens and Mercier 1993, Saver and Wilkens 1998), and, at high concentrations, the heart rate of *C. maenas* remains elevated for 4–5 h after infusion of the

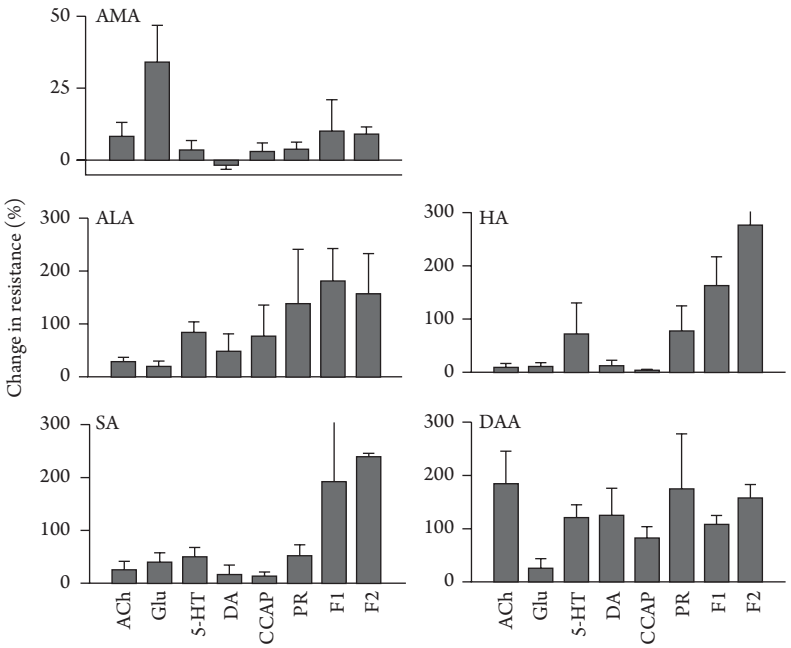


Fig. 7.5.
Relative change in resistance in flow in five arteries (mean + SEM) of the lobster *Homarus americanus* in response to perfusion of acetylcholine (ACh, 10^{-3} mol l^{-1}), glutamic acid (Glu, 10^{-3} mol l^{-1}), serotonin (5-HT, 10^{-6} mol l^{-1}), dopamine (DA, 10^{-6} mol l^{-1}), crustacean cardioactive peptide (CCAP, 10^{-6} mol l^{-1}), proctolin (PR, 10^{-8} mol l^{-1}), and the FMRFamide-like peptides (F1, F2, 10^{-8} mol l^{-1}). The actual magnitude of the change can be estimated by multiplying the percentage change shown here by the resistance at the same flow rate, 2 mL min^{-1}. Abbreviations: AMA, anterior median artery; ALA, anterior lateral artery; HA, hepatic artery; SA, sternal artery; DAA, dorsal abdominal artery. From Wilkens et al. (1997), with permission from The Company of Biologists, Inc.

peptide (Wilkens et al. 1985). Heart contractility also increases in *H. americanus* (Wilkens et al. 1996, Wilkens and Kuramoto 1998) and *C. maenas* (Saver and Wilkens 1998). In *C. magister*, a large increase in cardiac output via an increased stroke volume leads to an increase in hemolymph flow to the legs and mouthparts via the sternal artery (McGaw et al. 1994a).

Although proctolin appears to be cardioexcitatory in nature, it does exert inhibitory actions, primarily related to hemodynamics. A decreased outflow through the sternal artery occurs in the semi-isolated lobster heart (Wilkens et al. 1996). Decreases in flow rates in the abdominal artery of the spiny lobster *Panulirus japonicus* (Kuramoto and Ebara 1984) and the anterolateral arteries of *C. magister* also occur (McGaw et al. 1994a), presumably by the direct action of proctolin on the cardioarterial valves (Kuramoto and Ebara 1984, Tsukamoto and Kuwasawa 2003). In addition to controlling hemolymph flow into the major arteries, proctolin has also been implicated as a control agent in the peripheral vasculature where it causes an increased resistance in isolated arterial rings of the lobster (Wilkens et al. 2008).

FMRFamide-like Peptides (FLPs)

The FLPs are a superfamily of neurohormones that are important modulators of the cardiovascular and stomatogastric systems of crustaceans (Christie et al. 2010a). The FLPs have been identified in every invertebrate phylum, thus making them the largest and most widely distributed group of structurally similar peptides (Price and Greenberg 1989, Greenberg and Price 1992). In crustaceans,

the majority of the FLPs have been isolated from the pericardial organs or the X-organ complex in the eyestalks (Mercier et al. 2003).

Most experiments investigating hormonal actions on the cardiovascular system have been carried out using isolated or semi-isolated heart preparations. For the most part, the FLPs tend to be cardioexcitatory in nature when tested on such preparations. The hormones F1 (TNRNFLRFamide) and F2 (SDRNFLRFamide) consistently produce both chronotropic and inotropic effects in a variety of lobster, crab, and crayfish species at concentrations above 10^{-9} M (Mercier and Russenes 1992, Wilkens and McMahon 1992, Mercier et al. 1993, McGaw et al. 1995, Worden et al. 1995, Wilkens and Kuramoto 1998, Cruz-Bermudez and Marder 2007, Fort et al. 2007b). A number of other FLPs—FMRFamide and FLRFamide (Mercier and Russenes 1992), NRNFLRFa (Skerrett et al. 1995, Cruz-Bermudez and Marder 2007), APSGFLGMRamide, and TPSGFLGMRamide (Christie et al. 2008)—produce very similar effects. In addition to exerting both chronotropic and inotropic effects, application of synthetic sulfakinin (–$Y_{(SO_3H)}$ GHM/LRFamide) causes normalization of an irregular heart beat (Dickinson et al. 2007). The peptide GLDLGLGRGFSGSQAAKHLMGLAAANFAGGPamide (Homam-CLDH) modulates rate and contraction amplitude of the lobster heart at concentrations of 10^{-11} M, which is among the lowest for any cardioactive peptide (Christie et al. 2010b).

Not all the FLPs have dual cardioactive effects; GYNRSFLRFamide consistently increases heart rate but has no effect on contraction amplitude of isolated *C. sapidus* hearts (Krajniak 1991). In contrast, SYWKQCAFNAVSCFamide causes a large increase in amplitude of the heart beat of lobsters but no clear change in rate (Dickinson et al. 2009). The insect neuropeptides SchistoFLRFamide (PDVDHVFLRFamide) and leucomyosuppressin (PQDVDHVFLRFamide) markedly decrease the heart rate of crayfish, suppressing the cardiac rhythm for several minutes (Mercier and Russenes 1992).

The FLPs appear to modulate cardiac activity by acting on both the heart muscle and the cardiac ganglion (Saver et al. 1999, Fort et al. 2007a, Stevens et al. 2009). In addition to modulating the heart, the FLPs may also regulate blood flow through the arteries, probably by their direct action on the cardioarterial valves. F2 causes an increase in flow to anterior aorta but a decreased pressure and flow into the sternal artery of lobster *H. americanus* (Wilkens et al. 1996, Wilkens and Kuramoto 1998). Similar patterns occur in the Japanese lobster *P. japonicus*: flow rates into the anterior aorta and anterolateral arteries increase, whereas a decrease in flow to the sternal artery and hepatic arteries occurs (Wilkens and Kuramoto 1998). The FLPs may also influence blood flow in arteries and sinuses more distant from the heart by increasing resistance in these vessels (Wilkens and McMahon 1992, Wilkens 1997, Wilkens and Taylor 2003; Fig. 7.5).

Interestingly, in whole-animal preparations, a number of the FLPs appear to exert inhibitory responses. Although F1 and F2 are cardioexcitatory in isolated *C. magister* hearts, both elicit a dramatic and long-term decrease in heart rate and stroke volume and thus cardiac output in the whole animal at circulating concentrations between 10^{-9} and 10^{-7} M (McGaw et al. 1995). The decreases in rate are often accompanied by periods of cardiac arrest, and a regular rhythm is not regained for several hours (McGaw and McMahon 1995) and is accompanied by a decrease in hemolymph flow through all arterial systems (McGaw and McMahon 1995). Cardioinhibitory actions also occur during infusion of SchistoFLRFamide and leucomyosuppressin. Although neither hormone exerts chronotropic effects, stroke volume is significantly depressed, leading to a decrease in total cardiac output. Hemolymph flow through the sternal artery and anterolateral arteries also decreases. Threshold for these responses occurs at circulating concentration of 10^{-8} M (McGaw and McMahon 1999). Perfusion of the in vitro heart of lobsters with 10^{-8} M myosuppressin (pQDLDHVFLRFamide) causes a decrease in the frequency, with a concomitant increase in the amplitude of heart contractions. Evidence suggests that, in addition to modulating the cardiac ganglion, myosuppressin also acts on peripheral sites (Stevens et al. 2009). The difference between isolated

and whole-animal preparations suggests the FLPs may have multiple action sites in whole animals rather than a single unified effect. Multiple actions are exerted at separate locations in the cardiac system, leading to a cascade of secondary consequences mediated by intrinsic feed-forward and feedback coupling mechanisms (Fort et al. 2007b, Stevens et al. 2009).

Amines: Serotonin, Dopamine, Octopamine

In crustaceans, the primary source for the amines—dopamine, octopamine, and serotonin (5-HT)—are the pericardial organs and, to a lesser degree, the subesophageal and thoracic ganglia (Beltz 1999, Dickinson et al. 2008, Christie et al. 2010a, Christie 2011).

The effects of 5-HT are primarily cardioexcitatory in nature, causing an increase in heart rate and contraction amplitude in lobsters, crabs, crayfish, isopods, and stomatopods, with the threshold for these responses occurring between 10^{-9} and 10^{-7} M (Kuramoto and Ebara 1984, 1988, Wilkens et al. 1985, Airriess and McMahon 1992, Wilkens and McMahon 1992, Wilkens et al. 1996, Saver and Wilkens 1998, Listerman et al. 2000, Tsukamoto and Kuwasawa 2003, Ando and Kuwasawa 2004). Although primarily excitatory in nature, 5-HT causes a mild bradycardia when applied to the myogenic heart of *T. longicaudatus* (Yamagishi 2003). The chronotropic effects of 5-HT suggest that it acts directly on the cardiac ganglion, possibly increasing burst frequency, number of spikes per burst, and spike frequency in the burst (Miller et al. 1984, Kuramoto and Ebara 1988, Berlind 1998, 2001, Saver et al. 1999, Fort et al. 2004, Cruz Bermudez and Marder 2007). 5-HT imparts more variable effects on hemolymph flow. In isolated lobster hearts, it causes an increased flow pressure into the sternal and dorsal abdominal arteries (Wilkens et al. 1996). In intact *C. magister*, flow rates through the anterolateral and posterior arteries increase following perfusion of 10^{-7} M 5-HT (Airriess and McMahon 1992). In contrast, it constricts the cardioarterial valves of the isopod *Bathynomus doederleini*, resulting in a decreased pressure in all the arteries (Tsukamoto and Kuwasawa 2003). It is also known to act on the walls of arteries, constricting the lumen and increasing peripheral resistance (Wilkens and Taylor 2003).

Dopamine also exerts cardioexcitatory effects, with threshold for activity ranging between 10^{-9} M and 10^{-6} M (Saver and Wilkens 1998, Fort et al. 2004, Yamagishi et al. 2004). It increases heart rate in crayfish, lobsters, and crabs (Berlind and Cooke 1970, Florey and Rathmeyer 1978, Wilkens et al. 1985, Wilkens and Kuramoto 1998) and also causes an increase in both rate and amplitude of contraction of the heart of *T. longicaudatus* (Yamagishi 2003), *C. sapidus* (Fort et al. 2004), *L. exotica* (Yamagishi et al. 2004), and *C. maenas* (Saver and Wilkens 1998). Dopamine is slow acting on the latter two species, and the effects are sustained for several hours. In contrast, dopamine has no appreciable effect on heart rate and causes a decrease in pressure of isolated lobster hearts (Wilkens et al. 1996); in intact *C. magister*, it causes a bradycardia and/or cardiac arrest when applied in high concentrations (Airriess and McMahon 1992). Like 5-HT, dopamine exerts its action on the neurons of the cardiac ganglion (Cooke and Sullivan 1982, Miller et al. 1984, Berlind 1998, Saver and Wilkens 1998, Yazawa and Kuwasawa 1992, 1994, Tierney et al. 2003, Ando and Kuwasawa 2004, Cruz-Bermudez and Marder 2007). L-cell axons from the pericardial organs and the dorsal nerve cells of the CNS also regulate heart rate via modulatory feedback on the cardiac ganglion (Fort et al. 2004). The direct effects of the amine on the cardioarterial valves and the myocardium may also be important in regulation of blood flow (Wilkens et al. 1997, Tsukamoto and Kuwasawa 2003, Yamagishi et al. 2004). Administration of dopamine causes an increase in outflow through the sternal artery of lobsters (Wilkens et al. 1996). In *C. magister*, a reduction or cessation of flow in all arteries occurs, with effects lasting for up to 15 min (Airriess and McMahon 1992). In the isopod *B. doederleini*, dopamine decreases the pressure in the anterior aorta and five lateral arteries but increases the pressure in the paired anterolateral arteries. It is suggested that dopamine exerts its effects directly on the valve muscle, causing constriction

or relaxation of these structures (Tsukamoto and Kuwasawa 2003). Like 5-HT, dopamine causes increased resistance in the peripheral vessels of homarid and panulirid lobsters either by constricting the arterial valves or through its direct actions on the walls of the arteries and sinuses (Wilkens et al. 1997, Wilkens and Taylor 2003).

The effects of octopamine tend to be more variable compared with 5-HT and dopamine, with thresholds for effects occurring between 10^{-9} and 10^{-7} M. Octopamine exerts marked cardioexcitatory effects on crayfish, crab, lobster, and *Triops* hearts (Florey and Rathmayer 1978, Wilkens and Kuramoto 1998, Ando and Kuwasawa 2004, Yamagishi 2004). In isolated *C. maenas* hearts, it causes a sustained increase in rate (Wilkens et al. 1985) but has little effect on the intact heart rate of this species, suggesting that its actions are preparation-determinant (Saver and Wilkens 1998). It exerts biphasic effects in the crabs *Eriphia spinifrons* and *C. magister*, initially causing a short period of bradycardia followed by a more sustained and pronounced tachycardia (Florey and Rathmayer 1978, Airriess and McMahon 1992). In the lobster *P. japonicus*, it is inhibitory when perfused at minimal pressure, reducing heart rate and burst frequency of the cardiac ganglion, but it increases heart rate and contraction when the heart is perfused at higher pressures (Kuramoto and Ebara 1991). In other preparations, octopamine appears to produce minimal effects on the heart or cardiac ganglion (Grega and Sherman 1975, Wilkens et al. 1996, Cruz-Bermudez and Marder 2007). The effects of octopamine on the cardioarterial valves appear to be dependent on the preparation and species. Octopamine hyperpolarizes the resting membrane potential of muscle cells in the anterior valves and hepatic arteries of *P. japonicus*, causing them to relax, and it depolarizes the membrane potential in the posterior valve, causing contraction (Kuramoto and Ebara 1984, 1991, Wilkens and Kuramoto 1998). Although octopamine produces no clear effect on the sternal artery of *P. japonicus* (Wilkens and Kuramoto 1998), it does cause a decrease in flow into the sternal artery of *H. americanus* (Wilkens et al. 1996). In intact *C. magister*, octopamine infusion results in an increased flow rate through the anterolateral and posterior arteries, presumably by causing relaxation of the arterial valves (Airriess and McMahon 1992). In contrast, octopamine constricts the cardioarterial valves in the isopod heart, decreasing arterial pressure in all the arteries (Tsukamoto and Kuwasawa 2003). In conjuncture with the other two amines, octopamine causes an increased resistance in the peripheral arteries and infrabranchial sinuses (Wilkens and Taylor 2003)

PHYSIOLOGICAL RESPONSES

Modulation of cardiac function and regional blood flow optimizes delivery of O_2, nutrients, and hormones to the tissues and the subsequent removal of wastes. As such, cardiovascular mechanisms are closely coupled with respiratory and metabolic processes. These respiratory, osmoregulatory, and digestive processes are covered in detail elsewhere in this volume. Therefore, this section focuses on just the cardiovascular responses to environmental stressors.

Activity Patterns

Locomotor Activity

Crustaceans display a variety of locomotory patterns; in decapod crustaceans, slow walking in an aquatic environment (sideways or forward) is typical, interspersed with short bursts of fast running, the use of modified fifth pereopods for swimming, or the use of rapid escape mechanisms such as tail flips (McMahon et al. 1979, Hamilton and Houlihan 1992). Animals meet the increased metabolic demands associated with exercise by increasing O_2 uptake rates, thus increasing binding

or carrying capacity of the hemolymph and increasing tissue perfusion via the circulatory system (DeWachter and McMahon 1996a). As such, these physiological mechanisms tend to be closely coupled (Wilkens et al. 1985, Rose et al. 1998).

A number of articles have examined the cardiovascular responses of crustaceans to exercise; because these measurements were made at different walking speeds and temperatures using different experimental techniques, the relative rates and patterns of change rather than absolute values are discussed here. Cardiovascular responses to exercise are rapid, and heart rate plateaus within a minute or two of initiation of exercise (Gurguis and Wilkens 1995, Rose et al. 1998, O'Grady et al. 2001). In contrast, following periods of exercise, the heart rate takes considerably longer to drop to pre-exercise levels. Resting levels in *C. maenas* are reached in as little as 12 min (Hamilton and Houlihan 1992), but can take over 8 h following exhaustive exercise in *C. magister* (McMahon et al. 1979). These values represent repayment of an O_2 debt, the time being determined by the metabolic consequences of the exercise (Rose et al. 1998).

Cardiac output can be influenced by both heart rate and/or stroke volume of the heart. When *C. maenas* is exercised at sub-burst speed (5.8 m/min), there is a 1.8-fold increase in heart rate, coupled with a 1.5-fold increase in stroke volume leading to a 2.6-fold increase in cardiac output. These circulatory changes are accompanied by concomitant changes in ventilation and O_2 uptake (Hamilton and Houlihan 1992). Heart rate also influences cardiac output to a greater degree than stroke volume during activity in the blue crab *C. sapidus* (Booth et al. 1982) and the lobster *Homarus vulgaris* (Hamilton 1987). In contrast, when *C. magister* is forced to walk until exhausted, the heart rate only increases by 20%; however, cardiac output doubles, which suggests that for this species changes in cardiac output are afforded primarily by increases in stroke volume of the heart (McMahon et al. 1979). A similar pattern occurs during moderate walking activity in *C. magister*, but recovery from exercise takes less than 1 h as opposed to more than 8 h recovery from walking until exhaustion (McMahon et al. 1979, DeWachter and McMahon 1996a). In the lobster *H. americanus*, heart rate increases within 1 minute of exercise and remains elevated and steady while walking (Gurguis and Wilkens 1995, Rose et al. 1998), plateauing at 30–40% above resting levels while walking at speeds of 8 m/min. However, there is no observable increase in rate as a function of walking speed (Rose et al. 1998). Stroke volume, however, does increase as a function of walking speed and accounts for the bulk of the increased cardiac output (Rose et al. 2000). This underscores the limitation of relying on heart rate alone to interpret changes in cardiac output. In contrast, O'Grady et al. (2001) reported that the heart rate of lobsters does increase during forced walking activity; a 70% increase occurs at lower walking speeds of 0.9 m/min. Stroke volume has a greater influence on cardiac output in the amphibious crab *Pachygrapsus marmoratus* (Houlihan and Innes 1984) and the land crab *Cardisoma carnifex* (Wood and Randall 1981). Interestingly, when *C. carnifex* is exercised for prolonged periods, a bradycardia occurs, but this slowing of the heart likely allows increased filling time and a greater stroke volume (Herried et al. 1979). An increased cardiac output results in an increased hemolymph flow to the walking legs via the sternal artery, with smaller increases in flow through the anterolateral arteries (DeWachter and McMahon 1996a, McGaw and McMahon 1998). Digestion is impaired during activity (McGaw 2007) and is accompanied by a concomitant decrease in perfusion of the digestive gland via the hepatic arteries. Flow rates through the small posterior and anterior aortae are maintained during exercise (DeWachter and McMahon 1996a).

Although slow walking activity is the norm for most decapod crustaceans, some species exhibit specific behaviors such as rapid swimming or tail flips, which may be used for migration or escape from predators (McMahon et al. 1979, Hamilton and Houlihan 1992, Rose et al. 1998). When the portunid crab *Charybdis feriatus* swims, cardiac output increases by up to 60% due to an increased heart rate, whereas stroke volume actually decreases in this species (McMahon et al. 1996). This is accompanied by a major increase in blood flow to the leg muscles via the sternal artery, coupled

with lesser increases in the other major arterial systems. After prolonged swimming bouts of 2 h it takes 5 h for full recovery (McMahon et al. 1996). In macruran crustaceans, tail flips are associated with an increased flow through the sternal and large posterior aorta that supplies the tail muscles; these increases in flow persist for several minutes after tail flexion (Reiber et al. 1997). In lobsters, the heart rate remains stable during tail flexion, and so the 40% increase in cardiac output is driven by changes in stroke volume alone. The large increases in flows are not only the result of an increased cardiac output, but are also partially afforded by diverting hemolymph away from the anterior arteries and into the sternal artery and posterior aorta (Reiber et al. 1997). The rapid increase in flow through the posterior aorta supplies the O_2 demand of the muscles while a concomitant increase in flow through the sternal arteries supplies the scaphognathite muscles of the gill bailer (Reiber et al. 1997).

Burying

Representative species in 11 families of the Brachyura have been reported to exhibit burying behavior (Bellwood 2002, McGaw 2005b). The mechanics of burying are covered in detail elsewhere (Faulkes 2013). This mode of existence represents an unusual situation because, once buried, the animals body is supported in the sediment and is somewhat isolated from the surrounding environment. In addition, the respiratory openings are blocked, and animals may have to employ different ventilatory and cardiovascular mechanisms (Faulkes 2013).

Reports of cardiac function in crustaceans when they are buried are less common compared with ventilatory or respiratory responses. The heart rate of *Corystes cassivelaunus* decreases when buried (Bridges 1979), whereas in *C. maenas* the heart rate becomes erratic, interspersed with periods of cardiac arrest (Cumberlidge and Uglow 1978). Cardiac output increases significantly during the burying process in *C. magister* and *Cancer productus* (McGaw 2004). This increase in cardiac output is afforded primarily by an increased stroke volume and, to a lesser degree, an increase in heart rate. In both species, there is a subsequent decrease in cardiac output once the crabs are buried (McGaw 2004). This is due to a decrease in stroke volume in *C. productus*, but a decreased heart rate in *C. magister* (McGaw 2004). The decrease in cardiac output represents a reduced metabolic demand when buried, when only basal metabolic activities have to be supplied (Bridges 1979, Naylor and Taylor 1999). Spontaneous periods of cardiac arrest are common in settled animals (McDonald et al. 1977, Burnett and Bridges 1981), and the percentage of time spent in cardiac arrest increases in *C. productus* once buried (McMahon and Wilkens 1977, McGaw 2004). In general, cardiac and ventilatory parameters are usually tightly coupled in crustaceans (Wilkens et al. 1985, Jury et al. 1994a, Bock et al. 2001), with periods of cardiac arrest occurring in conjunction with ventilatory reversals (McMahon and Wilkens 1972, Wilkens et al. 1974, McGaw et al. 1994b). However, for buried crabs, the cardiac and scaphognathite pacemakers tend to uncouple, the reasons for which are unclear (McGaw 2004).

Hemolymph flows through the individual arterial systems of *C. magister* and *C. productus* also change during digging and subsequent burial (McGaw 2004). The burying process involves a large energy expenditure, and the greatest increase in flow rate occurs through the sternal artery that supplies the muscles of the thoracic sterna. Flow rates reach similar levels to those recorded during forced walking activity (DeWachter and McMahon 1996a). Once buried, hemolymph flows decrease substantially through the sternal artery, whereas hemolymph flow to the eyestalks and antennae via the anterior aorta increase (McGaw 2004). The antennae are usually the only structures protruding from the sand when the crabs are buried, and an increased blood flow to the musculature of these structures is reflective of their increased

flicking and sensory activity (McGaw 2005b). Hemolymph flow to the digestive organs via the anterolateral and hepatic arteries is maintained while the crabs are buried. This suggests that, in crabs, as in other organisms, digestion takes place during periods of inactivity (McGaw 2004). Occasionally, hemolymph flow through the sternal artery increases for approximately 30 sec before any movement is observed. This is followed by slight movements of the chelae, blowing sand out from between chelae and body by way of several ventilatory reversals and maintaining a clear pathway between the exostegal channels (Garstang 1897, McGaw 2004, 2005b). This suggests that cardiac function and locomotor activity may not be tightly coupled while animals are buried (O'Grady et al. 2001, Chabot and Webb 2008).

Feeding and Digestion

The consumption and subsequent digestion and assimilation of nutrients are associated with a general increase in metabolic parameters most commonly termed the specific dynamic action (SDA) of food (Secor 2009). In ectothermic invertebrates, this is represented as an increase in O_2 uptake or an increased CO_2 production. These increases in O_2 uptake represent the energy needed for feeding activity, mechanical digestion, production of enzymes, and the subsequent transport of nutrients and intracellular digestion (protein synthesis; Secor 2009). This increase in O_2 uptake and its subsequent delivery to the tissues is afforded by an increase in cardiac activity in *C. sapidus* (McGaw and Reiber 2000), *C. maenas* (Rovero et al. 2000), *C. magister*, and *Cancer gracilis* (McGaw 2005c, 2006b,c), which occurs immediately when the food is detected and before the animals have started feeding. The actual feeding process is associated with a sharp increase in heart rate, stroke volume, and cardiac output, the result of increased activity during food handling (McGaw and Reiber 2000, McGaw 2006b). This is accompanied by a doubling or tripling of flow rates through the sternal artery, branches of which supply the mouthparts and the chelae (McGaw and Reiber 2000). Increases in sternal artery flow rates are accompanied by increased flow through anterolateral arteries; this is the typical pattern observed during activity (McGaw et al. 1994b, DeWachter and McMahon 1996a). An increased flow in the anterolateral arteries between 1 and 3 h after feeding supplies blood to the cardiac stomach muscles aiding mechanical breakdown in the foregut (McGaw 2005c, 2006b, McGaw and Reiber 2000, 2002). Extracellular digestion by the hepatopancreas begins within 0.5–1 h after feeding (Dall 1967, Hopkin and Nott 1980), which corresponds closely with the initial increase in blood flow through these arteries. The total time (8 h) of increased perfusion of the hepatic arteries in *C. sapidus* correlates with the period of extracellular digestion (McGaw and Reiber 2000).

Heart rate in blue crabs typically remains elevated for up to 24 h following feeding, and there is a close correlation between the decline in heart rate to prefeeding levels and time for food clearance from the digestive system. In *C. magister* and *C. gracilis*, heart rate remains elevated for 10 h and 5 h, respectively (McGaw and Reiber 2000, McGaw 2005c, 2006b). In the latter two species, the changes in cardiac parameters and flow rates are not as pronounced compared with blue crabs; *C. magister* and *C. gracilis* were artificially fed a set meal size via a tube, whereas *C. sapidus* were allowed to forage freely and tended to consume larger meals (McGaw and Reiber 2000). Oxygen uptake usually remains elevated for longer periods (2–3 days) than cardiac parameters following feeding in crabs. This reflects increased energetic requirements for intracellular protein synthesis, which contributes to the bulk of the SDA response (Houlihan et al. 1990, Mente et al. 2003).

If crabs are deprived of food, a decrease in heart rate occurs between 5 and 10 days after the last feeding, which is associated with longer term decreases in O_2 uptake (Ansell 1973, Taylor and Naylor 1999). Heart rate also becomes more stable during this period with less variation between average day and nighttime heart rates (Ansell 1973).

Environmental Stressors

Temperature

Temperature is undoubtedly the most important environmental factor affecting the physiology, behavior, and distribution of aquatic ectotherms, and, in general, metabolic rate is positively correlated with temperature (Willmer et al. 2005). Crustaceans routinely experience seasonal, diel, or short-term changes in temperature (Lagerspetz and Vainio 2006) and exhibit different physiological and behavioral reactions depending on whether the temperature change experienced is acute or chronic (Whiteley et al. 1997, Cuculescu et al. 1998, Lagerspetz 2003). Because crustaceans are ectotherms and cannot regulate their body temperature, it will vary directly with the environment. The heart rate of many aquatic ectotherms is very sensitive to alterations in body temperature, and these changes in heart rate can be an important proxy for indicating animal stress (Iftikar et al. 2010). In crustaceans, delivery of O_2 to the tissues (via the circulatory system) in response to O_2 demand is essential for survival in temperature extremes, and a mismatch will determine the survival limits for many species (Frederich and Pörtner 2000). The temperature ranges within which an individual species can function is termed the *optimum* range, whereas temperatures outside this range, at which physiological mechanisms start to break down and do not recover, are referred to as the *pessimum* range (Frederich and Pörtner 2000).

In crustaceans, the majority of articles on cardiac modulation in response to temperature change concentrate on heart rate alone and report an increase in rate with increasing temperature and vice versa (Ahsanullah and Newell 1971, Spaargaren 1973, 1974, Cumberlidge and Uglow 1977, deFur and Mangum 1979, Burton et al. 1980, Depledge 1984, Morris and Taylor 1984, Mercaldo-Allen and Thurburg 1987, Reiber and Birchard 1993, Aagaard 1996, DePirro et al. 1999, DeWachter and McMahon 1996b, DeWachter and Wilkens 1996, Stillman and Somero 1996, Goudkamp et al. 2004, Stillman 2004, Camacho et al. 2006, Worden et al. 2006, Ungherese et al. 2008, Iftikar et al. 2010). Increases in rate are often accompanied by a reduction or abolition of spontaneous periods of cardiac arrest (DeWachter and McMahon 1996b, Camacho et al. 2006, Worden et al. 2006). Typical Q_{10} values for heart rate vary between 1.5 and 4; higher Q_{10} values are measured outside the thermal optimum range and tend to indicate a more stenothermal species (DeWachter and McMahon 1996b, Frederich and Pörtner 2000, McGaw and Whiteley 2012).

The rate and magnitude of temperature change may also affect heart rate. Lobsters respond to a rapid change in temperature (either an increase or decrease) with a short-term bradycardia (<1 min), followed by a tachycardia (Jury and Watson 2000). However, this response is not always observed if the temperature is changed more slowly (Jury and Watson 2000). In the freshwater prawn *Palaemonetes antennarius*, a rapid change of up to 8°C has no effect on heart rate (Ungherese et al. 2008). A hysteria of heart rate similar to that reported in reptiles has been observed in the crayfish *Cherax destructor*, whereby the change in heart rate is significantly higher during heating than during cooling. The increased rate during heating may act to dissipate some of the heat via the peripheral circulatory system (Goudkamp et al. 2004). Nevertheless, heart rate does not continue to rise indefinitely with increasing temperature. The heart rate increases up to a certain temperature, after which the rate starts to decrease and become erratic, followed by acardia at the upper critical maxima (Fig. 7.6). This break point, referred to as the *Arrhenius break temperature* (Stillman and Somero 1996) or the *pejus range* (Frederich and Pörtner 2000), is often used to determine the thermotolerance of a species (Stillman and Somero 1996, Camacho et al. 2006).

Acclimation to higher temperatures (20°C vs. 4°C) raises both the Arrhenius breakpoint temperature and the CT_{Max} in lobsters *H. americanus* by approximately 3°C and 5°C, respectively (Camacho et al. 2006). Likewise, in different species of temperate and tropical porcelain crabs, acclimation to high temperatures (10°C difference) increases the CT_{Max} in all species but is more

pronounced in the temperate species. The opposite occurs for acclimation to cooler temperatures; the CT_{Min} of the tropical species is extended to a greater degree (Stillman 2004). Although 2–3 weeks are usually considered the standard time for full acclimation to occur (McGaw and Whiteley 2012), in lobsters, cardiac function may be complete in as little as 3 days (Camacho et al. 2006). Nevertheless, there may be a longer term seasonal acclimation that is not abolished even after 4 weeks acclimation to laboratory conditions. A lower basal cardiac rate has been measured for lobsters collected in winter (Jury and Watson 2000). In contrast, the heart rate of winter-collected

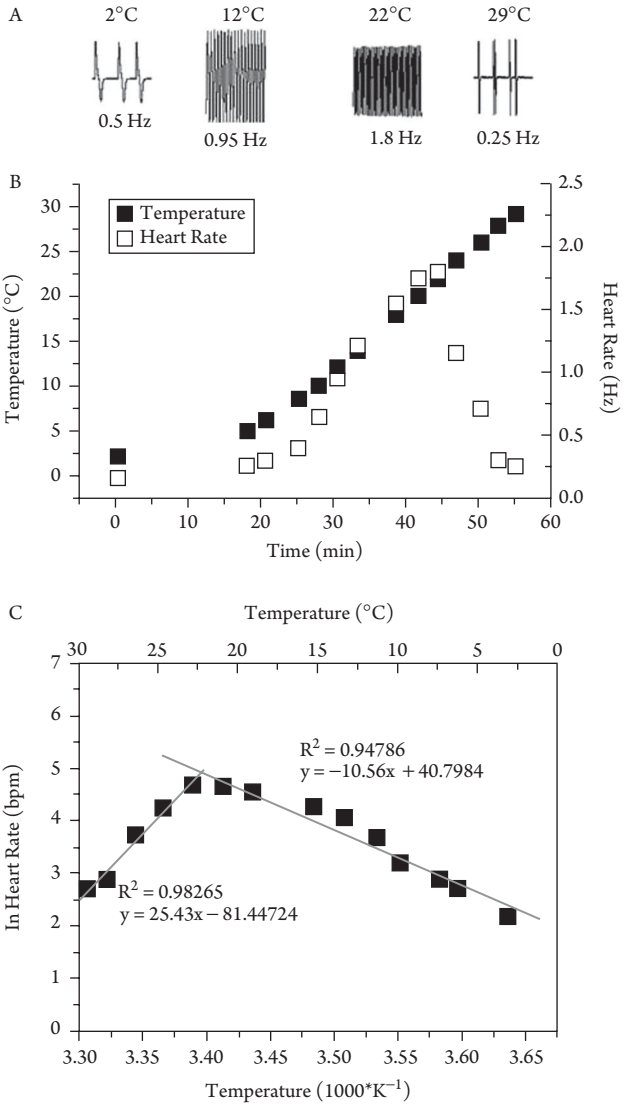


Fig. 7.6.
Heart rate changes in a single lobster, *Homarus americanus*, over a temperature range of 2–30°C. (A) Raw data traces. (B) Changes in heart rate over an increasing temperature range; here, heart rate reaches a maximum at 22°C before declining thereafter, with a CT_{Max} value of 29.3°C. (C) Arrhenius break plot temperature is the discontinuity in the slope determined by intercept of the linear fits to the rising and falling slopes. From Camacho et al. (2006), with permission from Springer.

crabs, *P. marmoratus*, is higher at any set temperature (DePirro et al. 1999), indicating a partial metabolic compensation (Hochachka and Somero 2002).

Although changes in heart rate in response to temperature change are well established, fewer studies have measured the contractility or stroke volume of the heart of crustaceans. This is important because it is the actual cardiac output that will determine delivery of O_2 to the tissues, and heart rate and stroke volume can vary independently to influence cardiac output (DeWachter and McMahon 1996, McGaw and McMahon 1996). The heart rate of lobsters is low and of large amplitude in cold water, becoming faster and of lower amplitude when the water is warmed (Worden et al. 2006). The cardiac output increases up to a temperature of approximately 10°C, only decreasing in strength toward the higher temperatures (22°C) tested (Worden et al. 2006). Likewise, in Dungeness crabs, the heart rate increases as the water is warmed from 4°C to 20°C; however, the increased rate shortens the diastolic filling period, and stroke volume decreases significantly up to about 12°C, with a slower decline thereafter, between 12°C and 20°C. These changes in heart rate and stroke volume result in an increase in cardiac output (DeWachter and McMahon 1996b). This increase in cardiac output is paralleled by a general increase in flow rates through each arterial system, although there is no differential hemolymph redistribution among each arterial system (DeWachter and McMahon 1996b). Although there is a general decrease in hemolymph flows when the spider crab *Maja squinada* is cooled from 12°C to 0°C, a break point occurs at 8°C, and hemolymph is diverted away from the lateral arteries and toward the muscles of the thoracic sterna and scaphognathites via the sternal artery; this may help maintain locomotor and ventilatory activity at lower temperatures. The diversion of flow to the sternal artery is accompanied by a concomitant diversion of hemolymph to the hepatopancreas via the hepatic arteries, possibly preventing hypoxia in this homeostatic organ (Frederich and Pörtner 2000, Bock et al. 2001). Flow rates in both the arterial and venous system decrease substantially at the lowest temperature, indicating that the critical thermal limits have been reached (Bock et al. 2001).

Although the exact location of the thermal receptors in crustaceans is still unknown, changes in cardiac function are thought to be mediated by the neurons rather than by the direct effects of temperature on the cardiac muscle (Jury and Watson 2000, Goudkamp et al. 2004, Worden et al. 2006). The lobster *H. americanus* may be able to respond to changes in temperature by as little as 0.15°C and can certainly detect changes of 1°C (Jury and Watson 2000). These authors suggest that the cardioregulatory nerves are responsible for regulating heart rate because lobsters with severed nerves do not respond to temperature change. In isolated lobster heart preparations, apart from a slightly lower heart rate, changes in cardiac parameters are the same as those observed in whole-animal preparations, and so it was suggested that the temperature effects are initiated via the cardiac ganglion rather than by peripheral regulatory nerves (Worden et al. 2006). In contrast, in isolated heart preparations of Dungeness crabs, the heart rate was only about one-third of that of intact animals, resulting in a decrease rather than an increase in cardiac output with increasing temperature (DeWachter and Wilkens 1996). These workers suggested that the heart must be regulated by neurohormones and/or the cardioregulatory nerves. In support of hormonal modulation of cardiac function, Kuramoto and Tani (1994) found that octopamine and serotonin are released from ligamental nerves of the pericardial organs of the spiny lobster *P. japonicas* during cooling. This may protect this species during low temperature exposure because spiny lobsters do not show as pronounced a bradycardia with decreasing temperature compared with other species (Nakamura et al. 1994, Kuramoto 1999).

Oxygen

Crustaceans encounter aquatic environments that are permanently or temporarily hypoxic, and this has resulted in the evolution of an array of behavioral and physiological responses. The physical characteristics of water, low O_2 capacitance, high viscosity, and slow diffusion rates, all

contribute to the stress of an animal in dealing with hypoxic exposure. Aquatic organisms must develop compensatory mechanisms to deal with a reduction in water O_2 levels because of the very nature of water as a medium (Toulmond 1987). The diverse array of habitats that crustaceans live in make their physiological responses toward hypoxia broad and difficult to categorize in a simple way. Animals are classically divided into two mutually exclusive groups: O_2 regulators, which maintain O_2 consumption (Mo_2) independent of environmental O_2 tension (Po_2), or O_2 conformers, in which Mo_2 varies in proportion to water Po_2. This division has been imposed on the system for easy categorization rather than to fit observed responses (Mangum and Van Winkle 1973).

A reduction in O_2 tension elicits a wide range of physiological responses, with O_2 conformation and regulation at opposite ends of a spectrum. The basic problem of maintaining O_2 delivery to metabolically active tissues with low hypoxic tolerance must remain in central focus when interpreting these physiological responses. Separating O_2 regulation from O_2 conformation is not always readily apparent, even within species. Many animals will regulate their O_2 consumption independent of water Po_2 down to some critical level below which they become O_2 conformers. The inflection point is known as P_{crit} and is used as the standard against which organisms are compared for hypoxic tolerance. P_{crit} for a given species is not constant. A variety of physical and physiological parameters influence the point at which an individual will switch from O_2 regulation to conformation (Maynard 1960, Taylor et al. 1977a, Herreid 1980). P_{crit} must be interpreted as an integrated homeostatic balance point that changes with internal and external conditions. The interpretation of P_{crit} remains under debate; however, conceptually, P_{crit} is a useful tool in evaluating an animal's adaptive responses toward hypoxia. If P_{crit} is considered a relative value that will be influenced by physical (temperature, salinity, etc.) and physiological (ventilatory, cardiovascular, metabolic, etc.) parameters, then one can make a prediction as to how a given parameter will influence this inflection point (Herreid 1980, Mangum and Van Winkle 1973).

Larger crustaceans (decapods) typically attempt to maintain O_2 consumption in the face of declining water Po_2. Oxygen regulation results from a combination of physiological adjustments leading to an increase in O_2 conductance. Oxygen conductance for the whole animal is the reciprocal function of total resistance (the summation of resistance in a series). This implies that conductance may be increased at any point between the external environment and the utilization of O_2 in the mitochondria. It appears that the primary mechanisms used by decapods to increase conductance are the modulation of ventilatory parameters, cardiovascular functions, and hemolymph pigment affinity (Dejours et al. 1970). Decapods exhibit a range of responses enabling them to regulate Mo_2 independent of water Po_2; however, not all of these are fully understood.

An initial increase (short-term response) in ventilation (V_w) accompanies a reduction in water Po_2 in most actively regulating decapods. Ventilatory volume can be increased as the result of increasing scaphognathite beat frequency (f_{sc}), and/or by adjusting the position of the scaphognathite (epipodites of the third maxillipeds) and the configuration of the pumping channel, thereby altering stroke volume (S_v; see McMahon and Wilkens 1983). Sole reliance on f_{sc} adjustments to compensate for a drop in O_2 availability does not appear to be a ubiquitous response in crustaceans. The typical ventilatory responses of decapod crustaceans (McMahon et al. 1974, Dejours and Beekenkamp 1977, Zanotto et al. 2005) to hypoxia is to increase both f_{sc} and adjust scaphognathite stroke volume concomitantly. This does not exclude the possibility that an increase in V_w could result entirely from an increase in f_{sc}. Increasing V_w brings more water over the exchange surfaces at the energetic expense of driving the scaphognathites (Wheatly and Taylor 1981, Wheatly 1993). If Mo_2 is maintained and V_w increases, then, by definition, the O_2 extracted from the water (E) will decrease, provided that Pvo_2 does not decrease substantially (Dejours and Beekenkamp 1977, Herreid 1980, Wheatly and Taylor 1981, Wilkes and McMahon 1982). The compensatory respiratory mechanism for short-term hypoxic exposure is to maintain O_2 delivery to the gills by increasing V_w, which is reflected by an increase in the convection requirement for water (V_w/Mo_2). The

increase in V_w (hyperventilation) also results in a respiratory alkalosis that increases hemocyanin (Hcy) O_2 affinity (Bohr shift). The decrease in extraction is compensated for, as reflected by maintenance of the arteriovenous O_2 content difference and maintenance of MO_2 (Wheatly and Taylor 1981, Wilkes and McMahon 1982).

The initial acute response toward hypoxic exposure is a transient increase in V_w, which maybe an avoidance response (Wilkes and McMahon 1982, Wheatly 1993). Sole reliance on respiratory adjustments does not appear to be responsible for the maintenance of MO_2 beyond short-term exposure (6–24 h). Long-term hypoxic adaptation appears to rely in part on mechanisms other than respiratory adjustments. Evidence for this comes from observation of a number of decapods (McMahon et al. 1974, deFur and Mangum 1979, Wilkes and McMahon 1982, deFur and Pease 1988, Harper and Reiber 2006a) during long-term hypoxic exposure (6–12 days). A diminution of f_{sc} and S_v is observed after approximately 24 h, with V_w dropping significantly compared to maximum hypoxic exposure values. The initial increase in convection requirement for water indicates a ventilatory compensation for the decrease in water PO_2. The convection requirement for water drops as V_w declines and MO_2 remains unchanged. Efficiency of gas exchange is increased through "other" long-term adaptive changes, as indicated by increases in convection requirements for hemolymph (V_b/MO_2), transfer factor (TO_2), and hemocyanin O_2 affinity, and a decrease in ventilation perfusion ratio (V_w/V_b; see McMahon et al. 1974, Wheatly and Taylor 1981, Wilkes and McMahon 1982). The decline in respiratory-mediated compensation is met by an increase in O_2 conductance, as indicated earlier. The transfer factor (TO_2) for O_2 through the gill (O_2 consumption as related to O_2 uptake per kPa O_2 pressure gradient across the gill) increases significantly in a number of crustaceans examined during prolonged hypoxic exposure (Wheatly and Taylor 1981, McMahon et al. 1974, Wilkes and McMahon 1982). The convection requirement for hemolymph (V_b/MO_2) and the ventilation perfusion ratio (V_w/V_b) both increase, the latter a result of declining V_w and a maintenance or increase in cardiac output (V_b). These changes would indicate a cardiovascular compensatory component toward increased O_2 conductance (Wheatly and Taylor 1981, McMahon et al. 1974, Wilkes and McMahon 1982, McMahon and Wilkens 1983, Reiber et al. 1992). Hemocyanin O_2 affinity also increases during both short- and long-term hypoxic exposure. These ventilatory-associated and/or nonventilatory components allow the animal to maintain MO_2 during hypoxic exposure for long periods of time.

The hypoxia-induced hyperventilation not only increases O_2 delivery, but also removes CO_2 from the hemolymph. The decrease in CO_2 results in a rise in hemolymph pH; that is, a respiratory alkalosis that causes an increase in hemocyanin O_2 affinity (Redmond 1955, Dejours and Beekenkamp 1977, Wilkes and McMahon 1982, Taylor 1982). The extreme hyperventilation observed early during hypoxic exposure initiates the rise in hemolymph pH. The alkalosis persists during long-term hypoxic exposure and is associated with a base deficit that is never fully compensated (Wilkes and McMahon 1982, Wheatly 1993). The increase in hemocyanin O_2 affinity appears to be mediated in part by a H^+ concentration-dependent increase (Bohr shift) resulting from the respiratory alkalosis. A second component to the increase in Hcy O_2 affinity is less understood and is H^+ independent. In several crustaceans, the increased Hcy O_2 binding allowed a significantly greater saturation of postbranchial blood. This increase in Hcy O_2 affinity during hypoxic exposure allowed 60% postbranchial saturation opposed to 20% saturation under normoxic conditions (Wilkes and McMahon 1982, Guadagnoli et al. 2005). The respiratory alkalosis resulting from hyperventilation is found to be offset slightly by a metabolic acidosis (L-lactate; Wheatly and Taylor 1981). L-lactate has been found to increase Hcy O_2 affinity and may play a role in increasing O_2 conductance through its interactions with hemocyanin (Mangum 1983). The increase in Hcy O_2 affinity plays a major role in increasing O_2 conductance maintaining the pressure gradient across the gills by removing O_2 from solution, thereby aiding maintenance of MO_2 (Booth et al. 1982, Mangum 1983, Wheatly 1993).

The cardiovascular response toward hypoxia has seen intense investigation in recent years, yet it is still the least understood of the compensatory mechanisms. Oxygen conductance increases with progressive hypoxia (as stated earlier) and is in part due to alterations in cardiovascular parameters (McMahon et al. 1974, Wheatly and Taylor 1981, Wilkes and McMahon 1982, McMahon and Wilkens 1983, Reiber et al. 1992). Decapod crustaceans exposed to progressive hypoxia tend to reduce heart rate (hypoxia induced bradycardia). This has been demonstrated in many decapods and appears to be a consistent response to hypoxic exposure in this group (Larimer and Gold 1961, Larimer 1962, McMahon et al. 1974, deFur and Mangum 1979, Wheatly and Taylor 1981, Reiber et al. 1992). As heart rate decreases, a concomitant increase in stroke volume takes place, thus maintaining cardiac output at or near normoxic levels down to a critical O_2 tension. Cardiac output in response to hypoxic exposure in several decapod species has been quantified using a variety of methods (Fick principle, dye dilution, and pulsed Doppler), and a hypoxia-induced bradycardia has been observed in each instance; however, cardiac output remains unchanged due to an increase in stroke volume (Wheatly and Taylor 1981, Wilkes and McMahon 1982, Reiber et al. 1997, Reiber and McMahon 1998, Harper and Reiber 1999, Guadagnoli et al. 2005, Guadangoli et al. 2007). The increase in stroke volume appears to be due to an increase in end diastolic volume resulting from both an increased filling time and filling pressure. During hypoxic exposure, mean pericardial sinus pressure was found to increase significantly over normoxic levels. Diastolic intracardiac pressure did not change during hypoxic exposure. The net effect is an increase in the pressure gradient between the pericardial sinus and the heart, which aids cardiac filling (Reiber et al. 1992, Reiber 1992, 1994, Harper and Reiber 1999, Guadangoli and Reiber 2005, Guadangoli et al. 2007).

The decrease in heart rate associated with hypoxic exposure has been of note for some decades and seen a great deal of discussion, but, until recently, no evidence has been produced to support the hypothesis that a benefit to the animal results from bradycardia. In 2007, Guadangoli, Tobita, and Reiber showed in grass shrimp that cardiac work is significantly decreased during periods of short-term hypoxic exposure and bradycardiac events (Fig. 7.7). Matched intracardiac pressures and cardiac volumes were used to develop pressure-volume loops with the area of the loop related to cardiac stroke work. Cardiac stroke work was seen to decrease significantly with progressive hypoxic exposure and decreased heart rates.

Along with the hypoxia-induced bradycardia and increased stroke volume comes a redistribution of cardiac output. Arterial hemolymph flow was measured using a pulsed Doppler technique in the anterior aorta, posterior aorta, and sternal artery of the crayfish *P. clarki* (Reiber et al. 1992, Reiber et al. 1997, Reiber and Wang 1997, Reiber and McMahon 1998; Fig. 7.8). As water P_{O_2} was decreased, arterial flow declined in the posterior aorta and sternal artery, whereas anterior aortic flow increased significantly. Control of arterial flow is thought to be neurohormonally modulated via the action of the cardioarterial valves (Kuramoto and Ebara 1984, McMahon and Burnett 1990, Reiber et al. 1992, Reiber 1994). Redistributing arterial flow toward the anterior region of the animal may have a protective function by maintaining O_2 delivery to hypoxic-sensitive nervous tissues. Decreasing hemolymph flow to the abdominal region in combination with maintaining cardiac output would effectively increase the minute volume of hemolymph circulating through the gills, which would confirm the hypothesis put forward by McMahon et al. (1974), Wheatly and Taylor (1981), and Wilkes and McMahon (1982). Altering hemolymph flow patterns through the gills to increase the functional exchange area has also been hypothesized as a mechanism to increase O_2 conductance at the gill (McMahon et al. 1974). There is no direct evidence for this in crayfish; however, Taylor and Taylor (1986) have found evidence of valve-like structures in the gills of other decapod crustaceans. Functionally, these structures may play a role in regulation of gill perfusion. The observed cardiovascular responses toward progressive hypoxia in these animals is very similar to the diving response seen in vertebrates and may serve many of the same functions. The role the cardiovascular responses play in increasing O_2 conductance during hypoxic exposure is only now

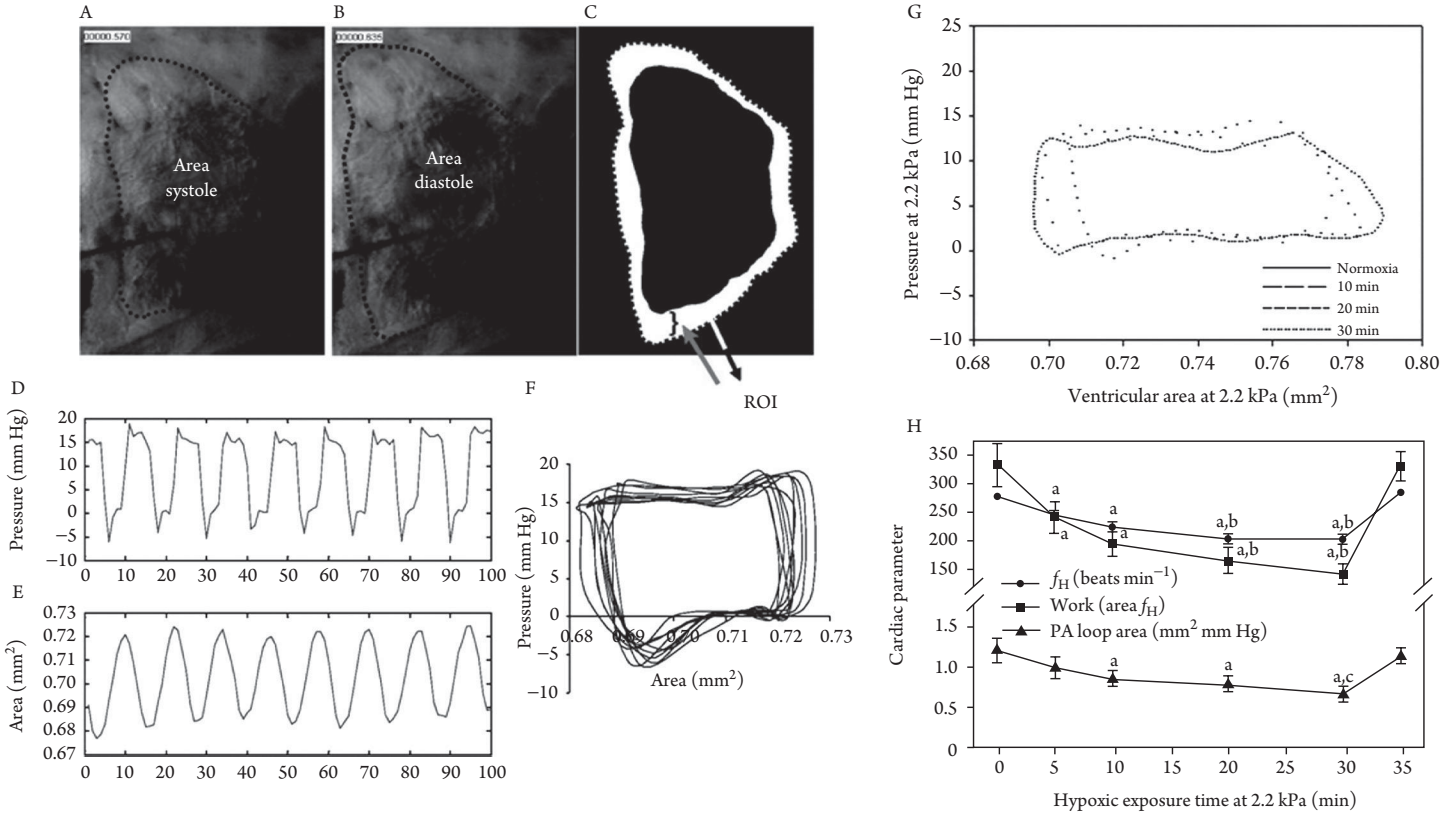


Fig. 7.7.
(A) An outline of the heart in end systole as it defines the minimal area of the heart. (B) An outline of the heart in end diastole as it defines the maximal area. (C) The difference between the maximal (end-diastolic volume [EDV]) and minimal (end-systolic volume [ESV]) areas defines the region of interest (ROI) or stroke volume used in automated area analysis. (D) Intraventricular pressure, as it coordinates with E. (E) Changes in cardiac area (multiple cardiac cycles are shown) calculated from ROI. (F) Eight pressure–area (P–A) loops generated by combining the values from D and E. (G) Representative P–A loops from a single animal under normoxic conditions (20.5 kPa) and after 10, 20, and 30 min of hypoxia at 2.2 kPa. The P–A loop areas are 1.175 for normoxia and 0.867 at 10 min, 0.854 at 20 min, and 0.848 at 30 min exposure to hypoxia (2.2 kPa). (H) Relationship between heart rate (f_H), P–A loop area, and minute cardiac work (CW). Measurements were made at 5, 10, 20, and 30 min after exposure to hypoxia, which was followed by exposure to normoxic water for 5 min and a final measurement taken at 35 min. The letter "a" denotes a standard deviation (s.d.) from time 0 at 20.5 kPa; the letter "b" denotes s.d. from 5 min at 2.2 kPa; and the letter "c" denotes s.d. from 10 min at 2.2 kPa ($P < 0.001$). From Guadangoli et al. (2011), with permission from The Company of Biologists, Inc.

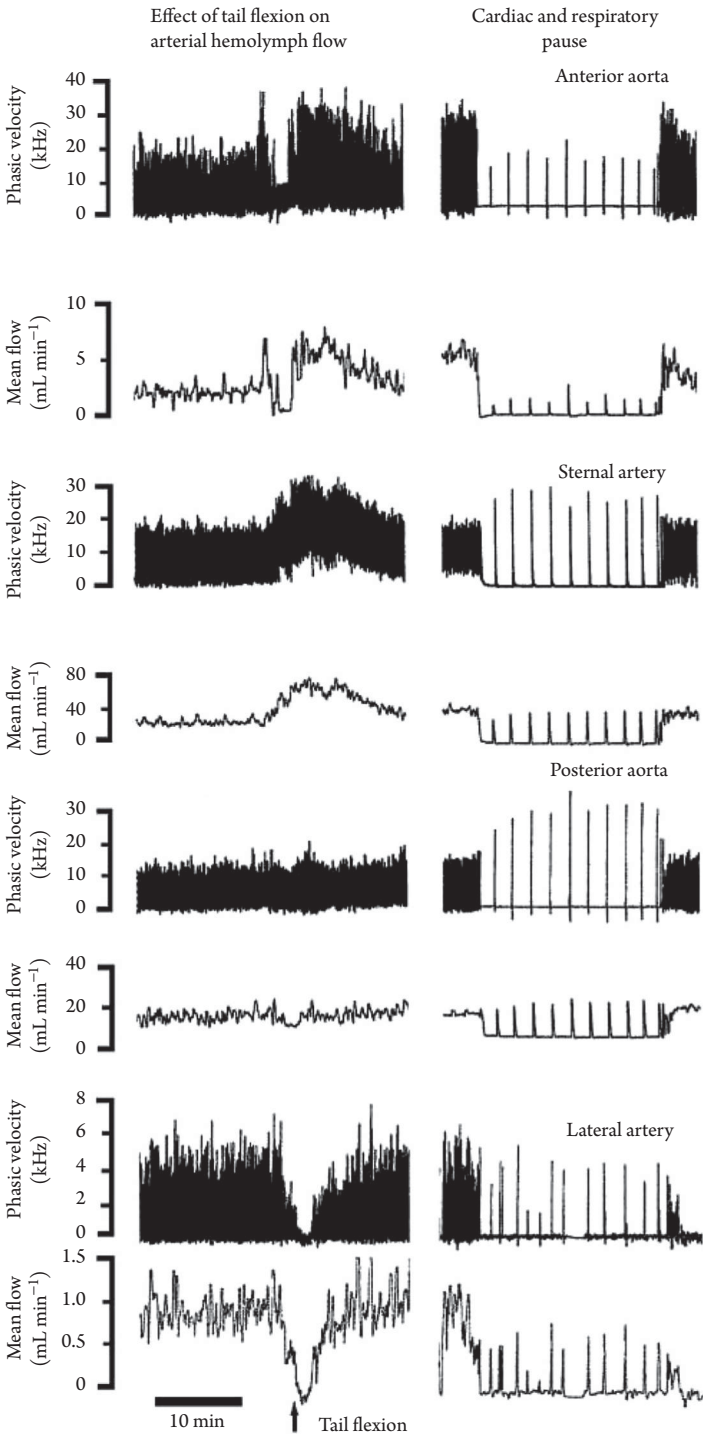


Fig. 7.8.
Composite recording from two lobsters of anterior aortic, sternal arterial, posterior aortic, and right lateral arterial hemolymph flow rates (both phasic velocity and mean flow rates are shown) during tail flexion (an arrow indicates the start of tail flexion; left panel) and during a cardiac and respiratory pause (right panel). From Reiber et al. (1997), with permission from The Company of Biologists, Inc.

beginning to be understood. It appears that modulation of cardiovascular parameters aids significantly the animals' ability to withstand prolonged hypoxic exposure.

Crustaceans exposed to environmental hypoxia are able to maintain aerobic metabolism down to a critical P_{O_2}, below which the animal must decrease M_{O_2} and switch to anaerobic metabolism. While exposed to water with a P_{O_2} above P_{crit}, the animal is able to mobilize a number of mechanisms that allow the maintenance of M_{O_2} by increasing O_2 conductance. Increasing ventilatory volume V_w via increasing f_{sc} and S_v brings more O_2 to the gill and allows short-term compensation. Modulation of Hcy O_2 affinity via the Bohr effect accounts for much of the long-term compensation. Cardiovascular parameters are also modulated, possibly increasing gill perfusion and altering gill perfusion patterns. Cardiac output is redirected toward the anterior region of the animal, presumably to maintain adequate O_2 delivery to hypoxic-sensitive nervous tissues while the hypoxia-induced bradycardia decreases cardiac work. The cardiovascular system plays a significant role in increasing O_2 conductance and allows the animal to survive severe hypoxic waters for extended periods of time. A limitation in any of the above-mentioned physiological parameters would result in an observed increase in P_{crit}.

Emersion

Crustaceans exhibit three main responses to aerial exposure depending on their lifestyle (deFur 1988): animals that are obligate water breathers and are rarely emersed tend not to be able to oxygenate the blood while exposed. Amphibious crustaceans that inhabit the intertidal zone or ephemeral pools vary greatly in their ability to tolerate aerial exposure, ranging from those that are essentially similar to subtidal species to the bimodal breathing crustaceans that can respire efficiently in both water and air. Finally, a number of groups (decapods and isopods) have made the transition to land and will drown if submerged in water for any period of time (deFur 1988). There are a number of articles on cardiorespiratory mechanisms in a variety of amphibious and terrestrial crustaceans. Interestingly, during emersion periods, there does not appear to be a close coupling in ventilation and cardiac parameters (Wilkens et al. 1985, deFur 1988, Rose et al. 1998). For example, in *C. productus, C. magister,* and *C. maenas*, although heart rate and cardiac output either remain stable or decline, there can be both short-term and longer term increases in scaphognathite beat frequency (Taylor and Butler 1978, deFur and McMahon 1984a,b, Airriess and McMahon 1996).

In *C. magister*, which is rarely exposed to air, there is a slow decline in heart rate with a concomitant drop in stroke volume and thus cardiac output (Airriess and McMahon 1996). Likewise, juvenile *C. productus* exhibit a drop in heart rate; however, this response is size-dependent, and in larger adults the heart rate remains unchanged when emersed (deFur and McMahon 1984a,b). In contrast, large *C. sapidus* respond with an increase, whereas small crabs exhibit a decrease in scaphognathite and heart rate during a 4 h period of emersion (deFur et al. 1988). There are substantial limitations to gas exchange during emersion in blue crabs because, like fish, the gills clump in air. These smaller individuals may survive aerial exposure by a reduction in metabolic demand or tolerance of the hypoxic conditions (deFur et al. 1988). A general metabolic depression occurs in *C. magister* coupled with a decrease in hemolymph flow rates through all arteries except the anterior aorta, where a substantial increase occurs. This artery serves the subesophageal ganglion (brain) of the animals, and doubling of flow through this vessel would serve to maintain oxygenation of this structure (Airriess and McMahon 1996).

Amphibious species, such as the green crab *C. maenas*, are better adapted to life in the intertidal zone. They show no change in heart rate and, unlike *C. productus*, are able to maintain blood oxygenation status, possibly via an increase in stroke volume (Taylor and Butler 1978). However, heart rate is also temperature-dependent, and it will decrease if they exit into cooler air or vice versa

(Wheatly and Taylor 1979, Taylor and Wheatly 1981, Depledge 1984). The intertidal crab *P. marmoratus* shows a similar response—a slight but consistent bradycardia in air, which is also dependent on the air temperature (DePirro et al. 1999). In the amphibious purple shore crab *Hemigrapsus nudus*, a tachycardia occurs when emerging, followed by a gradual but slight bradycardia in air, but no extended periods of cardiac arrest (Greenaway et al. 1996). The higher O_2 of air content and its high diffusion rate allows *H. nudus* to maintain an adequate PO_2 with only intermittent ventilation (Greenaway et al. 1996). The purple shore crab *Leptograpsus variegatus* exhibits both elevated heart and ventilation rates, as well as increased O_2 consumption in water compared to air. Part of this increase in rate may be due to increased activity in submerged crabs. The arterial and venous PO_2 are unchanged by immersion, whereas re-emersion promotes a transient increase in venous hemolymph oxygenation. Although immersion increases respiration rate, possibly via increased activity, this does not affect gas exchange or transport, and *L. variegatus* is able to maintain a high PO_2 in both air and water (Morris and Edwards 1996).

In bimodally breathing crustaceans such as *Cardisoma guanhumi*, which can live on land or in water, different responses are observed. These crabs have a reduced gill surface area and enlarged branchial chambers. There is no change in heart rate when crabs move from air into water, although after 7 days, heart rate decreases slightly. When re-emersed, a slight increase in rate occurs with a slow decline back to preimmersion levels (Gannon et al. 2001). In contrast, Shah and Herried (1978) report a 50% decrease in rate in this species because there is less O_2 in water, and a tachycardia occurs when re-emersed to repay the O_2 debt. In the land crab *Gecarcinus lateralis*, immersion leads to a depression of heart rate by almost 50%, with a supposed decrease in cardiac output. It is thought that this leads to a decrease in blood delivery to the gills and results in death if the animal is immersed for extended periods. Interestingly, there is no tachycardia to repay O_2 debt when re-emersion takes place; heart rate simply returns to normal (O'Mahoney-Damon 1984). In the terrestrial crab *Holthuisana transversa*, there can be a shunt of blood from gills to lungs (Taylor and Greenaway 1984). In water, more blood is sent to the gills, whereas when the animals are emersed the blood is then shunted to the lungs, so much so that they are capable of directing almost the entire respiratory flow to one structure or the other depending on the medium. The switch-over can take several hours, especially when emersed, suggesting that they retain some limited capacity for respiration in either medium (Taylor and Greenaway 1984).

Salinity

The osmoregulatory physiology of decapod crustaceans has been studied extensively (reviewed in Mantel and Farmer 1983, Pequeux 1995; see also Chapter 8 of this volume). The most common physiological response to low salinity is an increase in O_2 uptake (Engel et al. 1975, Taylor 1977, Guerin and Stickle 1992, Jury et al. 1994a), which is driven by an increased scaphognathite beat frequency (Cumberlidge and Uglow 1977, McGaw and McMahon 1996, Dufort et al. 2001). These changes are thought to reflect an increased energy requirement for active ion uptake (Taylor 1977, Jury et al. 1994a). Within the field of crustacean comparative physiology, there is a growing literature on the cardiovascular responses to low salinity, and the most common cardiac response to low salinity is a pronounced tachycardia (Hume and Berlind 1976, Cumberlidge and Uglow 1977, Spaargaren 1982, McGaw and McMahon 1996, 2003, McGaw and Reiber 1998, Dufort et al. 2001). Despite the fact that most crustaceans react to hyposaline exposure with an increase in cardiorespiratory parameters, earlier work by Kinne (1964) suggested that organisms respond to a dilution of the media by exhibiting an increase, a decrease, or no change in respiration levels. It was hypothesized that euryhaline organisms showed an increase, and stenohaline organisms exhibited a decrease in respiratory and cardiac parameters. However, in the past, this had been difficult to substantiate because most studies on crustaceans were limited to those that were classed as efficient

hyperosmoregulators (Wheatly 1988). With the increase in respiratory and cardiovascular studies in recent years, a general pattern is now emerging to which many decapod crustaceans conform.

In contrast to species that are able to hold the body fluid concentration above that of the medium, osmoconforming species exhibit a bradycardia (Spaargaren 1973, Cornell 1973, 1979, McGaw 2006b, Curtis et al. 2007) and a decrease in O_2 uptake (McGaw 2006b). The osmoconforming crab *C. gracilis* may be exposed to short periods of low salinity in its natural habitat and can only survive for a few hours in salinities below 55% seawater (Curtis et al. 2007). The decreases in cardiac function and hemolymph flow rates of *C. gracilis* in low salinity are a result of behavioral adjustments (McGaw 2006b, Curtis et al. 2007). *C. gracilis* becomes quiescent, closing its mouthparts and retracting the antennae as soon as the salinity starts to decrease. This closure response isolates the branchial chambers from the surrounding low-salinity water (Sugarman et al. 1983); because of this rapid isolation response, coupled with diffusive ion loss into a closed area, the water in the branchial chamber is held at a higher osmolality than the surrounding water (Curtis et al. 2007). At the same time, the decreased cardiac output results in a higher hemolymph residence time in the gills, thus reducing the average exchange gradient for inward movement of water and diffusive ion loss (Cornell 1973, Hume and Berlind 1976). Low salinity is known to cause an increased hemolymph O_2 binding affinity in *C. maenas* (Truchot 1973); therefore, a slowing of hemolymph flow at the tissue level could be beneficial in increasing O_2 extraction from the circulating hemolymph (Larimer 1964). There is a pronounced overshoot in cardiac and ventilatory parameters when *C. gracilis* is returned to 100% seawater. Thus, the short-term decrease in cardiac function and O_2 uptake is probably related to decrease in activity levels because an O_2 debt suggests repayment of energetic processes (McGaw 2006b).

Weaker regulators also tend to become inactive for short periods when they experience low salinity (Sugarman et al. 1983, McGaw et al. 1999). However, they eventually exhibit a halokinesis in an attempt to escape (Thomas et al. 1981 McGaw and Naylor 1992, McGaw et al. 1999). The weaker regulators tend to exhibit mixed cardiorespiratory responses to low salinity: *C. magister* is classified as a weak regulator and shows no change in O_2 uptake in low salinity (Brown and Terwilliger 1999, Curtis and McGaw 2010). Although this species exhibits an increase in heart rate during low-salinity exposure, a reduced time for filling results in a reduced stroke volume, which leads to a significant drop in cardiac output. This results in a depression in hemolymph flow rates through all arterial systems, except for the small posterior aorta, in which a flow increase is observed (McGaw and McMahon 1996, 2003, McGaw 2006a). These physiological responses are thought to be due primarily to behavioral adjustments, whereby the animal becomes quiescent, rather than a direct physiological response (McGaw et al. 1999). The American lobster, *H. americanus*, is also a weak hyperregulator and can inhabit estuaries. This species exhibits a biphasic cardiac pattern, responding to the initial drop in salinity with a tachycardia; as the salinity decreases below 22 ppt, a bradycardia ensues (Dufort et al. 2001) coupled with a transient increase in ventilation and a subsequent apnea. When the salinity drops below 10 ppt, O_2 uptake and heart rate increase (Jury et al. 1994a). For each of these weaker regulators, it is suggested that avoidance behavior may be the first response to low salinity; if they are forced to osmoregulate, changes in cardiac and respiratory processes may enhance their ability to survive hyposaline exposure. The magnitude and direction of these changes depend on the degree and duration of the low-salinity exposure (Jury et al. 1994b, McGaw et al. 1999).

The efficient osmoregulators increase activity levels in response to low salinity; this is termed *halokinesis* (Thomas et al. 1977). This behavior is accompanied by increases in respiratory and cardiovascular functions (Engel et al. 1975, Hume and Berlind 1976, Cumberlidge and Uglow 1977, Taylor 1977, Spaargaren 1973, 1982, Guerin and Stickle 1992, McGaw and Reiber 1998). Two efficient osmoregulators, which are essentially model organisms, are the green crab *C. maenas* and the blue crab *C. sapidus*. In *C. maenas*, there is an increase in heart and scaphognathite rate (Hume and Berlind 1976, Taylor 1977) associated with the increased energetic demand for osmoregulation (Taylor et al. 1978). In contrast, Taylor et al. (1977a) report that the heart rate of *C. maenas* does not change in 50% seawater (16 ppt); however, this study also examined temperature interactions

that could have affected the results. Spaargaren (1974) reports that blood flow rates are lowest at 20 ppt but increase in dilute or concentrated mediums where animals have to regulate body fluids. The heart rate of *C. sapidus* also increases when the salinity decreases (Sabourin 1984, McGaw and Reiber 1998), and, as in *C. maenas*, stroke volume is largely unaffected by low salinity, suggesting that the resulting increase in cardiac output is largely driven by heart rate (deFur and Mangum 1979, McGaw and Reiber 1998). The increased cardiac output is delivered through the sternal artery, anterior aorta, and anterolateral arteries, whereas flow rates in the hepatic arteries and posterior aorta remain stable. These increases in flow likely represent the increased activity but also an increased energetic demand for active ion uptake. Some of the flow is directed to the anterior aorta and anterolateral arteries, which have connections with the antennal gland, which is used for volume regulation (McGaw and Reiber 1998, 2000). The overall increase in cardiac output will eventually be directed through the branchial vessels, ensuring increased perfusion of the active uptake sites in the gills (McGaw and Reiber 1998). When *C. sapidus* is acclimated to low salinity, the heart rate, cardiac output, and flow rates drop back down to pretreatment levels (McGaw and Reiber 1998). This may related to an upregulation of Na^+/K^+-ATPase and the fact that hemolymph osmolality has reached new stable levels (Siebers et al. 1972).

The majority of articles on crustacean cardiovascular physiology have focused on the malacostracan crustaceans, probably because their large size and accessibility make them easier to study. Recent advances in optocardiographic methods have allowed fine-scale resolution in some smaller crustaceans (Calosi et al. 2003). Calosi et al. (2005) report an increased heart rate in the sandhopper *Talitrus saltator* when exposed to salinities as low as 5.5 ppt; at salinities below this level cardiac patterns tend to break down. This appears to be associated with a decrease in osmoregulatory capacity as time progressed, and it was surmised that modulation of heart rate and O_2 uptake levels in this species is directly related to their ability to osmoregulate.

Freshwater crustaceans tend to inhabit a more stable environment but can occasionally experience periods of increased salinity. The freshwater prawn *P. antennarius* reacts with a short-term alarm response and an increase in heart rate in salinities of 15 ppt and 30 ppt before rate decreases back to normal levels. A bradycardia occurs at 20 ppt, and this salinity is close to their iso-osmotic point and so less energy is required to maintain osmotic pressure differences within this range (Ungherese et al. 2008). Similar patterns are reported for freshwater crayfish *Astacus astacus*, where an increase in salinity depresses the normally high heart rates (Styrishave et al. 1995). In contrast, in the freshwater crab *Oziotelphusa senex*, minimal heart rates are observed in crabs adapted to 50% seawater, and rates increase in both 25% and 75% seawater (Subrahmanyam and Krishnamoorthy 1984).

FUTURE DIRECTIONS

The majority of physiological studies carried out over the past century have focused on the decapod crustaceans, probably because of the ease of use associated with their large size and their ability to be maintained in the laboratory environment. No doubt they will continue to be the main focus of further physiological research. Such work could include, but would not be limited to determining their responses to biotic and environmental stressors and the neural and hormonal control mechanisms that elicit these responses. Although hemolymph flows through the major arteries leaving the heart has been investigated in some detail, there is much less information on the control mechanisms used to shunt blood through the peripheral vessels and sinuses (Reiber and McGaw 2009). The gills are situated inside the branchial chambers and are difficult to access in intact animals; control of blood flow through each of the individual gills, as well as the function of the associated branchiostegal sinus, is still not understood. Thus, the determination of the exact role of the branchiostegal circulation and the partitioning of venous blood between the individual gills and this sinus represents a pressing question for crustacean physiologists (McGaw 2005a, McGaw and Stillman 2010).

With recent advances in technology, two areas will undoubtedly expand in the coming years. First, a comparison with other crustacean orders is long overdue, and, at present, information on any aspect of cardiorespiratory physiology of nondecapodan orders is very limited compared to the plethora of information available for decapods. This is important given the wide variation in anatomical and morphological complexity among crustacean cardiovascular systems, and this should be a fruitful area of research (Wirkner and Richter 2013). There is also a push toward carrying out experiments in a more natural setting or even in the field. The improvement and miniaturization of remote archival data tagging methods has accelerated so much in the past decade that tags can now be affixed to and used to gather data from relatively small animals (Curtis and McGaw 2008). As this technology continues to expand, it will open up an exciting new area for crustacean cardiorespiratory physiologists, one in which multiple stressors can be investigated in the field and compared with data derived from "controlled" laboratory experiments (Styrishave et al. 2003).

CONCLUSIONS

Crustaceans display a wide anatomical diversity of circulatory systems, ranging from simple tubular hearts with no vessels through to the highly developed system of the decapods. The majority of the work on crustaceans has focused on the decapods, probably because their large size, and the fact they are readily available and survive well in the laboratory setting makes them an easy subject to use. Historically, the cardiovascular system was regarded as a simple, low-pressure system with very little control over regional blood flow; however, during the past three decades, precise physiological control mechanisms have been shown to modulate cardiac output and partition regional blood flow, so much so that these mechanisms rival those of some of the simple vertebrate closed systems. Cardiac function and blood flow are modulated by both neural and hormonal control mechanisms. Neural control can occur via the action of the cardioregulatory nerves that influence both the rate and force of contraction of the heart by acting on the cardiac ganglion. Additionally, the tonus of the cardioarterial valves can be modulated through neuronal stimulation, which regulates hemolymph flow. A number of naturally occurring peptide and amine neurohormones modulate cardiac activity and differential hemolymph flow in crustaceans. These hormones act on the cardioarterial valves, artery walls, CNS, or the cardiac muscle itself. In addition, cardiac parameters and hemolymph flow rates have been found to change during development and in response to exercise, feeding, hypoxia, emersion, low salinity, and temperature change. These changes are not only important for efficient delivery of nutrients and gases, but the diversion of flow to metabolically active tissues also may enhance the ability of animals to cope with environmental changes. Future work will undoubtedly reveal further physiological control mechanisms, not only in the decapods, but also across the diverse array of organisms that comprise the subphylum Crustacea.

REFERENCES

Aagaard, A. 1996. *In situ* variation in heart rate of the shore crab *Carcinus maenas* in relation to environmental factors and physiological condition. Marine Biology 125:765–772.

Ahsanullah, M., and R.C. Newell. 1971. Factors affecting the heart rate of the shore crab *Carcinus maenas* (L.). Comparative Biochemistry and Physiology 39A:277–287.

Airriess, C.N., and B.R. McMahon. 1992. Aminergic modulation of circulatory performance in the crab *Cancer magister*. Pages 123–131 *in* R.B. Hill, K. Kuwasawa, B.R. McMahon, and T Kuramoto, editors. Comparative Physiology Vol. 11. Basel, Switzerland, Karger.

Airriess, C.N., and B.R. McMahon. 1994. Cardiovascular adaptations enhance tolerance of environmental hypoxia in the crab *Cancer magister*. Journal of Experimental Biology. 190:23–41.

Airriess, C.N., and B.R. McMahon. 1996. Short-term emersion affects cardiac function and regional haemolymph distribution in the crab *Cancer magister*. Journal of Experimental Biology 199:1–10.

Airriess, C.N., B.R. McMahon, I.J. McGaw, and G.B. Bourne. 1994. Application and in situ calibration of a pulsed-Doppler flowmeter for blood flow measurements in crustaceans. Journal of the Marine Biological Association U.K. 74:455–458.

Alexandrowicz, J.S. 1932. The innervation of the heart of the Crustacea. I. Decapoda. Quarterly Journal of Microscopy Science 75:181–249.

Ando, H., and K. Kuwasawa. 2004. Neuronal and neurohormonal control of the heart in the stomatopod crustacean, *Squilla oratoria*. Journal of Experimental Biology 207:4663–4677.

Ansell, A.D. 1973. Changes in oxygen consumption, heart rate and ventilation accompanying starvation in the decapod crustacean, *Cancer pagurus*. Netherlands Journal of Sea Research 7:455–475.

Barlow, D.I., and M.A. Sleigh. 1980. The propulsion and use of water currents for swimming and feeding in larval and adult Artemia. Pages 61–73 *in* G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers, editors. The brine shrimp Artemia, Vol. 1. Universa Press, Wetteren.

Barthe, J.Y., N. Mons, D. Cattaert, M. Geffard, and F. Clarac. 1989. Dopamine and motor activity in the lobster, *Homarus gammarus*. Brain Research 497:368–373.

Berlind, A. 1998. Dopamine and 5-hydroxytryptamine actions on the cardiac ganglion of the lobster *Homarus americanus*. Journal of Comparative Physiology A 182:363–76.

Berlind, A. 2001. Monoamine pharmacology of the lobster cardiac ganglion. Comparative Biochemistry and Physiology 128C:377–390.

Berlind, A., and I.M. Cooke. 1970. Release of a neurosecretory hormone as peptide by electrical stimulation of crab pericardial organs. Journal of Experimental Biology 53:679–686.

Bellwood, O. 2002. The occurrence, mechanics and significance of burying behaviour in crabs (Crustacea: Brachyura). Journal of Natural History 36:1223–1238.

Beltz, B.S. 1999. Distribution and functional anatomy of amine containing neurons in decapod crustaceans. Microscopy Research Techniques 44:105–120.

Bliss, D.E., and L.H. Mantel. 1983. Biology of the Crustacea. Academic Press, New York.

Blatchford, J.G. 1971. Haemodynamics of *Carcinus maenas* (L.). Comparative Biochemistry and Physiology 39A:193–202.

Bradford, S.M., and A.C. Taylor. 1982. The respiration of *Cancer pagurus* under normoxic and hypoxic conditions. Journal of Experimental Biology 97:273–288.

Bridges, C.R. 1979. Adaptations of *Corystes cassivelaunus* to an arenicolous mode of life. Pages 317–324 *in* Naylor, E., and R.G. Hartnoll, editors. Cyclic phenomena in marine plants and animals. Pergamon Press, Oxford.

Brody, M.S., and E.B. Perkins. 1930. The arterial system of *Palaemonetes*. Journal of Morphology 50:127–142.

Brown, A.C., and N.B. Terwilliger. 1999. Developmental changes in oxygen uptake in *Cancer magister* (Dana) in response to salinity and temperature. Journal Experimental Marine Biology Ecology 241:179–192.

Brown, R.W., T.S. Galloway, D. Lowe, M.A. Browne, A. Dissanayake, M.B. Jones, and M.H. Depledge. 2004. Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. Aquatic Toxicology 66:267–278.

Bock, C., M. Frederich, R.M. Wittig, and H.O. Pörtner. 2001. Simultaneous observations of haemolymph flow and ventilation in marine spider crabs at different temperatures: a flow weighted MRI study. Magnetic Resonance Imaging 19:1113–1124.

Booth, C.E., B.R. McMahon, and A.W. Pinder. 1982. Oxygen uptake and the potentiating effects of increased hemolymph lactate on oxygen transport during exercise in the blue crab, *Callinectes sapidus*. Journal Comparative Physiology B 148:111–121.

Bourne, G.B., and B.R. McMahon. 1989. Control of cardiac output and its distribution in crustacean open circulatory systems. Journal of Physiology London 418:134P.

Bouvier, E.L. 1891. Recherches anatomiques sur le systeme arterial des Crustaces Decapodes. Annales des Sciences Naturelles Zoologie et Biologie Animale 9:197–282.

Burggren, W.W., A. Pinder, B. McMahon, M. Doyle, and M. Wheatly. 1990. Heart rate and hemolymph pressure responses to hemolymph volume changes in the land crab *Cardisoma gauanhumi*: evidence for "Baroreflex" regulation. Physiological Zoology 63:167–181.

Burnett, L.E. 1979. The effects of environmental oxygen levels on the respiratory function of hemocyanin in the crabs, *Libinia emarginata and Ocypode quadrata*. Journal of Experimental Zoology 210:289–300.

Burnett, L.E., and C.M. Bridges. 1981. The physiological properties and function of ventilatory pauses in the crab *Cancer pagurus*. Journal Comparative Physiology B 145:81–88.

Burton, D.T., L.B. Richardson, and C.J. Moore. 1980. Cardiac frequency compensation responses of adult blue crabs *Callinectes sapidus* (Rathbun) exposed to moderate temperature increases. Comparative Physiology and Biochemistry 65A:259–263.

Calosi, P., G. Chelazzi, and A. Ugolini. 2003. Optocardiographic recording of heart rate in *Talitrus saltator* (Amphipoda: Talitridae). Physiological Entomology 28:344–348.

Calosi, P., A. Ugolini, and D. Morritt. 2005. Physiological responses to hyposmotic stress in the supralittoral amphipod *Talitrus saltator* (Crustacea: Amphipoda). Comparative Biochemistry and Physiology 136A:127–133.

Camacho, J., S.A. Qadri, H. Wang, and M.K. Worden. 2006. Temperature acclimation alters cardiac performance in the lobster *Homarus americanus*. Journal of Comparative Physiology A 192:1327–1334.

Carlson, A.J., and W.J. Meek. 1908. On the mechanism of the embryonic heart rhythm in *Limulus*. American Journal of Physiology 21:1–10.

Cavey, M.J., and J.L. Wilkens. 2000. F-actin in the amuscular arteries of the American lobster. American Zoologist 40:968A.

Cavey, M.J., K.S. Chan, and J.L. Wilkens. 2008. Microscopic anatomy of the thin-walled vessels leaving the heart of the lobster *Homarus americanus*: anterior median artery. Invertebrate Biology 127:189–200.

Chabot, CC., and L.K. Webb. 2008. Circadian rhythms of heart rate in freely moving and restrained American lobsters, *Homarus americanus*. Marine and Freshwater Behaviour and Physiology 41:29–41.

Chapman, S.L., and C.L. Reiber. 1998. Ontogeny of cardiac regulation in the crayfish. American Zoologist 37:142A.

Christie, A.E. 2011. Crustacean neuroendocrine systems and their signaling agents. Cell and Tissue Research 120:1011–1023.

Christie, A.E., C.R. Cashman, J.S. Stevens, C.M. Smith, K.M. Beale, E.A. Stemmler, S.J. Greenwood, D.W. Towle, and P.S. Dickinson. 2008. Identification and cardiotropic actions of brain/gut-derived tachykinin-related peptides (TRPs) from the American lobster *Homarus americanus*. Peptides 29:1909–1918.

Christie, A.E., E.A. Stemmler, and P.S. Dickinson. 2010a. Crustacean neuropeptides. Cellular and Molecular Life Sciences 67:4135–4169.

Christie, A.E., J.S. Stevens, M.R. Bowers, M.C. Chapline, D.A. Jensen, K.M. Schegg, J. Goldwaser, M.A. Kwiatkowski, T.K. Pleasant, L. Shoenfeld, L.K. Tempest, C.R. Williams, T. Wiwatpanit, C.M. Smith, K.M. Beale, D.W. Towle, D.A. Schooley, and P.S. Dickinson. 2010b. Identification of a calcitonin-like diuretic hormone that functions as an intrinsic modulator of the American lobster, *Homarus americanus*, cardiac neuromuscular system. Journal of Experimental Biology 213:118–127.

Chu, K.H., E.M.T. Mak, and B.R. McMahon. 1995. Effect of salinity on heart rate of larvae and postlarvae of the shrimp, *Metapenaeus ensis*. American Zoologist 35:64A.

Cooke, I.M. 1988. Studies on the crustacean cardiac ganglion. Comparative Biochemistry and Physiology 91:205–218.

Cooke, I.M., and R.E. Sullivan. 1982. Hormones and neurosecretion. Pages 205–290 *in* H.L. Atwood, and D.C. Sandemann, editors. The biology of Crustacea: neurobiology: structure and function. Vol. 3. Academic Press, New York.

Cornell, J.C. 1973. A reduction in water permeability in response to a dilute medium in the stenohaline crab *Libinia emarginata* (Brachyura, Majidae). Biological Bulletin 145:430–431.

Cornell, J.C. 1979. Salt and water balance in two marine spider crabs Libinia emarginata and *Pugettia producta*. II Apparent water permeability. Biological Bulletin 157:422–433.

Crozier, W.J., and T.J.B. Stier. 1927. Temperature and frequency of cardiac contractions in embryos of *Limulus*. Journal of General Physiology 10:501–518.

Cruz-Bermúdez, N.D., and E. Marder. 2007. Multiple modulators act on the cardiac ganglion of the crab, *Cancer borealis*. Journal of Experimental Biology 210:2873–2884.

Cuculescu, M., D. Hyde, and K. Bowler. 1998. Thermal tolerance of two species of marine crab, *Cancer pagurus* and *Carcinus maenas*. Journal of Thermal Biology 23:107–110.

Cumberlidge, N., and R.F. Uglow. 1977. Heart and scaphognathite activity in the shore crab *Carcinus maenas*. Journal of Experimental Marine Biology and Ecology 28:87–107.

Cumberlidge, N., and R.F. Uglow. 1978. Heart and scaphognathite activity during the digging behaviour of the shore crab *Carcinus maenas*. Pages 23–30 *in* D.S. McLusky and A.J. Berry, editors. Physiology and behaviour of marine organisms. Pergamon Press, Oxford.

Curtis, D.L., and I.J. McGaw. 2010. Respiratory and digestive responses of postprandial Dungeness crabs, *Cancer magister* and blue crabs, *Callinectes sapidus* during hyposaline exposure. Journal of Comparative Physiology B 180:189–198.

Curtis, D.L., E.K. Jensen, and I.J. McGaw. 2007. Behavioural influences on the physiological responses of the graceful crab, *Cancer gracilis* during hyposaline exposure. Biological Bulletin 212:222–231.

Dall W. 1967. Hypo-osmoregulation in Crustacea. Comparative Biochemistry and Physiology 21:653–678.

Davidson, G.W., and H.H. Taylor. 1995. Ventilatory and vascular routes in a sand burying swimming crab, *Ovalipes catharus* (White 1843) (Brachyura: Portunidae). Journal of Crustacean Biology 15:605–624.

Davidson, G.W., J.L. Wilkens, and P. Lovell. 1998. Neural control of the lateral abdominal arterial valves in the lobster *Homarus americanus*. Biological Bulletin 194:72–82.

Depledge, M.H. 1984. The influence of aerial exposure on gas exchange and cardiac activity in the shore crab, *Carcinus maenas* (L.). Comparative Biochemistry and Physiology 79A:339–344.

deFur, P.L. 1988. Systemic respiratory adaptations to air exposure in intertidal decapod crustaceans. American Zoologist 28:115–124.

deFur, P.L., and C.P. Mangum. 1979. The effects of environmental variables on the heart rates of invertebrates. Comparative Biochemistry and Physiology 62A:283–294.

deFur, P.L., and B.R. McMahon. 1984a. Physiological compensation to short term air exposure in red rock crabs, *Cancer productus Randall*, from littoral and sublittoral habitats. I. Oxygen uptake and transport. Physiological Zoology 57:137–150.

deFur, P.L., and B.R. McMahon. 1984b. Physiological compensation to short term air exposure in red rock crabs, *Cancer productus* Randall, from littoral and sublittoral habitats. II. Acid-base balance. Physiological Zoology 57:151–160.

deFur, P.L., and A.L. Pease. 1988. Metabolic and respiratory compensation during long term hypoxia in blue crabs, *Callinectes sapidus*. Virginia Sea Grant College Program VSG–89–78R:608–616.

deFur, P.L., A.S. Pease, A.M. Siebelink, and S. Elfers. 1988. Respiratory responses of blue crabs, *Callinectes sapidus*, to emersion. Comparative Biochemistry and Physiology 89A:97–101.

Dejours, P., and H. Beekenkamp. 1977. Crayfish respiration as a function of water oxygenation. Respiration Physiology 30:241–251.

Dejours, P., W.F. Garey, and H. Rahn. 1970. Comparison of ventilatory and circulatory flow rates between animals in various physiological conditions. Respiration Physiology 9:108–117.

DePirro, M., S. Cannicci, and G. Santini. 1999. A multi-factorial experiment on heart rate variations in the intertidal crab *Pachygrapsus marmoratus*. Marine Biology 135;341–345.

DeWachter, B., and B.R. McMahon. 1996a. Haemolymph flow distribution, cardiac performance and ventilation during moderate walking activity in *Cancer magister* (Dana) (Decapoda, Crustacea). Journal of Experimental Biology 199:627–633.

DeWachter, B., and B.R. McMahon. 1996b. Temperature effects heart performance and regional haemolymph flow in the crab *Cancer magister*. Comparative Biochemistry and Physiology 114A:27–33.

DeWachter, B., and J.L. Wilkens. 1996. Comparison of temperature effects on heart performance of the Dungeness crab, *Cancer magister, in vitro* and *in vivo*. Biological Bulletin 190:385–395.

Dickinson, P.S., J.S. Stevens, S. Rus, H.R. Brennan, C.C. Goiney, C.M. Smith, D.W. Towle, and A.E. Christie. 2007. Identification and cardiotropic actions of sulfakinin peptides in the American lobster *Homarus americanus*. Journal of Experimental Biology 210:2278–2289.

Dickinson, P.S., E.A. Stemmler, and A.E. Christie. 2008. The pyloric neural circuit of the herbivorous crab *Pugettia producta* shows limited sensitivity to several neuromodulators that elicit robust effects in more opportunistically feeding decapods. Journal of Experimental Biology 211:1434–1447.

Dickinson, P.S., T. Wiwatpanit, E.R. Gabranski, R.J. Ackerman, J.S. Stevens, C.R. Cashman, E.A. Stemmler, and A.E. Christie. 2009. Identification of SYWKQCAFNAVSCFamide: a broadly conserved crustacean C-type allatostatin-like peptide with both neuromodulatory and cardioactive properties. Journal of Experimental Biology 212:1140–1152.

Dufort, C.G., S.H. Jury, J.M. Newcomb, D.F. O'Grady, and W.H. Watson. 2001. Detection of salinity by the lobster *Homarus americanus*. Biological Bulletin 201:424–434.

Engel, D.W., R.L. Ferguson, and L.D. Eggert. 1975. Respiration rates and ATP concentrations in the excised gills of the blue crab as a function of salinity. Comparative Biochemistry and Physiology 52A:669–673.

Faulkes, Z. 2013. Morphological adaptations for digging and burrowing. Pages 276–295 *in* L. Watling and M. Thiel, editors. Natural history of the Crustacea, Vol. 1: functional morphology and diversity. Oxford University Press, New York.

Florey, E., and M. Rathmayer. 1978. The effects of octopamine and other amines on the heart and on neuromuscular transmission in decapod crustaceans: further evidence for a role as neurohormone. Comparative Biochemistry and Physiology 61C:229–237.

Fort, T.J., V. Brezina, and M.W. Miller. 2004. Modulation of an integrated central pattern generator-effector system: dopaminergic regulation of cardiac activity in the blue crab *Callinectes sapidus*. Journal of Neurophysiology 92:3455–3470.

Fort, T.J., V. Brezina, and M.W. Miller. 2007a. Regulation of the crab heartbeat by FMRFamide-like peptides: multiple interacting effects on center and periphery. Journal of Neurophysiology 98:2887–2902.

Fort, T.J., K. García-Crescioni, H.J. Agricola, V. Brezina, and M.W. Miller. 2007b. Regulation of the crab heartbeat by crustacean cardioactive peptide (CCAP): central and peripheral actions. Journal of Neurophysiology 97:3407–3420.

Freadman, M.A., and W.H. Watson. III. 1989. Gills as possible accessory circulatory pumps in *Limulus polyphemus*. Biological Bulletin 177:386–395.

Frederich, M., and H.O. Pörtner. 2000. Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab, *Maja squinado*. American Journal of Physiology 279:R1531–1538.

Freschi, J.E. 1989. Proctolin activates a slow, voltage-dependent sodium current in motoneurons of the lobster cardiac ganglion. Neuroscience Letters 106:105–111.

Freschi, J.E., and D.R. Livengood. 1989. Membrane current underlying muscarinic cholinergic excitation of motoneurons in the lobster cardiac ganglion. Journal of Neurophysiology 62:984–995.

Gannon, A.T., N. Arunakul, and R.P. Henry. 2001. Respiratory, cardiovascular, and hemolymph acid–base changes in the amphibious crab, *Cardisoma guanhumi*, during immersion and emersion. Marine and Freshwater Behaviour and Physiology 34:73–92.

Garstang, W. 1897. Contributions to marine bionomics. 2. The function of the anterolateral denticulations of the carapace of sand burrowing crabs. Journal of the Marine Biological Association U.K.:4:396–401.

Greenaway, P., and C. Farrelly. 1984. The venous system of the terrestrial crab *Ocypode cordimanus* (Desmarest 1825) with particular reference to the vasculature of the lungs. Journal of Morphology 181:133–142.

Greenaway, P., and C. Farrelly. 1990. Vasculature of the gas exchange organs in air breathing brachyurans. Physiological Zoology 63:117–139.

Greenaway, P., S. Morris, B.R. McMahon, C.A. Farrelly, and K.L. Gallagher. 1996. Air breathing by the purple shore crab *Hemigrapsus nudus* (Dana). I. Morphology, behavior and respiratory gas exchange. Physiological Zoology 69:785–805.

Greenberg, M.J., and D.A. Price. 1992. Relationships among the FMRFamide-like peptides. Progressive Brain Research 92:25–37.

Grega, D.S., and R.G. Sherman. 1975. Responsiveness of neurogenic hearts to octopamine. Comparative Biochemistry and Physiology 52C:5–8.

Guadagnoli, J.A., and C.L. Reiber. 2005. Changes in cardiac output and hemolymph flow during hypoxic exposure in the gravid grass shrimp, *Palaemonetes pugio*. Journal of Comparative Physiology B 175:313–322.

Guadagnoli, J.A., A.M. Braun, and C.L. Reiber. 2005a. Environmental hypoxia influences hemoglobin subunit structure in the Brachiopod crustacean, *Triops longicaudatus*. Journal of Experimental Biology 208:3543–3551.

Guadagnoli, J.A., L. Jones, and C.L. Reiber. 2005b. The influence of reproductive state on cardiac parameters and hypoxia tolerance in the grass shrimp *Palaemonetes pugio*. Journal of Functional Ecology 19:976–981.

Guadangoli, J.A., L. Tobita, and C.L. Reiber. 2007. Assessment of the pressure area relationship of the single ventricle of the grass shrimp *Palaemonetes pugio*. Journal of Experimental Biology 210:2192–2198.

Guadangoli, J.A., L. Tobita, and C.L. Reiber. 2011. Changes in cardiac performance during hypoxic exposure in the grass shrimp, *Palaemonetes pugio*. Journal of Experimental Biology 214:3906–3914.

Guerin, J.L., and W.B. Stickle. 1992. Effect of salinity gradients on the tolerance and bioenergetics of juvenile blue crabs (*Callinectes sapidus*) from waters of different environmental salinities. Marine Biology 114:391–396.

Goudkamp, J.E., F. Seebacher, M. Ahern, and C.E. Franklin. 2004. Physiological thermoregulation in a crustacean? Heart rate hysteresis in the freshwater crayfish *Cherax destructor*. Comparative Biochemistry and Physiology 138A:399–403.

Gurguis, M.S., and J.L. Wilkens. 1995. The role of the cardioregulatory nerves in mediating heart rate responses to locomotion, reduced stroke volume, and neurohormones in *Homarus americanus*. Biological Bulletin 188:179–85.

Haeckel, E. 1857. Uber die Gewebe des Flusskrebses. Pages 469–568 *in* J. Muller, editor. Archivfür Anatomieund Physiologie und Wissenschaftliche Medicin. Berlin, Germany.

Hamilton, N.M. 1987. Respiratory and circulatory changes accompanying aquatic treadmill exercise of *Carcinus maenas* (L.) and *Homarus vulgaris*. PhD thesis. University of Aberdeen, Scotland.

Hamilton, N.M., and D.F. Houlihan. 1992. Respiratory and circulatory adjustments during aquatic treadmill exercise in the European shore crab *Carcinus maenas*. Journal of Experimental Biology 162:37–54.

Handy, R.D., and M.H. Depledge. 1999. Physiological responses: their measurement and use as environmental biomarkers in ecotoxicology. Ecotoxicology 8:329–349.

Harper, S.L., and C.L. Reiber. 1999. Influence of hypoxia on cardiac functions in the grass shrimp (*Palaemonetes pugio* Holthuis). Comparative Biochemistry and Physiology A 124:569–573.

Harper, S.L., and C.L. Reiber. 2000. Developmental cardiac responses to GABA in the red swamp crayfish (*Procambarus clarkii*) and the relevance to crayfish burrow ecology. Arizona-Nevada Academy of Sciences 32:158–63.

Harper, S.L., and C.L. Reiber. 2001. Ontogeny of neurohormonal regulation of the cardiovascular system in crayfish (*Procambarus clarkii*). Journal of Comparative Physiology B:171:577–583.

Harper, S.L., and C.L. Reiber. 2004. Physiological development of the embryonic and larval crayfish heart. Biological Bulletin 206:78–86.

Harper, S.L., and C.L. Reiber. 2006a. Metabolic, respiratory and cardiovascular responses to acute and chronic hypoxic exposure in tadpole shrimp, *Triops longicaudatus*. Journal of Experimental Biology 209:1639–1650.

Harper, S.L., and C.L. Reiber. 2006b. Ontogeny of cardiac physiology and aerobic metabolism in the red swamp crayfish *Procambarus clarkii*. Journal of Comparative Physiology B:176:405–414.

Herreid, C.F. 1980. Hypoxia in invertebrates. Comparative Biochemistry and Physiology 67A:311–320.

Herried C.F., L.W. Lee, and G.M. Shah. 1979. Respiration and heart rate in exercising land crabs. Respiration Physiology 36:109–120.

Hill, R.B., and K. Kuwasawa. 1992. Phylogenetic models in functional coupling of the CNS and the cardiovascular system. Comparative Physiology. Basel, Karger, Vol. 11.

Hochachka P.W., and G.N. Somero. 2002. Biochemical adaptation: mechanism and process in physiological evolution. Oxford University Press, Oxford.

Hopkin, S.P., and J.A. Nott. 1980. Studies on the digestive cycle of the shore crab *Carcinus maenas* with special reference to the B cells of the hepatopancreas. Journal of the Marine Biological Association UK 60:891–907.

Houlihan, D.F., and A.J. Innes. 1984. The cost of walking in crabs: aerial and aquatic oxygen consumption during activity of two species of intertidal crab. Comparative Biochemistry and Physiology 77A:325–334.

Houlihan, D.F., C.P. Waring, E. Mathers, and C. Gray. 1990. Protein synthesis and oxygen consumption of the shore crab *Carcinus maenas* after a meal. Physiological Zoology 63:735–756.

Howse, H.D., V.J. Ferrans, and R.G. Hibbs. 1971. A light and electron microscopic study of the heart of a crayfish, *Procambarus clarkii* (Giraud). I. Histology and histochemistry. Journal of Morphology 131:237–252.

Hughes, G.M., B. Knight, and C.A. Scammell. 1969. The distribution of PO_2 and hydrostatic pressure changes within the branchial chambers in relation to gill ventilation in the shore crab *Carcinus maenas* (L). Journal of Experimental Biology 51:203–220.

Hume, R.I., and A. Berlind. 1976. Heart and scaphognathite changes in a euryhaline crab *Carcinus maenas* exposed to a dilute environmental medium. Biological Bulletin 150:241–254.

Iftikar, F.I., J. MacDonald, and A.J.R. Hickey. 2010. Thermal limits of portunid crab heart mitochondria: could more thermo-stable mitochondria advantage invasive species? Journal of Experimental Marine Biology and Ecology 395:232–239.

Johansen, K., C. Lenfant, and T.A. Mecklenburg. 1970. Respiration in the crab, *Cancer magister*. Journal of Comparative Physiology A 70:1–19.

Jury, S.H., and W.H. Watson III. 2000. Thermosensitivity of the lobster, *Homarus americanus*, as determined by cardiac assay. Biological Bulletin 199:257–264.

Jury, S.H., M.T. Kinnison, W.H. Howell, and W.H. Watson III. 1994a. The effects of reduced salinity on lobster (*Homarus americanus*) metabolism: implications for estuarine populations. Journal of Experimental Marine Biology and Ecology 176:167–185.

Jury, S.H., M.T. Kinnison, W.H. Howell, and W.H. Watson III. 1994b. The behavior of lobsters in response to reduced salinity. Journal of Experimental Marine Biology and Ecology 180:23–37.

Keller, R. 1992. Crustacean neuropeptides: structure, function and comparative aspects. Experientia 48:439–448.

Kihara, A., and K. Kuwasawa. 1984. A neuroanatomical and electrophysiological analysis of nervous regulation in the heart of an isopod crustacean, *Bathynomus doederleini*. Journal of Comparative Physiology 154A:883–894.

Kinne, O. 1964. The effects of temperature and salinity on marine and brackish water animals. II Salinity and salinity-temperature combinations. Pages 281–339 *in* Barnes, H., editor. Oceanography and marine biology—an annual review. Haefner Publishers, New York.

Krajniak, K. 1991. The identification and structure-activity relations of a cardioactive FMRFamide-related peptide from the blue crab *Callinectes sapidus*. Peptides 12:1295–1302.

Kuramoto, T. 1999. Cold-resistant changes in heartbeat of the Japanese spiny lobster. Comparative Biochemistry and Physiology 124A:553–559.

Kuramoto, T., and A. Ebara. 1984. Neurohormonal modulation of the cardiac outflow through the cardioarterial valve of the lobster. Journal of Experimental Biology 111:123–128.

Kuramoto, T., and A. Ebara. 1985. Effects of perfusion pressure on the bursting neurones in the intact or segmented cardiac ganglion of the lobster, *Panulirus japonicus*. Journal of Neuroscience Research 13:569–580.

Kuramoto, T., and A. Ebara. 1988. Combined effects of 5-hydroxy-tryptamine and filling pressure on the isolated heart of the lobster *Panulirus japonicus*. Journal of Comparative Physiology B 158:403–412.

Kuramoto, T., and A. Ebara. 1989. Contraction of flap muscle in the cardioarterial valve of *Panulirus japonicus*. Comparative Biochemistry and Physiology 93A:419–422.

Kuramoto, T., and A. Ebara. 1991. Combined effects of octopamine and filling pressure on the isolated heart of the lobster *Panulirus japonicus*. Journal of Comparative Physiology B 161:339–347.

Kuramoto, T., and K. Kuwasawa. 1980. Ganglionic activation of the myocardium of the lobster, *Panulirus japonicus*. Journal of Comparative Physiology 139:67–76.

Kuramoto, T., and M. Tani. 1994. Cooling-induced activation of the pericardial organs of the spiny lobster, *Panulirus japonicus*. Biological. Bulletin 186:319–327.

Kuramoto, T., and H. Yamagishi. 1990. Physiological anatomy, burst formation, and burst frequency of the cardiac ganglion of crustaceans. Physiological Zoology 63:102–116.

Kuramoto, T., J.L. Wilkens, and B.R. McMahon. 1995. Neural control of cardiac outflow through the sternal valve in the lobster *Homarus americanus*. Physiological Zoology 63:443–452.

Lagerspetz, K.Y.H. 2003. Thermal acclimation without heat shock, and motor responses to a sudden temperature change in *Asellus aquaticus*. Journal of Thermal Biology 28:421–427.
Lagerspetz, K.Y.H., and L.A. Vainio. 2006. Thermal behaviour of crustaceans. Biological Reviews 81:237–258.
Larimer, J.L. 1962. Responses of the crayfish heart during respiratory stress. Physiological Zoology 35:179–186.
Larimer, J.L. 1964. Sensory-induced modifications of ventilation and heart rate in crayfish. Comparative Biochemistry and Physiology 12:25–36.
Larimer, J.L., and A.H. Gold. 1961. Responses of the crayfish, *Procambarus simulans*, to respiratory stress. Physiological Zoology 134:167–176.
Larimer, J.L., and J.R. Tindel. 1966. Sensory modifications of heart rate in crayfish. Animal Behaviour 14:239–245.
Listerman, L.R., J. Deskins, H. Bradacs, and R.L. Cooper. 2000. Heart rate within male crayfish: social interactions and effects of 5-HT. Comparative Biochemistry and Physiology 125A:251–263.
Mangum, C.P. 1983. Oxygen transport in the blood. Pages 373–430 *in* L. Mantel, editor. Internal anatomy and physiological regulation. The biology of Crustacea, Vol. 5. Academic Press, New York.
Mangum C.P., and W. Van Winkle. 1973. Responses of aquatic invertebrates to declining oxygen conditions. American Zoologist 13:529–541.
Mantel, L.H., and H.H. Farmer. 1983. Osmotic and ionic regulation. Pages 54–143 *in* L. Mantel, editor. Internal anatomy and physiological regulation. The biology of Crustacea, Vol. 5. Academic Press, New York.
Martin, G.G., J.E. Hose, and C.J. Corzine. 1989. Morphological comparison of major arteries in the ridgeback prawn, *Sicyonia ingentis*. Journal of Morphology 200:175–183.
Maynard, D.M. 1960. Circulation and heart function. Pages 161–225 *in* Waterman, T.H., editor. The physiology of Crustacea. Academic Press. New York.
McDonald, D.G., B.R. McMahon, and C.M. Wood. 1977. Patterns of heart and scaphognathite activity in the crab *Cancer magister*. Journal of Experimental Zoology 202:33–44.
McGaw, I.J. 2004. Ventilatory and cardiovascular modulation associated with burying behaviour in two sympatric crab species, *Cancer magister* and *Cancer productus*. Journal of Experimental Marine Biology and Ecology 303:47–63.
McGaw, I.J. 2005a. The decapod crustacean cardiovascular system: a case that is neither open nor closed. Microscopy and Microanalysis 11:18–36.
McGaw, I.J. 2005b. Burying behaviour of two sympatric crab species, *Cancer magister* and *Cancer productus*. Scientia Marina 69:375–381.
McGaw, I.J. 2005c. Does feeding limit cardiovascular modulation in the Dungeness crab *Cancer magister* during hypoxia? Journal of Experimental Biology 208:83–91.
McGaw, I.J. 2006a. Prioritization or summation of events? Physiological responses of postprandial Dungeness crabs in low salinity. Physiological and Biochemical Zoology 79:169–177.
McGaw, I.J. 2006b. Feeding and digestion in low salinity in an osmoconforming crab, *Cancer gracilis*. I Cardiovascular and respiratory responses. Journal of Experimental Biology 209:3766–3776.
McGaw, I.J. 2006c. Feeding and digestion in low salinity in an osmoconforming crab, *Cancer gracilis*. II Gastric motility and evacuation. Journal of Experimental Biology 209:3777–3785.
McGaw, I.J. 2007. The interactive effects of exercise and feeding on oxygen uptake, activity levels and gastric processing in graceful crab, *Cancer gracilis*. Physiological and Biochemical Zoology 80:335–343.
McGaw, I.J., and S.D. Duff. 2008. Cardiovascular system of Anomuran crabs, Genus *Lopholithodes*. Journal of Morphology 269:1295–1307.
McGaw, I.J., and B.R. McMahon. 1995. The FMRFamide-related peptides F1 and F2 alter hemolymph distribution and cardiac output in the crab *Cancer magister*. Biological Bulletin 188:186–196.
McGaw, I.J., and B.R. McMahon. 1996. Cardiovascular responses resulting from variation in external salinity in the Dungeness crab *Cancer magister*. Physiological Zoology 69:1384–1401.
McGaw, I.J., and B.R. McMahon. 1998. Endogenous rhythms of haemolymph flow and cardiac performance in the crab *Cancer magister*. Journal of Experimental Marine Biology and Ecology 224:127–142.
McGaw, I.J., and B.R. McMahon. 1999. Actions of putative cardioinhibitory substances on the *in vivo* decapod crustacean cardiovascular system. Journal of Crustacean Biology 19:435–449.
McGaw, I.J., and B.R. McMahon. 2003. Balancing tissue perfusion demands: cardiovascular dynamics of *Cancer magister* during exposure to low salinity and hypoxia. Journal of Experimental Zoology 295A:57–70.

McGaw, I.J., and E. Naylor. 1992. Salinity preference of the shore crab *Carcinus maenas* in relation to coloration during intermoult and to prior acclimation. Journal of Experimental Marine Biology and Ecology 155:145–159.

McGaw, I.J., and C.L. Reiber. 1998. Circulatory modification in the blue crab, *Callinectes sapidus* during exposure and acclimation to low salinity. Comparative Biochemistry and Physiology 121A:67–76.

McGaw, I.J., and C.L. Reiber. 2000. Integrated physiological responses during feeding and digestion in the blue crab *Callinectes sapidus*. Journal of Experimental Biology 203:359–368.

McGaw, I.J., and C.L. Reiber. 2002. Cardiovascular system of the blue crab *Callinectes sapidus*. Journal of Morphology 251:1–21.

McGaw, I.J., and J.H. Stillman. 2010. Cardiovascular system of the Majidae (Crustacea: Decapoda). Arthropod Structure and Development 39:340–349.

McGaw, I.J., and N.M. Whiteley. 2012. Effects of acclimation and acute temperature change on specific dynamic action and gastric processing in the green shore crab, *Carcinus maenas*. Journal of Thermal Biology 37:570–578.

McGaw, I.J., C.N. Airriess, and B.R. McMahon. 1994a. Peptidergic modulation of cardiovascular dynamics in the Dungeness crab *Cancer magister*. Journal of Comparative Physiology B 164:103–111.

McGaw, I.J., C.N. Airriess, and B.R. McMahon. 1994b. Patterns of hemolymph flow variation in decapod crustaceans. Marine Biology 121:53–60.

McGaw, I.J., J.L. Wilkens, B.R. McMahon, and C.N. Airriess. 1995. Crustacean cardioexcitatory peptides may inhibit the heart in vivo. Journal of Experimental Biology 198:2547–2550.

McGaw, I.J., C.L. Reiber, and J.A. Guadagnoli. 1999. Behavioral physiology of four crab species in low salinity. Biological Bulletin 196:163–176.

McLaughlin, P.A. 1983. Internal anatomy. Pages l–41 *in* L.H. Mantel, editor. Biology of the Crustacea. Vol. 5: internal anatomy and physiological regulation. Academic Press, New York.

McMahon, B.R. 1992. Factors controlling the distribution of cardiac output in decapod crustaceans. Pages 51–61 *in* R.B. Hill, K. Kuwasawa, and T. Kuramoto, editors. Phylogenetic models of functional coupling of the CNS and the cardiovascular system. Comparative Physiology. Karger, Basel.

McMahon, B.R. 2001. Control of cardiovascular function and its evolution in Crustacea. Journal of Experimental Biology 204:923–932.

McMahon, B.R., and L.E. Burnett. 1990. The crustacean open circulatory system a reexamination. Physiological Zoology 63:35–71.

McMahon, B.R., and K.H. Chu. 1994. Cardiovascular development in crustaceans. The Physiologist. 37:A–44.

McMahon, B.R., and J. Doyle. 1995. In vivo effects of glutamate and GABA on Phyllopod rate and heart function in the brine shrimp *Artemia franciscana*. American Zoologist 35:34A.

McMahon, B.R., and J.L. Wilkens. 1972. Simultaneous apnoea and bradycardia in the lobster *Homarus americanus*. Canadian Journal of Zoology 50:165–170.

McMahon, B.R., and J.L. Wilkens. 1977. Periodic respiratory and circulatory performance in the red rock crab *Cancer productus*. Journal of Experimental Biology 202:363–374.

McMahon, B.R., and J.L. Wilkens. 1983. Ventilation, perfusion and oxygen uptake. Pages 289–372 *in* L. Mantel and D. Bliss, editors. The biology of Crustacea, Volume 6. Academic Press, New York.

McMahon, B.R., W.W. Burggren, and J.L. Wilkens. 1974. Respiratory responses to long-term hypoxic stress in the crayfish *Orconectes virilis*. Journal of Experimental Biology 60:195–206.

McMahon, B.R., D.G. McDonald, and C.M. Wood. 1979. Ventilation, oxygen uptake and haemolymph oxygen transport, following enforced exhausting activity in the Dungeness crab *Cancer magister*. Journal of Experimental Biology 80:271–285.

McMahon, B.R., C.L. Reiber, and W.W. Burggren. 1989. Arterial blood flows in normoxic and hypoxic lobster, *Homarus americanus*. American Zoologist 29:238A.

McMahon, B.R., C.N. Airriess, and R. Airriess 1996. Swimming in *Charybdis feriatus*: cardiovascular performance in a crustacean athlete. American Zoologist 36:63A.

McMahon, B.R., G.B. Bourne, and K.H. Chu. 1997. Invertebrate cardiovascular development. Pages 127–144 *in* W.W. Burggren and B.B. Keller, editors. Development of cardiovascular systems: molecules to organisms. Cambridge University Press, Cambridge.

Mente, E., A. Legeay, D.F. Houlihan, and J.C. Massabuau. 2003. Influence of oxygen partial pressure on protein synthesis in feeding crabs. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology 284:R500–R510.

Mercaldo-Allen, R., and F.P. Thurberg. 1987. Heart and gill ventilatory activity in the lobster, *Homarus americanus*, at various temperatures. Fisheries Bulletin 85:643–644.

Mercier, A.J., and R.T. Russenes. 1992. Modulation of crayfish hearts by FMRFamide-related peptides. Biological Bulletin 182:333–340.

Mercier, A.J., I. Orchard, V. TeBrugge, and M. Skerrett. 1993. Isolation of two FMRFamide-related peptides from crayfish pericardial organs. Peptides 14:137–143.

Mercier, A.J., R. Friedrich, and M. Boldt. 2003. Physiological functions of FMRFamide-like peptides (FLPs) in crustaceans. Microscopy Research and Technique 60:313–324.

Miller, M.W., J.A. Benson, and A. Berlind. 1984. Excitatory effects of dopamine on the cardiac ganglia of the crabs *Portunus sanguinolentus* and *Podophthalmus vigil*. Journal of Experimental Biology 108:97–118.

Morris, S., and T. Edwards. 1996. Circulatory, acid-base and respiratory responses of the purple shore crab *Leptograpsus variegatus* to immersion. Journal of Experimental Marine Biology and Ecology 196:189–211.

Morris, S., and A.C. Taylor. 1984. Heart rate responses of the intertidal prawn *Palaemon elegans* to stimulated and *in situ* environmental changes. Marine Ecology Progress Series 20:127–136.

Nakamura, M., M. Tani, and T. Kuramoto. 1994. Effects of rapid cooling on heart-rate of the Japanese lobster *in-vivo*. Zoological Science 11:375–379.

Naylor, J.K., and E.W. Taylor. 1999. Heart rate and gill ventilation in ovigerous and non-ovigerous edible crabs *Cancer pagurus*: the effects of disturbance substrate and starvation. Marine and Freshwater Behaviour and Physiology 32:129–145.

O'Grady, D.F., S.H. Jury, and W.H. Watson III. 2001. Use of a treadmill to study the relationship between walking, ventilation and heart rate in the lobster *Homarus americanus*. Marine and Freshwater Research 52:1387–1394.

Okada, J., K. Kuwasawa, A. Kihara, Y.F. Tsukamato, and T. Yazawa. 1997. Cholinergic inhibitory innervation of the cardioarterial valves in the isopod *Bathynomus doederleini*. Zoological Science 14:571–579.

O'Mahoney-Damon, P.M. 1984. Heart rate of the land crab, *Gecarcinus lateralis* during aquatic and aerial respiration. Comparative Biochemistry and Physiology 29A:621–624.

Patterson, N.E., and P.L. deFur. 1988. Ventilatory and circulatory responses of the crayfish *Procambarus clarkii* to low environmental pH. Physiological Zoology 61:396–406.

Paul, R.J., M. Colmorgen, S. Hüller, F. Tyroller, and D. Zinkler. 1997. Circulation and respiratory control in millimetre-sized animals (*Daphnia magna, Folsomia candida*) studied by optical methods. Journal of Comparative Physiology B 167:399–408.

Pearson, J. 1908. *Cancer*. Liverpool Marine Biology Committee. Memoirs XVI.

Pequeux, A. 1995. Osmotic regulation in crustaceans. Journal of Crustacean Biology 15:1–60.

Preiffer-Linn, C., and R.M. Glantz. 1989. Acetylcholine and GABA mediate opposing actions on neuronal chloride channels in crayfish. Science 245:1249–1251.

Price, D.A., and M.J. Greenberg. 1989. The hunting of the FLPs: the distribution of FMRFamide-related peptides. Biological Bulletin 177:198–205.

Pyle, R., and E. Cronin. 1950. The general anatomy of the blue crab *Callinectes sapidus*. State of Maryland, Board of Natural Resources. Publication No. 87.

Reiber, C.L. 1995a. Hemodynamics of the crayfish *Procambarus clarkii*. Physiological Zoology 67:449–467.

Reiber, C.L. 1995b. Physiological adaptations of crayfish to the hypoxic environment. American Zoologist 35:1–11.

Reiber, C.L. 1996. Ontogeny of cardiac regulation in the crayfish. American Zoologist 36:38A.

Reiber, C.L. 1997. Oxygen sensitivity in the crayfish *Procambarus clarkii*: peripheral O2 receptors and their effect on cardiorespiratory functions. Journal of Crustacean Biology 17:197–206.

Reiber, C.L., and G.F. Birchard. 1993. Effects of temperature on hemolymph pH and metabolism in the land crab *Stoliczia abbotti*. Journal of Thermobiology 18:49–152.

Reiber, C.L., and S.L. Harper. 2001. Aspects of cardiac physiological ontogeny in decapod crustaceans. (Zoologische Jahrbucher) (Zoology: Analysis of complex systems): Zoology of Complex Systems 104:103–113.

Reiber, C.L., and I.J. McGaw. 2009. A review of the "open" vs. "closed" circulatory systems: new terminology for complex invertebrate circulatory systems in light of current findings. International Journal of Zoology 2009:1–8.

Reiber, C.L., and B.R. McMahon. 1998. The effects of progressive hypoxia on the crustacean cardiovascular system: a comparison of the freshwater crayfish, *Procambarus clarkii* and the lobster, *Homarus americanus*. Journal of Comparative Physiology 168*B*:168–176.

Reiber, C.L., and T. Wang. 1997. Control of arterial blood gases: cardiovascular and ventilatory perspectives. American Zoologist 36:1–2.

Reiber, C.L., B.R. McMahon, and W.W. Burggren. 1992. Redistribution of cardiac output in response to hypoxia: a comparison of the freshwater crayfish *Procambarus clarkii* and the lobster *Homarus americanus*. Comparative Physiology. Karger, Basel.

Reiber, C.L., B.R. McMahon, and W.W. Burggren. 1997. Cardiovascular functions in two Macruran decapod crustaceans (*Procambarus clarkii* and *Homarus americanus*) during periods of inactivity, tail flexion and cardiorespiratory pauses. Journal of Experimental Biology 200:1103–1113.

Redmond, J.R. 1955. The respiratory function of hemocyanin in Crustacea. Journal of Cellular and Comparative Physiology 46:209–247.

Romney, A.L., and C.L. Reiber. 2013. Embryonic development and cardiac morphology of the grass shrimp, *Palaemonetes pugio* Holthuis, 1949 (Decapoda, Caridea, Palaemonidae): embryonic staging. Crustaceana 86:16–33.

Rose, R.A., J.L. Wilkens, and R.L. Walker. 1998. The effects of walking on heart rate, ventilation rate and acid-base status in the lobster *Homarus americanus*. Journal of Experimental Biology 201:2601–2608.

Rose, R.A., K. MacDougall, A. Patel, J.L. Wilkens, and R.L. Walker. 2000. Effects of walking on ventilatory and cardiac function in intact and cardiac-impaired lobsters. Physiological and Biochemical Zoology 74:102–110.

Rovero, F., R.N. Hughes, N.M. Whiteley, and G. Chelazzi. 2000. Estimating the energetic cost of fighting in shore crabs by non invasive monitoring of heartbeat rate. Animal Behaviour 59:705–713.

Sabourin, T.D. 1984. The relationship between fluctuating salinity and oxygen delivery in adult blue crabs. Comparative Biochemistry and Physiology 78A:109–118.

Saver, M.A., and J.L. Wilkens. 1998. Comparison of the effects of five hormones on intact and open heart cardiac ganglionic output and myocardial contractility in the shore crab *Carcinus maenas*. Comparative Biochemistry and Physiology 120A:301–310.

Saver, M.A., J.L. Wilkens, and C.N. Airriess. 1998. Proctolin affects the activity of the cardiac ganglion, myocardium, and cardioarterial valves in *Carcinus maenas* hearts. Journal of Comparative Physiology B 168:473–482.

Saver, M.A., J.L. Wilkens, and N.I. Syed. 1999. In situ and in vitro identification and characterization of cardiac ganglion neurons in the crab, *Carcinus maenas*. Journal of Neurophysiology 81:2964–2976.

Secor, S. 2009. Specific dynamic action, a review of the postprandial metabolic response. Journal of Comparative Physiology B 179:1–56.

Shadwick, R.E., C.M. Pollock, and S.A. Stricker. 1990. Structure and biomechanical properties of crustacean blood vessels. Physiological Zoology 63:90–101.

Shah, S.M., and H. Herried. 1978. Heart rate of the land crab *Cardiosoma guanhumi* during aquatic and aerial respiration. Comparative Biochemistry and Physiology 60A:335–341.

Siebers, D., C. Lucu, and K.R. Sperling. 1972. Kinetics of osmoregulation in the crab *Carcinus maenas*. Marine Biology 17:291–303.

Skerrett, M., P. Quigley, A. Peaire, and A.J. Mercier. 1995. Physiological targets of two FMRFamide-related peptides in crayfish. Journal of Experimental Biology 198:109–116.

Spaargaren, D.H. 1973. The effect of salinity and temperature on the heart rate of osmoregulating and osmoconforming shrimps. Comparative Biochemistry and Physiology 45A:773–786.

Spaargaren, D.H. 1974. Measurements of relative rate of blood flow in the shore crab, *Carcinus maenas*, at different temperatures and salinities. Netherlands Journal of Sea Research 8:399–406.

Spaargaren, D.H. 1982. Cardiac output in the shore crab *Carcinus maenas* in relation to solute exchange and osmotic stress. Marine Biology Letters 3:231–240.

Spicer, J.I. 1994. Ontogeny of cardiac function in the brine shrimp *Artemia franciscana* Kellogg 1906 (Branchiopoda: Anostraca). Journal of Experimental Zoology 270:508–516.

Spicer, J.I., and D. Morritt. 1996. Ontogenic changes in cardiac function in crustaceans. Comparative Biochemistry and Physiology A 114:81–89.

Stangier, J. 1991. Biological effects of crustacean cardioactive peptide (CCAP), a putative neurohormone/neurotransmitter from crustacean pericardial organs. Pages 201–210 *in* E. Florey and G.E. Stefanoe, editors. Comparative aspects of neuropeptide function. Manchester University Press, Manchester, UK.

Stangier, J., and R. Keller. 1990. Occurrence of the crustacean cardioactive peptide (CCAP) in the nervous system of the crayfish *Oronectes limosus*. Pages 394–400 *in* K. Wiese, W.-D. Krenz, J. Tautz, H. Reichert, and B. Mulloney, editors. Frontiers in crustacean neurobiology. Birkhauser, Basel.

Stangier, J., C. Hilbich, K. Beyreuther, and R. Keller. 1987. Unusual cardioactive peptide (CCAP) from the pericardial organ of the shore crab *Carcinus maenas*. Proceedings of the National Academy of Sciences USA 84:575–579.

Starratt, A.N., and B.E. Brown. 1975. Structure of the pentapeptide proctolin a proposed neurotransmitter in insects. Life Sciences 17:1253–1256.

Stevens, J.S., C.R. Cashman, C.M. Smith, K.M. Beale, D.W. Towle, A.E. Christie, and P.S. Dickinson. 2009. The peptide hormone pQDLDHVFLRFamide (crustacean myosuppressin) modulates the *Homarus americanus* cardiac neuromuscular system at multiple sites. Journal of Experimental Biology 212:3961–3976.

Stillman, J.H. 2004. A comparative analysis of plasticity of thermal limits in porcelain crabs across latitudinal and intertidal zone clines. International Congress Series 1275:267–274.

Stillman, J., and G. Somero. 1996. Adaptation to temperature stress and aerial exposure in congeneric species of intertidal porcelain crabs (genus Petrolisthes): correlation of physiology, biochemistry and morphology with vertical distribution, Journal of Experimental Biology 199:1845–1855.

Styrishave, B., A.D. Rasmussen, and M.H. Depledge. 1995. The influence of bulk and trace metals on the circadian rhythm of heart rates in the freshwater crayfish *Astacus astcus*. Marine Pollution Bulletin 31:87–92.

Styrishave, B., O. Andersen, and M.H. Depledge. 2003. In situ monitoring of heart rates of shore crabs *Carcinus maenas* in tow tidal estuaries: effects of physico-chemical parameters on tidal and diel rhythms. Marine and Freshwater Behaviour and Physiology 36:161–175.

Subrahmanyam, M.V.V., and R.V. Krishnamoorthy. 1984. Cardio-respiratory adaptations of the freshwater field crab to different salinities. Marine and Freshwater Behaviour and Physiology 11:229–238.

Sugarman, P.C., W.H. Pearson, and D.L. Woodruff. 1983. Salinity detection and associated behavior in the Dungeness crab, *Cancer magister*. Estuaries 6:380–386.

Sullivan, R.E., and M.W. Miller. 1984. Dual effects of proctolin on the rhythmic burst activity of the cardiac ganglion. Journal of Neurobiology 15:173–196.

Taylor, A.C. 1977. Respiratory responses of *Carcinus maenas* to changes in environmental salinity. Journal of Experimental Marine Biology and Ecology 29:197–210.

Taylor, E.W. 1982. Control and co-ordination of ventilation and circulation in crustaceans: responses to hypoxia and exercise. Journal of Experimental Biology 100:289–319.

Taylor, E.W., and P.J. Butler. 1978. Aquatic and aerial respiration in the shore crab, *Carcinus maenas* (L.), acclimated to 15 °C. Journal of Comparative Physiology B 127:315–323.

Taylor, E.W., and M.G. Wheatly. 1981. The effect of long-term aerial exposure on heart rate, ventilation, respiratory gas exchange and acid base status in the crayfish *Austropotamobius pallipes*. Journal of Experimental Biology 92:109–124.

Taylor, E.W., P. Butler, and A. Al-Wassia. 1977a. Some responses of the shore crab, *Carcinus maenas* (L.) to progressive hypoxia at different acclimation temperatures and salinities. Journal of Comparative Physiology 122:391–402.

Taylor, E.W., P.J. Butler, and A. Al-Wassia. 1977b. The effect of a decrease in salinity on respiration, osmoregulation and activity in shore crab *Carcinus maenas*. Journal of Comparative Physiology 119:155–170.

Taylor, E.W., P.J. Butler, and A. Al-Wassia. 1978. Some responses of the shore crab, *Carcinus maenas* (L.) to progressive hypoxia at different acclimation temperatures and salinities. Journal of Comparative Physiology B 122:391–402.

Taylor, H.H. 1989. Pressure flow characteristics of crab gills: implications for regulation of hemolymph pressure. Physiological Zoology 63:72–89.

Taylor, H.H., and P. Greenaway. 1979. The structure of the gills and lungs of the arid-zone crab, *Holthuisana transversa* (Brachyura: Sundathelphusidae) including observations on arterial vessels with the gills. Journal of Zoology London 189:359–384.

Taylor, H.H., and P. Greenaway. 1984. The role of the gills and branchiostegites in gas exchange in a bimodally air breathing crab *Holthusiana transversa*: evidence for a facultative change in the distribution of the respiratory circulation. Journal of Experimental Biology 111:103–121.

Taylor, H.H., and E.W. Taylor. 1986. Observations of valve-like structures and evidence for rectification of flow within the gill lamellae of the crab *Carcinus maenas* (Crustacea, Decapoda). Zoomorphology 106:1–11.

Taylor, H.H., and E.W. Taylor. 1992. Gills and lungs: the exchange of gases and ions. Pages 203–293 *in* F.W. Harrison and A.G. Humes, editors. Microscopic anatomy of the invertebrates, Vol 10. Wiley Liss, New York.

Thomas, N.J., T.A. Lasiak, and E. Naylor. 1981. Salinity preference behaviour in *Carcinus*. Marine Behaviour and Physiology 7:277–282.

Tierney, A.J., T. Kim, and R. Abrams. 2003. Dopamine in crayfish and other crustaceans: distribution in the central nervous system and physiological functions. Microscopy Research Techniques 60:325–335.

Toulmond, A. 1987. Adaptations to extreme environmental hypoxia in water breathers. Pages 123–136 *in* P. Dejours, editor. Comparative physiology of environmental adaptations, Vol. 2. Karger, Basel.

Truchot, J.P. 1973. Fixation et transport de l'oxygene par le sang de *Carcinus maenas*: variations en rapport avec diverses conditions de temperature et de salinitie. Netherlands Journal of Sea Research 7:482–485.

Tsukamoto, Y.F., and K. Kuwasawa. 2003. Neurohormonal and glutamatergic neuronal control of the cardioarterial valves in the isopod crustacean *Bathynomus doederleini*. Journal of Experimental Biology 206:431–443.

Tsukamoto, Y.F., K. Kuwasawa, and J. Okada. 1992. Anatomy and physiology of neural regulation of haemolymph flow in the lateral arteries of the isopod crustacean, *Bathynomus doederleini*. Comparative Physiology 11:70–85.

Ungherese, G.B., V. Boddi, and A. Ugolini. 2008. Eco-physiology of *Palaemonetes antennarius* (Crustacea, Decapoda): the influence of temperature and salinity on cardiac frequency. Physiological Entomology 3:151–161.

Wells, M.J. 1988. Respiratory and cardiac performance in *Lolliauncula brevis* (Cephalopoda, Myopsida): the effects of activity, temperature and hypoxia. 1988. Journal of Experimental Biology 138:17–36.

Wheatly, M.G. 1988. Integrated responses to salinity fluctuation. American Zoologist 28:65–77.

Wheatly, M.G. 1989. Physiological responses of the crayfish *Pacifastacus leniusculus* to environmental hyperoxia. I. Extracellular acid-base and electrolyte status and transbranchial exchange. Journal of Experimental Biology 143:33–51.

Wheatly, M.G. 1993. Physiological adaptations in decapodan crustaceans for life in fresh water. Pages 77–132 *in* R. Gilles, editor. Advances in comparative environmental physiology, Vol. 15. Springer-Verlag, Berlin.

Wheatly, M.G., and E.W. Taylor. 1979. Oxygen levels, acid-base status and heart rate during emersion of the shore crab *Carcinus maenas* (L) into air. Journal of Comparative Physiology B 132:305–311.

Wheatly, M.G., and E.W. Taylor. 1981. The effect of progressive hypoxia on heart rate, ventilation, respiratory gas exchange and acid-base status on the crayfish *Austropotamobius pallipes*. Journal of Experimental Biology 92:125–141.

Wheatly, M.G., and T. Toop. 1989. Physiological responses of the crayfish *Pacifastacus leniusculus* to environmental hyperoxia. II. Role of the antennal gland in acid-base and ion regulation. Journal of Experimental Biology 143:53–70.

Whiteley, N.M., E.W. Taylor, and A.J. El Haj. 1997. Seasonal and latitudinal adaptation to temperature in crustaceans. Journal of Thermal Biology 22:419–427.

Wiersma, C.A.G., and E. Novitiski. 1942. The mechanism of the nervous regulation of the crayfish heart. Journal of Experimental Biology 19:255–265.

Wilkes, P.R.H., and B.R. McMahon. 1982. Effect of maintained hypoxic exposure on the crayfish *Orconectes Rusticus*: I. Ventilatory, acid-base and cardiovascular adjustments. Journal of Experimental Biology 98:119–137.

Wilkens, J.L. 1995. Regulation of the cardiovascular system in crayfish. American Zoologist 35:37–48.

Wilkens, J.L. 1997. Possible mechanisms of control of vascular resistance in the lobster *Homarus americanus*. Journal of Experimental Biology 200:487–493.

Wilkens, J.L. 1999a. The control of cardiac rhythmicity and of blood distribution in crustaceans. Comparative Biochemistry and Physiology 124A:513–538.

Wilkens, J.L. 1999b. Evolution of the cardiovascular system in Crustacea. American Zoologist 39:199–214.

Wilkens, J.L., and G.M. Davidson. 1995. Peripheral circulation and its control in lobsters. Physiological Zoology 68:68A.

Wilkens, J.L., and T. Kuramoto. 1998. Comparison of the roles of neurohormones in the regulation of blood distribution from the hearts of the American and Japanese lobsters. Journal of Comparative Physiology B 168:483–490.

Wilkens, J.L., and B.R. McMahon. 1992. Intrinsic properties and extrinsic neurohormonal control of crab cardiac hemodynamics. Experientia 48:827–834.

Wilkens, J.L., and A.J. Mercier. 1993. Peptidergic modulation of cardiac performance in isolated hearts from the shore crab, *Carcinus maenas*. Physiological Zoology 66:237–256.

Wilkens, J.L., and H.H. Taylor. 2003. The control of vascular resistance in the southern rock lobster, *Jasus edwardsii* (Decapoda: Palinuridae). Comparative Biochemistry and Physiology 135A:369–376.

Wilkens, J.L., L. Wilkens, and B.R. McMahon. 1974. Central control of cardiac and scaphognathite pacemakers in the crab *Cancer magister*. Journal of Comparative Physiology B 90:89–104.

Wilkens, J.L., A.J. Mercier, and J. Evans. 1985. Cardiac and ventilatory responses to stress and to neurohormonal modulators by the shore crab, *Carcinus maenas*. Comparative Biochemistry and Physiology 82C:337–343.

Wilkens, J.L., T. Kuramoto, and B.R. McMahon. 1996. The effects of six pericardial hormones and hypoxia on the semi-isolated heart and sternal arterial valve of the lobster *Homarus americanus*. Comparative Biochemistry and Physiology 114C:57–65.

Wilkens, J.L., G.W. Davidson, and M.J. Cavey. 1997. Vascular peripheral resistance and compliance in the lobster *Homarus americanus*. Journal of Experimental Biology 200:477–485.

Wilkens, J L., T. Shinozaki, T. Yazawa, and H.E.D.J. ter Keurs. 2005. Sites and modes of action of proctolin and the FLP F2 on lobster cardiac muscle. Journal of Experimental Biology 208:737–747.

Wilkens, J.L., M.J. Cavey, I. Shovkivska, M.L. Zhang, and H.E.D.J. ter Keurs. 2008. Elasticity, unexpected contractility and the identification of actin and myosin in lobster arteries. Journal of Experimental Biology 21:766–772.

Willmer, P., G. Stone, and I. Johnston. 2005. Environmental physiology of animals. 2nd Ed. Blackwell Science, Malden, MA.

Wilson, C.B. 1904. A new species of *Argulus* with a complete account of two species already described. Proceedings of the U.S. National Museum 27:627–655.

Wirkner, C.S. 2009. The circulatory system in Malacostraca—evaluating character evolution on the basis of differing phylogenetic hypotheses. Arthropod Systematics 67:57–70.

Wirkner, C.S., and S. Richter. 2003. The circulatory system in Phreatoicidea: implication for the isopod ground pattern and peracarid phylogeny. Arthropod Structure and Development 32:337–347.

Wirkner, C.S., and S. Richter. 2007a. The circulatory system in Mysidacea—implications for the phylogenetic position of Lophogastrida and Mysida (Malacostraca, Crustacea). Journal of Morphology 268:311–328.

Wirkner, C.S., and S. Richter. 2007b. Comparative analysis of the circulatory system in Amphipoda (Malacostraca, Crustacea). Acta Zoologica 88:159–171.

Wirkner, C.S., and S. Richter. 2008. Morphology of the haemolymph vascular system in Tanaidacea and Cumacea: implications for the relationships of "core group" Peracarida (Malacostraca: Crustacea). Arthropod Structure and Development 37:141–154.

Wirkner, C.S., and S. Richter. 2013. Circulatory system and respiration. Pages 376–412 *in* L. Watling and M. Thiel, editors. Natural history of the Crustacea, volume 1: functional morphology and diversity. Oxford University Press, New York.

Wood, C.M., and D.J. Randall. 1981. Haemolymph gas transport, acid-base regulation, and anaerobic metabolism during exercise in the land crab (*Cardisoma carnifex*). Journal of Experimental Biology 218:23–35.

Worden, M., C. Clark, M. Conaway, and S. Qadri. 2006. Temperature dependence of cardiac performance in the lobster *Homarus americanus*. Journal of Experimental Biology 209:1024–1034.

Worden, M.K., E.A. Kravitz, and M.F. Goy. 1995. Peptide F1, and N-terminally extended analog of FMRFamide, enhances contractile activity in multiple target tissues in lobster. Journal of Experimental Biology 198:97–108.

Yamagishi, H. 1990. Physiological change of the heart beat in juvenile stage of the isopod crustacean, *Ligia exotica*. Zoological Science 7:1037.

Yamagishi, H. 2003. Aminergic modulation in the myogenic heart in the branchiopod crustacean *Triops longicaudatus*. Zoological Science 20:841–846.

Yamagishi, H., and E. Hirose. 1992. Nervous regulation of the myogenic heart in early juveniles of the isopod crustacean *Ligia exotica*. Pages 141–148 *in* R.B. Hill and K. Kuwasawa, editors. Phylogenetic models of functional coupling of the CNS and cardiovascular system. Comparative Physiology. Karger, Basel.

Yamagishi, H., and E. Hirose. 1997. Transfer of the heart pacemaker during juvenile development in the isopod crustacean *Ligia exotica*. Journal of Experimental Biology 200:2393–2404.

Yamagishi, H, H. Ando, and T. Makioka. 1997. Myogenic heartbeat in the primitive crustacean *Triops longicaudatus*. Biological Bulletin 193:350–358.

Yamagishi, H., T. Satoshi, and K. Tanaka. 2004. Dual effects of dopamine on the adult heart of the isopod crustacean *Ligia exotica*. Zoological Science 21:15–21.

Yazawa, T., and K. Kuwasawa. 1992. Intrinsic and extrinsic neural and neurohumoral control of the decapod heart. Experientia 48:834–840.

Yazawa, T., and K. Kuwasawa. 1994. Dopaminergic acceleration and GABAergic inhibition in extrinsic neural control of the hermit crab heart. Journal of Comparative Physiology A 174:65–75.

Zatta, P. 1987. Dopamine, noradrenaline and serotonin during hypo-osmotic stress of *Carcinus maenas*. Marine Biology 96:479–481.

Zanotto, F.P., M.G. Wheatly, C.L. Reiber, A.T. Gannon, and E. Jalles-Filho. 2004. Allometric relationship of postmolt net ion uptake, ventilation, and circulation in the freshwater crayfish *Procambarus clarkii*: intraspecific scaling. Physiological and Biochemical Zoology 77:275–284.

8

OSMOREGULATION AND EXCRETION

Jehan-Hervé Lignot and Guy Charmantier

Abstract

Most extant crustaceans live in aquatic habitats with diverse salinity conditions (stable or predictably or unpredictably variable). Therefore, some crustaceans are stenohaline osmoconformers (marine species), whereas others are steno- or euryhaline regulators (some marine and all freshwater species). The capacity to inhabit these various habitats can also differ through ontogeny. A great deal of information is currently available concerning the main optimized physiological cues, such as hemolymph composition, the efficiency of the different osmoregulatory strategies, hormonal control, and functional organization at tissue, cellular, and molecular levels of the key osmoeffectors. The effects of internal cues (gender, molting, nutrition, and ontogeny), as well as external factors (parasitism, season, temperature, dissolved oxygen, pollutants) are also key elements to consider. This broad review discusses these elements, focusing on different physiological strategies that allow osmoregulating species to maintain internal solute concentration at a level different from that of the external environment.

INTRODUCTION

Most of the extant crustaceans are aquatic (about 90%), living in the water column or as part of the benthic community. The crustaceans inhabit various habitats: coastal areas where salinity conditions can vary abruptly and unpredictably, the abysses (very stable environment), and hydrothermal vents (unstable) (Wirkner and Richter 2013). Crustaceans in different developmental stages may also have to move and adapt to very distinct haline media (parasites, ontogenetic migrators). This versatility makes crustaceans the most diverse aquatic invertebrates (Ruppert and Barnes 1994). Tolerance to changes in environmental salinity widely varies, and all types of osmoregulatory strategies are displayed within crustaceans. Euryhaline species can tolerate wide variations

in salinity, whereas others are intolerant to changes in salinity and are stenohaline. Some crustaceans are stenohaline osmoconformers (marine species), and others are stenohaline or euryhaline regulators (some marine and all freshwater species). A species living in a very stable environment with no salinity fluctuations (i.e., crayfish in freshwater) does not experience any change in salinity in the wild but can, nevertheless, tolerate salinity changes (up to 50% seawater) in experimental conditions. The freshwater prawns from the genus *Macrobrachium*, like crayfish, maintain high hemolymph osmolalities in freshwater and tolerate a range of salinities and are therefore recent colonizers of the freshwater habitat (Freire et al. 2003, Ordiano et al. 2005).

In osmoconformers, intracellular fluid is isosmotic to the extracellular fluid (Fig. 8.1). This limits the individuals to water bodies that do not vary in their ionic composition (strength). Although cell volume (water balance) is easily maintained in this situation, intracellular ionic homeostasis still requires finely tuned adjustments of the intracellular osmotic effectors (e.g., de novo synthesis and degradation of free amino acids [FAAs] and phosphoric compounds) in order to retain minimal osmotic gradients at the cell membrane surface (Schoffeniels and Gilles 1970, Péqueux 1995, Wehner et al. 2003). This type of regulation, which is presumed to be an ancestral trait, is relatively slow and thus is inefficient when rapid salinity changes occur (Anger 2001). Despite this, in some euryhaline crustaceans, the intracellular concentration of these FAAs may be 10 times higher than that observed in mammals (Gilles and Delpire 1997).

Crustacean regulators maintain their extracellular hemolymph osmolality with reduced variations, regardless of the salinity of the surrounding medium (Fig. 8.1). This anisosmotic extracellular regulation is based on several mechanisms, thus implying various permeability and salt transport properties within different ion-transporting epithelia. For example, in dilute seawater, crustaceans maintain their hemolymph at a higher solute concentration than their environment and thereby minimize osmotic water influx and diffusive salt loss across body surfaces by reducing drinking, epithelial water permeability, or urine production and by actively pumping in salts from their environment. These mechanisms are detailed later.

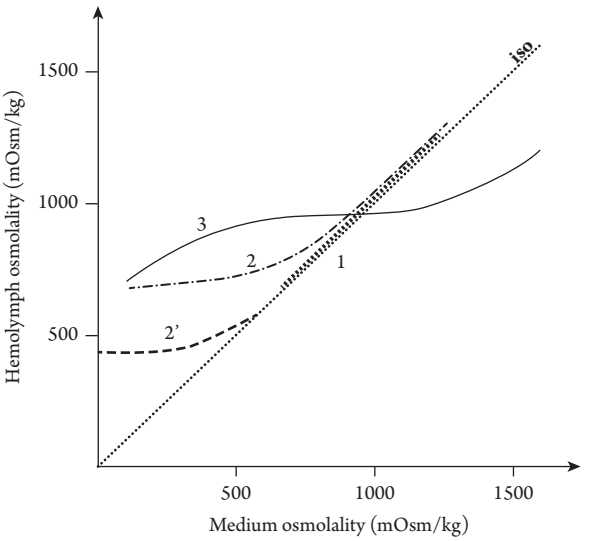


Fig. 8.1.
Patterns of osmoregulation in crustaceans: relation between medium and hemolymph solute concentration (mOsm/kg). 1: osmoconformers; 2: hyperisoregulators; 2′: hyperisoregulators in freshwater; 3: hyper-/hyporegulators. Iso: isosmotic line. Adapted from Charmantier et al. (2009).

A CENTURY OF RESEARCH

The pioneering work on osmoregulation was done by Hecht (1914), and, since then, many other studies have been published, culminating in the recent reviews by Péqueux (1995) and Charmantier et al. (2009). Most studies have dealt with the composition of hemolymph, the efficiency of osmoregulatory strategies, the hormonal control and functional organization of osmoregulatory effectors (at tissue, cellular, and molecular levels), and the effects of internal cues (molting, nutrition, ontogeny), as well as with external factors (temperature, dissolved oxygen, season, pollutants). Species of the order Decapoda have been the most studied because of their size and prevalence in coastal and freshwater environments. An estimated 32% of these decapods occupy freshwater habitats (De Grave et al. 2009, Coelho de Faria et al. 2011). Some only spend one or a few developmental stage(s) in this milieu, whereas others have become fully adapted to freshwater and they spend their entire life cycle there (several hololimnic caridean shrimps, trichodactylid, potamoid, and grapsid crabs). Therefore, comparative studies in these decapods contributed to a better understanding of the physiological mechanisms sustaining the invasion of the freshwater environment and enabled a clearer view of the ecological implications involved.

The first studies addressed the ionic and water hemolymph compositions in osmoconformers and regulators. The studies indicated that there are always (yet sometimes small) differences between the hemolymph and the external milieu and between the composition of the intra- and extracellular fluids. Intracellular isosmotic regulation has been particularly well studied by Gilles, Péqueux, and colleagues. In parallel, the structure and function of gills and other extrabranchial organs were explored using biochemistry, chemical blockers, isolated and perfused organs, electrophysiology, microscopy, and molecular techniques. One of the key and most-studied players is Na^+/K^+-ATPase, a membrane channel involved in the active pumping of sodium out of the cell in exchange for K^+ or NH_4^+. Among the numerous studies that focused on this enzyme, those by David Towle must be highlighted. From the early 1980s onward, he and his colleagues studied the physiological role of this pump and located it, as previously assumed, along the basolateral side of the ionocytes present in osmoregulating organs such as the posterior gills of brachyuran crabs. They then cloned and sequenced the α-subunit for the first time. David Towle was a true comparative physiologist who used molecular techniques and genomics as additional tools for the ecophysiological study of invertebrates.

Excretory physiology was poorly explored until the 1960s. After this date, the structure of antennal and maxillary glands, urine formation (ultrafiltration), and volume regulation were described (Péqueux 1995, Freire et al. 2008, Charmantier et al. 2009). Furthermore, the specific functional adaptations of these excretory organs when crustacean species are exposed to dilute seawater or concentrated seawater, and for terrestrial life have also been documented. It appears that the contribution of the excretory organs is geared toward the control of water balance (urine production) while total ionic balance remains minimal for most species. Therefore, the urine produced is usually isosmotic and shows similar sodium and chloride concentrations to the hemolymph. However, for some freshwater hyperregulators (crayfish) and freshwater and brackish water gammarids, compensatory ionic reabsorption occurs. Similarly, in terrestrial species, ionic reabsorption from the produced urine occurs in the gills and in the gut. As far as the digestive tract and its connected organs (hepatopancreas and midgut ceaca) are concerned, a direct role in osmoregulation was first demonstrated in *Artemia*, with careful measurements of the osmotic pressure of the gut fluid indicating that it is less concentrated than the medium but considerably more concentrated than the hemolymph (Croghan 1958a,b). The hepatopancreas has since been clearly identified as the main site for nutrient absorption (see studies by Ahearn and collaborators; e.g., Ahearn 1996), with some sodium-dependent transporters seen to be at work at least during nutrient assimilation. The midgut region has also been identified as a key player for water absorption at ecdysis (McNamara et al. 2005).

The neurohormonal control of hydromineral regulation took off with early studies on molting (McWhinnie 1962) and involved physiological experiments with organ ablation, extract injections, organ perfusion, and transepithelial ion transport. The organs involved are the antennal gland in isopods, and the brain, the "eyestalk complex," the thoracic ganglionic centers, and the pericardial organ in decapods. Various hormones, second-messengers like cyclic adenosine monophosphate (cAMP), and regulatory factors have also been detected.

Finally, advances during the past 20 years produced a deeper understanding of the cellular and molecular mechanisms and adaptive role of osmoregulation in crustaceans. Several membrane proteins (exchangers, cotransporters, other ion pumps or ion channels) involved in ionic and water transport have been revealed, along with their gene regulations, not only in the gills but also in other osmoregulating tissues such as the gut lining and hepatopancreas, as well as in the excretory organ. A more integrated understanding is now available at the gene and phenotypic level.

INTRINSIC FACTORS INFLUENCING THE HYDROMINERAL BALANCE IN CRUSTACEANS

Different internal factors such as gender, body size, nutritional status, and ontogenetic and non-ontogenetic migratory patterns, as well as the growth steps induced by molting, can directly or indirectly influence the osmo- and ionoregulatory capacity of a crustacean.

Gender

In *Gammarus roeseli*, females exposed to high salinity show the lowest tolerance and the highest and lowest hemolymph Na^+ and Cl^- concentrations, respectively (Sornom et al. 2010). This intersexual difference of sensitivity may strongly impair the population structure and could partly be explained by the increased energetic cost during oogenesis and egg incubation and the following increase of lipidic synthesis and mobilization, in comparison to spermatogenesis processes that are less demanding in terms of energy use (Buikema and Benfield 1979).

Age (Body Size/Weight)

A positive allometry has been documented for the freshwater crayfish *Procambarus clarkii*, with a net ion uptake of Ca^{2+}, Na^+, Cl^-, and NH_4^+ that increases with body mass (Zanotto et al. 2004). In this species, between 72% and 97% of variation in ionic regulation is related to body mass. However, in *G. roeseli*, interage difference has no impact on salinity stress tolerance (Sornom et al. 2010), although, with a larger age range, several studies have demonstrated a higher sensitivity of juvenile gammarids in comparison to adults (Naylor et al. 1995, Alonso et al. 2009).

The direct size effect may be due to different physiological factors, as well as to different surface-to-volume ratios and weight-to-gill surface area ratios. For example, compared to later life stages, juveniles could regulate their hemolymph at a lower osmolality, and the maximum salinity beyond which they cannot regulate their hemolymph osmolality could be lower (i.e., juveniles become osmoconformers at a lower salinity). Also, the maximum hemolymph osmolality that they can tolerate is lower than in adults (Kefford et al. 2007). Furthermore, for a hyperregulating species, juveniles with a proportionally greater gill surface area for their weights than adults experience larger water gain and salt loss and a higher metabolic rate per weight unit (Schmidt-Nielsen 1984). This has been demonstrated for postmetamorphic *Callinectes sapidus* hyperregulating in diluted seawater (Kinsey et al. 2003, Li et al. 2006). Small blue crabs experience a bigger challenge

than larger crabs, one that is incompletely compensated by lower integument permeability and higher levels of gill Na^+/K^+-ATPase compared to larger crabs. Finally, growth rates are higher in younger individuals than older ones with more frequent critical molting steps and shorter intermolt durations.

Nutrition

Feeding induces increased activity of the digestive tract (digestion, nutrient absorption, ion transport and secretion), and a large postprandial increase in oxygen uptake and in cardiovascular variables that correspond to the well-described specific dynamic action (SDA; Carefoot 1990, McGaw 2005). Cellular protein synthesis then follows (Houlihan et al. 1990, Mente et al. 2003). Therefore, the simultaneous demands of nutrition and osmoregulation can imbalance these physiological systems (prioritization or additive effects). For example, the osmoconforming *Cancer gracilis* can slow food processing in the gut and can even regurgitate food from the foregut at low salinity (McGaw 2006a). Increased mortality has also been reported for postprandial crabs (*Carcinus maenas, Cancer magister*) maintained at low salinity (Legeay and Massabuau 2000, McGaw 2006a,b). A strongly osmoregulating crab such as *C. sapidus* fed in seawater and then exposed to low salinity can coordinate the responses to both of these physiological challenges, whereas *C. magister*, a weak osmoregulator, under the same conditions prioritizes the osmoregulatory demand over digestion (Curtis and McGaw 2010). In the osmoconforming *C. gracilis*, exposure to low salinity immediately after feeding induces a temporary decrease in oxygen uptake, bradycardia, and reduced hemolymph flow rates. However, the crab soon recovers and prioritizes digestion over the metabolic response to low salinity.

Molting

Crustaceans present an incremental increase in size when molting but usually spend most of their time in intermolt, a period during which they feed and reproduce. Before shedding their confining exoskeleton (premolt), crustaceans stop feeding, and the exoskeletal calcium is solubilized and then transferred to the blood. It is temporarily stored in specialized organs such as the gastrolith (lobster, crayfish), anterior integumental sterites (isopods), or posterior midgut caeca (amphipods). It is then redeposited at postmolt into the new exoskeleton in order to increase its strength and rigidity. Therefore, large amounts of calcium are transferred through different epithelial cell layers during the molt cycle without altering the intracellular calcium activity (the cell signaling role of low intracellular calcium concentrations). To accomplish this massive transcellular movement of calcium in different directions through the cells, specific calcium transport proteins of the plasma membranes, the endoplasmic reticulum (ER) calcium ATPase (SERCA), the apical ryanoride receptor (RyR), and Na/Ca exchangers that use the transmembrane sodium gradient to drive calcium out of the cell can, together, regulate calcium flow. Furthermore, down- and upregulation of 26 different cuticle genes involved in the synthesis, breakdown, and resorption of chitin have been identified recently across the molt cycle (Seear et al. 2010).

During intermolt, transcellular calcium fluxes are minimal, and the plasma membrane Ca-ATPase largely controls cytoplasmic calcium activities by acting as a "housekeeping" protein. As a result of the transepithelial calcium transporting processes and ion compartmentalization occurring during premolt and postmolt, the ER may contain calcium activities greatly in excess to those in the bulk cytoplasm, thus allowing the cell to retain its cell signaling functions throughout these phases of the molt cycle.

Furthermore, before hardening by mineralization, the new exoskeleton is rapidly expanded through water uptake. This massive water absorption (that is equivalent to 60–80% of body

volume) occurs through the digestive tract, mostly. It accounts for 60–70% of the total water uptake (Chung et al. 1999), whereas the gills and the newly soft cuticle may be responsible for the rest (Neufeld and Cameron 1994).

Ontogenetic and Nonontogenetic Migrations

Some crustaceans spend their entire life cycle in the same environment, whereas others display complex migratory life history patterns, during which successive developmental stages are exposed to different osmotic conditions. For instance, salinity can affect egg production and development, as well as the development of post-hatch stages. In some cases, the salinity of the surrounding milieu during egg incubation can later impair the reproductive capacities of these individuals and can even induce sterility. Ontogenetic migrating crustaceans can have early life stages living in shallow coastal waters, lagoons, or freshwater environments while the adults live at sea, deeper in the water column and under more stable salinity conditions. The opposite ontogenetic migrating pattern also exists: semiterrestrial crabs for which hatching in water is compulsory, and freshwater palaemonid shrimps (*Macrobrachium* spp.) and brachyuran crabs (e.g., *Eriocheir sinensis*) migrate and release the larvae in saline waters. Salinity tolerance is usually higher in adults than in larvae, which generally show weaker regulatory capabilities than juveniles or adults (see the section "Ontogeny of Osmoregulatory Capacity"). Among the 200 extant species of the genus *Macrobrachium, M. amazonicum*, distributed along the northern and northeastern coasts of South America, typically displays ontogenetic migrations: freshwater-living berried females migrate downstream, and hatching occurs in brackish water of estuaries where larvae develop before recruitment to limnic populations through an upstream migration of juveniles to freshwater habitats. Adults are strong hyper-/hypo-osmoregulators. However, isolated populations, currently assigned to the same species, are found in freshwater habitats of the Pantanal in Brazil, where they have evolved a hololimnetic life cycle. Compared to the diadromous populations, the Pantanal shrimps have acquired an increased ability to hyperosmoregulate in freshwater, and, most strikingly, they show a complete loss of the ability to hypo-osmoregulate at high salinity (Charmantier and Anger 2011).

Nonontogenetic migration also occurs for species like spiny lobsters and homarid lobsters that move over large distances but in relatively stable environments. As weak regulators, they can adapt to only small salinity changes. Salinity fluctuation therefore may not be the major driving force for these migrations. For the brown crab *Cancer pagurus*, long-distance migrations of more than 260 km have also been identified from the European coastline, along specific directions and usually against the main oceanic currents (Bennett and Brown 1983). Although juveniles and adult males are more sedentary, inhabiting the coastal waters, females present a more migratory behavior. This may be linked to the stable salinity conditions found deeper offshore that allows better hatching success and larval survival. Vertical salinity gradients (haloclines) also affect the distribution and/or migration of planktonic crustaceans such as free-living copepods (Lougee et al. 2002). Both phototactic and geotactic behaviors can be altered by salinity. Indeed, various pelagic larvae of marine and estuarine crustaceans present downward movement when exposed to lower salinity seawater. This has been observed for copepods, branchiopods, barnacles and cirriped nauplii, and multiple decapod larvae (Grindley 1964, Hughes and Richard 1973). A change in sodium chloride concentration is the main environmental cue for detection of salinity variation (Forward 1989, 2009). In the supralittoral sandhopper amphipods, the salt concentration of the water influences their directional choice (Terracini-De Benedetti 1963, Scapini 2006). More specifically, Ca^{2+} concentration of seawater appears to be an important cue for the correct functioning of the sun compass mechanism to orientate along the sea–land axis (Ugolini et al. 2009).

OSMOREGULATORY STRATEGIES

Osmoconformers do not widely regulate their body fluid ionic composition and osmotic strength, although they have to face some fluctuations in salinity (Fig. 8.1). They allow body fluid osmolarity to vary directly with that of the environment. Osmoconformity is usually the result of a high permeability for monovalent ions and water through the integument (cuticle and underlying epithelium) therefore inducing an entirely passive influx or efflux of ions and water and lack of ion selectivity. Some species can reversibly switch from osmoconformation to osmoregulation depending on the salinity of their surrounding milieu and the time of exposure. Among regulating crustaceans, some hyperosmoregulate when exposed to a dilute environment, but they remain isosmotic when confronted with highly concentrated media ("weak" and "strong" hyperisoregulators). These individuals maintain their hemolymph at a higher solute concentration than the environment (i.e., more concentrated than the surrounding media). Strong hyperosmoregulators like *E. sinensis* are capable of maintaining their extracellular fluids at hyperosmotic levels even in freshwater (Péqueux and Gilles 1978, Cieluch et al. 2005). Crayfish, for instance, can maintain their hemolymph osmolality up to 400 times higher than their environment. Along with other freshwater decapods (e.g., *Pseudothelphusa jouyi* and *Metopaulias depressus*), crayfish are fully adapted to freshwater, have a high capacity for salt absorption in the gills and a very low permeability coefficient for the integument, and can tolerate increased salinity but are incapable of returning to full-strength seawater (Charmantier et al. 2009). Weak hyperosmoregulators (e.g., *Homarus gammarus*; Charmantier et al. 1984) are also able to cope with a hypo-osmotic environment but only for a restricted time and are not able to migrate to freshwater (Felder 1978).

Other regulators have the ability to maintain a constant extracellular fluid in a wide salinity range from concentrated seawater (>35‰) to freshwater (e.g., *Armases roberti, A. ricordi, A. miersii*; Schubart and Diesel 1998, Charmantier et al. 1998). They hyperosmoregulate in dilute seawater, but hyporegulate in seawater. Beachfleas (e.g., talitrid amphipods; Fig. 8.2) and terrestrial crabs are also very good osmoregulators and can even face desiccation. Upon return to seawater, they remain hypo-osmotic to the marine environment.

In light of these different osmoregulating strategies, complex mechanisms involving different organs, active uptake or active excretion of ions, water transport, and regulation of intracellular amino acid concentration have evolved. Water is not actively transported and can pass directly by osmosis across cell membranes following the ion concentration gradient. It is therefore indirectly controlled by the cellular response to changes in ion concentration. These strategies involve the "pumps and leaks" system through limiting and compensatory processes (Péqueux 1995). Limiting processes are mostly used by osmoconformers, which act on the permeability properties of the cell membrane in order to minimize the diffusive movements of osmotic effectors (Schoffeniels and Gilles 1970), whereas compensatory processes are mostly used by regulators and involve active movements of solutes to compensate for diffusive fluxes between spaces inside and outside the cell. The metabolic demands associated with this type of osmoregulation directly impact the animal's cardiac vascular system, oxygen uptake rate, and protein synthesis (Taylor et al. 1977, McGaw and Reiber 1998). For example, lobsters at low salinity increase their metabolism and heart and ventilation rates to help fuel the Na^+/K^+-ATPase necessary to keep their blood osmolarity higher than the ambient water (Charmantier et al. 2001). Also, in *Macrobrachium rosenbergii*, salinity change has a direct effect on protein synthesis in juveniles but not in postlarvae (Intanai et al. 2009). Osmotic stress also influences lipid synthesis and gluconeogenic activity in the gills, muscles, and hepatopancreas (Schein et al. 2004, Martins et al. 2011). For example, total lipid concentrations in gills and muscle of the crab *Neohelice granulata* (previously known as *Chasmagnathus granulata* and *C. granulatus*) are lower in crabs exposed to hypo-osmotic media (Luvizotto-Santos et al. 2003). A hyperosmotic stress also induces a decrease of the total lipid concentration in the posterior gills,

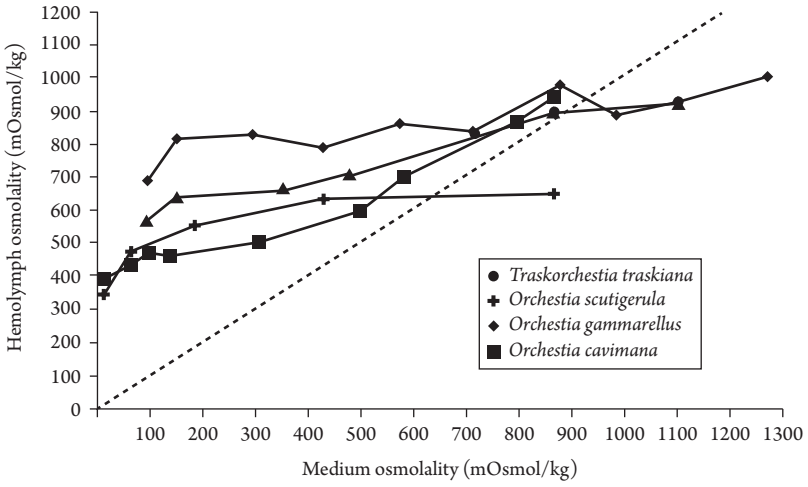


Fig. 8.2.
Hemolymph osmolality plotted against medium osmolality for different talitrid amphipods. Note the hyper-/hyporegulatory patterns displayed by these semiterrestrial beachfleas, except for *Orchestia cavimana*, which maintains its hemolymph hyperosmotic between external concentrations of 50 and 900 mOsmol/kg. Note also the regulation of *O. scutilerula* at ~635 mOsmol/kg during short-term exposure to a wide range of environmental salinities (0–900 mOsmol/kg). It is hypothesized that the potential wide range of osmotic stresses experienced on the high shore has selected the same osmoregulatory pattern. For *O. cavimana*, which is also the only one to produce hypo-osmotic urine from the antennary gland (all the other species examined having their urine isomotic with the hemolymph), the loss of hyporegulatory ability is explained by the fact that this species is unlikely to experience hypersaline stress at the freshwater end of temperate estuaries. The relatively high hemolymph concentration of ~350 mOsmol/kg for *O. cavimana* in freshwater and its euryhalinity may be conservative traits, indicating evolution from brackish water ancestors. Adapted from Morritt and Spicer (1998), with permission from NRC Research Press.

hepatopancreas, and muscles in this species (Chittó et al. 2009). Furthermore, during hypo-osmotic stress, the hepatopancreatic phosphoenolpyruvate carboxykinase (PEPCK) activity of the shrimp *Litopenaeus vannamei* (Rosas et al. 2001) and the PEPCK and glucose 6-phosphatase (G6Pase) activities in posterior gills of *N. granulata* are decreased.

IONIC HEMOLYMPH COMPOSITION

The presence or absence of ions in the bathing medium and their relative importance in the hemolymph can play key roles depending on the habitats used by the different developmental stages of a species. These ions are the main osmotic effectors (Na^+, Cl^- being the dominant ions, along with other major ions such as K^+, Ca^{2+}, and Mg^{2+}) accounting for up to 90% of the hemolymph osmolality, and the rest being due to organic compounds such as FAAs and carbohydrates. Na^+ and Cl^- concentrations account for 75–85% of the hemolymph osmotic pressure, as, for example, in penaeid shrimps (Castille and Lawrence 1981, Lin et al. 2000) and in the freshwater shrimp *Macrobrachium olfersii* when salinity of the external medium is more than 21 ppt (Lima et al. 1997).

The Mg^{2+} concentration is inversely related to the level of activity in different species of decapod crustaceans (Robertson 1953, 1960, Walters and Uglow 1981) and in the amphipod *Talitrus saltator* (Spicer et al. 1994). It has an anesthetic effect on marine invertebrates (Pantin 1946) and inhibits synaptic transmitter release at the neuromuscular junction (Mantel and Farmer 1983). Therefore, low magnesium concentrations along with low sulphate and calcium levels should positively influence

swimming activity (increase buoyancy) of active planktonic larval stages (Newton and Potts 1993). For instance, unlike Brachyura, Anomura, Palinura, and Astacidea (Reptantia), caridean shrimps (Natantia) have a high capacity for magnesium excretion and therefore maintain hemolymph Mg^{2+} concentrations far below that of seawater (Walters and Uglow 1981, Tentori and Lockwood 1990). This can have a direct consequence on species distribution. It may explain the presence of fewer decapods in the Antarctic continental shelf (caridean shrimps are present, but not brachyuran or anomuran crustaceans) compared to subantarctic regions (Wittmann et al. 2010, 2011).

Crustaceans also regulate their serum K^+ concentration over a wide range of salinities (Robertson 1960). However, a low K^+ concentration in inland saline waters that are abundant in Australia (with an otherwise similar inorganic ion concentration compared to the open sea) has been identified as the main cause for high mortalities in penaeid shrimp post-larvae (Tantulo and Fotedar 2006). Although the contribution of K^+ to the total hemolymph osmolality is small in these post-larvae, its role in maintaining Na^+/K^+-ATPase activity is crucial, and an imbalance between K^+ and Na^+ concentrations in the hemolymph can be the cause of the high mortality rate observed in these inland saline waters (Zhu et al. 2004, Sowers et al. 2005). Because K^+ concentration has a positive correlation with intracellular Na^+ (Burton 1995), low K^+ concentration may result in disproportionate Na^+ and K^+ concentrations in the hemolymph, which may in turn disturb the Na^+/K^+-ATPase activity.

The hemolymph Ca^{2+} concentration is significantly affected by molt stages and is usually strongly regulated over a wide range of salinities (Ferraris et al. 1986, Parado-Estepa et al. 1989, Neufeld and Cameron 1993). In freshwater with a low calcium concentration, for example, adapted crustaceans have a reduced integumental permeability, a high calcium affinity, and a net Ca^{2+} influx that occurs through active transport.

HORMONAL CONTROL

The Neuroendocrine System

The neuroendocrine control of water and ionic balance was recently reviewed (Chung et al. 2010, Mykles et al. 2010). It has been studied in a few isopods but mostly using decapod crustaceans. It involves the brain, the "eyestalk complex," the thoracic ganglionic centers, and the pericardial organ. In decapods, the brain is located between the two eyestalks and joins the thoracic ganglion (coalesced paired ganglia of all thoracic and abdominal segments) by two circumesophageal connectives, whereas the eyestalk complex is composed of the medulla terminalis X-organ (MTXO) and the sinus gland (SG). Initial studies focusing on animals with ablated eyestalks and injections of extracts from the different neuroendocrine centers indicated the presence of regulatory factors within the eyestalks (Zeleny 1905); their ablation impaired adaptation to different salinities (Mantel and Farmer 1983). One of the biogenic molecules is the crustacean hyperglycemic hormone neuropeptide (CHH; see Chapter 2 in this volume) produced by the MTXO (Charmantier-Daures et al. 1994). In *C. maenas*, a release of CHH in the hemolymph initiates molting and water uptake (Chung et al. 1999). A long-term acclimation to dilute seawater also shows elevation of circulating CHH (Chung and Webster 2005). Therefore, CHH and associated signaling pathways must have a central role in the overall response to hypo-osmotic conditions. Structurally identical CHH and CHH-like peptides have also been reported for non-eyestalk tissues, as, for example, in the gut and pericardial organ of the shore crab *C. maenas* and for estuarine and freshwater species (Freire and McNamara 1992, Tiu et al. 2007). Furthermore, in various decapod crustaceans, other biogenic molecules including serotonin (5-HT), dopamine, octopamine, and norepinephrine have been pinpointed as involved in the control of osmoregulation (Zatta 1987, Kuo et al. 1995). Finally, the

SG has been identified as a storage and release center for the different crustacean neurohormones synthesized in the brain and the MTXO complex.

Target Organs

The neuroendocrine extracts affect osmoregulation through increased circulation rate by the heart and movement of salt and water in the gills, stomach, intestine, and excretory organs (the antennal glands; Kamemoto 1991). CHH and ion transport peptide (ITP) are members of the same arthropod neuropeptide family. In insects, ITP regulates Cl^- transport and is involved in the gut water reabsorption (Phillips et al. 1998). In estuarine and freshwater decapods, CHH is involved in branchial ion transport and increases the transepithelial voltage and Na^+ influx (Serrano et al. 2003). Animals acclimated to low salinity also present high levels of CHH. Therefore, CHH has multiple functions and is associated with supplying the energy required for iono-/osmoregulation (glucose mobilization), molting, and reproduction.

OSMOSENSING AND OSMOREGULATORY EFFECTORS

The perception of salinity change and ionic composition of the external milieu, along with the regulation of water and ion concentrations in the body, relies on several osmoregulatory effectors and involves different epithelia (integument, gills, extrabranchial organs, gut lining, and excretory organs).

Osmosensing

Adult crustaceans and their larval stages can perceive salinity changes. The osmoreceptors are located in different areas: the walking legs, the branchial chamber, or near the excurrent opening of the branchial chamber (Larimer 1964, Davenport and Wankowski 1973, Schmidt 1989). Depending on the species, the antennae and antennules can also play an important role (Gleeson et al. 1996, 1997, Dufort et al. 2001). Finally, the system involved in salinity detection can be sensitive to the concentration of certain ions only, rather than to the overall osmolality. For example, the American lobster's receptors located in or near the branchial chamber are primarily sensitive to chloride ions (Dufort et al. 2001). Similarly, decreased concentration of sodium and cations of equivalent size allows the perception of a salinity decrease, whereas cation size is not an important parameter for detecting increased salinity (Harges and Forward 1982).

Integument

The permeability of the cuticle and underlying epithelium, because of its surface-to-body ratio, can represent the main exchanging site for water, ions, and other elements. For instance, direct integumental absorption of the exogenous amino acid glycine as a nutrient has recently been demonstrated in phyllosoma larvae of the Japanese spiny lobster *Panulirus japonicus* (Souza et al. 2010). Lowering integumental permeability limits water and ion fluxes. This is one of the key adaptations to freshwater and terrestrial life. Therefore, salt and water permeabilities are higher in marine and lower in freshwater species (Subramanian 1975), and acclimation to low salinity may induce a rapid decrease in water permeability. Furthermore, osmoregulators are less permeable than osmoconformers.

For brachyuran and anomuran terrestrial crustaceans, water and ion balance is related to their habitat and osmoregulatory pattern. Some regularly return to seawater for water uptake, excretion,

and reproduction, whereas others completely avoid immersion but live in moist environments or use inland freshwater, dew, and rainwater. These terrestrial crustaceans are capable of conserving salts by postrenal modification of the urine. For instance, they all retain gills and other osmoregulating tissues that are implicated in ion reabsorption (Taylor and Greenaway 2002). Therefore, although most marine and brackish water crustaceans generally produce urine isosmotic to their hemolymph, terrestrial crabs are able to modify the final composition of their urine in their branchial chambers. In some species, urine can also be reingested. Furthermore, evaporative water loss is strongly reduced. As a comparison, in intertidal hermit crabs (*Clibanarius*), the evaporative water loss rate is high and can be increased three times more if the animals are removed from their shells (Herreid 1969). However, in coenobitids such as the terrestrial *Coenobita scaevola*, removal from the shell increases evaporative loss by only 11% (Achituv and Ziskind 1985). For these terrestrial hermit crabs, which only migrate back to the sea for reproduction, producing urine that is only slightly hyperosmotic to the hemolymph generates increased drinking rates when drinking seawater (De Wilde 1973, Greenaway et al. 1990). Despite this increased drinking in seawater in order to compensate for the large quantity of urine produced, the net water gain still remains limited. Thus, when saline water is provided for drinking, intake must be considerably enhanced because much of the volume gained is needed to excrete the salt load, and the net gain of pure water, required to replace evaporative loss, is small.

Gills

The gills (pleurobranchs, arthrobranchs, and podobranchs, depending on the location of gill attachment) are the main organs for respiratory gas exchange and for homeostasis of the extracellular fluid (Wirkner and Richter 2013). A description of the structure, ventilation, microvasculature, histology, and ultrastructure of gills in various crustacean groups has recently been updated (Freire et al. 2008, Charmantier et al. 2009). They are designated as phyllobranchiate (brachyurans, carideans, and some anomurans), trichobranchiate (crayfish and lobsters), and dendrobranchiate gills (penaeid and sergestoid shrimps) depending on their degree of surface amplification (Freire et al. 2008). In the gills, osmoregulating sites can coexist with respiratory epithelial cells or can be restricted to the posterior ones, as observed in several estuarine crabs (Charmantier et al. 2009). In some species, gills are restricted to gas exchange, whereas epipodites and branchiostegites participate in osmoregulation (i.e., lobsters, see next paragraph). Furthermore, although gas diffusion is accomplished through thin and poorly differentiated cells, ion transport requires larger cells with well-characterized structures. These "ionocytes" possess apical infoldings and basolateral infoldings associated with numerous mitochondria. Adjacent ionocytes can be tightly linked with septate junctions and lateral infoldings, thus providing structural strength and permeability regulation through the intercellular space. A detailed review about the physiological and molecular data obtained for the combined cytosolic and membrane-bound proteins at work in these ionocytes is now provided in the most recent review (Charmantier et al. 2009). Therefore, a brief outline is detailed in the last paragraph of this review detailing new challenges along with the current working models for ion transport across the different osmoregulating tissues in crustaceans.

Extrabranchial Organs

Ionocytes and ion-permeable areas other than the gills have been described in several seawater, estuarine, and freshwater crustaceans (Figs. 8.3 and 8.4). Some are located in the branchial chambers: the pleurites, the inner epithelia of the branchiostegites and the epipodites (Bouaricha et al. 1994, Kikuchi and Matsumasa 1995, Lignot et al. 1999). A few others are situated outside these

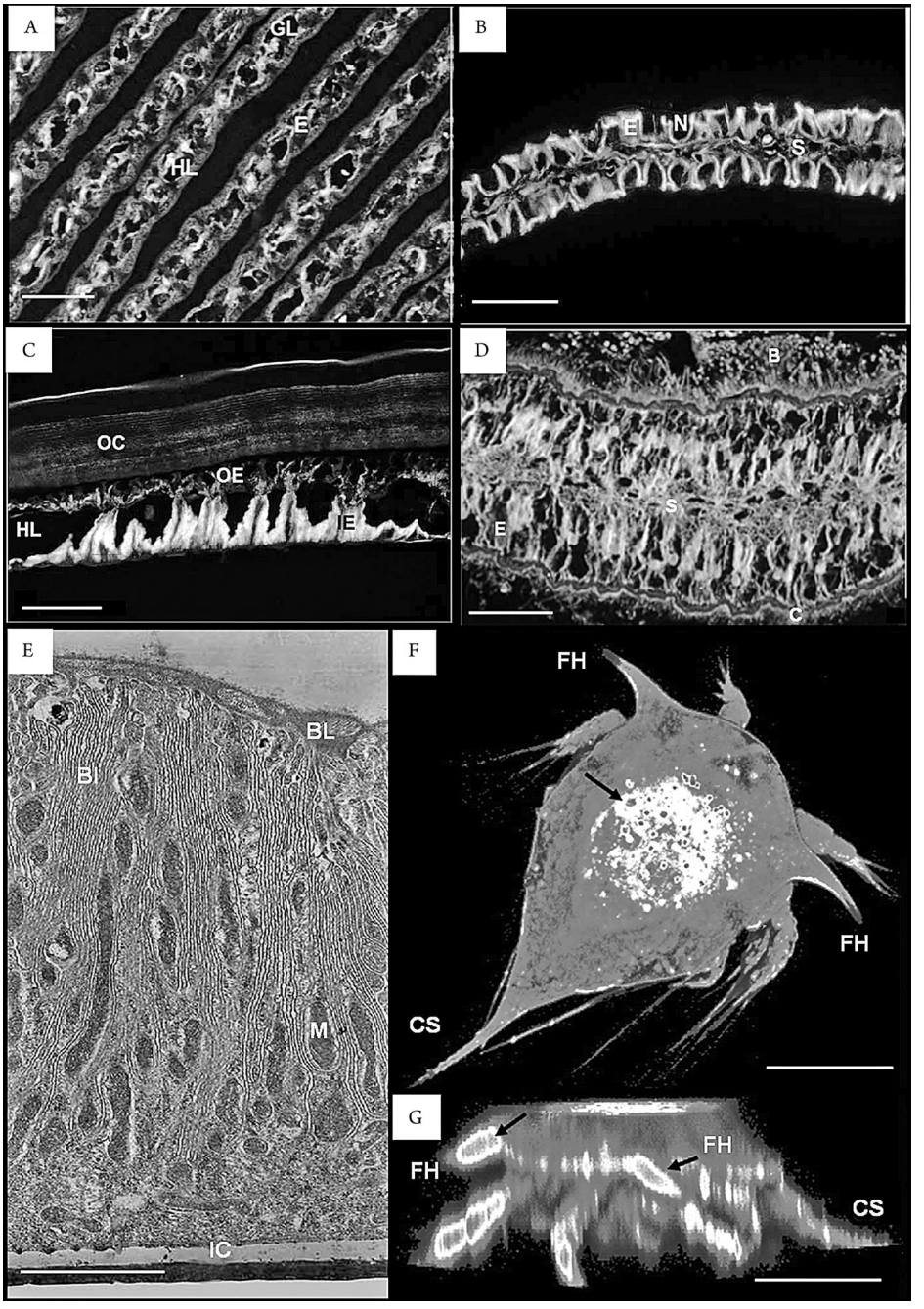


Fig. 8.3.
(A)–(D) Immunolocalization of Na^+/K^+-ATPase in the gills (A), epipodite (B), and branchiostegite (C) of the epibenthic *Palaemon adspersus* and in the epipodite of the deep-sea hydrothermal *Rimicaris exoculata* (D). Scale bars: 50 μm. (E) Transmission electron micrograph of the branchiostegite of *Palaemon adspersus*. Scale bar: 2.5 μm. (F) three-dimensional confocal stack showing the dorsal side of the *Amphibalanus amphitrite* nauplius. Black arrow indicates areas of intense fluorescent labeling in the naupliar tissue below the dorsal shield. (G) Lateral view of the nauplius. Black arrows denote the frontal horns where fluorescence intensity is high. Scale bars: 100 μm (F–G). Abbreviations: B, bacteria; BI, basal infoldings; BL, basal lamina; C, cuticle; CS, caudal spine (posterior); E, epithelium; FH, frontal horns; GL, gill lamellae; HL, hemolymph lacuna; IC: internal cuticle; IE, inner epithelium; M, mitochondria; N, nucleus; OC, outer cuticle; OE, outer epithelium; S, septum. See color version of this figure in the centerfold. Adapted from Martinez et al. (2005), with permission from Elsevier, and from Gohad et al. (2009), with permission from Elsevier.

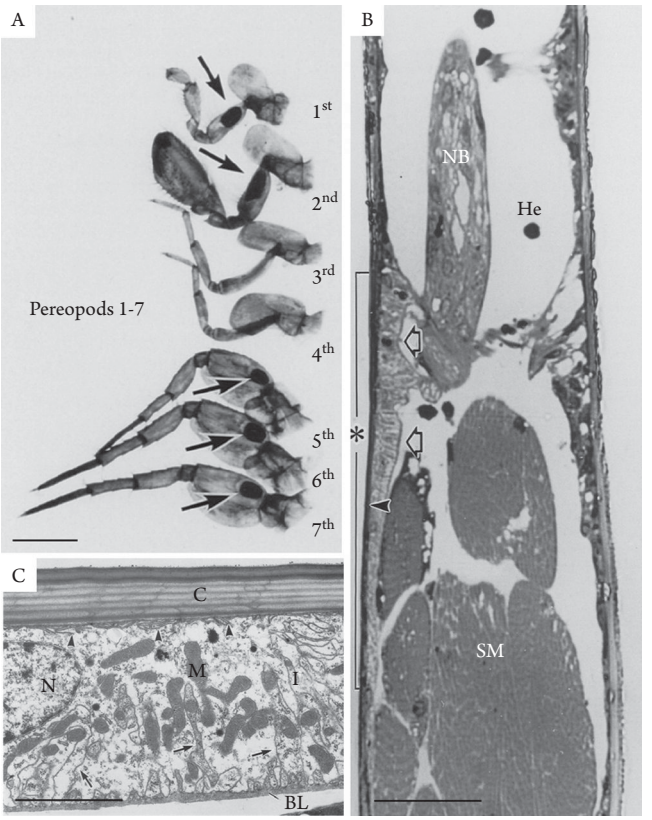


Fig. 8.4.
Pereopodal disks of the estuarine amphipod *Melita setiflagella*. (A) Medial view of the right pereopods treated by the silver nitrate nitric acid method. The arrows indicate the pereopodal disks as darkly stained discoid organ located on the medial surface of each basipodite, except the third and fourth pereopods. Scale bar: 500 μm. (B) Longitudinal section of a basipodite through the pereopodal disk. The disk (asterisk) is composed of a specialized thick epithelium (white block arrows) covered with a cuticle layer (arrowhead) thinner than that of the ordinary epithelium. Scale bar: 25 μm. (C) Ultrastructure of the pereopodal disk (cross-section of an epithelial cell). Note the presence of numerous mitochondria, deep basolateral infoldings (arrows), complicated interdigitations between the adjacent epithelial cells, and very shallow and sparse apical infoldings (arrowheads). Scale bar: 2.5 μm. Abbreviations: BL, basal lamina; C, cuticle layers; He, hemocoel; I, internal infoldings; M, mitochondria; N, nucleus; NB, nerve bundle; SM, striated muscle. From Kikuchi and Matsumasa (1995), with permission from Elsevier.

cavities in nondecapod crustaceans: the neck organ of the nauplii, the metepipodites of the phyllopodia, gut of the brine shrimp *Artemia salina*, the gut of terrestrial isopods, and the pereopodal disk on the medial surface of the basipodite in amphipods, as well as the sterna, sternal gills, and patch-like areas of the afferent blood vessels of the coxal gills in estuarine amphipods (Wägele 1992, Aladin and Potts 1995, Hosfeld and Schminke 1997, Kikuchi and Matsumasa 1997; see Fig. 8.4).

Digestive Tract

In arthropods, the gut is divided into three distinct parts: the fore-, mid-, and hindgut (see Chapter 9 in this volume). In some species, diverticula and anterior and posterior caeca showing considerable morphological diversity are also present (Icely and Nott 1985, Brunet et al. 1994). For example, in decapods, the hepatopancreas or midgut gland forms a large branching gut diverticulum and is the major site for nutrient absorption (Lockwood 1968).

Whether the gut of crustaceans plays a role in osmoregulation has been questioned in very early studies (Olmsted and Baumberger 1923, Drach 1939) and, since then, has been regularly addressed, taking into account the different parts of the gut, the hepatopancreas, and the midgut caeca, and using different osmoconforming and osmoregulating species as well as terrestrial species (for a review, see Charmantier et al. 2009). It appears that continuous or rhythmic antiperistaltic water uptake can occur through the mouth and anus, and this drinking acts as an enema, maintains intestinal antiperistalsis, and stretches the gut wall muscles (Fox 1952). Some of this water passes through the gut wall into the hemolymph and out of the body by way of the excretory organs (Greco et al. 1986). The water absorption through the gut varies according to the external salinity (De Wilde 1973, Greenaway et al. 1990) but is minor compared to the total water influx realized through the gills and cuticle. It is solute-dependent, and Na^+, Cl^- and K^+ are the key ions that drive this water uptake (Mantel and Farmer 1983). Furthermore, several ion and ionic-mediated nutrient transporters have been identified along the gut. For example, Na^+/K^+-ATPase has been detected in several parts of the gut. In the hepatopancreas, the Na^+/Cl^- exchanger, different isoforms of the electrogenic $2Na^+/1H^+$ antiporter, and the proton-gradient-dependent Cl^-/HCO_3^- have also been identified (Ahearn et al. 1987). In the Chinese mitten crab (*E. sinensis*), fatty acid-binding proteins (FABPs) have been identified in the hepatopancreas, intestine, and gills but were not detected in the stomach. Leptin receptor has also been identified in the intestine but is less expressed in the hepatopancreas and gills (Jiang et al. 2010, Gong et al. 2010). Whether this is related to osmoregulation remains to be investigated. Sodium-dependent glucose transport has also been reported in the hepatopancreas of the American lobster, *Homarus americanus* (Ahearn et al. 1985). The presence of glucose transporters (GLUT2-like, GLUT5-like, and SGLT1-like transporters) was later reported in this species (Sterling et al. 2009). With the sodium-dependent hexose SGLT1-like transporter, fructose is preferentially transported over glucose. It is potentially located in either the apical brush border or basolateral side of the hepatopancreatic cells, or possibly both.

Therefore, water and ion transport by the gut lining is related to physiological processes such as drinking, nutrient absorption, and molting. It is used by some marine hypo-osmoregulators and terrestrial species for limiting dehydration through active salt extrusion (Dall 1967a,b) and urine reingestion (Ahearn et al. 1999). For example, in the terrestrial crab *Gecarcinus lateralis*, permeability to water and ions by the foregut from lumen to hemolymph and in the reverse direction is hormonally driven and occurs during the intermolt period and immediately after ecdysis. It is therefore an important adaptation to conserve water (Mantel 1968). Similarly, in *C. maenas*, the massive and rapid release of CHH from the fore- and hindgut endocrine cells only during premolt induces water uptake by these tissues and, thus, size increase just after ecdysis (Chung et al. 1999). The midgut and the midgut caeca have also been reported as ion and water transporting sites in penaeids and various crab species exposed to dilute seawater (Dall 1967a,b, Mantel and Farmer 1983). Nevertheless, despite these data and the acute role of the digestive system for water intake during molting, differentiating the osmoregulatory function from digestive processes is still difficult, and additional research is necessary, particularly in conditions of hypo-osmoregulation.

Excretory Organs

Data available concerning the crustacean excretory organs (antennal, antennary, maxillary, and green or renal glands) indicate that they are functionally and structurally equipped for regulating volume and ionic composition of their hemolymph through urine production (Henry and Wheatly 1992, Péqueux 1995). These morphological and functional data have been reviewed recently and are briefly described (Freire et al. 2008, Charmantier et al. 2009). Among these different organs, the antennal glands have been the most studied (Webb 1940, Behnke et al. 1990, Gao and Wheatly 2004). Antennal glands possess the same basic structure as the maxillary glands and consist of an

end sac, a convoluted duct (or tubule) that can be divided into proximal and distal portions, and an exit duct that may differentiate as a bladder. In some species, the proximal part can be partially differentiated as a labyrinth. This structure can appear as a very convoluted, coiled tubule, a maze of anastomosing tubules, or a spongy tissue (Freire et al. 2008). Furthermore, different epithelial cell types can be found along the tubule: podocytes filled with small and large vacuoles, lysosomes and residual bodies in the end sac, typical transporting cells with an apical brushborder and basal infoldings associated with numerous mitochondria in the labyrinth and proximal tubule, and, last, epithelial cells with no or less dense apical microvilli in the distal tubule. Finally, the bladder not only functions as a storage compartment, but also possesses ionocytes. It is thus involved in absorptive and secretory processes and further modification of the urine (Freire et al. 2008, Charmantier et al. 2009). Functionally, different key ion-transporting enzymes can be found in the excretory organs, such as carbonic anhydrase and Na^+/K^+-ATPase, as well as Ca^{2+}-ATPase (Peterson and Loizzi 1974, Sarver et al. 1994, Khodabandeh et al. 2005a,b). Thus, the hemolymph filtrate in the end sac (Riegel 1961, Kirschner 1967) is modified by the reabsorption of some substances (i.e., organic molecules such as glucose and amino acids) and secretion of others into the fluid (Riegel and Kirschner 1960, Lin et al. 2000). As a result, most crustaceans living in seawater and/or subjected to dilute seawater produce urine that is isosmotic to their hemolymph. Only in a few freshwater species, including crayfish and some amphipods, are ions reabsorbed through the excretory glands, thus producing a hypotonic urine (review in Charmantier et al. 2009). For brachyuran and anomuran terrestrial crabs, specific adaptations allow them to conserve salts by postrenal modification of the urine. For crustaceans exposed to higher salinities than seawater, the urine produced is isosmotic.

ONTOGENY OF OSMOREGULATORY CAPACITY

Different postembryonic developmental strategies can be found in crustaceans (Anger 2001), with some species having numerous larval, postlarval, and juvenile stages (e.g., penaeid shrimps), whereas others possess only a few post-hatch juvenile stages (freshwater decapods). Physiological studies have clearly demonstrated that significant changes occur during the ontogenesis of osmoregulation in various crustaceans, including cladocerans (daphnids), isopods, amphipods, and decapods (crabs, lobsters, shrimps, and crayfish; Cieluch et al. 2004, Khodabandeh et al. 2005a,b; see Figs. 8.5 and 8.6).

Crustacean larvae are planktonic and swim actively in the water column. The larvae possess a very thin cuticle that is permeable to water and ions. Furthermore, they do not have gills, and salinity tolerance must therefore be achieved by actively regulating their internal concentration. This isosmotic intracellular regulation has been shown in the megalopa of the blue crab *C. sapidus* (Burton 1992) and in all the developmental stages of *H. gammarus* (Haond et al. 1999). Anisosmotic extracellular regulation is also possible in larvae (Kalber and Costlow 1968, Charmantier et al. 2009). Three patterns of ontogeny of osmoregulation have been recognized (Charmantier 1998, Charmantier et al. 2009). In osmoconformers, osmoregulation does not vary throughout development. The adult type of osmoregulation is established in the first postembryonic stage, thus osmoregulation occurs, usually late, during embryonic development. In addition, freshwater decapods display life cycles exhibiting varying degrees of abbreviated larval development, produce lecithotrophic eggs, and maintain low hemolymph osmolalities and diminished intracellular FAA titers (Lee and Bell 1999, Augusto et al. 2007). In a third pattern, the early stages of development, usually larvae, osmoconform or slightly osmoregulate; following metamorphosis, the juveniles display the adult type and capacity of osmoregulation, become more euryhaline, and are able to change their habitat (Fig. 8.7). These changes accompany ontogenetic migrations between sea and estuaries, as seen for example for the grapsid crab *C. granulata* (Charmantier et al. 2002).

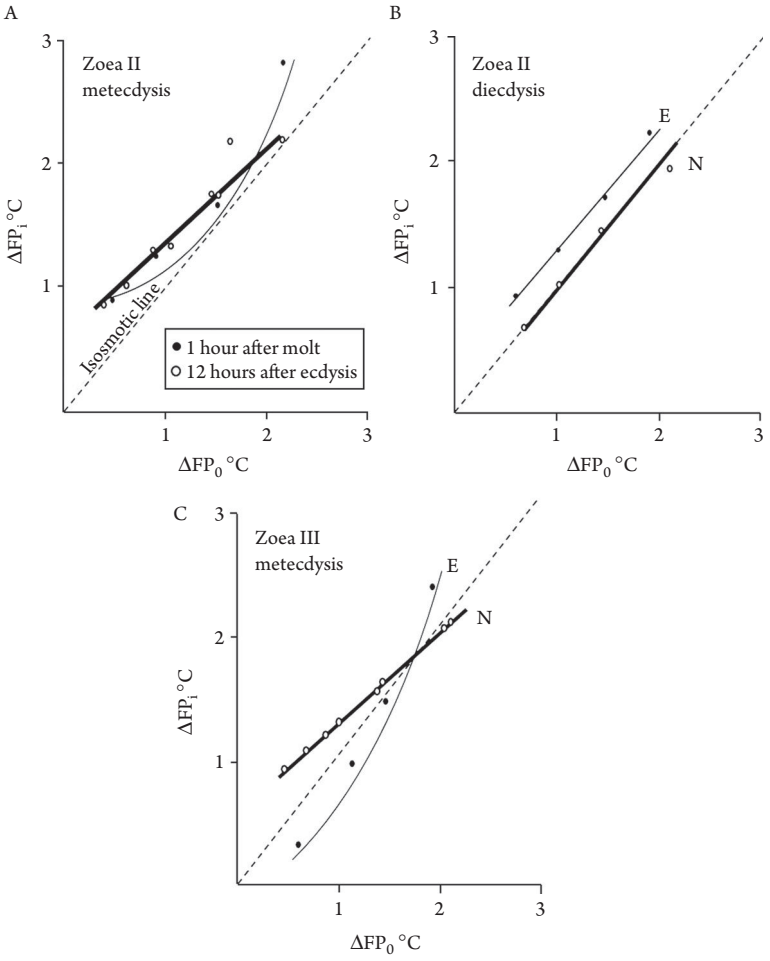


Fig. 8.5.
Osmoregulation curves for zoea II and III of *Rhithropanopeus harisii*. Adapted from Kalber and Costlow 1966, with permission from Oxford University Press. (A) Comparison of osmoregulation in normal metecdysial zoea II at 1 h following molt and at 12 h ecdysis. (B) Osmoregulatory responses of normal zoea II and the eyestalkless animals of the same age. (C) The effects of eyestalk removal on zoea III metecdysis at 12 h after molt. Abbreviations: E, eyestalkless; N, normal; ΔFPi, ΔFPo, hemolymph, medium freezing point depression. Osmotic pressure (mOsmol/kg) = (|ΔFP|/1.858) × 1,000 (1.858 is the freezing point constant for water). These results point out the effects of molting and of the sinus gland on the larval osmoregulatory capacity. Premolt (data not shown) and postmolt larvae (here, zoea II) immediately after molt are hyperosmotic above 33‰, most probably to ensure inflow of water and to establish a greater body volume during hardening of the new exoskeleton (A). Also, zoea II larvae do not hyperosmoregulate during diecdysis, but eyestalkless individuals show a significant gain to osmoregulate at all salinities (B). Normal zoea III hyperregulate at salinities below 30‰ and show a slight hyporegulation near 40‰ seawater (C). Eyestalkless larvae, however, hyporegulate at all salinities below 33‰ seawater and hyperosmoregulate against 40‰ seawater. Therefore, the sinus gland located in the eyestalk influences water uptake during molting.

Fig. 8.6. (*Continued*)
lings follow a hyperisosmotic regulation pattern. The established osmotic gradient required for the uptake of water results in the swelling of these embryos and occurs before the appearance of the coxal gills and possibly via the vitelline membrane and/or the dorsal organ. Stage 5, 6, and 7 embryos, however, exhibit a distinct hyper-/hypo-osmotic pattern of regulation. For all these developmental stages, the isosmotic point is similar (between 500 and 550 mOsmol/kg). It is also worth noting that for this species, the changes in osmoregulatory pattern are not associated with a profound metamorphosis. Adapted from Morritt and Spicer (1995), with permission from John Wiley and Sons.

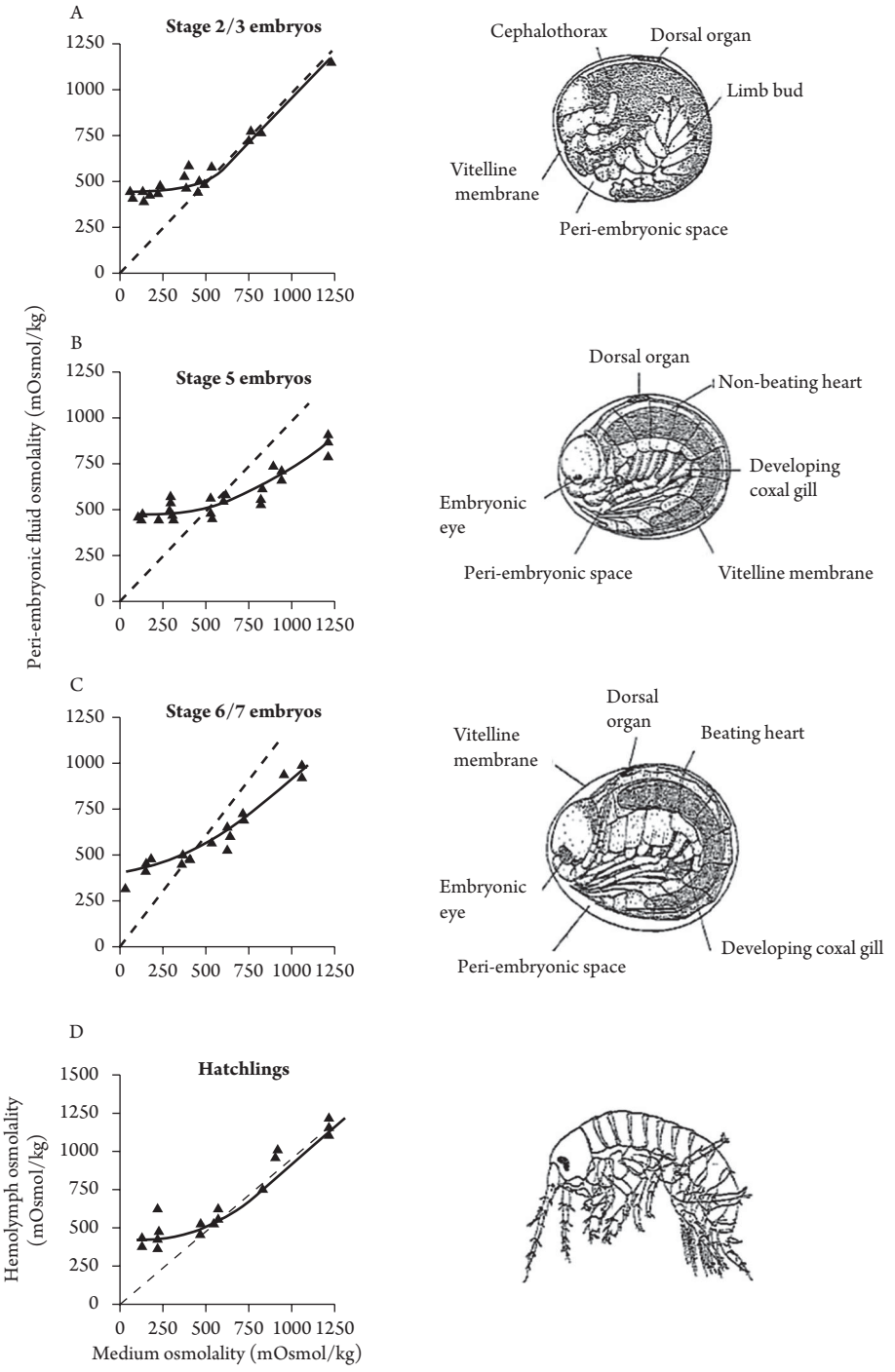


Fig. 8.6.
Changes in the pattern of osmoregulation in the embryonic development of the brackish water amphipod *Gammarus duebeni*. (A) Stage 2/3 embryos, reddening cephalothorax with prominent dorsal organ and limb bud development; (B) stage 5 embryos, appearance of eye pigmentation and nonbeating heart; (C) stage 7 embryos, heart beat present, initially irregular becoming regular; (D) hatchlings, immediately posteclosion. Periembryonic fluid (fluid from the space between the embryo and the vitelline membrane) or hemolymph osmotic pressure is plotted against the corresponding medium concentration. Stage 2 and 3 embryos and hatch

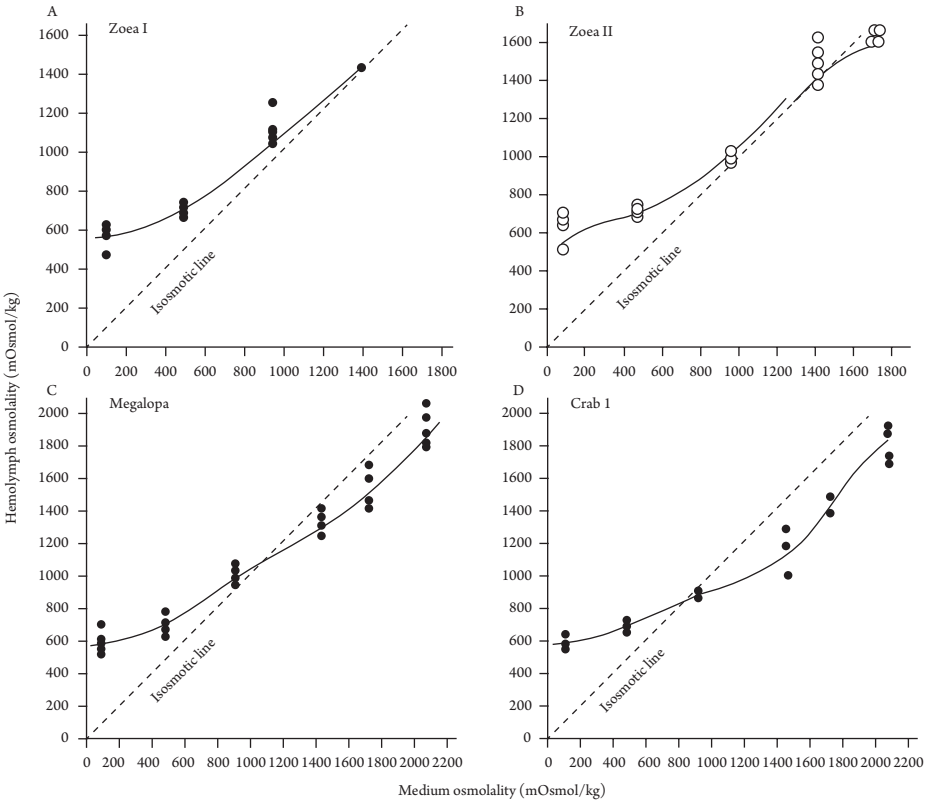


Fig. 8.7.
Osmoregulation curves for larvae and early postlarvae of *Uca subcylindrica*. This species living in semiarid habitats and facing regular and unpredictable salinity extremes present condensed larval development that is completed with only two brief zoeal stages followed by a postmetamorphic megalopa (2–3 days after hatching). The first crabs appear within 4.5–8 days after hatch. The ability to hyperosmoregulate is already present at hatching time (A), and tolerance to higher salinities increases with successive stages (B, C, and D) as the ability to hypo-osmoregulate improves (C, D). Adapted from Rabalais and Cameron (1985).

EXTERNAL CUES

Parasitism

Parasite-induced changes in physiology and behavior of the host appear adaptive for the parasite and can directly affect the osmoregulatory capacity (OC) of the host. For instance, hemolymph osmolality of hyperosmoregulating estuarine species, such as the blue crabs *Callinectes rathbunae* and *C. sapidus*, is lowered when parasitized respectively with the rhizocephalan barnacle *Loxothylacus texanus* (Alvarez et al. 2002) and microsporidian *Ameson michaelis* (Findley et al. 1981). In *C. sapidus*, hemolymph osmolality and sodium and chloride concentrations were reduced while hemolymph K^+ and amino acid concentrations increased. For *C. rathbunae*, which lives in tropical estuaries and is regularly subjected to sudden salinity changes, the OC of the parasitized crabs is significantly affected with only decreasing salinity, therefore limiting this crab to high-salinity areas. Protist parasites of the dinoflagellate-like genus *Hematodinium* also affect the OC of the osmoregulating *C. maenas*. No such alteration was detected in the osmoconforming *C. pagurus* and *Pagurus bernhardus*, although acid–base balance is affected (Hamilton et al. 2010). Supralittoral talitrid

sandhopper amphipods such as *Talorchestia quoyana* infected with the mermithid nematode parasite *Thaumamermis zealandica* burrow more deeply into sand layers than uninfected amphipods in order to reach the interstitial seawater level. This water-seeking behavior is induced by an increase in osmolality of body fluids and allows the parasite that is almost completely filling the body cavity of its host to emerge into moist sand, a necessary requisite for the free-living adult stage (Poinar et al. 2002, Williams et al. 2004). The presence of acanthocephalan parasites (e.g., *Polymorphus minutus*, in the cystacanth stage) inside the hemocoel of gammarid amphipods (*G. roeseli, G. pulex*) also changes hemolymph osmolality (Bentley and Hurd 1993), solute concentration (carotenoids, proteins, and glycogen; Plaistow et al. 2001), and salinity tolerance of the host (Piscart et al. 2007). Although the sodium pump activity is not affected in parasitized gammarids, reduced oxygen consumption, increased intra- and extracellular FAAs, and hemolymph hemocyanin concentration may explain the observed increased salinity tolerance (Patrick and Bradley 2000, Piscart et al. 2007). Devoting more resources to tolerate increased salinity may thus induce the death of the parasites and, subsequently, must decrease the parasitic-induced stress.

Season and Temperature

Temperature variations have been reported repeatedly to affect crustacean osmoregulation, with high and low temperatures generally decreasing the capacity to osmoregulate in temperate species. As observed for crab larvae and juvenile shrimp, the range of tolerance of salinity narrows when temperature deviates from the optimum and vice versa (Charmantier-Daures et al. 1988, Anger 2001). Temperature and salinity are therefore two extrinsic factors that can jointly evolve (Allan et al. 2006). Protein synthesis, amino acid concentration, and the position of the isosmotic point can also be modified according to the water temperature (Bückle et al. 2006, Intanai et al. 2009).

Also, in some species, the ionic composition of the hemolymph varies annually, and this may have direct physiological and behavioral consequences. For example, in the supralittoral amphipods, burrowing activity is seasonal and is related to the Mg^{2+} concentration in their hemolymph (Spicer et al. 1990). Mg^{2+} has a narcotizing effect on many marine invertebrates, and there is a general negative relationship between extracellular Mg^{2+} concentrations and activity in crustaceans (Morritt and Spicer 1993). In talitrid amphipods, Mg^{2+} is maintained at very low levels during the summer months, while in winter, when the amphipods have reduced activity and stay in deep burrows, the concentration of Mg^{2+} is significantly higher (Spicer et al. 1990).

Food Availability and Quality of the Diet

Diet quality also has a direct consequence on osmoregulation. For example, dietary *n*-3 highly unsaturated fatty acid (HUFA) has positive effects on the ability of penaeid shrimp to resist an osmotic shock (Rees et al. 1994, Palácios et al. 2004). It results in better tolerance to salinity stress in larval stages of *E. sinensis* and higher larval development (Sui et al. 2007). For that species, the ability to endure salinity stress (e.g., sudden transfer from 60% seawater to seawater and hypersaline seawater) for zoeal and megalopa stages is also improved when animals are fed diets with higher ratios of docosahexaenoic acid/eicosapentaenoic acid (DHA/EPA; Sui et al. 2007). However, in juvenile *L. vannamei*, although fatty acid composition in gill membranes is modified in response to their proportion in the diet, their osmotic pressure, FAA content, and gill Na^+/K^+-ATPase activity do not appear affected by HUFA supplementation (Hurtado et al. 2007). Marine crustaceans have a limited ability to elongate C18 *n*-3 poly-unsaturated fatty acids (*n*-3 PUFA) to *n*-3 HUFA (Kanazawa et al. 1977, 1979, Suprayudi et al. 2004), which therefore must be obtained from their food. In contrast to marine species, freshwater species have a lower requirement for *n*-3 HUFA and a higher capacity to elongate and desaturate these from shorter chain fatty acids. Therefore, the benefit of

HUFA complementation from the diet, although not completely identified, could be related to a modification of the fatty acid composition within cell membranes. Fatty acid composition of the cell membranes is modified by salinity in amphipods (Morris et al. 1982). It can change permeability (Haines et al. 1994) and can modulate the activity of Na^+/K^+-ATPase and other membrane-bound enzymes (Turner et al. 2003). The effects of a prebiotic used as a dietary supplement for growth and health management have also been tested on tolerance to low-salinity conditions in the Pacific white shrimp *L. vannamei* and proved to enhance survival (Li et al. 2009).

Environmental Stressors

A wide range of stressors affect crustacean osmotic and ion regulation (see Lignot et al. 2000, Charmantier et al. 2009), and, in most cases, exposure to stress results in a disruption of ionic regulation, mostly of Na^+ and Cl^- regulation, and therefore of osmotic regulation in osmoregulating crustaceans. The most studied stressors are pollutants such as metals (i.e., aluminium, cadmium, copper, lead, and zinc), oil, phenols, pesticides, and polychlorinated biphenyls (PCBs). Other stressing parameters, such as turbidity (Lin et al. 1992), low levels of dissolved ammonia and oxygen (Hagerman and Uglow 1982, 1983, Henry and Wheatly 1992), pathogenic agents (Souheil et al. 1999, Vinagre et al. 2002), acid stress (Felten et al. 2008), and the presence of antibiotic residues in the water (Tu et al. 2010) also disrupt ionic regulation.

Usually, the observed effects are size- and dose-dependent. The OC measured as hyper-OC in freshwater and diluted seawater or hypo-OC in seawater and concentrated seawater is markedly affected. Ion-transporting organs are also impacted (hemocytic congestion, necrotic blackening of the gills, intracellular vacuolization, nuclear pycnosis, enlarged mitochondria, and disruption of the basolateral infoldings of the ionocytes). Although most pollutants have a decreasing adverse effect on crustaceans as they develop, others, such as organophosphorus insecticides, act the opposite way by specifically inhibiting the activity of the acetylcholinesterase (AchE) that becomes fully functional in postmetamorphic stages and, then, by disrupting the osmotic capacity and the ionic hemolymph composition (Kobayashi et al. 1990, Lignot et al. 1997).

EVOLUTION FROM SEAWATER TO FRESHWATER AND TERRESTRIAL HABITATS

Invasions across environmental realms might have occurred directly (sea to freshwater and sea to land) or indirectly (sea to land via freshwater and sea to freshwater via land) and at different times since the Cambrian. Adaptive shifts in osmoregulatory strategies might have occurred, as is demonstrated, for example, by *Dilocarcinus pagei*, a neotropical hololimnetic crab (Augusto et al. 2007). Embryos, juveniles, and adults increase their total FAA content in muscles by 100% when acclimated from freshwater to 25 ppt saltwater. These authors therefore argue that anisosmotic extracellular regulation is replaced by isosmotic intracellular regulation in this species and that it can represent one of the main shifts that occurred in the first brachyurans that invaded freshwater.

Species that have invaded the freshwater environment in the recent past appear less efficient at osmoregulation than ancient freshwater species (Lee and Bell 1999), but this can vary according to species (Lee et al. 2012). For example, *Corophium curvispinum* requires more energy to regulate sodium in freshwater than do freshwater amphipods (Taylor and Harris 1986). Also, in order to be fully adapted to freshwater, crustacean species must have evolved different ontogenetic patterns because development in freshwater is associated with great physiological constraints (osmoregulation, but also tolerance to high pH, oxygen, and calcium concentrations; Powers and Bliss 1983). Therefore, for crustacean species that invaded freshwaters early in their history (i.e., crayfish,

palaemonids, and potamid crabs but also some Sesarminae species), exporting the pelagic larvae to marine waters, reducing the larval phase (with, e.g., two zoeal stages and one megalopa, as seen in freshwater *Sesarma* species), or laying only a few large eggs (i.e., reduced fecundity) from which fully developed, adult-like juveniles hatch, are the effective strategies (Anger 1995). Furthermore, brooding (as mostly seen in distantly related freshwater families such as gammarid and talitrid amphipods, crayfish, and potamid crabs) has been considered as another prerequisite for successful invasion of freshwater (Fig. 8.8), although it also occurs in some seawater species living at high latitudes or with extreme habitat specialization (Anger 1995, 2013, Vogt 2013). This parental care can limit predation in an environment with more limited feeding resources than in seawater. Reduction of the geographic range (endemism) also appears as a consequence of this shift to freshwater habitats. Some populations that have invaded freshwater appear to have lost their high salinity tolerance, a pattern found in more ancient freshwater inhabitants. This pattern suggests tradeoffs in osmoregulatory capability.

For terrestrial and semiterrestrial crustaceans, desiccation is overcome by different behavioral and physiological traits (Hoese 1981, Edney 1977). Among these species, the oniscoid isopods are the most successful terrestrial crustaceans today (Carefoot and Taylor 1995). The oniscoid isopods have developed key adaptations to minimize body evaporation in order to survive from the supralittoral zone up to arid deserts (Edney 1977). Some are endogenous and live in cool and humid areas, and those living in the most arid environments are nocturnal. Along the seashore, the semiterrestrial oniscoid *Ligia exotica* shows poor resistance to desiccation and cannot live without water (Tsai et al. 1997). They use closely apposed pereopods 6 and 7 (walking legs) to take water up from standing water (Horiguchi et al. 2007). The water moves upward by capillary action along grooves formed between the apposed legs. *L. exotica* also possess a water-conducting system that is coordinated with respiration and thermoregulation.

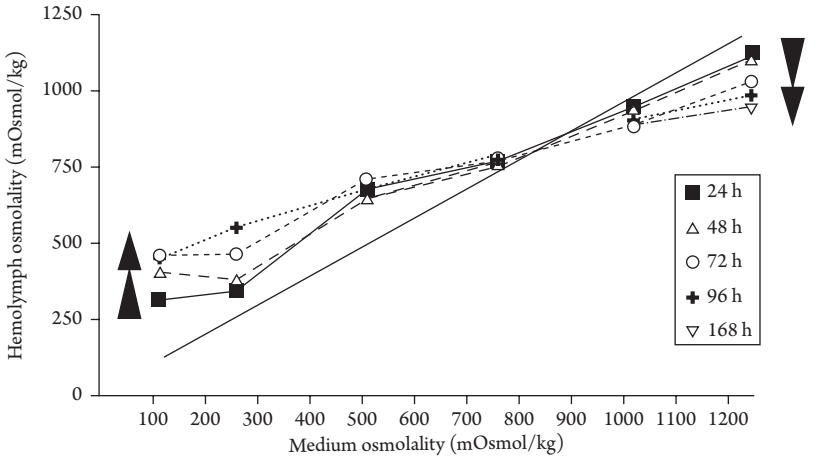


Fig. 8.8.
Relationship between the hemolymph osmolality for hatchling *O. gammarellus* at different ages post-hatching and medium osmolality. In Talitrid amphipods, hatchlings remain within a ventral groove that serves as a brood pouch or marsupium. These Talitrids are the only amphipod to have successfully invaded supralittoral habitats and colonized land, and the fluid resident in the ventral groove is isolated from the aquatic environment. In the experiment, the osmotic capacity (OC; osmotic difference between the hemolymph and external medium) is tested for hatchlings cultured in vitro at different salinities. Note the improvement of the OC (as indicated by the arrows) with time of development. These results support the hypothesis that hatchlings must attain a level of physiological competency in osmoregulation before leaving the marsupium. Adapted from Morritt and Spicer (1999), with permission from Elsevier.

NEW CHALLENGES: FROM MOLECULES TO MACROEVOLUTION AND BIOGEOGRAPHY, REDUCTIONIST AND HOLISTIC APPROACHES

The physiological, cellular, and molecular information obtained over the past 20 years or so on crustaceans' osmoregulating tissues and controlling sites allows the creation of hypothetical working models, such as those developed, for example, for NaCl uptake across the gills of hyperosmoregulating aquatic crustaceans. In these models, extracellular anisosmotic regulation appears controlled by specific morphological features (as already detailed for branchial and extrabranchial osmoregulating epithelia), but also by key apical, basolateral, and cytosolic proteins such as carbonic anhydrase, Na^+/K^+-ATPase, V-type H^+-ATPase, the $2Na^+/1H^+$ antiporter, the $Na^+/K^+/2Cl^-$ co-transporter, the Na/K exchanger, and the proton-gradient-dependent Cl^-/HCO_3^- antiport (Ahearn et al. 1987). Among these key proteins, a few are detailed here to highlight the most recent findings.

Cytosolic carbonic anhydrase is used to catalyze the formation of H^+ and HCO_3^-, and these counterions support Na^+ and Cl^- uptake by the gills (Na^+/H^+ and Cl^-/HCO_3^- exchange). Therefore, this pool of carbonic anhydrase provides support for the overall transport mechanisms and is specifically involved in the physiological and biochemical adaptation to low salinity, as seen by its transcriptional regulation and downstream translational and/or post-translational induction (Serrano and Henry 2008). This has been evaluated in the posterior gills of euryhaline crustaceans (*C. maenas, C. sapidus*) exposed to diluted seawater (Henry 1988, 2001, Serrano et al. 2007) and in the gills and epipodites of penaeid shrimps (*L. vannamei, Penaeus monodon*; Roy et al. 2007, Pongsomboon et al. 2009). Furthermore, carbonic anhydrase is under inhibitory regulation by a putative repressor present in the eyestalk (Henry and Campoverde 2006). Finally, branchial carbonic anhydrase activity is not modified by salinity transfer in *Cancer irroratus*, a stenohaline osmoconforming species (Henry and Campoverde 2006). Therefore, carbonic anhydrase may only function in hyperosmoregulating species acclimating to dilute media, such as crabs and the European lobster *H. gammarus* (Olsowski et al. 1995, Pavicic-Hamer et al. 2003).

Na^+/K^+-ATPase is the enzyme responsible for the exclusion of three sodium ions from the cell in exchange for two K^+ ions at the expense of the hydrolysis of one adenosine triphosphate (ATP) molecule. Its activity maintains cell-membrane potential, mediates transmembrane transport, and thus maintains osmotic equilibrium and cell volume (Lucu and Towle 2003). In crustaceans, it has been detected in the different organs containing ionocytes, such as the gills (e.g., in brachyuran crabs, caridean shrimp, crayfish), the epipodites (the European lobster, penaeid shrimps), the inner side of the branchiostegite (the European lobster), the *Artemia* salt gland, and the pleopod endopodites in isopods, as well as in the calcium-transporting sternal epithelium of the terrestrial isopod *Porcellio scaber*. For example, in brine shrimp living in hypersaline waters, metepipodites of the phyllopodia have very high activities of Na^+/K^+-ATPase, and chloride ions must be actively pumped out of the body against large concentration and charge gradients. Sodium ions also leave the shrimp against a large concentration gradient but reduce the net electrochemical (concentration and charge) gradient across the shrimp's body wall (Holliday et al. 1990). Also, in hyperosmoregulating species, when subjected to a hyposomotic stress (low salinity), the activity of Na^+/K^+-ATPase located along the basolateral membrane of the crustacean ionocytes is increased, but this response is less pronounced in osmoconformers and weak regulators (Spencer et al. 1979, Lucu et al. 2000). Therefore, Na^+/K^+-ATPase is largely involved in both hyperosmoregulation in dilute seawater and hypo-osmoregulation in concentrated seawater. It creates polarized cells with an intracellular Na+ gradient that drives secondary active transport.

The molecular characteristics of Na^+/K^+-ATPase have been well detailed in crustaceans, including the brine shrimp *Artemia franciscana*, the American lobster *H. americanus*, and the crabs *C. sapidus, Scylla paramamosain*, and *Pachygrapsus marmoratus* (Chung and Lin 2006, Charmantier et al. 2009).

The vacuolar type H^+-ATPase (V-type H^+-ATPase) is responsible for acid–base balance and nitrogen excretion (Weihrauch et al. 2001), and it plays a crucial role in the adaptation to freshwater, as seen in euryhaline crabs (Tsai and Lin 2007). It is also considered, with Na^+/K^+-ATPase, as a key enzyme during the transition from a marine environment to freshwater (Lee et al. 2011) and to land (Morris 2001, Weihrauch et al. 2004). It can be cytosolic or located along the apical or basolateral side of the ionocytes (Weihrauch et al. 2001, Ziegler et al. 2004, Tsai and Lin 2007). Also, in the calcium-transporting sternal epithelial cells of the terrestrial isopod *P. scaber*, a basolateral to apical shift occurs during the transition from calcium deposition to calcium resorption of intermittent $CaCO_3$ deposits (Ziegler et al. 2004).

The amiloride-sensitive $2Na^+/1H^+$ antiporter (or exchanger) is associated with pH regulation and appears present in crustaceans not only in the gills (Strauss and Graszynski 1992, Towle et al. 1997, Kimura et al. 1994), but also in the hepatopancreas and antennal glands (Ahearn et al. 1990). A role in osmoregulation has been demonstrated in *Daphnia magna* (Bianchini and Wood 2008), crayfish (Kirschner 2002), and crabs (Cameron 1979, Martinez et al. 1998, Onken and McNamara 2002, Siebers et al. 1987, Zeiske et al. 1992).

Despite the working models that are available in the most recent literature, a clear functional view of all the mechanisms at play is still incomplete, especially concerning the mechanisms of ion excretion in species that hypo-osmoregulate. Also, some transporters have been identified at the molecular level but still need to be precisely located. Large-scale gene expression studies using microarrays can also discriminate between transcriptional and post-transcriptional responses. As an example, one can take advantage of the recent study focusing on gene expression of posterior gills of *C. maenas* maintained in seawater and diluted seawater and analyzed by microarray (Towle et al. 2011). Expression ratios of selected transcripts of transporters and other membrane proteins revealed upregulation in diluted seawater-acclimated crabs of Na^+/K^+-ATPase and cytoplasmic carbonic anhydrase, both previously shown by quantitative polymerase chain reaction to respond positively to salinity reduction in *C. maenas* and other euryhaline crabs (Henry et al. 2002, Luquet et al. 2005, Serrano and Henry 2008). Other upregulated transcripts concerned an organic cation transporter, the sodium/glucose cotransporter, an endomembrane protein, a voltage-gated calcium channel, an anion exchanger, and an octopamine receptor that doubled in less than 2 h after transfer from seawater to low salinity. Many other transcripts encoding other transport-related proteins implicated in osmoregulatory ion transport in crustacean gill remained unaffected by the salinity challenge. These include the Na^+/H^+ exchanger, $Na^+/K^+/2Cl^-$ cotransporter, the V-type H^+-ATPase, the K^+/Cl^- symporter, the Rhesus-like ammonium transporter, the CFTR-like ABC transporter, and aquaporin. Therefore, for these proteins, gene regulation is not part of the adaptive process (Towle et al. 2011). The study also indicates no transcriptional response among the selected stress-related proteins but a significant upregulation of the transcript level for mitochondrial proteins. Although only a small number of the microarray positive hits have been analyzed, they clearly indicate that the integrated molecular and physiological response of the crab in front of an acute salinity transfer (from 100% to 50% and 32% seawater) does not represent a stressful challenge.

Finally, functional models should also integrate ontogeny and the molting cycle, although the molecular events occurring before, during, and after ecdysis have been tackled recently (Shechter et al. 2007, Shi et al. 2009, Seear et al. 2010).

Wide adaptive radiation occurred during crustacean evolutionary history that enabled them to inhabit different marine, brackish, freshwater, and terrestrial habitats. Some of these ecosystems correspond to transitory habitats (estuaries, sandy or rocky shores) with low species diversity due to large fluctuations of salinity and temperature. Therefore, being able to regulate internal solute concentration at a level different from that of the external environment using a suite of molecular, morphological, and physiological traits allowed some crustaceans to compensate for unpredictable fluctuations of the external environment. Evolutionary trends leading to increased independence

from fluctuations in the external environment include decreased permeability of the body surface to water and solutes, restrictions in the rate of water influx and ion loss, and enhanced ion uptake through modulation of transporter function.

The capacity to osmoregulate is also a key function for invasive species. The role of salinity tolerance of amphipod invaders was recently highlighted by Devin and Beisel (2007) who studied the biological and ecological traits of gammarid amphipods from Western Europe and North America that may explain their successful invasion. Their study has revealed a particular ecological profile for invaders, with a strong influence of salinity tolerance. This is also illustrated by the European green crab *C. maenas*, a strong hyper-/hyporegulator and one of the world's most successful aquatic invaders (Darling et al. 2008).

FUTURE DIRECTIONS

Since their early developments, investigations on crustacean ion and water regulation have been conducted in species differing in their ecology, thus establishing osmoregulation as one of the main adaptations to various habitats throughout development. However, future research is still necessary and concerns different levels of integration.

First, relatively few crustaceans are able to both hyper- and hypo-osmoregulate, and most studies on crustacean osmoregulation have been conducted under conditions of salinity decrease. This stems from the usually high isosmotic salinity that is close to seawater; that is, 1,000 mOsm kg^{-1} in marine crustaceans. As a result, most of what is known about the mechanisms of crustacean osmoregulation concerns hyperosmoregulation. It is thus necessary to focus on the mechanisms of hypo-osmoregulation in those species that are able to do so when salinity increases above the values of seawater. Also, as in marine fish, the gut is probably involved in water uptake, as are the gills for ion excretion. Therefore, the corresponding cells and molecular mechanisms should be studied along with Cl^- transport at the basal and apical sides of the cells.

As part of developmental biology in crustaceans, the origin of the osmoregulatory function should be addressed. The occurrence of ionocytes and their molecular functionality should be followed at different sites during embryonic and early postembryonic development in relation to the role of osmoregulation in ecophysiological strategies.

Finally, global climate change and environmental stressors should promote further investigations on their multilevel effects on osmoregulation.

CONCLUSIONS

This broad overview concerning mostly osmoregulating crustaceans clearly indicates that these species can maintain internal solute concentration at a different level from that of the external environment mostly because of a reduced permeability of their body surface to water and solutes, limited water influx and ion loss, and an enhanced ion uptake through modulation of the transporter function. Furthermore, ion and water regulation in these species is influenced by many internal and external factors (e.g., the effects of gender, molting, nutrition, ontogeny, parasitism, season, temperature, dissolved oxygen, pollutants, etc.). The way these factors influence the OC was discussed, as was the efficiency of the different osmoregulatory strategies; the functional organization at tissue, cellular, and molecular levels of the key osmoeffectors; and the hormonal system that controls the osmoregulatory function, each according to different ecological constraints. It appears therefore that these mechanisms are highly adaptive and at play in crustaceans living in environments with fluctuating salinities, for invasive crustaceans, and in populations colonizing new habitats.

ACKNOWLEDGMENTS

This chapter is dedicated to David W. Towle, who inspired several generations of carcinologists.

REFERENCES

Achituv, Y., and M. Ziskind. 1985. Adaptation of *Coenobita scaevola* (Crustacea: Anomura) to terrestrial life in desert-bordered shore line. Marine Ecology Progress Series 25:189–198.

Ahearn, G.A. 1996. The invertebrate $2Na^{+}$–$1H^{+}$ exchanger: polyfunctional epithelial workstation. News in Physiological Sciences 11:31–35.

Ahearn, G.A., M.L. Grover, and R.E. Dunn. 1985. Glucose transport by lobster hepatopancreatic brush-border membrane vesicles. American Journal of Physiology 248:R133–141.

Ahearn, G.A., M.L. Grover, R.T. Tsuji, and L.P. Clay. 1987. Proton-stimulated Cl-HCO_3 antiport by basolateral membrane vesicles of lobster hepatopancreas. American Journal of Physiology 252:R859–870.

Ahearn, G.A., P. Franco, and L.P. Clay. 1990. Electrogenic 2 Na^{+}/1 H^{+} exchange in crustaceans. Journal of Membrane Biology 116:215–226.

Ahearn, G.A., J.M. Duerr, Z. Zhuang, R.J. Brown, A. Aslamkhan, and D.A. Killebrew. 1999. Ion transport processes of crustacean epithelial cells. Physiological and Biochemical Zoology 72:1–18.

Aladin, N.V., and W.T.W. Potts. 1995. Osmoregulatory capacity of the Cladocera. Journal of Comparative Physiology 164B:671–683.

Allan, E.L., P.W. Froneman, and A.N. Hodgson. 2006. Effects of temperature and salinity on the standard metabolic rate (SMR) of the caridean shrimp *Palaemon peringueyi*. Journal of Experimental Marine Biology and Ecology 337:103–108.

Alonso, A., H.J. De Lange, and E.T.H.M. Peeters. 2009. Development of a feeding behavioural bioassay using the freshwater amphipod *Gammarus pulex* and the multispecies freshwater biomonitor. Chemosphere 75:341–346.

Alvarez, F., G. Alcaraz, and R. Robles. 2002. Osmoregulatory disturbances induced by the parasitic barnacle *Loxothylacus texanus* (Rhizocephala) in the crab *Callinectes rathbunae* (Portunidae). Journal of Experimental Marine Biology and Ecology 278:135–140.

Anger, K. 1995. The conquest of freshwater and land by marine crabs: adaptations in life-history patterns and larval bioenergetics. Journal of Experimental Marine Biology and Ecology 193:119–145.

Anger, K. 2001. The biology of decapod crustacean larvae. Page 420 *in* R. Vonk, editor. Crustacean issues 14. Balkema A.A..

Anger, K. 2013. Neotropical *Macrobrachium* (Caridea: Palaemonidae): on the biology, origin, and radiation of freshwater-invading shrimp. Journal of Crustacean Biology 33:151–183.

Augusto, A., L.J. Greene, H.J. Laure, and J.C. McNamara. 2007. Adaptive shifts in osmoregulatory strategy and the invasion of freshwater by brachyuran crabs: evidence from *Dilocarcinus pagei* (Trichodactylidae). Journal of Experimental Zoology 307A:688–698.

Behnke, R.D., R.K. Wong, S.M. Huse, S.J. Reshkin, and G.A. Ahearn. 1990. Proline transport by brush-border membrane vesicles of lobster antennal glands. American Journal of Physiology 258:F311–F320.

Bennett, D., and C. Brown. 1983. Crab (*Cancer pagurus*) migrations in the English Channel. Journal of the Marine Biological Association of the United Kingdom 63:371–398.

Bentley, C.R., and H. Hurd. 1993. *Pomphorhynchus laevis* (Acanthocephala): elevation of haemolymph protein concentrations in the intermediate host, *Gammarus pulex* (Crustacea: Amphipoda). Parasitology 107:193–198.

Bianchini, A., and C.M. Wood. 2008. Sodium uptake in different life stages of crustaceans: the water flea *Daphnia magna* Strauss. Journal of Experimental Biology 211:539–547.

Bouaricha, N., M. Charmantier-Daures, P. Thuet, J.-P. Trilles, and G. Charmantier. 1994. Ontogeny of osmoregulatory structures in the shrimp *Penaeus japonicus* Crustacea, Decapoda. Biological Bulletin 186:29–40.

Brunet, M., J. Arnaud, and J. Mazza. 1994. Gut structure and digestive cellular processes in marine Crustacea. Oceanography and Marine Biology Annual Review 32:335–367.

Bückle, L.F.R., S.B. Barón, and R.M. Hernández. 2006. Osmoregulatory capacity of the shrimp *Litopenaeus vannamei* at different temperatures and salinities, and optimal culture environment. Revista de Biología Tropical 54:745–753.

Buikema, A.L. Jr., and F. Benfield. 1979. Use of macroinvertebrate life history information in toxicity tests. Journal of the Fisheries Research Board of Canada 36:321–328.

Burton, R.F. 1995. Cation balance in crustacean haemolymph: relationship to cell membrane potentials and membrane surface charge. Comparative Biochemistry and Physiology 111A:125–131.

Burton, R.S. 1992. Proline synthesis during osmotic stress in megalopa stage larvae of the blue crab, *Callinectes sapidus*. Biological Bulletin 182:409–415.

Cameron, J.N. 1979. Effects of inhibitors on ion fluxes, trans-gill potential and pH regulation in freshwater blue crabs, *Callinectes sapidus* (Rathbun). Journal of Comparative Physiology 133:219–225.

Carefoot, T.H. 1990. Specific dynamic action (SDA) in the supralittoral isopod, *Ligia pallasii*—identification of components of apparent SDA and effects of dietary amino acid quality and content on SDA. Comparative Biochemistry and Physiology 95A:309–316.

Carefoot, T.H., and B.E. Taylor. 1995. Ligia: a prototypal terrestrial isopod. Pages 47–60 *in* M.A. Alikhan, editor. Terrestrial isopod biology. Balkema A.A., Rotterdam.

Castille, F.L. Jr., and A.L. Lawrence. 1981. The effect of salinity on the osmotic, sodium and chloride concentrations in the hemolymph of euryhaline shrimp of the genus *Penaeus*. Comparative Biochemistry and Physiology 68A:75–80.

Charmantier, G. 1998. Ontogeny of osmoregulation in crustaceans: a review. Invertebrate Reproduction and Development 33:177–190.

Charmantier, G., and K. Anger. 2011. Ontogeny of osmoregulatory patterns in the South American shrimp *Macrobrachium amazonicum*: loss of hypo-regulation in a land-locked population indicates phylogenetic separation from estuarine ancestor. Journal of Experimental Marine Biology and Ecology 396:89–98.

Charmantier, G., P. Thuet, and M. Charmantier-Daures. 1984. La régulation osmotique et ionique chez le Homard européen *Homarus gammarus* (L.) (Crustacea, Decapoda). Journal of Experimental Marine Biology and Ecology 76:191–199.

Charmantier, G., M. Charmantier-Daures, and K. Anger. 1998. Ontogeny of osmoregulation in the grapsid crab *Armases miersii* (Crustacea, Decapoda). Marine Ecology Progress Series 164:285–292.

Charmantier, G., C. Haond, J. Lignot, and M. Charmantier-Daures. 2001. Ecophysiological adaptation to salinity throughout a life cycle: a review in homarid lobsters. Journal of Experimental Biology 204:967–977.

Charmantier, G., L. Giménez, M. Charmantier-Daures, and K. Anger. 2002. Ontogeny of osmoregulation, physiological plasticity and larval export strategy in the grapsid crab *Chasmagnathus granulata* (Crustacea, Decapoda). Marine Ecology Progress Series 229:185–194.

Charmantier, G., M. Charmantier-Daures, and D.W. Towle. 2009. Osmotic and ionic regulation in aquatic arthropods. Pages 165–202 *in* D.H. Evans, editor. Osmotic and ionic regulation. Cells and animals. CRC Press, Taylor and Francis Group, Boca Raton.

Charmantier-Daures, M., P. Thuet, G. Charmantier, and J.-P. Trilles. 1988. Tolérance à la salinité et osmorégulation chez les post-larves de *Penaeus japonicus* et *P. chinensis*. Effet de la température. Aquatic Living Resources 1:267–276.

Charmantier-Daures, M., G. Charmantier, K.P.C. Janssen, D.E. Aiken, and F. Van Herp. 1994. Involvement of eyestalk factors in the neuroendocrine control of hydromineral metabolism in adult American lobster *Homarus americanus*. General and Comparative Endocrinology 94:281–293.

Chittó, A.L.F., V. Schein, R. Etges, L.C. Kucharski, and R.S.M. Da Silva. 2009. Effects of photoperiod on gluconeogenic activity and total lipid concentration in organs of crabs, *Neohelice granulata*, challenged by salinity changes. Invertebrate Biology 128:261–268.

Chung, J.S., and S.G. Webster. 2005. Dynamics of in vivo release of molt inhibiting hormone and crustacean hyperglycemic hormone in the shore crab, *Carcinus maenas*. Endocrinology 146:5545–5551.

Chung, J.S., H. Dircksen, and S.G. Webster. 1999. A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. Proceedings of the National Academy of Sciences USA 96:13103–13107.

Chung, J.S., N. Zmora, H. Katayama, and N. Tsutsui. 2010. Crustacean hyperglycemic hormone (CHH) neuropeptides family: functions, titer, and binding to target tissues. General and Comparative Endocrinology 166:447–454.

Chung, K.F., and H.C. Lin. 2006. Osmoregulation and Na,K-ATPase expression in osmoregulatory organs of *Scylla paramamosain*. Comparative Biochemistry and Physiology 144A:48–57.

Cieluch, U., K. Anger, F. Aujoulat, F. Buchholz, M. Charmantier-Daures, and G. Charmantier. 2004. Ontogeny of osmoregulatory structures and functions in the green crab *Carcinus maenas* (Crustacea, Decapoda). Journal of Experimental Biology 207:325–336.

Cieluch, U., G. Charmantier, E. Grousset, M. Charmantier-Daures, and K. Anger. 2005. Osmoregulation, immunolocalization of Na^+/K^+-ATPase, and ultrastructure of branchial epithelia in the developing brown shrimp, *Crangon crangon* (Decapoda, Caridea). Physiological and Biochemical Zoology 78:1017–1025.

Coelho de Faria, S., A. Silva Augusto, and J.C. McNamara. 2011. Intra- and extracellular osmotic regulation in the hololimnetic Caridea and Anomura: a phylogenetic perspective on the conquest of fresh water by the decapod Crustacea. Journal of Comparative Physiology 18:175–186.

Croghan, P.C. 1958a. The osmotic and ionic regulation of *Artemia salina* (L.). Journal of Experimental Biology 35:219–233.

Croghan, P.C. 1958b. The mechanism of osmotic regulation in *Artemia salina* (L.): the physiology of the branchiae. Journal of Experimental Biology 35:234–242.

Curtis, D.L., and I.J. McGaw. 2010. Respiratory and digestive responses of postprandial Dungeness crabs, *Cancer magister*, and blue crabs, *Callinectes sapidus*, during hyposaline exposure. Journal of Comparative Physiology B 180:189–198.

Dall, W. 1967a. Hypo-osmoregulation in Crustacea. Comparative Biochemistry and Physiology 21:653–678.

Dall, W. 1967b. The functional anatomy of the digestive tract of a shrimp *Metapenaeus bennettae* Racek & Dall (Crustacea: Decapoda: Peneidae). Australian Journal of Zoology 15:699–714.

Darling, J.A., M.J. Bagley, J. Roman, C.K. Tepolt, and J.B. Geller. 2008. Genetic patterns across multiple introductions of the globally invasive crab genus *Carcinus*. Molecular Ecology 17:4992–5007.

Davenport, J., and J. Wankowski. 1973. Pre-immersion salinity-choice behaviour in *Porcellana platycheles*. Marine Biology 22:313–316.

De Grave, S., N.D. Pentcheff, S.T. Ahyong, T.Y. Chan, K.A. Crandall, P.C. Dworschak, D.L. Felder, R.M. Feldmann, C.H.J.M. Fransen, L.Y.D. Goulding, R. Lemaitre, M.E.Y. Low, J.W. Martin, P.K.L. Ng, C.E. Schweitzer, S.H. Tan, D. Tshudy, and R. Wetzer. 2009. A classification of living and fossil genera of decapod crustaceans. Raffles Bulletin of Zoology 21:1–109.

De Wilde, P.A. 1973. On the ecology of Coenobita clypeatus in Curaçao. Pages 1–138 *in* P. Wagenaar Hummelinck and L. Van der Steen, editors. Studies on the fauna of Curaçao and other Caribbean Islands. Martinus Nijhoff, The Hague.

Devin, S., and J.-N. Beisel. 2007. Biological and ecological characteristics of invasive species: a gammarid study. Biological Invasions 9:13–24.

Drach, P. 1939. Mue et cycle d'intermue chez les Crustacés Décapodes. Annales de l'Institut Océanographique 19:103–391.

Dufort, C.G., S.H. Jury, J.M. Newcomb, D.F. O'Grady, and W.H. Watson. 2001. Detection of salinity by the lobster, *Homarus americanus*. Biological Bulletin 201:424–434.

Edney, E.B. 1977. Water balance in land arthropods. Springer-Verlag, Berlin.

Felder, D.L. 1978. Osmotic and ionic regulation in several western Atlantic Callianassidae (Crustacea, Decapoda, Thalassinidea). Biological Bulletin 54:409–429.

Felten, V., G. Charmantier, R. Mons, A. Geffard, P. Rousselle, M. Coquery, J. Garric, and O. Geffard. 2008. Physiological and behavioural responses of *Gammarus pulex* (Crustacea: Amphipoda) exposed to cadmium. Aquatic Toxicology 86:413–425.

Ferraris, R.P., F.D. Parado-Estepa, J.M. Ladj, and E.G. de Jesus. 1986. Effect of salinity on the osmotic, chloride, total protein and calcium concentrations in the hemolymph of the prawn *Penaeus monodon* (fabricius). Comparative Biochemistry and Physiology 83A:701–708.

Findley, A.M. Jr., E.W. Blakeney, and E.H. Weidner. 1981. *Ameson michaelis* (Microsporidia) in the blue crab *Callinectes sapidus*: parasite-induced alterations in the biochemical composition of host tissue. Biological Bulletin 161:115–125.

Forward, R.B. Jr. 1989. Behavioral responses of crustacean larvae to rates of salinity change. Biological Bulletin 176:229–238.

Forward, R.B. Jr. 2009. Larval biology of the crab *Rhithropanopeus harrisii* (Gould): a synthesis. Biological Bulletin 216:243–256.

Fox, H.N. 1952. Anal and oral intake of water by Crustacea. Journal of Experimental Biology 29:583–599.

Freire, C.A., and J.C. McNamara. 1992. Involvement of the central nervous system in neuroendocrine mediation of osmotic and ionic regulation in the freshwater shrimp *Macrobrachium olfersii* Crustacea, Decapoda. General and Comparative Endocrinology 88:316–327.

Freire, C.A., F. Cavassin, E.N. Rodrigues, A.H. Torres, and J.C. McNamara. 2003. Adaptive patterns of osmotic and ionic regulation, and the invasion of fresh water by the palaemonid shrimps. Comparative Biochemistry and Physiology 136A:771–778.

Freire, C.A., H. Onken, and J.C. McNamara. 2008. A structure-function analysis of ion transport in crustacean gills and excretory organs. Comparative Biochemistry and Physiology 51A:272–304.

Gao, Y., and M.G. Wheatly. 2004. Characterization and expression of plasma membrane Ca2+ ATPase (PMCA3) in the crayfish *Procambarus clarkii* antennal gland during molting. Journal of Experimental Biology 207:2991–3002.

Gilles, R., and E. Delpire. 1997. Variation in salinity, osmolarity and water availability: vertebrates and invertebrates. Pages 1523–1586 *in* W.H. Dantzler, editor. Handbook of comparative physiology. Oxford University Press, New York.

Gleeson, R.A., L.M. McDowell, and H.C. Aldrich. 1996. Structure of the aesthetasc (olfactory) sensilla of the blue crab, *Callinectes sapidus*: transformations as a function of salinity. Cell and Tissue Research 284:279–288.

Gleeson, R.A., M.G. Wheatly, and C. Reiber. 1997. Perireceptor mechanisms sustaining olfaction at low salinities: insight from the euryhaline blue crab *Callinectes sapidus*. Journal of Experimental Biology 200:445–456.

Gohad, N.V., G.H. Dickinson, B. Orihuela, D. Rittschof, and A.S. Mount. 2009. Visualization of putative ion-transporting epithelia in *Amphibalanus amphitrite* using correlative microscopy: potential function in osmoregulation and biomineralization. Journal of Experimental Marine Biology and Ecology 380:88–98.

Gong, Y.N., W.W. Li, J.L. Sun, F. Ren, L. He, H. Jiang, and Q. Wang. 2010. Molecular cloning and tissue expression of the fatty acid-binding protein (Es-FABP) gene in female Chinese mitten crab (*Eriocheir sinensis*). BMC Molecular Biology 11:71.

Greco, T.M., M.B. Alden, and C.W. Holliday. 1986. Control of hemolymph volume by adjustments in urinary and drinking rates in the crab, *Cancer borealis*. Comparative Biochemistry and Physiology 84A:695–701.

Greenaway, P., H.H. Taylor, and S. Morris. 1990. Adaptation to a terrestrial existence by the robber crab *Birgus latro*. VI. The role of the excretory system in fluid balance, Journal of Experimental Biology 64:767–786.

Grindley, J.R. 1964. Effects of low-salinity water on the vertical migration of estuarine plankton. Nature (Lond.) 203:781–782.

Hagerman, L., and R.F. Uglow. 1982. Effect of hypoxia on osmotic and ionic regulation in the brown shrimp *Crangon crangon* from brackish water. Journal of Experimental Marine Biology and Ecology 63:93–104.

Hagerman, L., and R.F. Uglow. 1983. Ventilatory behavior and chloride regulation in relation to oxygen tension in the shrimp *Palaemon adspersus* Rathke maintained in hypotonic medium. Ophelia 20:193–200.

Haines, C., M. Edmunds, and A.R. Pewsey. 1994. The pea crab, *Pinnotheres pisum* (Linnaeus, 1767) and its association with the common mussel, *Mytilus edulis* (Linnaeus, 1758), in the Solent (UK). Journal of Shellfish Research 13:5–10.

Hamilton, K.M., P.W. Shaw, and D. Morritt. 2010. Physiological responses of three crustacean species to infection by the dinoflagellate-like protist *Hematodinium* (Alveolata: Syndinea). Journal of Invertebrate Pathology 105:194–196.

Haond, C., L. Bonnal, R. Sandeaux, G. Charmantier, and J.P. Trilles. 1999. Ontogeny of intracellular isosmotic regulation in the European lobster *Homarus gammarus* (L.). Physiological and Biochemical Zoology 72:534–544.

Harges, P.L., and R.B. Forward, Jr. 1982. Salinity perception by larvae of the crab *Rhithropanopeus harrisii* (Gould). Marine Behavior & Physiology 8:311–331.

Hecht, S. 1914. Note on the absorption of calcium during the molting of the blue crab, *Callinectes sapidus*. Science 39:108.

Henry, R.P. 1988. Subcellular distribution of carbonic anhydrase activity in the gills of the blue crab, *Callinectes sapidus*. Journal of Experimental Zoology 245:1–8.

Henry, R.P. 2001. Environmentally mediated carbonic anhydrase induction in the gills of euryhaline crustaceans. Journal of Experimental Biology 204:991–1002.

Henry, R.P., and M. Campoverde. 2006. Neuroendocrine regulation of carbonic anhydrase expression in the gills of the euryhaline green crab, *Carcinus maenas*. Journal of Experimental Zoology 305A:663–668.

Henry, R.P., and M.G. Wheatly. 1992. Interaction of respiration, ion regulation and acid-base balance in the everyday life of aquatic crustaceans. American Zoology 32:407–416.

Henry, R.P., E.E. Garrelts, M.M. McCarty, and D.W. Towle. 2002. Differential time course of induction for branchial carbonic anhydrase and Na/K ATPase activity in the euryhaline crab, *Carcinus maenas*, during low salinity acclimation. Journal of Experimental Zoology 292:595–603.

Herreid, C.F. 1969. Integument permeability of crabs and adaptation to land. Comparative Biochemistry and Physiology 29:423–429.

Hoese, B. 1981. Morphologie und Funktion des Wasserleitungssystems der terrestrischen Isopoden (Crustacea, Isopoda, Oniscoidea). Zoomorphology 98:135–167.

Holliday, C.W., D.B. Roye, and R.D. Roer. 1990. Salinity-induced changes in branchial NA+/K+-ATPase activity and transepithelial difference in the brine shrimp *Artemia salina*. Journal of Experimental Biology 151:279–296.

Horiguchi, H., M. Hironaka, V.B. Meyer-Rochow, and T. Hariyama. 2007. Water uptake via two pairs of specialized legs in *Ligia exotica* (Crustacea, Isopoda). Biological Bulletin 213:196–203.

Hosfeld, B., and H.K. Schminke. 1997. The ultrastructure of ionocytes from osmoregulatory integumental windows of *Parastenocaris vicesima* (Crustacea, Copepoda, Harpacticoida). Archiv für Hydrobiologie 139:389–400.

Houlihan, D.F., C.P. Waring, E. Mathers, and C. Gray. 1990. Protein synthesis and oxygen consumption of the shore crab *Carcinus maenas* after a meal. Physiological Zoology 63:735–756.

Hughes, D.A., and J.D. Richard. 1973. Some current-directed movements of *Macrobrachium acanthurus* (Wiegman, 1836) (Decapoda, Palaemonidae) under laboratory conditions. Ecology 54:927–929.

Hurtado, M.A., I.S. Racotta, R. Civera, L. Ibarra, M. Hernández-Rodríguez, and E. Palacios. 2007. Effect of hypo- and hypersaline conditions on osmolality and Na^+/K^+-ATPase activity in juvenile shrimp (*Litopenaeus vannamei*) fed low- and high-HUFA diets. Comparative Biochemistry and Physiology 147A:703–710.

Icely, J.D., and J.A. Nott. 1985. Feeding and digestion in *Corophium volutator* (Crustacea, Amphipoda). Marine Biology 89:183–195.

Intanai, I., E.W. Taylor, and N.M. Whiteley. 2009. Effects of salinity on rates of protein synthesis and oxygen uptake in the post-larvae and juveniles of the tropical prawn *Macrobrachium rosenbergii* (de Man). Comparative Biochemistry and Physiology 152A:372–378.

Jiang, H., F. Ren, J. Sun, L. He, W. Li, Y. Xie, and Q. Wang. 2010. Molecular cloning and gene expression analysis of the leptin receptor in the Chinese mitten crab *Eriocheir sinensis*. PLoS One 5:e11175.

Kalber, F.A., and J.D.J. Costlow. 1966. The ontogeny of osmoregulation and its neurosecretory control in the Decapod crustacean, *Rhithropanopeus harisii* (Gould). American Zoologist 6:221–229.

Kalber, F.A., and J.D.J. Costlow. 1968. Osmoregulation in larvae of the land crab, *Cardisoma guanhumi* Latreille. American Zoologist 8:411–416.

Kamemoto, F.I. 1991. Neuroendocrinology of osmoregulation in crabs. Zoological Science 8:827–833.

Kanazawa, A., S. Teshima, and S. Tokiwa. 1977. Nutritional requirements of prawn-VII. Effect of dietary lipids on growth. Bulletin of the Japanese Society of Fisheries 43:849–856.

Kanazawa, A.S., S. Teshima, H.J. Tokiwa, and Ceccaldi. 1979. Effects of dietary linoleic and linolenic acids on growth of prawn. Oceanologica Acta 2:41–47.

Kefford, B.J., D. Nugegoda, L. Zalizniak, E.J. Fields, and K.L. Hassell. 2007. The salinity tolerance of freshwater macroinvertebrate eggs and hatchlings in comparison to their older life-stages: a diversity of responses. Aquatic Ecology 41:335–348.

Khodabandeh, S., G. Charmantier, and M. Charmantier-Daures. 2005a. Ultrastructural studies and Na^+, K+-ATPase immunolocalization in the antennal urinary glands of the lobster *Homarus gammarus* (Crustacea, Decapoda). Journal of Histochemistry and Cytochemistry 53:1203–1214.

Khodabandeh, S., M. Kutnik, F. Aujoulat, G. Charmantier, and M. Charmantier-Daures. 2005b. Ontogeny of the antennal glands in the crayfish Astacus leptodactylus (Crustacea, Decapoda). Anatomical and cell differentiation. Cell and Tissue Research 319:153–165.

Kikuchi, S., and M. Matsumasa. 1995. Pereopodal disk: a new type of extrabranchial ion-transporting organ in an estuarine amphipod, *Melita setiflagella* (Crustacea). Tissue and Cell 27:635–643.

Kikuchi, S. and M. Matsumasa. 1997. Ultrastructural evidence for osmoregulatory function of the sternal epithelia in some gammaridean amphipods. Journal of Crustacean Biology 17:377–388.

Kimura, C., G.A. Ahearn, L. Busquets-Turner, R.S. Haley, C. Nagao, and H.G. De Couet. 1994. Immunolocalization of an antigen associated with the invertebrate electrogenic 2Na/1H antiporter. Journal of Experimental Biology 189:85–104.

Kinsey, S.T., E. Buda, and J. Nordeen. 2003. Scaling of gill metabolic potential as a function of salinity in the euryhaline crab, *Callinectes sapidus* Rathbun. Physiological and Biochemical Zoology 76:105–114.

Kirschner, L.B. 1967. Comparative physiology: invertebrate excretory organs. American Journal of Physiology 29:169–196.

Kirschner, L.B. 2002. Sodium-proton exchange in crayfish. Biochimica et Biophysica Acta 1566:67–71.

Kobayashi, K., Rompas, R.M., Meakawa, T., Imada, N., and Y. Oshima. 1990. Changes in metabolic activity of tiger shrimp larvae at different stages to fenitrothion, an organophosphate insecticide. Bulletin of the Japanese Society of Scientific Fisheries 56:489–496.

Kuo, C.M., Hsu, C.R., and C.Y. Lin. 1995. Hyperglycaemic effects of dopamine in tiger shrimp, *Penaeus monodon*. Aquaculture 135:161–172.

Larimer, J.L. 1964. Sensory-induced modifications of ventilation and heart rate in crayfish. Comparative Biochemistry and Physiology 12:25–36.

Lee, C., and M.A. Bell. 1999. Causes and consequences of recent freshwater invasions by saltwater animals. Trends in Ecology & Evolution 14:284–287.

Lee, C.E., M. Kiergaard, G.W. Gelembiuk, B.D. Eads, and M. Posavi. 2011. Pumping ions: rapid parallel evolution of ionic regulation following habitat invasions. Evolution 65:1–16.

Lee, C.E., M. Posavi, and G. Charmantier. 2012. Rapid evolution of body fluid regulation following independent invasions into freshwater habitats. Journal of Evolutionary Biology 25:625–633.

Legeay, A., and J.C. Massabuau. 2000. Effect of salinity on hypoxia tolerance of resting green crabs, *Carcinus maenas*, after feeding. Marine Biology 136:387–396.

Li, P., X.X. Wang, S. Murthy, D.M. Gatlin III, F.L. Castille, and A.L. Lawrence. 2009. Effect of dietary supplementation of brewer's yeast and GroBiotic®-A on growth, immune responses, and low-salinity tolerance of Pacific white shrimp *Litopenaeus vannamei* cultured in recirculating systems. Journal of Applied Aquaculture 21:110–119.

Li, T., R. Roer, M. Vana, S. Pate, and J. Check. 2006. Gill area, permeability and Na^+,K^+-ATPase activity as a function of size and salinity in the blue crab, *Callinectes sapidus*, Journal of Experimental Zoology 305A:233–245.

Lignot, J.-H., J.P. Trilles, and G. Charmantier. 1997. Effect of an organophosphorus insecticide, fenitrothion, on survival and osmoregulation of various developmental stages of the shrimp *Penaeus japonicus* (Crustacea: Decapoda). Marine Biology 128:307–316.

Lignot, J.-H., M. Charmantier-Daures, and G. Charmantier. 1999. Immuno-localization of Na^+, K^+-ATPase in the organs of the branchial cavity of the European lobster *Homarus gammarus* (Crustacea, Decapoda). Cell and Tissue Research 296:417–426.

Lignot, J.-H., C. Spanings-Pierrot, and G. Charmantier. 2000. Osmoregulatory capacity as a tool in monitoring the physiological condition and effect of stress in crustaceans. Aquaculture 191:209–245.

Lima, A.G., J.C. McNamara, and W.A. Terra. 1997. Regulation of hemolymph osmolytes and gill Na^+/K^+-ATPase activities during acclimation to saline media in the freshwater shrimp *Macrobrachium olfersii* (Wiegman, 1863) (Decapoda, Palaemonidae). Journal of Experimental Marine Biology and Ecology 215:81–91.

Lin, H.P., G. Charmantier, P. Thuet, and J.-P. Trilles. 1992. Effects of turbidity on survival, osmoregulation and gill Na^+-K^+ ATPase in juvenile shrimp *Penaeus japonicus.* Marine Ecology Progress Series 90:31–37.

Lin, S.C., C.-H. Liou, and J.H. Cheng. 2000. The role of the antennal glands in ion and body volume regulation of cannulated *Penaeus monodon* reared in various salinity conditions. Comparative Biochemistry and Physiology 127A:121–129.

Lockwood, A.P.M. 1968. Aspects of the physiology of Crustacea. Oliver & Boyd, Edinburg and London.
Lougee, L.A., S.M. Bollens, and S.R. Avent. 2002. The effects of haloclines on the vertical distribution and migration of zooplankton. Journal of Experimental Marine Biology and Ecology 278:111–134.
Lucu, C., and D.W. Towle. 2003. Na^+/K^+-ATPase in gills of aquatic crustacea. Comparative Biochemistry and Physiology 135A:195–214.
Lucu, C., M. Devescovi, B. Skaramuca, and V.V. Kozul. 2000. Gill Na,K-ATPase in the spiny lobster *Palinurus elephas* and other marine osmoconformers. Adaptiveness of enzymes from osmoconformity to hyperregulation. Journal of Experimental Marine Biology and Ecology 246:163–178.
Luquet, C.M., D. Weihrauch, M. Senek, and D.W. Towle. 2005. Induction of branchial ion transporter mRNA expression during acclimation to salinity change in the euryhaline crab *Chasmagnathus granulatus*. *Journal of Experimental Biology* 208:3627–3636.
Luvizotto-Santos, R., J. Lee, Z. Branco, A. Bianchini, and L. Nery. 2003. Lipids as energy source during salinity acclimation in the euryhaline crab *Chasmagnathus granulata* dana, 1851 (crustacea-grapsidae). Journal of Experimental Zoology 295A:200–205.
Mantel, L.H. 1968. The foregut of *Gecarcinus lateralis* as an organ of salt and water balance. American Zoologist 8:433–442.
Mantel, L.H., and L.L. Farmer. 1983. Osmotic and ionic regulation. Pages 53–161 *in* L.H. Mantel, editor. The biology of Crustacea, Vol. 5: Internal anatomy and physiological regulation. Academic Press, New York.
Martinez, A.S., G. Charmantier, P. Compère, and M. Charmantier-Daures. 2005. Branchial chamber tissues in two caridean shrimps: the epibenthic *Palaemon adspersus* and the deep-sea hydrothermal *Rimicaris exoculata*. Tissue and Cell 37:153–165.
Martinez, C.B., R.R. Harris, and M.C. Santos. 1998. Transepithelial potential differences and sodium fluxes in isolated perfused gills of the mangrove crab (*Ucides cordatus*). Comparative Biochemistry and Physiology 120A:227–236.
Martins, T.L., A.L.F. Chittó, C.L. Rossetti, C.K. Brondani, L.C. Kucharski, and R.S.M. Da Silva. 2011. Effects of hypo- or hyperosmotic stress on lipid synthesis and gluconeogenic activity in tissues of the crab *Neohelice granulata*. Comparative Biochemistry and Physiology 158A:400–405.
McGaw, I.J. 2005. Does feeding limit cardiovascular modulation in the Dungeness crab *Cancer magister* during hypoxia? Journal of Experimental Biology 208:83–91.
McGaw, I.J. 2006a. Prioritization or summation of events? Cardiovascular physiology of postprandial Dungeness crabs in low salinity. Physiological and Biochemical Zoology 79:169–177.
McGaw, I.J. 2006b. Feeding and digestion in low salinity in an osmoconforming crab, *Cancer gracilis*. I. Cardiovascular and respiratory responses. Journal of Experimental Biology 209:3766–3776.
McGaw, I.J., and C.L. Reiber. 1998. Circulatory modification in the blue crab *Callinectes sapidus*, during exposure and acclimation to low salinity. Comparative Biochemistry and Physiology 121A:67–76.
McNamara, J.C., F.P. Zanotto, and H. Onken. 2005. Adaptation to hypoosmotic challenge in brachyuran crabs: a microanatomical and electrophysiological characterization of the intestinal epithelia. Journal of Experimental Zoology 303A:880–893.
McWhinnie, M.A. 1962. Gastrolith growth and calcium shifts in the freshwater crayfish *Orconectes virilise*. Comparative Biochemistry and Physiology 7:1–14.
Mente, E., A. Legeay, D.F. Houlihan, and J.C. Massabuau. 2003. Influence of oxygen partial pressures on protein synthesis in feeding crabs. American Journal of Physiology 284:R500–R510.
Morris, R.J., A.P.M. Lockwood, and M.E. Dawson. 1982. An effect of acclimation salinity on the fatty acid composition of the gill phospholipids and water flux of the amphipod crustacean *Gammarus duebeni*. Comparative Biochemistry and Physiology 72A:497–503.
Morris, S. 2001. Neuroendocrine regulation of osmoregulation and the evolution of air-breathing in decapod crustaceans. Journal of Experimental Biology 204:979–989.
Morritt, D., and J.I. Spicer. 1993. A brief re-examination of the function and regulation of extracellular magnesium and its relationship to activity in crustacean arthropods. Comparative Biochemistry and Physiology 106A:19–23.
Morritt, D., and J.I. Spicer. 1995. Changes in the pattern of osmoregulation in the brackish water amphipod *Gammarus duebeni* Lilljeborg (Crustacea) during embryonic development. Journal of Experimental Zoology 273:271–281.

Morritt, D., and J.I. Spicer. 1998. The physiological ecology of talitrid amphipods: an update. Canadian Journal of Zoology 76:1965–1982.

Morritt, D., and J.I. Spicer. 1999. Developmental ecophysiology of the beachflea *Orchestia gammarellus* (Pallas) (Crsutacea: Amphipoda: Talitrida) III. Physiological competency as a possible explanation for timing of hatchling release. *Journal of Experimental Biology and Ecology* 232:275–283.

Mykles, D.L., M.E. Adams, G. Gäde, A.B. Lange, H.G. Marco, and I. Orchard. 2010. Neuropeptide action in insects and crustaceans. Physiological and Biochemical Zoology 83:836–846.

Naylor, C., L. Pindar, and P. Calow. 1995. Inter- and intraspecific variation in sensitivity to toxins: the effects of acidity and zinc on the freshwater crustaceans *Asellus aquaticus* (L.) and *Gammarus pulex* (L.). Water Research 24:757–762.

Neufeld, D.S., and J.N. Cameron. 1993. Transepithelial movement of calcium in crustaceans. Journal of Experimental Biology 184:1–16.

Neufeld, D.S., and J.N. Cameron. 1994. Effect of the external concentration on the postmoult uptake of calcium in blue crabs (*Callinectes sapidus*). Journal of Experimental Biology 188:1–9.

Newton, C., and W.T.W. Potts. 1993. Ionic regulation and buoyancy in some planktonic organisms. Journal of the Marine Biological Association of the United Kingdom 73:15–23.

Olmsted, J.M.D., and J.P. Baumberger. 1923. Form and growth of grapsoid crabs. A comparison of the form of three species of grapsoid crabs and their growth at molting, Journal of Morphology 38:279–294.

Olsowski, A.M. Putzenlechner, K. Böttcher, and K. Graszynski. 1995. The carbonic anhydrase of the Chinese crab *Eriocheir sinensis*: effects of adaptation from tap to salt water, Helgolànder Wissenschaftliche Meeresunter 49:727–735.

Onken, H., and J.C. McNamara. 2002. Hyperosmoregulation in the red freshwater crab *Dilocarcinus pagei* (Brachyura, Trichodactylidae): structural and functional asymmetries of the posterior gills. Journal of Experimental Biology 205:167–175.

Ordiano, A., F. Alvarez, and G. Alcaraz. 2005. Osmoregulation and oxygen consumption of the hololimnetic prawn, *Macrobrachium tuxtlaense* at varying salinities (Decapoda, Palaemonidae). Crustaceana 78:1013–1022.

Palácios, E., A. Bonilla, A. Pérez, I.S. Racotta, and R. Civera. 2004. Influence of highly unsaturated fatty acids on the responses of white shrimp *Litopenaeus vannamei* (Boone) postlarvae to low salinity. Journal of Experimental Marine Biology and Ecology 299:201–215.

Pantin, C.F.A. 1946. Notes on microscopical techniques for zoologists. Cambridge University Press, Cambridge.

Parado-Estepa, J.M., E.G. Ladja, and R.P. Ferraris. 1989. Effect of salinity on hemolymph calcium concentration during the molt cycle of the prawn *Penaeus monodon*. Marine Biology 102:189–193.

Patrick, M.L., and T.J. Bradley. 2000. The physiology of salinity tolerance in larvae of two species of culex mosquitoes: the role of compatible solutes. Journal of Experimental Biology 203:821–830.

Pavicic-Hamer, D., M. Devescovi, and C. Lucu. 2003. Activation of carbonic anhydrase in branchial cavity tissues of lobsters (*Homarus gammarus*) by dilute seawater exposure. Journal of Experimental Marine Biology and Ecology 287:79–92.

Péqueux, A. 1995. Osmotic regulation in crustaceans. Journal of Crustacean Biology 15:1–60.

Péqueux, A., and R. Gilles. 1978. Osmoregulation of the euryhaline Chinese crab Eriocheir sinensis. Ionic transports across isolated perfused gills as related to the salinity of the environment. Pages 105–111 *in* D.S. McLusky and A.J. Berry, editors. Physiology and behaviour of marine organisms. Pergamon Press, Oxford.

Peterson, D.R., and R.F. Loizzi. 1974. Ultrastructure of the crayfish kidney, coelomosac, labyrinth, and nephridial canal. Journal of Morphology 142:241–264.

Phillips, J.E., J. Meredith, N. Audsley, N. Richardson, A. Macins, and M. Ring. 1998. Locust ion transport peptide (ITP): a putative hormone controlling water and ionic balance in terrestrial Insects. American Zoologist 38:461–470.

Piscart, C., A. Manach, G.H. Copp, and P. Marmonier. 2007. Distribution and microhabitats of native and non-native gammarids (Amphipoda, Crustacea) in Brittany, with particular reference to the endangered endemic subspecies *Gammarus duebeni celticus*. Journal of Biogeography 34:524–533.

Plaistow, S.J., J.P. Troussard, and F. Cézilly. 2001. The effect of acanthocephalan parasite *Pomphorhynchus laevis* on the lipid and glycogen content of its intermediate host *Gammarus pulex*. International Journal for Parasitology 31:346–351.

Poinar, G.O., A.D.M. Latham, and R. Poulin. 2002. *Thaumamermis zealandica* n. sp. (Mermithidae: Nematoda) parasitizing the intertidal marine Amphipod *Talorchestia quoyana* (Talitridae: Amphipoda) in New Zealand, with a summary of mermithids infecting amphipods. Systematic Parasitology 53:227–233.

Pongsomboon, S., S. Udomlertpreecha, P. Amparyup, S. Wuthisuthimethavee, and A. Tassanakajon. 2009. Gene expression and activity of carbonic anhydrase in salinity stressed *Penaeus monodon*. Comparative Biochemistry and Physiology 152A:225–233.

Powers, L.W., and D.E. Bliss. 1983. Terrestrial adaptations. Pages 271–333 *in* F.J. Vernberg and W.B. Vernberg, editors. The biology of Crustacea. Environmental adaptations. Vol. 8. Academic Press, New York.

Rabalais, N.N., and J. Cameron. 1985. The effects of factors important in semi-arid environments on the early development of *Uca subcylindrica*. Biological bulletin 168:147–160.

Rees, J.F., K. Curé, S. Piyatiratitivorakul, P. Sorgeloos, and P. Menasveta. 1994. Highly unsaturated fatty acid requirements of *Penaeus monodon* postlarvae: an experimental approach based on *Artemia* enrichment. Aquaculture 122:193–207.

Riegel, J.A. 1961. The influence of water-loading and low temperature on certain functional aspects of the crayfish antennal gland. Journal of Experimental Biology 38:291–299.

Riegel J.A., and L.B. Kirschner. 1960. The excretion of inulin and glucose by the crayfish antennal gland. Biological Bulletin 118:296–307.

Robertson, J.D. 1953. Further studies on ionic regulation in marine invertebrates. Journal of Experimental Biology 30:277–296.

Robertson, J.D. 1960. Osmotic and ionic regulation. Pages 317–339 *in* T.H. Waterman, editor. The physiology of Crustacea, Vol. 1: The metabolism and growth. Academic Press, New York.

Rosas, C., G. Cuzon, G. Gaxiola, Y. Le Priol, C. Pascual, J. Rossignyol, F. Contreras, A. Sanchez, and A. van Wormhoudt. 2001. Metabolism and growth of juveniles of *Litopenaeus vannamei*: effect of salinity and dietary carbohydrate levels. Journal of Experimental Marine Biology and Ecology 259:1–22.

Roy, L.A., D.A. Davis, I.P. Saoud, and R.P. Henry. 2007. Effects of various levels of aqueous potassium and magnesium on survival, growth, and respiration of the pacific white shrimp, *Litopenaeus vannamei*, reared in low salinity waters. Aquaculture 262:461–469.

Ruppert, E.E., and R.D. Barnes. 1994. Invertebrate zoology. 6th ed. Saunders College Publishing, Harcourt Brace and Company, Orlando, Florida.

Sarver, G.L., M.A. Flynn, and C.W. Holliday. 1994. Renal Na,K-ATPase and osmoregulation in the crayfish *Procambarus clarkia*. Comparative Biochemistry and Physiology 107A:349–356.

Scapini, F. 2006. Keynote papers on sandhopper orientation and navigation. Marine and Freshwater Behaviour and Physiology 39:73–85.

Schein, V., Y. Waché, R. Etges, L.C. Kucharski, A. Wormhoudt, and R.S.M. Da Silva. 2004. Effect of hyperosmotic shock on phosphoenolpyruvate carboxykinase gene expression and gluconeogenic activity in the crab muscle. FEBS Letters 561:202–206.

Schmidt, M. 1989. The hair-peg organs of the shore crab, *Carcinus maenas* (Crustacea, Decapoda): ultrastructure and functional properties of sensilla sensitive to changes in seawater concentration. Cell and Tissue Research 257:609–621.

Schmidt-Nielsen, K. 1984. Scaling: why is animal size so important? Cambridge University Press, New York.

Schoffeniels, E., and R. Gilles. 1970. Osmoregulation in aquatic arthropods. Pages 255–286 *in* M. Florkin and B.T. Scheer, editors. Chemical Zoology, Vol. V. Arthropoda, Part A. Academic Press, New York and London.

Schubart, C.D., and R. Diesel. 1998. Osmoregulatory capacities and penetration into terrestrial habitats: a comparative study of Jamaican crabs of the genus *Armases* Abele (Brachyura: Grapsidae: Sesarminae). Bulletin of Marine Science 63:743–752.

Seear, P.J., G.A. Tarling, G. Burns, W.P. Goodall-Copestake, E. Gaten, Ö. Özkaya, and E. Rosato. 2010. Differential gene expression during the moult cycle of Antarctic krill (*Euphausia superba*). BMC Genomics 11:582.

Serrano L., and R.P. Henry. 2008. Differential expression and induction of two carbonic anhydrase isoforms in the gills of the euryhaline green crab, *Carcinus maenas*, in response to low salinity. Comparative Biochemistry and Physiology 3D:186–193.

Serrano, L., G. Blanvillain, D. Soyez, G. Charmantier, E. Grousset, F. Aujoulat, and C. Spanings-Pierrot. 2003. Putative involvement of crustacean hyperglycemic hormone isoforms in the neuroendocrine mediation of osmoregulation in the crayfish *Astacus leptodactylus*. Journal of Experimental Biology 206:979–988.

Serrano, L., K.M. Halanych, and R.P. Henry. 2007. Salinity-stimulated changes in expression and activity of two carbonic anhydrase isoforms in the blue crab, *Callinectes sapidus*. Journal of Experimental Biology 210:2320–2332.

Shechter, A., M. Tom, Y. Yudkovski, S. Weil, S.A. Chang, E.S. Chang, V. Chalifa-Caspi, A. Berman, and A. Sagi. 2007. Search for hepatopancreatic ecdysteroid-responsive genes during the crayfish molt cycle: from a single gene to multigenicity. Journal of Experimental Biology 210:3525–3537.

Shi, X.Z., Q. Ren, X.F. Zhao, and J.X. Wang. 2009. Expression of four trypsin-like proteases from the Chinese shrimp, *Fenneropenaeus chinensis*, as regulated by pathogenic infection. Comparative Biochemistry and Physiology 153B:54–60.

Siebers, D., C. Winkler, A. Lucu, U. Grammersdorf, and H. Wille. 1987. Effects of amiloride on sodium chloride transport across isolated perfused gills of shore crabs *Carcinus maenas* acclimated to brackish water. Comparative Biochemistry and Physiology 87A:333–340.

Sornom, P., V. Felten, V. Médoc, S. Sroda, P. Rousselle, and J.N. Beisel. 2010. Effect of gender on physiological and behavioural responses of *Gammarus roeseli* (Crustacea Amphipoda) to salinity and temperature. Environmental Pollution 158:1288–1295.

Souheil, H., A. Vey, P. Thuet, and J.-P. Trilles. 1999. Pathogenic and toxic effects of *Fusarium oxysporum* (Schlecht) on survival and osmoregulatory capacity of *Penaeus japonicus* (Bate). Aquaculture 178:209–224.

Souza, J.C.R., C.A. Strüssmann, F. Takashima, H. Satoh, S. Sekine, Y. Shima, and H. Matsuda. 2010. Oral and integumental uptake of free exogenous glycine by the Japanese spiny lobster *Panulirus japonicus* phyllosoma larvae. Journal of Experimental Biology 213:1859–1867.

Sowers, A.D., D.M. Gatlin, S.P. Young, J.J. Isely, C.L. Browdy, and J.R. Tomasso. 2005. Responses of *Litopenaeus vannamei* (Boone) in water containing low concentrations of total dissolved solids. Aquatic Resources 36:819–823.

Spencer, A.M., A.H. Fielding, and F.I. Kamemoto. 1979. The relationship between gill Na K-ATPase activity and osmoregulatory capacity in various crabs. Physiological Zoology 52:1–10.

Spicer, J.I., A.C. Taylor, and B.R. McMahon. 1990. O2-binding properties of haemocyanin from the sandhopper *Talitrus saltator* (Montagu, 1808) (Crustacea: Amphipoda). Journal of Experimental Marine Biology and Ecology 135:213–228.

Spicer, J.I., D. Morritt, and A.C. Taylor. 1994. Effect of low temperature on oxygen uptake and haemolymph ions in the sandhopper *Talitrus saltator* (Crustacea: Amphipoda). Journal of the Marine Biological Association of the United Kingdom 74:313–321.

Sterling, K.M., C.I. Cheeseman, and G.A. Ahearn. 2009. Identification of a novel sodium-dependent fructose transport activity in the hepatopancreas of the Atlantic lobster *Homarus americanus*. Journal of Experimental Biology 212:1912–1920.

Strauss, O., and K. Graszynski. 1992. Isolation of plasma membrane vesicles from the gill epithelium of the crayfish, *Orconectes limosus* Rafinesque, and properties of the Na^+/H^+ exchanger. Comparative Biochemistry and Physiology 102A:519–526.

Subramanian, A. 1975. Sodium and water permeabilities in selected Crustacea. Physiological Zoology 48:398–403.

Sui, L., M. Wille, Y. Cheng, and P. Sorgeloos. 2007. The effect of dietary n-3 HUFA levels and DHA/EPA ratios on growth, survival and osmotic stress tolerance of Chinese mitten crab *Eriocheir sinensis* larvae. Aquaculture 273:139–150.

Suprayudi, M.A., T. Takeuchi, and K. Hamasaki. 2004. Essential fatty acids for larval mud crab *Scylla serrata*: implications of lack of the ability to bioconvert C18 unsaturated fatty acids to highly unsaturated fatty acids. Aquaculture 231:403–416.

Tantulo, U., and R. Fotedar. 2006. Comparison of growth, osmoregulatory capacity, ionic regulation and organosomatic indices of black tiger prawn (*Penaeus monodon* Fabricius, 1798) juveniles reared in potassium fortified inland saline water and ocean water at different salinities. Aquaculture 258:594–605.

Taylor, A.C., and P. Greenaway. 2002. Osmoregulation in the terrestrial Christmas Island red crab *Gecarcoidea natalis* (Brachyura: Gecarcinidae): modulation of branchial chloride uptake from the urine. Journal of Experimental Biology 205:3251–3260.

Taylor, P.M., and R.R. Harris. 1986. Osmoregulation in *Corophium curvispinum* (Crustacea: Amphipoda), a recent coloniser of freshwater. Journal of Comparative Physiology 156:323–329.

Taylor, A.C., P.J. Butler, and A. Al-Wassia. 1977. The effect of a decrease in salinity on respiration, osmoregulation and activity of the shore crab *Carcinus maenas* (L.) at different acclimation temperatures. Journal of Comparative Physiology 119:155–170.

Tentori, E., and A.P.M. Lockwood. 1990. Haemolymph magnesium levels in some oceanic Crustacea. Comparative Biochemistry and Physiology 95A:545–548.

Terracini-De Benedetti, E. 1963. Preliminary observations on the orientation of *Talitrus saltator* in fresh and sea water. Naturwissenschaften 50:25–26.

Tiu, S.H.K., J.G. He, and S.M. Chan. 2007. The LvCHH-ITP gene of the shrimp (*Litopenaeus vannamei*) produces a widely expressed putative ion transport peptide (LvITP) for osmo-regulation. Gene 396:226–235.

Towle, D.W., M.E. Rushton, D. Heidysch, J.J. Magnani, M.J. Rose, A. Amstutz, M.K. Jordan, D.W. Shearer, and W.S. Wu. 1997. Sodium-proton antiporter in the euryhaline crab *Carcinus maenas*: molecular cloning, expression and tissue distribution. Journal of Experimental Biology 200:1003–1014.

Towle, D.W., R.P. Henry, and N.B. Terwilliger. 2011. Microarray-detected changes in gene expression in gills of green crabs (*Carcinus maenas*) upon dilution of environmental salinity. Comparative Biochemistry and Physiology. D Genomics Proteomics. 6:115–125.

Tsai, J.-R., and H.-C. Lin. 2007. V-type H^+-ATPase and Na^+,K^+-ATPase in the gills of 13 euryhaline crabs during salinity acclimation. Journal of Experimental Biology 210:620–627.

Tsai, M.L., C.F. Dai, and H.C. Chen. 1997. Responses of two semiterrestrial isopods, *Ligia exotica* and *Ligia taiwanensis* (Crustacea) to osmotic stress. Comparative Biochemistry and Physiology 118A:141–146.

Tu, H.T., F. Silvestre, N.T. Phuong, and P. Kestemont. 2010. Effects of pesticides and antibiotics on penaeid shrimp with special emphases on behavioral and biomarker responses. Environmental Toxicology and Chemistry 29:929–938.

Turner, H.V., D.L. Wolcott, T.G. Wolcott, and A.H. Hines. 2003. Postmating behavior, intramolt growth, and onset of migration to Chesapeake Bay blue crabs, *Callinectes sapidus* Rathbun. Journal of Experimental Marine Biology and Ecology 295:107–130.

Ugolini, A., G. Ungherese, L. Mercatelli, D. Saer, and L. Lepri. 2009. Seawater Ca^{2+} concentration influences solar orientation in *Talitrus saltator* (Crustacea, Amphipoda). Journal of Experimental Biology 212:797–801.

Vinagre, T.M., J.C. Alciati, J.S. Yunes, A. Bianchini, J. Richards, and J.M. Monserrat. 2002. Effects of extracts from the cyanobacterium *Microcystis aeruginosa* on ion regulation and gill Na^+, K^+-ATPase and phosphatase K^+ dependent activities of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). Physiological and Biochemical Zoology 75:600–608.

Vogt, G. 2013. Abbreviation of larval development and extension of brood care as key features of the evolution of freshwater Decapoda. Biological Reviews 88:81–116.

Wägele, J.W. 1992. Isopoda. Pages 529–617 *in* F.W. Harrison and A.G. Humes, editors. Microscopic anatomy of invertebrates. Wiley-Liss, Inc., New York.

Walters, N.J., and R.F. Uglow. 1981. Haemolymph magnesium and relative heart activity of some species of marine decapod crustaceans. Journal of Experimental Marine Biology and Ecology 55:255–265.

Webb, D.A. 1940. Ionic regulation in *Carcinus maenas*. Proceedings of the Royal Society B 129:107.

Wehner, F., H. Olsen, H. Tinel, E. Kinne-Saffran, and R.K. Kinne. 2003. Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. Reviews of Physiology, Biochemistry and Pharmacology 148:1–80.

Weihrauch, D., A. Ziegler, D. Siebers, and D.W. Towle. 2001. Molecular characterization of V-type H^+-ATPase (B-subunit) in gills of euryhaline crabs and its physiological role in osmoregulatory ion uptake. Journal of Experimental Biology 204:25–37.

Weihrauch, D., S. Morris, and D.W. Towle. 2004. Ammonia excretion in aquatic and terrestrial crabs. Journal of Experimental Biology 207:4491–504.

Williams, C.M., R. Poulin, and B.J. Sinclair. 2004. Increased haemolymph osmolality suggests a new route for behavioural manipulation of *Talorchestia quoyana* (Amphipoda: Talitridae) by its mermithid parasite. Functional Ecology 18:685–691.

C.S. Wirkner, and S. Ritcher. 2013. Circulatory System and Respiration. Pages 376–412 *in* L. Watling and M. Thiel, editors. The natural history of the Crustacea, Vol. 1. Oxford University Press, New York.

Wittmann, A.C., C. Held, H.O. Pörtner, and F.J. Sartoris. 2010. Ion regulatory capacity and the biogeography of Crustacea at high southern latitudes. Polar Biology 33:919–928.

Wittmann, A.C., D. Storch, K. Anger, H.O. Pörtner, and F.J. Sartoris. 2011. Temperature-dependent activity in early life stages of the stone crab *Paralomis granulosa* (Decapoda, Anomura, Lithodidae): a role for ionic and magnesium regulation? Journal of Experimental Marine Biology and Ecology 397:27–37.

Zanotto, F.P., M.G. Wheatly, C.L. Reiber, A.T. Gannon, and E. Jalles-Filho. 2004. Allometric relationship of postmolt net ion uptake, ventilation, and circulation in the freshwater crayfish *Procambarus clarkii*: intraspecific scaling. Physiological and Biochemical Zoology 77:275–284.

Zatta, P. 1987. Dopamine, noradrenaline and serotonin during hypo-osmotic stress of *Carcinus maenas*. Marine Biology 96:479–481.

Zeiske, W., H. Onken, H.-J. Schwarz, and K. Graszynski. 1992. Invertebrate epithelial Na+ channels: amiloride-induced current noise in crab gills. Biochimica et Biophysica Acta 1105:245–252.

Zeleny, C. 1905. Compensatory regulation. Journal of Experimental Zoology 2:1–102.

Zhu, C., S. Dong, F. Wang, and G. Huang. 2004. Effects of Na/K ratio in seawater on growth and energy budget of juvenile *Litopenaeus vannamei*. Aquaculture 234:485–496.

Ziegler, A., D. Weihrauch, M. Hagedorn, D.W. Towle, and R. Bleher. 2004. Expression and polarity reversal of V-type H^+-ATPase during the mineralization-demineralization cycle in *Porcellio scaber* sternal epithelial cells. Journal of Experimental Biology 207:1749–1756.

9

NUTRITION AND DIGESTION

Reinhard Saborowski

Abstract

The crustaceans colonized all aquatic habitats. In the oceans they are present from the coasts down to the deep sea basins. They are also frequent in freshwater systems and some species even live on land. Accordingly, they adapted to utilize a variety of food items. These items comprise organic matter from dead or alive animals, vascular plants, detritus, macro- and microalgae, microbial films, and organic sediment compounds. This food is utilized in the digestive organs with the aid of highly active enzymes. The enzymes are synthesized in specific cells along the midgut lining or, in the higher crustaceans, in the complex midgut diverticula. From there they are released into the stomach where they initiate food digestion. According to the biochemical nature of the major food compounds the crustaceans possess a variety of endo- and exopeptidases to hydrolyze proteins and peptides, amylases to cleave glycogen, and lipases and esterases to utilize lipids. The liberated nutrients are resorbed by specific cells of the midgut or the midgut diverticula. Besides, crustaceans can also digest organic materials which are known for their chemical stability, namely chitin, cellulose, and hemicelluloses. The acting enzymes are for the most part of endogenous origin. Bacteria may be present in the gut in quite high numbers but their contribution to the digestive processes is not mandatory. The digestive enzymes of Crustacea show extraordinary catalytic properties which form the basis to efficiently utilize a variety of food items and, thus, to meet the nutritive demands of the organisms.

INTRODUCTION

Feeding and assimilation of food are cardinal physiological processes in heterotrophic organisms. They comprise the uptake as well as the chemical degradation of organic material. The purpose is to gain nutrients and energy for maintaining homeostasis and facilitating growth

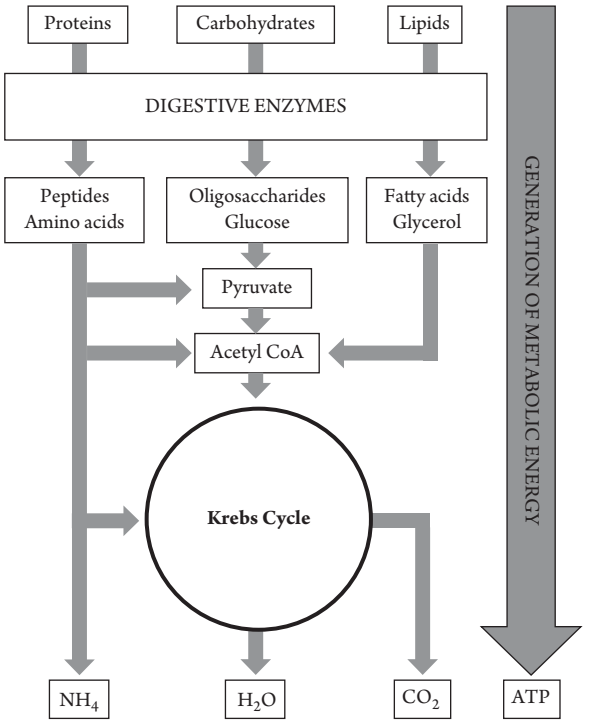


Fig. 9.1.
Simplified diagram of the catabolic pathways of major storage products.

and reproduction (Fig. 9.1). The digestive organs of crustaceans and their function have been researched for more than 100 years. The first detailed anatomical studies on the stomach of crayfish and on the basic enzymatic processes within the digestive organs were published in the 19th century.

During the past 30–40 years, research on crustacean digestive physiology increased considerably. This interest is based on both emerging knowledge about the ecological significance of crustaceans in various habitats and also on their relevance in food production. The ecological aspects deal with the trophic interactions of crustaceans within their habitats and their ecophysiological adaptations and specializations. The economic aspects mostly target the optimization of feeds to increase yield and thus earnings.

This chapter is aimed at providing an overview of the feeding and utilization of food in crustaceans from the physiological and biochemical point of view. More details about crustacean feeding biology can be found in extended reviews from the past decades. The anatomy of the crustacean digestive system is presented by Watling (2013). The decapod hepatopancreas and its function in digestion and food storage were thoroughly described by Gibson and Barker (1979). The gut structure and the detailed cellular processes of digestion in Malacostraca and other crustaceans were presented by Brunet et al. (1994). Dall and Moriarty (1983) contributed a chapter entitled "Functional Aspects of Nutrition and Digestion" to the series *The Biology of Crustacea*. Metabolism and transport processes were reviewed by Chang and O'Connor (1983). A comprehensive overview on crustacean nutrition was edited and published by D'Abramo et al. (1997). A recent review on the digestive tract of crustaceans was contributed by Ceccaldi (2006).

CRUSTACEAN NUTRITION

Crustaceans occupy a variety of habitats. To utilize a variety of food from these habitats, crustaceans show specific morphological and anatomical adaptations to handle and to process their food (Watling 2013). Moreover, they also developed physiological and biochemical properties to utilize the food.

Food Sources and Feeding Habits

The various morphotypes of crustaceans are generally capable of utilizing almost all kinds of organic material. Food may originate from live or dead animals, higher plants and their remains, macro- and microalgae, and organic surface layers or the organic fraction of sediments (Fig. 9.2). Some species evolved to extract nutrients from other living organisms, thus acting as exo- or endoparasites. Other species process hardly digestible food items such as cellulose and lignocellulose with the aid of endogenous enzymes (Linton et al. 2006, King et al. 2010).

Feeding frequencies, gut passage times, and assimilation efficiencies differ widely between species and also depend on the quantity and the chemical composition of the food (Waddington 2008).

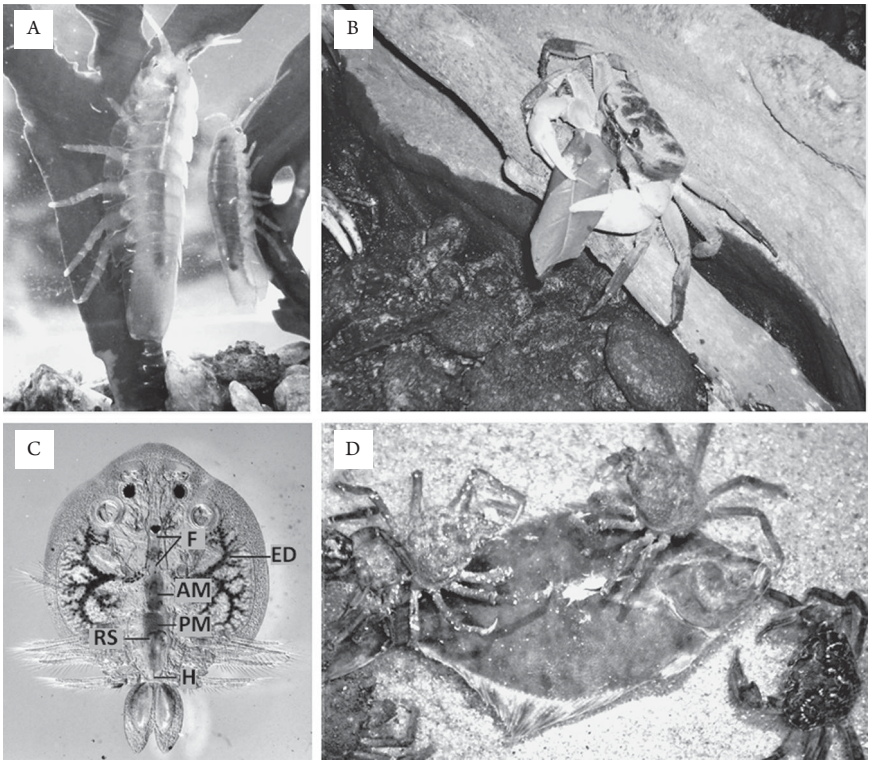


Fig. 9.2.
Examples of crustaceans feeding on different food items. (A) Herbivorous isopods *Idotea emarginata* feeding on seaweed. Photo by Reinhard Saborowski. (B) Christmas Island land crab *Discoplax hirtipes* feeding on a leaf. Photo by Reinhard Saborowski. (C) Parasitic fish louse *Argulus japonicus*. The digestive system is filled with the blood of the host. Abbreviations: AM, anterior midgut; ED, central diverticulum; F, foregut; H, hindgut; PM, posterior midgut ventral to reproductive system (RS). The length of the animal is 4 mm. From Tam and Avenant-Oldewage (2009), with permission from Elsevier. (D) *Hyas araneus* and *Carcinus maenas* feeding on a dead flatfish. Photo by Uwe Nettelmann.

The gut passage may last for several minutes or up to hours and days. It depends on the size of the animal, the feeding strategy, and on the anatomy of the digestive tract. For example, the spiny lobster *Jasus edwardsii* showed poor growth on formulated diets due to limitations in food uptake and low gut transition times (Simon and Jeffs 2008). Feeding experiments showed that food uptake was rapid, but satiation occurred quickly due to low foregut capacity. The filling time of the foregut amounted to 1–2 h, whereas the clearance time lasted for 10 h. The gut throughput was slow (34–42 h), and appetite revival was evident after 18 h (Simon and Jeffs 2008). The scavenging Antarctic isopod *Natatolana obtusata* has a very plastic mode of food uptake. The mouth opening is very flexible, and the anterior hindgut is extremely variable in its lumen and very distensible. The gut is perfectly suited to store huge amounts of food and to overcome long periods of food deficiency (Storch et al. 2002).

Nutritional Requirements

The nutritional requirements of crustaceans were most intensively studied and are best documented for species with high economic value and relevance in aquaculture. In this respect, the nutritional requirements for *Litopenaeus vannamei* were reviewed by Cuzon et al. (2004). A general and comprehensive overview on crustacean nutrition was presented by D'Abramo et al. (1997).

The chemical gross compositions of food were reported for a range of different crustaceans and their ontogenetic stages. The amounts of protein, lipids, carbohydrates, and inorganic compounds vary widely, depending on the ability of the species to accumulate storage products and on the degree of calcification of the shell. As a rough overview, the following numbers may be given. The amount of water in the food may range from 75% to 85% of the total fresh mass. The inorganic fraction (ash) may amount to 10–20% of dry weight and may increase significantly when species are strongly calcified. The protein content can account for up to 70% of ash-free dry mass and total lipids for less than 10% to up to 60% of ash-free dry weight.

Water

The uptake of water happens predominantly through the epithelia of the digestive system and, to a lesser extent, through the gills. In some species, highly specialized adaptations may have evolved that allow those animals to cope with exceptional environmental conditions. The most common method of water uptake is to swallow it through the mouth and esophagus into the stomach. Land crabs have been observed drinking water from a small creek. They dipped the tips of their claws into the water, moved the claws toward the mouth, and allowed the adhering water to drip onto and behind the maxillipeds that cover the mouth. In addition to oral water uptake, uptake through the anus into the hindgut was reported (Fox 1952). Other groups, such as some isopods, developed highly specialized cuticular microstructures on their legs for water uptake (Horiguchi et al. 2007).

Proteins and Essential Amino Acids

The requirements for protein vary greatly between species and also depend on feeding habits. Carnivorous and omnivorous species will easily meet their demand with their normal diet, whereas herbivorous species need to optimize their ability to assimilate proteins. In addition to protein, chitin, which is present in arthropod shells, provides another valuable source for nitrogen. Herbivorous crustaceans adapted to low-nitrogen diets by elevating their feeding rates, keeping gut retention times short, and increasing the assimilation rates for soluble cellular materials (Linton and Greenaway 2007). In aquaculture, fish meal is a common source for proteins. The dietary amounts of proteins should range between 30% and 50%. Supplements of carbohydrate can reduce the demand of proteins.

Table 9.1. Essential amino acid ratio (percentage of all essential amino acids) of whole animals at different stages of growth and tail muscle (*).

Amino acid	*Litopenaeus vannamei*	*Penaeus monodon*	*Penaeus japonicus*	*Macrobrachium rosenbergii* *
Arginine	13.7	15.3	15.2	20.6
Histidine	3.8	4.7	4.5	4.5
Isoleucine	10.8	8.5	8.6	7.2
Leucine	16.3	14.6	15.0	14.8
Lysine	14.4	14.5	15.8	17.3
Methionine	1.4	7.4[a]	7.5[a]	6.5
Phenylalanine	11.3	15.5	16.8[b]	7.4[b]
Threonine	9.1	7.6	8.2	7.6
Tryptophan	2.1	2.1	–	–
Valine	10.6	9.9	8.3	7.3

[a] Methionine and cysteine.
[b] Phenylalanine and tyrosine.
After Mente et al. (2002).

The dietary amino acid composition should be correlated with the amino acid profile of the whole body. Ten amino acids cannot be synthesized by crustaceans. These essential amino acids and their relative amount are listed in Table 9.1. Shrimp tail muscle is rich in arginine. Therefore, arginine was often the first limiting factor when experimental diets were administered (Cuzon et al. 2004).

Lipids and Essential Fatty Acids

Lipids are both important structural compounds and valuable energy stores in crustaceans. Phospholipids (PL) represent the majority of polar lipids and are essential elements of cellular membranes. Storage lipids are predominantly deposited as neutral lipids in the form of wax esters or triacyl glycerols, with both of these helping to overcome periods of food deprivation. The preference for either of these storage lipids varies between species (Kreibich et al. 2010; Fig. 9.3). Wax esters serve as long-term lipid stores and may remain in the body for several months, as, for example, in high-latitude species. Triacyl glycerols, in contrast, are considered short-term depot lipids. They are used to meet metabolic energy requirements and are subjected to continuous turnover. Lipids are predominantly stored in the midgut gland. Some species of euphausids deposit lipids in a connective tissue forming the so-called fat body. High-latitude species of copepods may store lipids within oil sacs.

Fatty acids, the principal compounds of most of the crustacean storage lipids, are synthesized by elongation of acetyl CoA primers. Crustaceans are capable of synthesizing saturated fatty acids and monounsaturated fatty acids. However, they cannot synthesize significant amounts of *n*-3 and *n*-6 polyunsaturated fatty acids. Accordingly, essential fatty acids like eicosapentaenoic acid (EPA, 20:5(*n*-3)) and docosahexaenoic acid (DHA, 22:6(*n*-3)) need to be extracted from the diet (Dalsgaard et al. 2003). Elevated dietary levels of linoleic acid (18:2*n*-6) and *n*-3 highly unsaturated fatty acids increased fecundity, egg hatching efficiency, and larval quality of *Macrobrachium rosenbergii* (Cavalli et al. 1999). Supplements of sterols and polyunsaturated fatty acids had beneficial effects on *Daphnia magna* (Martin-Creuzburg and von Elert 2009). Sterols seem to be important for unconstrained

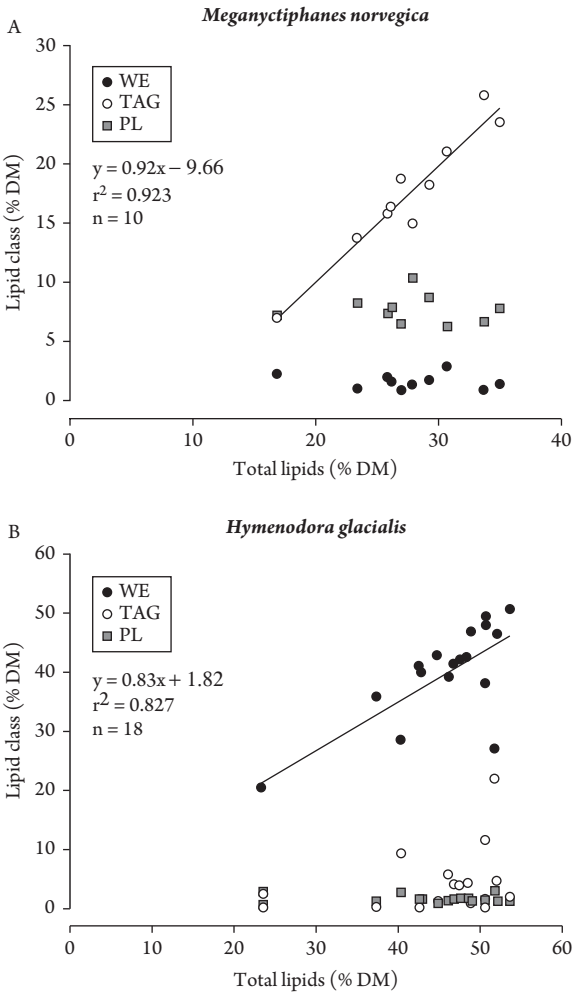


Fig. 9.3.
Correlation between the amount of wax esters (WE), triacylglycerols (TAG), phospholipids (PL), and the amount of total lipids in % of the dry mass for (A) *Meganyctiphanes norvegica* (Euphausiacea) and (B) *Hymenodora glacialis* (Caridea). Note the different scaling of the axes. From Kreibich et al. (2010), with permission from Inter-Research.

somatic growth, whereas polyunsaturated fatty acids support egg production. Cholesterol is essential and needs to be administered with the diets to meet demands (Cuzon et al. 2004).

Carbohydrates

Crustaceans possess a variety of digestive glucanases. They are able to digest polysaccharides such as starch, cellulose, or laminarin. Polysaccharides are sources for sugars that can be directly converted into energy, stored as glycogen, or used for the synthesis of chitin, fatty acids, or sterols, for example. Dietary requirements for carbohydrates are not defined. Glucose is only poorly utilized by crustaceans. Glucose-supplemented diets yield lower growth rates in shrimps than do diets with polyglucans like starch or glycogen (Shiau 1998). Rock lobsters (*J. edwardsii*) were fed diets with

different carbohydrate-to-lipid ratios (Johnston et al. 2003). Maximum growth and highest levels of lipid and dry matter in midgut glands and whole body were present at a diet of 27% carbohydrate and 13.5% lipid (2:1 ratio). Balanced supplements of carbohydrates may improve the growth performance of crustaceans because they provide immediate energy for metabolic processes.

Vitamins

Vitamin requirements were studied in several, predominantly commercial species. The recommended doses vary distinctly between species but also between ontogenetic stages within a species. Many vitamins are cofactors for enzymes or important metabolites. Others act as antioxidants, growth factors, or structural components (Table 9.2).

Antioxidants

L-Ascorbic acid (vitamin C) and tocopherol (vitamin E) are potent antioxidants. Both vitamins are traditionally used in shrimp feeds to improve the animals' health. Vitamin C deficiency caused "black death disease" in penaeid shrimps (Magarelli Jr. et al. 1979). Dietary levels of 20–130 mg vitamin C per kg diet supplied as a stable and bioavailable source allow a normal growth. Elevated

Table 9.2. The physiological functions of vitamins and their recommended feed levels.

Vitamin	Physiological function	Recommended feed level ($mg \cdot kg^{-1}$ diet)
Thiamin	Co-factor for metabolic enzymes	60
Riboflavin	FMN and FAD are intermediates in electron transfer in biological redox reactions	25
Niacin	Precursor for NAD^+/NADH and $NADP^+$/NADPH	40
Vitamin B_6	Co-factor for metabolic enzyme	50
Pantothenic acid	Required for CoA synthesis	75
Biotin	Prosthetic group of enzymes, epigenetic regulation of gene function	1
Folate	Deficiency causes poor growth	10
Vitamin B_{12}	Cobalt-containing cofactors of enzymes involved in nucleic acid and protein synthesis	0.2
Choline	Compound of cell membranes (phosphatidylcholine)	600
myo-inositol	Basis for signaling and second messenger molecules	400
Vitamin C	Antioxidant, synthesis possible in some species	200
Vitamin A	Many physiological functions, compound of eye pigments	250
Vitamin E	Antioxidant, protection of membrane-bound PUFAs	100
Vitamin D	Mediator in calcium metabolism	0.1
Vitamin K	Blood coagulation, calcium transport, co-factor for enzymes	5

After Conklin (1997).

levels of 1,500 mg/kg or more enhance the resistance to stress and diseases. Because ascorbic acid is quite vulnerable to oxidation, more stable derivatives with sulfate and phosphate moieties show beneficial effects when used in feeds.

Vitamin E in the diet can provide immediate protection against free radicals. Although the hepatopancreatic tissue has a strong intrinsic antioxidant capability, addition of vitamin E distinctly increased the radical scavenging ability (Díaz et al. 2004).

Carotenoids

Carotenoids fulfill various physiological functions in crustaceans (Meyers and Latscha 1997). In aquaculture, they were administered to improve growth, reproduction, oxidative defense, pigmentation, and cellular protection from light damage (Liñán-Cabello et al. 2002). However, the effects reported were often not consistent. Dietary carotenoids are the sole biological precursors of retinoids and thus pro-vitamin A in crustaceans. Retinoids, in turn, are involved in the activation of nuclear hormone receptors. They play important roles in many developmental processes, including embryogenesis and cell differentiation. Administration of retinol palmitate caused induction of the primary vitellogenic phase in crayfish females (*Cherax quadricarinatus*). In the shrimp *L. vannamei*, the concentrations of carotenoids as well as vitamin A were higher in the digestive glands of wild catches than in captive animals (Liñán-Cabello et al. 2003). The authors suggested that supplementation of carotenoids is important for captive shrimps to improve cellular protection at oocyte differentiation, for example.

Minerals

Minerals fulfill a variety of biochemical functions in cell homeostasis and signaling, as cofactors for enzymes, and in skeletal and tissue formation. The minerals representing the highest proportions are denoted macrominerals and comprise calcium, phosphorus, potassium, sodium, and magnesium. Microminerals are represented by copper, iron, manganese, selenium, and zinc (Table 9.3).

Calcium carbonate ($CaCO_3$) is the most important inorganic compound of the mineral phase of crustacean shells. Depending on the degree of calcification, animals lose a significant amount of calcium with the molt. Marine species are capable of extracting calcium from seawater, predominantly via the gill epithelia. The net influx amounts to 2 $mmol \cdot kg^{-1} \cdot h^{-1}$ (Zanotto and Wheatly 2003). Unlike marine crustaceans, animals from freshwater and terrestrial habitats are more restricted in their calcium availability. In these species, the uptake of calcium occurs predominantly via the diet, and, accordingly, calcium influx is facilitated through the digestive organs. The ingestion of calcium may range between 10 and 20 $mmol \cdot kg^{-1} \cdot h^{-1}$ over a calcification period of 72 h. The physiological processes of calcium transport in crustacean digestive organs are reviewed in detail by Zanotto and Wheatly (2003). In addition to calcium uptake from water or food, most species also developed mechanisms to retain calcium in the body over ecdysis. Crayfishes and some crabs retain most of the calcium as calcified concretions (gastroliths) in the cardiac stomach (Fig. 9.4). After ecdysis, the gastroliths are released into the digestive tract, and the calcium is rapidly reabsorbed. Other storage sites are the midgut gland that, however, predominantly accumulates calcium phosphate and sulfate rather than calcium carbonate. The calcium concentrations in the R and E cells of the midgut gland were higher during premolt than in the intermolt stage (Chavez-Crooker et al. 2003).

Probiotics, Prebiotics, and Other Supplements

Probiotics and prebiotics are increasingly used in fish and crustacean aquaculture to improve the health and survival of the hosts (Yousefian and Amiri 2009, Ganguly et al. 2010). For example, the

Table 9.3. Minerals in crustaceans and their function.

Mineral	Physiological function
Macrominerals	
Calcium	Component of enzymes, signaling, support of skeletal structures
Phosphorus	Component of membranes, nucleic acids, and various other biomolecules; structural support
Potassium	Maintenance of membrane potential, osmoregulation, acid–base regulation
Sodium	Same as potassium
Magnesium	Muscle function
Microminerals	
Copper	Integral part of the respiratory pigment hemocyanin (most Crustacea)
Iron	Component of hemoglobin (e.g., *Daphnia*), cytochrome, and enzymes
Manganese	Component of some enzymes
Selenium	Component of antioxidative enzyme glutathione peroxidase
Zinc	Component of some enzymes (zinc endopeptidases)

After Mente et al. (2002).

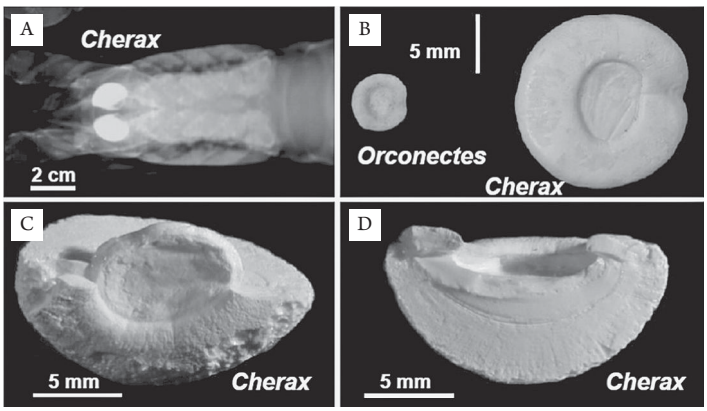


Fig. 9.4.
Localization, shape, and structure of gastroliths from crayfishes. (A) Radiography of the cephalothorax of *Cherax quadricarinatus* showing the X-ray dense gastroliths. (B) Dissected gastroliths from *Orconectes limosus* and *Cherax quadricarinatus*. The size is proportional to the size of the animals. (C) Broken gastrolith showing its internal aspects. (D) Cross-section showing the dense and striated structure of the gastrolith. From Luquet et al. (2013), with permission from MDPI AG.

supplement of probiotics (photosynthetic bacteria and *Bacillus* sp.) to basal shrimp diets led to significantly better growth than did unsupplemented feed (Wang 2007). Supplement of *Lactobacillus plantarum* modified the bacterial microbiota in shrimp digestive tracts and increased resistance to *Vibrio harveyi* infection (Vieira et al. 2010). In addition to dietary supplement of bacteria, administration of soybean isoflavones showed positive effects on the growth of shrimps *L. vannamei* as well (Chen et al. 2011). Immunological parameters like total hemocyte counts, activities of antioxidant enzymes, and resistance against *Vibrio alginolyticus* also increased. The defense mechanisms of crustaceans depend completely on the innate immune system. It is activated when pathogen-associated molecular patterns are recognized by soluble or cell surface host proteins (Vazquez et al. 2009). In order to understand the effect of probiotics in crustaceans, more intensive studies are needed on the function of their immune reactions.

Reactions to Starvation

Crustaceans experience starvation during periods of food scarcity. However, they also starve during and after molt when the exoskeleton, and, thus, the feeding appendages and masticatory structures of the foregut have to harden before the animals can continue feeding. Starvation affects the organism on different organizational levels ranging from behavioral changes to alterations of subcellular structures and molecular properties (Sánchez-Paz et al. 2006).

The reactions to prolonged starvation depend, on one hand, on the ability of the animal to reduce its metabolic rates. On the other hand, it depends on the amounts and availability of nutrient reserves in the body. Small planktonic copepods without significant lipid reserves (e.g., *Temora longicornis*) show first reactions to starvation very rapidly, within 24 h. They successively decrease digestive and metabolic enzyme activities, reduce egg production rates, and catabolize remaining body lipids (Kreibich et al. 2008).

Species that accumulate higher amounts of storage products can consequently better cope with starvation. Short-term starvation of up to 5 days in whiteleg shrimp (*L. vannamei*) caused a rapid reduction of plasma and hepatopancreas glucose and a decrease in hepatopancreatic glycogen levels (Sánchez-Paz et al. 2007). Triacylglycerol levels decreased first at the end of the starvation period (5 days), whereas protein levels remained unchanged.

The life cycle of Antarctic krill *Euphausia superba* is characterized by a strong seasonality of biological production. Krill may face severe starvation during the low-productive Antarctic winter. To withstand these conditions, *E. superba* is capable of reducing its metabolic activity. Laboratory experiments revealed a reduction of metabolic rates by 30% after 10 days of starvation (Auerswald et al. 2009). Moreover, *E. superba* is capable of catabolizing muscle proteins, which entails shrinkage of the animal (McGaffin et al. 2002).

Cytological changes appeared in the midgut gland of larval spider crabs *Hyas aranaeus* and postlarval prawns *Penaeus monodon* after starvation for a few days (Storch and Anger 1983, Storch et al. 1984). The R cells were most sensitive and showed reduction in size, depletion of lipid stores, and pronounced swelling of mitochondria. In contrast to R cells, B cells showed only slight reactions, and F and E cells remained almost unchanged (Vogt et al. 1985). However, after a starvation of 13 days, all cell types disintegrated, and after 15 days the postlarvae died.

Effects of starvation on the immune response and oxidative stress were studied in *Carcinus aestuarii* (Matozzo et al. 2011). Crabs that starved for 7 days showed elevated numbers of hemocytes. Glucose concentrations increased in the cell-free hemolymph, but protein values declined. Phenoloxidase activity was elevated in hemocyte lysates. Matozzo et al. (2011) concluded that starvation influenced immune parameters in crabs and that the animals can modulate their cellular and biochemical parameters to cope with starvation.

Natural and Anthropogenic Toxins and Their Effects

Many micro- and macroalgae, bacteria, fungi, and protists are capable of producing and releasing adverse metabolites. Exposure of crustaceans to harmful chemicals can have various effects on all organizational levels (Brouwer and Lee 2008).

Tannins and phlorotannins, for example, are polyphenolic compounds from terrestrial plants and kelp, respectively. They appear predominantly in coastal areas and are nutritionally adverse because they can precipitate proteins, inhibit digestive enzymes, and impair the utilization and uptake of vitamins and minerals (Chung et al. 1998, Sotka and Whalen 2008). On the other hand, it was reported that tannins may be beneficial by reducing mutagenicity, showing anticarcinogenic activity, and inhibiting growth of fungi, bacteria, and viruses. Exposure of freshwater crustaceans (Cladocera, Copepoda, and Ostracoda) to tannic acids showed a sequential degradation of the midgut epithelium. The strength of the effect differed between species and depended on the duration of exposure and the concentration assayed (Pautou et al. 2000). The resistance to tannic acid of the crustacean was correlated with qualitative and quantitative features of detoxification enzymes, cytochrome P450 enzymes, esterases, and glutathione S-transferase (Rey et al. 2000).

During blooms of cyanobacteria, elevated amounts of biologically active secondary metabolites are released. Among these metabolites, proteinase inhibitors are present that are capable of inhibiting the digestive proteinases of the cladoceran *D. magna* (Schwarzenberger et al. 2010). The cladocerans react to the presence of cyanobacterial proteinase inhibitors by upregulation of proteinase expression. They increase their capacity for protein digestion and thus may compensate for the adverse effects of the inhibitors (Schwarzenberger et al. 2010).

In addition to natural toxins, numerous anthropogenic xenobiotics pollute aquatic and terrestrial habitats. In coastal regions, industrial activities, ship traffic, or oil drilling activities liberate, for example, petroleum components (hydrocarbons), heavy metals, or antifouling agents. Exposure to water-soluble petroleum constituents significantly impairs the reception of food in European lobsters *Homarus gammarus* (Walter et al. 2008). Moreover, Lavarías et al. (2006) observed an increase in the activities of lipid-catabolizing enzymes in shrimps *Macrobrachium borellii* after exposure to sublethal concentrations of water-soluble petroleum compounds. Ultimately, exposure to these pollutants adversely affects the shrimp by raising its energy demand.

Some crustaceans, however, may also make use of environmental toxins. For example, the hydrothermal vent crabs *Xenograpsus testudinatus* are adapted to feed on poisoned planktonic organisms. The crabs live in crevices very close to shallow water hydrothermal vents off of Taiwan (Jeng et al. 2004). The discharges of the vents are highly acidic and rich in sulfur. The crabs can resist elevated temperatures, low pH, and high concentrations of dissolved toxic gases. Moreover, they feed on planktonic organisms that were killed by the toxic plumes and fell to the bottom (Hu et al. 2011). During slack water, huge numbers of crabs leave their crevices and pick up fallen dead organisms. With the onset of the tidal currents, they seek shelter again. Accordingly, these hydrothermal vent crabs are exposed to toxins in two ways: the surrounding water and the ingested food. Apparently, the crabs are capable of coping with toxins and high metal ion loads and thus might serve as suitable models in crustacean toxicology and, particularly, in toxin resistance studies.

Dietary Shifts During Ontogenesis

Ontogenetic shifts in feeding habits and nutrition may appear in different ways. The animals may simply grow and, consequently, change the size spectrum of their prey. As they grow, they change

their morphology or anatomy. During development, the ontogenetic stages may change their habitat and thus food sources. They may also change between lecithotrophy and planktotrophy, as demonstrated for the larvae of some species.

Size gain and habitat shift often appear concomitantly. For example, lobster larvae feed on small zooplankton or phytoplankton. As the lobsters metamorphose, the food originates from the benthic environment but still includes small organisms that can be handled by the early post-larvae. When the post-larvae mature and grow, they develop strong claws and start hunting for larger prey organisms. They are able to crack other crabs and mollusks with their strong claws and can even catch bottom-living fishes. Stomach content analysis of early benthic stages of American lobsters revealed a progressive dietary shift with increasing size (Sainte-Marie and Chabot 2002). Small lobsters fed on soft or readily available food such as fish carrion, small bivalves, macroalgae, and meiobenthic crustaceans. Larger ones preferred more mobile and more nutritious prey including other crustaceans and fish (Sainte-Marie and Chabot 2002).

Significant morphological and anatomical changes are frequent in lecithotrophic larvae from higher latitudes. These larvae do not start feeding after hatching but utilize yolk reserves that they maintained from the embryonic stages. In this respect, the larvae of the king crab *Lithodes santolla* do not feed during the first larval stage but catabolize their yolk (Calcagno et al. 2004). These larvae also lack a functional digestive tract and do not express digestive enzymes such as endopeptidases, which are required for the digestion of external food (Saborowski et al. 2006). In the subsequent stages, however, they start feeding and develop a functional digestive system to utilize external food (Fig. 9.5).

Most crustacean species undergo a more continuous larval development through a sequence of predetermined larval stages. Between these stages, however, distinct differences in enzyme expression and thus capacity of food utilization may also occur. For example, larval development of the pink shrimp *Farfantepenaeus paulensis* passes through five to six nonfeeding naupliar stages, followed by three protozoeal stages and three mysid stages prior to the postlarval stages. Lemos et al. (1999) found a significant increase of endopeptidase activities in the protozoea stages, activity that declined again in the subsequent mysis and postlarval stages. The authors conclude that the elevated enzyme activities may be related to higher energy demands due to increased swimming behavior and feeding activity.

DIGESTIVE ENZYMES

Studies on digestive enzymes of crustaceans can be traced back to the 1870s. Hoppe-Seiler (1877) and Krukenberg (1878) were the first to report about digestive processes and the proteolytic properties of the gastric fluid of the crayfish *Astacus astacus*. Thereupon, in the early 20th century, a number of excellent physiologists and biochemists established the cytological and biochemical fundamentals of crustacean digestive physiology and biochemistry which, for the most part, are still valid. However, the detailed factors that determine the properties and composition of the expressed enzymes are poorly understood and thus still under debate. A certain genetic disposition seems apparent that determines the basic expression patterns of enzymes, such as the predominance for cysteine- or serine-proteinases (Teschke and Saborowski 2005, Navarrete del Toro et al. 2006). But, beyond that, the composition of enzymes seems also to favor specific feeding behavior and may indicate trophic resource utilization, as shown in brachyuran crabs (Johnston and Freeman 2005).

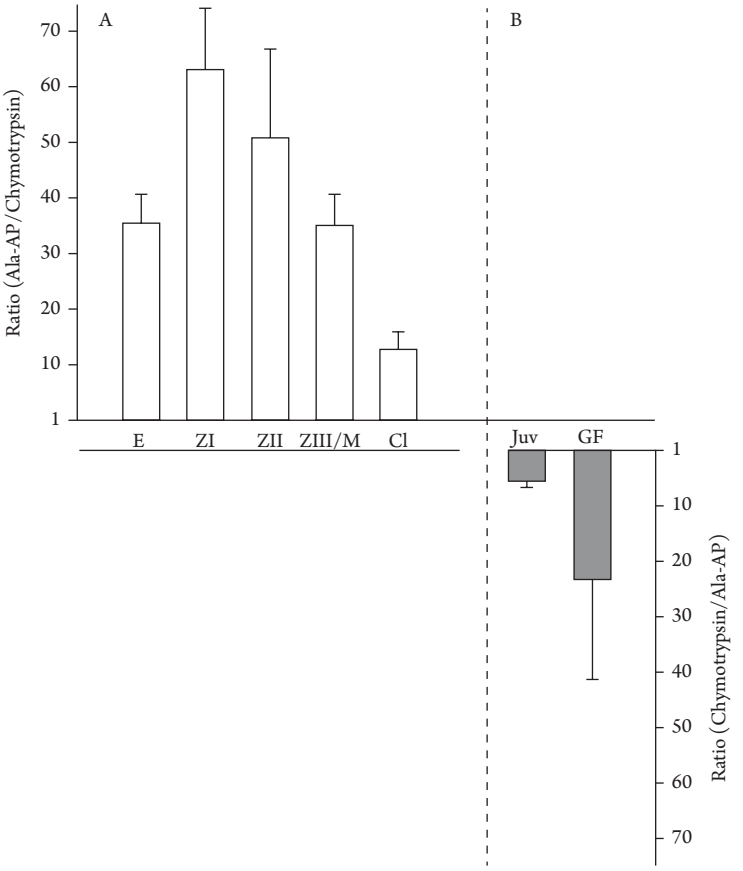


Fig. 9.5.
Activity ratios of alanine-aminopeptidase (Ala-AP) and chymotrypsin in different larval stages, juveniles, and the gastric fluid of adult *Lithodes santolla*. (A) Eggs (E), zoea I (ZI), zoea II (ZII), zoea III, and megalopa (ZIII/M), crab I (CI). (B) Juvenile midgut gland (juv), gastric fluid of adults (GF). From Saborowski et al. (2006), with permission from Springer.

Sites of Enzyme Synthesis and Enzyme Action

In all crustaceans studied so far, enzyme synthesis and enzyme secretion take place in specialized cells in the midgut region or in the midgut caeca (Fig. 9.6). The digestive cellular processes in higher and in lower Crustacea were reviewed in detail by Brunet et al. (1994).

In those crustaceans that do not possess distinct midgut diverticula, the midgut lining forms specialized cells that may produce and secrete enzymes. Cladocera, for example, show a quite simple cellular organization of their gut. However, they also show variations in cell structure along the midgut that can be related to particular functions, such as enzyme secretion and nutrient resorption (Schultz and Kennedy 1976). Digestive enzymes are secreted in a holocrine fashion, preferably by the cells in the mid-regions of the gut. Posteriorly, the midgut cells become smaller and seem to be more responsible for the resorption of nutrients.

In copepods, the epithelium of the midgut regions consists of different cell types called R, F, and B cells, according to those in the higher crustaceans. The F cells show structures such as abundant rER and active dictyosomes, which indicate enzyme synthesis and secretion.

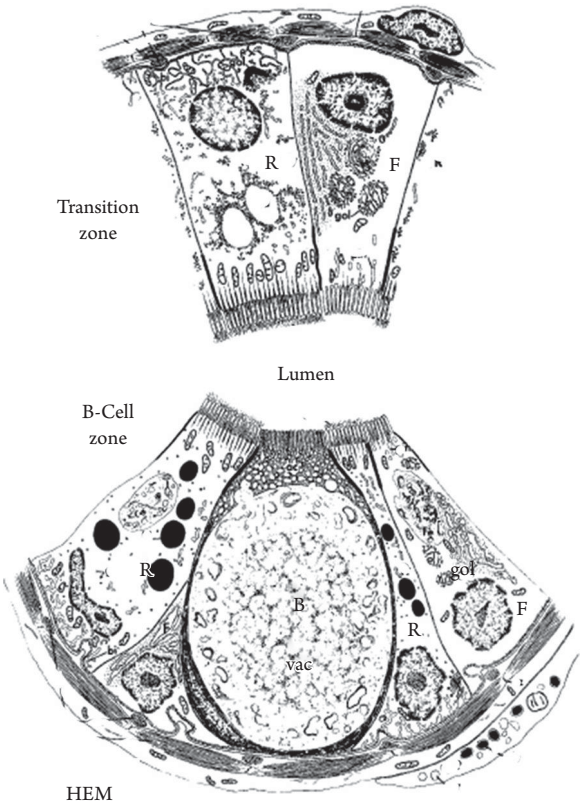


Fig. 9.6.
Schematic drawing of the different mature cell types (R, F, and B cells) within the hepatopancreatic tubules of a decapod. Abbreviations: Golgi body, gol; hemolymph surrounding tubule, HEM; VAC, vacuoles. From Loizzi (1971), with permission from Springer.

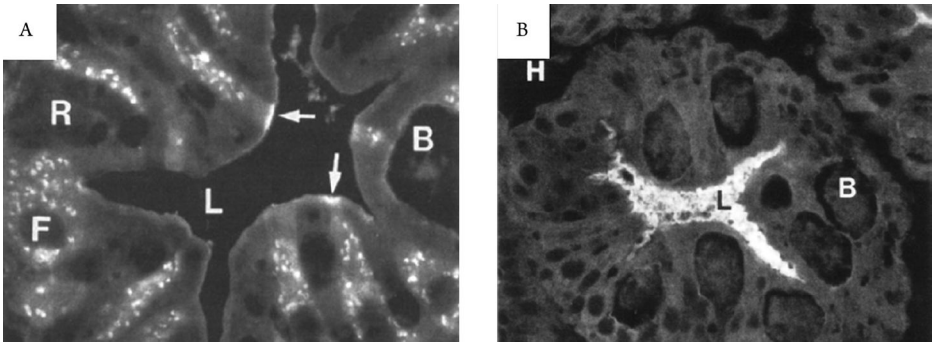


Fig. 9.7.
Immunohistochemical localization of protease in the hepatopancreatic tubules of the crayfish *Astacus astacus*. (A) Intracellular labeling of protease in F cells and predominant appearance in the apical part (arrows) of the cells and around the cell nucleus, ×240. (B) Strong fluorescence is located in the lumen of the midgut tubule hours after induction of enzyme production and release of enzymes into the lumen, ×400. B cells (B), R cells (R), F cells (F), lumen (L), hemolymph space (H). From Vogt et al. (1989), with permission from Springer.

In the higher crustaceans, the midgut gland forms the principal site of digestive enzyme synthesis and enzyme secretion. The tubules of the midgut gland contain three cell types that are directly involved in the digestion and assimilation of nutrients: the R, F, and B cells. The R cells perform the resorption of nutrients, whereas the F cells are reported as the sites of enzyme synthesis. Vogt et al. (1989) confirmed the production of digestive proteases in the F cells of crayfish by immunohistochemistry and incorporation of radiolabeled amino acids (Fig. 9.7). Toullec et al. (1992) separated the different cell types of the midgut gland of the caridean shrimp *Palaemon serratus* and found maximum amylase activities within the fractions also containing the F cells. Lehnert and Johnson (2002), as well as Hu and Leung (2007), detected the mRNA for cathepsin L and other digestive enzymes solely within the cytoplasm of the F cells in the shrimps *P. monodon* and *Metapenaeus ensis*. No enzyme synthesis was detected in the cytoplasm of B cells. However, several studies revealed the presence of the mature digestive enzymes within the B cells and also in the lumen of the midgut gland tubules. A plausible model for the function of the midgut gland and the interplay of the different cell types was given by Vogt (1994). In brief, the mRNAs, as well as the enzymes, are synthesized within the F cell. The enzymes are released through subapical vesicles into the tubule lumen of the midgut gland, seep through the tubules and the ducts, and finally accumulate in the stomach. After ingestion and mechanical processing of food within the stomach, the chyme consisting of enzymes and predigested foods is pressed through the pyloric filter and back into the midgut gland tubules. Part of the nutrients is resorbed by the R cells. B cells incorporate the chyme by endocytosis and continue to process the nutrients intracellularly in the huge vacuoles of the cells (Lehnert and Johnson 2002). This process is terminated by apocrine or holocrine extrusion of the B cells into the lumen of the tubules. Accordingly, it can be considered that the B cells fulfill two functions: intracellular digestion of nutrients and elimination of waste material (Vogt 1994).

Recent comparative studies gave evidence that the composition of digestive enzymes varies considerably between species and, probably, may show specific patterns for certain groups or taxa. Most distinctly, group-specific separation becomes evident in the expression of proteolytic enzymes (endopeptidases). Most of the endopeptidases in the crustacean digestive tract belong to a group of serine-, cysteine-, aspartate-, or metallo-endopetidases. The serine-endopeptidases comprise trypsin, chymotrypsin, and the recently established group of brachyurins. These enzymes form the major share of gastric endopeptidases in many of the decapod taxa such as the Anomura, Brachyura, Palinura, and Penaeidea.

The extracellular degradation of food items within the lumen of the digestive tract is the initial step of enzymatic degradation. It releases oligomers, monomers, or other fragments of biological molecules that, in the second step of enzymatic degradation, can be incorporated by the resorptive cells of the midgut gland. The resorbed nutrients are finally processed by the digestive cells. They can be catabolized to provide metabolic energy, used for somatic growth or reproduction, or deposited as storage material.

For a long time, it was not clear whether crustacean digestive enzymes and, particularly, proteinases, are synthesized and released by the secretory cells in an already active form or as an inactive pro-enzyme. The first description of a putative zymogen sequence was provided by Klein et al. (1998) for trypsin from the shrimp *L. vannamei*. The inactive precursor, trypsinogen, was detected in the cells and the tubule lumen of the midgut gland of *L. vannamei* (Sainz et al. 2004). Cathepsin L, a cysteine endopeptidase, was also shown to be synthesized as an inactive pro-enzyme (Le Boulay et al. 1998). Further studies were carried out on astacin, a novel metalloprotease from the crayfish *A. astacus*. Astacin is produced as a zymogen (Geier at al. 1997). The pro-enzyme has an extension of 49 amino acids, which is removed during the maturation process of the enzyme. Pro-astacin was detected within the F cells. After release, the pro-enzyme was also present within the tubules of the midgut gland but not in the cardiac stomach. The activation of astacin happens by stepwise autoproteolytic degradation of the 49 amino acid extension (Möhrlen et al. 2001, Guevara

et al. 2010). It seems likely that many, if not all, proteolytic enzymes are synthesized as pro-enzymes but are rapidly activated after secretion.

Extracellular and Intracellular Digestion

The digestive enzymes secreted by the hepatopancreatic cells accumulate in the stomach. Upon starvation, the gastric fluid contains almost no food items but consists solely of an aqueous solution of digestive enzymes, some emulsifiers, and some inorganic compounds. The amount of protein comprises about 5%. As soon as food is ingested and further minced with the aid of the gastric mill, the enzymes start to degrade the food compounds.

The enzymatic cocktail in the stomach consists predominantly of endo-hydrolases. These enzymes cleave internal bounds of their targets, which are dietary macromolecules such as proteins, polysaccharides, or nucleic acids. The resulting mono- and oligomers are absorbed by the resorptive cells of the midgut region. In the higher crustaceans, the entire chyme including the predigested nutrients is pressed through the pyloric filter into the tubules of the midgut gland. There, the nutrients are incorporated by the R cells and subjected to further metabolic processing.

Studies on membrane transport within the gastrointestinal tract of crustaceans were most intensively carried out on isolated brush border membrane vesicles (BBMV; Ahearn 1987). BBMV from American lobster (*Homarus americanus*) midgut glands possess at least seven distinct transport proteins for nutrient absorption (Ahearn et al. 1992). Four of them are sodium dependent (carrier systems for glucose, inositol, L-leucine, L-glutamate) and three are sodium independent (L-alanine, L-proline, and a second system for L-leucine; Berra et al. (2006). Reduction of the pH (down to pH 4) stimulated the uptake rates of both Na^+-dependent and Na^+-independent systems.

Glucose transport mechanisms in crustaceans were reviewed by Verri et al. (2001). The hepatopancreatic brush border membranes of shrimps (*L. vannamei*) and lobsters (*H. americanus*) have sodium-dependent D-glucose transporters of the GLUT-family (Zhao and Keating 2007) and, at least in lobsters, also for fructose. The transporters showed immuno-crossreactivity with antibodies raised against mammalian GLUT transporters and high sequence similarity with mammalian and insect transporters (Ahearn et al. 1985, Verri et al. 2001, Sterling et al. 2010).

Uptake and transport of other dietary requirements were studied for only a few substances. The transport of myo-inositol was shown to be Na^+- but not K^+-dependent. However, it was sensitive to pH, showing optimal influx at neutral pH inside and outside the vesicles (Siu and Ahearn 1988). The transport system of folic acid (Pte-Glu) was studied in BBMV isolated from the midgut gland of the prawn *Marsupenaeus japonicus*. The transport into the vesicles appeared to be carrier mediated. It also was stimulated by an inward proton gradient (pH 5.5 outside, 7.4 inside) and was unaffected by a sodium gradient (Blaya et al. 1998).

The Major Digestive Enzymes

Digestive enzymes can be studied from midgut gland extracts or directly from the gastric fluid, which can be simply and repeatedly obtained from living animals (Fig. 9.8). Most of the digestive enzymes belong to the enzyme class of hydrolases (EC 3). They are capable of cleaving various molecular bonds such as ester bonds, glycosidic bonds, or peptide bonds by reactions involving the addition of water.

From the beginning, research on digestive enzymes of Crustacea was focused on the proteolytic enzymes and, particularly, on trypsin. The reasons for this fascination may be the fact that trypsin was one of the first proteases identified, and it also plays an important role in digestion, as well as in the regulation of various physiological processes (Neurath 2001). The proteinases that have been characterized so far belong predominantly to four major groups: the serine-, cysteine-,

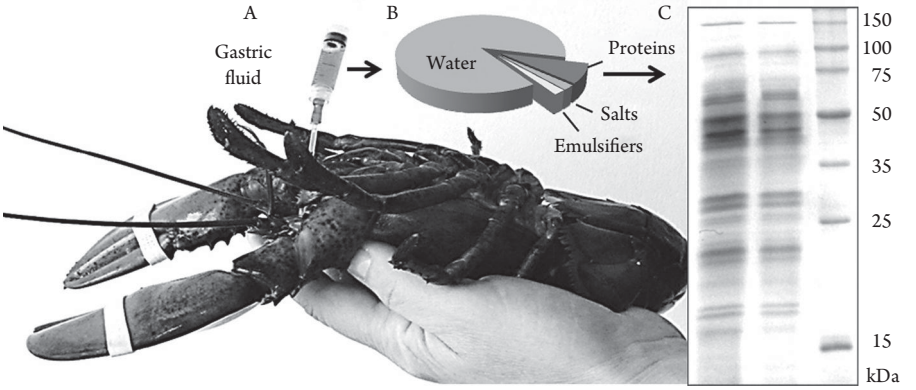


Fig. 9.8.
Gastric fluid from lobster *Homarus americanus*. (A) Sampling of gastric fluid from live lobsters using a syringe and a flexible Teflon tube. The sampling does not harm the animal and can be repeated after a few days. Photo by Kristine Reuter. (B) The gastric fluid contains about 5–6% protein, 2–3% inorganic salts, and a similar amount of emulsifiers. The majority (>90%) of the gastric contents consists of water. (C) Electrophoretic separation of gastric proteins. When the animals have starved for 2–3 days before sampling, these proteins represent solely the digestive enzymes but no alimentary proteins.

and metallo-endopeptidase families, and, recently, aspartic proteases were isolated from the gastric fluid of lobsters (Rojo et al. 2010).

Trypsin

Trypsin shows a distinct catalytic specificity, preferably hydrolyzing proteins and peptides at the carboxylic site of arginine and lysine residues. Trypsin-like enzymes were considered by many authors to be the most important proteolytic enzymes in crustaceans. In fact, these enzymes were identified in a large number of species including ancient forms such as the "living fossil" *Triops* sp. (Maeda-Martínez et al. 2000) or the brine shrimp *Artemia salina* (Pan et al. 1991). Other crustaceans, such as the cladoceran *D. magna* (von Elert et al. 2004), several copepod species (Hallberg and Hirche 1980, Mayzaud et al. 1998, Lischka et al. 2007, França et al. 2010), and the nauplius larvae of the cirriped *Elminius modestus* (Le Vay et al. 2001) possess high trypsin activities as well. Tryptic enzymes were studied most intensively in Malacostraca and, particularly, in those species with significant commercial importance. For example, Klein et al. (1998) obtained the coding sequences of three trypsin genes from the whiteleg shrimp *L. vannamei*. Two of them were expressed in the midgut gland. The iso-enzymes were isolated and genetically characterized by Sainz et al. (2004, 2005). A detailed review of invertebrate trypsins and some of their properties was recently provided by Muhlia-Almazan et al. (2008).

Chymotrypsin

Chymotrypsin, another serine endopeptidase in crustaceans, appears in lower numbers of isoforms but it often exhibits a higher catalytic activity than does trypsin. Chymotrypsin is less specific, preferentially cleaving peptide bonds at the carboxyl side of the aromatic amino acids tyrosine, tryptophan, and phenylalanine. Probably due to analytical difficulties, it was long considered to be absent in crustacean digestive organs. However, Kimoto et al. (1985) reported high activities of

chymotrypsin in the Antarctic krill *E. superba*. Using a new and specific substrate, Tsai et al. (1986, 1991) detected and isolated chymotrypsin-like enzymes in three penaeid species. However, only marginal activity was present in the caridean shrimp *M. rosenbergii*, which, in turn, indicated that chymotrypsin may not be ubiquitous among Crustacea. Subsequently, van Wormhoudt et al. (1992) isolated chymotrypsin isoforms from *L. vannamei* and cloned the cDNAs encoding these enzymes (Sellos and van Wormhoudt 1992). To date, chymotrypsin-like enzymes were identified in a variety of crustacean species such as the squat lobster *Pleuroncodes planipes* and the crayfish *Pacifastacus astacus* (García-Carreño et al. 1994), the edible crab *Cancer pagurus* (Saborowski et al. 2004), and the branchiopod *D. magna* (von Ehlert et al. 2004). Chymotrypsin-like enzymes are, however, not ubiquitous in crustaceans. Comparative studies among some eucarid crustaceans revealed that crustaceans belonging to the Brachyura, Anomura, and Euphausiacea express high chymotrypsin activities. In contrast, representatives of the Astacura and Caridea often express only low activities (Saborowski, unpublished). It seems as if the appearance of chymotrypsin-like enzymes might be a group-specific characteristic within the Crustacea.

Collagenases, Brachyurins

Since the 1960s and 1970s, proteolytic enzymes were isolated from crustacean midgut glands and were reported to possess unique catalytic properties. In addition to showing trypsin and chymotrypsin activities, they were highly active in degrading native triple-helix collagen under physiological conditions. The prototype of these collagenolytic enzymes was isolated from the midgut gland of the fiddler crab *Uca pugilator* (Eisen and Jeffrey 1969, Eisen et al. 1973). The amino acid sequence revealed its nature as a serine endopeptidase and, consequently, its divergence from mammalian collagenases, which are zinc-metalloenzymes (Grant et al. 1980). The enzyme from *U. pugilator*, denoted as serine collagenase 1, was recognized as a novel member of the chymotrypsin protease family (Tsu and Craik 1996, Perona et al. 1997). Soon, proteases with similar collagenolytic properties were identified in a shrimp (*L. vannamei*), the shore crab *Carcinus maenas*, and in the Kamtschatka crab *Paralithodes camtschaticus* (Sellos and van Wormhoudt 1992, Roy et al. 1996, Rudenskaya et al. 2004). A cold-adapted analogue from Antarctic krill *E. superba*, named euphaulysin, shares many of these characteristics (Gudmundsdóttir 2002). Since 1992, the new term "brachyurins" (EC 3.4.21.32) was recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) for these collagenolytic serine endopeptidases (Rudenskaya 2003). The brachyurins are of interest for studies of structure–function relationships and for understanding of the evolution of serine proteases. Due to their catalytic properties, they appear interesting for dermatological applications in terms of wound care.

Astacin

Astacins are another example of a new group of digestive enzymes that were first discovered and studied in crustaceans. These enzymes are zinc endopetidases (Stöcker et al. 1988) that were first isolated from the gastric fluid of the crayfish *Astacus fluviatilis*. Although already described by Pfleiderer et al. (1967), the detailed analyses of the enzyme started with the identification of the amino acid sequence by Titani et al. (1987). The endopeptidase, with a molecular mass of 22.6 kDa, showed unusual specificities and was not affected by the hitherto known inhibitor for the established families of proteases. An enzyme from the crayfish *A. astacus* served as the prototype for the new astacin family. The preferred cleavage sites of astacin are peptide bonds in front of small aliphatic residues. The crystal structure of the enzyme was discovered by Bode et al. (1992), and the catalytic mechanism was investigated by Stöcker et al. (1993) and Stöcker and Bode (1995).

Today, the astacin family of metalloproteases comprise a versatile and diverse group of enzymes with respect to function and evolutionary history. They are present in bacteria and animals but were not found in plants and fungi. Their physiological roles span a variety of key functions beyond digestion (Becker-Pauly et al. 2009).

Cathepsin L

The term *cathepsin* arose from the ancient Greek meaning "digestion," and cathepsins comprise a number of proteolytic enzymes with different structural and catalytic characteristics. Cathepsins are involved in a variety of intracellular metabolic reactions. About a dozen cathepsins are known today, which, however, belong to the different groups of serine-, cysteine-, and aspartate-proteinases. Laycock et al. (1989, 1991) isolated a cysteine protease that accounted for 80% of the proteolytic activity in the gastric fluid of the American lobster. It showed high similarity to papain and, particularly, to the L cathepsins of vertebrates. Le Boulay et al. (1996, 1998) cloned the cathepsin L from *L. vannamei* and analyzed its gene organization, which is homologous to that of rat cathepsin L. Accordingly, the shrimp cathepsin L was categorized as a member of the cysteine proteinase of the papain superfamily. Other digestive enzymes of this family were identified and studied in the penaeid shrimp *M. ensis* (Hu and Leung 2004, 2007), and the caridean shrimps *Pandalus borealis* (Aoki et al. 2003, 2004), *Crangon crangon*, and *C. allmani* (Teschke and Saborowski 2005). Cathepsin L is known as an intracellular lysosomal enzyme in many tissues. Thus, its role in extracellular digestion within the crustacean stomach is unusual and remains to be investigated in more detail.

Exopeptidases

From a functional point of view, endopeptidases should be secreted by the midgut gland and should perform protein digestion in the stomach, whereas exopeptidases are supposed to be highly active in the midgut gland tubules and the brush border membranes of the resorbing cells. Early studies on exopeptidases, including carboxypeptidase A and B, arylamidases, and dipeptidases, are summarized by Dall and Moriarty (1983). Recent studies addressed various physiological and biochemical aspects with respect to digestive exopeptidases. Carboxypeptidases were observed in the Atlantic blue crab *Callinectes sapidus* (Dendinger 1987) and leucine-aminopeptidases were isolated and characterized from the midgut gland of the crayfish *Procambarus clarkii* (de la Ruelle et al. 1992). Fernandez et al. (1997) showed variation in the activities of amino- and carboxypeptidases in *Farfantepenaeus notialis* during the reproduction and molting cycles, and Ezquerra et al. (1999) showed that different diets can influence the activities of a set of exopeptidases in the whiteleg shrimp *L. vannamei*. Sakharov and Prieto (2000) characterized carboxypeptidases from the midgut gland of the king crab *P. camtschatica*, which is the ingredient of a commercial enzyme preparation. The concerted interplay, however, between endo- and exopeptidases in crustaceans is still poorly understood. Recent comparative investigation showed that high activities of exopeptidases may also occur in the gastric fluid of the crayfish *Astacus leptodactylus* but not in the clawed lobster *H. gammarus* and the crab *C. pagurus* (Weber and Saborowski, unpublished). The physiological function of the enzyme in the gastric fluid, as well as the cellular origin and expression pattern of the enzyme, demands deeper investigation.

Lipases/Esterases

Digestive lipases catalyze the hydrolysis of triacylglycerols and thus the liberation of fatty acids and glycerol. Lipases known to date vary considerably in size and in primary structure. However,

they all belong to the α/β-hydrolase superfamily and they have in common a catalytic triad of His, Ser, and Asp (Rivera-Pérez et al. 2011). Crustacean lipases do not depend on colipase or bile salts as mammalian lipases do. Cherif et al. (2007) and Cherif and Gargouri (2009) isolated a thermostable lipase with a molecular mass of 65 kDa from the midgut gland of the shore crab *C. maenas*. The larval stages of the shrimp *L. vannamei* show lipase activity already in the first stages, indicating that these stages are capable of degrading dietary lipids or storage lipids (Rivera-Pérez et al. 2010).

Chitinases

The process of biochemical chitin degradation demands a set of enzymes that first produce oligomers of the chitin chain by an endochitinase, commonly referred to as *chitinase*. The oligomers, in turn, serve as substrates for an exochitinase that, in concert with unspecific β-glucosidases, liberates the monomers, acetylated amino sugars (Saborowski et al. 1993). The exochitinase is commonly termed N-acetyl-β-D-glucosaminidase (NAGase). Chitinolytic enzymes are involved in different physiological processes such as molting, pathogen suppression, and digestion. In the euphausiids *E. superba* and *Meganyctiphanes norvegica*, Peters et al. (1998, 1999) distinguished enzymes that are involved in molting and those that are digestive enzymes. Digestive chitinases are not only suitable in degrading and utilizing chitinous arthropod shells; they can also help to utilize crystalline chitin from the spines of phytoplankton, as shown in Antarctic krill (Saborowski and Buchholz 1999). Several studies in various species identified genes encoding for digestive chitinase (Watanabe et al. 1998, Tan et al. 2000, Proespraiwong et al. 2010) suggesting that the utilization of dietary chitin is an ancient intrinsic characteristic of crustaceans.

Amylases

Amylases hydrolyze the α(1,4)-glycosidic bonds of starch and glycogen and liberate glucose oligomers and monomers. The distribution and activities of amylases from crustaceans were studied in detail by van Wormhoudt et al. (1995). Among 40 species of decapods, highest activities were present in shrimps and crabs. The molecular mass of the enzymes ranged between 30 and 55 kDa, and a high degree of polymorphism was apparent in many species. The sequences of amylase genes in the shrimp *L. vannamei* were studied by van Wormhoudt and Sellos (2003). It is not fully understood yet whether the polymorphic amylase genes are of advantage for the animal. However, this knowledge might help in the formulation of carbohydrate-enriched feeds for shrimp aquaculture. The activities of amylase and, particularly, the ratios between amylase and protease activities have often been used to interpret dietary preferences of shrimps and their ontogenetic stages, respectively (Ribeiro and Jones 2000, Johnston et al. 2004).

Cellulases and Laminarinases

One of the most remarkable findings of the past decade's research is the verification of endogenous cellulolytic enzymes in many crustaceans. Cellulolytic activity was reported earlier in several species, but the activity could not be unambiguously assigned to the crustaceans and was possibly contributed by symbiotic bacteria. Byrne et al. (1999) obtained a complete cDNA open reading frame for an endo-β-1,4-glucanase of 469 amino acids from a hepatopancreas cDNA library of the crayfish *C. quadricarinatus*. The endogenous origin of the enzyme was confirmed by amplification of a polymerase chain reaction (PCR)-product from genomic DNA. The gene, belonging to the glycosyl hydrolase family 9 (GHF9), showed conserved

regions, which supports the ancestral nature of this gene (Crawford et al. 2004, Crawford 2005). Cellulase fragments were also sequenced from two other crayfishes (*Cherax destructor, Euastacus* sp.) and the terrestrial brachyuran *Austrothelphusa transversa* (Linton et al. 2006). The high cellulolytic activities in terrestrial anomurans also support the presence of endogenous enzymes (Greenaway 2003, Wilde et al. 2004). Recent studies on marine crustaceans confirmed the presence of the cellulase genes in various species of branchiopods, isopods, amphipods, euphausiids, and decapods (King et al. 2010, Saborowski unpublished). The ecological significance of cellulose digestion in marine crustaceans remains to be investigated in more detail. In coastal areas, detritivorous species may benefit from endogenous cellulases when feeding on terrestrial plant material washed into the sea or on kelp. In terrestrial crustaceans like the woodlouse *Porcellio scaber*, the benefits of endogenous cellulose digestion are obvious because these animals are important decomposers of plant material (Kostanjšek et al. 2010). Cellulose degradation in land crabs differs from the hitherto existing model (Fig. 9.9). It depends on a two-enzyme catalysis (endo-β-1,4-glucanase and glucohydrolase) rather than the generally proposed three-enzyme process (Allardyce et al. 2010). Studies on the digestion of storage products from kelp, such as laminarin or mannitol, are rare. Laminarinase enzymes from the freshwater crayfish *C. destructor* and land crab *Gecarcoidea natalis* were isolated and characterized by Allardyce and Linton (2008).

Properties of Enzymes

Proteolysis Resistance

The digestive enzymes that are released into the stomach are exposed to various proteinases and esterases that were simultaneously synthesized and secreted. Although it was shown that some proteolytic enzymes appear within the cell as inactive zymogenes after synthesis, they mature to fully

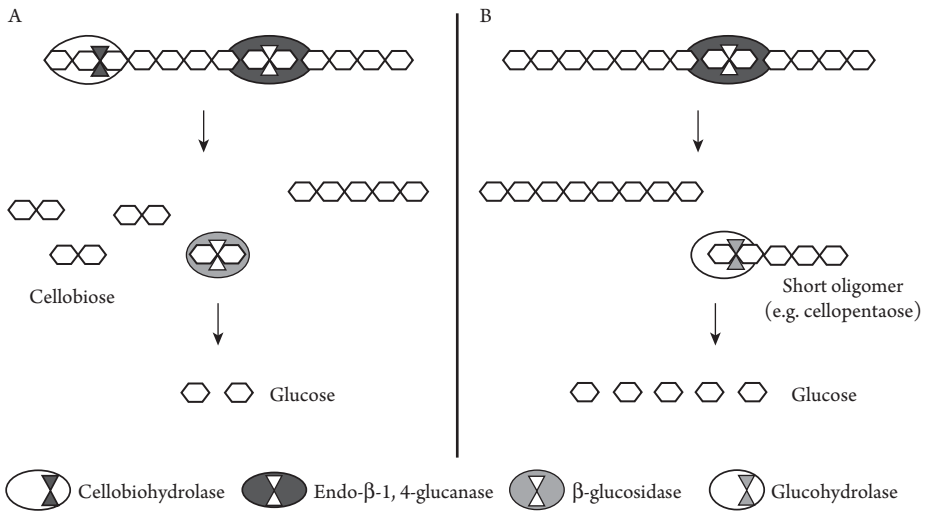


Fig. 9.9.
Scheme of cellulose degradation in (A) fungi and in (B) crustaceans. The conventional reactions involved in the degradation of cellulose comprise the catalytic action of an endo-β-1,4-glucanase, a cellobiohydrolase, and a β-glucosidase. In crustaceans, the glucohydrolase is capable of hydrolyzing glucose from both cellobiose (dimer) and short oligomers. From Allardyce et al. (2010), with permission from The Company of Biologists, Inc.

active enzymes after secretion and release into the stomach. Many of these extracellular enzymes show a remarkable stability and may resist degradation for extended periods (Saborowski et al. 2004). The enhanced stability may be related to structural properties such as the location of hydrolytic cleavage sites and stabilization through Ca^{2+}-incorporation and electrostatic interactions. For example, a trypsin isoform from the edible crab *C. pagurus* has a lower number of autolytic cleavage sites at the surface of the molecule compared to vertebrate trypsin (Fig. 9.10). And although a C-terminal fragment of about 11 amino acids was removed, the enzyme retained almost full activity (Hehemann et al. 2008).

Enhanced stabilities were also reported for other digestive enzymes, such as an endochitinase from the Antarctic krill *E. superba* (Saborowski et al. 1993) and a chymotrypsin from *C. pagurus* (Saborowski et al. 2004). These properties of the enzymes may help to avoid rapid degradation and thus may contribute to saving metabolic energy for enzyme synthesis and secretion. Depending on the feeding habits of the species or on the trophic environment (i.e., the supply of food), specimens may starve for extended periods. However, as soon as food appears, they must be able to digest it efficiently. An example for such a strategy of food utilization may be the Antarctic krill *E. superba*, which shows high digestive enzyme activities also on starvation and successfully exploits patchy food environments (Saborowski and Buchholz 1999).

Effects of pH

The gastric fluids at the extracellular sites of the digestive systems of crustaceans may range from slightly acidic to slightly alkaline. In the gut of the copepod *Calanus helgolandicus*, the pH was determined within the foregut and in the hindgut with a microinjection technique (Pond et al. 1995). The pH ranged between 6.11 and 7.25 in starved animals and up to 9.35 in animals that were fed with phytoplankton (Fig. 9.11). In the stomachs of Malacostraca, the pH ranges between approximately 5 and 6. The lowest pH values were measured in lobsters, at around 4.7 (Navarrete del Toro et al. 2006). Upon feeding, the pH rises 0.5 to almost 1 unit. The shift in pH favors the catalytic action of

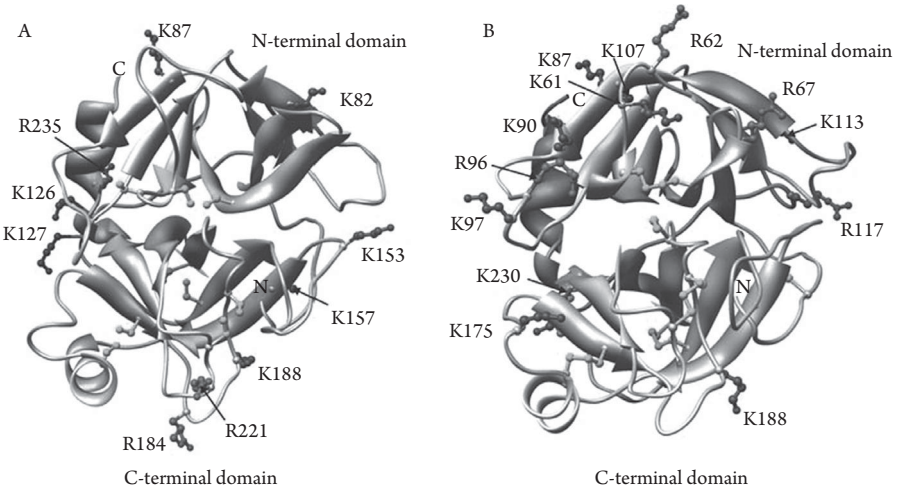


Fig. 9.10.
Computer-generated model of the three-dimensional structure of a trypsin isoform of the marine crab *Cancer pagurus* (A) compared with a trypsin from rat (B). Arginine (R) and lysine (K) residues are indicated and labeled according to the common chymotrypsin numbering. From Hehemann et al. (2008), with permission from Elsevier.

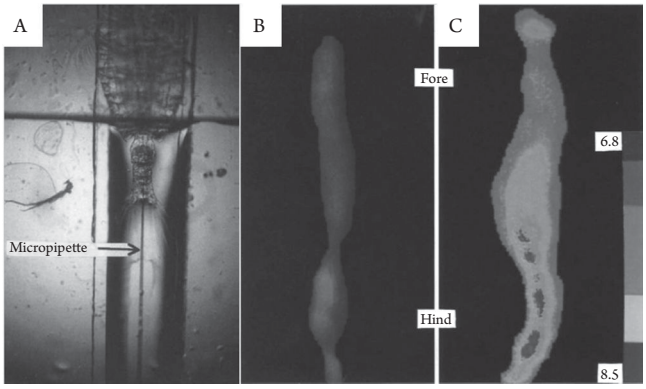


Fig. 9.11.
Calanus helgolandicus. (A) Restrained female with a micropipette inserted into the hindgut via the anus to facilitate the injection of the pH-sensitive dye BCECF into the gut. (B and C) Gray-shaded images of the guts of individual females indicating the pH after starvation (B) and after feeding (C). The gray scale represents the pH from 6.8 to 8.5. From Pond et al. (1995), with permission from Springer.

proteolytic enzymes that show their maximum activity at neutral and slightly alkaline conditions. This passive pH alteration may act as an efficient mechanism to control proteolytic activity. During periods of starvation, the pH in the stomach drops to lower values that, in turn, cause a reduction of proteolytic activity. Lowered proteolytic activity also prevents hydrolysis and thus destruction of other enzymes that accumulate simultaneously with the proteolytic enzymes in the stomach. After feeding, the pH rises again, and the proteolytic enzymes gain activity and capability of digesting alimentary proteins.

Thermal Properties

The thermodynamic properties of crustacean digestive enzymes and their catalyzed reactions, respectively, were predominantly studied in view of ecophysiological adaptations (Somero 2004). Most enzymes exhibit a distinct thermal stability and remain fully active up to temperatures of 40–50°C or even higher. The increase of activity with rising temperature from 0° to about 30°C generally follows an exponential relationship according to van't Hoff's law. At higher temperatures, the enzymes become progressively degraded. The optimum curve reaches its maximum before the activity drastically declines and drops to zero. The "thermal optimum" by itself has no physiological meaning but mirrors the physicochemical properties of the protein. However, the initial activity at low temperatures may provide some indication about the cold tolerance of animals, and the course of the exponential increase can be used to calculate the activation energy of the reaction according to the Arrhenius equation.

Polymorphism of Digestive Enzymes

Polymorphism of digestive enzymes has been reported in many crustacean species and for a number of enzymes. Van Wormhoudt and Sellos (2003) found eight electromorphs of α-amylase in the whiteleg shrimp *L. vannamei*. The authors characterized three genes encoding for mature proteins of 495 amino acids. All of the three genes were expressed in the midgut gland of the shrimps. The sequences of the genes suggested an ancient duplication event and a long evolutionary development between the present and ancestral genes. Similarly, several

isoforms for amylase, but also for endopeptidases and unspecific esterases, were reported in the spiny lobster *Panulirus argus* by Perera et al. (2008). The ecological benefit of polymorphic enzyme expression may be seen as an adaptation to better cope with variable food supply. According to Nelson and Hedgecock (1980), species with a broad food spectrum showed a higher degree of heterozygosity than did feeding specialists. Moreover, among the specialists, carnivorous species were more often heterozygous than herbivorous species. Perera et al. (2010) studied the in vitro digestibility of food by spiny lobsters with three different major endopeptidase isoenzyme patterns. It was apparent that the different phenotypes released different amounts of amino acids from various protein sources. The different activities of trypsin phenotypes in *L. vannamei* are also related to different kinetic properties of isoenzyme (Sainz Hernández and Cordova Murueta 2009). Accordingly, enzyme polymorphism contributes a surplus of catalytic potential due to slight structural and thus kinetic variations of the isoforms. These slight differences improve the concerted degradation of the natural target molecules and the yield of bioavailable products.

Dietary Effects on Enzyme Expression and Activities

During the past decades, a huge number of studies were published dealing with the effects of natural or formulated diets or nutrients on the expression of digestive enzymes. The general aims were, on one hand, to improve formulated diets for aquaculture and, on the other hand, to define physiological proxies for the trophic preferences of a species or a population. The studies revealed a huge variety of results, of which only a few examples can be presented here. Le Moullac et al. (1996) reported a significant dose–response effect between the amount of dietary casein supplement and activity and, particularly, the amount of trypsin in *L. vannamei*. Chymotrypsin and amylase levels showed high scatter but no distinct trends. In contrast to casein, gelatin-supplemented diet inhibited trypsin activity. Muhlia-Almazán et al. (2003) showed that the same species of shrimp (*L. vannamei*) fed with a diet containing 30% of proteins exhibited higher trypsin and chymotrypsin activities than did animals fed with lower or higher protein contents. Moreover, differential expression of trypsin mRNA was observed in the midgut gland of *L. vannamei* under starvation (Sánchez-Paz et al. 2003). In another species, *L. stylirostris*, saturation levels for midgut gland glucogenesis and amylase activity were apparent at a diet containing 21% of carbohydrates (Rosas et al. 2000). Simon (2009) fed juvenile spiny lobsters *J. edwardsii* with natural and formulated diet. The latter caused a decrease of digestive enzyme activities, nutritional conditions, and changes of midgut cytology. To reduce the amount of protein from fish meal, diets with soybean meal and blood meal were tested. Both showed low in vitro digestibility due to high inhibitory effects (Lemos et al. 2004). However, feeding shrimps (*L. vannamei*) with diets containing different amounts of soybean trypsin inhibitor over the course of 10 weeks had no effect on overall weight gain (Sessa and Lim 1992).

Amphipods from different habitats and with different food preferences were analyzed for their digestive enzyme activities (Johnston et al. 2005). According to their habitats, the amphipods showed high laminarinase and lipase activities (supralittoral kelp-feeder); high α- and β-glucosidase, cellobiase, and xylanase activities (low shore intertidal feeder); or high activities of all of these enzymes (forest litter feeder). These results support a relationship between dietary preferences and digestive enzyme complements (Johnston et al. 2005). Very rapid responses to starvation or change of diet were exhibited by small copepods (*T. longicornis*). Starvation caused significant reductions in digestive and metabolic enzyme activities within 24 h. Change in food altered rapidly the fatty acid composition and the expression pattern of digestive enzymes (Kreibich et al. 2008, 2011). A fast

response of enzyme expression may reflect an adaptive strategy to cope with a variable and changing food supply.

Effect of Hormones

Vertebrate gastrointestinal hormones were found to stimulate the release of enzymes from midgut gland tissue of the crayfish *Orconectes limosus* (Resch-Sedlmeier and Sedlmeier 1999). The authors concluded that crustaceans possess endogenous factors similar to vertebrate hormones or, at least, receptors that accept these hormones. Putative endocrine cells were already described by Mykles (1979) in the midgut epithelium of the crab *Cancer magister*. However, only recently was their hormone content investigated and SIFamide- and tachykinin-related peptide (TRP)-like immunopositive cells were identified (Christie et al. 2007). SIFamide-like labeling prevailed in the anterior part of the midgut and the paired anterior midgut caeca. The TRP-like immunoreactivity predominated in the posterior midgut and posterior midgut caecum. Moreover, TRPs were detected in the hemolymph of starved animals but not in fed animals. Christie et al. (2007) suggested that midgut-derived TRP in *Cancer* spp. may function in the paracrine/hormonal control of feeding behavior.

Symbiotic Bacteria and Other Microbes

The variety of potential food items of Crustacea also include materials that are hard to digest. Such materials are chitin from the shell of crustaceans or other invertebrates, cellulose and hemicelluloses from plants, and other structural compounds such as lignin. The biochemical ability to degrade these materials was often attributed to symbiotic bacteria or other microorganisms. However, limnorid wood-boring isopods do not rely on symbiotic microbes for lignocellulose digestion but are capable of producing the relevant enzymes by themselves (King et al. 2010). Nevertheless, the presence of external and internal bacteria was reported for various taxa and species of crustaceans such as copepods, decapods, or isopods. Terrestrial isopods show bacteria within their midgut glands that seem beneficial in the digestion of plant material. In contrast, marine species showed no significant microbiota in their midgut glands (Zimmer et al. 2001). Low numbers of bacteria in the midgut gland are not surprising because the midgut diverticula are separated from the stomach by a fine pyloric filter system. Unlike the midgut gland, microbes have direct access to the hindgut. The physiological conditions in the gut lumen of terrestrial isopods would allow for the coexistence of aerobic and anaerobic microorganisms with fermentative activities (Zimmer and Brune 2005). In addition to direct contribution to digestion, high bacterial proliferation rates in the posterior hindgut promote dense colonization of feces with bacteria. After egestion, these feces may serve as partly fermented and high-quality supplements to leaf litter. However, even if high numbers of bacteria are present in the animal's intestines, their contribution to digestion is not mandatory. Nordic krill *M. norvegica*, which omnivorously feeds on microalgae and zooplankton, was studied for bacterial contribution in chitin degradation (Donachie et al. 1995). Chitinolytic bacteria were successfully isolated from the digestive organs of krill. However, the enzymes of these bacteria showed much lower activities and different chromatographic properties than the putative endogenous enzymes of the krill. It is self-evident that bacteria that are somehow associated with the foods are ingested by crustaceans and accumulate and proliferate in their guts. However, rapid gut transit times, as well as the formation of peritrophic membranes around the undigested food, are detrimental in the establishment of a persistent and potent microbial community. Nevertheless, the microbes present in the gut may certainly produce or liberate important micronutrients.

FUTURE DIRECTIONS

During the past decades, significant progress was achieved in the research on digestive processes in crustaceans. Morphological and anatomical studies made use of groundbreaking inventions such as raster electron microscopy or laser scanning microscopy. This progress in analytical techniques allows for biochemical measurements in very small crustaceans, larvae, and isolated organs or tissues (Kreibich et al. 2011), and the results provide new and exciting insights into the biology and digestive physiology of crustaceans. Now the "omics" are entering the field of crustacean digestive physiology and may open new dimensions in the understanding of these cardinal living processes (Ward et al. 2010).

Despite progress in research, today, the entirety of the gathered knowledge still does not provide a clear and comprehensive picture of the digestive physiology of Crustacea. There are still open questions, apparently contradicting findings, and unsolved regulatory pathways. For example, there is no consistent view on the genesis and development of the R, F, and B cells in the midgut gland. A general and often presented view is that B cells develop from F cells. However, there is also evidence that all of the three cell types directly arise from the embryonic E cells and develop independently. Concerted cytological and biochemical studies are necessary to answer this question. Basic research is also important to reveal the background for the different preferences of taxa in expressing proteolytic enzymes. Certainly, this knowledge will also be helpful for understanding the cytological processes within the midgut gland (Hu and Leung 2007). The transfer of nutrients through the membranes of the midgut gland cells, as well as hormonal control of feeding activity and digestion, are further important targets of basic research.

The major branches of applied crustacean biology and digestive physiology are aquaculture and, to a lesser extent, biotechnology. Aquaculture of crustaceans increased significantly in the past decades. In order to improve assimilation of the diets and, in turn, to reduce expenses for feeds, a line of crustacean digestive research evolved to study the effects of food additives on the performance of shrimp stocks, for example. Moreover, optimized food can help to lower excretion and thus reduce pollution in and around aquaculture facilities. Another promising field in aquaculture is the improvement of larval rearing. Optimized feeds and supplements are suitable to improve growth, health, and survival of larvae and early juvenile stages (Simon 2009).

Biotechnological approaches make use of different products derived from crustaceans such as chitin, chitosan, or astaxanthin. In addition, the versatile enzymes from the digestive tract of crustaceans often show exceptional catalytic properties. Such exceptional enzymes are suitable tools for biotechnological, analytical, or clinical applications. For example, shrimp alkaline phosphatase, which is isolated from the northern shrimp *P. borealis*, became an important tool in molecular biology and genetics. There is evidence that crustacean enzymes may have a high potential for applications either as byproducts directly obtained from crustaceans or as recombinant proteins (Saborowski et al. 2006, Hehemann et al. 2008).

Crustacea represent one of the most important and versatile group of invertebrates on Earth. Their relevance in both human alimentation as well as a source for new biotechnological products is rising. Accordingly, nutrition of Crustacea, along with digestive physiology, biochemistry, and molecular biology, will provide challenging and exciting fields of future research.

CONCLUSIONS

Crustacean fossil records can be back-dated to the Middle Cambrian. During the 500 million years that have passed since then, the crustaceans evolved to occupy almost all aquatic and also some terrestrial habitats. Due to their wide geographic distribution, but also due to their huge span in size,

they are capable of feeding on a variety of food items ranging from microscopic plankton organisms to fish and huge vertebrate carrion. Food uptake is facilitated by the aid of the primary feeding appendages, the mandible and maxillae. Higher species developed additional feeding appendages such as maxillipeds, gnathopods, or filter baskets that derived from the thoracopods. They use various feeding modes such as filter feeding, deposit feeding, or scavenging. Several taxa also developed parasitic forms that feed on the body fluids of their hosts.

The extraction and utilization of the nutrients occur in the digestive organs. In primitive taxa, these organs can have the apparently simple appearance of a tube-like gut pervading the body from the esophagus to the rectum. The gut is separated into different sections that bear epithelial cells for enzyme secretion and nutrient resorption. Advanced taxa show a progressive septation of the ectodermal foregut and hindgut and an entodermal midgut. The foregut forms the esophagus and the complex chitinous stomach, whereas the diverticula of the midgut are the principal sites of digestive enzyme synthesis and nutrient resorption. In order to cover the nutritive demands for growth and reproduction, crustaceans express a variety of digestive enzymes. These enzymes fulfill the general tasks of hydrolyzing the common biopolymers proteins, carbohydrates, and lipids. However, recent studies revealed a unique variety of structural characteristics and thus functional properties. In particular, many of these enzymes are very resistant to proteolysis as an adaption to their concomitant appearance with active peptidases in the lumen of the stomach. Crustaceans are even capable of hydrolyzing quite inert biological substances like cellulose or chitin by the aid of their endogenous enzymes.

In conclusion, the Crustacea developed a complex but efficient digestive system. The functional match between anatomy and biochemistry is unique among the animal kingdom. It forms the key for the successful utilization of a large and diverse range of food items to meet their nutritional demands.

REFERENCES

Ahearn, G.A. 1987. Nutrient transport by the crustacean gastrointestinal tract: recent advances with vesicle techniques. Biological Reviews 62:45–63.

Ahearn, G.A., M.L. Grover, and R.E. Dunn. 1985. Glucose transport by lobster hepatopancreatic brush border membrane vesicles. American Journal of Physiology 248:R133–R141.

Ahearn, G.A., G.A. Gerencser, M. Thamotharan, R.D. Behnke, and T.H. Lemme. 1992. Invertebrate gut diverticula are nutrient absorptive organs. American Journal of Physiology 263:472–481.

Allardyce, B.J., and S.M. Linton. 2008. Purification and characterization of endo-ß-1,4-glucanase and laminarinase enzymes from the gecarcinid land crab *Gecarcoidea natalis* and the aquatic crayfish *Cherax destructor*. The Journal of Experimental Biology 211:2275–2287.

Allardyce, B.J., S.M. Linton, and R. Saborowski. 2010. The last piece of the cellulase puzzle: the characterization of ß-glucosidase from the herbivorous gecarcinid land crab *Gecarcoidea natalis*. The Journal of Experimental Biology 213:2950–2957.

Aoki, H., M.N. Ahsan, and S. Watabe. 2003. Molecular cloning and functional characterization of crustapain: a distinct cysteine proteinase with unique substrate specificity from Northern Shrimp *Pandalus borealis*. Journal of Biochemistry 133:799–810.

Aoki, H., M.N. Ahsan, and S. Watabe. 2004. Molecular and enzymatic properties of a cathepsin L-like proteinase with distinct substrate specificity from northern shrimp (*Pandalus borealis*). Journal of Comparative Physiology B 174:59–69.

Auerswald, L., C. Pape, D. Stübing, A. Lopata, and B. Meyer. 2009. Effect of short-term starvation of adult Antarctic krill, *Euphausia superba*, at the onset of summer. Journal of Experimental Marine Biology and Ecology 381:47–56.

Becker-Pauly, C., B.C. Bruns, O. Damm, A. Schütte, K, Hammouti, T. Burmester, and W. Stöcker. 2009. News from an ancient world: two novel astacin metalloproteases from the horseshoe crab. Journal of Molecular Biology 385:236–248.

Berra, E., M. Forcella, R. Giacchini, and P. Parenti. 2006. Leucine transport across plasmamembranes from the scud *Echinogammarus stammeri* (Amphipoda: Gammaridae). Annales de Limnologie—International Journal of Limnology 42:79–85.

Blaya, J.A., F.J.G. Muriana, V. Ruiz-Gutierrez, C.M. Vazquez, and J. Bolufer. 1998. Folate transport by prawn hepatopancreas brush-border membrane vesicles. Bioscience Reports 18:9–17.

Bode, W., F.X. Gomis-Rüth, R. Huber, R. Zwilling, and W. Stöcker. 1992. Structure of astacin and implication for activation of astacins and zinc-ligation of collagenases. Nature 358:164–167.

Brouwer, M., and R. Lee. 2008. Response to toxic chemicals at the molecular, cellular, tissue, and organismal level. Pages 405–432 *in* V.S. Kennedy and L.E. Cronin, editors. The blue crab *Callinectes sapidus*. Maryland Sea Grant, Maryland.

Brunet, M., J. Arnaud, and J. Mazza. 1994. Gut structure and digestive cellular processes in marine Crustacea. Oceanography and Marine Biology: an Annual Review 32:335–367.

Byrne, K.A., S.A. Lehnert, S.E. Johnson, and S.S. Moore. 1999. Isolation of a cDNA encoding a putative cellulase in the red claw crayfish *Cherax quadricarinatus*. Gene 239:317–324.

Calcagno, J.A., K. Anger, G.A. Lovrich, S. Thatje, and A. Kaffenberger. 2004. Larval development of the subantarctic King crabs *Lithodes santolla* and *Paralomis granulosa* reared in the laboratory. Helgoland Marine Research 58:11–14.

Cavalli, R.O., P. Lavens, and P. Sorgeloos. 1999. Performance of *Macrobrachium rosenbergii* broodstock fed diets with different fatty acid composition. Aquaculture 179:387–402.

Ceccaldi, H.J. 2006. The digestive tract: anatomy, physiology, and biochemistry. Pages 85–203 *in* J. Forest, J.C. von Vaupel Klein, and F.R. Schram, editors. The Crustacea—treatise on zoology—anatomy, taxonomy, biology, Vol. 2. Brill, Leiden.

Chang, E.S., and J.D. O'Connor. 1983. Metabolism and transport of carbohydrate and lipids. Pages 263–287 *in* L.H. Mantel, editor. The biology of Crustacea, Vol. 5. Internal anatomy and physiological regulation. Academic Press, New York.

Chavez-Crooker, P., P. Pozo, H. Castro, M.S. Dice, I. Boutet, A. Tanguy, D. Moraga, and G.A. Ahearn. 2003. Cellular localization of calcium, heavy metals, and metallothionein in lobster (*Homarus americanus*) hepatopancreas. Comparative Biochemistry and Physiology C 136:213–224.

Chen, X.R., B.P. Tan, K.S. Mai, W.B. Zhang, X.J. Wang, Q.H. Ai, W. Xu, Z.G. Liufu, and H.M. Ma. 2011. Dietary administration of soybean isoflavones enhanced the immunity of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*. Aquaculture Nutrition 17:24–32.

Cherif, S., and Y. Gargouri. 2009. Thermoactivity and effects of organic solvents on digestive lipase from hepatopancreas of green crab. Food Chemistry 116:82–86.

Cherif, S., A. Fendri, N. Miled, H. Trabelsi, H. Mejdoub, and Y. Gargoury. 2007. Crab digestive lipase acting at high temperature: purification and biochemical characterization. Biochimie 89:1012–1018.

Christie, A.E., K.K. Kutz-Naber, E.A. Stemmler, A. Klein, D.I. Messinger, C.C. Goiney, A.J. Conterato, E.A. Bruns, Y.-W.A. Hsu, L. Li, and P. Dickinson. 2007. Midgut epithelial endocrine cells are a rich source of the neuropeptides APSGFLGMRamide (*Cancer borealis* tachykinin-related peptide Ia) and GYRKPPFNGSIFamide (Gly^1-SIFamide) in the crabs *Cancer borealis, Cancer magister* and *Cancer productus*. The Journal of Experimental Biology 210:699–714.

Chung, K.-T., C.-I. Wei, and M.G. Johnson. 1998. Are tannins a double-edged sword in biology and health? Trends in Food Science and Technology 9:168–175.

Conklin, D.E. 1997. Vitamins. Pages 123–149 *in* L.R. D'Abramo, D.E. Conklin, and D.M. Akiyama, editors. Crustacean Nutrition. Advances in World Aquaculture, Vol. 6. The World Aquaculture Society, Baton Rouge, Louisiana.

Crawford, A.C., J.A. Kricker, A.J. Anderson, N.R. Richardson, and P.B. Mather. 2004. Structure and function of a cellulase gene in redclaw crayfish, *Cherax quadricarinatus*. Gene 340:267–274.

Crawford, A.C., N.R. Richardson, and P.B. Mather. 2005. A comparative study of cellulose and xylanase activity in freshwater crayfish and marine prawn. Aquaculture Research 36:586–592.

Cuzon, G., A. Lawrence, G. Gaxiola, C. Rosas, and J. Guillaume. 2004. Nutrition of *Litopenaeus vannamei* reared in tanks or in ponds. Aquaculture 235:513–551.

D'Abramo, L., D.E. Conklin, and D.M. Akiyama. 1997. Crustacean nutrition. Advances in world aquaculture. Volume 6. The World Aquaculture Society, Baton Rouge, Louisiana.

Dalsgaard, J., M. St. John, G. Kattner, D. Müller-Navarra, and W. Hagen. 2003. Fatty acid trophic markers in the pelagic marine environment. Advances in Marine Biology 46:225–340.

Dall, W., and D.J.W. Moriarty. 1983. Functional aspects of nutrition and feeding. Pages 215–261 *in* L.H. Mantel, editor. The biology of Crustacea, Vol. 5. Internal anatomy and physiological regulation. Academic Press, New York.

de la Ruelle, M., M. Hajjou, F. van Herp, and Y. Le Gal. 1992. Aminopeptidase activity from the hepatopancreas of *Procambarus clarkii*. Biochemical Systematics and Ecology 20:331–337.

Dendinger, J.E. 1987. Digestive proteases in the midgut gland of the Atlantic Blue crab, *Callinectes sapidus*. Comparative Biochemistry and Physiology B 88:503–506.

Díaz, A.C., A.V. Fernández Gimenez, S.N. Mendiara, and J.L. Fenucci. 2004. Antioxidant activity in hepatopancreas of the shrimp (*Pleoticus muelleri*) by electron paramagnetic spin resonance spectrometry. Journal of Agricultural and Food Chemistry 52:3189–3193.

Donachie, S.P., R. Saborowski, G. Peters, and F. Buchholz. 1995. Bacterial digestive enzyme activity in the stomach and hepatopancreas of *Meganyctiphanes norvegica* (M. Sars, 1857). Journal of Experimental Marine Biology and Ecology 188:151–165.

Eisen, A.Z., and J.J. Jeffrey. 1969. An extractable collagenase from crustacean hepatopancreas. Biochimica et Biophysica Acta 191:517–526.

Eisen, A.Z., K.O. Henderson, J.J. Jeffrey, and R.A. Bradshaw. 1973. A collagenolytic protease from the hepatopancreas of the fiddler crab, *Uca pugilator*. Purification and properties. Biochemistry 12:1814–1822.

Ezquerra, J.M., F.L. García-Carreño, M.G. Arteaga, and N.F. Haard. 1999. Effects of feed diet on aminopeptidase activities from the hepatopancreas of white shrimp (*Penaeus vannamei*). Journal of Food Biochemistry 23:59–74.

Fernández, I., M. Oliva, O. Carrillo, and A. van Wormhoudt. 1997. Digestive enzyme activities of *Penaeus notialis* during reproduction and moulting cycle. Comparative Biochemistry and Physiology A 118:1267–1271.

Fox, H.M. 1952. Anal and oral water intake by Crustacea. Nature 169:1051–1052.

França, R.C.P., I.P.G. Amaral, W.M. Santana, L.P. Souza-Santos, L.B. Carvalho Jr., and R.S. Bezerra. 2010. Proteases from the harpacticoid copepod *Tisbe biminiensis*: comparative study with enzymes from farmed aquatic animals. Journal of Crustacean Biology 30:122–128.

Ganguly, S., I. Paul, and S.K. Mukhopadhayay. 2010. Application and effectiveness of immunostimulants, probiotics, and prebiotics in aquaculture: a review. Israeli Journal of Aquaculture—Bamidgeh 62:130–138.

García-Carreño, F.L., M.P. Hernandez Cortes, and N. Haard. 1994. Enzymes with peptidase and proteinase activity from the digestive systems of a fresh water and a marine decapod. Journal of Agricultural and Food Chemistry 42:1456–1461.

Geier, G., E. Jacob, W. Stöcker, and R. Zwilling. 1997. Genomic organization of the zinc-endopeptidase astacin. Archives of Biochemistry and Biophysics 337:300–307.

Gibson, R., and P.L. Barker. 1979. The decapod hepatopancreas. Oceanography and Marine Biology: an Annual Review 17:285–346.

Grant, G.A., K.O. Henderson, A.Z. Eisen, and R.A. Bradshaw. 1980. Amino acid sequence of a collagenolytic protease from the hepatopancreas of the fiddler crab, *Uca pugilator*. Biochemistry 19:4653–4659.

Greenaway, P. 2003. Terrestrial adaptations in the Anomura (Crustacea: Decapoda). Memoirs of Museum Victoria 60:13–26.

Gudmundsdóttir, A. 2002. Cold-adapted and mesophilic brachyurins. Biological Chemistry 383:1125–1131.

Guevara, T., I. Yiallouros, R. Kappelhoff, S. Bissdorf, W. Stöcker, and F.X. Gomis-Rüth. 2010. Proenzyme structure and activation of astacin metallopeptidase. The Journal of Biological Chemistry 285:13958–13965.

Hallberg, E., and H.-J. Hirche. 1980. Differentiation of mid-gut in adults and over-wintering copepodids of *Calanus finmarchicus* (Gunnerus) and *C. helgolandicus* Claus. Journal of Experimental Marine Biology and Ecology 48:283–295.

Hehemann, J.-H., L. Redecke, J. Murugaiyan, M. von Bergen, C. Betzel, and R. Saborowski. 2008. Autoproteolytic stability of a trypsin from the marine crab *Cancer pagurus*. Biochemical and Biophysical Research Communications 370:566–571.

Hoppe-Seyler, F. 1877. Ueber Unterschiede im chemischen Bau und in der Verdauung höherer und niederer Thiere. Pflüger's Archiv für die gesamte Physiologie des Menschen und der Thiere 14:395–400.

Horiguchi, H., M. Hironaka, V.B. Meyer-Rachow, and T. Hariyama. 2007. Water uptake via two pairs of specialized legs in *Ligia exotica* (Crustacea, Isopoda). Biological Bulletin 213:196–203.

Hu, K.-J., and P.C. Leung. 2004. Shrimp cathepsin L encoded by an intronless gene has predominant expression in hepatopancreas, and occurs in the nucleus of oocyte. Comparative Biochemistry and Physiology B 137:21–33.

Hu, K.-J., and P.-C. Leung. 2007. Food digestion by cathepsin L and digestion-related rapid cell differentiation in shrimp hepatopancreas. Comparative Biochemistry and Physiology B 146:69–80.

Hu, M.Y.-A., W. Hagen, M.-S. Jeng, and R. Saborowski. 2012. Metabolic energy demand and food utilization of the hydrothermal vent crab *Xenograpsus testudinatus* (Crustacea: Brachyura). Aquatic Biology 15:11–25.

Jeng, M.-S., N.K. Ng, and P.K.L. Ng. 2004. Hydrothermal vent crabs feast on sea "snow." Nature 432:969.

Johnston, D., and J. Freeman. 2005. Dietary preference and digestive enzyme activities as indicators of trophic resource utilization by six species of crab. Biological Bulletin 208:36–46.

Johnston, D.J., K.A. Calvert, B.J. Crear, and C.G. Carter. 2003. Dietary carbohydrate/lipid ratios and nutritional condition in juvenile southern rock lobster, *Jasus edwardsii*. Aquaculture 220:667–682.

Johnston, D., A. Ritar, C. Thomas, and A. Jeffs. 2004. Digestive enzyme profiles of spiny lobster *Jasus edwardsii* phyllosoma larvae. Marine Ecology Progress Series 275:219–230.

Johnston, M., D. Johnston, and A. Richardson. 2005. Digestive capabilities reflect the major food sources in three species of talitrid amphipods. Comparative Biochemistry and Physiology B 140:251–257.

Kimoto, K., T. Yokoi, and K. Murakami. 1985. Purification and characterization of chymotrypsin-like proteinase from *Euphausia superba*. Agricultural and Biological Chemistry 49:1599–1603.

King, A.J., S.M. Cragg, Y. Li, J. Dymond, M.J. Guille, D.J. Bowles, N.C. Bruce, I.A. Graham, and S.J. McQueen-Mason. 2010. Molecular insight into lignocelluloses digestion by a marine isopod in the absence of gut microbes. Proceedings of the National Academy of Sciences USA 107:5345–5350.

Klein, B., D. Sellos, and A. van Wormhoudt. 1998. Genomic organisation and polymorphism of a crustacean trypsin multi-gene family. Gene 216:123–129.

Kostanjšek, R., M. Milatovič, and J. Štrus. 2010. Endogenous origin of endo-ß-1,4-glucanase in common woodlouse *Porcellio scaber* (Crustacea, Isopoda). Journal of Comparative Physiology B 180:1143–1153.

Kreibich, T., R. Saborowski, W. Hagen, and B. Niehoff. 2008. Short-term variation of nutritive and metabolic parameters in *Temora longicornis* females (Crustacea, Copepoda) as a response to diet shift and starvation. Helgoland Marine Research 62:241–249.

Kreibich, T., W. Hagen, and R. Saborowski. 2010. Food utilization of two pelagic crustaceans in the Greenland Sea: *Meganyctiphanes norvegica* (Euphausiacea) and *Hymenodora glacialis* (Decapoda, Caridea). Marine Ecology Progress Series 413:105–115.

Kreibich, T., R. Saborowski, W. Hagen, and B. Niehoff. 2011. Influence of short-term nutritional variations on digestive enzyme and fatty acid patters of the calanoid copepod *Temora longicornis*. Journal of Experimental Marine Biology and Ecology 407:182–189.

Krukenberg, F.W. 1878. Zur Verdauung bei den Krebsen. Untersuchungen des physiologischen Instituts der Universität Heidelberg 2:262–272.

Lavarías, S., R.J. Pollero, and H. Heras. 2006. Activation of lipid metabolism by the water-soluble fraction of petroleum in the crustacean *Macrobrachium borellii*. Aquatic Toxicology 77:190–196.

Laycock, M.V., T. Hirama, S. Hasnain, D. Watson, and A.C. Storer. 1989. Purification and characterization of a digestive cysteine proteinase from the American lobster (*Homarus americanus*). Biochemical Journal 263:439–444.

Laycock, M.V., R.M. MacKay, M. Di Fruscio, and J.W. Gallant. 1991. Molecular cloning of three cDNAs that encode cysteine proteinases in the digestive gland of the American lobster (*Homarus americanus*). FEBS Letters 292:115–120.

Le Boulay, C., A. van Wormhoudt, and D. Sellos. 1996. Cloning and expression of cathepsin L-like proteinases in the hepatopancreas of the shrimp *Penaeus vannamei* during the intermoult cycle. Journal of Comparative Physiology B 166:310–318.

Le Boulay, C., D. Sellos, and A. van Wormhoudt. 1998. Cathepsin L gene organization in crustaceans. Gene 218:77–84.

Lehnert, S.A., and S.E. Johnson. 2002. Expression of hemocyanin and digestive enzyme messenger RNAs in the hepatopancreas of the black tiger shrimp *Penaeus monodon*. Comparative Biochemistry and Physiology B 133:163–171.

Lemos, D., M.P. Hernández-Cortés, A. Navarrete, F.L. García-Carreño, and V.N. Phan. 1999. Ontogenetic variation in digestive proteinase activity of larvae and postlarvae of the pink shrimp *Farfantepenaeus paulensis* (Crustacea: Decapoda: Penaeidae). Marine Biology 135:653–662.

Lemos, D., A. Navarrete del Toro, J.H. Córdova-Murueta, and F.L. Garcia-Carreño. 2004. Testing feeds and feed ingredients for juvenile pink shrimp *Farfantepenaeus paulensis*: in vitro determination of protein digestibility and proteinase inhibition. Aquaculture 239:307–321.

Le Moullac, G., B. Klein, D. Sellos, and A. van Wormhoudt. 1996. Adaptation of trypsin, chymotrypsin and *a*-amylase to casein level and protein source in *Penaeus vannamei* (Crustacea Decapoda). Journal of Experimental Marine Biology and Ecology 208:107–125.

Le Vay, L., D.A. Jones, A.C. Puello-Cruz, R.S. Sangha, and C. Ngamphongsai. 2001. Digestion in relation to feeding strategies exhibited by crustacean larvae. Comparative Biochemistry and Physiology A 128:623–630.

Liñán-Cabello, M.A., J. Paniagura-Michel, and P.M. Hopkins. 2002. Bioactive roles of carotenoids and retinoids in crustaceans. Aquaculture Nutrition 8:299–309.

Liñán-Cabello, M.A., J. Paniagura-Michel, and T. Zenteno-Savín. 2003. Carotenoids and retinal levels in captive and wild shrimp, *Litopenaeus vannamei*. Aquaculture Nutrition 9:383–389.

Linton, S.M., and P. Greenaway. 2007. A review of feeding and nutrition of herbivorous land crabs: adaptations to low quality plant diets. Journal of Comparative Physiology B 177:269–286.

Linton, S.M., P. Greenaway, and D. Towle. 2006. Endogenous production of endo-β-1,4-glucanase by decapod crustaceans. Journal of Comparative Physiology B 176:339–348.

Lischka, S., L. Giménez, W. Hagen, and B. Ueberschär. 2007. Seasonal changes in digestive enzyme (trypsin) activity of the copepods *Pseudocalanus minutus* (Calanoida) and *Oithona similis* (Cyclopoida) in the Arctic Kongsfjorden (Svalbard). Polar Biology 30:1331–1341.

Loizzi, R.F. 1971. Interpretation of crayfish hepatopancreatic function based on fine structural analysis of epithelial cell lines and muscle network. Zeitschrift für Zellforschung und mikroskopische Anatomie 113:420–440.

Luquet, G., M.S. Fernández, A. Badou, N. Guichard, N. Le Roy, M. Corneillat, G. Alcaraz, and J.L. Arias. 2013. Comparative ultrastructure and carbohydrate composition of gastroliths from Astacidae, Cambaridae and Parastacidae freshwater crayfish (Crustacea, Decapoda). Biomolecules 3:18–38.

Maeda-Martínez, A.M., V. Obregón-Barboza, M.A. Navarrete del Toro, H. Obregón-Barboza, and F.L. García-Carreño. 2000. Trypsin-like enzymes from two morphotypes of the 'living fossil' Triops (Crustacea: Branchiopoda: Notostraca). Comparative Biochemistry and Physiology B 126:317–323.

Martin-Creuzburg, D., and E. von Elert. 2009. Good food versus bad food: the role of sterols and polyunsaturated fatty acids in determining growth and reproduction of *Daphnia magna*. Aquatic Ecology 43:943–950.

Magarelli, P.C. Jr., B. Hunter, D.V. Lightner, and B. Colvin. 1979. Black death: an ascorbic acid deficiency disease in penaeid shrimp. Comparative Biochemistry and Physiology A 63:102–108.

Matozzo V., C. Gallo, and M.G. Marin. 2011. Can starvation influence cellular and biochemical parameters in the crab *Carcinus aestuarii*? Marine Environmental Research 71:207–212.

Mayzaud P., V. Tirelli, J.M. Bernard, and O. Roche-Mayzaud. 1998. The influence of food quality on the nutritional acclimation of the copepod *Acartia clausi*. Journal of Marine Systems 15:483–493.

McGaffin A.F., S. Nicol, and D.A. Ritz. 2002. Changes in muscle tissue of shrinking Antarctic krill. Polar Biology 25:180–186.

Mente E., P. Coutteau, D. Houlihan, I. Davidson, and P. Sorgeloos. 2002. Protein turnover, amino acid profile and amino acid flux in juvenile shrimp *Litopenaeus vannamei*: effects of dietary protein source. Journal of Experimental Biology 205:3107–3122.

Meyers S.P., and T. Latscha. 1997. Carotenoids. Pages 164–193 *in* L.R. D'Abramo, Conklin D.E., and D.M. Akiyama, editors. Crustacean nutrition. Advances in world aquaculture, Volume 6. The World Aquaculture Society Baton Rouge, Louisiana.

Möhrlen F., S. Baus, A. Gruber, H.-R. Rackwitz, M. Schnölzer, G. Vogt, and R. Zwilling. 2001. Activation of pro-astacin. Immunological and model peptide studies on the processing of immature astacin, a zinc-endopeptidase from the crayfish *Astacus astacus*. European Journal of Biochemistry 268:2540–2546.

Muhlia-Almazán A., F.L. García-Carreño, J.A. Sánchez-Paz., G. Yepiz-Plascencia, and A.B. Peregrino-Uriarte. 2003. Effects of dietary protein on the activity and mRNA level of trypsin in the midgut gland of the white shrimp *Penaeus vannamei*. Comparative Biochemistry and Physiology B 135:373–383.

Muhlia-Almazán A., A. Sánchez-Paz, and F.L. García-Carreño. 2008. Invertebrate trypsins: a review. Journal of Comparative Physiology B 178:655–672.

Mykles, D.L. 1979. Ultrastructure of alimentary epithelia of lobsters, *Homarus americanus* and *H. gammarus*, and crab, *Cancer magister*. Zoomorphologie 92:201–215.

Navarrete del Toro, M.A., F.L. García-Carreño, M. Díaz López, L. Celis-Guerrero, and R. Saborowski. 2006. Aspartic proteinases in the digestive tract of marine decapod crustaceans. Journal of Experimental Zoology 305A:645–654.

Nelson, K., and D. Hedgecock. 1980. Enzyme polymorphism and adaptive strategy in the decapod Crustacea. American Naturalist 116:238–280.

Neurath, H. 2001. From proteases to proteomics. Protein Science 10:892–904.

Pan, B.S., C.C. Lan, and T.Y. Hung. 1991. Changes in composition and proteolytic enzyme activities of *Artemia* during early development. Comparative Biochemistry and Physiology A 100:725–730.

Pautou, M.-P., D. Rey, J.P. David, and J.-C. Meyran. 2000. Toxicity of vegetable tannins on Crustacea associated with Alpine mosquito breeding sites. Ecotoxicology and Environmental Safety 47:323–332.

Perera, E., F.J. Moyano, M. Díaz, R. Perdomo-Morales, V. Montero-Alejo, E. Alonso, O. Carrillo, and G.S. Galich. 2008. Polymorphism and partial characterization of digestive enzymes in the Spiny lobster *Panulirus argus*. Comparative Biochemistry and Physiology B 150:247–254.

Perera, E., F.J. Moyano, L. Rodriguez-Viera, A. Cervantes, G. Martínez-Rodriguéz, and J.M. Mancera. 2010. In vitro digestion of protein sources by crude enzyme extracts of the Spiny lobster *Panulirus argus* (Latreille, 1804) hepatopancreas with different trypsin isoenzyme patterns. Aquaculture 310:178–185.

Perona, J.J., C.A. Tsu, C.S. Craik, and R.J. Fletterick. 1997. Crystal structure of an ecotin-collagenase complex suggests a model for recognition and cleavage of the collagen triple helix. Biochemistry 36:5381–5392.

Peters, G., R. Saborowski, R. Mentlein, and F. Buchholz. 1998. Isoforms of an N-acetyl-ß-D-glucosaminidase from the Antarctic krill, *Euphausia superba*: purification and antibody production. Comparative Biochemistry and Physiology B 120:743–751.

Peters, G., R. Saborowski, F. Buchholz, and R. Mentlein. 1999. Two distinct forms of the chitin-degrading enzyme N-acetyl-ß-D-glucosaminidase in the Antarctic krill, *Euphausia superba*: specialists in digestion and moult. Marine Biology 134:697–703.

Pfleiderer, G., R. Zwilling, and H.-H. Sonneborn. 1967. Zur Evolution der Endopeptidasen, III. Eine Protease vom Molekulargewicht 11000 und eine trypsinähnliche Fraktion aus *Astacus fluviatilis* Fabr. Hoppe-Seyler's Zeitschrift für Physiologische Chemie 348:1319–1331.

Pond, D.W., R.P. Harris, and C. Brownlee. 1995. A microinjection technique using a pH-sensitive dye to determine the gut pH of *Calanus helgolandicus*. Marine Biology 123:75–79.

Proespraiwong, P., A. Tassanakajon, and V. Rimphanitchayakit. 2010. Chitinases from the black tiger shrimp *Penaeus monodon*: phylogenetics, expression and activities. Comparative Biochemistry and Physiology B 156:86–96.

Resch-Sedlmeier, G., and D. Sedlmeier. 1999. Release of digestive enzymes from the crustacean hepatopancreas: effects of vertebrate gastrointestinal hormones. Comparative Biochemistry and Physiology B 123:187–192.

Rey, D., J.P. David, A. Cuany, M. Amichot, and J.C. Meyran. 2000. Differential sensitivity to vegetable tannins in planktonic Crustacea from alpine mosquito breeding sites. Pesticide Biochemistry and Physiology 67:103–113.

Ribeiro, F.A.L.T., and D.A. Jones. 2000. Growth and ontogenetic change in activities of digestive enzymes in *Fennero Penaeus indicus* postlarvae. Aquaculture Nutrition 6:53–64.

Rivera-Pérez, C., M.d.l.A. Navarrete del Toro, and F.L. García-Carreño. 2010. Digestive lipase activity through development and after fasting and re-feeding in the whiteleg shrimp *Penaeus vannamei*. Aquaculture 300:163–168.

Rivera-Pérez, C., F.L. García-Carreño, and R. Saborowski. 2011. Purification and biochemical characterization of digestive lipase from whiteleg shrimp. Marine Biotechnology 13:284–295.

Rojo, L., A. Muhlia-Almazan, R. Saborowski, and F.L. García-Carreño. 2010. Aspartic cathepsin D endopeptidase contributes to extracellular digestion in clawed lobsters *Homarus americanus* and *Homarus gammarus*. Marine Biotechnology 12:696–707.

Rosas, C., G. Cuzon, G. Gaxiola, L. Arena, P. Lemaire, C. Soyez, and A. van Wormhoudt. 2000. Influence of dietary carbohydrate on the metabolism of juvenile *Litopenaeus stylirostris*. Journal of Experimental Marne Biology and Ecology 249:181–198.

Roy, P., B. Colas, and P. Durand. 1996. Purification, kinetical and molecular characterizations of a serine collagenolytic protease from green shore crab (*Carcinus maenas*) digestive gland. Comparative Biochemistry and Physiology B 115:87–95.

Rudenskaya, G.N. 2003. Brachyurins, serine collagenolytic enzymes from crabs. Russian Journal of Bioorganic Chemistry 29:101–111.

Rudenskaya, G.N., Y.A. Kislitsin, and D.V. Rebrikov. 2004. Collagenolytic serine protease PC and trypsin PC from king crab *Paralithodes camtschaticus*: cDNA cloning and primary structure of the enzymes. BMC Structural Biology 4:2.

Saborowski, R., and F. Buchholz. 1999. A laboratory study on digestive processes in the Antarctic krill, *Euphausia superba*, with special regard to chitinolytic enzymes. Polar Biology 21:295–304.

Saborowski, R., F. Buchholz, R.-A.H. Vetter, S.J. Wirth, and G.A. Wolf. 1993. A soluble, dye-labeled chitin derivative adapted for the assay of krill chitinase. Comparative Biochemistry and Physiology B 105:673–678.

Saborowski, R., G. Sahling, M.A. Navarrete del Toro, I. Walter, and F.L. García-Carreño. 2004. Stability and effects of organic solvents on endopetidases from the gastric fluid of the marine crab *Cancer pagurus*. Journal of Molecular Catalysis B: Enzymatic 30:109–118.

Saborowski, R., S. Thatje, J.A. Calcagno, G.A. Lovrich, and K. Anger. 2006. Digestive enzymes in the ontogenetic stages of the southern king crab, *Lithodes santolla*. Marine Biology 149:865–873.

Sainte-Marie, B., and D. Chabot. 2002. Ontogenetic shifts in natural diet during benthic stages of American lobster (*Homarus americanus*), off the Magdalen Islands. Fisheries Bulletin 100:106–116.

Sainz, J.C., F.L. García-Carreño, A. Sierra-Beltrán, and P. Hernández-Cortés. 2004. Trypsin synthesis and storage as zymogen in the midgut gland of the shrimp *Litopenaeus vannamei*. Journal of Crustacean Biology 24:266–273.

Sainz, J.C., F.L. García-Carreño, J.H. Córdova-Murueta, and P. Cruz-Hernández. 2005. Whiteleg shrimp (*Litopenaeus vannamei*, Boone, 1931) isotrypsins: their genotype and modulation. Journal of Experimental Marine Biology and Ecology 326:105–113.

Sainz Hernández, J.C., and J.H. Cordova Murueta. 2009. Activity of trypsin from *Litopenaeus vannamei*. Aquaculture 290: 190–195.

Sakharov, I., and G.A. Prieto. 2000. Purification and some properties of two carboxypeptidases from the hepatopancreas of the crab *Paralithodes camtschatica*. Marine Biotechnology 2:259–266.

Sánchez-Paz, A., F.L. García-Carreño, A. Muhlia-Almazán, N.Y. Hernández-Saavedra, and G. Yepiz-Plascencia. 2003. Differential expression of trypsin mRNA in the white shrimp (*Penaeus vannamei*) midgut gland under starvation conditions. Journal of Experimental Marine Biology and Ecology 292:1–17.

Sánchez-Paz, A., F.L. García-Carreño, A. Muhlia-Almazán, A.B. Peregrino-Uriarte, J. Hernández-López, and G. Yepiz-Plascencia. 2006. Usage of energy reserves in crustaceans during starvation: status and future directions. Insect Biochemistry and Molecular Biology 36:241–249.

Sánchez-Paz, A., F.L. García-Carreño, J. Hernández-López, A. Muhlia-Almazán, and G. Yepiz-Plascencia. 2007. Effects of short-term starvation on hepatopancreas and plasma energy reserves of the Pacific white shrimp (*Litopenaeus vannamei*). Journal of Experimental Marine Biology and Ecology 340:184–193.

Schultz, T.W., and J.R. Kennedy. 1976. The fine structure of the digestive system of *Daphnia pulex* (Crustacea: Cladocera). Tissue and Cell 8:479–490.

Schwarzenberger, A., A. Zitt, P. Kroth, S. Mueller, and E. von Elert. 2010. Gene expression and activity of digestive proteases in *Daphnia*: effects of cyanobacterial protease inhibitors. BMC Physiology 10:6.

Sellos, D., and A. van Wormhoudt. 1992. Molecular cloning of a cDNA that encodes a serine protease with chymotryptic and collagenolytic activities in the hepatopancreas of the shrimp *Penaeus vannamei* (Crustacea, Decapoda). FEBS Letters 309:219–224.

Sessa, D.J., and C. Lim. 1992. Effect of feeding soy products with varying trypsin inhibitor activities on growth of shrimp. Journal of the American Oil Chemists Society 69:209–212.

Shiau, S.-Y. 1998. Nutrient requirements of penaeid shrimps. Aquaculture 164:77–93.

Simon, C., and A. Jeffs. 2008. Feeding and gut evacuation of cultured juvenile spiny lobster, *Jasus edwardsii*. Aquaculture 280:211–219.

Simon, C.J. 2009. Digestive enzyme response to natural and formulated diets in cultured juvenile Spiny lobster, *Jasus edwardsii*. Aquaculture 294:271–281.

Siu, T., and G.A. Ahearn. 1988. Inositol transport by hepatopancreatic brush-border membrane vesicles of the lobster *Homarus americanus*. The Journal of Experimental Biology 140:107–121.

Somero, G.N. 2004. Adaptation of enzymes to temperature: searching for the basic "strategies." Comparative Biochemistry and Physiology B 139:321–333.

Sotka, E.E., and K.E. Whalen. 2008. Herbivore offense in the sea: the detoxification and transport of secondary metabolites. Pages 203–228 *in* C.D. Amsler, editor Algal chemical ecology. Springer, Berlin.

Sterling, K.M., B. Roggenbeck, and G.A. Ahearn. 2010. Dual control of cytosolic metals by lysosomal transporters in lobster hepatopancreas. The Journal of Experimental Biology 213:769–774.

Stöcker, W., and W. Bode. 1995. Structural features of a superfamily of zinc-endopeptidases: the metzincins. Current Opinion in Structural Biology 5:383–390.

Stöcker, W., R.L. Wolz, and R. Zwilling. 1988. *Astacus* protease, a zinc metalloenzyme. Biochemistry 27:5026–5032.

Stöcker, W., F.-X. Gomis-Rüth, W. Bode, and R. Zwilling. 1993. Implications for the three-dimensional structure of astcin for the structure and function of the astcin family of zinc-endopeptidases. European Journal of Biochemistry 214:215–231.

Storch, V., and K. Anger. 1983. Influence of starvation and feeding on the hepatopancreas of larval *Hyas aranaeus* (Decapoda, Majidae). Helgoländer Meeresuntersuchungen 36:67–75.

Storch, V., J.V. Juario, and F.P. Pascual. 1984. Early effects of nutritional stress on the liver of milkfish, *Chanos chanos* (Forsskal), and on the hepatopancreas of the tiger prawn, *Penaeus monodon* (Fabricius). Aquaculture 36:229–236.

Storch, V., J. Strus, and A. Brandt. 2002. Microscopic anatomy and ultrastructure of the digestive system of *Natatolana obtusata* (Vanhöffen, 1914) (Crustacea, Isopoda). Acta Zoologica 83:1–14.

Tam, Q., and A. Avenant-Oldewage. 2009. The ultrastructure of the digestive cells of *Argulus japonicus*, Thiele 1900 (Crustacea: Branchiura). Arthropod Structure and Development 38:45–53.

Tan, S.H., B.M. Degnan, and S.A. Lehnert. 2000. The *Penaeus monodon* chitinase 1 gene is differentially expressed in the hepatopancreas during the molt cycle. Marine Biotechnology 2:126–135.

Teschke, M., and R. Saborowski. 2005. Cysteine proteinases substitute for serine proteinases in the midgut glands of *Crangon crangon* and *Crangon allmani* (Decapoda: Caridea). Journal of Experimental Marine Biology and Ecology 316:213–229.

Titani, K., H.-J. Torff, S. Hormel, S. Kumar, K.A. Walsh, J. Rödl, H. Neurath, and R. Zwilling. 1987. Amino acid sequence of a unique protease from the crayfish *Astacus fluviatilis*. Biochemistry 26:222–226.

Toullec, J.Y., M. Chikhi, and A. van Wormhoudt. 1992. In vitro protein synthesis and *a* amylase activity in F cells from hepatopancreas of *Palaemon serratus* (Crustacea; Decapoda). Experientia 48:272–277.

Tsai, I.-H., K.-L. Chuang, and J.L. Chuang. 1986. Chymotrypsins in digestive tracts of crustacean decapods (shrimps). Comparative Biochemistry and Physiology B 85:235–239.

Tsai, I.-H., P.-J. Lu, and J.-L. Chuang. 1991. The midgut chymotrypsins of shrimps (*Penaeus monodon, Penaeus japonicus* and *Penaeus penicillatus*). Biochimica et Biophysica Acta 1080:59–67.

Tsu, C.A., and C.S. Craik. 1996. Substrate recognition by recombinant serine collagenase 1 from *Uca pugilator*. The Journal of Biological Chemistry 271:11563–11570.

Vazquez, L., J. Alpuche, G. Maldonado, C. Agundis, A. Pereyra-Morales, and E. Zenteno. 2009. Review: immunity mechanisms in crustaceans. Innate Immunity 15:179–188.

Verri, T., A. Mandal, L. Zilli, D. Bossa, P.K. Mandal, L. Ingrosso, V. Zonno, S. Vilella, G.A. Ahearn, and C. Storelli. 2001. D-glucose transport in decapod crustacean hepatopancreas. Comparative Biochemistry and Physiology A 130:585–606.

Vieira, F.N., C.C. Buglione, J.P.L. Mouriño, A. Jatobá, M.L. Martins, D.D. Schleder, E.R. Andreatta, M.A. Baracco, and L.A. Vinatea. 2010. Effect of probiotic supplemented diet on marine shrimp survival after challenge with *Vibri harrveyi*. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 62:631–638.

Vogt, G. 1994. Life-cycle and functional cytology of the hepatopancreatic cells of *Astacus astacus* (Crustacea, Decapoda). Zoomorphology 114:83–101.

Vogt, G., V. Storch, E.T. Qinitio, and F.P. Pascual. 1985. Midgut gland as monitor organ for the nutritional value of diets in *Penaeus monodon* (Decapoda). Aquaculture 48:1–12.

Vogt, G., W. Stöcker, V. Storch, and R. Zwilling. 1989. Biosynthesis of *Astacus* protease, a digestive enzyme from crayfish. Histochemistry 91:373–381.

van Wormhoudt, A., and D. Sellos. 2003. Highly variable polymorphism of the *α*-amylase gene family in *Litopenaeus vannamei* (Crustacea Decapoda). Journal of Molecular Evolution 57:659–671.

van Wormhoudt, A., P. Le Chevalier, and D. Sellos. 1992. Purification, biochemical characterization and N-terminal sequence of a serine-protease with chymotrypsic and collagenolytic activities in a tropical shrimp, *Penaeus vannamei* (Crustacea, Decapoda). Comparative Biochemistry and Physiology B 103:675–680.

van Wormhoudt, A., G. Bourreau, and G. Le Moullac. 1995. Amylase polymorphism in Crustacea Decapoda: electrophoretic and immunological studies. Biochemical Systematics and Ecology 23:139–149.

von Elert, E., M.K. Agrawal, C. Gebauer, H. Jaensch, U. Bauer, and A. Zitt. 2004. Protease activity in gut of *Daphnia magna*: evidence for trypsin and chymotrypsin enzymes. Comparative Biochemistry and Physiology B 137:287–296.

Waddington, K. 2008. Variation in evacuation rates of different foods skew estimates of diet in the Western Rock lobster *Panulirus cygnus*. Marine and Freshwater Research 59:347–350.

Walter, I., M. Schmidt, and F. Buchholz. 2008. Der Einfluss von Erdöl auf das Verhalten von Hummern. Effekte auf Nahrungssuche und Aggression. VDM Verlag Dr. Müller, Saarberücken.

Wang, Y.-B. 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. Aquaculture 269:259–264.

Watanabe, T., M. Kono, K. Aida, and H. Nagasawa. 1998. Purification and molecular cloning of a chitinase expressed in the hepatopancreas of the penaeid prawn *Penaeus japonicus*. Biochimica et Biophysica Acta 1382:181–185.

Watling, L. 2013. Feeding and digestive system. Pages 237–260 *in* L. Watling and M. Thiel, editors. Natural history of the Crustacea, Vol. 1. Oxford University Press, New York.

Ward, D.A., E.M. Sefton, M.C. Prescott, S.G. Webster, G. Wainwright, H.H. Rees, and M.J. Fisher. 2010. Efficient identification of proteins from ovaries and hepatopancreas of the unsequenced edible crab, *Cancer pagurus*, by mass spectrometry and homology-based, cross-species searching. Journal of Proteomics 73:2354–2364.

Wilde, J.E., S.M. Linton, and P. Greenaway. 2004. Dietary assimilation and the digestive strategy of the omnivorous anomuran land crab *Birgus latro* (Coenobitidae). Journal of Comparative Physiology B 174:299–308.

Yousefian, M., and M.S. Amiri. 2009. A review of the use of prebiotic in aquaculture for fish and shrimp. African Journal of Biotechnology 8:7313–7318.

Zanotto, F.P., and M.G. Wheatly. 2003. Calcium balance in crustaceans: nutritional aspects of physiological regulation. Comparative Biochemistry and Physiology A 133:645–660.

Zhao, F.-Q., and A.F. Keating. 2007. Functional properties and genomics of glucose transporters. Current Genomics 8:113–128.

Zimmer, M., and A. Brune. 2005. Physiological properties of the gut lumen of terrestrial isopods (Isopoda: Oniscidea): adaptive to digesting lignocellulose? Journal of Comparative Physiology B 175:275–283.

Zimmer, M., J.P. Danko, S.C. Pennings, A.R. Danford, A. Ziegler, R.F. Uglow, and T.H. Carefoot. 2001. Hepatopancreatic endosymbionts in coastal isopods (Crustacea: Isopoda), and their contribution to digestion. Marine Biology 138:955–963.

10

RESPONSES TO ENVIRONMENTAL STRESSES: OXYGEN, TEMPERATURE, AND pH

Nia M. Whiteley and Edwin (Ted) W. Taylor

Abstract

Although crustaceans are primarily a marine group, they have diversified into and adapted to a wide range of environments, including the deep sea, where oxygen and sulfite levels may vary; the littoral zone, with its daily fluctuations in water levels, temperature, and respiratory gas concentrations; and freshwater and terrestrial habitats, either as facultative air breathers or as fully terrestrial species resisting desiccation. The ability of crustaceans to compensate for anthropogenic factors such as global warming and acidification of aquatic habitats is poorly understood. Early studies on the effects of ocean acidification imply that adult crustaceans' survival is affected by their ability to compensate for acid–base disturbances. Future studies should concentrate on integrated crustacean responses to complex environmental changes, such as the relationship between physiological plasticity and the transcriptome, as well as their ability to undergo genetic adaptations to progressive anthropogenic factors such as those resulting from climate change.

INTRODUCTION

The subphylum Crustacea comprises primarily aquatic animals inhabiting marine environments. Although the conditions in oceanic waters have changed over geological time, they can be characterized within recorded time as having relatively stable temperatures well within the biological temperature range, a relatively alkaline pH of around 8, and sufficient oxygen (O_2) to support aerobic metabolism. This generalization does not apply to isolated arms of the sea (such as the Baltic Sea) and is currently being challenged by the increase in atmospheric carbon dioxide generated by progressive industrialization, with its associated reduction in the pH

and salinity of surface waters, and increase in temperature due to global warming. Relatively small changes in these variables are likely to have disproportionate effects on the distribution and survival of animal populations, including crustaceans, over the long term. A large number of crustacean species are found in shallow seas where many are important constituents of plankton, either as larvae or adults, and where they may show diel vertical migrations through the thermocline, thus experiencing rapid changes in temperature and sometimes in O_2 levels. Others form part of the benthos where O_2 levels may be very low, and, in extremely anaerobic conditions, sulfides accumulate. Many species have left the oceans to invade freshwater and even terrestrial habitats where they are likely to encounter various combinations of highly variable temperatures, areas or periods of hypoxia, and, in some circumstances, changes in pH. For example, in small pools of salt- or freshwater, daytime temperatures can rise and plant photosynthesis can generate very high levels of dissolved O_2 accompanied by high pH levels due to consumption of carbon dioxide. By contrast, at night, the same pools can cool and become hypoxic and acidotic due to plant and animal respiration (Taylor and Butler 1973, Truchot and Duhamel-Jouve 1980).

Any consideration of the effects of temperature, O_2 availability, and pH on animals has to take account of the fact that these environmental variables are interrelated. In water, an increase in temperature will reduce the solubility coefficient for O_2, effectively reducing its availability, and will result in a reduction in the pH at neutrality. Similar effects will be experienced by the body fluids and tissues of an animal exposed to temperature change, although they can actively combat these changes. As well as the physical solubility of O_2 in body fluids being reduced by increased temperatures, most crustaceans show a reduction in the O_2 affinity of the respiratory blood pigment hemocyanin with temperature. This is in part related to the direct allosteric effect of temperature on O_2 binding, but it also relates to the reduction in the pH of body fluids as temperature increases, which typically reduces O_2 affinity. So, as O_2 demand increases with temperature, the supply of O_2 to the metabolizing tissues may be compromised.

Much of our current understanding of respiratory physiology in crustaceans, including acid–base balance, comes from studies on decapodan crustaceans because of their convenient size for sampling and their value as a food source. More recently microtechniques have been developed that enable the study of respiratory responses in smaller crustaceans such as the branchiopods *Daphnia* spp. and *Triops cancriformis* (Paul et al. 2004, Weber and Pirow 2009). The transparency of *Daphnia*, for instance, has allowed optical techniques to be used to determine respiratory variables such as the O_2-binding properties of respiratory pigments, the use of fluorescent microspheres for investigating ventilatory flows, and O_2-sensitive phosphorescent dyes to examine the distribution of partial pressure of O_2 (Po_2) within the hemolymph (Paul et al. 2004). Microspectral fluorometry, along with pH-sensitive dyes, has been used to determine acid–base balance from microliter hemolymph samples, whereas fluorescence techniques have also been used as highly sensitive methods for measuring small changes in O_2 levels in water samples in order to determine rates of O_2 uptake of individual crustaceans weighing less than 100 mg (Rastrick and Whiteley 2011).

The following account features the integrated metabolic, physiological, and behavioral responses shown by a range of crustacean species to aquatic hypoxia (which can include the facultative use of air as a source of O_2) and to environmental temperatures ranging from near freezing in polar environments to the relatively high temperatures found in the tropics via the variable temperatures encountered with changes in latitude and height on the seashore. The combination of relatively high temperatures and aquatic hypoxia is considered to have been associated with the evolution of air breathing, and the adaptations necessary for survival in air are discussed. Finally, we consider the responses of crustaceans to anthropogenic effects such as global warming and environmental acidification.

RESPIRATORY GAS EXCHANGE WITH WATER

Oxygen Exchange

The gills in typical aquatic decapod crustaceans are contained within branchial chambers on either side of the body and are ventilated with a forwardly directed stream of water driven by a pair of balers or scaphognathites (Taylor 1982). These create subambient, oscillating hydrostatic pressures around the gills. When an animal is active, it ventilates large volumes of water in order to maintain a high O_2 partial pressure of 15–20 kPa in the water contained in the branchial chambers (P_WO_2). This in turn can generate high O_2 levels in the arterialized hemolymph (Pa_{O_2}) of 10–13 kPa. However, despite the potential for countercurrent or cross-current exchange, P_{O_2} levels in arterialized hemolymph rarely exceed those in expired water. This was long considered to be due to a reduced diffusion coefficient for O_2 over crustacean gills caused by the presence of a relatively impermeable layer of chitin covering the gill lamellae, although this has been questioned (Innes and Taylor 1986). In settled, inactive crustaceans, the O_2 levels in the hemolymph can apparently be far lower than have been previously reported (Massabuau and Forgue 1996), with recorded values for arterialized hemolymph of less than 1 kPa that are well below the partial pressures for saturation of crab hemocyanin described by Truchot (1975). In the prebranchial (venous) hemolymph, P_{VO_2} levels in routinely active animals vary between 0.67 and 2.67 kPa. This ensures a relatively high diffusion gradient for O_2 transfer across the gills from normoxic water, giving a partial pressure difference (ΔP_{GO_2}) of up to 12 kPa. Despite their limited O_2 carrying capacity due to the respiratory pigment hemocyanin being carried in colloidal suspension in the hemolymph, decapods are able to maintain a substantial venous reservoir of O_2 by virtue of their large circulating hemolymph volume and the relatively high affinity for O_2 of hemocyanin. Values for these variables that govern respiratory gas exchange and transport were given for several crustacean species in a review by Taylor and Innes (1988).

In the cladoceran *Daphnia*, O_2 exchange takes place across the entire body surface because the carapace is relatively permeable. Gas exchange also occurs between the hemolymph and the ambient medium at specific sites: the carapace lacuna and the posterior part of the head (Pirow et al. 1999). Ventilatory currents are maintained to the inner walls of the carapace by the thoracic appendages that are also responsible for filtering food particles from the water stream. Hemolymph P_{O_2} immediately after the carapace lacuna is 0.4–0.5 kPa below ambient and ranges from 0.9 to 6.1 kPa (Pirow et al. 2004). The respiratory pigment in *Daphnia*, as in other cladocerans, is hemoglobin (Hb; Kobayashi and Hoshi 1982). Both ventilation and heart rate vary with body size and in response to algal concentrations and O_2 levels in the ambient medium (Paul et al. 2004).

Carbon Dioxide Exchange

Crustaceans, like all other organisms, maintain pH levels in the body fluids within set limits. This occurs against a continuous production of CO_2 by cell metabolism and, in some species, against fluctuations in CO_2 in the surrounding water. The high ventilation rates of routinely active aquatic gill-breathers relate to the relatively low availability of O_2 in water, which is limited by its solubility, and is affected by temperature or salinity. Consequently, there is an effective hyperventilation with respect to the elimination of carbon dioxide so that partial pressure of carbon dioxide (Pa_{CO_2}) levels in the hemolymph are very low (<0.67 kPa; Cameron 1986). Hemolymph buffer base levels are correspondingly low and range between 4 and 10 mmol l^{-1} (Wheatly and Henry 1992), with the exception of daphnids, in which hemolymph [HCO_3^-] reaches 21 mmol l^{-1} (see summary table of acid–base status in *Crustacea* by Weber and Pirow

2009). Elevated HCO_3^- levels in *Daphnia* hemolymph are associated with an alkaline pH despite elevated Pa_{CO_2} levels due to relatively high metabolic rates. In all crustaceans, hemolymph pH regulation involves the passive buffering of CO_2 changes by the carbonate system and other nonbicarbonate buffers (e.g., proteins) and the electroneutral exchange of acid–base equivalents (Fig. 10.1). The most dominant mechanism is electroneutral exchange of H^+ and HCO_3^- ions across ion transporting epithelia of the gills in decapods (Wheatly and Henry 1992) and the epipodites in daphnids (Glover and Wood 2005). In decapods, it is generally accepted that electroneutral ion exchange involves branchial Cl^-/HCO_3^- and Na^+/NH_4^+ or H^+ exchangers, active ion transporters, and the hydration/dehydration of CO_2 by the enzyme carbonic anhydrase (Wheatly and Henry 1992, Whiteley 1999, Freire et al. 2008). Although the interrelationships among the various components of this exchange system are still unclear, it appears that inward HCO_3^- fluxes are dependent on apical Cl^-/HCO_3^- exchange driven by an apical V-type H^+-pump following the hydration of CO_2 into H^+ and HCO_3^- by carbonic anhydrase (Onken and Putzenlechner 1995, Henry 2001, Freire et al. 2008). The outward flux of acid equivalents depends on apical Na^+/H^+ exchangers driven by a basolateral Na^+/K^+ ATPase (Taylor and Taylor 1992, Towle and Weihrauch 2001). As a result, acid–base regulation in decapods is closely associated with ion regulation because both homeostatic processes share the same mechanisms (Whiteley et al. 2001b). These relationships

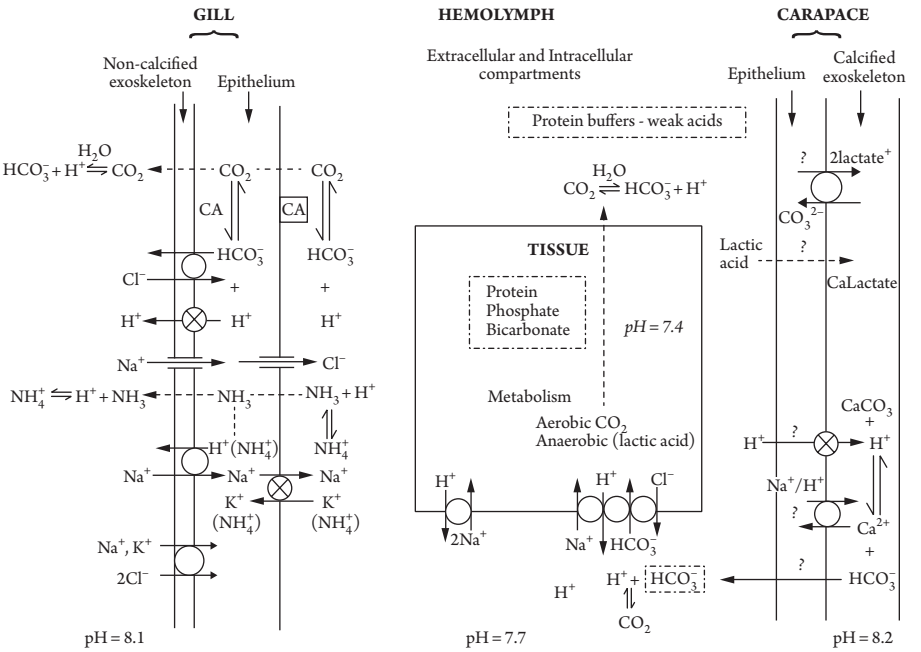


Fig. 10.1.
Schematic diagram of the proposed mechanisms involved in regulating acid–base status in the extra- and intracellular compartments of a typical aquatic crustacean. Acid–base regulation takes place against a continuous production of carbon dioxide by the cells and is dominated by passive buffering due to the presence of bicarbonate and nonbicarbonate buffers and by the exchange of acidic/basic equivalents (H^+/HCO_3^-) between body compartments and the surrounding water. All of the mechanisms responsible for branchial acid–base exchange are also involved in ion regulation. See text for further details. The broken lines represent passive diffusion. CA, carbonic anhydrase. Circles with crosses represent electrogenic ion pumps, and open circles represent electroneutral ion exchange. Mechanisms labeled with a question mark are speculative. Modified from Whiteley (1999).

are summarized in Fig. 10.1. Although the ion exchange mechanisms responsible for hemolymph pH regulation in *Daphnia* appear to be similar to those in decapods, the involvement of carbonic anhydrase (CA) has not been established. However, recent molecular investigations have identified 31 genes with CA-like coding regions in the *D. pulex* genome (Weber and Pirow 2009). Twenty-five of the CA sequences had sequence similarities to secretory proteins, indicating that CA is secreted into the hemolymph, unlike in decapods, which lack circulating CA (Cameron 1986). The presence of CA activity in the hemolymph of *Daphnia* could help to explain the transport of CO_2 in the hemolymph as HCO_3^- rather than as dissolved CO_2 as modeled by Weber and Pirow (2009). Overall, resting aquatic crustaceans are either in acid–base balance or they show net base excretion (acid uptake) due to a largely herbivorous diet (Wheatly and Henry 1992).

As described for hemolymph, pH regulation in the intracellular compartment (designated as pH_i) is primarily regulated by electroneutral ion exchange (Taylor et al. 1991, Wheatly and Henry 1992). Early experiments reveal that intracellular pH in crustaceans is regulated by a Na^+-dependent Cl^-/HCO_3^- exchanger and an electrogenic antiporter that exchanges Na^+ for H^+ (Wheatly and Henry 1992, Towle and Weihrauch 2001). Closer examination of the sodium/proton antiporter using brush border membrane vesicles from the hepatopancreas in marine (*Homarus americanus*) and freshwater (*Macrobrachium rosenbergii*) crustaceans has revealed that two Na^+ ions are transferred in exchange for one H^+ (Ahearn et al. 1990 and see Fig. 10.1). This uneven stoichiometry results in considerable Na^+ uptake across a relatively small transmembrane pH gradient and is responsible for a wide array of functions in addition to pH_i regulation (Ahearn et al. 2001). Within the intracellular compartment, buffering capacities are higher than in the hemolymph due to the higher concentrations of proteins and phosphates (Wheatly and Henry 1992). Intracellular bicarbonate levels are relatively low at around 2 mmol l^{-1}, whereas P_{CO_2} levels match those in the venous hemolymph. Intracellular pH values are typically 0.2–0.6 pH units lower than the pH values in the hemolymph, apart from the exoskeletal compartment where pH levels are 0.3 pH units higher in order to maintain the integrity of its calcium carbonate (Wheatly et al. 1991, Wheatly and Henry 1992). This compartment has an important role in acid–base regulation because it constitutes a source of buffer base and is the site for the sequestration of protons and lactate ions during anaerobic metabolism (Jackson et al. 2001) (Fig. 10.1).

AQUATIC HYPOXIA

Aquatic crustaceans can encounter environmental hypoxia and even anoxia in their natural environment. In the marine environment, hypoxia occurs naturally in O_2-minimum zones, deep basins, upwelling areas of eastern boundary currents, and fjords (Helly and Levin 2004, Rabalais et al. 2010). Over the past 50 years, the number of reported hypoxic sites in the global ocean has increased, mainly in coastal regions but also in the open ocean due to anthropogenic activities such as increased nutrient pollution leading to eutrophication (Diaz and Rosenberg 1995, 2008, Gilbert et al. 2010, Rabalais et al. 2010). Climate-related changes are also involved because the rise in surface water temperatures is increasing the likelihood of strengthened stratification, and increased precipitation is likely to increase freshwater discharge and associated influx of nutrients (Rabalais et al. 2010). The sensitivity of estuarine systems to hypoxia is also on the rise due to climate-driven changes in ocean circulation patterns that have driven the upwelling of O_2-poor but nutrient-rich water from the deep ocean into coastal habitats (Howarth et al. 2011). The interstitial water in tidal mudflats and the fluid seeping from hydrothermal vents are both anoxic and contain elevated levels of hydrogen sulfide due to the activity of anaerobic bacteria. Finally, in freshwater ecosystems, O_2 deprivation is characteristic of eutrophic lakes and streams, especially where water flow is low or nonexistent and subject to eutrophication.

Crustaceans can respond to variations in O_2 supply by changing their behavior and/or their physiology. Behavioral changes are summarized here and usually involve movement away from hypoxia in order to find more oxygenated water or emersion into air. Physiological responses range from changes in ventilation and heart rate to variations in the factors determining the functional range of respiratory pigments and metabolic responses. Particular attention is given to the provision of O_2 to developing eggs and the adaptations shown by burrowing crustaceans and those living at great depths. Finally, the facultative or obligate use of air as a source of O_2 is considered.

Behavioral Responses to Hypoxia

Mobile aquatic crustaceans avoid chronic hypoxia by seeking out normoxic waters. In general, crustaceans increase their activity rates up to threefold as P_{O_2} levels start to decline both in the laboratory and in the field (Hagerman and Uglow 1985, deFur et al. 1990, Bell et al. 2003, 2009, Haselmair et al. 2010). At P_{O_2} levels down to 2 kPa, benthic species show avoidance behavior as they abandon their secluded benthic habitats and move vertically to find oxygenated water (Hagerman and Uglow 1985, McMahon 2001, Haselmair et al. 2010). Increased activity occurs regardless of the threat of exposure to predation and the existence of entrained diurnal activity patterns. During severe hypoxia (0.04–2.0 kPa), benthic crustacean species become less active, presumably to reduce O_2 demand when O_2 supply is limiting (Johansson 1997), and can demonstrate a number of atypical behaviors. For example, territorial species are no longer able to avoid each other but will aggregate together in areas where P_{O_2} levels are higher (Haselmair et al. 2010). Predator–prey interactions are also reduced, as well as feeding rates, as shown in the isopod *Saduria entomon*, which feeds on the benthic amphipod *Monoporeia affinis*. During moderate hypoxia, the interactions between *S. entomon* and *M. affinis* decline mainly due to a decrease in activity rates of the latter (Sandberg and Bonsdorff 1996). During anoxia, most species become immobile and moribund, although tolerances do vary because those species with a burrowing lifestyle are more active under such conditions (Haselmair et al. 2010). In addition, crayfish have been shown to actively seek out colder environments during hypoxia to reduce metabolic rates and consequently O_2 demand (Morris 2004). This behavior is known as hypoxia-induced behavioral hypothermia. Other species, such as the northern krill *Meganyctiphanes norvegica*, use cooler temperatures to enable migration down into severely hypoxic waters during the day (Spicer and Saborowski 2010). Migrations to the surface during the night are essential to pay off the O_2 debt. Other extensive diel migrations are observed in the zooplankton. In the coastal upwelling regions off the coast of Chile, for example, the copepod *Eucalanus inermis* and the euphausiid *Euphausia mucronata* are closely associated with the O_2 minimum zone (Escribano et al. 2009). *E. mucronata* in particular performs extensive diel migrations between the surface waters and the core of the O_2 minimum zone. If the temperatures increase as expected due to global warming, then the depth of vertical migrations in these crustacean species could be limited, subjecting them to greater predation risk.

Despite collective behavioral responses to hypoxia, crustacean species demonstrate different threshold levels. Those species with higher tolerances will remain active down to lower P_{O_2} values. To date, it has been shown that species-specific thresholds for reduced activity are lower in situ than they are in the laboratory (Haselmair et al. 2010). They are also lower in adults compared with juveniles (Eriksson and Baden 1997). It has also been demonstrated that activity thresholds can vary within species (Bell et al. 2009). For example, nutritional state can influence an individual's response to hypoxia because recently fed individuals will behave differently to unfed individuals. In *Cancer magister*, declining P_{O_2} levels (21–1.5 kPa) reduced both the amount of food eaten and the amount of time spent feeding (Bernatis et al. 2007). In the laboratory, it was observed that feeding had no effect on the behavior of the crabs because both fed and unfed individuals preferred the highest P_{O_2} levels when placed into an O_2 gradient. Out in the field, however, unfed crabs remained

mobile and migrated up to 1.4 km in 6 h. Crabs that had recently fed settled in well-oxygenated areas to digest their meal for at least 48 h (Bernatis et al. 2007). Hypoxia also affects foraging behavior in *Cancer setosus* because hypoxic crabs consume less prey of smaller body sizes than normoxic crabs (Cisterna et al. 2008).

Physiological Responses to Hypoxia

The differences just described between species in behavioral thresholds to hypoxia can be accounted for by differences in their respiratory physiology. Generally speaking, crustaceans either regulate rates of O_2 uptake down to a critical value of ambient O_2 partial pressure (P_{crit}) or conform to declining O_2 levels in their aquatic environment. In the former case, rates of O_2 uptake are maintained during moderate hypoxia by changes in cardiorespiratory responses and in the ability of the circulatory system to transport O_2 to the metabolizing tissues. Both act to improve O_2 uptake and delivery at a time when ambient O_2 levels are declining. In general, crustaceans maintain O_2 uptake rates down to P_{crit} by increasing ventilation rates (Wheatly and Taylor 1981, Taylor 1982, Airriess and McMahon 1994, McMahon 2001). This effective hyperventilation serves to "blow-off" CO_2 and results in a respiratory alkalosis (Fig. 10.2 B,D). In contrast, in the cladoceran *Daphnia*, which uses its appendages to filter feed, ventilation rates increase but only when the animals are maintained in nutrient-free conditions. When nutrients are available to *Daphnia*, ventilation by the thoracic appendages remains unchanged or even decreases (Paul et al. 1997, Pirow and Buchen 2004). Heart rate either increases, as observed in *Daphnia* (Paul et al. 1997), or declines, as observed in crayfish, lobsters, and crabs (Wheatly and Taylor 1981, Airriess and McMahon 1994, Reiber and McMahon 1998). During hypoxia, cardiac output is typically maintained by increased cardiac stroke volume so that perfusion of the respiratory gas exchange surfaces remains unchanged or may even increase (Taylor 1982, Airriess and McMahon 1994, Reiber and McMahon 1998). Hemolymph flow may also be adjusted during hypoxia, resulting in its redistribution to tissues with higher energy demands. In the crab *C. magister*, for example, hemolymph flow is redistributed from the anterodorsal regions to the posterior and anteroventral regions by the differential operation of valves on the arterial outflow from the heart (Airriess and McMahon 1994). Below P_{crit}, ventilation and heart rate decrease and the O_2 content of the hemolymph decreases (Fig. 10.2 C). At this stage, crustaceans resort to anaerobic metabolism, resulting in the accumulation of lactic acid as the main metabolic end product (McMahon 2001) (Fig. 10.2 E).

The values for P_{crit} in crustaceans vary with aquatic habitat. Table 10.1 summarizes P_{crit} values obtained from crustacean species inhabiting a range of freshwater and marine environments. Generally speaking, those species more likely to encounter hypoxia and anoxia in their natural environment have lower P_{crit} values. In mid-water crustacean species, for instance, it appears that P_{crit} values of less than 3 kPa are associated with specific adaptations for hypoxia in the O_2 minimum layer (Childress and Seibel 1998). Species with the highest P_{crit} values are mid-water species living at high environmental O_2 levels or those living in burrows. Intertidal crustaceans have intermediate P_{crit} values (Table 10.1; for squat lobsters see Lovrich and Thiel 2011). The responses to hypoxia in water breathing crustaceans are affected by temperature because, in the short term at least, it affects rates of aerobic metabolism and consequently O_2 demand. In the shore crab *Carcinus maenas*, P_{crit} for the reduction in both rate of O_2 uptake and heart rate during progressive hypoxia varied from around 8 kPa at 20°C to 5.3 kPa at 10°C (Taylor et al. 1977, Taylor 1981). In the crayfish *Pacifastacus leniusculus*, P_{crit} of O_2 uptake was reduced from 5 kPa at 20°C to 2.5 kPa at 5°C (Gorr et al. 2010). At the lower acclimation temperature, crayfish accumulated less lactic acid, suggesting that aerobic adenosine triphosphate (ATP) production was unaffected by hypoxia at 5°C. In northern krill *M. norvegica*, P_{crit} decreased from 8–11 kPa at 15°C to 4–6 kPa at 10°C (Strömberg and Spicer 2000). This shift in P_{crit} has important implications because *M. norvegica* has a moderate ability to regulate

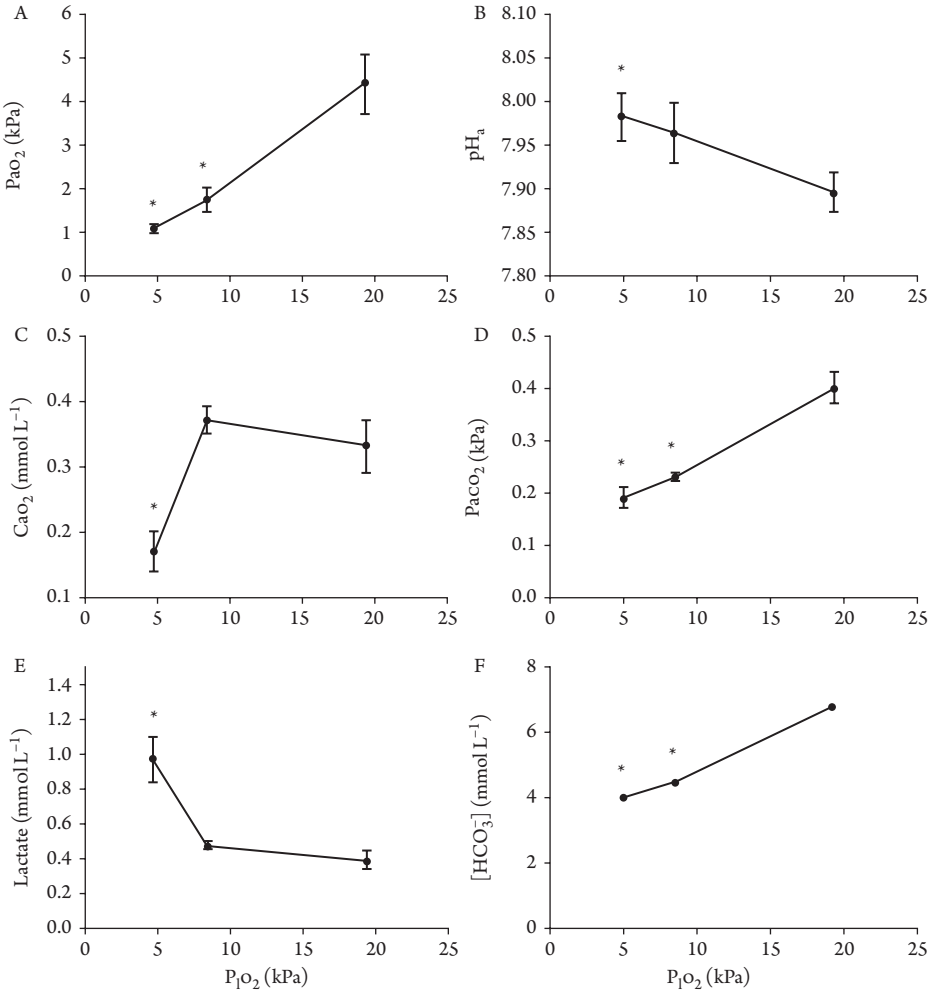


Fig. 10.2.
Changes in postbranchial hemolymph oxygen and acid–base variables during exposure to hypoxia in the freshwater crayfish *Austropotamobius pallipes*. Aquatic hypoxia caused a proportional reduction in (A) hemolymph oxygen partial pressure (Pao_2), (B) an increase in hemolymph pH (pH_a) due to a respiratory alkalosis, (C) the maintenance of hemolymph oxygen content (Cao_2) in moderate hypoxia, (D) a reduction in hemolymph CO_2 partial pressure ($Paco_2$), (E) an accumulation of lactate during severe hypoxia, and (F) a reduction in hemolymph bicarbonate concentration ($[HCO_3^-]$). Values taken from Wheatly and Taylor (1981).

rates of O_2 uptake in the face of environmental hypoxia and rapidly accumulates large concentrations of L-lactate during anoxia (Spicer 1999). Temperature changes during migration into hypoxic layers during diel vertical migration of this mesopelagic species are hypothesized to be crucial for survival (Spicer and Saborowski 2010).

The Role of Respiratory Pigments

The ability of crustaceans to tolerate hypoxia relies to a large extent on the functional properties of their respiratory pigment. This is hemocyanin in most crustaceans, although Hb is present in branchiopod crustaceans such as *Daphnia, Artemia*, and *Chirocephalus* (Terwilliger and Ryan 2001).

Table 10.1. Critical oxygen partial pressures (P_{crit}) for rates of oxygen uptake in crustacean taxa occupying a range of aquatic habitats. Amphipods classified to suborder, decapods to family (apart from thalassinidean shrimps, which are classified to infraorder).

	P_{crit} (kPa)	Temp (°C)	Taxa	Source
Mid-water (Mesopelagic)				
Polar (Antarctic)	3.9–5.6	0.5	Gammarid and hyperiid amphipods, euphausiids, ostracod	Torres et al. (1994)
Temperate	4.0–6.0	10	Euphausiid	Strömberg and Spicer (2000)
	8.0–11.0	15	Euphausiid	Strömberg and Spicer (2000)
Subtropical	2.7–4.0	7	Sergestid and oplophorid shrimps, penaeid prawns	Donnelly and Torres (1988)
	3.3–4.7	14	Sergestid and oplophorid shrimps, penaeid prawns	Donnelly and Torres (1988)
	4.0–6.7	20	Sergestid and oplophorid shrimps, penaeid prawns	Donnelly and Torres (1988)
Oxygen minimum layer	1.2–1.5	4	Mysid and oplophorid shrimps, copepod, ostracod	Childress (1975)
	1.0–1.1	5	Mysid, oplophorid and pandalid shrimps, copepod, gammarid amphipod	Childress (1975)
	1.3–2.4	7	Pandalid shrimp, copepod	Childress (1975)
	2.1–2.7	10	Sergestid shrimp, hyperiid amphipod, euphausiid	Childress (1975)

(*continued*)

Table 10.1. (*Continued*)

	P_{crit} (kPa)	Temp (°C)	Taxa	Source
Deep-sea hydrothermal vents	1.7	5	Bythograeid crab (vent crab)	Mickel and Childress (1982)
Burrow environments	1.3–6.7	10–25	Thalassinidean shrimps	James et al. (2005)
Intertidal	5.3	10	Portunid crab	Taylor (1981)
	~8.0	20	Portunid crab	Taylor et al. (1977)
	~2.0	?	Palaemonid shrimp	Morris and Taylor (1985)
Freshwater	2.5	5	Astacid crayfish	Gorr et al. (2010)
	4.0	15	Astacid crayfish	Wheatly and Taylor (1981)
	6.0	25	Cambarid crayfish	Reiber and McMahon (1998)
Hypogeal	3.3–3.9	22	Cambarid crayfish	Gannon et al. (1999)

During hypoxia, alterations in both carrying capacity and O_2 affinity can occur. Above P_{crit}, the rise in ventilation rate in moderate hypoxia results in a hyperventilation with respect to CO_2 and causes a respiratory alkalosis that left shifts the O_2 equilibrium curve for hemocyanin (Wheatly and Taylor 1981, McMahon 2001). This maintains O_2 loading at the respiratory surfaces, conserving a high O_2 content in postbranchial hemolymph that sustains O_2 supply to the tissues despite reductions in hemolymph Po_2 (Fig. 10.2). Below P_{crit}, it is possible that other modulators increase in importance, such as increases in hemolymph $[Ca^{2+}]$ and $[Mg^{2+}]$ associated with the mobilization of HCO_3^- from internal stores (Taylor and Whiteley 1989), increases in hemolymph [L-lactate] (Truchot 1980, Mangum 1997), and increases in hemolymph urate (Lallier and Truchot 1989). All of these modulators increase O_2 affinity of crustacean hemocyanins. Further details of the modulation of the binding properties of respiratory pigments and how these relate to hypoxia are covered in Chapter 11 of this volume. Increases in the O_2 carrying capacity of hemolymph due to increased hemocyanin content have been observed in *Callinectes sapidus* but only after prolonged exposure to hypoxia (deFur et al. 1990), and in *C. maenas* on exposure to hypoxia after feeding (Legeay and Massabuau 2000). Hb concentrations rise in response to declining O_2 levels in *Daphnia* and *Artemia* (Kobayashi and Hoshi 1982, Mangum 1997, Paul et al. 2004). The induction of Hb is a standard response, and the regulation of Hb gene expression is central to the medium-term survival of *Daphnia* during hypoxia because it leads to the synthesis of Hb subunits with high O_2 affinities (Paul et al. 2004). Between-individual variation in P_{crit} is related to the properties of the respiratory pigments, with increases in the O_2 affinity of hemocyanin associated with lower P_{crit} in *C. sapidus* (Bell et al. 2009) and an increase in both Hb concentration and O_2 affinity being responsible for lower P_{crit} in *Daphnia* (Kobayashi and Hoshi 1984). In the branchiopod crustacean *Chirocephalus diaphanus*, an inhabitant of temporary ponds in which temperatures and respiratory gas concentrations are highly variable, this response is gender-specific because females show the induction of Hb during chronic hypoxia whereas the males do not. Poisoning with carbon monoxide increased the

P_{crit} in females to the level shown by normal males (Taylor 1965). This difference between the sexes may relate to the high cost of egg production and to behavioral differences because the females were observed to swim close to the bottom of temporary pools, where they may avoid predation but be exposed to low O_2 levels, whereas the males swam close to or even at the surface, except for periods when they swam in tandem with the females during insemination.

Metabolic Responses to Hypoxia

A number of metabolic adjustments at the cellular and molecular level also take place during hypoxia, either to protect the tissues from O_2 deprivation or to provide alternative pathways for ATP production. During hypoxia, animals can show several defense mechanisms against O_2 deprivation, including a reduction in energy (measured as ATP) turnover. Studies on vertebrate tissues, mainly hepatocytes and brain tissue, have shown that the defense system in hypoxia-tolerant animals involves an O_2 sensor and a signal transduction pathway, including protein kinase cascades that regulate preferential expression of several proteins (Hochachka 1997). Prolonged hypoxia or anoxia leads to downregulation of ATP turnover rates to achieve a new hypometabolic steady state. Extremely low levels of ATP turnover are achieved via the downregulation of protein turnover, urea synthesis, glucose synthesis, and the maintenance of electrochemical gradients (Wu 2002). The main molecular responses to hypoxia are regulated by the transcription factor hypoxia-inducible factor 1 (HIF-1), which leads to differential gene expression under hypoxia and a series of biochemical and physiological responses (Wu 2002). To date, it has been shown that the synthesis of Hb in *Daphnia magna* during hypoxia is dependent on HIF-1 (Gorr et al. 2004). It has also been suggested that HIF-1α is involved in the regulation of gene expression in crustaceans during the molt and in response to infection (Gorr et al. 2010). In mammals, HIF-1α is even upregulated during normoxia and is associated with cell proliferation, apoptosis, vascularization, and a strong immune response.

Provision of Oxygen for Developing Eggs

Female decapod crustaceans typically hold a mass of fertile eggs on the underside of their abdomens to be released into the plankton as motile larvae following a period of development. Conditions within the egg mass may become markedly hypoxic, especially in brachyuran crabs where the egg mass is relatively large in proportion to body size (Naylor et al. 1999, Fernández et al. 2000, Baeza and Fernández 2002). There is evidence that the female edible crab *Cancer pagurus* may induce development and synchronized release of the eggs after an overwintering dormant period by actively ventilating the egg mass with water. Female crabs attach a mass of up to 3 million fertile eggs to their abdominal pleopods in October/November, then move offshore into deeper, calmer water where they stay half buried in the sand or silt, neither moving nor feeding for about 6–9 months (Naylor et al. 1997). During this inactive period, they show reduced rates of O_2 uptake and decreased heart rates compared to their nonovigerous counterparts (Naylor and Taylor 1999). They also do not appear to aerate or agitate the egg mass. In the spring, these ovigerous female crabs adopt a characteristic stance, which takes the form of a raised posture and increased pumping actions by the abdomen and pleopods, which serves to clean and aerate the eggs (Naylor and Taylor 1999). This was confirmed by measurements of O_2 levels within the egg mass that ranged from 4.5 kPa during early developmental stages to 13.1 kPa at stages close to hatching (Naylor et al. 1999). These increased P_{O_2} levels within the egg mass coincided with a rise in O_2 demand of the eggs and the O_2 sensitivity of the egg masses; P_{crit} increased from 8.0 to 11 kPa with development (Naylor et al. 1999). Preliminary experiments on ovigerous *C. pagurus* revealed that an injection of potassium cyanide in the vicinity of the egg mass increased ventilatory activity by the ovigerous female, indicating that her O_2 receptors were sampling water collected from around the eggs (Naylor et al.

1999) so that she is able to measure the effectiveness of her ventilation of the egg mass. The final stages of egg development in *C. pagurus* can be fairly rapid, taking only 2–3 weeks, and is associated with the active ventilation of the egg mass by the female. Accordingly, the behavioral change leading to the onset of active irrigation of the egg mass in spring enables its synchronized release by the ovigerous female.

The success of in vitro cultivation of decapod eggs has been shown to rely on the degree of agitation of the eggs (Helluy and Beltz 1991). Continuous agitation of eggs isolated from *C. maenas* resulted in higher survival rates and faster rates of embryonic development compared to eggs still attached to adults under similar conditions (Hartnoll and Paul 1982). These observations suggest that circulation of well-aerated water around the eggs is important for development. Oxygen uptake rates in *C. pagurus* eggs were shown to drop by 76% between stirred and stationary conditions, thus indicating that the availability of O_2 is limiting to aerobic metabolism (Naylor et al. 1997). Because the eggs are extremely small, O_2 uptake of individual eggs is unlikely to be diffusion limited. However, they are closely packed within the egg mass so that their O_2 supply is strongly convection limited, with PO_2 in the vicinity of the eggs determined by the brooding behavior of the ovigerous female. Brooding behaviors in brachyuran crabs range from movements of the abdomen to grooming of the egg mass with the chelae (Naylor and Taylor 1999, Naylor et al. 1999, Fernández et al. 2000, Baeza and Fernández 2002, McCleary 2006). Active brood behavior can represent a substantial parental investment due to the costs associated with ventilating the large egg mass (Fernández et al. 2000). Costs increase during development as the O_2 demand of the embryos increases. Movements of the abdomen, for example, increase in frequency during development accompanied by an increase in rates of O_2 consumption of the brooding females (Baeza and Fernández 2002). Furthermore, abdominal movements are continuous during later stages of development and are not subject to diurnal activity rhythms (Ruiz-Tagle et al. 2002). Active brood care has also been observed in freshwater (*Crangonyx pseudogracilis*) and intertidal (*Apherusa jurinei*) amphipods that normally encounter wide variations in O_2 levels in their natural environment (Dick et al. 1998, 2002). Ovigerous females ventilate the embryos by using a flexing motion to flush water through the brood pouch. The time spent by the female ventilating the brood pouch increases when the O_2 levels in the surrounding water are reduced. Brooding behaviors, however, decline at more advanced stages of development when the embryos have functional cardiovascular and respiratory systems.

Hypoxia and Larval Development

Hypoxia has been shown to affect hatching in copepods. Hatching success was suppressed by up to 50% when the eggs were exposed to hypoxia for 12 days, although newly spawned eggs survived exposure to anoxia longer than fully developed eggs (Marcus and Lutz 1994). Experiments on spider crab eggs showed that the eggs are resistant to periods of anoxia and can tolerate low O_2 levels for much of the embryonic development until 1–2 months before hatching (Peterson and Anger 1997). This latter phase is likely to coincide with increases in protein synthesis rates, growth, and differentiation as observed in *C. pagurus* embryos (McCleary 2006). An extreme case is shown by *Artemia* eggs, which can tolerate anoxia during desiccation, surviving as dehydrated cysts for up to 4 years. When water supply is limited, they undergo profound metabolic depression, accompanied by a dramatic drop in intracellular pH (Hand 1998). Both O_2 depletion and a drop in pH depress protein synthesis due to the presence of a molecular O_2 receptor and proton sensitive translational components (Kwast and Hand 1996). The biochemical and molecular adjustments needed to sustain extended metabolic depression are considered elsewhere (see Chapter 12 in this volume). In developing larvae exposed to progressive hypoxia, maintenance of metabolic rate with hypoxia is less well developed than in either post-larvae or adults. Regulation of O_2 uptake rates in

N. norvegicus improves in post-larvae as gas exchange shifts from the extrabranchial surfaces (telson and uropods) to the highly specialized gills (Spicer and Eriksson 2003). This transition occurs as the general exoskeleton surface calcifies and becomes less permeable to O_2 and CO_2. Larvae, however, can improve tolerances to hypoxia by increasing hemocyanin O_2 affinity and possibly circulating hemocyanin concentrations (Spicer and Eriksson 2003).

Living at Depth

Hypoxia and anoxia are features of the open ocean where low ambient O_2 levels occur at intermediate depths as O_2 minimum layers (400–1,000 m) and in the deep ocean at depths below 1,000 m at hydrothermal vents and cold-seeps. In the absence of physical turnover of large masses of seawater, as happens during autumnal cooling in temperate regions, reaeration from the atmosphere of water in the deep ocean is inhibited, and oceanic O_2 minimum layers develop (Childress and Seibel 1998). Knowledge of the ability of crustaceans to live in O_2 minimum layers comes from studies on the bathypelagic mysid *Gnathophausia ingens*. Morphological changes to the gills involve an increase in surface area and a reduction in diffusion distances (Childress 1971). *G. ingens* is also highly effective at extracting O_2 from the ventilatory stream due to relatively high ventilation rates and increased circulatory capacity (Belman and Childress 1976). In addition, hemocyanin from *G. ingens* has a high affinity for O_2, a high cooperativity, and a large Bohr effect (Sanders and Childress 1990). Marine species living within the O_2 minimum layer tend to have poor anaerobic abilities, suggesting that changes in aerobic capacity are sufficient for survival in conditions where P_{O_2} levels are consistently low at only a fraction of 1 kPa (Childress and Seibel 1998).

Deep-sea habitats present further challenges to marine life due to increases in barometric pressure, decreases in temperature, and the lack of light apart from bioluminescence. Conditions can be even more unfavorable at hydrothermal vents and cold-seeps where ambient seawater at 2°C mixes with anoxic and acidic (pH 3.0) vent fluid at 350°C; this fluid contains high concentrations of heavy metals, CO_2, and sulfide (Hourdez and Lallier 2007). Cold-seeps occur where hypersaline brine containing reduced sulphur, methane, and often mineral oils emerge from the sediments on the seafloor without an appreciable increase in temperature (Levin 2005). Despite these harsh and variable conditions, deep-sea habitats support thriving marine communities characterized by low species diversity but high species abundance. Crustaceans are well represented in both communities, with 125 species of decapod crustaceans from more than 33 families being reported in 2005 (Martin and Haney 2005). Amphipods, isopods, and copepods have also been reported to occur in cold-seeps (Levin 2005). Although these deep-sea sites are largely inaccessible, studies on animals successfully brought to the surface and kept under high pressure are beginning to explore the respiratory adaptations of deep-sea crustaceans. Currently most of our knowledge comes from work on hydrothermal vent species as opposed to cold-seep species and those that occupy O_2 minimum zones where hypoxic conditions are more stable.

In general, rates of O_2 uptake in deep-sea species at low P_{O_2} levels are similar to those of related shallow water species at higher P_{O_2} values (Seibel and Drazen 2007). A reduction in metabolic rate with depth in pelagic species is thought to be associated with a reduction in the use of visual predation and the requirements for high activity rates (Childress and Seibel 1998). Metabolic rates in benthic decapods and amphipods do not change with depth when values were standardized for temperature and body size. Therefore, it appears that neither pressure nor hypoxia has an effect on metabolic rates in deep-sea benthic crustaceans (Childress and Seibel 1998). To date, physiological studies have shown that metabolic rates are maintained during hypoxia in deep-sea crustaceans via a series of morphological and respiratory adaptations. It appears that only those species that frequently encounter hot, anoxic conditions show morphological differences in their branchial chambers. Both the vent shrimp *Rimicaris exoculata* and the vent crab *Bythograea thermydron* show

a significant increase in scaphognathite surface area when compared with related littoral species (Decelle et al. 2010). Presumably, this morphological adaptation serves to improve water flow through the branchial chambers in order to increase O_2 supply to the gills. Gill surface areas in both vent species are also significantly higher due to a doubling of the number of gill lamellae. Despite the increase in gill surface area observed in *R. exoculata*, the branchial chambers are enlarged to contain chemoautotrophic bacteria that oxidize sulfur from the hydrothermal vents to fix carbon and provide a direct food source for the shrimps. This is a highly successful arrangement because *R. exoculata* can live in very large swarms at densities of 1,000–3,000 individuals m^{-2} (Cottin et al. 2010). Impairment of respiratory gas exchange by the bacteria is avoided because they are restricted to distinct sites within the branchial chambers. Each branchial chamber is divided into three parts, with the bacteria housed in two areas, one with and one without mineral deposits of iron oxides (Schmidt et al. 2008). The gills occupy the third part of the branchial chamber and remain free of both bacteria and mineral formations. In contrast, scaphognathite and gill surface areas in those species with access to colder more oxygenated waters in the vicinity of hydrothermal vents are similar to those in littoral species (Decelle et al. 2010). None of the deep-sea species examined by Decelle et al. (2010) showed a reduction in diffusion distances across the gills. Overall deep-sea crustaceans have a good capacity for anaerobic respiration that enables them to survive bouts of severe hypoxia and/or anoxia (Hourdez and Lallier 2007).

The respiratory pigments, hemocyanin or Hb, of deep-sea crustaceans are characterized by a high affinity for O_2 and a large Bohr effect (Hourdez and Lallier 2007). These characteristics facilitate O_2 loading at the gill and O_2 release at the tissues. Lactate increases the O_2 affinity of hemocyanin in some species but not others, such as the vent-chimney crab *Cyanagraea praedator* (Chausson et al. 2001), although thiosulphate increases hemocyanin O_2 affinity in the vent crab *B. thermydron* (Vetter et al. 1987). Interestingly, temperature has no or little effect on the O_2 affinity of hemocyanins in the vent crustaceans sampled to date (Lallier and Truchot 1997, Chausson et al. 2001). This insensitivity to temperature may represent an adaptation to living in the highly variable temperatures of hydrothermal vent systems. Hb concentrations in the two deep-sea vent copepods studied to date indicate that concentrations are relatively low. It appears that Hb serves to facilitate O_2 uptake during hypoxia because its O_2 affinity is so high, as discussed by Hourdez and Lallier (2007).

Burrowing Crustaceans

A number of aquatic crustaceans burrow into the substratum in order to shelter from extreme environmental perturbations and to protect themselves from predators (Taylor and Atkinson 1991, James et al. 2005). Species either bury themselves into the sediment for short-term concealment or construct semipermanent burrows within the sediment that they rarely leave unless they have to move to feed and to breed. Burying behavior occurs in many Brachyura but also in anomurans and has been observed in reptant decapods such as Astacidea and Thalassinidea, as well as in natant decapods such as the Alpheidae (Taylor and Atkinson 1991, James et al. 2005).

Burrowing aquatic decapods can encounter very severe hypoxia or even anoxia in their burrows on a regular basis, especially species that inhabit tidal mud flats where levels of hydrogen sulphide may be elevated. In burrows inhabited by two species of upogebiid mud-shrimp (Thalassinidea), Po_2 values varied between 10.7 and 13.3 kPa at the burrow mouth but dropped to 1.3–6 kPa in the deeper, more poorly irrigated parts (Astall et al. 1997). The first response of many burrowing species is to increase the rates of beating of their pleopods that are used to irrigate the burrows and to increase the time spent irrigating (Taylor and Atkinson 1991). However, if Po_2 of the burrow water falls below P_{crit} (Table 10.1), pleopod beating will cease and the animal will either leave the burrow to find oxygenated water, as observed in *N. norvegicus* and burrowing crayfish (Hagerman

and Uglow 1985, McMahon 2001), or it will tolerate anoxia by resorting to anaerobic metabolism (James et al. 2005, Holman and Hand 2009).

In general, burrowing aquatic decapod crustaceans are highly tolerant of hypoxia, although juveniles and larger adults are more sensitive (Taylor and Atkinson 1991, Eriksson and Baden 1997, Holman and Hand 2009). Burrowing crustaceans show the same hypoxic response as nonburrowing species: ventilation rates increase and heart rate remains unchanged or decreases but fails to show bradycardia during severe hypoxia/anoxia (Astall et al. 1997, James et al. 2005). Burrowing decapods such as callianassid species, *Upogebia* mud-shrimps and *N. norvegicus* are able to maintain rates of O_2 uptake down to relatively low critical P_{O_2} levels during progressive hypoxia (Table 10.1; see, e.g., Hagerman and Uglow 1985, Eriksson and Baden 1997). They achieve this by possessing respiratory pigments with higher O_2 affinities than those found in other aquatic decapod species and, in burrowing crayfish at least, by having higher O_2 carrying capacities (Taylor et al. 2000, McMahon 2001). An increase in hemocyanin O_2 affinity facilitates O_2 uptake at the gills despite the presence of relatively low circulating P_{O_2} levels.

In those species that can tolerate anoxia, such as the mud-shrimp *Calocaris macandreae* (lethal time for 50% mortality, LT_{50} = 43 h at 10°C) and the ghost shrimp *Lepidophthalmus louisianensis* (LT_{50} = 64–113 h at 25°C), animals accumulated L-lactate as the metabolic end product (Anderson et al. 1994, Holman and Hand 2009). Recovery from anoxia was slow in *C. macandreae*, and anaerobic metabolism was only utilized in extreme cases when burrow P_{O_2} levels dropped below 0.9 kPa (Anderson et al. 1994). Moreover, it appears that metabolic depression, a strategy employed to reduce O_2 demand during deficiencies in O_2 supply, is delayed in the ghost shrimp for up to 48 h of anoxia (Holman and Hand 2009). ATP production between 6 and 12 h anoxia was sustained up to 55–77% of the aerobic rate from lactate and arginine phosphate metabolism, which could help to explain why this species is so tolerant of anoxia. Alternatively, those species with access to the air–water interface at the burrow openings, such as species occupying intertidal mud flats, will utilize air as a source of O_2. A similar response has been observed in burrowing crayfish, which can undergo total emergence into air for approximately 75% of the hypoxic period (McMahon 2001). Access to O_2 increases circulating P_{O_2} levels, but a concomitant accumulation of hemolymph P_{CO_2} results in a small Bohr shift and subsequent reduction in hemocyanin O_2 affinity. This allows O_2 delivery at the tissues despite the increase in circulating P_{O_2} (McMahon 2001).

Facultative Air Breathing

Some species of crustaceans live routinely at the air–water interface. The shore crab *C. maenas* inhabits the littoral zone along the Atlantic coast of Europe, as well as the Mediterranean and increasingly the Pacific coast of North America. It can often be found in rock pools that can become markedly hypoxic at night. Under these circumstances, the crab raises its exhalent openings above the water surface and, by reversing the normal direction of ventilation, bubbles air through the water surrounding the gills in the branchial chambers, a behavior termed the *emersion response* (Taylor and Butler 1973, Taylor et al. 1973). The O_2 partial pressure at which this behavior is elicited varies with metabolic rate, which is affected by temperature, salinity (Taylor et al. 1977), and the consumption of food (Robertson et al. 2002). The behavior serves to aerate the water surrounding the gills and to raise the O_2 content of the arterialized hemolymph (Taylor et al. 1973) while retaining the aquatic route for the excretion of carbon dioxide. Female crabs carrying eggs direct the stream of bubbles backward over the egg mass, and they show this specific emersion behavior, which seemingly relates to the O_2 requirements of the developing eggs, at higher O_2 levels than do nonovigerous females (Wheatly 1981). This response is highly temperature dependent because an increase in acclimation temperature from 15°C to 25°C increased the percentage time spent aerating the egg mass by the ovigerous females. Over the same temperature range, the P_{crit} values for O_2

uptake of the eggs increased from 6.7 to 10.7 kPa (Wheatly 1981). Collectively, these observations imply that the ovigerous female responds to the O_2 requirements of her eggs despite them being in a mass external to her body.

Many primarily aquatic crustaceans are able to survive relatively long periods out of water. This ability is exploited by marine littoral species that can feed on the shore between tides and by some freshwater species that can migrate onto land to feed at night and to migrate between bodies of water during drought. This behavior can be elicited by increased temperature. When the shore crab was exposed to a progressive increase in ambient temperature, it moved into air at a mean temperature of 28°C. In air, it is able to maintain a similar rate of O_2 consumption as when it is submerged in normoxic water despite being reliant on gas exchange over chitin-covered gills (Taylor and Butler 1978, Taylor and Wheatly 1979). The freshwater crayfish can survive in air for up to 72 hours and routinely leaves water to forage for food or to change habitats. Under experimental conditions, it can be driven into air from shallow water by progressive hypoxia or increasing temperature (Taylor and Wheatly 1980). When in air, its gills collapse, resulting in a pronounced reduction in the effective surface area for gas exchange. As a result, the animal becomes markedly hypoxic and hypercapnic (Fig. 10.3A,E). However, an initial respiratory and metabolic acidosis is countered by elevation of buffer base in the form of bicarbonate ions (Fig. 10.3D). Evidence that these are mobilized from the calcified exoskeleton was provided by the simultaneous elevation of calcium levels in the hemolymph (not shown in Fig. 10.3). Oxygen supply to the tissues falls to zero, then is restored during aerial exposure, initially by the reversal of a Bohr shift due to compensation for the acidosis (Taylor and Wheatly 1981) and subsequently by an increase in the affinity of hemocyanin for O_2 resulting from the accumulation of lactate and calcium ions in the hemolymph (Morris et al. 1986a,b) (Fig. 10.3F). Both protons and lactate are then effectively buffered/sequestered in the calcified exoskeleton (Jackson et al. 2001). Following resubmersion there is a lactate washout into the hemolymph, likely from the exoskeleton (Fig. 10.3F). So, the exoskeleton buffers protons, releases bicarbonate and calcium, and sequesters lactate during aerial exposure (Fig. 10.1).

These adaptations to survival out of water are exploited by the commercial fishing industry that transports live crustaceans long distances in air. The adaptations enabling survival have been studied in some detail in the lobster *Homarus gammarus* and are described by means of a pH-bicarbonate or Davenport diagram (Fig. 10.4; Taylor and Whiteley 1989, Whiteley and Taylor 1989, 1992). The effects of aerial exposure and recovery have also been described in other commercial species such as the Norway lobster *N. norvegicus* (Lund et al. 2009, Albalat et al. 2010) and the South African Cape lobster *Jasus lalandii* (Haupt et al. 2006).

The ability of aquatic gill-breathing decapods to survive aerial exposure appears to be related to the differences in habitat and to the degree of exposure that species normally encounter (Whiteley 1999). Subtidal species such as *C. sapidus* (Cameron and Batterton 1978) and the red rock crab *Cancer productus* (deFur et al. 1983) bury into the substratum if exposed on rare occasions and remain quiescent. Species distributed further up the shore can function as facultative air breathers, as mentioned earlier for *C. maenas*, and can survive longer periods of aerial exposure.

Terrestrial, Air-breathing Crustaceans

The combination of relatively high temperatures and frequent periods of hypoxia in tropical habitats such as mangrove swamps likely presented the environmental pressures that led to the evolution of air breathing in crustaceans (Innes and Taylor 1986). Regular exposure in air is often associated with a reduction in gill area although some air-breathing crabs may continue to use the gill surfaces for gas exchange and in particular for the excretion of CO_2. They achieve this by holding a small volume of water in the branchial chambers while in air, and they flick this water over the gill surfaces. This reservoir of water is used as a sink for CO_2 and ammonia (Wood and Randall

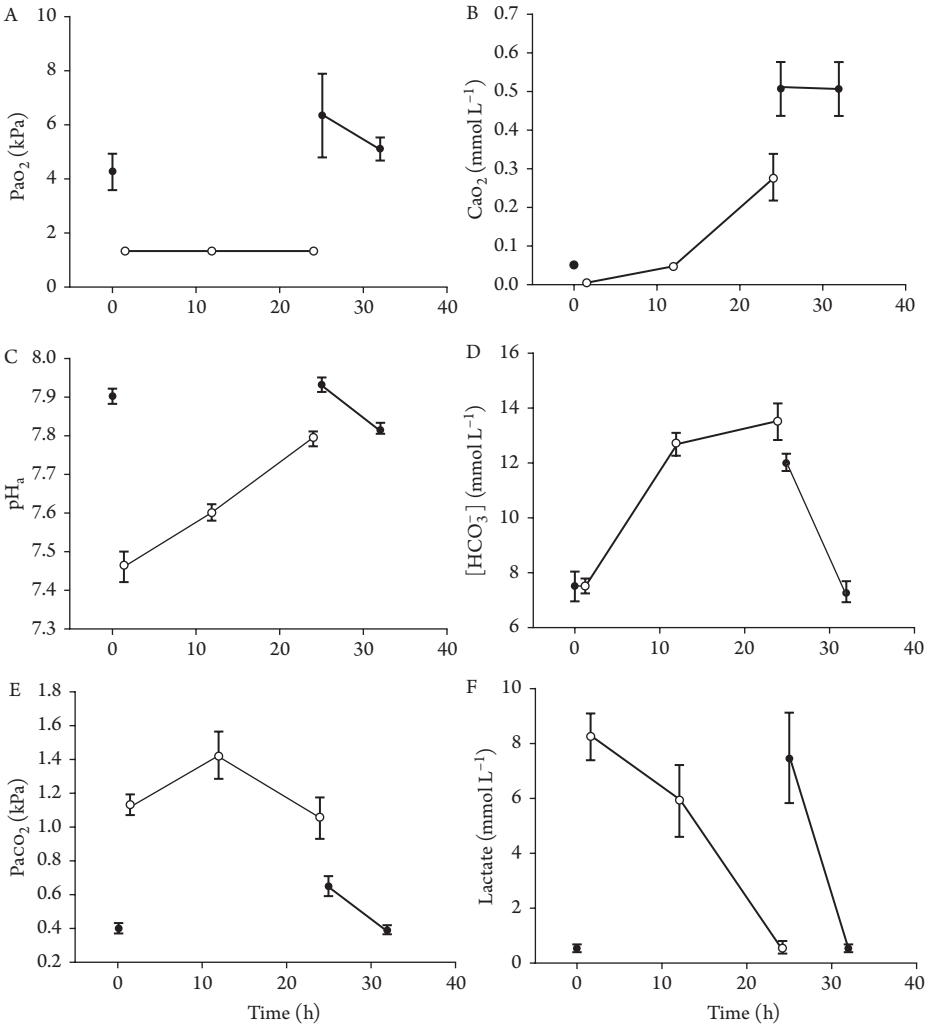


Fig. 10.3.
Changes in postbranchial hemolymph oxygen and acid–base variables during 24 h of exposure to air (open circles) and subsequent recovery in aerated water (filled circles) in the freshwater crayfish *Austropotamobius pallipes*. Values are (A) oxygen partial pressure (Pao_2), (B) oxygen content (Cao_2), (C) pH (pH_a), (D) bicarbonate concentration ($[HCO_3^-]$), (E) CO_2 partial pressure ($Paco_2$), and (F) lactate concentration. Values taken from Taylor and Wheatly (1981).

1981). This adaptation ties them to a local supply of water to refresh their reservoir when it becomes saturated with CO_2 or ammonia, so that they cannot stray far from the margins of the sea or from their water-filled burrows. Many of these land crabs continue to release larvae into the plankton, which is an additional factor tying them to the seashore. Oxygen is typically obtained by diffusion over a lung-like structure elaborated from the wall of the branchial chamber; this structure is characterized by an increased surface area and a thin cuticle, resulting in short diffusion distances (Greenaway and Taylor 1976, Taylor and Taylor 1992). Thus, these structures satisfy the requirements of Krogh's law of diffusion, and land crabs can be characterized as maintaining O_2 levels of between 6.66 and 13.33 kPa and CO_2 levels below 1.33 kPa by the combined use of gills and lungs (Fig. 10.5).

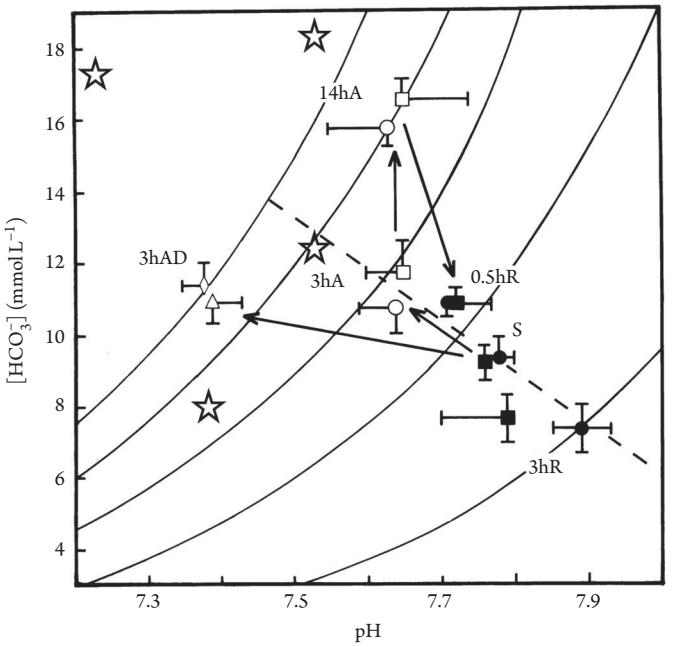


Fig. 10.4.
A pH-bicarbonate (Davenport) diagram of the changes in acid-base variables in the postbranchial hemolymph during 14 h of exposure in air and subsequent recovery in aerated seawater in the lobster *Homarus gammarus*. On exposure in air for 3 h, the submerged animal (S) showed an increase in P_{aCO_2} along the nonbicarbonate buffer line, accompanied by a mild acidosis when undisturbed (3hA), but a much greater acidosis when disturbed in air (3hAD). After 14 h in air (14hA), pH was restored to submerged levels by elevation of bicarbonate at constant P_{aCO_2}. Resubmergence resulted in a recovery alkalosis (0.5hR and 3hR). The ☆ symbol denotes animals sampled on arrival at market following transport in air. Values taken from Taylor and Whiteley (1989) and Whiteley and Taylor (1992).

A few species are more independent of water, living for periods remote from a source of standing water. Preeminent among this group is the Trinidadian mountain crab *Pseudothelphusa germani*. This South American crab lives in a tropical rainforest and never returns to the sea to breed. During the dry season, the female aestivates in a burrow, holding a small batch of eggs that she releases as fully formed crabs into freshwater streams at the onset of the rainy season. She then molts and joins the males in a period of active feeding and mating. During this period, both sexes are active air breathers that can, however, utilize freshwater as a sink for CO_2 and ammonia by irrigating the gills, using the scaphognathites. At the onset of the dry season, both sexes retreat back into their burrows on the banks of streams that dry up and deprive them of access to free water. The animals then rely on respiratory gas exchange with air over their elaborately formed lungs. These are diffuse structures that are invaginated into the walls of the branchial chambers and present a large surface area of thin epithelium to a stream of deoxygenated hemolymph that perfuses large sinuses (Innes and Taylor 1986, Taylor and Innes 1988, Taylor and Taylor 1992). Use of these invaginated lungs can generate very high O_2 partial pressures (up to 20 kPa) in arterialized hemolymph (Fig. 10.5). These O_2 levels seem disproportionate in relation to the binding properties of hemocyanin because the respiratory pigment will be saturated at lower O_2 levels. The answer to this apparent paradox lies in the function of the lungs in excreting CO_2 in the absence of an aquatic route. When the respiratory gas exchange properties of the gills and lungs of a range of animals are plotted as a CO_2/O_2 diagram (Fig. 10.5), it shows that the mountain crab is effectively blowing off CO_2 by

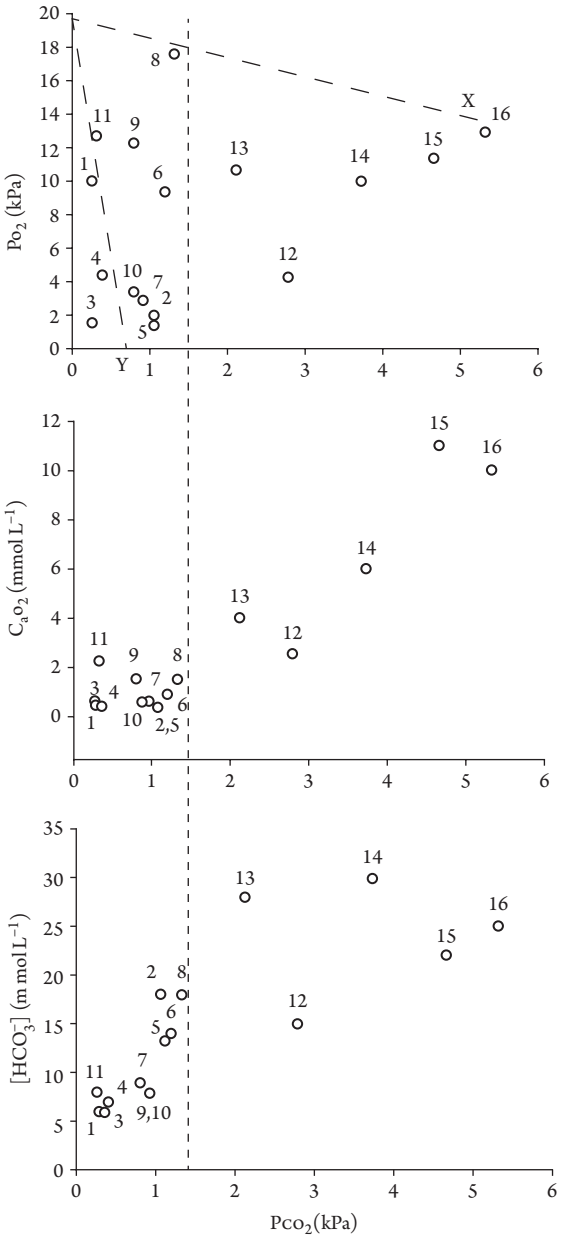


Fig. 10.5.
Comparison of the relationships between crustacean hemolymph and vertebrate blood values of: oxygen partial pressure (P_{O_2}), oxygen content (Ca_{O_2}), and bicarbonate concentration ($[HCO_3^-]$), plotted against carbon dioxide partial pressure. The two oblique lines on the plot of P_{O_2} against P_{CO_2} labeled X and Y are the respiratory gas exchange (R) lines for a ratio of 1 in air breathers and water breathers, respectively (see Rahn 1966). All of the crustaceans, whether in water or air (numbered 1–10), have CO_2 partial pressures below 1.33 kPa (10 mm Hg), indicated by the vertical broken line. This relates to the low nonbicarbonate buffering capacity of their hemolymph that reflects its low protein content, which in turn determines its low oxygen content. Air-breathing vertebrates (numbered 12–16) have elevated CO_2 partial pressures and oxygen contents in the blood that relate to their increased blood protein levels, with hemoglobin packaged into red cells. Bicarbonate concentrations are elevated in air-breathing crustaceans to about the level shown by lungfish but not as high as the committed air breathers among the vertebrates. These relationships are further discussed in the text. The numbered points represent shore crab, *Carcinus maenas*: 1, submerged in seawater; 2, in air; 3, in shallow seawater with access to air; crayfish, *Austropotamobius pallipes*: 4, submerged in freshwater; 5, in air; land crab,

apparent hyperventilation at a respiratory exchange ratio of unity (R = 1). In fact, its rate of ventilation is very low, being generated by small, slow changes in pressure in the branchial chambers, in order to reduce respiratory water loss. This ventilation rate, however, is high relative to the flow of hemolymph, and this, coupled with a high diffusion coefficient over the lung, results in this highly effective exchange of respiratory gases. The primary role of the lung under these circumstances is clearly to reduce levels of CO_2 in the hemolymph below a critical level of 1.33 kPa in order to maintain acid–base balance (Innes and Taylor 1986, Taylor and Innes 1988). In comparison to vertebrate blood with its red cells, the hemocyanin level in crustacean hemolymph is limited by the colloid osmotic pressure it exerts (Mangum and Johansen 1975). This represents a potential limit on its O_2 carrying capacity and also on its buffering capacity against the accumulation of CO_2 that, in most land crabs, is countered by retaining the aquatic route for CO_2 excretion but in the mountain crab is achieved by exchange of respiratory gases over its highly effective lung (Fig. 10.5).

TEMPERATURE

Animals in general live within a biological temperature range set by the freezing point of water and the upper thermal limits for protein stability, but, within these limits, species vary according to their thermal histories. The body temperatures of ectotherms including crustaceans vary with those of their environment. However, thermal ranges differ considerably and reflect evolutionary adaption to particular thermal niches. Those species that inhabit relatively constant temperatures, such as polar and tropical marine species as well as deep-sea and cave-dwelling species, are stenothermal and sensitive to temperature change. In general, those that inhabit more variable thermal environments, such as freshwater and shallow coastal species, are eurythermal and are less sensitive to temperature change. Furthermore, the latter can vary within genetically fixed limits over temporal and spatial scales (Hochachka and Somero 2002). Temperature has a profound effect on ectotherms at all levels of biological organization from influencing biological rate functions to influencing the biogeographical distribution patterns of a species. Although crustaceans are unable to control body temperatures independently of the external environment, they can show a number of behavioral responses to avoid temperature extremes. Many can also acclimate to temperature change in the laboratory and acclimatize in the natural environment to simultaneous changes in multiple environmental variables. Ecophysiological responses to temperature change have been studied in crustaceans since the early 1950s, and there is a current interest in the physiological characteristics of marine species distributed along natural thermal gradients, such as those that occur with latitude and vertical distance up the shore, because of the growing concern over the effects of increasing sea surface temperatures occurring as a result of climate change. Physiological responses can indicate thermal tolerances, thus enabling us to understand factors affecting the survival, distribution, and abundance of marine invertebrates (including crustaceans) and predict how they will respond to increasing temperatures and be able to adjust their thermal tolerances to survive climate change (Stillman 2003, Somero 2010).

Thermal Limits

The degree of eurythermy shown by ectotherms is ultimately affected by physiological responses at the level of the whole organism (Pörtner 2002). Early experiments on the spiny spider crab *Maja squinado* showed that thermal tolerances were influenced by limitations in O_2 supply (Federich and

Fig. 10.5. (*Continued*)

Cardisoma carnifex: 6, in air; 7, in water; Trinidad mountain crab, *Pseudothelphusa germani*: 8, in air; 9, in shallow water with access to air; 10, submerged in freshwater; 11, fish, *Scyliorhinus canicula*; 12, lungfish, *Protopterus*; 13, amphibian, *Bufo marinus*; 14, reptile, *Pseudemys*; 15, bird, *Gallus*; 16, mammal, *Homo sapiens*.

Pörtner 2000). Measurements of heart and ventilation rate between 0°C and 40°C showed that both rate functions increased with temperature to compensate for the rise in O_2 demand. Above the "pejus temperature," which is the early limit to temperature tolerance, both heart and ventilation rate stabilized, and arterial Po_2 started to decrease even though the crabs were in fully aerated seawater. As temperatures reached the upper critical limit, systemic hypoxia preceded anaerobic metabolism as O_2 demand outstripped O_2 supply. At the lower limit of the temperature range, both rate processes decreased to such an extent that O_2 uptake and transport was insufficient. This concept of O_2 and capacity limitation demonstrates that optimal aerobic performance in *M. squinado* is only maintained between 6°C and 16°C (Federich and Pörtner 2000). Above and below these thermal limits, performance decreases to unsustainable levels. Thermal limits differ between species and populations and can be influenced by concomitant changes in another environmental variable. The simultaneous exposure of two crab species, *C. pagurus* and *Hyas araneus*, to hypercapnia plus an increase in temperature reduced the upper thermal limits and increased mortality rates in both species (Metzger et al. 2007, Walther et al. 2009).

Physiological Responses to Thermal Gradients in the Littoral Zone

Differences in thermal sensitivities of crustacean species distributed along a vertical environmental gradient in the intertidal zone have been attributed to physiological variation with height on the shore (Somero 2002). Thermal adaptations on the shore are necessary because, during emersion, organisms are no longer under the influence of the buffering effects of the seawater but are exposed to higher air temperatures, solar insolation (radiation), and changes in humidity and wind speed. As a result, there is considerable spatial and temporal variation in temperatures on the shore, further influenced by the timing of low tide, climatic conditions, aspect, cloud cover, and occurrence of microclimates (reviewed by Helmuth et al. 2006). This can result in rapidly fluctuating and often extreme temperatures, particularly in sessile intertidal crustaceans such as barnacles, and especially in those species distributed higher up on the shore. Whereas sessile species are unable to avoid thermal extremes, mobile intertidal crustaceans, such as intertidal decapods, isopods, and amphipods, can escape to favorable microclimates by hiding in crevices or under stones and seaweed where temperatures are more moderate. Some also rely on behavioral thermoregulation, such as the semiterrestrial isopod *Ligia oceanica*, which uses evaporative cooling to keep body temperatures several degrees lower than ground temperatures (Edney 1953). This behavioral response, however, is limited because *L. oceanica* needs to return to humid conditions at intervals to prevent death by desiccation.

Physiological adaptation to increased thermal stress is of key importance to the survival of crustacean species on the shore. To date, studies are limited to observations on barnacles and porcelain crabs. High shore barnacles are able to keep their filter-feeding cirri beating at markedly higher temperatures than are individuals from further down the beach. Thermal limits for the circulatory system also vary. Two sympatric species of the porcelain crab (*Petrolisthes*) living in different vertical zones of the intertidal were examined by Stillman and Somero (1996). Upper and lower thermal limits were compared over the range of temperatures normally encountered in the field. They varied with the thermal conditions in the intertidal habitats of crabs from different biogeographic regions (Stillman and Somero 1996). These thermal tolerances are relatively plastic, although adjustments in LT_{50} were lower in the upper intertidal species compared with low intertidal and subtidal species (Stillman 2002). There was a strong positive correlation between LT_{50} and maximum habitat temperature that varied with vertical position on the shore. Arrhenius break temperatures (ABT) of heart rate varied with the upper shore species, *Petrolisthes cinctipes*, characterized by an ABT of 31.5°C and the low intertidal/subtidal species, *Petrolisthes eriomerus*, characterized by an ABT of 26.6°C. The upper shore species, which normally experiences a maximum habitat temperature

of 33°C, is more eurythermal and is remarkably tolerant to a wide range of temperatures. *P. eriomerus* could not survive at the temperatures experienced by the upper shore species. The upper thermal limits of heart function (CT_{max}) after temperature acclimation showed a similar response because CT_{max} decreased linearly among species in inverse proportion to maximal habitat temperature (Stillman 2003). In *P. cinctipes*, heart failure was one of the proximate causes of heat death in its native habitat (Stillman and Somero 1996). It is possible that thermal limits were set by differences in the properties of the cardiac muscle or differing sensitivities in the nerves innervating the heart. It was concluded that upper shore species with the greatest tolerance to high temperatures are the most threatened by increases in habitat temperature because they are already living at temperatures close to their LT_{50}s and so have reduced ability to adjust their thermal limits (Stillman 2002).

Cold temperatures can also be a problem in the intertidal zone, but the physiological effects are rarely mentioned. Coastal areas at high latitudes are subject to freezing conditions in the winter months for part of the tidal cycle. Intertidal invertebrates in such areas are freeze tolerant because they cannot avoid the numerous ice crystals present in the environment and have little time to protect themselves from ice formation. The barnacle *Semibalanus balanoides* can tolerate freezing and thawing in the extracellular compartment on a regular basis (Murphy 1983). Freeze tolerance and lower lethal temperatures also vary with season because summer *S. balanoides* are unable to survive at −6°C when about 40–45% body water freezes. In winter, death in barnacles occurs at −18.3°C when more than 80% of the body water freezes. Other species, such as the gammarid amphipod *Gammarus duebeni*, can avoid freezing by inhabiting hypersaline rock pools where sea water remains unfrozen even when temperatures reach −8°C (Davenport 1992). Physiological responses to cold acclimatization involve classic strategies, such as changes in lipid composition in the membranes and changes in enzyme activities. For example, crabs undergo increases in membrane fluidity due to a decrease in cholesterol levels within the membrane, with the more stenothermal crab *C. pagurus* having slightly more fluid membranes and lower cholesterol/phospholipid ratios than the eurythermal species *C. maenas* (Cuculescu et al. 1995).

Physiological Responses to Latitudinal Thermal Gradients

Metabolic Rate

Temperature typically has a marked effect on physiological and biochemical rate processes in ectothermic animals, causing a two- to threefold increase in rate over a 10°C rise in temperature. Nevertheless, responses to temperature vary tremendously because many species are able to compensate biological rate processes such as metabolic rate. Comparisons within species demonstrate that crustaceans from latitudinally separate populations usually compensate for environmental temperature gradients. The overall effect is the maintenance of similar rates of O_2 uptake when measured at the habitat temperatures experienced by the different populations (e.g., metabolic rates in species such as the fiddler crab *Uca rapax* and the prawn *Pandalus montagui* are higher in northern versus southern populations, when measured at the same temperature; Fox and Wingfield 1937, Vernberg 1962). A similar response has been observed in northern krill *M. norvegica* from three geographically separate populations (Saborowski et al. 2002) and in boreal/temperate and temperate gammarid amphipod species from different latitudes (Rastrick and Whiteley 2011). However, lack of any metabolic compensation with latitude has been observed in the porcelain crab *Petrolisthes granulosus* sampled from three sites along the Chilean coast (20–36°S; Monaco et al. 2010) and in the subarctic/boreal gammarid species at high polar latitudes (Rastrick and Whiteley 2011). Metabolic compensation was confined to the more eurythermal species that may benefit from the maintenance of a consistency in biological rate processes across latitudes. In those species where metabolic compensation does take place with latitude, there is some evidence of local adaptation. In *Uca*

pugilator, for instance, individuals from three latitudinal populations were hatched and reared under identical laboratory conditions (Vernberg and Costlow 1966). Metabolic rates were higher in cold water populations from North Carolina and Massachusetts compared with warm water populations from Florida. The persistence of differences in metabolic rate across generations suggests they are genetically fixed, resulting in differences in acclimatory ability with latitude. Moreover, a southern population of the copepod *Euterpina acutifrons* from Brazil (24°S) reduced metabolic rate at high acclimation temperatures more effectively than did a population from South Carolina (35°N), thus reflecting better adaptation to mean habitat temperatures (Vernberg and Moreira 1974).

Species-related comparisons of standard metabolic rate indicate that marine crustaceans do not compensate metabolic rates in order to live in the extreme cold, as previously suggested by the concept of metabolic cold adaptation. Antarctic isopod crustaceans do not upregulate their metabolic rates in the cold and stable conditions of the Southern Ocean (Luxmoore 1984, Chapelle and Peck 1995, Whiteley et al. 1996). Instead, they maintain relatively low rates of O_2 uptake resulting in low maintenance costs, which is a significant advantage in the cold because food resources may be limiting (Clarke 1993, Pörtner et al. 2005). Arctic amphipods also maintain relatively low rates of O_2 consumption because rates were lower in the subarctic/boreal species *Gammarus oceanicus* and *Gammarus setosus* compared with the temperate species *G. locusta* (Rastrick and Whiteley 2011). It has been suggested that lower metabolic rates and therefore O_2 demand, as well as the increase in O_2 solubility at low temperatures, will allow for larger body sizes (a feature of many Antarctic marine ectotherms) because sufficient O_2 uptake from O_2-rich waters is possible despite the reductions in O_2 gradients and diffusion distances that accompany an increase in body size (Chapelle and Peck 1999, Pörtner et al. 2007). This suggestion ignores the role of the circulatory system.

Protein Synthesis Rates

Protein synthesis rates are reported to be closely related to rates of O_2 uptake mainly because protein synthesis is energetically costly and can account for up to 42% of basal metabolic rate (Houlihan et al. 1995, Whiteley and Fraser 2009). Rates of protein synthesis generally increase with temperature, but values are influenced by the rate of feeding, which also increases with temperature. Under these conditions, whole-animal rates of protein synthesis in a range of crustacean species collected from different thermal regimes and acclimated to a constant temperature for 4 weeks showed an exponential increase with temperature (Fig. 10.6). In all cases, animals were fed *ad libitum* so that protein synthesis rates were not limited by lack of food. All values were also standardized for differences in body mass, which is also known to influence protein synthesis rates (Whiteley and Fraser 2009). Figure 10.6 shows that the lowest rates of protein synthesis were observed in the Antarctic isopod *Glyptonotus antarcticus*. The relatively low rate of protein synthesis plus the observation that Antarctic invertebrates are characterized by a low protein retention efficiency, helps to explain the lower than average growth rates observed in Antarctic versus temperate and tropical species (Pörtner et al. 2007).

Despite these broad species-related differences in protein synthesis rates and presumably growth potential, there are also examples of compensatory responses in which protein synthesis rates vary independently from temperature. The first example of this comes from a study on the semiterrestrial, intertidal isopod *L. oceanica* (Whiteley and Faulkner 2005). *L. oceanica* inhabits rocky shores at the high water mark of spring tides where it experiences a wide range of temperatures. In summer, the surface of rocks facing direct sunlight, even in temperate regions, can rise to over 40°C, but *L. oceanica* inhabits crevices and the undersurface of loose rock, which provide shade and increased humidity (Edney 1953). In winter, temperatures drop to around 5°C. In acclimatized animals straight from the shore, whole-animal rates of protein synthesis are maintained at a relatively low but constant level despite acute changes in temperature. Summer and winter animals

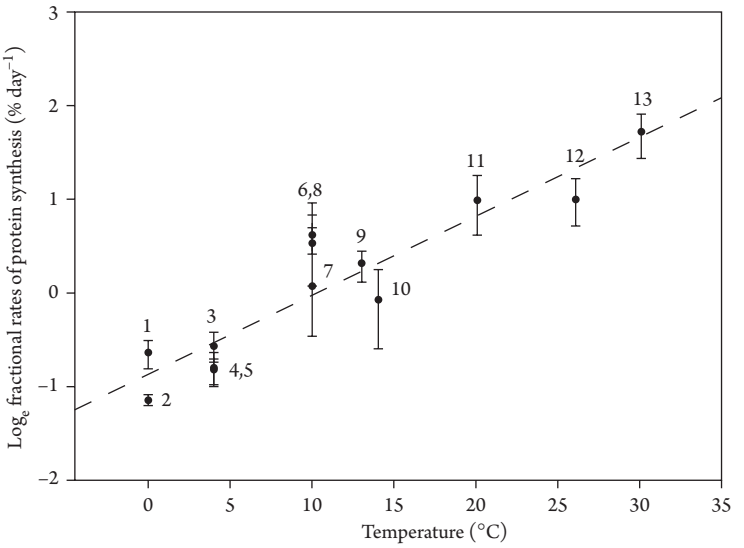


Fig. 10.6.
Relationship between fractional whole-animal rates of protein synthesis (k_s) and acclimation temperature in crustaceans from different thermal habitats. All protein synthesis values scaled to a standard body mass of 1g wet mass using a weight exponent of −0.2. Numbers represent the giant Antarctic isopod *Gyptonotus antarcticus* (1–3), the temperate isopod *Idotea rescata* (4 & 10), the Baltic isopod *Saduria entomon* (5 & 9), boreal/temperate amphipod *Gammarus duebeni* from two different populations (6 & 7), the subarctic/boreal amphipod *Gammarus oceanicus* (8), and the tropical prawn *Macrobrachium rosenbergii* (11–13). In all cases, the animals were acclimated to their respective experimental temperatures for 1 month and fed *ab libitum*. The fitted regression line is described by the exponential function: Log k_s = log 0.415 + 0.085.log temp, r^2= 0.874. Data taken from Whiteley et al. (1996) for *G. antarcticus* and *I. rescata*; Robertson et al. (2001a, 2001b) for *G. antarcticus* and *S. entomon*; Rastrick and Whiteley (2013) for the gammarid amphipods and for *M. rosenbergii*.

showed a similar response. A relatively low and constant rate of protein synthesis was also observed in the boreal/temperate amphipod species *Gammarus duebeni* (Rastrick and Whiteley 2013). This high shore species occupies areas under the influence of freshwater runoff and is highly tolerant of environmental change (Bulnheim 1979). Whole-animal rates of protein synthesis did not differ between acclimatized individuals from populations at different latitudes experiencing different thermal regimes. The abilities of both *L. oceanica* and *G. duebeni* to maintain relatively low rates of protein synthesis could be an adaptation for life in a highly changeable environment. It is possible that this response represents a strategy for enhancing survival by reducing the energetic costs associated with the replacement, repair, and synthesis of proteins.

Specific Dynamic Action

In addition to its effects on rates of metabolism and protein synthesis in crustaceans, temperature can also influence duration and timing of the rise in O_2 uptake rate after feeding or the specific dynamic action (SDA). Comparisons between species from different thermal habitats revealed that the SDA response in the Antarctic isopod *G. antarcticus* is considerably slower than in temperate isopod species, lasting for 8 days instead of 10 h and taking 3 days instead of several hours to reach peak values (Whiteley et al. 2001a). Differences in SDA responses are largely explained by differences in temperature, as demonstrated by acclimating the shore crab *C. maenas* to a range of temperatures. A reduction in acclimation temperature from 22°C to 7°C decreased the magnitude of the SDA response in *C. maenas* by increasing SDA duration but decreasing the SDA factorial scope

(Robertson et al. 2002). Further comparisons between cold water isopod species such as *G. antarcticus* and the Baltic isopod *Saduria entomon*, which inhabits the Baltic and Bothnia Seas as well as the Arctic Ocean, revealed marked differences in SDA response. *G. antarcticus* had a lower absolute rate of O_2 uptake at peak SDA, but the SDA duration was extended when compared to *S. entomon* at the same acclimation temperature (Robertson et al. 2001a,b). Whole-animal rates of protein synthesis increased in proportion to the postprandial increase in O_2 uptake rate in both species. Estimates on the costs of protein synthesis after a meal gave similar values in both species, despite their differences in SDA response and thermal habitat (Whiteley et al. 2001a). The extended SDA duration observed in the Antarctic isopod species when compared to either *S. entomon* or *C. maenas* held at the same temperature is thought to be caused by the extreme stenothermy of the Antarctic environment, where the longer SDA duration reflects lower aerobic scopes and a marked sensitivity to temperature change (Whiteley et al. 2001a). Overall, the rates at which Antarctic species process their food is much slower, further contributing to the observed reductions in growth rate.

Seasonal Temperature Change

A long-term study of the effects of seasonal temperature variation between 1°C and 21°C on a population of the freshwater crayfish revealed that both water and hemolymph pH varied with temperature during the spring warming. However, following the summer molt, the hemolymph pH increased and remained virtually unchanged as water temperatures cooled toward winter. This relative independence of pH with variation in temperature was explained by a complex of seasonal changes in CO_2 solubility, bicarbonate concentrations, and P_{CO_2}, as well as by measured values for the carbonic acid dissociation constant, pK_1 (Whiteley and Taylor 1993). This apparent regulation of hemolymph pH was accompanied by active regulation of intracellular pH (pH_i; Fig. 10.7). Tissues of functional importance in the winter months, such as the abdominal muscles responsible for the escape response, had a pH_i that varied inversely with body temperature in order to maintain the integrity of protein function (alphastat hypothesis; Reeves 1972). The tissues that are less active in the winter, when the crayfish do not feed, such as claw muscle and the hepatopancreas, maintained a constant pH_i as temperature fell. As a result, these tissues became relatively acidotic, which may serve to depress the activity of metabolic enzymes during the cold winter months (Whiteley et al. 1995). Under laboratory conditions, pH_i normally varies with temperature according to the alphastat hypothesis, but different tissues can maintain very different pH levels (Wheatly and Henry 1992).

ACIDIFICATION OF AQUATIC ENVIRONMENTS

Acidification of Freshwater Environments

Acidification of freshwater ecosystems can occur naturally or as a result of anthropogenic activities (Dangles et al. 2004, Weber and Pirow 2009). In contrast to naturally occurring acidification, the anthropogenic input of acidified material into freshwater lakes and rivers is a recent event that began in Europe and North America in the middle of the past century and peaked in the 1970s and 1980s. The major causes of anthropogenic acidification include "acid rain" and the effect of acid mine drainage. Sulfuric and nitric acids can reduce the pH of uncontaminated rainwater in equilibrium with atmospheric CO_2 from pH 5.6 to less than 4.3, whereas freshwater areas influenced by acid mine drainage can have pH values of less than 3.0 (Deneke 2000). Their impact is dependent on the capacity of the surrounding geology and soils to neutralize acids, with granite and gneiss regions being the most vulnerable.

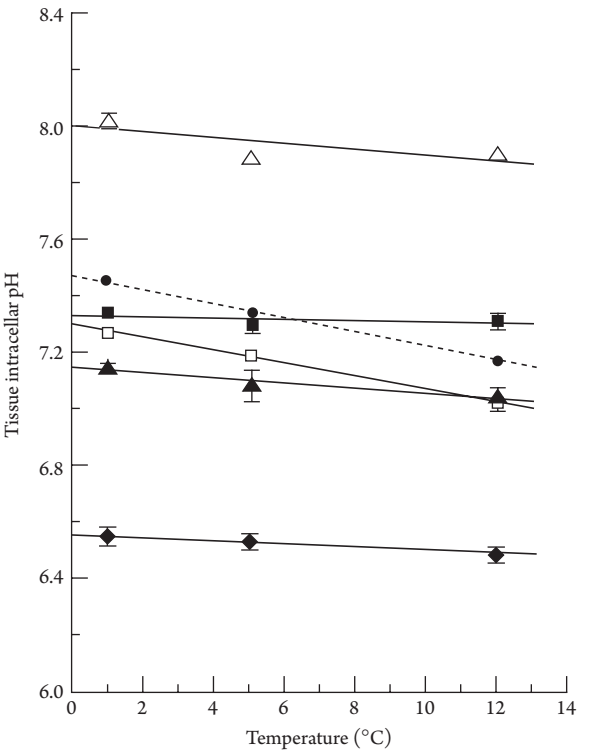


Fig. 10.7.
Intracellular pH (pH_i) in the heart (▲), claw muscle (■), abdominal muscle (□), and hepatopancreas (♦) of the freshwater crayfish *Austropotamobius pallipes* caught in the winter and acclimated to 1°C, 5°C, and 12°C. Values are means ± SE. Equivalent changes in hemolymph pH (Δ) and the changes in pH of pure water or pN (● and broken line) are also given. There was little change in pH_i in the claw muscles and hepatopancreas with temperature, but heart pH_i changed in parallel to the changes in the hemolymph at −0.010 pH units °C^{-1}. The largest temperature-related change in pH_i occurred in the abdominal muscles at −0.025 pH units °C^{-1}. From Whiteley et al. (1995).

Survival rates of freshwater crayfish declined as pH levels dropped below pH 6.0, but crayfish showed a remarkable ability to tolerate extremely low pH levels for short periods. For example, 50% of *Procambarus clarkii* survived for 4 days at pH 3.0 and for 60 days at pH 4.0, although increased mortality was observed in *Astacus astacus* kept for extended periods at pH 5.6 (McMahon and Stuart 1989). Initial exposure of *A. astacus* and *P. clarkii* to freshwater acidification caused a drop in hemolymph pH and a substantial loss of Na^+ and Cl^- ions from both the hemolymph and the tissues due to the large uptake of acid equivalents from the water (McMahon and Stuart 1989, Jensen and Malte 1990). However, both species were able to recover by increasing ventilation rates to reduce hemolymph P_{CO_2} levels rather than depending on ion regulation. Nevertheless, crayfish exposed to acid conditions in the medium term reached a new ionic equilibrium (McMahon and Stuart 1989). Other species, such as the amphipod *Gammarus pulex*, were more sensitive to pH levels of less than 6.0 because they experienced a reduction in hemolymph osmolarity, alterations in Na^+ regulation, and reductions in ventilatory and locomotory activity (Felten et al. 2008). Survival in *Daphnia*, which is an important member of freshwater zooplankton, was ensured by improved tolerance to acid stress despite alterations in acid–base balance, ventilation, and energy metabolism (Weber and Pirow 2009). More specifically, chronic exposure of *Daphnia* to acidic conditions (ΔpH of 0.16–0.23 pH units) resulted in a slight hemolymph acidosis and a decrease in $[HCO_3^-]$

by up to 65%. When external pH was reduced to 6.0, the hemolymph acidosis was accompanied by an increase in nonbicarbonate buffering capacity, as well as by tachycardia, hyperventilation, and hypermetabolism (Weber and Pirow 2009). The highest tolerance to acid stress was observed at an external pH of 5.5, which was attributed to the activation of defense mechanisms that occurred at the level of transcription.

Alterations in freshwater pH have also been demonstrated to affect larval survival and development. An interesting example is provided by the crab *Metopaulias depressus*, which occupies the water retained in the leaf axils of large bromeliads (Diesel and Schuh 1993). Although the axil water provides stable and reliable breeding sites, water pH ranges from 3.8 to 6.8, and it can be both hypoxic and hypercapnic (Diesel 1992). Maternal manipulation of the environment helps to improve larval survival by increasing median water pH from 4.8 to 6.8 (Diesel and Schuh 1993). This is achieved by the removal of dead leaf litter and other organic material, along with the supply of small shells to increase buffering capacities of the axil water. The exposure of freshwater crustaceans to long-term reductions in water pH of less than 6.0 is known to alter species distribution and abundance (e.g., France 1993), species richness (Walseng et al. 2003), and community structure and function (e.g., Dangles et al. 2004). As a result, planktonic crustacean species (cladocerans and copepods) are currently being used to gauge recovery of freshwater lakes from past anthropogenic acidification events and to assess the evolutionary reversal of acid tolerance (Walseng et al. 2003). Freshwater acidification effects remain valid examples of the negative impacts of acidification on aquatic communities because of the relatively long periods of time required for recovery once pollution controls have been put into place and anthropogenic emissions have been reduced. However, freshwater communities exposed to natural acidification processes appear to be more tolerant of reductions in external pH, indicating that communities can adapt to protracted low pH if given sufficient time (Dangles et al. 2004).

Ocean Acidification

Since preindustrial times, more than a third of the CO_2 released into the atmosphere has been absorbed by the oceans, thus reducing both pH and carbonate ion concentrations in the surface waters (Feely et al. 2004, Sabine et al. 2004, Fabry et al. 2008). These changes in carbonate chemistry are causing some concern, especially as the saturation of the oceans with respect to aragonite, a soluble form of calcium carbonate required by marine calcifiers for their shells and exoskeletons, is also decreasing (Caldeira and Wickett 2003, Fabry et al. 2008). If atmospheric P_{CO_2} continues to rise according to the "business as usual" CO_2 emission scenario (Houghton et al. 2001), then the pH of the oceans is predicted to fall from current pH levels of 8.1 by a further 0.3–0.4 pH units by the end of the century and by 0.7 pH units by 2300 (Caldeira and Wickett 2003, Feely et al. 2004). The effects of CO_2 emissions in the 21st century may also be strongly delayed. For example, if CO_2 emissions were reduced or stopped by 2100, their effects would still be experienced in 2500 when changes in ocean pH are predicted to extend from the surface down to a depth of 2,000 m (Frölicher and Joos 2010).

The ability to compensate for the effects of ocean acidification (OA) varies between crustacean species and is linked to the ability to buffer the resulting changes in P_{CO_2} and pH in the hemolymph (Whiteley 2011). Strongly iono- and osmoregulating crabs are able to fully compensate for exposure to extremely high CO_2 levels (hypercapnia) by elevating hemolymph HCO_3^- levels to reach a new acid–base equilibrium (Cameron and Iwama 1987). The majority of the HCO_3^- ions (93%) originate from the seawater, but a small percentage comes from internal stores (7%; Cameron 1985). Species that maintain a higher hemolymph HCO_3^- are more tolerant of hypercapnia (Pörtner et al. 2004, Melzner et al. 2009), although it appears that $[HCO_3^-]$ elevation is limited to a threshold value of approximately 50 mmol l^{-1} (Cameron and Iwama 1987, Spicer et al. 2007). The inability to increase $[HCO_3^-]$ beyond this value is thought to be a compromise between acid–base and ion

regulation (Cameron and Iwama 1987), in which ion regulation takes priority over acid–base balance to maintain cell volume control (Whiteley et al. 2001b). When exposed to lower external CO_2 levels (i.e., 0.10–0.20 kPa) for 24 h, even poor ion regulators such as the subtidal crabs *Necora puber* (Spicer et al. 2007) and *C. magister* (Pane and Barry 2007) are able to buffer the resulting hemolymph acidosis by elevating $[HCO_3^-]$. However, HCO_3^- buffering was inadequate in *N. puber* after 16 and 30 days exposure at a P_{CO_2} of 0.20 kPa and after 4–5 days exposure at a P_{CO_2} of 2.0 kPa (Spicer et al. 2007, Small et al. 2010). In all three cases, the elevation of hemolymph $[HCO_3^-]$ was limited below 55 mmol l^{-1} and led to the development of an internal acidosis. The inability to compensate an internal acidosis can lead to hypoxemia and death from asphyxiation because the subsequent increase in $[H^+]$ suppresses the O_2 affinity of hemocyanin to such an extent that O_2 delivery to the tissues is severely disrupted (Taylor and Whiteley 1989, Whiteley and Taylor 1992). In contrast, the strongly iono- and osmoregulating prawn species *Palaemon elegans* and *P. serratus* fully compensated for the effects of 30 days exposure to a P_{CO_2} of 0.30 kPa (Dissanayake and Ishimatsu 2011).

The ability to compensate for acid–base disturbances during hypercapnia is also related to circulating levels of the nonbicarbonate buffer, hemocyanin. The level of this respiratory pigment varies among crabs according to routine levels of activity (Watt et al. 1999). Active crabs tend to have higher hemocyanin levels when compared with slow-moving species. Higher hemocyanin levels increase nonbicarbonate buffering and O_2 carrying capacities to match the higher production of metabolic CO_2 and to satisfy the higher aerobic requirements. Slow-moving crustaceans with low hemocyanin levels are therefore less likely to be able to buffer an acidosis resulting from elevated seawater P_{CO_2}. The deep-sea tanner crab *Chionoecetes tanneri* is unable to compensate for exposure to a P_{CO_2} of 1.28 kPa for 24 h (Pane and Barry 2007). Its relatively low hemolymph protein levels, which are mainly hemocyanin, plus the fact that this species is unable to elevate hemolymph HCO_3^- levels beyond 3 mmol l^{-1} (Pane and Barry 2007), means that this species is poorly adapted for acid–base compensation and is likely to be more sensitive to OA. In contrast, the deep-sea stenothermal prawn *Pandalus borealis* is able to partially compensate for the effects of exposure to a P_{CO_2} of 0.90 kPa by increasing $[HCO_3^-]$ levels threefold within the first day of exposure up to 15 mmol l^{-1} (Hammer 2012). The ability to partially compensate for the resulting extracellular acidosis was attributed to the higher rates of activity characteristic of this species because it undergoes diel migrations in order to feed. The diversity of acid–base responses in deep-sea crustaceans to hypercapnia further confirms the importance of routine activity levels in determining the compensatory capacities of crustaceans to OA. Low activity rates are generally characteristic of Antarctic benthic marine species because of the low and stable temperatures and a lack of resources (Whiteley 2011). From the few studies carried out to date on the giant Antarctic isopod *G. antarcticus*, it appears that polar crustaceans also have very poor hemolymph buffering capacities (Whiteley et al. 1997). The circulating protein levels in *G. antarcticus* are up to 7.5 times lower than the values characteristic of temperate crustaceans. In addition, hemocyanin O_2 affinity in *G. antarcticus* is highly sensitive to a reduction in pH (Jokumsen et al. 1981). Both characteristics suggest that OA could potentially disrupt both acid–base balance and O_2 transport in the hemolymph of *G. antarcticus* and lead to death by asphyxiation.

FUTURE DIRECTIONS

Many of the experiments described in this chapter have adopted the historical approach to physiological experimentation whereby crustacean species have been exposed to acute changes in single environmental variables under controlled conditions for relatively short periods of time; their responses are averaged then subjected to routine statistical analysis. The purpose of these experiments was to explore the physiological mechanisms by which species adjust to environmental

variation. More recently, attention has shifted from the mechanisms themselves to the metabolic costs of short- and long-term adjustments. This approach is of ecological importance because costs can affect tolerances and therefore the abundance and distribution patterns of populations or species (Monaco and Helmuth 2011). In addition, there has been a growing interest in the study of multiple environmental variables on the ability of individuals to adjust to change. This has come about in part in response to the increasing interest in climate change, in which environmental variables are changing simultaneously. Researchers in the field of OA for instance, have realized the importance of studying the effects of more than one variable, such as OA and either temperature, O_2 levels, or salinity (Whiteley 2011). To date, it has been shown that OA and temperature change have a synergistic effect (Melzner et al. 2009). There is also a growing interest in exposing crustaceans to longer term changes in environmental variables (months to years), which raises interesting questions about the experimental regime and whether experimental temperatures, for example, should change naturally with the seasons or remain constant. The effect of variable temperatures has also attracted attention in recent years with early experiments showing that an increase in the amplitude of daily temperature variation can decrease thermal sensitivity and increase metabolic rate in the terrestrial isopod *Porcellio laevis* (Folguera et al. 2011). However, it is still unknown how crustaceans will be able to cope physiologically with the changes in temperature variability expected from global climate change. Finally, there is a great deal of interest in the role of adaptation at the molecular level in determining physiological responses to environmental change. As present, climate changes are occurring at rates that make it difficult for marine species to adapt; future survival of populations and their constituent individuals will depend to a large extent on the plasticity available within the existing genotype (Somero 2010). Physiological experiments are needed to define the degree of plasticity, whereas multiple generational studies are required to determine the role of genetic adaptation. Other molecular approaches involve the use of genomic technologies and the growing availability of DNA sequence data (both genomic and expressed sequence tag) to improve our understanding of the effects of environmental change on the transcriptome of crustaceans (Stillman et al. 2008). Such an approach has yielded novel genes and identified genes that were previously unknown to be involved in temperature acclimatization responses (Tagmount et al. 2010). Overall, contemporary crustacean physiology needs to consider the responses of a range of organisms living in a range of habitats to more clearly define the processes that constrain physiological adaption to change. Crustaceans remain a rich source of material for such studies because they demonstrate the full spectrum of responses, from those that are unable to respond to environmental change to those that are highly adaptable, as demonstrated in this chapter.

CONCLUSIONS

Crustaceans occupy a range of aquatic and terrestrial habitats where they encounter variable temperature, O_2, and pH levels. They manage to survive by changing their behavior and by undergoing physiological and metabolic adjustments. During environmental hypoxia, aquatic crustaceans move away to find more oxygenated water and, in the case of berried females, spend more time ventilating their eggs. Adults typically maintain their ventilation rates and cardiac output down to a critical P_{O_2} value, and they maintain O_2 transport in the hemolymph via increases in carrying capacity and O_2 affinity of the blood pigment. Early stages of development tend to be more resistant to hypoxia and anoxia, with eggs tolerating hypoxia for most of embryonic development. Crustaceans living at depth and in burrows survive hypoxia by increasing aerobic capacity. Burrowing crustaceans can also tolerate anoxia by resorting to anaerobic metabolism. Several species can resort to air breathing by raising the exhalent openings above the surface of the water and reversing the normal direction of ventilation to bubble air through the branchial chambers. Some species can also live

for several days out of water, despite becoming markedly hypoxic, by buffering the accumulating hemolymph acidosis with HCO_3^- ions from the exoskeleton and improving O_2 transport to the tissues by increasing hemocyanin O_2 affinity. Hypoxia combined with warmer temperatures in the tropics has probably led to the evolution of air breathing in crustaceans. Terrestrial crabs are characterized by reduced gills that are responsible for the excretion of CO_2, and elaborately formed lungs that exchange O_2.

The thermal ranges experienced by crustaceans differ considerably. Thermal limits are determined at the level of the whole organism by O_2 and capacity limitation and are associated with physiological variability. Along natural thermal gradients, there is evidence of metabolic compensation but only in more eurythermal species. Protein synthesis rates in crustaceans generally increase with acclimation temperature, but in those species that occupy the high intertidal, protein synthesis rates can be independent of temperature, which is beneficial in a highly fluctuating environment. Temperature can also affect the duration and magnitude of the SDA response in crustaceans, and the relationship between pH_i and temperature can be influenced by season. Freshwater crayfish can survive for 4 days at an environmental pH of 3.0 but suffer from a substantial loss of Na^+ and Cl^- ions. Improved tolerance to acid stress is possible. The ability of crustaceans to survive OA depends on their compensatory capacities. Strong iono- and osmoregulators are more likely to survive, although the added effects of elevated temperatures and/or reduced salinities are unknown.

In summary, aquatic crustaceans respond to environmental change in a number of ways. In order to avoid the most immediate effects, individuals move away from the problem and show a range of unusual behaviors, such as the aggregation of both predators and prey to avoid bouts of hypoxia. Physiological responses further enhance the ability of individuals to survive relatively short-term changes in either O_2, temperature, or pH levels in the surrounding water. Compensatory responses are observed in a range of crustacean species and are focused on adjustments in respiratory physiology, including acid–base homeostasis and ion regulation. Survival depends on the ability to maintain the most appropriate conditions for protein function. The physiological responses and consequent survival of aquatic crustaceans to simultaneous changes in environmental pH and either temperature or O_2 over the longer term remain to be studied.

REFERENCES

Ahearn, G.A., P. Franco, and L.P. Clay. 1990. Electrogenic $2Na^+/H^+$ exchange in crustaceans. Journal of Membrane Biology 116:215–226.

Ahearn, G.A., P.K. Mandal, and A. Mandal. 2001. Biology of the $2Na^+/H^+$ antiporter in invertebrates. Journal of Experimental Zoology 289:232–244.

Airriess, C., and B.R. McMahon. 1994. Cardiovascular adaptations enhance tolerance of environmental hypoxia in the crab *Cancer magister*. Journal of Experimental Biology 190:23–41.

Albalat, A., S. Sinclair, J. Laurie, A.C. Taylor, and D. Neil. 2010. Targeting the live market: recovery of Norway lobsters *Nephrops norvegicus* (L.) from trawl-capture as assessed by stress-related parameters and nucleotide breakdown. Journal of Experimental Marine Biology and Ecology 395:206–214.

Anderson, S.J., A.C. Taylor, and R.J.A. Atkinson. 1994. Anaerobic metabolism during anoxia in the burrowing shrimp *Calocaris macandreae* Bell (Thalassinidea). Comparative Biochemistry and Physiology 108A:515–522.

Astall, C. 1997. Behavioural and physiological implications of a burrow-dwelling lifestyle for two species of upogebiid mud-shrimp (Crustacea: Thalassinidea). Estuarine, Coastal and Shelf Science 44:155–168.

Baeza, J.A., and M. Fernández. 2002. Active brood care in *Cancer setosus* (Crustacea: Decapoda): the relationship between female behaviour, embryo oxygen consumption and the cost of brooding. Functional Ecology 16:241–251.

Bell, G.W., D.B. Eggleston, and T.G. Wolcott. 2003. Behavioural responses of free-ranging blue crabs to episodic hypoxia. Marine Ecology Progress Series 259:215–225.

Bell, G.W., D.B. Eggleston, and E J. Noga. 2009. Environmental and physiological controls of blue crab avoidance behavior during exposure to hypoxia. Biological Bulletin 217:161–172.

Belman, B.W., and J.J. Childress. 1976. Circulatory adaptations to the oxygen minimum layer in the bathypelagic mysid *Gnathophausia ingens*. Biological Bulletin 150:15–37.

Bernatis, J.L., S.L. Gerstenberger, and I.J. McGaw. 2007. Behavioural responses of the Dungeness crab, *Cancer magister*, during feeding and digestion in hypoxic conditions. Marine Biology 150:941–951.

Bulnheim, H.P. 1979. Comparative studies on the physiological ecology of five euryhaline *Gammarus* species. Oecologia 44:80–86.

Caldeira, K., and M.E. Wickett. 2003. Anthropogenic carbon and ocean pH. Nature 425:365–365.

Cameron, J.N. 1985. Molting in the blue crab. Scientific American 252:102–109.

Cameron, J.N. 1986. Acid base equilibria in invertebrates. Pages 368–371 *in* N. Heisler, editor. Acid-base regulation in animals. Elsevier Press, Oxford.

Cameron, J.N., and C.V. Batterton. 1978. Temperature and blood acid-base status in the blue crab, *Callinectes sapidus*. Respiration Physiology 35:101–110.

Cameron J.N., and G.K. Iwama. 1987. Compensation of progressive hypercapnia in channel catfish and blue crabs. Journal of Experimental Biology 133:183–197.

Chapelle, G., and L.S. Peck. 1995. The influence of acclimation and substratum on the metabolism of the Antarctic amphipods *Waldeckia obesa* (Chevreux 1905) and *Bovallia gigantea* (Pfeffer 1888). Polar Biology 15:225–232.

Chapelle G., and L.S. Peck. 1999. Polar gigantism dictated by oxygen availability. Nature 399:114–115.

Chausson, F., C.R. Bridges, P.-M. Sarradin, B.N. Green, R. Riso, J.-C. Caprais, and F.H. Lallier. 2001. Structural and functional properties of hemocyanin from *Cyanagraea praedator*, a deep-sea hydrothermal vent crab. Proteins 45:351–359.

Childress, J.J. 1971. Respiratory adaptation to the oxygen minimum layer in the bathypelagic mysid *Gnathophausia ingens*. Biological Bulletin 141:109–121.

Childress, J.J. 1975. The respiratory rates of mid-water crustaceans as a function of depth of occurrence and relative to the oxygen minimum layer off southern California. Comparative Biochemistry and Physiology 50A:787–799.

Childress, J.J., and B.A. Seibel. 1998. Life at stable low oxygen levels: adaptations of animals to oxygen minimum layers. Journal of Experimental Biology 201:1223–1232.

Cisterna, A.J., G.S. Saldias, and C.W. Caceres. 2008. Hypoxia effect on foraging behavior of *Cancer setosus* (Molina, 1782) (Crustacea: Decapoda) feeding on *Mytilus chilensis* (Hupé, 1854). Revista de Biología Marina y Oceanografía 43:419–423.

Clarke, A. 1993. Seasonal acclimatization and latitudinal compensation in metabolism—do they exist? Functional Ecology 7:139–149.

Cottin, D., B. Shillito, T. Chertemps, S. Thatje, N. Léger, and J. Ravaux. 2010. Comparison of heat-shock responses between the hydrothermal vent shrimp *Rimicaris exoculata* and the related coastal shrimp *Palaemonetes varians*. Journal of Experimental Marine Biology and Ecology 393:9–16.

Cuculescu, M., D. Hyde, and K. Bowler. 1995. Temperature acclimation of marine crabs: changes in plasma membrane fluidity and lipid composition. Journal of Thermal Biology 20:207–222.

Dangles, O., B. Malmqvist, and H. Laudon. 2004. Naturally acid freshwater ecosystems are diverse and functional: evidence from boreal streams. Oikos 104:149–155.

Davenport, J.A. 1992. Animal life at low temperature. Chapman & Hall, London.

Decelle, J., A.C. Andersen, and S. Hourdez. 2010. Morphological adaptations to chronic hypoxia in deep-sea decapod crustaceans from hydrothermal vents and cold seeps. Marine Biology 157:1259–1269.

deFur, P.L., C.P. Mangum, and J.E. Reese. 1990. Respiratory responses of the blue crab to long-term hypoxia. Biological Bulletin 178:46–54.

deFur, P.L., B.R. McMahon, and C.E. Booth. 1983. Analysis of haemolymph oxygen levels and acid-base status during emersion in situ in the red rock crab *Cancer productus*. Biological Bulletin 165:582–590.

Deneke, R. 2000. Review of rotifers and crustaceans in highly acidic environments of pH values ≤3. Hydrobiologia 433:167–172.

Diaz, R.J., and R. Rosenberg. 1995. Marine benthic hypoxia: a review of its ecological effects and the behavioural responses of benthic macrofauna. Oceanography and Marine Biology, an Annual Review 33:245–303.

Diaz, R.J., and R. Rosenberg. 2008. Spreading dead zones and consequences for marine ecosystems. Science 321:926–929.

Dick, J., S. Faloon, and R. Elwood. 1998. Active brood care in an amphipod: influences of embryonic development, temperature and oxygen. Animal Behaviour 56:663–672.

Dick, J.T., R.J.E. Bailey, and R.W. Elwood. 2002. Maternal care in the rockpool amphipod *Apherusa jurinei*: developmental and environmental cues. Animal Behaviour 63:707–713.

Diesel, R. 1992. Managing the offspring environment: brood care in the bromeliad crab, *Metopaulias depressus*. Behavioral Ecology and Sociobiology 30:125–134.

Diesel, R., and M. Schuh. 1993. Maternal care in the bromeliad crab *Metopaulias depressus* (Decapoda): maintaining oxygen, pH and calcium levels optimal for the larvae. Behavioral Ecology and Sociobiology 32:11–15.

Dissanayake, A., and A. Ishimatsu. 2011. Osmoregulatory ability and salinity tolerance in several decapod crustaceans (Palaemonidae & Penaeidae) of the East China Sea. Plankton Benthos 6:135–140.

Edney, E.B. 1953. The temperature of woodlice in the sun. Journal of Experimental Biology 30:331–349.

Eriksson, S.P., and S.P. Baden. 1997. Behaviour and tolerance to hypoxia in juvenile Norway lobster (*Nephrops norvegicus*) of different ages. Marine Biology 128:49–54.

Escribano, R., P. Hidalgo, and C. Krautz. 2009. Zooplankton associated with the oxygen minimum zone system in the northern upwelling region of Chile during March 2000. Deep Sea Research Part II: Topical Studies in Oceanography 56:1083–1094.

Fabry V.J., B.A. Seibel, R.A. Feely, and J.C. Orr. 2008. Impacts of ocean acidification on marine fauna and ecosystem processes. ICES Journal of Marine Science 65:414–432.

Federich, M., and H.O. Pörtner. 2000. Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab, *Maja squinado*. American Journal of Physiology—Integrative and Comparative Physiology 279:R1531–R1538.

Feely, R.A., C.L. Sabine, K. Lee, W. Berelson, J. Kleypas, V.J. Fabry, and F.J. Millero. 2004. Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. Science 305:362–366.

Felten, V., G. Charmantier, M. Charmantier-Daures, F. Aujoulat, J. Garric, and O. Geffard. 2008. Physiological and behavioural responses of *Gammarus pulex* exposed to acid stress. Comparative Biochemistry and Physiology 147C:189–197.

Fernández, M., C. Brock, and H.O. Pörtner. 2000. The cost of being a caring mother: the ignored factor in the reproduction in marine invertebrates. Ecology Letters 3:487–494.

Folguera, G., D.A. Bastías, J. Caers, J.M. Rojas, M.-D. Piulachs, X. Bellés, and F. Bozinovic. 2011. An experimental test of the role of environmental temperature variability on ectotherm molecular, physiological and life-history traits: implications for global warming. Comparative Biochemistry and Physiology 159A:242–246.

Fox, H.M., and C.A. Wingfield. 1937. The activity and metabolism of poikilothermic animals in different latitudes. II. Proceedings of the Zoological Society of London Series A 107: 275–282.

France, R.L. 1993. Influence of lake pH on the distribution, abundance and health of crayfish in Canadian Shield lakes. Hydrobiologia 271:65–70.

Freire, C.A., H. Onken, and J.C. McNamara. 2008. A structure-function analysis of ion transport in crustacean gills and excretory organs. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 151:272–304.

Frölicher, T.L., and F. Joos. 2010. Reversible and irreversible impacts of greenhouse gas emissions in multi-century projections with the NCAR global coupled carbon cycle-climate model. Climate Dynamics 35:1439–1459.

Gannon, A.T., V.G. Demarco, T. Morris, M.G. Wheatly, and Y.-H. Kao. 1999. Oxygen uptake, critical oxygen tension, and available oxygen for three species of cave crayfishes. Journal of Crustacean Biology 19:235–243.

Gilbert, D., N.N. Rabalais, R.J. Díaz, and J. Zhang. 2010. Evidence for greater oxygen decline rates in the coastal ocean than in the open ocean. Biogeosciences 7:2283–2296.

Glover, C.N., and C.A. Wood. 2005. Physiological characterisation of a pH- and calcium-dependent sodium uptake mechanisms in the freshwater crustacean, *Daphnia magna*. Journal of Experimental Biology 208:951–959.

Gorr, T.A., J.D. Cahn, H. Yamagata, and H.F. Bunn. 2004. Hypoxia-induced synthesis of hemoglobin in the crustacean *Daphnia magna* is hypoxia-inducible factor-dependent. The Journal of Biological Chemistry 279:36038–47.

Gorr, T.A., D. Wichmann, J. Hu, M. Hermes-Lima, A.F. Welker, N. Terwilliger, J.F. Wren, M. Viney, S. Morris, G.E. Nilsson, A. Deten, J. Soliz, and M. Gassmann. 2010. Hypoxia tolerance in animals: biology and application. Physiological and Biochemical Zoology 83:733–752.

Greenaway, P., and H.H. Taylor. 1976. Aerial gas exchange in Australian arid-zone crab, *Parathelphusa transversa* Vonmartens. Nature 262:711–713.

Hagerman, L., and Uglow, R.F. 1985. Effects of hypoxia on the respiratory and circulatory regulation of Nephrops norvegicus. Marine Biology 87:273–278.

Hammer, K.M., and S.A. Pedersen 2013. The deep-water prawn Pandalus borealis displays a relatively high pH regulatory capacity in response to CO_2-induced acidosis. Marine Ecology Progress Series 492:139–151.

Hand, S.C. 1998. Quiescence in *Artemia franciscana* embryos: reversible arrest of metabolism and gene expression at low oxygen levels. Journal of Experimental Biology 201:1233–42.

Hartnoll, R.G., and R.G.K. Paul. 1982. The development of attached and isolated eggs of *Carcinus maenas*. International Journal of Invertebrate Reproduction 5:247–252.

Haselmair, A., M. Stachowitsch, M. Zuschin, and B. Riedel. 2010. Behaviour and mortality of benthic crustaceans in response to experimentally induced hypoxia and anoxia *in situ*. Marine Ecology Progress Series 414:195–208.

Haupt, P., S. Brouwer, G. Branch, and G. Gade. 2006. Effects of exposure to air on the escape behaviour and haemolymph chemistry of the South African Cape lobster, *Jasus lalandii*. Fisheries Research 81:210–218.

Helluy, S.M., and B.S. Beltz. 1991. Embryonic development of the American lobster (*Homarus americanus*): quantitative staging and characterisation of an embryonic molt cycle. Biological Bulletin 180:355–371.

Helly, J.J., and J.A. Levin. 2004. Global distribution of naturally occurring of marine hypoxia on continental margins. Deep-Sea Research Part 1–Oceanographic Research Papers 51:1159–1168.

Helmuth, B., N. Mieszkowska, P. Moore, and S.J. Hawkins. 2006. Living on the edge of two changing worlds: forecasting the responses of rocky intertidal ecosystems to climate change. Annual Review of Ecology Evolution and Systematics 37:373–404.

Henry, R.P. 2001. Environmentally mediated carbonic anhydrase induction in the gills of euryhaline crustaceans. Journal of Experimental Biology 204:991–1002.

Hochachka, P.W. 1997. Oxygen-A key regulatory metabolite in metabolic defense against hypoxia. American Zoology 37:595–603.

Hochachka, P.W., and G.N. Somero. 2002. Biochemical adaptation: mechanisms and processes in physiological evolution. Oxford University Press, New York.

Holman, J.D., and S.C. Hand. 2009. Metabolic depression is delayed and mitochondrial impairment averted during prolonged anoxia in the ghost shrimp, *Lepidophthalmus louisianensis* (Schmitt, 1935). Journal of Experimental Marine Biology and Ecology 376:85–93.

Houghton, J.T., Y. Ding, D.J. Griggs, M. Noguer, P.J. van der Linden, and D. Xiaosu. 2001. Climate change 2001: the scientific basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge.

Houlihan D.F., C.G. Carter, and I.D. McCarthy. 1995. Protein turnover in animals. Pages 1–32 *in* P. Walsh and P. Wright, editors. Nitrogen metabolism and excretion. CRC Press, Boca Raton, Florida.

Hourdez, S., and F.H. Lallier. 2007. Adaptations to hypoxia in hydrothermal-vent and cold-seep invertebrates. Reviews in Environmental Science and Biotechnology 6:143–159.

Howarth, R., F. Chan, D.J. Conley, J. Garnier, S.C. Doney, R. Marino, and G. Billen. 2011. Coupled biogeochemical cycles: eutrophication and hypoxia in temperate estuaries and coastal marine ecosystems. Frontiers in Ecology and the Environment 9:18–26.

Innes, A.J., and E.W. Taylor. 1986. The evolution of air-breathing in crustaceans: a functional analysis of branchial, cutaneous and pulmonary gas exchange. Comparative and Biochemical Physiology A 85:621–637.

Jackson, D.C., T. Wang, P. Koldkjaer, and E.W. Taylor. 2001. Lactate sequestration in the carapace of the crayfish *Austropotamobius pallipes* during exposure in air. Journal of Experimental Biology 204:941–946.

James, R., A. Atkinson, and A.C. Taylor. 2005. Aspects of the biology, physiology and ecology of thalassinidean shrimps in relation to their burrow environment. Oceanography and Marine Biology 43:173–210.

Jensen, F.B., and H. Malte. 1990. Acid-base and electrolyte regulation, and hemolymph gas-transport in crayfish *Astacus astacus*, exposed to soft, acid water with and without aluminium. Journal of Comparative Physiology B 160:483–490.

Johansson, B. 1997. Behavioural responses to gradually declining oxygen concentration by Baltic Sea macrobenthic crustaceans. Marine Biology 129:71–78.

Jokumsen, A., R.M.G. Wells, H.D. Ellerton, and R.E. Weber. 1981. Hemocyanin of the giant Antarctic isopod, *Glyptonotus antarcticus*—structure and effects of temperature and pH on its oxygen affinity. Comparative and Biochemical Physiology 70A:91–95.

Kobayashi, M, and T. Hoshi. 1982. Relationship between the haemoglobin concentration of *Daphnia magna* and the ambient oxygen concentration. Comparative Biochemistry and Physiology 72A:247–249.

Kobayashi, M., and T. Hoshi. 1984. Analysis of respiratory role of haemoglobin in *Daphnia magna*. Physiological Zoology 59:35–42.

Kwast, K.E., and S.C. Hand. 1996. Acute depression of mitochondrial protein synthesis during anoxia. Contributions of oxygen sensing, matrix acidification, and redox state. The Journal of Biological Chemistry 271:7313–7319.

Lallier, F.H., and J.P. Truchot. 1989. Modulation of hemocyanin oxygen-affinity by L-lactate and urate in the prawn *Penaeus japonicas*. Journal of Experimental Biology 147:133–146.

Lallier, F.H., and J.P. Truchot. 1997. Hemocyanin oxygen-binding properties of a deep-sea hydrothermal vent shrimp: evidence for a novel co-factor. Journal of Experimental Zoology 277:357–364.

Legeay, A., and J.-C. Massabuau. 2000. The ability to feed in hypoxia follows a seasonally dependent pattern in shore crab *Carcinus maenas*. Journal of Experimental Marine Biology and Ecology 247:113–129.

Levin, L.A. 2005. Ecology of cold seep sediments: interactions of fauna with flow, chemistry and microbes. Oceanography and Marine Biology: An Annual Review 43:1–46.

Lovrich, G.A., and M. Thiel. 2011. Ecology, physiology, feeding and trophic role of squat lobsters. Pages 183–222 *in* G.C.B. Poore, S.T. Ahyong, and J. Taylor, editors. The biology of squat lobsters. CSIRO Publishing, Victoria, Australia.

Lund, H.S., T. Wang, E.S. Chang, L.F. Pedersen, E.W. Taylor, P.B. Pedersen, and D.J. McKenzie. 2009. Recovery by the Norway lobster *Nephrops norvegicus* (L.) from the physiological stresses of trawling: influence of season and live-storage position. Journal of Experimental Marine Biology and Ecology 373:124–132.

Luxmoore, R.A. 1984. A comparison of the respiration rate of some Antarctic isopods with species from lower latitudes. British Antarctic Survey Bulletin 62:53–66.

Mangum, C.P. 1997. Adaptation of the oxygen transport system to hypoxia in the blue crab, *Callinectes sapidus*. American Zoologist 37:604–611.

Mangum, C.P., and K. Johansen. 1975. The colloid osmotic pressure of invertebrate body fluids. Journal of Experimental Biology 63:661–671.

Marcus, N.H., and R.V. Lutz. 1994. Effects of anoxia on the viability of subitaneous eggs of planktonic copepods. Marine Biology 121:83–87.

Martin, J.W., and T.A. Haney. 2005. Decapod crustaceans from hydrothermal vents and cold seeps: a review through 2005. Zoological Journal of the Linnean Society 145:445–522.

Massabuau, J.C., and J. Forgue. 1996. A field versus laboratory study of blood oxygen status in normoxic crabs at different temperatures. Canadian Journal of Zoology 74:423–430.

McCleary, S.J. 2006. Role of oxygen in the control of embryonic growth and metabolism in the edible crab Cancer pagurus. PhD thesis, Bangor University.

McMahon, B.R. 2001. Respiratory and circulatory compensation to hypoxia in crustaceans. Respiration Physiology 128:349–64.

McMahon, B.R., and S.A. Stuart. 1989. Physiological problems of crayfish in acid waters. Pages 171–199 *in* R. Morris, E.W. Taylor, D.J.A. Brown, and J.A. Brown, editors. Acid toxicity and aquatic animals. Society for Experimental Biology Seminar Series 34. Cambridge University Press, Cambridge.

Melzner, F., M.A. Gutowska, M. Langenbuch, S. Dupont, M. Lucassen, M.C. Thorndyke, M. Bleich, and H.O. Pörtner. 2009. Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6:2313–2331.

Metzger, R., F.J. Sartoris, M. Langenbuch, and H.O. Pörtner. 2007. Influence of elevated CO2 concentrations on thermal tolerance of the edible crab *Cancer pagurus*. Journal of Thermal Biology 32:144–151.

Mickel, T.J., and J.J. Childress. 1982. Effects of temperature, pressure, and oxygen concentration on the oxygen consumption rate of the hydrothermal vent crab *Bythograea thermydron* (Brachyura). Physiological Zoology 55:199–207.

Monaco, C.J., and B. Helmuth. 2011. Tipping points, thresholds and the keystone role of physiology in marine climate research. Advances in Marine Biology 60:120–160.

Monaco, C., K. Brokordt, and C. Gaymer. 2010. Latitudinal thermal gradient effect on the cost of living of the intertidal porcelain crab *Petrolisthes granulosus*. Aquatic Biology 9:23–33.

Morris, S. 2004. HIF and anapyrexia: a case for crabs. International Congress Series 1275:79–88.

Morris, S., and A.C. Taylor. 1985. The respiratory response of the intertidal prawn *Palaemon elegans* (Rathke) to hypoxia and hyperoxia. Comparative Biochemistry and Physiology 81A:633–639.

Morris, S., R. Tyler-Jones, and E.W. Taylor. 1986a. The regulation of haemocyanin oxygen affinity during emersion of the crayfish, *Austropotamobius pallipes*. I. An *in vitro* investigation of the interactive effects of calcium and L-lactate on oxygen affinity. Journal of Experimental Biology 121:315–326.

Morris, S., R. Tyler-Jones, C.R. Bridges, and E.W. Taylor. 1986b. The regulation of haemocyanin oxygen affinity during emersion of the crayfish, *Austropotamobius pallipes* II. An investigation of *in vivo* changes in oxygen affinity. Journal of Experimental Biology 121:327–338.

Murphy, D.J. 1983. Freezing resistance in intertidal invertebrates. Annual Review of Physiology 45:289–99.

Naylor, J.K., and E.W. Taylor. 1999. Heart rate and gill ventilation in ovigerous and non-ovigerous edible crabs, *Cancer pagurus*: the effects of disturbance, substrate and starvation. Marine and Freshwater Behaviour and Physiology 32:129–145.

Naylor, J.K., E.W. Taylor, and D.B. Bennett. 1997. The oxygen uptake of ovigerous edible crabs (*Cancer pagurus*) (L.) and their eggs. Marine and Freshwater Behaviour and Physiology 30:29–44.

Naylor, J.K., E.W. Taylor, and D.B. Bennett. 1999. Oxygen uptake of developing eggs of *Cancer pagurus* (Crustacea: Decapoda: Cancridae) and consequent behaviour of the ovigerous females. Journal of the Marine Biological Association UK 79:305–315.

Onken H., and M. Putzenlechner. 1995. A V-ATPase drives active, electrogenic and Na^+-independent Cl^- absorption across the gills of *Eriocheir sinensis*. Journal of Experimental Biology 198:767–774.

Pane, E.F., and J.P. Barry. 2007. Extracellular acid-base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab. Marine Ecology Progress Series 334:1–9.

Paul, R.J., M. Colmorgen, S. Hüller, F. Tyroller, and D. Zinkler. 1997. Circulation and respiratory control in millimetre-sized animals (*Daphnia magna, Folsomia candida*) studied by optical methods. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 167:399–408.

Paul, R.J., B. Zeis, T. Lamkemeyer, M. Seidl, and R. Pirow. 2004. Control of oxygen transport in the microcrustacean *Daphnia*: regulation of haemoglobin expression as central mechanism of adaptation to different oxygen and temperature conditions. Acta Physiologica Scandinavica 182:259–275.

Peterson, S., and K. Anger. 1997. Chemical and physiological changes during the embryonic development of the spider crab, *Hyas araneus* L (Decapoda: Majidae). Comparative and Biochemical Physiology B 117:299–306.

Pirow, R., and I. Buchen. 2004. The dichotomous oxyregulatory behavior of the planktonic crustacean *Daphnia magna*. Journal of Experimental Biology 207:683–696.

Pirow, R., F. Wollinger, and R.J. Paul. 1999. The sites of respiratory gas exchange in the planktonic crustacean *Daphnia magna*: an in vivo study employing blood haemoglobin as an internal oxygen probe. Journal of Experimental Biology 202:3089–3099.

Pirow, R., C. Bäumer, and R.J. Paul. 2004. Crater landscape: two-dimensional oxygen gradients in the circulatory system of the microcrustacean *Daphnia magna*. Journal of Experimental Biology 207:4393–4405.

Pörtner, H.O. 2002. Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comparative and Biochemical Physiology 132A:739–761.

Pörtner, H.O., M. Langenbuch, and A. Reipschlager. 2004. Biological impact of elevated ocean CO_2 concentrations: lessons from animal physiology and earth history. Journal of Oceanography 60:705–718.

Pörtner, H.O., D. Storch, and O. Heilmayer. 2005. Constraints and trade-offs in climate-dependent adaptation: energy budgets and growth in a latitudinal cline. Scientia Marina. 69:271–285.

Pörtner, H.O., L.S. Peck, and G.N. Somero. 2007. Thermal limits and adaptation in marine Antarctic ectotherms: an integrative view. Philosophical Transactions of the Royal Society London B 362:2233–2258.

Rabalais, N.N., R.J. Díaz, L.A. Levin, R.E. Turner, D. Gilbert, and J. Zhang. 2010. Dynamics and distribution of natural and human-caused hypoxia. Biogeosciences 7:585–619.

Rahn, H. 1966. Aquatic gas exchange: theory. Respiration Physiology 1:1–12.

Rastrick, S.P.S., and N.M. Whiteley. 2011. Congeneric amphipods show differing abilities to maintain metabolic rates with latitude. Physiological and Biochemical Zoology 84:154–165.

Rastrick, S.P.S., and N.M. Whiteley. 2013. Influence of natural thermal gradients on whole animal rates of protein synthesis in marine gammarid amphipods. PLoS ONE 8:e60050.

Reeves, R.B. 1972. An imidazole alphastat hypothesis for vertebrate acid-base regulation: tissue carbon dioxide content and body temperature in bullfrogs. Respiratory Physiology 14:219–236.

Reiber, C.L., and B.R. McMahon. 1998. The effects of progressive hypoxia on the crustacean cardiovascular system: a comparison of the freshwater crayfish (*Procambarus clarkii*), and the lobster (*Homarus americanus*). Journal of Comparative Physiology B 168:168–176.

Robertson, R.F., A.J. El Haj, A. Clarke, and E.W. Taylor. 2001a. Effects of temperature on specific dynamic action and protein synthesis rates in the Baltic isopod crustacean, *Saduria entomon*. Journal of Experimental Marine Biology and Ecology 262:113–129.

Robertson, R.F., A.J. El Haj, A. Clarke, L.S. Peck, and E.W. Taylor. 2001b. The effects of temperature on metabolic rate and protein synthesis following a meal in the isopod *Glyptonotus antarcticus* Eights (1852). Polar Biology 24:677–686.

Robertson, R.F., J. Meagor, and E.W. Taylor. 2002. Specific dynamic action in the shore crab, *Carcinus maenas* (L.), in relation to acclimation temperature and to the onset of the emersion response. Physiological and Biochemical Zoology 75:350–359.

Ruiz-Tagle, N., M. Fernández, and H.O. Pörtner. 2002. Full time mothers: daily rhythms in brooding and nonbrooding behaviors of Brachyuran crabs. Journal of Experimental Marine Biology and Ecology 276:31–47.

Sabine, C.L., R.A. Feely, N. Gruber, R.M. Key, K. Lee, J.L. Bullister, R. Wanninkhof, C.S. Wong, D.W.R. Wallace, B. Tilbrook, F.J. Millero, T.-H. Peng, A. Kozyr, T. Ono, and A.F. Rios. 2004. The oceanic sink for anthropogenic CO_2. Science 305:367–371.

Saborowski, R., S. Brohl, G.A. Tarling, and F. Buchholz. 2002. Metabolic properties of Northern krill, *Meganyctiphanes norvegica*, from different climatic zones. I. Respiration and excretion. Marine Biology 140:547–556.

Sandberg, E., and E. Bonsdorff. 1996. Effects of predation and oxygen deficiency on different age classes of the amphipod *Monoporeia affinis*. Journal of Sea Research 35: 345–351.

Sanders, N.K., and J.J. Childress. 1990. Adaptations to the deep-sea oxygen minimum layer: oxygen binding by the hemocyanin of the bathypelagic mysid, *Gnathophausia ingens* Dohrn. Biological Bulletin 178:286–294.

Schmidt, C., N.L. Bris, and F. Gaill. 2008. Interactions of deep-sea vent invertebrates with their environment: the case of *Rimicaris exoculata*. Journal of Shellfish Research 27:79–90.

Seibel, B.A., and J.C. Drazen. 2007. The rate of metabolism in marine animals: environmental constraints, ecological demands and energetic opportunities. Philosophical Transactions of the Royal Society of London B 362:2061–2078.

Small, D., P. Calosi, D. White, J.I. Spicer, and Widdicombe, S. 2010. Impact of medium-term exposure to CO_2 enriched seawater on the physiological functions of the velvet swimming crab *Necora puber*. Aquatic Biology 10:11–21.

Somero, G.N. 2002. Thermal physiology and vertical zonation of intertidal animals: optima, limits and costs of living. Integrative and Comparative Biology 42:780–789.

Somero, G.N. 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine "winners" and "losers." The Journal of Experimental Biology 213:912–920.

Spicer, J.I. 1999. Possessing a poor anaerobic capacity does not prevent the diel vertical migration of Nordic krill *Meganyctiphanes norvegica* into hypoxic waters. Journal of Comparative Physiology 185:181–187.

Spicer, J.I., and S.P. Eriksson. 2003. Does the development of respiratory regulation always accompany the transition from pelagic larvae to benthic fossorial postlarvae in the Norway lobster *Nephrops norvegicus*. Journal of Marine Biology and Ecology 295:219–243.

Spicer, J.I., and R. Saborowski. 2010. Physiology and metabolism of Northern Krill (*Meganyctiphanes norvegica* Sars). Advances in Marine Biology 57:91–126.

Spicer, J.I., A. Raffo, and S. Widdicombe. 2007. Influence of CO_2-related seawater acidification on extracellular acid-base balance in the velvet swimming crab *Necora puber*. Marine Biology 151:1117–1125.

Stillman, J.H. 2002. Causes and consequences of thermal tolerance limits in rocky intertidal porcelain crabs, genus Petrolisthes. Integrative and Comparative Biology 42:790–796.

Stillman, J.H. 2003. Acclimation capacity underlies susceptibility to climate change. Science 301:65.

Stillman, J.H., and G.N. Somero, G. 1996. Adaptation to temperature stress and aerial exposure in congeneric species of intertidal porcelain crabs (genus Petrolisthes): correlation of physiology, biochemistry and morphology with vertical distribution. The Journal of Experimental Biology 199:1845–1855.

Stillman, J.H., J.K. Colbourne, C.E. Lee, N.H. Patel, M.R. Phillips, D.W. Towle, B.D. Eads, G.W. Gelembuik, R.P. Henry, E.A. Johnson, M.E. Pfrender, and N.B. Terwilliger. 2008. Recent advances in crustacean genomics. Integrative and Comparative Biology 48:852–868.

Strömberg, J.O., and J.I. Spicer. 2000. Cold comfort for krill? Respiratory consequences of diel vertical migration by *Meganyctiphanes norvegica* into deep hypoxic waters. Ophelia 53:213–217.

Tagmount, A., M. Wang, E. Lindquist, Y. Tanaka, K.S. Teranishi, S. Sunagawa, M. Wong, and J.H. Stillman. 2010. The porcelain crab transcriptome and PCAD, the porcelain crab microarray and sequence database. PloS One 5:e9327.

Taylor, A.C., and R.J.A. Atkinson. 1991. Respiratory adaptations of aquatic decapod crustaceans and fish to a burrowing mode of life. Pages 211–234 *in* A.J. Woakes, M.K. Grieshaber, and C.R. Bridges, editors. Physiological strategies for gas exchange and metabolism. Society for Experimental Biology Seminar Series 41. Cambridge University Press, Cambridge.

Taylor, A.C., C M. Astall, and R.J.A. Atkinson. 2000. A comparative study of the oxygen transporting properties of the haemocyanin of five species of thalassinidean mud-shrimps. Journal of Experimental Marine Biology and Ecology 244:265–283.

Taylor, E.W. 1965. Respiration in species inhabiting temporary freshwater habitats. Ph.D. thesis, University of Southampton.

Taylor, E.W. 1981. Some effects of temperature on respiration in decapodan crustaceans. Journal of Thermal Biology 6:239–248.

Taylor, E.W. 1982. Control and co-ordination of ventilation and circulation in crustaceans: responses to hypoxia and exercise. Journal of Experimental Biology 100:289–319.

Taylor, E.W., and P.J. Butler. 1973. The behaviour and physiological responses of the shore crab *Carcinus maenas* during changes in environmental oxygen tension. Netherlands Journal of Sea Research 7:496–505.

Taylor, E.W., and P.J. Butler. 1978. Aquatic and aerial respiration in the shore crab, *Carcinus maenas* L. at 150C. Journal of Comparative Physiology 127:315–323.

Taylor, E.W., and A.J. Innes. 1988. A functional analysis of the shift from gill to lung breathing during the evolution of land crabs (Crustacea, Decapoda). Biological Journal of the Linnean Society 34:229–247.

Taylor, E.W., and M.G. Wheatly. 1979. The behaviour and respiratory physiology of the shore crab, *Carcinus maenas* L. at moderately high temperatures. Journal of Comparative Physiology 130:309–316.

Taylor, E.W., and M.G. Wheatly. 1980. Ventilation, heart rate and respiratory gas-exchange in the crayfish *Austropotamobius pallipes* (Lereboullet) submerged in normoxic water and following 3 h exposure in air at 15°C. Journal of Comparative Physiology 138:67–78.

Taylor, E.W., and M.G. Wheatly. 1981. The effect of long-term aerial exposure on heart rate, ventilation, respiratory gas exchange and acid-base status in the crayfish, *Austropotamobius pallipes*. Journal of Experimental Biology 92:109–124.

Taylor, E.W., and N.M. Whiteley. 1989. Oxygen transport and acid-base balance in the haemolymph of the lobster, *Homarus gammarus*, during aerial exposure and resubmersion. Journal of Experimental Biology 144:417–436.

Taylor, E.W., P.J. Butler, and P.J. Sherlock. 1973. The respiratory and cardiovascular changes associated with the emersion response of *Carcinus maenas* (L.) during environmental hypoxia, at three different temperatures. Journal of Comparative Physiology 86:95–115.

Taylor, E.W., P.J. Butler, and A. Al-Wassia. 1977. Some responses of the shore crab, *Carcinus maenas* L. to progressive hypoxia at different acclimation temperatures and salinities. Journal of Comparative Physiology 122:391–402.

Taylor, E.W., N.M. Whiteley, and M.G. Wheatly. 1991. Respiratory gas exchange and the regulation of acid-base status in decapod crustaceans. Pages 79–106 *in* A.J. Woakes, M.K. Grieshaber, and C.R. Bridges, editors. Physiological strategies for gas exchange and metabolism. Society for Experimental Biology, Seminar Series 41. Cambridge University Press, Cambridge.

Taylor, H.H., and E.W. Taylor. 1992. Gills and lungs: the exchange of gases and ions. Pages 203–293 *in* F.W. Harrison and A.G. Humes, editors. Decapod Crustacea. Vol. 10 of Microscopic Anatomy of Invertebrates. Wiley-Liss, New York.

Terwilliger, N.B., and M. Ryan. 2001. Ontogeny of Crustacean respiratory proteins. Integrative and Comparative Biology 41:1057–1067.

Torres, J.J., A.V. Aarset, J. Donnelly, T.L. Hopkins, T.M. Lancraft, and D.G. Ainley. 1994. Metabolism of Antarctic micronektonic Crustacea as a function of depth of occurrence and season. Marine Ecology Progress Series 113:207–219.

Towle, D.W., and D. Weihrauch. 2001. Osmoregulation by gills of euryhaline crabs: molecular analysis of transporters. American Zoologist 41:770–780.

Truchot, J.P. 1975. Factors controlling *in vitro* and *in vivo* oxygen affinity of the haemocyanin in the crab, *Carcinus maenas* (L.). Respiration Physiology 223:351–360.

Truchot, J.P. 1980. Lactate increases the oxygen affinity of crab hemocyanin. Journal of Experimental Zoology 214:205–208.

Truchot, J.-P., and A. Duhamel-Jouve. 1980. Oxygen and carbon dioxide in the marine intertidal environment: diurnal and tidal changes in rock pools. Respiration Physiology 39:241–254.

Vernberg, F.J. 1962. Comparative physiology—latitudinal effects on physiological properties of animal populations. Annual Reviews of Physiology 24:517–544.

Vernberg, F.J., and J.D. Costlow. 1966. Studies on physiological variation between tropical and temperate-zone fiddler crabs of the genus Uca. 4. Oxygen consumption of larvae and young crabs reared in the laboratory. Physiological Zoology 39:36–52.

Vernberg F.J., and G.S. Moreira. 1974. Metabolic-temperature responses of the copepod *Euterpina acutifrons* (Dana) from Brazil. Comparative Biochemistry Physiology 42A:867–876.

Vetter, R.D., M.E. Wells, A.L. Kurtsman, and G.N. Somero. 1987. Sulfide detoxification by the hydrothermal vent crab *Bythograea thermydron* and other decapod crustaceans. Physiological Zoology 60:121–137.

Walseng, B., N.D. Yan, and A.K. Schartau. 2003. Littoral microcrustacean (Cladocera and Copepoda) indicators of acidification in Canadian Shield lakes. AMBIO 32:208–213.

Walther, K., F.J. Sartoris, C. Bock, and H.O. Pörtner. 2009. Impact of anthropogenic ocean acidification on thermal tolerance of the spider crab *Hyas araneus*. Biogeosciences 6:2207–2215.

Watt, A.J.S., N.M. Whiteley, and E.W. Taylor. 1999. An *in situ* study of respiratory variables in three British sublittoral crabs with different routine rates of activity. Journal Experimental Marine Biology and Ecology 239:1–21.

Weber, A.K., and R. Pirow. 2009. Physiological responses of *Daphnia pulex* to acid stress. BMC Physiology 9:1–25.

Wheatly, M.G. 1981. The provision of oxygen to developing eggs by female shore crabs (*Carcinus maenas*). Journal of the Marine Biological Association UK 61:117–128.

Wheatly, M.G., and R.P. Henry. 1992. Extracellular and intracellular acid-base regulation in crustaceans. Journal of Experimental Zoology 263:127–142.

Wheatly, M.G., and E.W. Taylor. 1981. The effect of progressive hypoxia on heart rate, ventilation, respiratory gas exchange and acid-base status in the crayfish, *Austropotamobius pallipes*. Journal of Experimental Biology 92:125–141.

Wheatly, M.G., T. Toop, and R.J. Morrison, and L.C. Yow. 1991. Physiological responses of the crayfish *Pacifastacus leniusculus* (Dana) to environmental hyperoxia. 3. Intracellular acid-base balance. Physiological Zoology 64:323–343.

Whiteley, N.M. 1999. Acid-base regulation in aquatic crustaceans: role of bicarbonate ions. Pages 233–255 *in* S. Egginton, E.W. Taylor, and J.A. Raven, editors. Regulation of acid-base status in animals and plants. Society for Experimental Biology Seminar Series 68. Cambridge University Press, Cambridge.

Whiteley, N.M. 2011. Physiological and ecological responses of crustaceans to ocean acidification. Marine Ecology Progress Series 430:257–271.

Whiteley, N.M., and L.S. Faulkner. 2005. Temperature influences whole animal rates of metabolism but not protein synthesis in a temperate intertidal isopod. Physiological Biochemistry and Zoology 78:227–238.

Whiteley, N.M., and K.P.P. Fraser. 2009. The effects of temperature on ectotherm protein metabolism. Pages 249–265 *in* T.E. Esterhouse, and L.B. Petrinos, editors. Protein biosynthesis. Nova Science Publishers Inc., New York.

Whiteley, N.M., and E.W. Taylor. 1989. The acid-base consequences of aerial exposure in the lobster, *Homarus gammarus* (L.) at 10 and 20°C. Journal of Thermal Biology 15:47–56.

Whiteley, N.M., and E.W. Taylor. 1992. Oxygen and acid-base disturbances in the haemolymph of the lobster, *Homarus gammarus*, during commercial transport and storage. Journal of Crustacean Biology 12:19–30.

Whiteley, N.M., and E.W. Taylor. 1993. The effects of seasonal variations in temperature on extracellular acid-base status in a wild population of the crayfish. *Austropotamobius pallipes*. Journal of Experimental Biology 181:295–311.

Whiteley, N.M., J.K. Naylor, and E.W. Taylor. 1995. Extracellular and intracellular acid-base status in the freshwater crayfish, *Austropotamobius pallipes*, between 1 and 12°C. Journal of Experimental Biology 198:567–576.

Whiteley, N.M., E.W. Taylor, and A.J. El Haj. 1996. A comparison of the metabolic cost of protein synthesis in stenothermal and eurythermal isopod crustaceans. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology 40:R1295–R1303.

Whiteley, N.M., E.W. Taylor, A. Clarke, and A.J. El Haj. 1997. Haemolymph oxygen transport and acid-base status in *Glyptonotus antarcticus* Eights. Polar Biology 18:10–15.

Whiteley N.M., R.F. Robertson, J. Meagor, A.J. El Haj, and E.W. Taylor. 2001a. Protein synthesis and specific dynamic action in crustaceans: effects of temperature. Comparative Biochemistry and Physiology A 128:595–606.

Whiteley N.M., J.L. Scott, S.J. Breeze, and L. McCann. 2001b. Effects of water salinity on acid-base balance in decapod crustaceans. Journal of Experimental Biology 204:1003–1011.

Wood, C.M., and D.J. Randall. 1981. Oxygen and carbon dioxide exchange during exercise in the land crab (*Cardisoma carnifex*). Journal of Experimental Zoology 218:7–22.

Wu, R.S.S. 2002. Hypoxia: from molecular responses to ecosystem responses. Marine Pollution Bulletin 45:35–45.

11

OXYGEN TRANSPORT PROTEINS IN CRUSTACEA: HEMOCYANIN AND HEMOGLOBIN

Nora B. Terwilliger

Abstract

In crustaceans, oxygen transport proteins hemocyanin and hemoglobin increase oxygen transport capacity of hemolymph and sustain metabolic needs. Hemocyanins are large, multi-subunit molecules containing highly conserved copper-based oxygen binding sites; their structure and subunit heterogeneity have been explored using techniques that enhance our understanding of their evolution and the role of specific amino acid residues in their assembly and function. Hemocyanin also functions as a phenoloxidase, a carrier protein, and in immune response. Increasing evidence implicates hemocyanin in sclerotization of new exoskeleton at ecdysis. Additional gene family members are cryptocyanin, a copperless hexamer expressed during premolt that functions in exoskeleton formation, and a hemocyte phenoloxidase involved in wound repair, sclerotization, melanin synthesis, and immune system. Future directions call for understanding the regulation and coordination of expression of multiple hemocyanin or hemoglobin genes in crustaceans and the possibility of transgenerational epigenetic inheritance in response to development, molting, and environmental change.

INTRODUCTION

Challenges of Obtaining Oxygen

Small organisms have high surface-to-volume ratios that allow them to obtain O_2 through simple diffusion. As an animal grows, metabolic needs tend to increase cubically, while the surface of the animal, the window by which an organism obtains O_2, only increases as the square. Thus, the surface-to-volume ratio decreases with increasing size. Organisms also

must contend with the diffusion rate of gases, which is equal to the cross-sectional area exposed to the gas multiplied by the diffusion properties of the gas (in either air or water) multiplied by the concentration gradient of the gas. Only the area and the concentration gradients are subject to modification by the organism. Anatomical modifications to satisfy O_2 needs through increasing the surface area are seen in many soft-bodied animals. In Annelida, for example, certain oligochaetes and leeches take up O_2 directly across the thin body wall to underlying capillaries; capitellids assume flattened string-like shapes; nereid and glycerid polychaetes extend thin-walled flaps or cirri from their segmentally arrayed parapodia, and tube-dwelling terebellids sport corkscrew gills that protrude into the oxygenated seawater from the most anterior segments and contribute up to one-third of the total body surface. These thin-walled "gills" all function to increase the general surface area and minimize the diffusion path for O_2 from outside to inside the organism, the first step in aerobic respiration.

Crustaceans and other Arthropoda, whose bodies are encased in exoskeletons, have limited opportunities to increase their gas exchange areas and must rely on the gills and epithelial lining of the branchial chambers where the exoskeleton coverings are markedly thinner. Crustaceans do possess numerous behavioral and biochemical strategies to improve the O_2 flux and steepen the O_2 gradient between the outside milieu and inside the chitin-enclosed gill. Externally, ventilatory movements by appendages like the scaphognathite or gill bailer, that push O_2-depleted water out of the gill chambers and allow well-oxygenated water to flow in and around the gills, enhance the O_2 gradient (McMahon 2001). Internally, the highly regulated circulatory system of crustaceans effectively removes O_2 from inside the thin barrier gill, both steepening the diffusion gradient and delivering O_2 elsewhere (see Chapter 7 in this volume). Finally, O_2 transport proteins that are specialized to bind O_2 in regions of high concentration and release it in regions of lower concentration circulate in the hemolymph, thus providing a molecular means to sustain the metabolic needs of the crustaceans. The proteins markedly increase the O_2 transport capacity of the hemolymph in comparison to the very low solubility of dissolved O_2. Like ventilation and circulation, O_2 transport proteins effectively improve the O_2 flux and enhance the O_2 gradient.

Arthropods abound in the Middle Cambrian fossils from the Burgess Shale. This site in the Canadian Rockies, renowned for its excellent preservation of soft-bodied animals that are more than 500 million years old, also has exceptional diversity of early arthropod species. The exoskeletons covering these mobile, multicellular bilaterians suggest that the O_2 needs of these arthropods could not have been met through simple diffusion; they would have required proteins that could bind the O_2 molecules and transport them via a circulatory system to the respiring tissues. Phylogenetic studies estimate the O_2 transport proteins evolved prior to the Cambrian, perhaps between 600 and 700 million years ago, and preceded the appearance of fossils with hard exoskeletons and shells (Decker and van Holde 2011). *Marrella splendens*, a basal arthropod frequently found in the Burgess Shale, is often preserved with a dark stain under or around the fossil (Fig. 11.1). This stain probably represents the fossilized traces of a copper-based hemocyanin that circulated in the hemolymph. Elevated concentrations of copper identified specifically in the dark stain, but also in other regions of the fossilized *Marrella* using synchrotron X-ray fluorescence imaging, provide intriguing evidence for a most ancient hemocyanin (Pratt et al. 2010).

Today's crustaceans are among the most sensitive taxa to O_2 levels, showing the highest LC_{50} and the shortest LT_{50} when compared with fish, mollusks, annelids, echinoderms, cnidarians, and priapulids in a survey of 872 published experiments (Vaquer-Sunyer and Duarte 2008). Obtaining O_2 is a critical task for a crustacean. This chapter focuses on recent findings on the structures, functional properties, and phylogenetic relationships of the crustacean respiratory proteins, hemocyanins and hemoglobins.

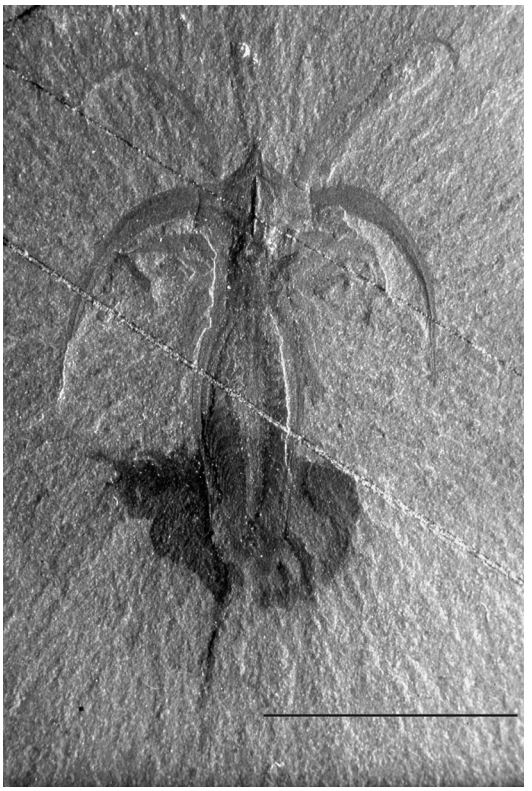


Fig. 11.1.
Marrella splendens Walcott, a Middle Cambrian basal arthropod from the Burgess Shale. The dark stain visible toward the posterior of the ~500-million year old fossil is rich in copper and probably represents an ancient hemocyanin. Scale bar = 1 cm. Copyright Peabody Museum of Natural History, Yale University, New Haven, CT. YPM catalog no. 5861. Photography by W.K. Sacco.

Oxygen Transport Proteins: Hemocyanin, Hemoglobin, Hemerythrin

Three types of O_2 binding proteins reversibly transport O_2 within an organism. First, hemocyanins, found exclusively in Arthropoda and Mollusca, are large, multi-subunit molecules composed of individual polypeptide chains or subunits containing highly conserved copper-based O_2-binding sites (Van Holde and Miller 1995, Decker et al. 2007a, Decker and van Holde 2011). Each of the two copper atoms in the active site is covalently bound by three histidine residues (Fig. 11.2). Upon oxygenation, a di-O_2 is bound as a peroxide in a side-on (μ-η^2:η^2) coordination between the two copper atoms (type III copper binding site). When O_2 is bound, the coppers oxidize from Cu(I) to Cu(II) and the hemocyanin becomes blue; this is reversed when O_2 is released in the deoxygenated state. Hemocyanins are extracellular and circulate dissolved in the hemolymph, in contrast to vertebrate hemoglobins in erythrocytes. Arthropod hemocyanins are hexameric arrays of individual approximately 75 kDa subunits, and each subunit has one O_2-binding site. The hexamers assemble into multiples of hexamers depending on the arthropod species, as described below. The largest, the 8-hexamer oligomer of horseshoe crab hemocyanin, has 48 O_2-binding sites. Molluskan hemocyanins are cylindrical structures whose 10 or 20 subunits are much larger than arthropod hemocyanin subunits and assemble into decamers, didecamers, or multidecamers. A molluskan subunit of 350–400 kDa contains 7–8 paralogous functional units (FU). Each 50 kDa FU contains one active site, so that intact hemocyanin of a mollusk may have 160 O_2-binding sites (Cuff et al. 1998, Miller

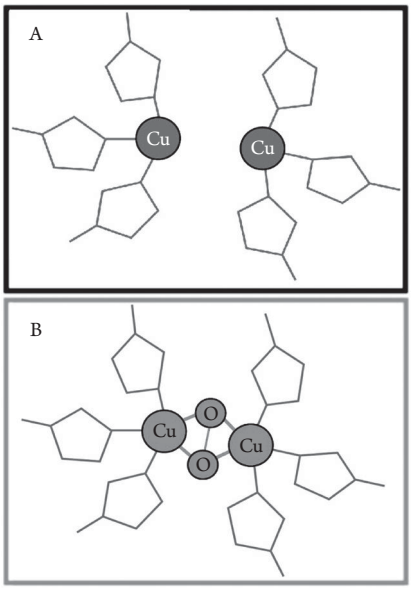


Fig. 11.2.
Diagram of active site of oxy and deoxy hemocyanin showing each copper atom complexed by a set of three histidines, which form the connection to the protein backbone. (A) Deoxygenated state without any ligands. (B) Oxygenated state with oxygen bound as peroxide in a side-on coordination. From Panzer et al. (2010), with permission from the American Chemical Society.

et al. 1998, Perbandt et al. 2003). The protein sequences of arthropod and molluskan hemocyanins appear to be phylogenetically unrelated except perhaps at the functional site (Van Holde et al. 2001, Burmester 2002), yet overall structural aspects and functional properties show hints of shared history (Durstewitz and Terwilliger 1997, Jaenicke et al. 2010). Whether these circulating copper proteins have evolved independently or in fact share a common ancestor is an ongoing topic of investigation and conjecture (see later discussion).

The other two O_2 transport proteins, hemoglobins and hemerythrins, both have iron-based O_2-binding sites. Hemoglobins are nearly ubiquitous, being widely distributed in animals, plants, protists, and bacteria, and are expressed in multiple tissues, although they are not all involved in reversible O_2 transport (Terwilliger 1992, Terwilliger 1998, Weber and Vinogradov 2001, Royer et al. 2005, Brunori and Vallone 2007, Burmester and Hankeln 2007). The single iron in the heme of a hemoglobin subunit is coordinated on its proximal side to a histidine of the globin protein. On its distal side, the heme iron reversibly binds a di-O_2 molecule within the protective globin framework. Vertebrates contain the well-known $\alpha_2\beta_2$ tetrameric hemoglobin, each subunit with a single heme, in a circulating red blood cell or erythrocyte. Across the phyla, however, circulating hemoglobins range from intracellular to extracellular, single to multiple hemes per subunit, and monomeric to multimeric subunits per molecule, and they self-assemble into a wide range of beautiful three-dimensional arrays.

The third O_2 transport protein, hemerythrin, has been functionally described in only four protostome groups: Sipuncula, Annelida, Brachiopoda, and Priapulida (Kurtz 1992, Vanin et al. 2006). In contrast to hemoglobin, two iron atoms in the O_2 binding site of a hemerythrin subunit are coordinated directly to the hemerythrin protein itself, there is no heme group, and the protein sequence has no resemblance to a globin (Vanin et al. 2006, Meyer and Lieb 2010). Oxygenated hemerythrin is deep pink-purple, whereas deoxyhemerythrin is colorless. Hemerythrins are intracellular in circulating hemerythrocytes as trimeric or octomeric assemblages that may have

evolved from a monomeric ancestor, and hemerythrins are also present in muscle and nervous tissue. Hemerythrin-related sequences have been determined in Cnidaria and Bacteria, but not in Arthropoda—nor in any deuterostomes (Xiong et al. 2000, Bailly et al. 2008, French et al. 2008, Traverso et al. 2010). Although future genomic analyses may reveal a more extensive distribution of hemerythrins and a cosmopolitan occurrence of hemocyanins and hemoglobins, at the moment, expression of circulating O_2 transport proteins in the Crustacea is limited to hemocyanin and hemoglobin.

CRUSTACEAN OXYGEN BINDING PROTEINS: HEMOCYANIN

Hemocyanins have been identified in all four subphyla of Arthropoda, Crustacea, and Chelicerata (Markl and Decker 1992, Van Holde and Miller 1995, Rehm et al. 2012), Myriapoda (Mangum et al. 1985), and Hexapoda (Hagner-Holler et al. 2004, Pick et al. 2008). They are also found in the related ecdysozoan, Onychophora (Kusche et al. 2002). Within the Crustacea, hemocyanin occurs in Remipedia (Ertas et al. 2009) and is expressed extensively in Malacostraca, from the most basal Phyllocarida, *Nebalia* (Vierthaler et al. 2003), to the shrimps, lobsters, and crabs, but it has not been described in other Crustacea.

Hemocyanin Structure

Detailed knowledge of the subunit structure of a crustacean hemocyanin is the basis for understanding the complex quaternary assemblages and functional properties of the circulating proteins, as well as the evolution of the arthropod hemocyanin gene family. Crystal structure analyses of single subunits from hemocyanins of a crustacean *Panulirus interruptus* (spiny lobster) and a chelicerate *Limulus polyphemus* (horseshoe crab) have shown that the arthropod hemocyanin subunit is folded into three distinct regions or domains (Gaykema et al. 1984, Volbeda and Hol 1989, Hazes et al. 1993, Magnus et al. 1994). The N-terminal domain 1, predominantly α-helical, is thought to form a narrow channel for the O_2 to enter and exit the molecule. Upon oxygenation, an 8-degree tilt of domain 1 occurs, which may block the channel. Thus, domain 1 shields the entrance to the active site. The central domain 2, a four-α-helix bundle, contains the O_2-binding site, where each of the two copper atoms is complexed to three histidines. The C-terminal domain 3 has a seven-stranded β-barrel folding motif. Six of these approximately 75 kDa subunits assemble into a sandwich of two trimers. The resulting approximately 450 kDa hexamer is the smallest physiologically active circulating hemocyanin.

Hemocyanin hexamers and multiples of hexamers are composed of species-specific combinations of unique subunits, rather than multiple copies of a single protein. This diversity, referred to as *subunit heterogeneity*, enhances the assembly and functional properties of the oligomers. Subunit heterogeneity, as well as phylogenetic relationships among the hemocyanin subunits and among the Arthropoda, have been established through numerous studies at the protein, mRNA, and gene levels (Markl et al. 1979, Markl and Decker 1992, Burmester 2001, 2002, Terwilliger and Ryan 2006). Determination of the complete cDNA sequences of a species' array of hemocyanin subunits, as in the seven subunits of the tarantula *Eurypelma californicum* and the six subunits of the brachyuran crab *Cancer (Metacarcinus) magister*, confirms each subunit's identity as a unique gene product (Voit et al. 2000, Terwilliger et al. 2006).

Within the malacostracan crustaceans, hemocyanin subunits appeared to group into several types, α, β, and γ, based on immunogenicity and roles in aggregation (Markl 1986). As hemocyanin sequences representing more malacostracans have become available, additional subunit types and support for new interpretations of crustacean phylogenetic relationships are emerging (Terwilliger

and Ryan 2006, Ertas et al. 2009, Scherbaum et al. 2010). For example, hemocyanin is expressed in *Nebalia kensleyi*, a leptostracan in the subclass Phyllocarida, considered the most basal of the Malacostraca (Fig. 11.3). *Nebalia* hemocyanin, present only as hexamers, combines reversibly with O_2, indicating that it functions as an O_2 transporter (Vierthaler et al. 2003). Its sequence clusters with other malacostracan hemocyanins, but separate from the α, β, and γ types, consistent with the basal position of the Phyllocarida (Fig. 11.4). In contrast, four hemocyanin subunits of the stomatopod *Odontodactylus scyllarus* (Malacostraca, subclass Hoplocarida) all belong to the branch of β-type subunits (Scherbaum et al. 2010). Eucaridan decapods (Malacostraca, subclass Eumalacostraca) also express β subunits; in the brachyuran *C. magister*, two of the six hemocyanin subunits are β whereas the other four are γ, with no α representatives (Terwilliger and Ryan 2006), and hemocyanin in the anomuran *Petrolisthes cinctipes* is composed of β and γ subunits (Scherbaum et al. 2010). Thus, an early hemocyanin gene duplication seems to have given rise to the β subunit lineage as early as 520–480 mya, near the time when the Eumalacostraca and Phyllocarida diverged, followed by the Hoplocarida at approximately 404 mya (Fig. 11.5; Scherbaum et al. 2010). Hemocyanin sequences from amphipod and isopod species (all eumalacostracan Peracarida) cluster within the malacostracan hemocyanins and cryptocyanins but in a separate branch distinct from the α and γ type hemocyanin sequences of the eumalacostracan Eucarida (Figs. 11.4 and 11.5; Hagner-Holler et al. 2005, Terwilliger and Ryan 2006, Jaenicke et al. 2009). This may reflect a gene duplication, separation, and subsequent evolution of hemocyanin types as eumalacostracan crustaceans evolved into separate superorders of Eucarida and Peracarida, and this theory is substantiated by functional

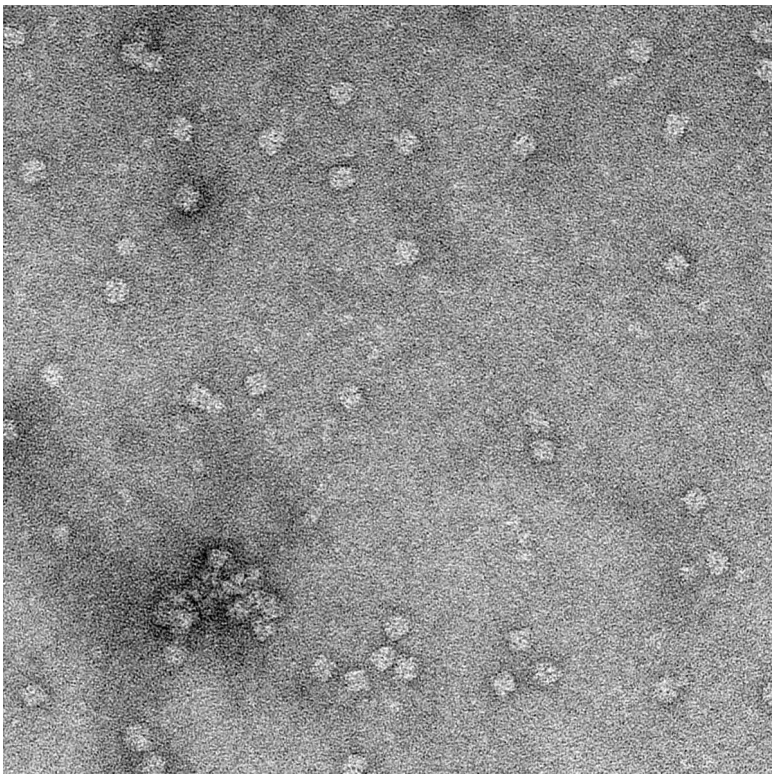


Fig. 11.3.
Electron micrograph of *Nebalia kensleyi* hemocyanin shows the presence of single hexamers. Hemolymph negatively stained with 1% uranyl acetate. Microscopy by E. Schabtach.

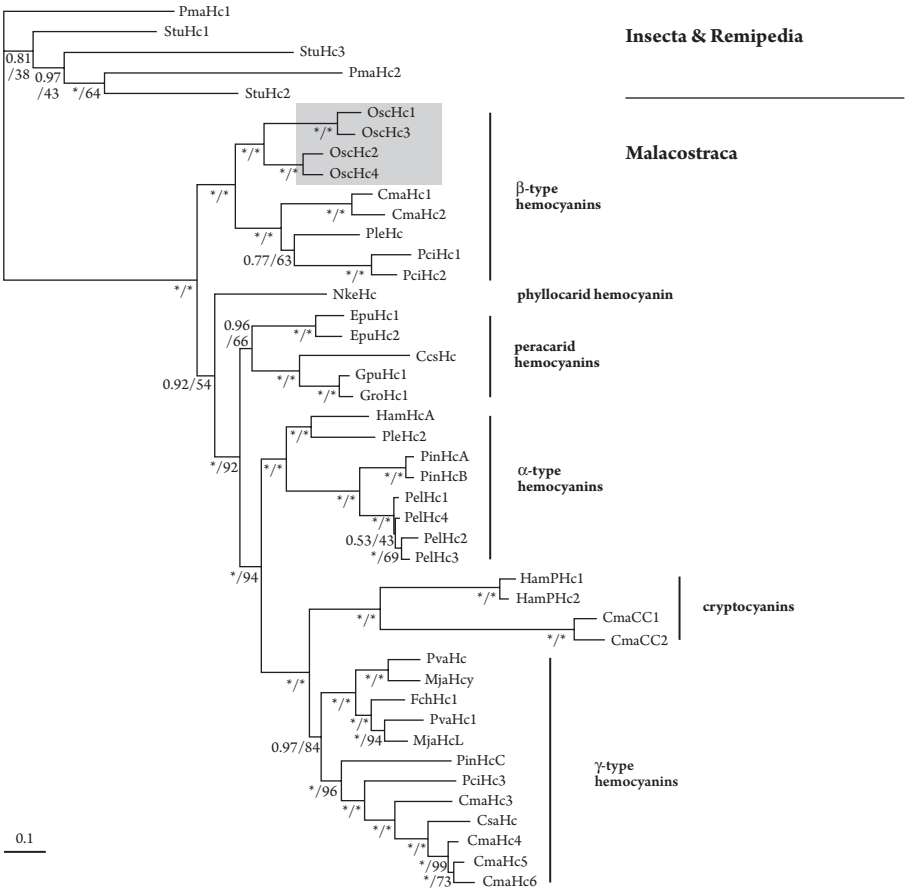


Fig. 11.4.
Phylogeny of crustacean hemocyanins, including remipedian and malacostracan (phyllocaridan, hoplocaridan, eumalacostracan eucaridan, and eumalacostracan peracaridan) hemocyanin and eumalacostracan eucaridan cryptocyanin (pseudohemocyanin) sequences. Sister-group relation of hexapodan and remipedian hemocyanins to malacostracans is included. See Table 11.1 for abbreviations. The numbers at the nodes are Bayesian posterior probabilities (first number) and ML nonparametric bootstrap support values (second number). Asterisks indicate 1.0 Bayesian posterior probability and 100% ML bootstrap support, respectively. The bar represents 0.1 PAM distance. From Scherbaum et al. (2010), with permission from Springer Science.

differences between eucaridan and peracaridan hemocyanins (phenoloxidase function). Further gene duplications and subunit evolution into α and γ type subunits, including the cryptocyanins, occurred as the eucaridans diversified (Hagner-Holler et al. 2005, Scherbaum et al. 2010). Thus, the hemocyanin molecular phylogenies inform the crustacean phylogenies and vice versa.

The discovery of hemocyanin in the Remipedia *Speleonectes tulumensis* is the first unambiguous evidence for crustacean hemocyanin expression outside the Malacostraca (van der Ham and Felgenhauer 2007, Ertas et al. 2009). The Remipedia hemocyanin is most similar to Hexapoda hemocyanins, which places both in a sister-group relation to the hemocyanins of malacostracan crustaceans (Fig. 11.4; Ertas et al. 2009). Evidence indicates that the Remipedia hemocyanin subunits are orthologs of hexapod hemocyanin subunits; therefore, the hemocyanin lineages would have split before the Remipedia and Hexapoda groups diverged. A hexapod-crustacean clade has support from both morphological and molecular analyses, although there have been several

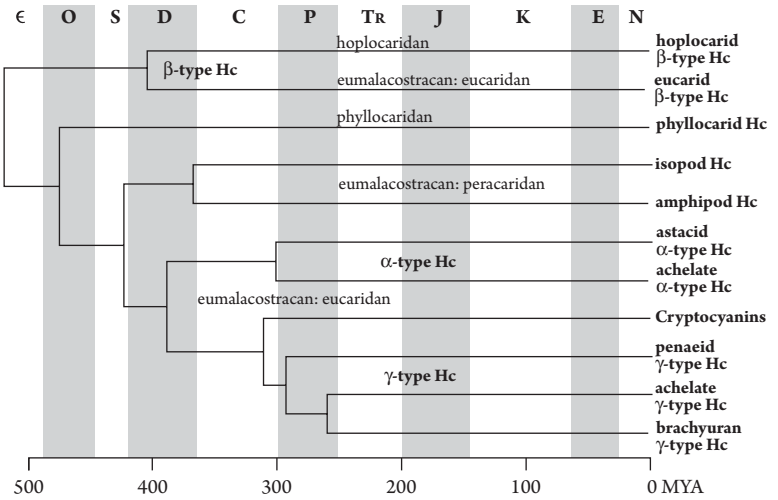


Fig. 11.5.
Molecular clock analysis of malacostracan hemocyanin subunits based on Fig. 11.4. The geological periods are indicated at the top. Є, Cambrian; O, Ordovician; S, Silurian; D, Devonian; C, Carboniferous; P, Permian; T_R, Triassic; J, Jurassic; K, Cretaceous; E, Paleogene; N, Neogene. From Scherbaum et al. (2010), with permission from Springer Science.

candidates for the crustacean sister group, including Malacostraca, Branchiopoda, Copepoda, Remipedia, and Cephalocarida, and the debates on arthropod phylogeny still continue (Kusche et al. 2003, Dunn et al. 2008, Edgecombe 2010, von Reumont et al. 2012).

A major consequence of subunit heterogeneity in arthropod hemocyanin is the ability of hexamers to assemble into larger oligomers (Giomi and Beltramini 2007). Hemocyanin hexamers self-assemble into 2-, 4-, 6-, and 8-hexameric aggregates in a species-specific distribution, presumably based on availability of amino acid residues providing intra- and interhexameric contacts. Of the malacostracan crustaceans, for example, phyllocaridans, caridean shrimp, spiny lobsters, and amphipods have 1-hexamer hemocyanins, whereas crabs, clawed lobsters, and isopods usually have both 1- and 2-hexamer forms. The 2-hexamer hemocyanins typically require an additional subunit type, a linker, not present in the 1-hexamer form, and the two oligomer populations are not in a simple equilibrium mixture (Markl et al. 1979, Terwilliger 1982, Terwilliger and Terwilliger 1982). Hemocyanins of thalassinid crustaceans circulate predominantly as aggregates of 4-hexamers, along with a low percentage of 1-hexamers and 2-hexamers (Fig. 11.6). Within the Chelicerata, 1-, 2-, 4-, and 8-hexamer hemocyanins have been described. However, the 4-hexamer hemocyanins of scorpions and tarantulas differ from the 4-hexamer hemocyanin of thalassinid crustaceans in the three-dimensional arrangement of the hexamers (Hartmann and Decker 2002, Paoli et al. 2007, Micetic et al. 2010). Unique 6-hexamer aggregates occur in the Myriapoda, and the largest hemocyanins, 8-hexamers, are found in horseshoe crabs (Chelicerata). Hemocyanin structures have been studied intensively for many years as models for protein-protein interaction and subunit assembly using electron microscopy, ultracentrifugation, and electrophoresis (Van Holde and Miller 1982, Salvato and Beltramini 1990, Markl and Decker 1992, Van Holde and Miller 1995). Numerous sequence comparisons have shown that important secondary structure features of arthropod hemocyanin subunits are highly conserved, and this has led to homology-based molecular modeling of the giant hexameric aggregates. Integration of models with high-resolution cryoelectron microscopy images and small-angle X-ray scattering (SAXS) provides new ways of understanding the role of specific amino acid residues in the intra- and interhexameric contacts that promote both

Table 11.1. Hemocyanin (Hc), cryptocyanin (Cc), and pseudohemocyanin (PH) sequences used in Fig. 11.4.

Abbr	Species	Subclass	Superorder	Order	Infraorder	Acc. No.	Protein
Subphylum Hexapoda, Class Insecta							
PmaHc1	*Perla marginata*			Plecoptera		AJ555403	Hc1
PmaHc2	*Perla marginata*			Plecoptera		AJ555404	Hc2
Subphylum Crustacea, Class Remipedia							
StuHc1	*Speleonectes tulumensis*			Nectiopoda		FM863709	Hc1
StuHc2	*Speleonectes tulumensis*			Nectiopoda		FM863710	Hc2
StuHc3	*Speleonectes tulumensis*			Nectiopoda		FM863711	Hc3
Subphylum Crustacea, Class Malacostraca							
NkeHc	*Nebalia kensleyi*	Phyllocarida		Leptostraca		GQ279108	Hc
OscHc1	*Odontodactylus scyllarus*	Hoplocarida		Stomatopoda		FM99928	Hc1
OscHc2	*Odontodactylus scyllarus*	Hoplocarida		Stomatopoda		FM99929	Hc2
OscHc3	*Odontodactylus scyllarus*	Hoplocarida		Stomatopoda		FM99930	Hc3
OscHc4	*Odontodactylus scyllarus*	Hoplocarida		Stomatopoda		FM99931	Hc4
GroHc1	*Gammarus roeseli*	Eumalacostraca	Peracarida	Amphipoda		AJ937836	Hc1
GpuHc1	*Gammarus pulex*	Eumalacostraca	Peracarida	Amphipoda		EST data	Hc 2
CcsHc	*Cyamus scammoni*	Eumalacostraca	Peracarida	Amphipoda		DQ230983	Hc
EpuHc1	*Euridice pulcra*	Eumalacostraca	Peracarida	Isopoda		GQ153951	Hc1
EpuHc2	*Euridice pulcra*	Eumalacostraca	Peracarida	Isopoda		GQ153952	Hc2
PleHc	*Pacifastacus leniusculus*	Eumalacostraca	Eucarida	Decapoda	Astacidea	AF522504	Hc1
PleHc2	*Pacifastacus leniusculus*	Eumalacostraca	Eucarida	Decapoda	Astacidea	AY193781	Hc2

(*continued*)

Table 11.1. *(Continued)*

Abbr	Species	Subclass	Superorder	Order	Infraorder	Acc. No.	Protein
HamHcA	*Homarus americanus*	Eumalacostraca	Eucarida	Decapoda	Astacidea	AJ272095	HcA
PinHcB	*Panulirus interruptus*	Eumalacostraca	Eucarida	Decapoda	Palinura	P10787	Hcb
PinHcA	*Panulirus interruptus*	Eumalacostraca	Eucarida	Decapoda	Palinura	P04254	Hca
PinHcC	*Panulirus interruptus*	Eumalacostraca	Eucarida	Decapoda	Palinura	S21221	Hcc
PvuHc	*Palinurus vulgaris*	Eumalacostraca	Eucarida	Decapoda	Palinura	P80888	Hc
PelHc1	*Palinurus elephas*	Eumalacostraca	Eucarida	Decapoda	Palinura	AJ344361	Hc1
PelHc2	*Palinurus elephas*	Eumalacostraca	Eucarida	Decapoda	Palinura	AJ344362	Hc2
PelHc3	*Palinurus elephas*	Eumalacostraca	Eucarida	Decapoda	Palinura	AJ344363	Hc3
PelHc4	*Palinurus elephas*	Eumalacostraca	Eucarida	Decapoda	Palinura	AJ516004	Hc4
CmaHc6	*Cancer magister*[a]	Eumalacostraca	Eucarida	Decapoda	Brachyura	U48881	Hc6
CmaHc1	*Cancer magister*	Eumalacostraca	Eucarida	Decapoda	Brachyura	AY861676	Hc1
CmaHc2	*Cancer magister*	Eumalacostraca	Eucarida	Decapoda	Brachyura	AY861677	Hc2
CmaHc3	*Cancer magister*	Eumalacostraca	Eucarida	Decapoda	Brachyura	AY861678	Hc3
CmaHc4	*Cancer magister*	Eumalacostraca	Eucarida	Decapoda	Brachyura	AY861679	Hc4
CmaHc5	*Cancer magister*	Eumalacostraca	Eucarida	Decapoda	Brachyura	AY861680	Hc5
CsaHc	*Callinectes sapidus*	Eumalacostraca	Eucarida	Decapoda	Brachyura	AF249297	Hc
PciHc1	*Petrolisthes cinctipes*	Eumalacostraca	Eucarida	Decapoda	Anomura	EST data	Hc1
PciHc2	*Petrolisthes cinctipes*	Eumalacostraca	Eucarida	Decapoda	Anomura	EST data	Hc2
PciHc3	*Petrolisthes cinctipes*	Eumalacostraca	Eucarida	Decapoda	Anomura	EST data	Hc3
PvaHc1	*Penaeus vannamei*[b]	Eumalacostraca	Eucarida	Decapoda	Penaeoidea	AJ250830	Hc1
PvaHc	*Penaeus vannamei*	Eumalacostraca	Eucarida	Decapoda	Penaeoidea	X82502	Hc

FchHc	*Fenneropenaeus chinensis*	Eumalacostraca	Eucarida	Decapoda	Penaeoidea	FJ594414	Hc
MjaHcL	*Marsupenaeus japonicus*	Eumalacostraca	Eucarida	Decapoda	Penaeoidea	EF375711	HcL
MjaHcY	*Marsupenaeus japonicus*	Eumalacostraca	Eucarida	Decapoda	Penaeoidea	EF375712	HcY
HamPHc1	*Homarus americanus*	Eumalacostraca	Eucarida	Decapoda	Astacidea	AJ132141	PH
HamPHc2	*Homarus americanus*	Eumalacostraca	Eucarida	Decapoda	Astacidea	AJ132142	PH
CmaCC1	*Cancer magister*	Eumalacostraca	Eucarida	Decapoda	Brachyura	AF091261	Cc
CmaCC2	*Cancer magister*	Eumalacostraca	Eucarida	Decapoda	Brachyura	DQ230982	Cc

[a] *Cancer (Metacarcinus) magister*.
[b] *Penaeus (Litopenaeus) vannamei*.

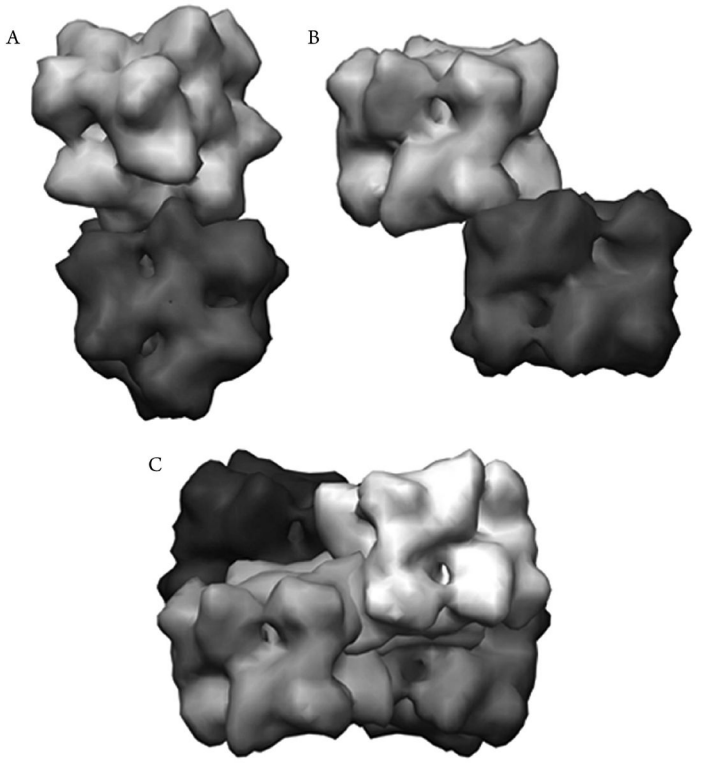


Fig. 11.6.
Comparison of oligomers of three hemocyanins using small angle X-ray scattering (SAXS) refinement of structural models derived by negative-stain electron microscopy. (A) Brachyuran crab *Carcinus estuarii* 2-hexamer hemocyanin is typical of most crustacean 2-hexamer hemocyanins. (B) Peculiar stacking of stomatopod *Squilla mantis* 2-hexamer hemocyanin resembles 2-hexameric substructure of larger cheliceratan hemocyanins. (C) Thalassinid *Upogebia pusilla* 4-hexamer hemocyanin has a unique tetrahedral packing in contrast to square planar shape of 4-hexamer hemocyanins of chelicerates. Structures of final, best-fit models. The trimeric axes of the hexameric units are oriented differently with respect to one another in the three hemocyanin oligomers. See figure 6 in Micetic et al. for structural parameters and geometries. From Micetic et al. (2010), figures 2, 3, 4 with permission from Elsevier.

the assembly and the complex functional properties of the hemocyanins. Thus, the structures of the 1-hexamer hemocyanin of the spiny lobster *Palinurus elephas* (Meissner et al. 2003) and the 2-hexamer hemocyanins of the clawed lobster *Homarus americanus* (Hartmann et al. 2001), the brachyuran crab *Carcinus aestuarii*, and the stomatopod *Squilla mantis* (Micetic et al. 2010) have been clarified. The structure of *C. aestuarii* hemocyanin is similar to those of most other crustacean 2-hexamer hemocyanins, with the two hexamers aligned along their short rough sides, but it differs from the arrangement of the two uniquely linked hexamers of *S. mantis* and *O. scyllarus*, stacked on their long flat sides, which more closely resemble a 2-hexameric substructure from a cheliceran 4- or 8-hexamer hemocyanin (Fig. 11.6; Bijlholt and van Bruggen 1986, Micetic et al. 2010, Scherbaum et al. 2010). Myriapod hemocyanin, with its unique 6-hexamer structure, was first described in centipedes (Mangum et al. 1985) and diplopods (Jaenicke et al. 1999a). A high-resolution analysis modeling 6-hexamer hemocyanins with strikingly different functional properties, one from a slow-moving diplopod *Spirostreptus* (h ~1.3, P_{50} ~5 torr) and the other from a speedy scutigeromorph centipede *Scutigera coleoptrata* (h ~10; P_{50} ~50 torr) has led the authors to suggest how

detailed differences in amino acids at the interhexameric interfaces explain the striking divergence in functional allostery within the 6-hexamer framework of these myriapod hemocyanins (Markl et al. 2009). The giant structures of 4-hexamer hemocyanin in the scorpion *Pandinus imperator* (Cong et al. 2009) and 8-hexamer hemocyanin in the horseshoe crab *L. polyphemus* (Martin et al. 2007) have also been finely resolved. These intricate multi-subunit hemocyanins provide excellent opportunities to explore the effects of gene duplications and subsequent independent evolutionary trajectories of heterogeneous subunits within a given structural framework.

Hemocyanin Gene Family

Gene duplications and mutations have resulted not only in hemocyanin subunit heterogeneity, but also in the evolution of several phylogenetically related hexameric proteins. The arthropod hemocyanin gene family includes hemocyanins, cryptocyanins, phenoloxidases, and hexamerins (Terwilliger 1998, Burmester 2001). The four proteins show strong conservation of primary and quaternary structure, but each has unique features that have led to diverse functions. *Cryptocyanin* (pseudohemocyanin), like hemocyanin, is synthesized in the hepatopancreas of crabs and lobsters and circulates in the hemolymph. Its hexamers are so similar to hemocyanin hexamers that early researchers were unaware of its presence, and often it was inadvertently co-purified with hemocyanin from the hemolymph. Its active site has a reduced number of the six histidine residues critical for copper binding, and therefore it lacks copper and has no O_2 transport or oxidase functions (Terwilliger et al. 1999, Burmester 1999). It is produced in high concentrations during premolt and helps form the new exoskeleton (Terwilliger et al. 2005, Kuballa et al. 2007, Terwilliger 2011). The possibility that there are tissue-specific cryptocyanins with unique functions in some species should be studied further. *Phenoloxidase* is a hexameric enzyme synthesized in hemocytes that circulate in the hemolymph of some crustaceans and insects (Aspán et al. 1995, Terwilliger and Towle 2007). The active site of the phenoloxidase subunit contains the two copper atoms and six histidine residues similar to hemocyanin, and therefore it binds O_2 (Lerch and German 1988, Decker and Terwilliger 2000, Terwilliger and Ryan 2006, Cong et al. 2009). Rather than transporting O_2 reversibly, however, phenoloxidase functions in the arthropod immune response (Söderhäll and Cerenius 1998) and participates in crosslinking the new exoskeleton after molting, in exoskeleton repair, and in encapsulating foreign material (Sugumaran 1998). Hemocyanin can be converted to enzymatically active phenoloxidase (see the section "Hemocyanin Function: Phenoloxidase Activity"). *Hexamerins*, the fourth member of the gene family, are extracellular hexameric proteins found in insect hemolymph. A hexamerin subunit, like cryptocyanin, lacks one or more of the six histidines in the highly conserved active site and binds neither copper nor O_2 (Telfer and Massey 1987, Beintema et al. 1994, Pick et al. 2008, Pick and Burmester 2009). Although a number of functions have been demonstrated for different hexamerins, they are frequently referred to as storage proteins.

Hemocyanin Function

Oxygen Binding and Transport

Arthropod hemocyanins demonstrate variable O_2 affinities and high cooperativities. The dramatically wide range of allosteric functional properties is another major consequence of subunit heterogeneity and assembly into multiple oligomers. Hemocyanins have high O_2 transport capacities due to their copious O_2-binding sites and high concentrations in the hemolymph. Numerous biochemical and physiological studies have enhanced our understanding of how crustaceans are able to live

in widely different habitats and obtain sufficient O_2 under potentially stressful conditions (Morris 1990, Truchot 1992, Mangum 1997a, Morris and Airriess 1998, Terwilliger 1998, Bridges 2001, Giomi and Beltramini 2007). Crustacean hemocyanins, like other respiratory proteins, have the ability to adopt different conformations that have different affinities for O_2. The conformational changes are regulated by allosteric effectors, including inorganic ions, such as H^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-, the organic molecules lactate and urate, sulfide and thiosulfate, and neurohormones (Truchot 1980, Morris et al. 1985, Burnett 1992, Truchot 1992, Sanders and Childress 1992, Van Holde and Miller 1995, Hagerman and Vismann 1999). Hemocyanin O_2 affinities are affected by external environmental factors as well, such as temperature and salinity. However, O_2 binding of hemocyanin from crustaceans regularly exposed to highly variable temperatures, including deep-sea hydrothermal vent species and certain shallow-water species, show little or no temperature sensitivity (Hourdez and Lallier 2007).

Conformational changes in the tertiary and quaternary structure of hemocyanins affect both subunit interactions and the active site of the subunit. Most hemocyanins exhibit highly cooperative O_2 binding, and this extraordinary cooperativity often is stronger in the more complex multihexamer structures with higher molecular masses. Cooperativity in the higher aggregation forms, from 2-hexamer to 8-hexamer, has been described as hierarchies of the equilibrium between different types of allosteric units, a nesting model, in which one level, a 1-hexamer for example, is embedded in the next, a 2- or 4-hexamer unit (Robert et al. 1987, Decker and Sterner 1990, Decker et al. 2007a). Although hemocyanins with increased degrees of subunit heterogeneity and oligomers tend to exhibit a broader range of functional properties, this is not always true. Recent work on hemocyanin of *Upogebia pusilla*, a burrowing mud shrimp, has shown that, despite the 4-hexamer structure, the functional properties in this species are based on the hexamer as the allosteric unit without additional interactions between the hexamers (Hellmann et al. 2010). Furthermore, unlike 4-hexamer hemocyanins from several other thalassinid shrimps, the O_2 affinity in this species increases in the presence of L-lactate. The authors suggest that possibly the lactate compensates even at resting conditions for a low intrinsic O_2 affinity in *U. pusilla*. This exception is a reminder that although model organisms can provide critical templates for understanding biological patterns, evolution occurs at the species level. Studies characterizing structure and function of an unusual female-specific hemocyanin in the crab *Scylla olivacea* (Chen et al. 2007) and previously undescribed hemocyanins in the crab *Calappa granulata* (Olianas et al. 2006) and spiny lobster *Palinurus gilchristi* (Olianas et al. 2009) continue to enhance our knowledge base of both typical and unusual systems.

Some of the parameters that affect the binding of O_2 to the copper atoms at the active site and the transition from deoxy Cu(I) to oxy Cu(II)-O_2-Cu(II) include the geometries of the Cu-Cu coordination and access to them by the binding ligands, as well as the distance between the two coppers. The Bohr effect, the well-known sensitivity of O_2 binding in respiratory proteins to fluctuations in pH, permits the proteins to vary their affinities in response to changes in the physiological demands of the organism. An increase in proton concentration generally decreases hemocyanin O_2 affinity and cooperativity (Truchot 1992). Studies on the molecular basis of the effect of hydrogen ions on hemocyanin, comparing three well-characterized hemocyanins from *P. interruptus, C. aestuarii*, and *L. polyphemus*, used flash photolysis and X-ray edge measurements to focus on the coordination geometry of the copper at the active site (Hirota et al. 2008). Findings indicate that the Bohr effect results from changes in the constant k_{on}, the rate of O_2 binding to the protein, and not from changes in the k_{off}. Thus, there is a structural rearrangement in the deoxygenated protein that produces a change in the rate of binding O_2. This structural effect of pH is on the coordination geometry of the individual Cu(I) sites of the binuclear center, which may in turn affect the redox potential of the copper ions, as well as on a change in the Cu-Cu distance, as others have suggested.

In contrast to the Bohr effect, the binding of lactate, a metabolic byproduct of glycolysis under anaerobic conditions, usually increases O_2 affinity and cooperativity of hemocyanin (Truchot 1980, Graham et al. 1983). The lactate effect appears to counterbalance the potential decrease in O_2 affinity caused by the Bohr effect under conditions of acidosis. In the portunid crab *Carcinus maenas*, lactate increases O_2 affinity of hemocyanin by raising the association equilibrium constant of the low-affinity Tense state without affecting that of the high-affinity Relaxed state (Weber et al. 2008). The same study shows the important dual roles for lactate and proton ions in affecting the temperature sensitivity of hemocyanin O_2 binding in crustaceans. The structural basis of the lactate effect on O_2 binding in the closely related *C. estuarii* is a modulation of the global conformation of the protein, but lactate binding does not affect the active site, in contrast to the Bohr effect (Hirota et al. 2010). Based on SAXS measurements, at pH 8.3, L-lactate had little effect, but at pH 6.5, lactate caused a concentration-dependent shift in the interhexameric distances. Changes in quaternary structure of both hexamers and 2-hexamers were affected by lactate binding, supporting the binding of lactate at an intersubunit site rather than an exterior site. The dual functional roles of a circulating 6-hexamer hemocyanin and a tracheal system in Myriapoda have been explored in the huge African diplopod *Archispirostreptus gigas* (Damsgaard et al. 2013). The authors compare the mechanisms of allosteric control, including effects of lactate, Ca^{2+}, and pH on this diplopod hemocyanin with other O_2 transport proteins.

Another study, using L-edge X-ray absorption spectroscopy (XAS) under physiological conditions to detail the changes in the active copper sites in hemocyanin of *H. americanus*, has demonstrated that O_2 binding does not simply switch the copper valence state between Cu I and Cu II (Panzer et al. 2010). Rather, the authors show that, upon deoxygenation, water can replace the di-O_2. When O_2 is added, the water molecules keep the copper atoms partially oxidized. This new finding promises to stimulate further studies on water-O_2-protein functions.

Hemocyanin Function: Phenoloxidase Activity

In addition to binding O_2 reversibly and functioning as O_2 transport molecules, hemocyanins of arthropods and mollusks can be activated to function as phenoloxidases under certain in vitro conditions (Zlateva et al. 1996, Salvato et al. 1998, Decker et al. 2001, Siddiqui et al. 2006, Terwilliger and Ryan 2006, Decker et al. 2007b; and see also the section "Hemocyanin Gene Family"). Phenoloxidases, found in many organisms, are enzymes that catalyze the hydroxylation of monophenols, such as tyrosine, to *o*-diphenols, and their subsequent oxidation to highly reactive *o*-quinones. The quinones are on the pathway to melanin synthesis, a compound critical in the arthropod immune response (Sánchez-Ferrer et al. 1995). "Phenoloxidase" is a generic term that includes tyrosinases, capable of catalyzing both reactions, and catecholoxidases, able to catalyze the second reaction only. The enzymes are responsible for the browning of fruits, coloring of mammalian skin and hair, and wound healing, immune defense, and sclerotization in arthropods. Because melanin has antimicrobial, antifungal, and antiviral properties, phenoloxidases are important components of the innate immune system. In many crustaceans and insects, phenoloxidases occur as prophenoloxidases in circulating hemocytes, and their activation is regulated by a tight system of enzymatic reactions (the prophenoloxidase cascade; Ashida and Yamazaki 1990, Söderhäll and Cerenius 1998). Hemocyanin is closely related to phenoloxidase phylogenetically, and the two share the binuclear copper active site structure of type 3 copper proteins. That hemocyanin can function as both an O_2 transporter and phenoloxidase raises intriguing questions.

First, dramatic evidence for the structural mechanism of activation of arthropod hemocyanin and phenoloxidase has been presented by Decker and colleagues (Cong et al. 2009). Sodium dodecyl sulfate (SDS) has been commonly used in lieu of natural activators to convert prophenoloxidase

and hemocyanin into enzymatically active phenoloxidases and measure phenoloxidase activity, but how SDS worked was unknown. Analysis of electron cryomicroscopy and pseudoatomic models of the 4-hexameric hemocyanin from the scorpion *P. imperator* in resting and SDS-activated states validates an activation model proposed earlier (Decker and Tuczek 2000). At the individual subunit level, SDS induces a conformational change in which the flexible domain I twists away from domains II and III, exposing the entrance to the two coppers in the subunit's active site and allowing access by bulky phenol or diphenol substrates (see the section "Hemocyanin Structure"). The shape change in individual subunits results in a cooperative structural rearrangement of the entire 4-hexamer oligomer. SDS, instead of acting as a denaturant, functions as an allosteric modulator of the hemocyanin megamolecule. The results provide a new example of how arthropod hemocyanin hexamers interact and behave cooperatively during oxygenation or phenoloxidase activation. Because the active site of type 3 copper proteins is well conserved, these results may explain the mechanism of action of other systems as well.

Second, the relative contributions of hemocyte phenoloxidase versus activated hemocyanin have been investigated in a number of crustaceans (Decker et al. 2001, Terwilliger and Ryan 2006, García-Carreno et al. 2008, Nillius et al. 2008, Jaenicke et al. 2009). At the physiological ratio at which they occur in the hemolymph, hemocyte phenoloxidase in the crab *C. magister* has high activity, but hemocyanin is present in a much higher concentration (Terwilliger and Ryan 2006). Hemocyte phenoloxidase is able to catalyze both reactions—the conversion of monophenols to diphenols and the oxidation to *o*-quinones—but the activated hemocyanin of a chelicerate or crustacean often functions only as a catecholase, depending on the species and/or experimental conditions (Decker et al. 2001, Nillius et al. 2008).

A third important question is whether hemocyanins function as phenoloxidases in vivo. Two groups of arthropods, chelicerates and peracarid crustaceans, have the same needs for immune defense and hardening of the exoskeleton after molting as do other arthropods. Surprisingly, species of horseshoe crabs, tarantulas, isopods, and amphipods examined to date lack a hemocyte phenoloxidase, but their hemocyanins do function as phenoloxidases in vitro (Pless et al. 2003, Arellano and Terwilliger 2004, Terwilliger 2007, Jaenicke et al. 2009). Anionic phospholipids such as phosphatidylserine, as well as coagulation factors and antimicrobial peptides, have been implicated as natural activators of horseshoe crab hemocyanin in vivo (Nagai et al. 2001, Coates et al. 2011). Hemocyanins in chelicerates and peracarid crustaceans, then, must be able to switch back and forth between O_2 transport and phenoloxidase, although there is no information on how the two functions are regulated or how circulating hemocyanin molecules are allocated to a particular function. Whether all chelicerates and peracarid crustaceans, including the tanaids, cumaceans, and mysids, share a reliance on hemocyanin as the sole source of phenoloxidase activity remains to be explored.

In those eucaridan malacostracans expressing both hemocyte phenoloxidase and hemocyanin, regulation of hemocyanin function in vivo between reversible O_2 transport and oxidative enzyme also needs to be addressed (Terwilliger and Ryan 2006, Cerenius et al. 2008). It has been reported that crab hemocyanin may be activated into a functional phenoloxidase by hemocyte components (Adachi et al. 2003, Fan et al. 2009). Perhaps hemocyte phenoloxidases of crabs, shrimps, and lobsters function as rapid responders to microbial or viral challenge. Hemocyanin may be activated to phenoloxidase activity only under extreme stress, as in prolonged hypoxia when hemocyte phenoloxidase activity is suppressed (Tanner et al. 2006) or immediately postmolt when there is a need for concerted, global sclerotization (Terwilliger 2007). Support for this hypothesis comes from work on the crayfish *Cherax quadricarinatus*. Hemocyanin with phenoloxidase activity has been localized in the chitin matrix of the crayfish gastrolith, a transient calcium deposit secreted similarly to the crayfish exoskeleton by an epithelium continuous with that forming the exoskeleton (Glazer et al. 2013). Hemocyanin transcript expression was specific

to the hepatopancreas during premolt, consistent with other studies on hemocyanin synthesis in crayfish and crabs. The authors suggest the hemocyanin may indeed be functioning as a sclerotizing agent in hardening the chitinous layers of the gastrolith as calcium is deposited during premolt. Problems resulting from a crustacean immune response with overactive or unregulated hemocyanin and/or hemocyte phenoloxidase activity are manifested in the extreme melanization of lobster and crab shells in response to bacterial infection, "black shell disease," as well as postmortem darkening of whiteleg shrimp *Penaeus vannamei*, both of which reduce marketability (Vogan et al. 2002, Terwilliger 2007, García-Carreno et al. 2008). Although uncommon, albino *C. magister* and *H. americanus* do occur, and these may be due in part to alterations in the pathway of melanin synthesis (Protas et al. 2011). In vertebrates, defects in melanin regulation range from albinism to melanomas. Contamination of the marine environment with alkylphenols that competitively interact with the phenoloxidase-dependent incorporation of tyrosine into the exoskeleton may exacerbate problems associated with crustacean molting, metamorphosis, and survival (Laufer et al. 2005) and provide added impetus for understanding the balance between O_2 transport and phenoloxidase activity in hemocyanin.

Additional Functions of Hemocyanin

Circulating hemocyanin oligomers may function as specific or nonspecific carrier proteins for a variety of molecules in a role similar to that played by serum albumin in vertebrate blood (Carter and Ho 1994, Terwilliger 2011). High hemolymph concentrations and approximately 75 Kda subunits of hemocyanin, as well as cryptocyanin and insect hexamerins, potentially offer multiple binding sites for steroids, peptide hormones, fatty acids, and other ligands. The steroid hormone ecdysone is bound with low affinity by hemocyanin from the tarantula *E. californica* (Jaenicke et al. 1999b), similar to the ecdysone binding affinity seen in some insect hexamerins (Enderle et al. 1983). Furthermore, antimicrobial, antifungal, and antiviral properties have been reported for several arthropod hemocyanins and their peptides (Bachere 2000, Destoumieux-Garzon et al. 2001, Lee et al. 2003, Lei et al. 2008). Because the first and third domains of the hemocyanin subunit, rather than the copper-containing second domain, appear to be responsible for these protective functions, cryptocyanin might also have these immune functions. In the shrimp *Litopenaeus vannamei*, hemocyanin showed hemolytic activity (Zhang et al. 2009), and in the shrimp *Penaeus japonicus*, differences in antiviral activity among its hemocyanin subunits have been reported (Lei et al. 2008), which offers more evidence for the adaptive advantage of subunit heterogeneity. Other functions proposed for hemocyanins include cation transport and exoskeleton formation (Jaenicke et al. 1999b, Jaenicke and Decker 2004, Terwilliger 2011).

Hemocyanin Synthesis and Regulation

Site of Synthesis

Hemocyanin subunits of brachyuran crabs are synthesized in R cells of the hepatopancreas, as shown by in situ hybridization, Northern blots, and immunohistology of the crab *C. magister* (Terwilliger et al. 1999, 2005, Terwilliger 2011). Cryptocyanin is also synthesized in the hepatopancreas R cells. Both proteins are secreted basally into the blood vessels surrounding the hepatopancreas tubules, consistent with the ultrastructure of the hepatopancreas R cells in *Penaeus semisulcatus* (Al-Mohanna and Nott 1989). Self-assembly of subunits into hexameric quaternary structures probably occurs either in the hepatopancreas cells prior to secretion or in the hemolymph immediately after secretion. Monomers of each protein have

not been identified in the circulating hemolymph. Earlier studies had proposed that reserve cells (Cuenot 1893, Johnson 1980) in the connective tissue were the site of hemocyanin synthesis, based in part on electron micrographs of large crystalline inclusions in the cells (Ghiretti-Magaldi et al. 1973, 1977). Our studies showed, however, that reserve cells express neither hemocyanin nor cryptocyanin mRNA. The reserve cells accumulate and increase in size in the connective tissue during premolt. They become strongly immunoreactive against cryptocyanin-specific monoclonal antibodies immediately prior to ecdysis and then decrease in size and disappear in early postmolt. Instead of synthesizing hemocyanin or cryptocyanin, it appears that reserve cells endocytose and metabolize excess cryptocyanin from the hemolymph at ecdysis. Hemocyanin synthesis has been reported to take place in the hepatopancreas of the shrimps *Penaeus monodon* and *P. japonicus* (Lehnert and Johnson 2001, Lei et al. 2008), and both hemocyanin and cryptocyanin mRNA have been amplified in the hepatopancreas of many brachyuran and anomuran crabs and lobsters (Terwilliger and Ryan, unpublished). We and others have also found hemocyanin mRNA in the hepatopancreas of the cheliceran horseshoe crab *L. polyphemus*. Cyanocytes of *L. polyphemus*, like the reserve cells of crabs, probably are also involved in hemocyanin catabolism and not its synthesis, contrary to previous interpretation (Fahrenbach 1970).

Phenotypic Plasticity: Regulation of Hemocyanin Subunit Expression

Hemocyanin subunit heterogeneity is responsive to developmental trajectories and environmental challenges. Ontogenic regulation of expression of the six genes coding for the six hemocyanin subunits of the crab *C. magister* occurs during development from megalopa to adult (Terwilliger and Brown 1993, Terwilliger and Ryan 2001, Terwilliger et al. 2006). Hemocyanin hexamers circulating in the early megalopa include three different subunits, whereas the 2-hexamer hemocyanins include the same three plus a linker subunit. Shortly after metamorphosis from megalopa to juvenile crab, a fifth unique subunit (Hc subunit 4) appears in each oligomer, and, after several more juvenile crab molts, the sixth subunit type is present. These structural changes in expression of hemocyanin subunits are accompanied by increases in intrinsic O_2 affinity (Brown and Terwilliger 1992, 1998). In synchrony with these shifts is an increased capacity for ion regulation, especially Mg^{2+}, an ion that allosterically raises the hemocyanin O_2 affinity. Developmentally correlated decreases of hemolymph Mg^{2+} to adult levels counterbalance the increased intrinsic O_2 affinity of the hemocyanin, effectively maintaining a constant O_2 affinity of whole hemolymph from megalopa to adult. Ontogenic changes in hemocyanin subunits and changes in hexamer/2-hexamer ratios have also been reported in the American lobster *H. americanus* (Olson 1991).

Changes in expression of specific hemocyanin subunits, with a concomitant shift in O_2 affinity, occur in response to nondevelopmental, external stimuli as well and have been studied at the protein level. In the amphipod *Chaetogammarus marinus*, an increase in one and maybe more of the eight hemocyanin subunits in response to environmental salinity caused an increase in O_2 affinity (Spicer and Hodgson 2003). Hemocyanin of the blue crab *Callinectes sapidus* showed a decrease in three of its six subunit types and a resulting increase in the ratio of 1-hexamers to 2-hexamers after exposure to environmental hypoxia (deFur et al. 1990, Mangum et al. 1991, Mangum 1997b). The 1-hexamers had a higher O_2 affinity and lower cooperativity than did the 2-hexamers. Molecular studies on *C. sapidus* have confirmed the effect of environmental hypoxia as a stimulus of hemocyanin subunit expression (Brouwer et al. 2004, Brown-Peterson et al. 2005). Environmental temperature may be another effector of hemocyanin subunit expression, as evidenced by a shift in expression of the linker subunit in hemocyanin of the crayfish *Astacus leptodactylus* (Decker and

Foll 2000). Animals acclimatized to higher temperatures showed a decrease in both linker subunit and 2-hexamer hemocyanin. When juvenile *C. magister* were raised in varying regimes of temperature and food level, those raised in warm water had higher 1-hexamer hemocyanin concentrations than did those in cold water (Terwilliger and Dumler 2001). Levels of the 2-hexamer hemocyanin were relatively unresponsive to differences in food and temperature. The instar timing of the developmental switch from juvenile to adult hemocyanin subunit composition in *C. magister* was altered by both nutritional state and water temperature, indicating that ontogeny of gene expression can be altered by environmental conditions. External parameters clearly can affect hemocyanin subunit expression, even though identifying the precise stimulus or combination of factors—O_2 level, pH, organic or inorganic ions, temperature—can be difficult. The epigenetic mechanisms of phenotypic plasticity in crustacean hemocyanins are unknown, although in *Drosophila* (another arthropod), piRNAs have been shown to shut down genes, and modifier proteins add methyl groups to histones (Huang et al. 2013, Saey 2013). Whether environmentally induced epigenetic changes in hemocyanin expression can be transgenerationally inherited is also unknown. Global climate change and ocean acidification will prove challenging to crustaceans trying to obtain O_2 and maintain sufficient calcium in their exoskeletons. Both genetic and epigenetic processes may participate in the evolution of hemocyanin structure and function.

Hemocyanin Concentration

Many measurements of changes in total hemocyanin concentration have been reported (Giomi and Beltramini 2007). Major determinants of hemocyanin concentration in the hemolymph are molt stage and nutrition, and both factors must be accommodated into the experimental design for meaningful interpretation of results. In addition, valid concentration measurements must distinguish between hemocyanin and cryptocyanin hexamers. During the molt cycle, circulating levels of hemocyanin and cryptocyanin vary greatly from a gradual increase in premolt to their highest levels just before ecdysis, followed by almost undetectable levels immediately after ecdysis in both juvenile and adult crabs (Terwilliger et al. 2005, Terwilliger 2011). In the adult crab, hemolymph cryptocyanin disappears after ecdysis until the following premolt, months later, whereas hemocyanin levels resume almost immediately after molting. These changes in hemocyanin and cryptocyanin expression as a function of the molt cycle have been confirmed at the subunit level in *C. magister* by M.R. Phillips using a cDNA microarray and characterizing global transcription patterns (Phillips 2007, Stillman et al. 2008), as have gene expression studies in *Portunus pelagicus* (Kuballa et al. 2011). The potent influence of the molt cycle on hemocyanin concentration is widespread across crustaceans, including krill *Meganyctiphanes norvegica* (Spicer and Stromberg 2002). Adaptive changes in hemocyanin levels are usually attributed toward maintaining O_2 transport needs. In this context, the sudden drop in hemocyanin levels just before ecdysis seems counterproductive. Perhaps some of the high premolt levels of hemocyanin are destined for epidermal sequestration. There, the hemocyanin molecules could be mobilized to provide phenoloxidase-related functions for the new postmolt exoskeleton. Localization of a prophenoloxidase-activating enzyme in the epidermis of the blue crab *C. sapidus* (Buda and Shafer 2005) is consistent with this provocative model, as is the discovery of a phenoloxidase-active hemocyanin in the crayfish gastrolith (Glazer et al. 2013, see the section "Hemocyanin Function"). Alternatively, the decrease in synthesis and concentration of hemocyanin at ecdysis might protect against excess O_2 uptake and reactive O_2 species production, since O_2 could more readily diffuse across the unsclerotized new exoskeleton. In addition to molt stage and food availability, other factors invoked as modifying hemocyanin concentration include gender, collecting site, hypoxic exposure, low salinity, and temperature (Giomi and Beltramini 2007).

CRUSTACEAN OXYGEN TRANSPORT PROTEINS: HEMOGLOBINS

Hemoglobin Distribution

Hemoglobin expression is limited in the Arthropoda. Neither Chelicerata nor Myriapoda are known to express circulating hemoglobin. Hemoglobins are present in Hexapoda, but they occur mostly in noncirculating cells and rarely as O_2 transporters (Burmester and Hankeln 2007). In the Crustacea, hemoglobins are extensively distributed in Branchiopoda, and they are present in Copepoda, Ostracoda, some parasitic Cirrepedia, and several Malacostraca (Terwilliger 1992, 1998, Hourdez et al. 2000, Weber and Vinogradov 2001, Zeis et al. 2003).

The predominance of hemocyanins in Malacostraca versus hemoglobins in Branchiopoda has long intrigued biologists. A resurgence of interest in crustacean hemoglobins since 2001, especially with publication of the draft genome of *Daphnia pulex* and related studies (Colbourne et al. 2011), warrants a full review. Here, space allows only a summary of some of the more tantalizing findings. Like hemocyanins, crustacean hemoglobins that function in O_2 transport are large, multi-subunit molecules that circulate in the hemolymph. No crustacean erythrocytes have been described. The hemoglobin complexes exhibit much variability in O_2 affinities, cooperativities, Bohr effects, and responses to allosteric modulators including divalent cations. The only Cirripedia with a significant amount of circulating O_2 transport protein are the parasitic rhizocephalan barnacles, primarily *Briarosaccus callosus*, whose host is the king crab (Shirley et al. 1986, Terwilliger et al. 1986). Both the externa and interna of this large rhizocephalan are filled with a bright red hemoglobin. The giant hemoglobin molecules circulate between the externa that protrudes outward from the ventral thoracic-abdominal junction of the crab, and the interna, whose thin-walled rootlets are bathed in crab hemocyanin inside the crab (Terwilliger 1998). Hemoglobin is also present in smaller rhizocephalans *Peltogaster pagurus* and *Peltogasterella gracilis*, whose host, the hermit crab *Pagurus samuelis*, like the king crab, has a circulating hemocyanin (Torchin 1994). Oxygen affinities of the rhizocephalan hemoglobins are higher than those of their hosts' hemocyanins, indicating an O_2 transfer system from host to parasite that ensures an ample supply of O_2 for the rhizocephalan embryos developing in the externa.

Hemoglobin Phenotypic Plasticity, Hypoxia, and HIF

Crustacean hemoglobins have been studied most thoroughly in the Branchiopoda, one of the classes touted as a potential sister group to the Hexapoda, along with Remipedia and Malacostraca. Branchiopods and their hemoglobins are models for phenotypic responses to abiotic environmental fluctuations, especially hypoxia (Kimura et al. 1999, Guadagnoli et al. 2005, Zeis et al. 2009), and, in *Daphnia*, the rise and fall of hemoglobin levels in response to O_2 availability has been shown to be hypoxia-inducible factor (HIF) dependent (Gorr et al. 2004, Hoogewijs et al. 2007). Genomic analysis of the 11 hemoglobin genes in *D. pulex* and seven in *D. magna* teases apart the tandem gene duplication and subsequent differentiation, in concert with proteome analysis of the acclimatory responses of *D. pulex* to environmental changes (Colbourne et al. 2011). Demonstration of the eco-responsiveness of these magnificently coordinated multi-subunit hemoglobins and other expanded gene families in *Daphnia* promise to fuel more comparative physiological genomics studies in Crustacea and enhance our comprehension of how and why subunit heterogeneity and phenotypic plasticity are maintained.

CO-EXPRESSION OF HEMOCYANIN AND HEMOGLOBIN

The majority of crustaceans express either hemocyanin or hemoglobin, but there are exceptions. First, although all Branchiopods have an extracellular hemoglobin, *Artemia franciscana* and *Triops longicaudatus* (and probably others) also express phenoloxidase, evidence they have

retained the hemocyanin gene family in addition to hemoglobin (Terwilliger and Ryan 2006). Second, the cyamid amphipod *Cyamus scammoni* (Malacostraca, subclass Eumalacostraca, Peracarida), commensal on the gray whale, has both circulating hemoglobin and hemocyanin (Terwilliger 1991, 2008b). This is the first example of hemoglobin in a Malacostraca. Most amphipods have only hemocyanin. Similar to other peracaridans, the "whale rider" lacks a separate hemocyte prophenoloxidase, so the hemocyanin probably also functions as a phenoloxidase. Third, a membrane-bound hemoglobin has been described in the gills of the green crab *C. maenas* (Ertas et al. 2011). Just like other brachyuran crabs, *C. maenas* has 1- and 2-hexamer hemocyanin circulating in its hemolymph. The hemoglobin does not function in O_2 transport, but the authors propose that it may protect cell membrane lipids from reactive O_2 species, similar to bacterial membrane-bound hemoglobins. Phylogenetic analysis indicates that the *C. maenas* membrane-bound hemoglobin does not cluster with the extracellular circulating hemoglobins of the Branchiopoda, insect globins, or expression sequence tagged (EST) tick (Chelicerata) globins. Its location in the gills may be analogous to hemoglobins in insect tracheal cells. This surprising finding should stimulate further database searches and experiments that may reveal the widespread presence of similar membrane-bound hemoglobins among the crustaceans, insects, and chelicerates and lead to functional analyses. Today, multiple functions in addition to O_2 transport are recognized for both hemoglobins and hemocyanins (Decker and van Holde 2011). Genes for both families of O_2-binding proteins in one species are probably present throughout the Arthropoda, including the crustaceans, and will be identified along with potential expression patterns in synchrony with the development of emerging technologies.

FUTURE DIRECTIONS

New insights about the phylogenetic relatedness of hemocyanin between arthropods and mollusks stem from recognition of structural similarities among an arthropod hemocyanin subunit (*Limulus* hemocyanin subunit II), an unusual C-terminal extension of one functional unit of a molluskan hemocyanin, KLH-h (*Megathura crenulata*, keyhole limpet), and a type 1 copper protein from a cucumber (Jaenicke et al. 2010). The cucumber basic protein (CBC) has one copper-binding site and is a member of the cupredoxin family, a phytocyanin or plantacyanin (Guss et al. 1996). Another cupredoxin, the bacterial CopC protein, has two monocopper-binding sites (Arnesano et al. 2002) and is structurally correlated with domain 3 of arthropod hemocyanin. Jaenicke and colleagues propose that both arthropod and molluskan hemocyanins evolved from ancestral tyrosinases, each with two copper-binding sites, to which the cupredoxin-like domains were added to enhance copper loading onto the active sites (Fig. 11.7). The ancestral tryosinases were considered to have evolved from a single last common ancestor. An additional domain (domain 1 in arthropod hemocyanin) was added for specificity of O_2 binding. As the two hemocyanins continued to evolve in the two phyla, acquiring multiple subunits and functional properties, the cupredoxin-like domain acquired new functions and lost its monocopper binding site. Each arthropod hemocyanin subunit still maintains the cupredoxin-like domain 3, whereas the molluskan hemocyanin subunit adds it only to the final functional unit of the long subunit. This co-evolutionary pattern of addition and subsequent functional loss of a copper-loading domain is remarkable because it is based on the concept of the two hemocyanins evolving congruently from ancestral tyrosinases that had themselves evolved from a last common ancestor. The model is consistent with the patterns of gene duplication and preservation by entrainment that describe the *Daphnia* genome (Colbourne et al. 2011) and the concept of protein evolution by domain rearrangements, the emergence of new domains, and the loss of old ones (Moore and Bornberg-Bauer 2012).

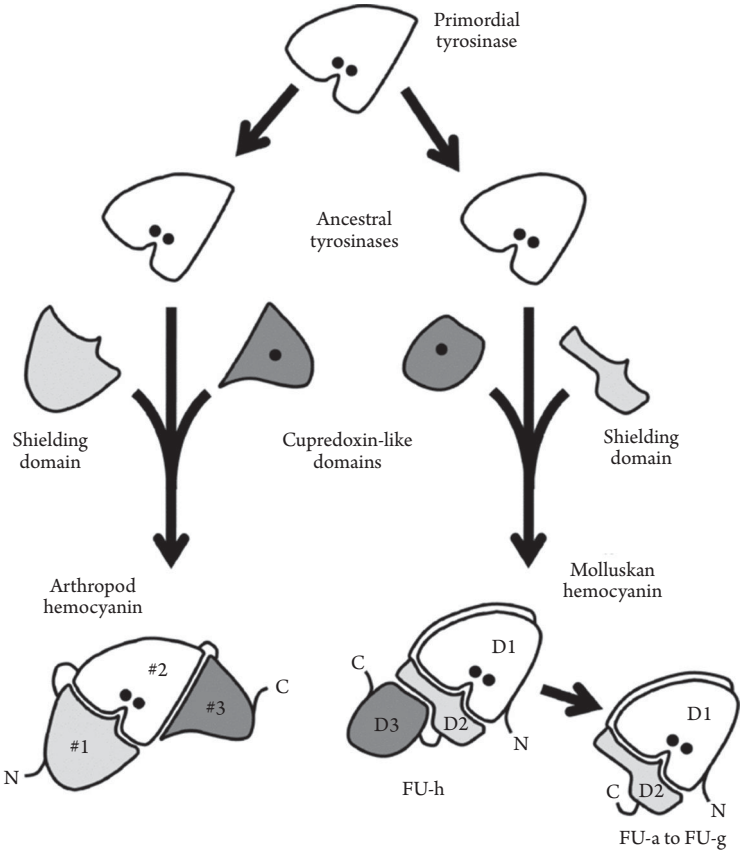


Fig. 11.7.
Tracing the evolution of arthropod and molluskan hemocyanins from their putative common ancestor, a primordial tyrosinase. The oxygen-binding domain (white) of arthropod and molluskan hemocyanin presumably evolved independently from ancestral tyrosinases. To enhance efficient copper loading to the active site in the oxygen-binding domain, a cupredoxin-like domain (dark gray) with an additional monocopper center was added. A high specificity for oxygen was established by an additional domain (light gray), which shields the binuclear copper site from regular tyrosinase substrates but still allows entry and exit of oxygen molecules. Slight tilting movements of this shielding domain, which opens and closes the narrow oxygen diffusion channel, added allosteric regulation. The cupredoxin-like domain eventually assumed new functions in assembly and allosterism of the hemocyanin multimers, and its monocopper center was lost. Today, the cupredoxin-like domain is still ubiquitous in the 75 kDa arthropod hemocyanin subunits, whereas in molluskan hemocyanin it is restricted to FU-h, the tail of the 400 kDa subunit. Black dots, copper-binding units. Reproduced from Jaenicke et al. (2010), with permission from The Biochemical Society.

Integrating the new tools of contemporary biology, genomics, proteomics, metabolomics, microarrays, and modern technologies with ecologically relevant studies that address multiple environmental factors promises a treasure box full of potential answers about how animals adapt and evolve, as the *Daphnia* genome study has revealed. We anticipate similar studies in more crustaceans, recognizing that relying on only a few model organisms in the past has been both beneficial and limiting. Explanations about regulation of these processes are tantalizingly close as well. Regulation of phenotypic plasticity in crustacean hemoglobin expression by HIF has been described, and we are moving toward a better understanding of a regulatory role of HIF in hemocyanin expression and

function (Li and Brouwer 2007, Hoogewijs et al. 2007, Terwilliger 2008a, Soñanez-Organis et al. 2009, Gorr et al. 2010).

CONCLUSIONS

Oxygen transport proteins assist in the challenge of obtaining sufficient O_2, especially in an arthropod whose body, including the gills, is covered by a chitinous exoskeleton. Hemocyanins and hemoglobins, specialized to bind O_2 in regions of high concentration and release it in regions of lower concentration, circulate in the hemolymph of crustaceans and provide a molecular means to sustain the metabolic needs of the organism. In Crustacea, both the copper-based hemocyanin and the iron-heme-based hemoglobin are large extracellular molecules composed of individual polypeptide chains that self-assemble into species-specific aggregates. Creative studies using technological advances have enhanced our understanding of key structural features in both the assembly and function of these molecules. High degrees of subunit heterogeneity in the two types of O_2 transport proteins provide opportunities for highly cooperative O_2 binding, a wide range of O_2 affinities, and phenotypic plasticity. New models have resulted from studies that integrate conformational changes in structure with functional properties. Other roles for hemocyanin and hemoglobin are surfacing as well.

Although crustacean O_2 transport proteins provide excellent examples of gene duplication and diversification, we await precise descriptions of how the functional expression of multiple hemocyanin or hemoglobin genes is coordinated during the molt cycle, in response to temporary "dead zones" of hypoxic waters, or under immune challenge. As we search to understand the roles of phenotypic plasticity, as well as the initiation of novel phenotypes in response to issues such as climate change, ocean acidification, global nutritional needs, and health issues, hemocyanin and hemoglobin will continue to provide experimental model systems.

ACKNOWLEDGMENTS

I acknowledge Charlotte Mangum, Steve Morris, Bob Terwilliger, and David Towle for their inspiration and appreciation of balancing form and function, molecule and organism. I am grateful to students and colleagues around the world who have contributed to these studies on invertebrate hemocyanins and hemoglobins through stimulating discussions, collaborative research, and valued friendships. I thank the editors, Ernie Chang and Martin Thiel, for their invitation to participate in this project.

REFERENCES

Adachi, K., T. Hirata, T. Nishioka, and M. Sakaguchi. 2003. Hemocyte components in crustaceans convert hemocyanin into a phenoloxidase-like enzyme. Comparative Biochemistry and Physiology B 134:135–141.

Al-Mohanna, S.Y., and J.A. Nott. 1989. Functional cytology of the hepatopancreas of *Penaeus semisulcatus* (Crustacea: Decapoda) during the moult cycle. Marine Biology 101:535–544.

Arellano, S., and N. Terwilliger. 2004. Hemocyanin, cryptocyanin and phenoloxidase in deep sea (*Bathynomus giganteus*) and intertidal (*Cirolana harfordi*) isopods. American Zoologist 43:961A.

Arnesano, F., L. Banci, I. Bertini, and A. Thompsett. 2002. Solution structure of CopC: a cupredoxin-like protein involved in copper homeostasis. Structure 10:1337–1347.

Ashida, M., and H.I. Yamazaki. 1990. Biochemistry of the phenoloxidase system in insects: with special reference to its activation. Pages 239–265 *in* E. Ohnishi and H. Ishizaki, editors. Molting and metamorphosis. Springer-Verlag, Berlin.

Aspán, A., T.-S. Huang, L. Cerenius, and K. Söderhäll. 1995. cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation. Proceedings of the National Academy of Sciences, USA 92:939–943.

Bachere, E. 2000. Penaeidins, antimicrobial peptides of shrimp: a comparison with other effectors of innate immunity. Aquaculture 191:71–88.

Bailly, X., S. Vanin, C. Chabasse, K. Mizuguchi, and S. Vinogradov. 2008. A phylogenomic profile of hemerythrins, the nonheme diiron binding respiratory proteins. BMC Evolutionary Biology 8:244.

Beintema, J.J., W.T. Stam, B. Hazes, and M.P. Smidt. 1994. Evolution of arthropod hemocyanins and insect storage proteins (hexamerins). Molecular Biology Evolution 11:493–503.

Bijlholt, M., and E. van Bruggen. 1986. A model for the architecture of the hemocyanin from the arthropod *Squilla mantis* (Crustacea, Stomatopoda). European Journal of Biochemistry 155:339–344.

Bridges, C. 2001. Modulation of haemocyanin oxygen affinity: properties and physiological implications in a changing world. Journal of Experimental Biology 204:1021–1032.

Brouwer, M., P. Larkin, N. Brown-Peterson, C. King, S. Manning, and N. Denslow. 2004. Effects of hypoxia on gene and protein expression in the blue crab, *Callinectes sapidus*. Marine Environmental Research 58:787–792.

Brown, A.C., and N.B. Terwilliger. 1992. Developmental changes in ionic and osmotic regulation in the Dungeness crab, *Cancer magister*. Biological Bulletin 182:270–277.

Brown, A.C., and N.B. Terwilliger. 1998. Ontogeny of hemocyanin function in the Dungeness crab *Cancer magister*: hemolymph modulation of hemocyanin oxygen-binding. Journal of Experimental Biology. 201:819–826.

Brown-Peterson, N.J., P. Larkin, N. Denslow, C. King, S. Manning, and M. Brouwer. 2005. Molecular indicators of hypoxia in the blue crab Callinectes sapidus. Marine Ecology Progress Series 286:203–215.

Brunori, M., and B. Vallone. 2007. Neuroglobin, seven years after. Cellular and Molecular Life Sciences 64:1259–1268.

Buda, E.S., and T.H. Shafer. 2005. Expression of a serine proteinase homolog prophenoloxidase-activating factor from the blue crab, *Callinectes sapidus*. Comparative Biochemistry and Physiology B 140:521–673.

Burmester, T. 1999. Identification, molecular cloning, and phylogenetic analysis of a non-respiratory pseudo-hemocyanin of *Homarus americanus*. The Journal of Biological Chemistry 274:13217–13222.

Burmester, T. 2001. Molecular evolution of the arthropod hemocyanin superfamily. Molecular Biology and Evolution 18:184–195.

Burmester, T. 2002. Origin and evolution of arthropod hemocyanins and related proteins. Journal of Comparative Physiology B 172:95–107.

Burmester, T., and T. Hankeln. 2007. The respiratory proteins of insects. Journal of Insect Physiology 53:285–294.

Burnett, L.E. 1992. Integrated function of the respiratory pigment hemocyanin in crabs. American Zoologist 32:438–446.

Carter, D., and J. Ho. 1994. Structure of serum albumin. Pages 153–196 *in* V. Schumaker, editor. Advances in protein chemistry. Academic Press, San Diego.

Cerenius, L., B. Lee, and K. Soderhall. 2008. The proPO-system: pros and cons for its role in invertebrate immunity. Trends in Immunology 29:263–271.

Chen, H., S. Ho, T. Chen, K. Soong, I. Chen, and J. Cheng. 2007. Identification of a female-specific hemocyanin in the mud crab, *Scylla olivacea* (Crustacea: Portunidea). Zoological Studies 46:194–202.

Coates, C., S. Kelly, and J. Nairn. 2011. Possible role of phosphatidylserine-hemocyanin interaction in the innate immune response of *Limulus polyphemus.* Developmental and Comparative Immunology 35:155–163.

Colbourne, J., M.E. Pfrender, D. Gilbert, W.K. Thomas, A. Tucker, T.H. Oakley, S. Tokishita, A. Aerts, G.J. Arnold, M.K. Basu, D.J. Bauer, C.E. Cáceres, L. Carmel, C. Casola, J.-H. Choi, J.C. Detter, Q. Dong, S. Dusheyko, B.D. Eads, T. Fröhlich, K.A. Geiler-Samerotte, D. Gerlach, P. Hatcher, S. Jogdeo, J. Krijgsveld, E.V. Kriventseva, D. Kültz, C. Laforsch, E. Lindquist, J. Lopez, J.R. Manak, J. Muller, J.

Pangilinan, R.P. Patwardhan, S. Pitluck, E.J. Pritham, A. Rechtsteiner, M. Rho, I.B. Rogozin, O. Sakarya, A. Salamov, S. Schaack, H. Shapiro, Y. Shiga, C. Skalitzky, Z. Smith, A. Souvorov, W. Sung, Z. Tang, D. Tsuchiya, H. Tu, H. Vos, M. Wang, Y.I. Wolf, H. Yamagata, T. Yamada, Y. Ye, J.R. Shaw, J. Andrews, T.J. Crease, H. Tang, S.M. Lucas, H.M. Robertson, P. Bork, E.V. Koonin, E.M. Zdobnov, I.V. Grigoriev, M. Lynch, and J.L. Boore. 2011. The ecoresponsive genome of *Daphnia pulex*. Science 331:555–561.

Cong, Y., Q. Zhang, D. Woolford, T. Schweikardt, H. Khant, M. Dougherty, S. Ludtke, W. Chiu, and H. Decker. 2009. Structural mechanism of SDS-induced enzyme activity of scorpion hemocyanin revealed by electron cryomicroscopy. Structure 17:749–758.

Cuenot, L. 1893. Etudes physiologiques sur les Crustaces Decapodes. Archives de Biologie Lieges 13:245–303.

Cuff, M.E., K.I. Miller, K.E. van Holde, and W.A. Hendrickson. 1998. Crystal structure of a functional unit from *Octopus* hemocyanin. Journal of Molecular Biology 278:855–870.

Damsgaard, C., A. Fago, S. Hagner-Holler, H. Malte, T. Burmester, and R.E. Weber. 2013. Molecular and functional characterization of hemocyanin of the giant African millipede, *Archispirostreptus gigas*. Journal of Experimental Biology 216:1616–1623.

Decker, H., and R. Foll. 2000. Temperature adaptation influences the aggregation state of hemocyanin from *Astacus leptodactylus*. Comparative Biochemistry and Physiology A 127:147–154.

Decker, H., and R. Sterner. 1990. Nested allostery of arthropodan hemocyanin (*Eurypelma californicum* and *Homarus americanus*). The role of protons. Journal of Molecular Biology 211:281–293.

Decker, H., and N. Terwilliger. 2000. COPs and robbers: putative evolution of copper oxygen binding proteins. Journal of Experimental Biology 203:1777–1782.

Decker, H., and F. Tuczek. 2000. Tyrosinase/catecholoxidase activity of hemocyanins: structural basis and molecular mechanism. Trends in Biochemical Sciences 25:392–397.

Decker, H., and K. van Holde. 2011. Oxygen and the evolution of life. Springer-Verlag Berlin Heidelberg.

Decker, H., M. Ryan, E. Jaenecke, and N. Terwilliger. 2001. SDS-induced phenoloxidase activity of hemocyanins from *Limulus polyphemus, Eurypelma californicum*, and *Cancer magister*. Journal of Biological Chemistry 276:17796–17799.

Decker, H., N. Hellmann, E. Jaenicke, B. Lieb, U. Meissner, and J. Markl. 2007a. Recent progress in hemocyanin research. Integrative and Comparative Biology 47:631–644.

Decker, H., T. Schweikardt, D. Nillius, U. Salzbrunn, E. Jaenicke, and F. Tuczek. 2007b. Similar enzyme activation and catalysis in hemocyanins and tyrosinases. Gene 398:183–191.

deFur, P., C. Mangum, and J. Reese. 1990. Respiratory responses of the blue crab *Callinectes sapidus* to long-term hypoxia. Biological Bulletin 178:46–54.

Destoumieux-Garzon, D., D. Saulnier, J. Garniert, C. Jouffrey, P. Bulet, and E. Bachere. 2001. Crustacean immunity. Antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challenge. The Journal of Biological Chemistry 276:47070–47077.

Dunn, C., A. Hejnol, D. Matus, K. Pang, W. Browne, S. Smith, E. Seaver, G. Rouse, M. Obst, G. Edgecombe, M. Sorensen, S. Haddock, A. Schmidt-Rhaesa, A. Okusu, R. Kristensen, W. Wheeler, M. Martindale, and G. Giribet. 2008. Broad taxon sampling improves resolution of the Animal Tree of Life. Nature 452:745–749.

Durstewitz, G., and N.B. Terwilliger. 1997. cDNA cloning of a developmentally regulated hemocyanin subunit in the crustacean *Cancer magister* and phylogenetic analysis of the hemocyanin gene family. Molecular Biology and Evolution 14:266–276.

Edgecombe, G. 2010. Arthropod phylogeny: an overview from the perspectives of morphology, molecular data and the fossil record. Arthropod Structure and Development 39:74–87.

Enderle, U., G. Kauser, L. Reum, K. Scheller, and J. Koolman. 1983. Ecdysteroids in the haemolymph of blowfly larvae are bound to calliphorin. Pages 40–49 *in* K. Scheller, editor. The larval serum proteins of insects. Georg Thieme Verlag, Stuttgart.

Ertas, B., B. von Reumont, J. Wagele, B. Misof, and T. Burmester. 2009. Hemocyanin suggests a close relationship of Remipedia and Hexapoda. Molecular Biology and Evolution 26:2711–2718.

Ertas, B., L. Kiger, M. Blank, M. Marden, and T. Burmester. 2011. A membrane-bound hemoglobin from gills of the green shore crab *Carcinus maenas*. The Journal of Biological Chemistry 286:3185–3193.

Fahrenbach, W.H. 1970. The cyanoblast: hemocyanin formation in *Limulus polyphemus*. Journal of Cell Biology 44:445–453.

Fan, T., Y. Zhang, L. Yang, X. Yang, G. Jiang, M. Yu, and R. Cong. 2009. Identification and characterization of a hemocyanin-derived phenoloxidase from the crab *Charybdis japonica.* Comparative Biochemistry and Physiology B 152:144–149.

French, C., J. Bell, and F. Ward. 2008. Diversity and distribution of hemerythrin-like proteins in prokaryotes. FEMS Microbiology Letters 279:131–145.

García-Carreno, F., K. Cota, and M. Navarrete del Toro. 2008. Phenoloxidase activity of hemocyanin in whiteleg shrimp *Penaeus vannamei*: conversion, characterization of catalytic properties, and role in postmortem melanosis. Journal of Agricultural and Food Chemistry 56:6454–6459.

Gaykema, W., W. Hol, J. Vereijken, N. Soeter, H. Bak, and J. Beintema. 1984. 3.2 A structure of the copper-containing, oxygen-carrying protein *Panulirus interruptus* haemocyanin. Nature 309:23–29.

Ghiretti-Magaldi, A., C. Milanesi, and B. Salvato. 1973. Identification of hemocyanin in the cyanocytes of *Carcinus maenas*. Experientia 29:1265–1267.

Ghiretti-Magaldi, A., C. Milanesi, and G. Tognon. 1977. Hemopoiesis in Crustacea decapoda: origin and evolution of hemocytes and cyanocytes of *Carcinus maenas.* Cell Differentiation 6:167–186.

Giomi, F., and M. Beltramini. 2007. The molecular heterogeneity of hemocyanin: its role in the adaptive plasticity of Crustacea. Gene 398:192–201.

Glazer, L., M. Tom, S. Weil, Z. Roth, I. Khalaila, B. Mittelman, and A. Sagi. (2013) Hemocyanin with phenoloxidase activity in the chitin matrix of the crayfish gastrolith. Journal of Experimental Biology 216: 1898–1904.

Gorr, T.A., J.D. Cahn, H. Yamagata, and H.F. Bunn. 2004. Hypoxia-induced synthesis of hemoglobin in the crustacean *Daphnia magna* is hypoxia-inducible factor-dependent. Journal of Biological Chemistry 279:36038–36047.

Gorr, T., D. Wichmann, J. Hu, M. Hermes-Lima, A. Welker, N. Terwilliger, J. Wren, M. Viney, S. Morris, G. Nilsson, A. Deten, J. Soliz, and M. Gassmann. 2010. Hypoxia tolerance in animals: biology and application. Physiological and Biochemical Zoology 83:733–752.

Graham, R.A., C.P. Mangum, R.C. Terwilliger, and N.B. Terwilliger. 1983. The effect of organic acids on oxygen binding of hemocyanin from the crab *Cancer magister*. Comparative Biochemistry and Physiology 74A:45–50.

Guadagnoli, J., A. Braun, S. Roberts, and C. Reiber. 2005. Environmental hypoxia influences hemoglobin subunit composition in the branchiopod crustacean *Triops longicaudatus*. Journal of Experimental Biology 208:3543–3551.

Guss, J., E. Merrit, R. Phizackerley, and H. Freeman. 1996. The structure of a phytocyanin, the basic blue protein from cucumber, refined at 1.8 angstrom resolution. Journal of Molecular Biology 262:686–705.

Hagerman, L., and B. Vismann. 1999. Effects of thiosulphate on haemocyanin oxygen affinity in the isopod *Saduria entomon* (L.) and the brown shrimp *Crangon crangon* (L.). Journal of Comparative Physiology B 169:549–554.

Hagner-Holler, S., A. Schoen, W. Erker, J. Marden, R. Rupprecht, H. Decker, and T. Burmester. 2004. A respiratory protein from an insect. Proceedings of the National Academy of Sciences of the USA 101:871–874.

Hagner-Holler, S., K. Kusche, A. Hembach, and T. Burmester. 2005. Biochemical and molecular characterisation of hemocyanin from the amphipod *Gammarus roeseli*: complex pattern of hemocyanin subunit evolution in Crustacea. Journal of Comparative Physiology B 175:445–452.

Hartmann, H., and H. Decker. 2002. All hierarchical levels are involved in conformational transitions of the 4x6-meric tarantula hemocyanin upon oxygenation. Biochimica et Biophysica Acta 1601:132–137.

Hartmann, H., B. Lohkamp, N. Hellmann, and H. Decker. 2001. The allosteric effector l-lactate induces a conformational change of 2x6-meric lobster hemocyanin in the oxy state as revealed by small angle x-ray scattering. The Journal of Biological Chemistry 276:19954–19958.

Hazes, B., K.A. Magnus, C. Bonaventura, J. Bonaventura, Z. Dauter, K.H. Kalk, and W.G.J. Hol. 1993. Crystal structure of deoxygenated *Limulus polyphemus* subunit II hemocyanin at 2.18Å resolution: clues for a mechanism for allosteric regulation. Protein Science 2:597–619.

Hellmann, N., M. Paul, F. Giomi, and M. Beltramini. 2010. Unusual oxygen binding behavior of a 24-meric crustacean hemocyanin. Archives of Biochemistry and Biophysics 495:112–121.

Hirota, S., T. Kawahara, M. Beltramini, P. DiMuro, R. Magliozzo, J. Peisach, L. Powers, N. Tanaka, S. Nagao, and L. Bubacco. 2008. Molecular basis of the Bohr effect in arthropod hemocyanin. The Journal of Biological Chemistry 283:31941–31948.

Hirota, S., N. Tanaka, I. Micetic, P. DiMuro, S. Nagao, H. Kitagishi, K. Kano, R. Magliozzo, J. Peisach, M. Beltramini, and L. Bubacco. 2010. Structural basis of the lactate-dependent allosteric regulation of oxygen binding in arthropod hemocyanin. The Journal of Biological Chemistry 285:19338–19345.

Hoogewijs, D., N.B. Terwilliger, K.A. Webster, J.A. Powell-Coffman, S. Tokishita, H. Yamagata, T. Hankeln, T. Burmester, K.T. Rytkonen, M. Nikinmaa, D. Abele, K. Heise, M. Lucassen, J. Fandrey, P.H. Maxwell, S. Pahlman, and T.A. Gorr. 2007. From critters to cancer: bridging trajectories between comparative and clinical research of oxygen sensing, HIF signaling and adaptations towards hypoxia. Integrative and Comparative Biology 47:552–577.

Hourdez, S., and F. Lallier. 2007. Adaptations to hypoxia in hydrothermal vent and cold-seep invertebrates. Reviews in Environmental Science and Biotechnology 6:143–159.

Hourdez, S., J. Lamontagne, P. Peterson, R.E. Weber, and C.R. Fisher. 2000. Hemoglobin from a deep-sea hydrothermal-vent copepod. Biological Bulletin 199:95–99.

Huang, X.A., H. Yin, S. Sweeney, D. Raha, M. Snyder, and H. Lin. 2013. A major epigenetic programming mechanism guided by piRNAs. Developmental Cell 24: 502–516.

Jaenicke, E., and H. Decker. 2004. Functional changes in the family of type 3 copper proteins during evolution. ChemBioChem 5:163–176.

Jaenicke, E., H. Decker, W. Gebauer, J. Markl, and T. Burmester. 1999a. Identification, structure, and properties of hemocyanins from diplopod Myriapoda. The Journal of Biological Chemistry 274:29071–29074.

Jaenicke, E., R. Foll, and H. Decker. 1999b. Spider hemocyanin binds ecdysone and 20-OH-ecdysone. Journal of Biological Chemistry 274:34267–34271.

Jaenicke, E., S. Fraune, S. May, P. Irmak, R. Augustin, C. Meesters, H. Decker, and M. Zimmer. 2009. Is activated hemocyanin instead of phenoloxidase involved in immune response in woodlice? Developmental & Comparative Immunology 33:1055–1063.

Jaenicke, E., K. Buchler, J. Markl, H. Decker, and T. Barends. 2010. Cupredoxin-like domains in hemocyanins. Biochemical Journal 426:373–378.

Johnson, P.T. 1980. Histology of the blue crab, Callinectes sapidus: a model for the Decapoda. Praeger, New York.

Kimura, S., S. Tokishita, T. Ohta, M. Kobayashi, and H. Yamagata. 1999. Heterogeneity and differential expression under hypoxia of two-domain hemoglobin chains in the water flea, *Daphnia magna*. Journal of Biological Chemistry 274:10649–10653.

Kuballa, A., D. Merritt, and A. Elizur. 2007. Gene expression profiling of cuticular proteins across the moult cycle of the crab *Portunus pelagicus*. BMC Biology 5:45.

Kuballa, A., T. Holton, B. Paterson, and A. Elizur. 2011. Moult cycle specific differential gene expression profiling of the crab *Portunus pelagicus*. BMC Genomics 12:147.

Kurtz, D.J. 1992. Molecular structure and function relationships of hemerythrins. Pages 151–171 *in* C. Mangum, editor. Advances in comparative environmental physiology. Springer Velag, New York.

Kusche, K., H. Ruhberg, and T. Burmester. 2002. A hemocyanin from the Onychophora and the emergence of respiratory proteins. Proceedings of the National Academy of Sciences, USA 99:10545–10548.

Kusche, K., A. Hembach, S. Hagner-Holler, W. Gebauer, and T. Burmester. 2003. Complete subunit sequences, structure and evolution of the 6x6-mer hemocyanin from the common house centipede, *Scutigera coleoptrata*. European Journal of Biochemistry 270:2860–2868.

Laufer, H., N. Demir, and X. Pan. 2005. Shell disease in the American lobster and its possible relations to alkyphenols. New England Aquarium Journal 5:73–75.

Lee, S.Y., B.L. Lee, and K. Soderhall. 2003. Processing of an antibacterial peptide from hemocyanin of the freshwater crayfish *Pacifastacus leniusculus*. The Journal of Biological Chemistry 278:7927–7933.

Lehnert, S.A., and S.E. Johnson. 2001. Expression of hemocyanin and digestive enzyme messenger RNAs in the hepatopancreas of the black tiger shrimp *Penaeus monodon*. Comparative Biochemistry and Physiology 133:163–171.

Lei, K., F. Li, M. Zhang, H. Yang, T. Luo, and X. Xu. 2008. Difference between hemocyanin subunits from shrimp *Penaeus japonicus* in anti-WSSV defense. Developmental & Comparative Immunology 32:808–813.

Lerch, K., and U.A. German. 1988. Evolutionary relationships among copper proteins containing coupled binuclear copper sites. Pages 331–348 *in* T.E. King, H.S. Mason, and M. Morrison, editors. Oxidases and related redox systems. Alan R. Liss, New York.

Li, T., and M. Brouwer. 2007. Hypoxia-inducible factor, gsHIF, of the grass shrimp *Palaemonetes pugio*: molecular characterization and response to hypoxia. Comparative Biochemistry and Physiology B 147:11–19.

Magnus, K., B. Hazes, H. Ton-That, C. Bonaventura, J. Bonaventura, and W.G.J. Hol. 1994. Crystallographic analysis of oxygenated and deoxygenated states of arthropod hemocyanin shows unusual differences. Proteins 19:302–309.

Mangum, C.P. 1997a. Invertebrate blood oxygen carriers. Pages 1097–1131 *in* W. Danzler, editor. Handbook of physiology. Oxford University Press, New York.

Mangum, C.P. 1997b. Adaptation of the oxygen transport system to hypoxia in the blue crab, *Callinectes sapidus*. American Zoologist 37:604–611.

Mangum, C., J. Scott, R. Black, K. Miller, and K. van Holde. 1985. Centipedal hemocyanin: its structure and its implications for arthropod phylogeny. Proceedings of the National Academy of Sciences, USA 82:3721–3725.

Mangum, C.P., J. Greaves, and J.S. Rainer. 1991. Oligomer composition and oxygen binding of the hemocyanin of the blue crab *Callinectes sapidus*. Biological Bulletin 181:453–458.

Markl, J. 1986. Evolution and function of structurally diverse subunits in the respiratory protein hemocyanin from arthropods. Biological Bulletin 171:90–115.

Markl, J., and H. Decker. 1992. Molecular structure of the arthropod hemocyanins. Pages 325–376 *in* C.P. Mangum, editor. Advances in comparative and environmental physiology. Springer Verlag, New York.

Markl, J., A. Hofer, G. Bauer, A. Markl, B. Kempter, M. Brenzinger, and B. Linzen. 1979. Subunit heterogeneity in arthropod hemocyanins. II. Crustacea. Journal of Comparative Physiology B 133:167–175.

Markl, J., A. Moeller, A. Martin, J. Rheinbay, W. Gebauer, and F. Depoix. 2009. 10-Å cryoEM structure and molecular model of the myriapod (*Scutigera*) 6x6mer hemocyanin: understanding a giant oxygen transport protein. Journal of Molecular Biology 392:362–380.

Martin, A., F. Depoix, M. Stohr, U. Meissner, S. Hagner-Holler, K. Hammouti, T. Burmester, J. Heyd, W. Wriggers, and J. Markl. 2007. *Limulus polyphemus* hemocyanin: 10 A cryo-EM structure, sequence analysis, molecular modelling and rigid-body fitting reveal the interfaces between the eight hexamers. Journal of Molecular Biology 366:1332–1350.

McMahon, B. 2001. Respiratory and circulatory compensation to hypoxia in crustaceans. Respiration Physiology 128:349–364.

Meissner, U., M. Stohr, K. Kusche, T. Burmester, H. Stark, R. Harris, E. Orlova, and J. Markl. 2003. Quaternary structure of the European spiny lobster (*Panulirus elephas*) 1x6-mer hemocyanin from cryoEM and amino acid sequence data. Journal of Molecular Biology 325:99–109.

Meyer, A., and B. Lieb. 2010. Respiratory proteins in *Sipunculus nudus*—implications for phylogeny and evolution of the hemerythrin family. Comparative Biochemistry and Physiology B 155:171–177.

Micetic, I., C. Losasso, P. DiMuro, G. Tognon, P. Benedetti, and M. Beltramini. 2010. Solution structure of 2x6-meric and 4x6-meric hemocyanins of crustaceans *Carcinus aestuarii, Squilla mantis* and *Upogebia pusilla*. Journal of Structural Biology 171:1–10.

Miller, K.E., M.E. Cuff, W.F. Lang, P. Varga-Weisz, K.G. Field, and K.E. van Holde. 1998. Complete sequence of a molluscan hemocyanin: structural and evolutionary implications. Journal of Molecular Biology 278:827–842.

Moore, A., and E. Bornberg-Bauer. 2012. The dynamics and evolutionary potential of domain loss and emergence. Molecular Biology and Evolution 29:787–796.

Morris, S. 1990. Organic ions as modulators of respiratory function during stress. Physiological Zoology 63:253–287.

Morris, S., and C.N. Airriess. 1998. Integration of physiological responses of crustaceans to environmental challenge. South African Journal of Zoology 33:87–106.

Morris, S., C.R. Bridges, and M.K. Grieshaber. 1985. A new role for uric acid: modulator of haemocyanin oxygen affinity in crustaceans. Journal of Experimental Zoology 235:135–139.

Nagai, T., T. Osaki, and S. Kawabata. 2001. Functional conversion of hemocyanin to phenoloxidase by horseshoe crab antimicrobial peptides. Journal of Biological Chemistry 276:27166–27170.

Nillius, D., E. Jaenicke, and H. Decker. 2008. Switch between tryosinase and catecholoxidase activity of scorpion hemocyanin by allosteric effectors. FEBS Letters 582:749–754.

Olianas, A., M. Sanna, I. Messana, M. Castagnola, D. Masia, B. Manconi, A. Cau, B. Giardina, and M. Pelligrini. 2006. The hemocyanin of the shamefaced crab *Calappa granulata*: structural-functional characterization. Journal of Biochemistry 139:957–966.

Olianas, A., B. Manconi, D. Masia, M. Sanna, M. Castognola, S. Salvadori, I. Messana, B. Giardina, and M. Pellegrini. 2009. The oxygen-binding modulation of hemocyanin from the Southern spiny lobster *Palinurus gilchristi*. Journal of Comparative Physiology B 179:193–203.

Olson, K.S. 1991. Developmental changes in the structure and function of lobster hemocyanin. Ph.D. thesis. Massachusetts Institute of Technology and Woods Hole Oceanographic Institution.

Panzer, D., C. Beck, J. Hahn, J. Maul, G. Schonhense, H. Decker, and E. Aziz. 2010. Water influences on the copper active site in hemocyanin. The Journal of Physical Chemistry Letters 1:1642–1647.

Paoli, M., F. Giomi, N. Hellmann, E. Jaenicke, H. Decker, P.D. Muro, and M. Beltramini. 2007. The molecular heterogeneity of hemocyanin: structural and functional properties of the 4x6-meric protein of *Upogebia pusilla* (Crustacea). Gene 398:177–182.

Perbandt, M., E. Guthohrlein, W. Rypniewski, K. Idakieva, S. Stoeva, W. Voelter, N. Genov, and C. Betzel. 2003. The structure of a functional unit from the wall of a gastropod hemocyanin offers a possible mechanism for cooperativity. Biochemistry 42:6341–6346.

Phillips, M.R. 2007. Functional genetic analysis of two non-model marine invertebrates: physiologically and environmentally induced changes in gene expression. PhD Dissertation in Biology, University of Oregon, Eugene, Oregon.

Pick, C., and T. Burmester. 2009. A putative hexamerin from a *Campodea* sp. suggests an independent origin of haemocyanin-related storage proteins in Hexapoda. Insect Molecular Biology 18:673–679.

Pick, C., S. Hagner-Holler, and T. Burmester. 2008. Molecular characterization of hemocyanin and hexamerin from the firebrat *Thermobia domestica* (Zygentoma). Insect Biochemistry and Molecular Biology 38:977–983.

Pless, D., M. Aguilar, A. Falcon, E. Lozano-Alvarez, and E. de la Cotera. 2003. Latent phenoloxidase activity and N-terminal amino acid sequence of hemocyanin from *Bathynomus giganteus*, a primitive crustacean. Archives of Biochemistry and Biophysics 409:402–410.

Pratt, B.R., M. Pushie, I. Pickering, and G. George. 2010. Synchrotron imaging of Burgess Shale fossils: evidence for biochemical copper (hemocyanin) in the Middle Cambrian arthropod *Marrella splendens*. Geological Society of America 42:11.

Protas, M.E., P. Trontelj, and N.H. Patel. 2011. Genetic basis of eye and pigment loss in the cave crustacean, *Asellus aquaticus*. Proceedings of the National Academy of Sciences, USA 108: 5702–5707.

Rehm, P., C. Pick, J. Borner, J. Markl, and T. Burmester. 2012. The diversity and evolution of chelicerate hemocyanins. BMC Evolutionary Biology 12:19.

Robert, C., H. Decker, B. Richey, S. Gill, and J. Wyman. 1987. Nesting: hierarchies of allosteric interactions. Proceedings of the National Academy of Sciences, USA 84:1891–1895.

Royer, W., H. Zhu, T. Gorr, J. Flores, and J. Knapp. 2005. Allosteric hemoglobin assembly: diversity and similarity. The Journal of Biological Chemistry 280:27477–27480.

Saey, T.H. 2013. From Great-Grandma to You. Science News 183:18–21.

Salvato, B., and M. Beltramini. 1990. Hemocyanins: molecular architecture, structure and reactivity of the binuclear copper active site. Life Chemistry Reports 8:1–47.

Salvato, B., M. Santamaria, M. Beltramini, G. Alzuet, and L. Casella. 1998. The enzymatic properties of *Octopus vulgaris* hemocyanin: o-diphenol oxidase activity. Biochemistry 37:14065–14077.

Sánchez-Ferrer, Á., J.N. Rodríguez-López, F. García-Cánovas, and F. García-Carmona. 1995. Tyrosinase: a comprehensive review of its mechanism. Biochimica et Biophysica Acta 1247:1–11.

Sanders, N., and J. Childress. 1992. Specific effects of thiosulphate and L-lactate on hemocyanin-O2 affinity in brachyuran hydrothermal vent crab. Marine Biology 113:175–180.

Scherbaum, S., B. Ertas, W. Gebauer, and T. Burmester. 2010. Characterization of hemocyanin from the peacock mantis shrimp *Odontodactylus scyllarus* (Malacostraca: Hoplocarida). Journal of Comparative Physiology B 180:1235–1245.

Shirley, S.M., T.C. Shirley, and T. Meyers. 1986. Hemolymph responses of Alaskan king crabs to rhizocephalan parasitism. Canadian Journal of Zoology 64:1774–1781.

Siddiqui, N., R. Akosung, and C. Gielens. 2006. Location of intrinsic and inducible phenoloxidase activity in molluscan hemocyanin. Biochemical and Biophysical Research Communications 348:1138–1144.

Söderhäll, K., and L. Cerenius. 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. Current Opinions in Immunology 10:23–28.

Soñanez-Organis, J., A. Peregrino-Uriarte, S. Gómez-Jiménez, A. López-Zavala, H. Forman, and G. Yepiz-Plascencia. 2009. Molecular characterization of hypoxia inducible factor-1 (HIF-1) from the white shrimp *Litopenaeus vannamei* and tissue-specific expression under hypoxia. Comparative Biochemistry and Physiology C 150:395–405.

Spicer, J., and E. Hodgson. 2003. Structural basis for salinity-induced alteration in oxygen binding by haemocyanin from the estuarine amphipod *Chaetogammarus marinus* (L). Journal of the Marine Biological Association of the UK 83:945–947.

Spicer, J., and J. Stromberg. 2002. Diel vertical migration and the hemocyanin of krill *Meganyctiphanes norvegica*. Marine Ecology Progress Series 238:153–162.

Stillman, J., J. Colbourne, C. Lee, N. Patel, M. Phillips, D. Towle, B. Eads, G. Gelembuik, R. Henry, E. Johnson, M. Pfrender, and N. Terwilliger. 2008. Recent advances in crustacean genomics. Integrative and Comparative Biology 48:852–868.

Sugumaran, M. 1998. Unified mechanism for sclerotization of insect cuticle. Advances in Insect Physiology 27:229–334.

Tanner, C., L. Burnett, and K. Burnett. 2006. The effects of hypoxia and pH on phenoloxidase activity in the Atlantic blue crab, *Callinectes sapidus*. Comparative Biochemistry and Physiology A 144:218–223.

Telfer, W.H., and H.C.J. Massey. 1987. A storage hexamer from *Hyalophora* that binds riboflavin and resembles the apoprotein of hemocyanin. Pages 305–314 *in* J.H. Law, editor. Molecular entomology. Alan R. Liss, New York.

Terwilliger, N.B. 1982. Effect of subunit composition on quaternary structure of isopod (*Ligia pallasii*) hemocyanin. Biochemistry 21:2579–2586.

Terwilliger, N.B. 1991. Arthropod (Cyamus scammoni, Amphipoda) hemoglobin structure and function. Pages 59–63 *in* S. Vinogradov and O. Kapp, editors. Structure and function of invertebrate oxygen carriers. Springer, Berlin, Heidelberg, New York.

Terwilliger, N.B. 1992. Molecular structure of the extracellular heme proteins. Pages 193–229 *in* C.P. Magnum, editor. Advances in comparative and environmental physiology. Springer-Verlag, Berlin.

Terwilliger, N.B. 1998. Functional adaptations of oxygen-transport proteins. Journal of Experimental Biology 201:1085–1098.

Terwilliger, N.B. 2007. Hemocyanins and the immune response: defense against the dark arts. Integrative and Comparative Biology 47:662–665.

Terwilliger, N.B. 2008a. HIF and stress responses in crustaceans in normoxia. Pages 249–258 *in* S. Morris, A. Vosloo, editors. Proceedings of the 4th comparative physiology and biochemistry in Africa: Mara 2008. Molecules to migration: the pressures of life. Medimond, Monduzzi Editore International, Bologna, Italy.

Terwilliger, N.B. 2008b. Whale rider: the co-occurrence of hemoglobin and hemocyanin in Cyamus scammoni. Pages 203–209 *in* C. Verde, editor. Protein reviews: dioxygen binding and sensing proteins. Springer, Italy.

Terwilliger, N.B. 2011. Gene expression profile, protein production, and functions of cryptocyanin during the crustacean molt cycle. Invertebrate Reproduction and Development 56:229–235.

Terwilliger, N.B., and A.C. Brown. 1993. Ontogeny of hemocyanin function in the Dungeness crab *Cancer magister*: the interactive effects of developmental stage and divalent cations on hemocyanin oxygenation properties. Journal of Experimental Biology 183:1–13.

Terwilliger, N.B., and K. Dumler. 2001. Ontogeny of decapod crustacean hemocyanin: effects of temperature and nutrition. Journal of Experimental Biology 204:1013–1020.

Terwilliger, N.B., and M. Ryan. 2001. Ontogeny of crustacean respiratory proteins. American Zoologist 41:1057–1067.

Terwilliger, N.B., and M. Ryan. 2006. Functional and phylogenetic analyses of phenoloxidases from brachyuran (*Cancer magister*) and branchiopod (*Artemia franciscana, Triops longicaudatus*) crustaceans. Biological Bulletin 210:38–50.

Terwilliger, N.B., and R.C. Terwilliger. 1982. Changes in the subunit structure of *Cancer magister* hemocyanin during larval development. Journal of Experimental Biology 221:181–191.

Terwilliger, N.B., and D. Towle. 2007. Prophenoloxidase mRNA expression in hemocytes of the green crab *Carcinus maenas*. Mt. Desert Island Biological Laboratory Bulletin 46:79–80.

Terwilliger, R.C., N.B. Terwilliger, and E. Schabtach. 1986. Hemoglobin from the parasitic barnacle, Briarosaccus callosus. Pages 125–127 *in* B. Linzen, editor. Invertebrate oxygen carriers. Springer, New York.

Terwilliger, N.B., L.D. Dangott, and M.C. Ryan. 1999. Cryptocyanin, a crustacean molting protein: evolutionary link with arthropod hemocyanins and insect hexamerins. Proceedings of the National Academy of Sciences of the USA 96:2013–2018.

Terwilliger, N.B., M. Ryan, and D. Towle. 2005. Evolution of novel functions: cryptocyanin helps build new exoskeleton in Cancer magister. Journal of Experimental Biology 208:2467–2474.

Terwilliger, N.B., M. Ryan, and M. Phillips. 2006. Crustacean hemocyanin gene family: oxygen probes of the global gene scene. Integrative and Comparative Biology 46:991–999.

Torchin, M. 1994. The effect of parasitism on the hemocyanin of an intertidal hermit crab Pagurus samueli. MS. Thesis, University of Oregon, Eugene, Oregon.

Traverso, M., P. Subramanian, R. Davydov, B. Hoffman, T. Stemmler, and A. Rosenzweig. 2010. Identification of a hemerythrin-like domain in a P_{1B}-type transport ATPase. Biochemistry 49:7060–7068.

Truchot, J.-P. 1980. Lactate increases the oxygen affinity of crab hemocyanin. Journal of Experimental Zoology 214:205–208.

Truchot, J.-P. 1992. Respiratory function of arthropod hemocyanins. Pages 377–410 *in* C.P. Mangum, editor. Blood and tissue oxygen carriers. Springer-Verlag, Heidelberg.

van der Ham, J., and B. Felgenhauer. 2007. The functional morphology of the putative injecting apparatus of *Speleonectes tanumekes* (Remipedia). Journal of Crustacean Biology 27:1–9.

van Holde, K.E., and K.I. Miller. 1982. Hemocyanins. Quarterly Review of Biophysics 15:1–129.

van Holde, K.E., and K.I. Miller. 1995. Hemocyanins. Advances in Protein Chemistry 47:1–81.

van Holde, K., K. Miller, and H. Decker. 2001. Hemocyanins and invertebrate evolution. Journal of Biological Chemistry 276:15563–15566.

Vanin, S., E. Negrisolo, X. Bailly, L. Bubacco, M. Beltramini, and B. Salvato. 2006. Molecular evolution and phylogeny of sipunculan hemerythrins. Journal of Molecular Evolution 62:32–41.

Vaquer-Sunyer, R., and C. Duarte. 2008. Thresholds of hypoxia for marine biodiversity. Proceedings of the National Academy of Sciences of the USA 105:15452–15457.

Vierthaler, L., M. Ryan, and N. Terwilliger. 2003. A functional hemocyanin in the leptostracan *Nebalia*: insights into molecular and arthropod phylogenies. The Society for Integrative and Comparative Biology Meeting, P2.108.

Vogan, C., C. Costa-Ramos, and A. Rowley. 2002. Shell disease syndrome in the edible crab, *Cancer pagurus*—isolation, characterization and pathogenicity of chitinolytic bacteria. Microbiology 148:743–754.

Voit, R., G. Feldmaier-Fuchs, T. Schweikardt, H. Decker, and T. Burmester. 2000. Complete sequence of the 24mer hemocyanin of the tarantula *Eurypelma californicum*: structure and intramolecular evolution of the subunits. The Journal of Biological Chemistry 275:39338–39344.

Volbeda, A., and W.G.J. Hol. 1989. Crystal structure of hexameric hemocyanin from *Panulirus interruptus* refined at 3.2Å resolution. Journal of Molecular Biology 209:249–279.

von Reumont, B., R. Jenner, M. Wills, E. Dell'ampio, G. Pass, I. Ebersberger, B. Meyer, S. Koenemann, T. Iliffe, A. Stamatakis, O. Niehuis, K. Meusemann, and B. Misof. 2012. Pancrustacean phylogeny in the light of new phylogenomic data: support for Remipedia as the possible sister group of hexapoda. Molecular Biology and Evolution 29:1031–1045.

Weber, R., and S. Vinogradov. 2001. Nonvertebrate hemoglobins: functions and molecular adaptations. Physiological Reviews 81:589–628.

Weber, R., J. Behrens, H. Malte, and A. Fago. 2008. Thermodynamics of oxygenation-linked proton and lactate binding govern the temperature sensitivity of O_2 binding in crustacean (*Carcinus maenas)* hemocyanin. Journal of Experimental Biology 211:1057–1062.

Xiong, J., D.J. Kurtz, J. Ai, and J. Sanders-Loehr. 2000. A hemerythrin-like domain in a bacterial chemotaxis protein. Biochemistry 39:5117–5125.

Zeis, B., B. Becher, T. Lamkemeyer, S. Rolf, R. Pirow, and R. Paul. 2003. The process of hypoxic induction of *Daphnia magna* hemoglobin: subunit composition and functional properties. Comparative Biochemistry and Physiology B 134:243–252.

Zeis, B., T. Lamkemeyer, R. Paul, F. Nunes, S. Schwerin, M. Koch, W. Schutz, J. Madlung, C. Fladerer, and R. Pirow. 2009. Acclimatory responses of the *Daphnia pulex* proteome to environmental changes. 1. Chronic exposure to hypoxia affects the oxygen transport system and carbohydrate metabolism. BMC Physiology 9:7.

Zhang, Y., F. Yan, Z. Hu, X. Zhao, S. Min, Z. Du, S. Zhao, X. Ye, and Y. Li. 2009. Hemocyanin from shrimp *Litopenaeus vannamei* shows hemolytic activity. Fish Shellfish Immunology 27:330–335.

Zlateva, T., P. Di Muro, B. Salvato, and M. Beltramini. 1996. The *o*-diphenol oxidase activity of arthropod hemocyanin. FEBS Letters 384:251–254.

12

ENERGETICS AND METABOLIC REGULATION

Ana Gabriela Jimenez and Stephen T. Kinsey

Abstract

Crustaceans exploit diverse habitats and face numerous environmental challenges. This chapter reviews mechanisms by which crustaceans maintain energy homeostasis in the face of physical and physiological stresses. The basic metabolic pathways of energy metabolism present in higher vertebrates also function in crustaceans, with some important differences. Crustaceans store major metabolic fuels in the form of glycogen, protein, and several types of lipids, and muscle and hepatopancreas are their most important depots. Biological energetic and environmental challenges cause an increase in ATP demand. Although maintaining homeostasis under variable environmental conditions may lead to increased energy expenditure, lack of tolerance to environmental changes is not necessarily due to energetic constraints. Current understanding of crustacean energy homeostasis provides a framework for future work addressing the likely effects of climate change on species distributions and invasions and novel approaches for enhancing aquaculture production of commercially viable species.

INTRODUCTION

The maintenance of energetic homeostasis is a prerequisite for all organisms. Crustaceans, like other animals, use carbohydrates, lipids, and proteins as principal fuel molecules, as well as building blocks for intra- and extracellular structural components. These molecules are either synthesized or acquired by ingestion, and some can be oxidized to produce adenosine triphosphate (ATP). Storage forms of carbohydrates, lipids, and proteins play an important role in maintaining energy balance, particularly during periods of reduced ATP supply or increased ATP demand. Changes in synthesis or mobilization of these fuels can therefore occur during changes in physiological state, such as during locomotion, reproduction, or molting, which increase ATP demand, or during

starvation, which limits fuel supply. Similarly, the cellular and whole-body energy state can be altered by changing environmental conditions, including fluctuations in temperature, O_2 availability, or salinity.

Many of the mechanisms by which crustaceans respond to the specific physiological states and environmental conditions just mentioned are covered in detail in other chapters of this volume. Therefore, this chapter considers only aspects of energy homeostasis, with an emphasis on the consequences and metabolic costs associated with physiological challenges, and how fuel reserves are allocated to maintain or restore energy balance at the cellular, tissue, and whole-animal level. We focus largely, but not exclusively, on decapod crustaceans and avoid substantial discussion of model crustaceans such as *Artemia* or *Daphnia*, for which there is an abundance of literature. To limit the scope of this chapter, we also concentrate on more recent literature. Older studies are discussed in detailed reviews elsewhere (Cameron and Mangum 1983, Chang and O'Connor 1983, Claybrook 1983, Gilles and Pequeux 1983).

ENERGY METABOLISM

Early work on crustacean biochemistry centered on characterizing metabolic pathways, generally by determining whether enzymes and regulatory mechanisms identified in mammals were also present in crustaceans, and many of these studies focused on the oxidation and synthesis of metabolic fuel molecules. Chang and O'Connor (1983) reviewed the synthesis and breakdown of carbohydrates and lipids, while Claybrook (1983) evaluated early studies on protein and amino acid metabolism. Although there are some obvious differences in crustacean biochemistry compared to that in mammals (e.g., crustacean cuticle formation), the major enzymatic pathways of energy metabolism and biosynthesis of fuel molecules found in mammals are present in crustaceans (Fig. 12.1).

Major ATP-Producing and Consuming Pathways

Fuel Molecules

Carbohydrates

As in most vertebrates, D-glucose is the principal monosaccharide present in the hemolymph of crustaceans, and it serves several purposes, including the synthesis of mucopolysaccharides, chitin, nicotinamide adenine dinucleotide phosphate (NADPH), and glycogen, as well as the formation of pyruvate (Fig. 12.1). Glucose in hemolymph comes from the direct absorption of dietary glucose through hepatopancreatic and intestinal epithelial cells or from peripheral tissues such as the hepatopancreas and muscle, where it is stored as glycogen or synthesized by the gluconeogenic pathway. Glucose levels in hemolymph are tightly controlled, particularly by the crustacean hyperglycemic hormone (CHH), a neuropeptide produced by the sinus gland of eyestalks (Verri et al. 2001). Relatively little free glucose occurs in cells because it is rapidly converted to glucose-6-phosphate by hexokinase (HK). Then, glucose-6-phosphate follows one of three primary fates: glycogenesis, glycolysis, or the pentose-phosphate pathway (Chang and O'Connor 1983, Santos and Keller 1993, Oliveira et al. 2004). Glucose in crustaceans is stored in the form of glycogen, and the largest depots are in the hepatopancreas and muscle (Vinagre and Da Silva 1992, Oliveira et al. 2003, Buckup et al. 2008). The net storage mobilization of glucose depends on a variety of biotic and abiotic factors, including molt stage, diet, nutritional state, season, salinity, and dissolved O_2, which are discussed in later sections (Vinagre and Da Silva 1992, Buckup et al. 2008). However, in general,

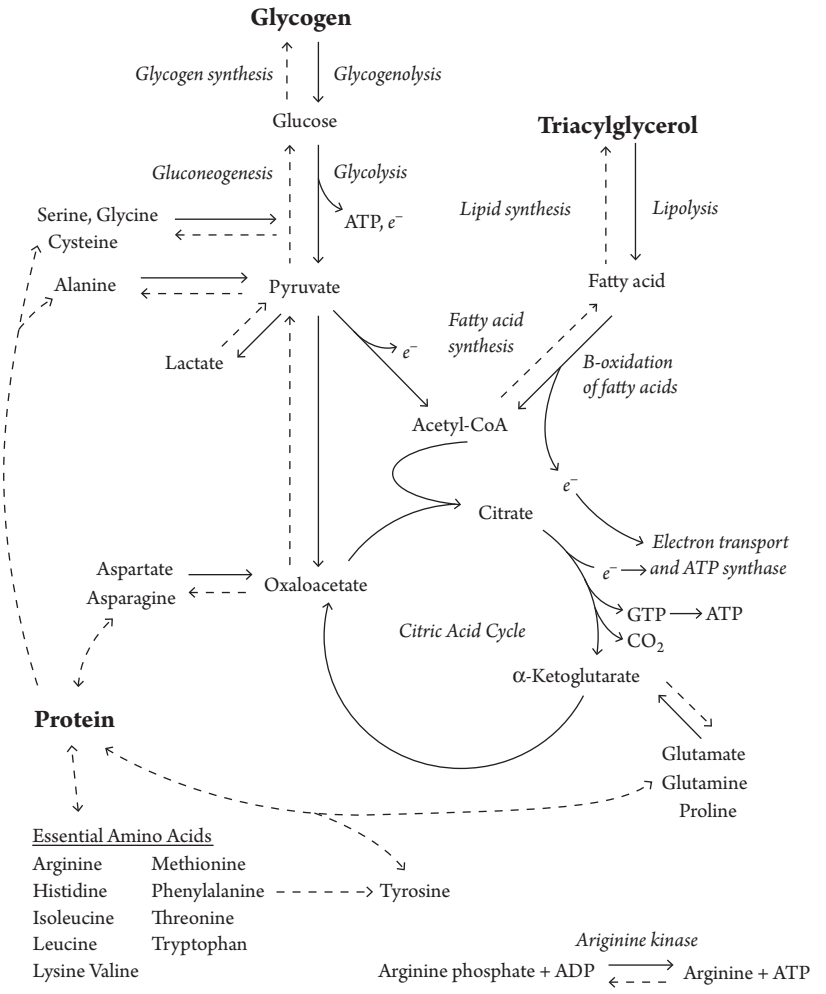


Fig. 12.1.
Overview of energy metabolism in crustaceans. Storage forms of major fuel molecules are in large type, metabolic pathways are in italics, and major metabolites are also shown. Pathways associated with adenosine triphosphate (ATP) production are designated with solid lines. These pathways include glycolysis and fatty acid oxidation, both of which partially oxidize carbon substrates and feed acetyl-CoA into the citric acid cycle, where the acetyl groups are fully oxidized to CO_2. These pathways yield the direct production of ATP as well as electrons (e^-) that are carried to the electron transport system by nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). Electron transport in the mitochondria leads to a proton motive force, which is used to make ATP via ATP synthase. The dashed lines show biosynthetic pathways, as well as other pathways that can interconvert molecules in crustaceans. Amino acids are oxidized to produce ATP by being converted to an intermediate of glycolysis or the citric acid cycle. In addition to these pathways, the arginine kinase reaction can provide ATP by transferring a phosphate from the phosphagen arginine phosphate to ADP.

when hemolymph glucose levels are low, glycogen stored in tissues is broken down into glucose and mobilized into the hemolymph, whereas when glucose levels are high, glycogen synthesis occurs via gluconeogenesis (Santos and Keller 1993).

Under aerobic conditions, glucose is fully oxidized to CO_2 through glycolysis and the citric acid cycle, and ATP is produced via oxidative phosphorylation (Chang and O'Connor 1983). Under anaerobic conditions, lactate is the exclusive metabolic end-product in crustaceans, as in vertebrates

(Gade and Grieshaber 1986). Lactate accumulates when ATP demand exceeds the supply available by oxidative metabolism, either because of increased demand, such as occurs in muscles during intensive exercise (England and Baldwin 1983, Morris and Adamczewska 2002, Johnson et al. 2004, Kinsey et al. 2005, Hardy et al. 2006, Jimenez et al. 2008), or due to reduced supply, such as during anoxia (Oliveira et al. 2001, Abe et al. 2007, Holman and Hand 2009).

Lipids

Lipids play a major role in crustacean metabolism and in cellular and subcellular membrane structure. The principal site of lipid storage in crustaceans appears to be the hepatopancreas (Chang and O'Connor 1983, Kucharski and Da Silva 1991a, Muriana et al. 1993, Garcia et al. 2002). The catabolism of fatty acids appears to occur via β-oxidation, whereas the synthesis of fatty acids, triacylglycerols, and phospholipids also occurs via the same pathways found in mammals (Fig. 12.1; Chang and O'Connor 1983). The principal circulating lipids in these animals are phospholipids, in contrast with vertebrates, and the hemolymph also contains triglycerides and sterols, all of which are packaged in high-density lipoproteins or very high-density lipoproteins, although there is only a small fraction of free fatty acids (Yepiz-Plascencia et al. 2000, 2002). Additionally, the principal storage form of lipids in some crustaceans is wax esters, although in most crustaceans, including decapods, it is triacylglycerols (Chang and O'Connor 1983, Chang 1995). Lipid synthesis follows the same pathways as in vertebrates, and lipid droplets accumulate in specific tissues to serve as energy stores, particularly during reproduction.

Amino Acids

Biosynthetic and degradation pathways for amino acids in crustaceans are largely the same as those of mammals (Fig. 12.1; Claybrook 1983). Although most amino acids are in proteins, the concentration of free amino acids in most crustaceans is several-fold higher than in vertebrate tissues. There are 10 essential amino acids for crustaceans, nine of which are the same as in humans (in crustaceans, arginine is also considered essential). Glucose can supply carbon skeletons for nine of the nonessential amino acids via intermediates of glycolysis and the citric acid cycle. In glycolysis, 3-phosphoglycerate and pyruvate are precursors to amino acids, whereas in the citric acid cycle, α-ketoglutarate and oxaloacetate are substrates for amino acid synthesis. Tyrosine is derived from phenylalanine, which is an essential amino acid. The pathways by which amino acids are degraded also are thought to be closely related to those found in vertebrates, where glucogenic amino acid carbon skeletons are converted to pyruvate or a citric acid cycle intermediate, while ketogenic amino acid carbon skeletons are converted to acetyl-CoA or acetoacetyl-CoA, thus facilitating their oxidation for ATP production or conversion to glucose or lipids. Nitrogen from amino acids is excreted as ammonia, uric acid, or urea, although there does not appear to be a true urea cycle in crustaceans (Claybrook 1983).

Tissue Specificity

Hepatopancreas and muscle are the major storage depots for glycogen owing to the relatively large mass of these tissues, whereas gills and gonads are smaller reservoirs (although gonad glycogen reserves are dependent on the reproductive stage; Fig. 12.2; Chang and O'Connor 1983, Vinagre and Da Silva 1992, 2002, Oliveira and Da Silva 1997, Oliveira el al. 2003, Antunes et al. 2010). In the absence of adipose tissue, the hepatopancreas is the principal storage site for lipids (Chang and O'Connor 1983, Schmitt and Santos 1993), whereas muscle is the main protein storage location in crustaceans (Claybrook 1983, Buckup et al. 2008). Although most of the metabolic pathways associated with energy metabolism are similar to those in vertebrates, the metabolic

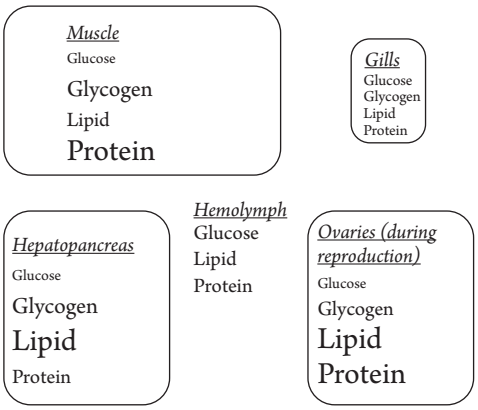


Fig. 12.2.
Generalized schematic of the relative importance of major tissues in fuel storage. The size of the box indicates the relative size of the tissue, and the size of the type indicates the importance of that tissue for each type of fuel molecule. These relationships are highly species specific, and this diagram is not meant to be quantitative.

capacity for these processes is sometimes more evenly distributed among the major tissues in crustaceans. For instance, gluconeogenesis appears to occur in hepatopancreas, gills, muscle, and hemocytes, rather than being confined to specific tissues (Lallier and Walsh 1991, Oliveira et al. 2004). However, the hepatopancreas does appear to be the principal site of lipid and lipoprotein synthesis (Walker et al. 2003).

Mechanisms of Regulation

Energy metabolism responds to environmental challenges in part through changes in gene expression. Hyperglycemia is a response to various kinds of stress, such as changes in temperature and pH, and is linked to increased expression of CHH (Chang et al. 1999). Changes in temperature, O_2, or metal ion concentrations can lead to elevation in the expression of heat shock proteins (HSPs), which help preserve the function of other proteins (Ryan and Hightower 1994), as well as elevate the expression of other stress proteins (Willsie and Clegg 2001, Gorr et al. 2004). Hypoxia leads to significant decreases in transcription of superoxide dismutase, hemocyanin, and ribosomal genes in blue crabs (*Callinectes sapidus*) after 5 days of exposure. The decrease in two of the three ribosomal cDNAs analyzed suggests that protein synthesis may have been slowing down in these animals during prolonged hypoxia (Brouwer et al. 2004). In the grass shrimp, *Palaemonetes pugio*, severe chronic hypoxia leads to an initial upregulation of the mitochondrial genes associated with electron transport, whereas long-term exposure leads to a downregulation of both transcript and protein levels (Brouwer et al. 2008). Hyposmotic stress causes an increased expression of the enzyme carbonic anhydrase, which plays a role in osmoregulation in gills of the green crab *Carcinus maenas* (Serrano and Henry 2008) and the tiger shrimp *Penaeus monodon* (Pongsomboon et al. 2009), as well as the activity of the citric acid cycle enzyme citrate synthase in gills of *C. sapidus* (Kinsey et al. 2003).

Changes in physiological state also alter the expression of genes involved in the energy metabolism of crustaceans. In the shrimp *Metapenaeus ensis*, oocyte maturation is associated with increased expression of glyceraldehyde-3-phosphate dehydrogenase and arginine kinase (AK) transcripts, which are involved with ATP production via glycolysis and phosphagen hydrolysis, respectively (Sze Lo et al. 2007). During molting, there is a substantial increase in expression of transcripts for cuticular proteins in the swimming crab *Portunus pelagicus* (Kuballa et al. 2011), and in the Antarctic krill *Euphausia superba* (Seear et al. 2010) and *P. pelagicus*, the increased demand for ATP during

cuticle formation is associated with an increased expression of mitochondrial genes (Kuballa et al. 2011). Jiang et al. (2009) examined the relationship between the expression of genes that are associated with nutritional status and those that are associated with reproduction in hepatopancreas and testis of the mitten crab, *Eriocheir sinensis*. With respect to energy metabolism, these authors found that nutritional state altered the expression of AK, which is an ATP buffer important during periods of increasing ATP demand.

In addition to changes in gene expression, energy metabolism is also controlled hormonally and by allosteric regulation of enzyme activity, the latter usually in the same manner as in mammals (Claybrook 1983, Chang and O'Connor 1983). For instance, CHH plays a key role in regulating tissue synthesis and release of glucose and lipids from hepatopancreas and muscle (Chang and O'Connor 1983, Santos et al. 1997). Glycogen breakdown in crustaceans is regulated by the enzyme glycogen phosphorylase, and activity of this enzyme is controlled by phosphorylation state and by adenosine monophosphate (AMP; Kamp 1989). The glycolytic enzymes, phosphofructokinase and pyruvate kinase, are allosterically regulated by the adenylates, AMP and adenosine diphosphate (ADP) (activators) and ATP (inhibitor) (England and Baldwin 1985). The citric acid cycle enzyme, citrate synthase, is allosterically inhibited by ATP in crustaceans, providing a negative feedback that slows metabolism when energy needs are met (Vetter 1995). Glutamate dehydrogenase, which regulates the production of ammonia during amino acid breakdown, is inhibited by nicotinamide adenine dinucleotide (NADH), the citric acid cycle intermediate α-ketoglutarate, and guanosine triphosphate (GTP), but is activated by ADP (Claybrook 1983). These and other examples of allosteric regulation are essentially the same as in mammals, indicative of the highly conserved nature of pathways of energy metabolism.

Conditional Responses of Metabolism

Responses to Physiological State

Locomotion/Activity

Locomotion modes for crustaceans include walking (aquatic and terrestrial), swimming, and tail-flipping (in lobsters and shrimp). For crustaceans, walking and swimming have been divided into short-term high-speed bursts leading to rapid fatigue, prolonged high- to moderate-speed movement leading to eventual fatigue, and long-term low-speed activity that can be sustained for extended periods (Wood and Randall 1981). Tail-flipping entails one or more abdominal flexions that are used as an escape response and therefore always are rapid, high-speed movements for short durations. As in other taxa, burst locomotion is primarily fueled by anaerobic metabolism and powered by muscle fibers with few mitochondria (usually <3% of cell volume is mitochondria). Prolonged locomotion in crustaceans is usually characterized by an increase in O_2 consumption and some accumulation of lactate and is powered by aerobic muscle fibers that may have more than 25% of their cell volume devoted to mitochondria (Houlihan and Innes 1984, Boyle et al. 2003, Johnson et al. 2004, Hardy et al. 2009, Kinsey et al. 2011).

Most species of crustaceans have a diversity of muscle fiber types that reflect functional demand (Tse et al. 1983, Silverman et al. 1987, Stokes and Josephson 1992, Hardy et al. 2009, 2010). The metabolic range of muscle function has been well characterized in the blue crab *C. sapidus*, which is a fast and efficient swimmer that exhibits both burst-escape locomotion to avoid predators and sustainable aerobic locomotion (Booth and McMahon 1992). Both types of locomotion are powered by a large mass of "backfin" muscles attached to the paddle-like fifth pereopods, which move in a sculling-type motion to propel the animal sideways while swimming (White and Spirito 1973). The swimming muscle responsible for these distinct forms of locomotion are classified into two major

metabolic categories: so-called "dark" aerobic fibers that power sustained swimming and a large mass of anaerobic "light" fibers that are used for burst swimming (Tse et al. 1983; see Chapter 4 in this volume). The aerobic fibers are noticeably yellow-brown in color due to the presence of a dense population of mitochondria and are highly perfused (Johnson et al. 2004, Hardy et al. 2009). The dark and light fibers in crustaceans are functionally analogous to the red and white fibers of fishes, but the aerobic fibers are not red in color since crustaceans lack myoglobin. The dark fibers are highly subdivided and appear to have arisen from light fiber precursors during the evolution of sustained exercise in the swimming crabs (Tse et al. 1983, Hardy et al. 2009, 2010). To accommodate the demands of aerobic swimming, the fiber sarcolemmal membrane became highly invaginated, permitting intrafiber perfusion and forming small, isolated metabolic functional units (fiber subdivisions) that have high mitochondrial density and short O_2 diffusion distances. However, the contractile functional unit remains the fiber as a whole because innervation patterns are identical in the dark and light fibers. This separation of the metabolic (fiber subdivision) and contractile (fiber) functional units in dark muscle reflect the importance of O_2 diffusion in governing fiber structure, whereas contractile function is not constrained by diffusion (Hardy et al. 2009, 2010). Similarly, subdivided fibers are present in many crustaceans, and the extent of subdivision and mitochondrial density reflects the demand for sustained contraction.

Burst contraction in crustacean muscles relies initially on AK, which catalyzes the transfer of a phosphate from the phosphagen arginine phosphate (AP) to ADP, thus forming ATP (Fig. 12.1). The AK/AP system is analogous to the creatine kinase/creatine phosphate system in vertebrates and some other invertebrates (Ellington 2001). There is typically 30–40 mM AP in crustacean light muscle, and, during successive burst contractions, the AP pools are nearly depleted (Fig. 12.3; England and Baldwin 1983, Baldwin et al. 1999, Kinsey et al. 2005, Hardy et al. 2006, Jimenez et al. 2008). For additional contractions, ATP is supplied by anaerobic glycogenolysis, which is reflected by the accumulation of lactate and depletion of glycogen, as well as by a reduced contractile speed (Fig. 12.1; Booth and McMahon 1992, Milligan et al. 1989, Morris and Adamczeskwa 2002, Johnson et al. 2004, Kinsey et al. 2005). The ATP, AMP, and ADP concentrations in decapod crustacean light muscle are higher than in insects, and AP concentration is generally higher than is creatine phosphate in vertebrates, perhaps indicative of the highly anaerobic poise of these muscles (Beis and Newsholme 1975, England and Baldwin 1983, Hill et al. 1991, Speed et al. 2001). Previous work has demonstrated that factors limiting anaerobic capacity include the size of the phosphagen and glycogen stores and sensitivity to anaerobic end-products (Baldwin et al. 1999). The glycogen content of leg muscle in the terrestrial Christmas Island red crab *Gecarcoidea natalis* and other land crabs, can become extraordinarily high when adequate food is available (Henry et al. 1994, Adamczewska and Morris 1994, 2000) and appears to be crucial in supporting the elevated level of anaerobiosis in *G. natalis* and several other species of crab (Henry et al. 1994). The large depletion of glycogen and the ensuing glycolysis ultimately require the large flux of lactate from the muscles into the hemolymph, as is also the case in other exercising decapods (Fig. 12.3; Henry et al. 1994). On the other hand, large amounts of glucose mobilized from glycogen remained as glucose-6-phosphate to directly fuel muscle glycolysis during exercise (Morris and Adamczewska 2002).

Although the metabolic processes that power burst contraction in crustaceans are similar to those in vertebrates, metabolic recovery following contraction in crustaceans does not always follow the vertebrate paradigm. Vertebrates rely exclusively on aerobic metabolism to power resynthesis of creatine phosphate, and lactate does not accumulate following contractions (Kushmerick 1983, Meyer 1988), even in highly anaerobic fish white muscle (Curtin et al. 1997). In contrast, postcontractile restoration of AP pools in crustaceans is largely powered by anaerobic glycogenolysis, leading to glycogen depletion and lactate accumulation *after* contraction (Fig. 12.3; England and Baldwin 1983, Boyle et al. 2003, Johnson et al. 2004, Kinsey et al. 2005). Postcontractile lactate accumulation in crustacean muscle appears to be a mechanism for accelerating certain phases of

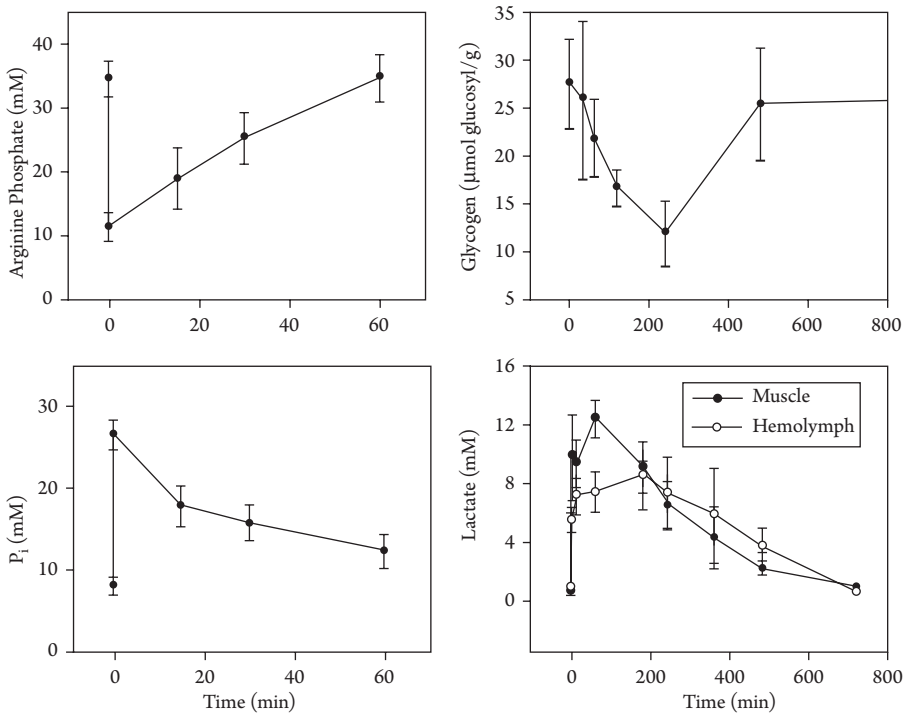


Fig. 12.3.
Changes in locomotor muscle and hemolymph metabolites following a short-burst contraction in the blue crab, *Callinectes sapidus*. Note the rapid initial change in arginine phosphate and inorganic phosphate (P_i) due to high adenosine triphosphate (ATP) demand during the burst contractile period and then the slower recovery. Glycogen is depleted, and lactate accumulates over a longer time course primarily after contraction, indicating that anaerobic glycogenolysis is largely powering the recovery of arginine phosphate and P_i, as well as other processes such as the restoration of intracellular pH (not shown). Aerobic metabolism is ultimately responsible for the slow restoration of glycogen and lactate levels. Data from Boyle et al. (2003), Johnson et al. (2004), and Kinsey et al. (2005).

the recovery process to facilitate additional high-force contractions because aerobic capacity is so low in light muscle (Boyle et al. 2003, Johnson et al. 2004, Kinsey et al. 2005, Jimenez et al. 2008). Furthermore, postcontractile lactate accumulation is much greater in the very large muscle fibers of adults compared to the small fibers of juveniles, again indicating the increasing importance of O_2 diffusion in limiting aerobic metabolic flux as fibers grow and diffusion distances increase (Boyle et al. 2003, Johnson et al. 2004, Hardy et al. 2006, Kinsey et al. 2011).

Despite their reliance on anaerobic metabolism to power specific recovery processes, complete recovery ultimately must depend on aerobic pathways. Furthermore, crustacean muscle fibers do not appear to express a lactate transporter (Kinsey and Ellington 1996), and lactate efflux from crustacean muscle is extremely slow (Milligan et al. 1989, Kinsey and Ellington 1996). The subsequent resynthesis of muscle glycogen, presumably from lactate (Milligan et al. 1989), and the restoration of intracellular pH (pH_i) occurs in situ over a protracted time course (several hours) and constitutes the aerobic phase of recovery (Kamp 1989, Milligan et al. 1989, Henry et al. 1994). The restoration of high-energy phosphates and depleted O_2 stores generally occurs more rapidly than the removal of lactate (Morris and Adamczewska 2002). Although the fate of lactate in crustaceans is still unclear, some possibilities for metabolic regulation of lactate accumulation after exercise include its reincorporation into glycogen or oxidation in a variety of tissues (Ellington 1983, Hill

et al. 1991, Lallier and Walsh 1992, Oliveira and da Silva 2000, Hervant et al. 1999), excretion into the environment (Head and Baldwin 1986, Hervant et al. 1999a), or endogenous glyconeogenesis within muscles (Henry et al. 1994, Hervant et al. 1999).

Exercise at low rates can be powered almost exclusively by aerobic metabolism in crustaceans, and, as intensity increases, the contribution of anaerobic glycolysis increases. Terrestrial crabs generally respond to exercise with a combination of elevated aerobic metabolism, further accompanied by anaerobic lactate accumulation (Herreid and Full 1988, Full and Weinstein 1992). For example, air-breathing crabs like the ghost crabs of the genus *Ocypode* exhibit large and rapid elevations in aerobic metabolism matched to high endurance, whereas fiddler crabs of the genus *Uca* fatigue more quickly and are unable to elevate O_2 uptake to the extent of ghost crabs (Herreid and Full 1988). Thus, as a general rule in crustaceans, the intensity of exercise is largely governed by the ability to accelerate aerobic metabolism and glycolysis and to tolerate accumulated anaerobic end-products (Full and Weinstein 1992, Adamczewska and Morris 2000, Weinstein 2001). Disruption of aerobic metabolism directly influences exercise capacity. For example, *C. sapidus* injected with a sublethal dose of *Vibrio campbellii* had a reduced aerobic capacity that led to a lower rate of O_2 consumption and higher lactate accumulation during exercise (Thibodeaux et al. 2009).

An interesting feature of crustacean muscles is that they are typically composed of very large fibers (cells), and because this leads to large O_2 diffusion distances that may constrain aerobic metabolism, this property has long puzzled crustacean biologists (Kinsey et al. 2007, 2011). Jimenez et al. (2011) recently showed that large fibers provide a means of reducing basal metabolic cost due to a lower surface area-to-volume ratio (SA:V) and therefore less membrane over which to maintain the membrane potential via Na^+/K^+-ATPase (NAK) transporter. In the American lobster, *Homarus americanus*, adults have abdominal muscle fibers that have a twofold larger diameter (and twofold higher SA:V) than juveniles, and this leads to a proportional twofold lower NAK activity and cost of operating the NAK in resting muscle (Fig. 12.4). We have recently found that NAK cost is proportional to fiber SA:V in muscle from a broad range of crustaceans and fishes, suggesting that fibers are large to reduce whole-animal metabolic rate (Jimenez et al. 2013).

Reproduction

The cost of reproduction can be direct, such as that associated with gamete production, or indirect, such as that associated with courtship and mating behaviors or parental care/brooding. The energetic expenditure associated with reproduction in female decapods is reflected by an increased metabolic demand (Guadagnoli et al. 2005), a doubling of food intake (Teshima et al. 1986), decreased growth rate, and increased susceptibility to predators (Berglund and Rosenqvist 1986). Lipids and proteins appear to be the principal metabolic reserves mobilized during reproduction, whereas glycogen is more important in fueling activity (Harrison 1990, Quackenbush 1994, Lee and Walker 1995, Rosa and Nunes 2003, Vinagre et al. 2007, Antunes et al. 2010). For instance, in the blue crab *C. sapidus*, lipid droplets, which form a minor component in immature ovaries, constitute nearly a third of the total lipids in mature ovaries (Lee and Walker 1995). The origin of lipids reaching the ovary is not fully understood. Lipids stored in the hepatopancreas have been shown to be transported to the ovary during vitellogenesis (Harrison 1990). However, the amount of lipids accumulated within the ovaries is greater than that stored in the hepatopancreas, suggesting some lipid synthesis likely occurs within the ovaries and developing oocytes (Millamena and Pascual 1990, Khayat et al. 1994, Palacios et al. 2000). In addition, some lipid requirements of the developing ovary seem to be more dependent on the ingestion of dietary lipids than on hepatopancreatic reserves.

Synthesis of several proteins, including enzymes and egg yolk proteins, are also important in maturation and reproduction, and vitellogenesis is accompanied by yolk protein synthesis largely in the form of high-density lipoproteins (HDL) and glycoproteins (Yehezkel et al. 2000). In decapods,

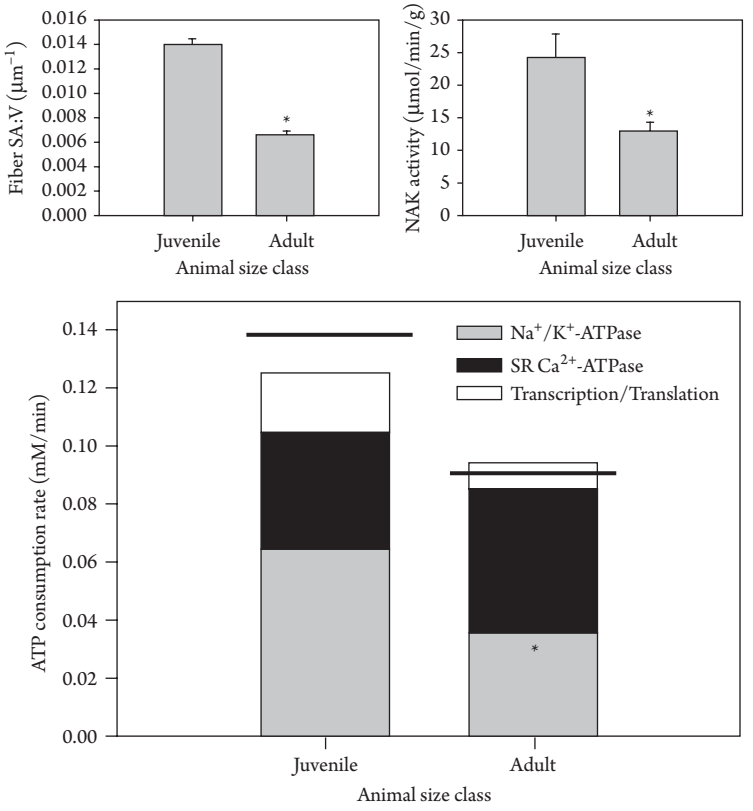


Fig. 12.4.
The effect of fiber size on metabolic maintenance costs in abdominal muscle from the American lobster, *Homarus americanus*. Fiber surface area-to-volume ratio (SA:V) was twofold lower in adults, and this resulted in a twofold lower Na^+/K^+-ATPase (NAK) activity, and a twofold lower adenosine triphosphate (ATP) cost associated with NAK function. In the lower panel, the black horizontal lines represent the total basal ATP demand in the muscle, and three processes—transcription/translation, the sarcoplasmic reticulum Ca^{2+}-ATPase, and the NAK—account for nearly all of this. Of these processes, only the NAK cost is significantly different between the size classes and has dependence on the SA:V because it is a sarcolemmal membrane protein. The difference in the NAK cost accounts for most of the difference in cost between the juveniles and adults, indicating the savings associated with the larger fibers in adults. The * indicate significant differences between size classes. Data are from Jimenez et al. (2011).

the dominant HDLs are LP-I and LP-II (also known as lipovitellin), which are important in transporting lipids from the hepatopancreas to peripheral tissues (Harrison 1990, Lee and Walker 1995, Walker et al. 2003). In *C. sapidus*, LP-I has been shown to be synthesized in the hepatopancreas in both larvae and adults. Free sterols also are abundant in developing ovaries, where they contribute to membrane structure and are precursors of hormones and steroids (Rosa and Nunes 2003, Antunes et al. 2010).

However, in addition to the biosynthetic costs associated with reproduction, there may be costs associated with parental care. Crustaceans may release eggs into the environment, brood the embryos until hatching, or carry offspring in brood pouches after hatching. The cost of brooding embryos has been examined because, among brooders, crustaceans are large animals and must expend energy in maintaining an adequate O_2 supply to their relatively large egg masses. In some brachyuran crabs, the rate of O_2 consumption can nearly double in brooding females, suggesting

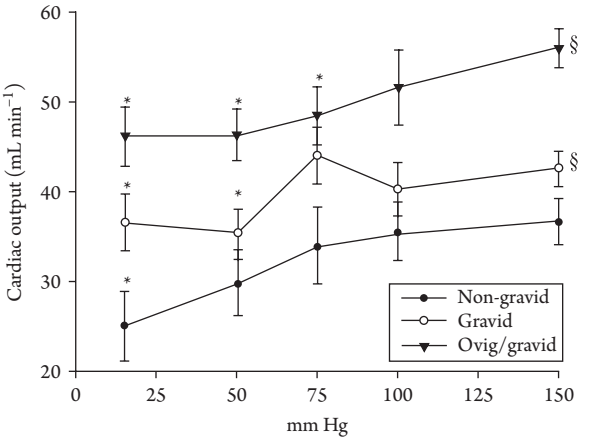


Fig. 12.5.
The effect of reproductive state and oxygen partial pressure (P_{O_2}) on cardiac output in the grass shrimp, *Palaemonetes pugio*. Ovigerous/gravid females had a higher cardiac output than gravid females, which had in turn higher rates than nongravid females, indicating the high aerobic cost of egg production and brooding. Cardiac output also decreased with declining P_{O_2}, and the higher demand of egg production and brooding reduces hypoxia tolerance because ovigerous/gravid females could maintain cardiac output at a P_{O_2} as low as 75 mm Hg, gravid females as low as 50 mm Hg, and nongravid females as low as 15 mm Hg. The § indicates significant differences between reproductive groups, and the * indicates significant differences from normoxia (150 mm Hg). Data from Guandagnoli et al. (2005), with permission from John Wiley and Sons.

that the cost of brooding can be high, whereas in other species the costs are negligible (Fernández et al. 2000, Taylor and Leelapiyanart 2001). Guadagnoli et al. (2005) showed that in the grass shrimp *P. pugio* cardiac output and pleopod fanning frequency was highest in ovigerous females, indicating the elevated cost of egg production and brooding. Ovigerous females were intermediate in cardiac output, whereas nonovigerous females had the lowest metabolic demand (Fig. 12.5).

Molting

Molting is controlled hormonally, and the high energetic cost of molting is reflected by changes in fuel storage and mobilization, as well as by changes in metabolic rate prior to and during the molt. Oxygen consumption and heart rate increases during premolt and declines following ecdysis (Penkoff and Thurberg 1982, Cockcroft and Wooldridge 1985, Kuramoto 1993), although metabolism may remain elevated in early postmolt (Mangum et al. 1985). The increased metabolic costs associated with molting are largely related to the net synthesis of protein, new cuticle formation, and remodeling of tissues. For instance, in the swimming crab *P. pelagicus*, an analysis of transcript expression revealed that genes associated with energy metabolism, such as NADH dehydrogenase, cytochrome *c* oxidase, and ATP synthase, were upregulated during the premolt period, presumably to meet the energy demands associated with molting. This is consistent with the increased expression of cuticular protein transcripts during molting (Fig. 12.6; Kuballa et al. 2011). A similar large increase in transcript expression of 23 cuticular proteins was observed during molting in Antarctic krill, *E. superba*, indicating the extensive metabolic cost associated with protein synthesis (Seear et al. 2010).

Changes in protein, lipid, and carbohydrate content of the hepatopancreas have been seen during the course of the molt cycle in several different species. Most notable is the pre-ecdysial increase in lipid content and shifts in lipid classes in the hepatopancreas that have been recorded in a number of species (Chang and O'Connor 1983, Jeckel et al. 1990, Chang 1995). This increase in

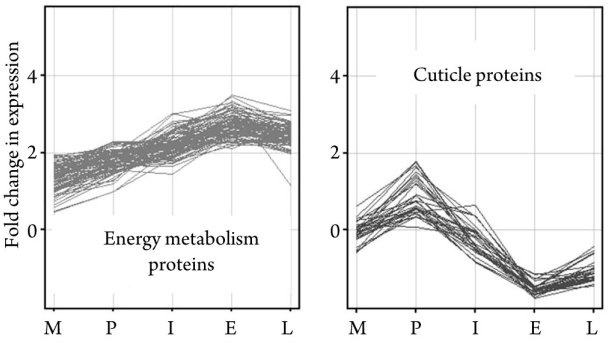


Fig. 12.6.
Changes in transcript expression during the molt cycle (M, molt; P, postmolt; I, intermolt; E, early premolt; L, late premolt). Transcripts for mitochondrial proteins involved with energy metabolism, such as the adenosine triphosphate (ATP) synthase nicotinamide adenine dinucleotide (NADH) dehydrogenase and cytochrome *c* oxidase increase during premolt, presumably to provide the ATP needed for molting, whereas cuticle protein transcripts are upregulated largely during the molt and in postmolt. Data from Kuballa et al. (2011), with permission from BioMed Central.

premolt lipid content is likely due to an increased rate of fatty acid synthesis and an increased capacity for esterification of ingested fatty acids forming triacylglycerols. For example, Bollenbacher et al. (1972) demonstrated several decades ago that the premolt increase in lipid storage in the hepatopancreas paralleled an increase in enzyme activity associated with fatty acid synthesis. There is also an increase in hemolymph glucose and hepatopancreas glycogen during premolt, which may reflect both the energy needs associated with ecdysis as well as the usage of glucose as a precursor to chitin (Chang 1995, Galindo et al. 2009). Large changes in protein expression and free amino acid levels have also been observed in the premolt hepatopancreas (Chang 1995). The amount of DNA in the hepatopancreas also increased during premolt and may be a mechanism to increase the capacity for transcription as needed for the molt (Chang 1995).

In addition to the synthesis of new cuticle proteins, there is also extensive tissue degradation in some muscles of decapod crustaceans during premolt, which allows the animal to escape from the confines of its previous exoskeleton at ecdysis (Mykles 1999). This is consistent with reduced muscle proteins during molting in the Pacific white shrimp *Litopenaeus vannamei* (de Oliveira Cesar et al. 2006). This process likely contributes to the increase in protein turnover that occurs during ecdysis in the American lobster, *H. americanus*, as reflected by higher protein synthesis rates in muscle during the molt cycle (El Haj et al. 1996).

Starvation

Unlike the conditions just described, which increase ATP demand, starvation constitutes a limitation of oxidizable fuels used to make ATP. Fuel reserves used during starvation in crustaceans have been reviewed by Sanchez-Paz et al. (2006). Several studies of crustacean metabolism have shown high variability of energy reserve mobilization during starvation, making it difficult to outline a general metabolic profile. Experimental results have led to the long-standing view that the primary source of energy in crustaceans is protein (Neiland and Scheer 1953, Claybrook 1983, Anger 2001), contrasting with the paradigm for mammals and birds, which utilize mainly carbohydrates and lipids as energy sources while sparing protein (Cherel et al. 1992). However, some crustaceans may also minimize protein depletion during starvation. For example, the glycogen and lipid stores in the hepatopancreas and muscle were depleted during starvation in the shrimp species *Penaeus japonicus* (Cuzon et al. 1980), *P. duorarum* (Schafer 1968), and *Crangon crangon* (Cuzon and Ceccaldi 1973).

In the Pacific white shrimp, *L. vannamei*, a strict reliance on carbohydrate metabolism was observed for short bouts of starvation, followed by use of plasma protein (Fig. 12.7; Sanchez-Paz et al. 2007). Similarly, for the copepod *Calanus finmarchicus* (Helland et al. 2003) and in the isopod *Stenasellus virei* (Hervant and Renault 2002), carbohydrate and lipid reserves were used early in starvation, and protein was used in the later stages. Once the animals were allowed to feed again, energy reserves were fully recovered after a period of 7–15 days of refeeding (Hervant and Renault 2002).

Experiments that reduced dietary protein by replacing it with carbohydrates suggested that the ability of shrimp to utilize carbohydrates is limited as a consequence of both the low storage capacity and the low capability of enzymatic processing (Rosas et al. 2000). However, polysaccharides may be more useful dietary sources of energy than simple sugars because they may have protein- and lipid-sparing effects. For instance, *P. monodon* shrimp fed starch or dextrin had significantly higher weight gain and survival than those fed glucose (Shiau and Peng 1992). These authors suggested that a diet high in glucose may lead to a rapid elevation of plasma glucose, leading to excretion of glucose. The ability of certain crustaceans to utilize protein for fuel may further reduce the need for simple sugars (Sanchez-Paz et al. 2006). For example, crabs fed a high-protein diet had a lower hemolymph glucose level and lower hepatopancreas and muscle glycogen levels than animals fed a high-carbohydrate diet (Oliveira et al. 2004, Pellegrino et al. 2008). During starvation, hemolymph glucose was maintained largely by hydrolysis of hepatopancreatic glycogen stores in the high-carbohydrate diet group, whereas gluconeogenic

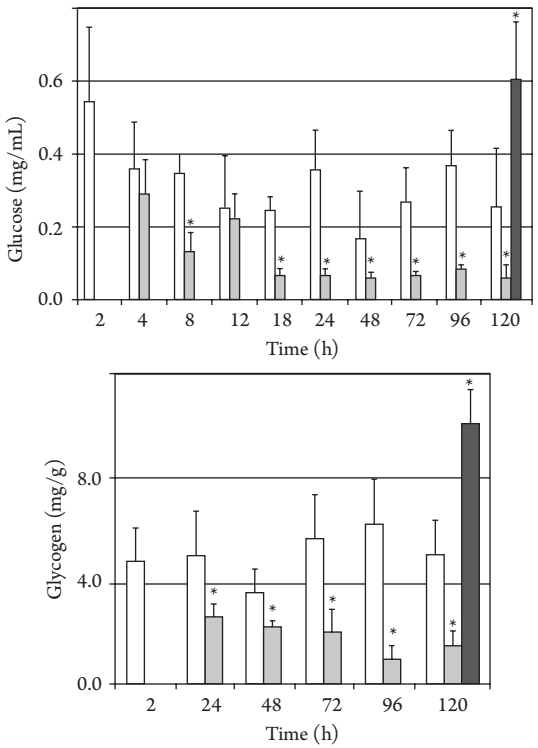


Fig. 12.7.
Hemolymph glucose and hepatopancreas glycogen during short-term starvation and refeeding in the Pacific white shrimp, *Litopenaeus vannamei*. Both glucose and glycogen decrease indicate the critical role of carbohydrates in supporting short-term starvation. Empty bars, control; gray bars, starved; black bar, refed. The * indicates significant difference from control. Data from Sanchez-Paz et al. (2007), with permission from Elsevier.

conversion of alanine to glucose and muscle glycogen synthesis from lactate appears to be the major mechanism for maintaining hemolymph glucose (and lactate) in the high-protein diet group (Oliveira et al. 2004, Pellegrino et al. 2008).

It is generally thought that the activities of digestive enzymes parallel food availability and that these enzymes are inactive until secretion, although there is limited supporting data (Sanchez-Paz et al. 2006). The crustacean hepatopancreas produces and secretes several digestive enzymes, including proteases like trypsin and chymotrypsin, lipases, and carbohydrate-degrading enzymes (Dall et al. 1990). Trypsin and chymotrypsin activities in *L. vannamei* hepatopancreas were 40–60% lower after 120 h of starvation (Muhlia-Almazan and Garcıa-Carreno 2002), whereas trypsin mRNA was 30% lower (Sanchez-Paz et al. 2003). Lipase activity has been found in crustaceans such as *L. vannamei* (Gamboa-Delgado et al. 2003), red claw crayfish *Cherax quadricarinatus* (Lopez-Lopez et al. 2003), the shrimp *Macrobrachium borellii* (Gonzalez-Baro et al. 2000), and terrestrial isopods (Zimmer 2002). However, the role and regulation of these enzymes under starvation periods is unknown. As dietary fuel sources are reduced and intracellular stores are mobilized, there is some evidence of upregulation of glycolytic enzymes. In the hepatopancreas of *L. vannamei*, the glycolytic enzyme phosphofructokinase mRNA levels increased 120-fold after 96 h of starvation, suggesting an increased reliance on cellular carbohydrate stores to maintain ATP supply in this tissue (Sanchez-Paz et al. 2007).

Responses to Environment

Temperature

Reduced temperatures make it less likely that enzymatic reactions will exceed their activation energies, thus reducing the metabolic rate. The aggregate effect of temperature on many reactions is often encapsulated by the operational term Q_{10}, where a 10°C change in temperature is associated with about a twofold change in metabolic rate, thus producing a Q_{10} of 2 (Schmidt-Nielsen 1997). During thermal stress, crustaceans must either address the temperature change by metabolically compensating, if the animal needs to remain active, or avoid the thermal stressor by drastically reducing the metabolic rate, a process called *estivation* or *brumation* (Vernberg and Vernberg 1968). Thus, there are clear energetic consequences associated with temperature acclimation associated either with compensatory remodeling of tissues or a need to cease activity.

Colson-Proch et al. (2009) found in the subterranean aquatic amphipod *Niphargus rhenorhodanensis* that cold stress caused a large reduction in ventilatory rate and locomotion, although there was no increase in lactate production, suggesting that aerobic metabolism was able to meet energy demand. This is consistent with the view that anaerobic metabolism is only invoked when temperatures drop below a critical level at which aerobic metabolism cannot keep pace with resting ATP demand (Pörtner et al. 2006). However, the glucose and glycerol pools were reduced (glycerol can be used as a precursor for antifreeze compounds or as a precursor to glucose to maintain stable hemolymph levels), and there were significant increases in the free amino acid pool, largely due to changes in concentrations of alanine, glutamine, lysine, and arginine, which all seem to be important in conferring cryoprotection (Issartel et al. 2005, Colson-Proch et al. 2009). Seasonal changes in temperature appear to alter the fuel utilization in decapods, and there can be large changes in glycogen and lipid storage in the hepatopancreas and muscle, although the seasonal patterns are variable and species-specific (Kucharski and Da Silva 1991b, Oliveira et al. 2004, Vinagre et al. 2007, Buckup et al. 2008, Pellegrino et al. 2008). These shifts in fuel storage likely reflect changes not only due to temperature, but also to seasonal differences in other factors such as activity level, reproductive status, and food intake.

When the temperature drops below a threshold level, some species can undergo brumation, which is a dramatic decrease in metabolic rate associated with a cessation in activity. For instance,

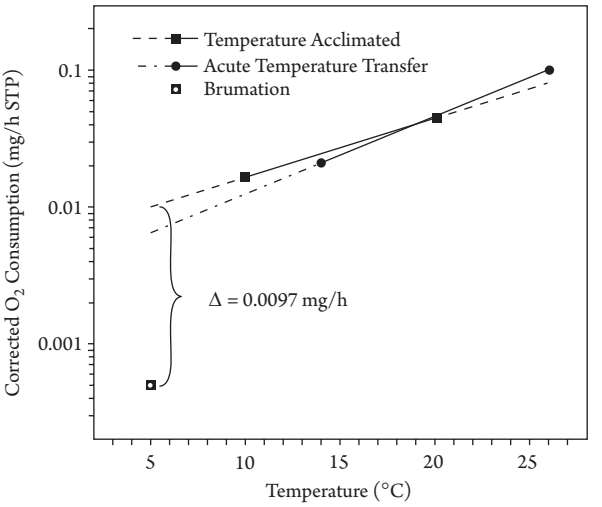


Fig. 12.8.
Oxygen consumption rates as a function of temperature in the fiddler crab, *Uca pugilator* (STP, standard temperature and pressure). The acute temperature transfer groups (circles) were acclimated to 20°C and then transferred to 14°C or 26°C. The temperature acclimated groups (squares) were acclimated to 5°C, 10°C, or 20°C. At 5°C, there was a 90-fold reduction of metabolic rate compared to crabs acclimated to 20°C, indicative of brumation, during which crabs were inactive and nonresponsive to stimuli. Data from Jimenez and Bennett (2007), with permission from Elsevier.

brumation is common in the fiddler crab *Uca pugilator* (Jimenez and Bennett 2007) and the beach flea *Talochestia megalopthalma* (Edwards and Irving 1943). This compensatory change in metabolic rate allows the animal to survive unfavorable thermal conditions in an energetically efficient manner. For example, when acclimated to 5°C, metabolic demand in brumating *U. pugilator* populations decreased 90-fold compared to active crabs acclimated to 20°C (Fig. 12.8; Jimenez and Bennett 2007).

Thermal tolerance range is particularly important in dictating vertical and latitudinal zonation patterns in the intertidal zone (Somero 2002). For example, among porcelain crabs in the genus *Petrolisthes*, tolerance to high habitat temperatures is associated with a capacity to maintain heart rate, O_2 consumption rates, and nerve action potentials and to limit lactate production during a high-temperature challenge (Stillman and Somero 1996, Somero 2002). At the cell level, some of these thermal adaptations likely reflect tradeoffs between protein flexibility and substrate binding affinity and alterations in membrane fluidity that preserve enzyme and membrane function under different temperature regimes (Hochachka and Somero 2002). For instance, there is some evidence that citrate synthase and pyruvate kinase substrate affinities are adjusted in response to acclimation temperature in the krill *Meganyctiphanes norvegica* and in the isopod genus *Idotea* over a seasonal temperature range (Vetter and Buchholz 1997, Buchholz and Saborowski 2000).

Hypoxia and Anoxia

Tolerance of hypoxia or anoxia in crustaceans is highly species-specific, and although some representatives are O_2 conformers, most complex species have the ability to regulate O_2 consumption even at low partial pressures by increasing physiological parameters such as heart rate and ventilation rate (McMahon 2001). However, when O_2 levels fall below the O_2 regulating range, three types of adaptation appear to permit survival: maintenance of large stores of glycogen and AP in tissues, utilization of anaerobic pathways to produce ATP and to maintain redox balance in anaerobic

conditions, and the reduction of metabolic rate (Oliveira et al. 2001). Unlike other invertebrates that may generate a variety of anaerobic end-products (Hochachaka and Somero 2002), crustaceans employ only classical anaerobic glycolysis, leading to lactate as the sole end-product (Oliveira et al. 2001, Abe et al. 2007). For example, the ghost shrimp *Lepidophthalmus louisianensis* maintains an ATP production rate at near-aerobic levels during the first 12 h of anoxia, and between 12 h and 48 h of anoxia still supports about 50% of its ATP demand via aerobic metabolism. However, after 48 h, there is a large accumulation of lactate, as well as a major depression of metabolism (Holman and Hand 2009).

The phosphagen AP provides an immediate but fairly rapidly depleted source of ATP during anaerobiosis and, in addition, helps limit acidification because AP hydrolysis is a proton-consuming process (Ellington 2001). Hypoxia or anoxia in muscles of the shrimp species *Marsupenaeus japonicus* and *L. louisianensis* led to a near depletion of AP during the early stages of hypoxia, whereas ATP levels remained fairly constant. As AP was depleted, glycogenolysis became the dominant source of ATP, and lactate gradually accumulated (Abe et al. 2007, Holman and Hand 2009). However, in *M. japonicus* muscle, the glycolytic enzyme fructose bisphosphate aldolase was downregulated after 6 h of hypoxia, suggesting a suppression of glycolysis perhaps due to cellular acidification or a whole-organism level metabolic downregulation (Abe et al. 2007). In fact, metabolic rate depression is probably essential for extended bouts of severe hypoxia or anoxia (Hill et al. 1991, Holman and Hand 2009). It has even been proposed that crustaceans intentionally maintain low arterial blood O_2 (even when environmental O_2 is high), which limits metabolic rate somewhat but also prevents excessive reactive O_2 species production associated with high cellular O_2 (Massabuau 2001, Corbari et al. 2004).

Recovery from hypoxia or anoxia entails replenishment of ATP, AP, and glycogen stores, as well as a restoration of pH. These processes may be powered exclusively by aerobic metabolism without the accumulation of additional lactate (Oliveira et al. 2001, Abe et al. 2007), or there may be additional glycogen depletion and lactate accumulation during recovery, similar to the case for burst contraction (Hill et al. 1991). The latter case likely reflects a mechanism to speed up key phases of recovery in tissues such as muscle, which in crustaceans often has a very low aerobic capacity as well as possible diffusion constraints on aerobic metabolism (Kinsey et al. 2007, 2011). Three pathways have been proposed for the clearance of lactate: complete oxidation, conversion into products such as glycogen, and excretion (Ellington 1983), and it appears that crustaceans rely on all three processes to eliminate lactate (Gade and Grieshaber 1986, Hill et al. 1991, Henry et al. 1994, Hervant et al. 1999, Oliveira et al. 2001, 2004, Marqueze et al. 2006, Maciel et al. 2008).

Although transient exposure to hypoxia is common in some coastal ecosystems, midwater crustaceans that reside in the O_2 minimum zones of the oceanic water column may be chronically exposed to hypoxia. The mysid shrimp *Gnathophausia ingens* resides in the O_2 minimum zone and has been the subject of considerable study (Childress and Seibel 1998). This species has a number of traits that allow it to maintain aerobic function in the O_2 minimum layer, including a high ventilatory rate and circulatory capacity; a high gill surface area; short O_2 diffusion distances across the gills; and hemocyanin with a high affinity for O_2, high cooperativity, a large Bohr effect to aid delivery of O_2 to tissues, and a low concentration, which presumably lowers the circulatory costs. These traits lead to O_2 extraction from the water that may be as great as 90%, thus allowing O_2 consumption rates to be regulated even at the lowest O_2 pressures encountered in the environment (Childress and Seibel 1998). However, as O_2 falls below critical levels, a reduction in metabolic rate or reliance on anaerobic metabolism is necessary to maintain energy homeostasis (Seibel 2011). Oxygen minimum zones also intercept the benthos at continental margins and can create vast regions of essentially permanent, severely hypoxic benthic habitats (Levin 2003). Squat lobsters are highly tolerant of hypoxia and are one of the relatively few large invertebrates that can exploit these habitats (Zainal et al. 1992, Matabos et al. 2012). Adult squat lobsters in the genus *Munida* have been

shown to use the same increased ventilator response just described to regulate O_2 consumption over a broad range of O_2 tensions (Zainal et al. 1992). The larvae of the squat lobster *Pleuroncodes monodon*, which are often released into the O_2 minimum zone, are also highly tolerant to hypoxia, although less so than the adult. As the larvae develop from zoeal stages to megalopae, the capacity to regulate O_2 consumption increases, which corresponds to a shift toward lower O_2 environments (Yannicelli et al. 2013, Yannicelli and Castro 2013). The expected increase in hypoxic zones on continental shelves associated with global climate change will favor the relatively few hypoxia-tolerant species, leading to a reduction in community diversity (Matabos et al. 2012).

The molecular basis of the hypoxic response in crustaceans is not well resolved. Crustaceans express a hypoxia inducible factor-1 (HIF-1) that has homology with vertebrate HIF (Hoogewijs et al. 2007), but the regulation of expression may not follow the vertebrate paradigm (Li and Brouwer 2007, Soñanez-Organis et al. 2009, Head 2010). For example, in the Pacific white shrimp *L. vannamei*, HIF-1α has a decreased transcript level during hypoxia in gill, muscle, and hepatopancreas (Soñanez-Organis et al. 2009). In mammals, HIF-1α is typically regulated post-translationally, and the protein levels increase during hypoxia, so the surprising downregulation of transcript expression led these authors to suggest that elevated HIF-1α protein may invoke a decrease in mRNA levels. A follow-up study on *L. vannamei*, Soñanez-Organis et al. (2011) found that activity of the glycolytic enzyme HK was upregulated during hypoxia, and this effect was blocked in gills and reduced in muscle, when the HIF-1α or -β expression was silenced (Fig. 12.9). Head (2010) further suggested that HIF-1 may regulate the increase in blood hemocyanin content and changes in isoform expression that accompany hypoxia in the crab *Cancer magister*.

Salinity

The energetic challenge associated with osmoregulation in euryhaline crustaceans appears to be largely associated with (i) anisosmotic regulation of the extracellular fluid, where the osmolality of the hemolymph is independent of the osmolality of the external medium, and (ii) isosmotic regulation of the intracellular fluid (Gilles and Delphire 1997). Hemolymph osmolality is regulated during hyper- or hyposmotic regulation in crustaceans primarily by a suite of ion transporters, which generally occur in specialized tissues such as the gills (McNamara and Faria 2012). For instance, in decapods such as the blue crab *C. sapidus*, which has been a principal subject in studies of osmoregulation, the posterior gills are specialized for ion transport whereas the anterior gills are largely respiratory (Mantel and Farmer 1983, Towle and Weihrauch 2001). The energetic cost associated with ion transport in *C. sapidus* is reflected by the greater capacity of the posterior gills to produce ATP (Piller et al. 1995, Kinsey et al. 2003) and to upregulate ion transport proteins and increase gill O_2 consumption under hypo-osmotic stress (Péqueux 1995). These responses lead, in part, to increases in whole-animal respiration in response to reduced salinity, although the effects may be relatively small in some species (Guerin and Stickle 1997, McGaw and McMahon 2003). For instance, the small increase in cardiac output during low salinity exposure in *C. sapidus* was associated with increased blood flow to the legs and mouthparts and behavioral changes, rather than to ion pumping per se (Fig. 12.10; McGaw and Reiber 1998), whereas blood flow rates and cardiac dynamics were only modestly influenced by salinity in the Dungeness crab, *C. magister* (McGaw and McMahon 2003). This suggests that ion transport associated with osmoregulation may incur a relatively small energetic cost, particularly in highly euryhaline species because it is often confined to the posterior gills, which represent a very small fraction of body mass. Nevertheless, crustaceans maintained in suboptimal salinities do often show reduced growth rates and feed conversion rates, and the view that this effect is due to energetic stress cannot be ruled out (Romano and Zeng 2012).

Although ion pumps in the gills play a central role in osmoregulation, there can still be significant variation in hemolymph osmolarity, and the intracellular fluid must remain isosmotic with the hemolymph to minimize cell volume changes. This is accomplished by increasing the concentration

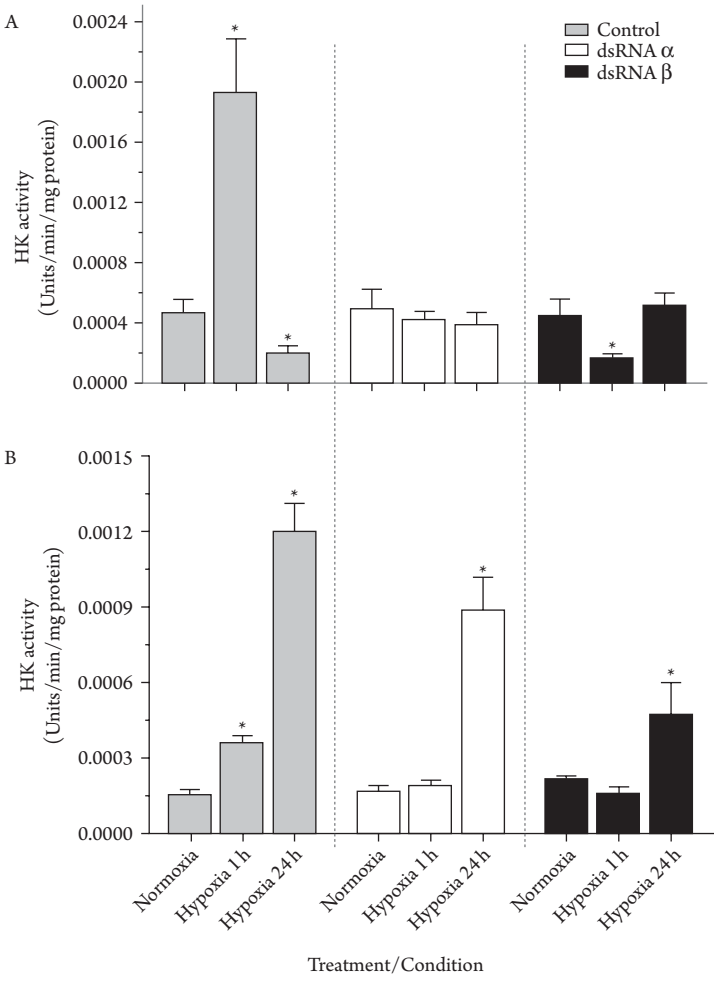


Fig. 12.9.
Hexokinase (HK) activity during hypoxia in (A) gills and (B) muscle from the shrimp *Litopenaeus vannamei*, as a function of silencing of hypoxia inducible factor-1 (HIF-1α and HIF-1β) with double stranded RNA (dsRNA). HK activity increases under hypoxia in controls, whereas silencing of either HIF-1 subunit eliminates or reduces the response, indicating control of HK activity by HIF-1. The * indicates a significant difference from the normoxia group. Data from Soñanez-Organis et al. (2011).

of certain free amino acids and other compatible or counteracting solutes that, unlike some inorganic ions, do not interfere with protein or DNA structure (Gilles and Delpire 1997, Yancey 2001). For instance, Holt and Kinsey (2002) showed that in isolated superfused muscle from *C. sapidus*, extracellular osmolarity increases caused a reduction in AK function, presumably due to the influx of inorganic ions. However, in a follow-up study, changes in salinity of a similar magnitude did not alter AK function in vivo, suggesting that the ion pumping capacity of the gills moderated changes in blood osmolarity (Kinsey and Lee 2003). Thus, the integrated responses—immediate increases in gill ion pumping followed by a slower accumulation of compatible solutes—allowed *C. sapidus* to preserve enzyme function in vivo despite large acute changes in salinity.

Much of our understanding of the sources of amino acids and other compatible solutes and the mechanisms of removal from both the intracellular fluid and hemolymph is derived from the

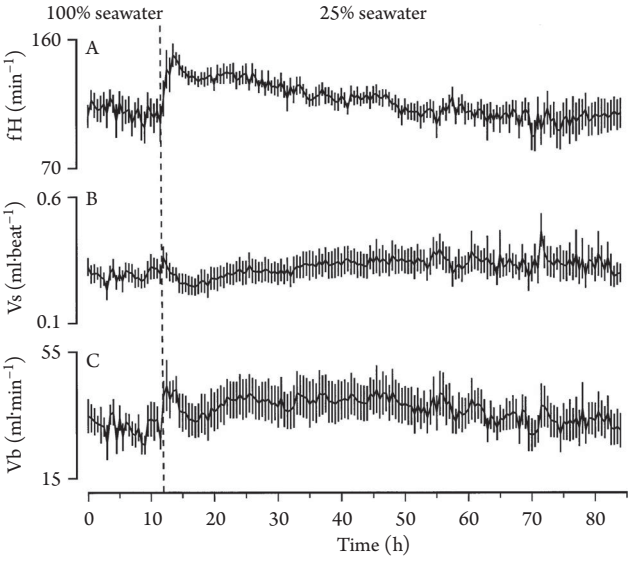


Fig. 12.10.
The effect of reduced salinity on (A) heart rate, (B) cardiac stroke volume, and (C) cardiac output in the blue crab, *Callinectes sapidus*. Note the elevation in heart rate and cardiac output that occurs due to hyposmotic regulation, suggesting a modest energetic cost that gradually decreases during 2–3 days of acclimation. Data from McGaw and Reiber (1998), with permission from Elsevier.

numerous metabolic studies that were conducted several decades ago (Claybrook 1983). In the crab *Neohelice granulata*, hyperosmotic stress leads to a decrease in lipid concentrations in the hepatopancreas, muscle, and posterior gills suggesting that increased lipid oxidation is associated with hyper- and hypo-osmotic regulation (Luvizzotto-Santos et al. 2003, Chitto et al. 2001). In addition, hepatopancreatic and muscular gluconeogenesis is involved in osmoregulatory adjustments (Oliveira and Da Silva 2000, Schein et al. 2005), suggesting that free amino acids released from different organs during hypo-osmotic stress are deaminated in the hepatopancreas, and the carbon chains are used as a substrate for the gluconeogenic pathway (Oliveira and Da Silva 2000). Consistent with this view, Martins et al. (2011) also found an upregulation of gluconeogenic enzyme activities in the gills and hepatopancreas of *N. granulata* during changes in salinity.

COMPARISON WITH OTHER TAXA

The basic metabolic pathways and mechanisms of regulation of energy metabolism in crustaceans are largely the same as in other invertebrates and vertebrates, although there are some notable differences. For instance, the first line of defense in defending ATP levels in response to an energetic challenge in most animals is phosphagen hydrolysis. However, crustaceans rely exclusively on the phosphagen AP, whereas other invertebrate groups have a variety of phosphagens, and vertebrates have only creatine phosphate (Ellington 2001). Similarly, crustaceans produce only lactate as an anaerobic end-product, as do vertebrates, whereas other invertebrates produce a wide range of metabolic end-products in order to maintain cellular redox balance during anaerobiosis (Hochachka and Somero 2002). Crustaceans produce urea, but lack a formal urea cycle analogous to that in mammals, and they also excrete uric acid and ammonia (Claybrook 1983). The fact that crustaceans, like other arthropods, grow by molting means that growth is highly pulsatile. This

leads to major swings in energy metabolism and fuel storage/utilization associated with the molt cycle, which is different from vertebrates and most other invertebrates that do not grow incrementally. There is also substantial energy devoted to the synthesis of the cuticle in crustaceans (Chang 1995). Many crustaceans also undergo an increase in body mass during growth and development that encompasses several orders of magnitude. In addition, the transition from planktonic larvae to benthic organisms represents a major reorganization of the body plan. Among the vertebrates, only fishes experience such a broad size range within a species. Thus, many crustaceans experience very different respiratory challenges during growth as cellular and egg mass O_2 transport distances increase with animal size (Fernández et al. 2000, Taylor and Leelapiyanart 2001, Kinsey et al. 2011).

FUTURE DIRECTIONS

Prior work summarized here and in earlier reviews has provided a sound basis for our understanding of crustacean energetics and metabolic regulation and has given us a platform for extending this work to areas that are likely to be increasingly important. In our view, there are three major areas where advancements are needed. First, climate change is likely to alter species distributions in a dramatic fashion. This will lead to species composition changes that are likely to alter entire ecosystems. Understanding the manner in which climate change impacts individual species will aid our understanding of which populations are likely to extend their range, perhaps leading to a wide range of new invasive species, and which species are likely to have their range reduced or to even face extinction. Second, population growth and further development of coastal and terrestrial habitats are likely to lead to an increased frequency and duration of deleterious events such as hypoxic episodes, harmful algal blooms, or toxin loading. A better understanding of the metabolic consequences of these challenges will aid our ability to manage ecosystems, and key species may be useful indicators of impending deleterious conditions. Finally, much of the current and future work is devoted to understanding the energetic and nutritional conditions that maximize aquaculture production. As coastal crustacean populations are increasingly under duress and natural stocks face increasing harvesting pressure, the need for efficient aquaculture operations will likely continue to grow.

CONCLUSIONS

Much of the early work on crustacean energetics and metabolic regulation centered on basic aspects of biochemistry and physiology. These studies demonstrated that most of the biochemistry of crustaceans was similar to that of higher vertebrates, although there are notable departures. Most recent work has probed mechanisms by which crustaceans respond energetically to different physiological states, as well as to abiotic environmental challenges. We have attempted to summarize some of the work conducted in the past two to three decades in an effort to point out both consistent patterns among groups as well as species specific responses. Much of the future work on crustacean energetics will likely focus on issues such as the effects of climate change on species distributions and invasions, as well as means of enhancing the productivity of marketable species in an aquaculture setting.

ACKNOWLEDGMENTS

This work was supported by a National Science Foundation grant to S.T.K. (IOS-0719123).

REFERENCES

Abe, H., S. Hirai, and S. Okada. 2007. Metabolic responses and arginine kinase expression under hypoxic stress of the kuruma prawn *Marsupenaeus japonicus*. Comparative Biochemistry and Physiology 146:40–46.

Adamzczewska, A.M., and S. Morris. 1994. Exercise in the terrestrial Christmas Island red crab, *Gecarcoidea natalis*. Journal of Experimental Biology 188:235–256.

Adamczewska, A.M., and S. Morris. 2000. Respiratory gas transport, metabolic status and locomotor capacity of the Christmas Island red crab *Gecarcoidea natalis* assessed in the field with respect to dichotomous seasonal activity levels. Journal of Experimental Zoology 286:552–562.

Anger, K. 2001. The biology of decapod crustacean larvae. Pages 1–419 *in* R. Vonk, editor. Crustacean issues 14. A.A. Balkema Publishers, Lisse, The Netherlands.

Antunes, G.F., A.P. Nunes do Amaral, F.P. Ribarcki, E.F. Wiilland, D.M. Zancan, and A.S Vinagre. 2010. Seasonal variations in the biochemical composition and reproductive cycle of the ghost crab *Ocypode quadrata* (Fabricius, 1787) in Southern Brazil. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 313:A280–291.

Baldwin, J., A. Gupta, and X. Iglesias. 1999. Scaling of anaerobic energy metabolism during tail flipping behaviour in the freshwater crayfish, *Cherax destructor*. Marine and Freshwater Research 5:183–187.

Beis, I.D., and E.A. Newsholme. 1975. The content of adenine nucleotides, phosphagens and some glycolytic intermediates in resting muscle from vertebrates and invertebrates. Biochemical Journal 152:23–32.

Berglund, A., and G. Rosenqvist. 1986. Reproductive costs in the prawn *Palaemon adspersus*: effects on growth and predator vulnerability. Oikos 46:349–354.

Bohenbacher, W.E., S.M. Flechner, and J.D. O'Connor. 1972. Regulation of lipid synthesis during early premolt in decapod crustaceans. Comparative Biochemistry and Physiology 42B:157–165.

Booth, C.E., and B.R. McMahon. 1992. Aerobic capacity of the blue crab, *Callinectes sapidus*. Physiological Zoology 65:1074–1091.

Boyle, K.L., R.M. Dillaman, and S.T. Kinsey. 2003. Mitochondrial distribution and glycogen dynamics suggest diffusion constraints in muscle fibers of the blue crab, *Callinectes sapidus*. Journal of Experimental Zoology 297A:1–16.

Brouwer, M., P. Larkin, N. Brown-Peterson, C. King, S. Manning, and N. Denslow. 2004. Effects of hypoxia on gene and protein expression in the blue crab, *Callinectes sapidus*. Marine Environmental Research 58:787–792.

Brouwer, M., M.J. Brown-Peterson, T. Hoexum-Brouwer, S. Manning, and N. Denslow. 2008. Changes in mitochondrial gene and protein expression in grass shrimp, *Palaemonetes pugio*, exposed to chronic hypoxia. Marine Environmental Research 66:143–145.

Buchholz, F., and R. Saborowski. 2000. Metabolic and enzymatic adaptations in northern krill, *Meganyctiphanes norvegica*, and Antarctic krill, *Euphausia superba* Canadian Journal of Fisheries and Aquatic Sciences 57:115–129.

Buckup, L., B.K. Dutra, F.P. Ribarcki, F.A. Fernandes, C.K. Noro, G.T. Oliveira, and A.S. Vinagre. 2008. Seasonal variations in the biochemical composition of the crayfish *Parastacus defossus* (Crustacea, Decapoda) in its natural environment. Comparative Biochemistry and Physiology—Part A: Molecular & Integrative Physiology 149:59–67.

Cameron, J.N., and C.P. Mangum. 1983. Environmental adaptations of the respiratory system: ventilation, circulation, and oxygen transport. Pages 43–63 *in* D.E. Bliss, editor-in-chief. The biology of Crustacea. Vol. 8. F.J. Vernberg and W.B. Vernberg, editors. Environmental adaptations. Academic Press, New York.

Chang, E.S. 1995. Physiological and biochemical changes during the molt cycle in decapod crustaceans: an overview. Journal of Experimental Marine Biology and Ecology 193:1–14.

Chang E.S., and J.D. O'Connor. 1983. Metabolism and transport of carbohydrates and lipids. Pages 263–289 *in* L.H. Mantel, editor. The biology of Crustacea. Vol. 5. Academic Press, New York.

Chang, E.S., S.A. Chang, R. Keller, P.S. Reddy, M.J. Snyder, and J.L. Spees. 1999. Quantification of stress in lobsters: crustacean hyperglycemic hormone, stress proteins, and gene expression. American Zoologist 39:487–495.

Cherel, Y., J.P. Robin, A. Heitz, C. Calgari, and Y. Le Maho. 1992. Relationships between lipid availability and protein utilization during prolonged fasting. Journal of Comparative Physiology 4B:305–313.

Childress, J.J., and B.A. Seibel. 1998. Life at stable low oxygen: adaptations of animals to oceanic oxygen minimum layers. Journal of Experimental Biology. 201:1223–1232.

Chitto, A.L., L.C. Kucharski, and R.S.M. Da Silva. 2001. Atividade da fosfoenolpiruvato carboxiquinase (PEPCK) e o efeito do acido picolı´nico sobre a gliconeogenese em branquias de caranguejo Chasmagnathus granulata. XVI Reuniao Anual da Federacao de Sociedades de Biologia Experimental, Caxambu, MG, Brazil, Abstract, p. 416.

Claybrook, D.L. 1983. Nitrogen metabolism. Pages 163–202 *in* Mantel, L.H., editor. The biology of Crustacea. Internal anatomy and physiological regulation, Vol. 5. Academic Press, New York.

Cockcroft, A.C., and T. Wooldridge. 1985. The effects of mass, temperature and molting on the respiration of *Macropetasma africanus* Balss (Decapoda: Penaeoidea). Comparative Biochemistry and Physiology 8lA:143–148.

Colson-Proch, C., A. Morales, F. Hervant, L. Konecny, C. Moulin, and C.J. Douady. 2009. First cellular approach of the effects of global warming on groundwater organisms: a study of the HSP70 gene expression. Cell Stress Chaperones 3:259–270.

Corbari, L., P. Carbonel, and J.-C. Massabuau. 2004. How a low tissue O_2 strategy could be conserved in early crustaceans: the example of the podocopid ostracods. Journal of Experimental Biology 207:4415–4425.

Curtin, N.A., M.J. Kushmerick, R.W. Wiseman, and R.C. Woledge. 1997. Recovery after contraction of white muscle fibres from the dogfish, *Scyliorhinus canicula*. Journal of Experimental Biology 200:1061–1071.

Cuzon, G., and H.J. Ceccaldi. 1973. Influence of the fasting stabulation on the metabolism of the shrimp *Crangon crangon* (L.). Compte Rendu Social Biologies 167:66–69.

Cuzon, G., C. Caha, J.F. Aldrin, J.L. Messager, G. Stephan, and M. Mevel. 1980. Starvation effect on metabolism of *Penaeus japonicus*. Proceedings of the World Mariculture. Society 11:410–423.

Dall, W., B.J. Hill, P.C. Rothlisberg, and D.J. Staples. 1990. The biology of the Penaeidae. Advances in Marine Biology 27:1–489.

de Oliveira Cesar, J.R., B. Zhao, S. Malecha, H. Ako, and J. Yang. 2006. Morphological and biochemical changes in the muscle of the marine shrimp *Litopenaeus vannamei* during the molt cycle. Aquaculture 261:688–694.

Edwards, A., and L. Irving. 1943. The influence of temperature and season upon the oxygen consumption of the sand crab. *Emerita tufpoida* (Say). Journal of Cellular and Comparative Physiology 21:161–182.

El Haj, A.J., S.R. Clarke, P. Harrison, and E.S. Chang. 1996. *In vivo* muscle protein synthesis rates in the American lobster *Homarus americanus* during the moult cycle and in response to 20-hydroxyecdysone. Journal of Experimental Biology 199:579–585.

Ellington, W.R. 1983. The recovery from anaerobic metabolism in invertebrates. Journal of Experimental Zoology 228:431–444.

Ellington, W.R. 2001. Evolution and physiological roles of phosphagen systems. Annual Reviews of Physiology 63:289–325.

England, W.R., and J. Baldwin. 1983. Anaerobic energy metabolism in the tail musculature of the Australian yabby, *Cherax destructor*: role of phosphagens and anaerobic glycolysis during escape behavior. Physiological Zoology 56:614–622.

England W.R., and J. Baldwin. 1985. Anaerobic energy metabolism in the tail musculature of the Australian yabby *Cherax destructor* (Crustacea, Decapoda, Parastacidae). Regulation of anaerobic glycolysis. Comparative Biochemistry and Physiology 80B:327–335.

Fernández, M., C. Bock, and H. Pörtner. 2000. The cost of being a caring mother: the ignored factor in the reproduction of marine invertebrates. Ecology Letters 3:487–494.

Full, R.J., and R.B. Weinstein. 1992. Integrating the physiology, mechanics and behavior of rapid running ghost crabs: slow and steady doesn't always win the race. American Zoologist 32:382–395.

Gade, G., and M.K. Grieshaber. 1986. Pyruvate reductases catalyze the formation oflactate and opines in anaerobic invertebrates. Comparative Biochemistry and Physiology 83B:255–272.

Galindo, C., G. Gaxiola, G. Cuzon, and X. Chiappa-Carrara. 2009. Physiological and biochemical variations during the molt cycle in juvenile *Litopenaeus vannamei* under laboratory conditions. Journal of Crustacean Biology 29:544–549.

Gamboa-Delgado, J., C. Molina-Poveda, and C. Cahu. 2003. Digestive enzyme activity and food ingesta in juvenile shrimp *Litopenaeus vannamei* (Boone, 1931) as a function of body weight. Aquaculture Research 15:1403–1411.

Garcia, F., M. Gonzalez-Baro, and R. Pollero. 2002. Transfer of lipids between hemolymph and hepatopancreas in the shrimp *Macrobrachium borellii*. Lipids 37:581–585.

Gilles, R., and E. Delpire. 1997. Variations in salinity, osmolarity, and water availability: vertebrates and invertebrates. Pages 1523–1586 *in* W.H. Dantzler, editor. Handbook of comparative physiology, Vol. 2. Oxford University Press, New York.

Gilles, R., and A. Pequeux. 1983. Interactions of chemical and osmotic regulation with the environment. Pages 109–165 *in* D.E. Bliss, editor-in-chief. The biology of Crustacea. F. Vernberg and W.B. Vernberg, editors. Vol. 8: Environmental adaptations. Academic Press, New York, New York.

Gonzalez-Baro, M.R., H. Heras, and R.J. Pollero. 2000. Enzyme activities involved in lipid metabolism during embryonic development of *Macrobrachium borellii*. Journal of Experimental Zoology 286:231–237.

Gorr, T.A., J.D. Cahn, H. Yamagata, and H.F. Bunn. 2004. Hypoxia induced synthesis of hemoglobin in the crustacean *Daphnia magna* is hypoxia-inducible factor-dependent. Journal of Biological Chemistry 279:36038–36047.

Guadagnoli, J.A., L.A. Jones, and C.L. Reiber. 2005. The influence of reproductive state on cardiac parameters and hypoxia tolerance in the grass shrimp, *Palaemonetes pugio*. Functional Ecology 19:976–981.

Guerin, J.L., and W.B. Stickle. 1997. Effect of salinity on survival and bioenergetics of juvenile lesser blue crabs, *Callinectes similis*. Marine Biology 129:63–69.

Hardy, K.M., B.R. Locke, M. Da Silva, and S.T. Kinsey. 2006. A reaction-diffusion analysis of energetics in large muscle fibers secondarily evolved for aerobic locomotor function. Journal of Experimental Biology 209:3610–3620.

Hardy, K.M., R.M. Dillaman, B.R. Locke, and S.T. Kinsey. 2009. A skeletal muscle model of extreme hypertrophic growth reveals the influence of diffusion on cellular design. American Journal of Physiology, Integrative Comparative and Regulatory Physiology 296:R1855–R1867.

Hardy, K.M., S.C. Lema, and S.T. Kinsey. 2010. The metabolic demands of swimming behavior influence the evolution of skeletal muscle fiber design in the brachyuran crab family Portunidae. Marine Biology 157:221–236.

Harrison, K.E. 1990. The role of nutrition in maturation, reproduction and embryonic development of decapod crustaceans, a review. Journal of Shellfish Research 9:l–28.

Head, G., and J. Baldwin, J. 1986. Energy metabolism and the fate of lactate during recovery from exercise in the Australian freshwater crayfish, *Cherax destructor*. Australian Journal of Marine and Freshwater Research 37:641–646.

Head, J.M. 2010. The effects of hypoxia on hemocyanin regulation in *Cancer magister*: possible role of Hypoxia-Inducible Factor-1. Journal of Experimental Marine Biology and Ecology 386:77–85.

Helland, S., B.F. Terjesen, and L. Berg. 2003. Free amino acid and protein content in the planktonic copepod *Temora longicornis* compared to *Artemia franciscana*. Aquaculture 215:213–228.

Henry, R.P., C.E. Booth, F.H. Lallier, and P.J. Walsh. 1994. Postexercise lactate production and metabolism in three species of aquatic and terrestrial decapod crustaceans. Journal of Experimental Biology 186:215–234.

Herreid, C.F., and R.J. Full. 1988. Energetics and locomotion. Pages 333–377 *in* W.W. Burggren and B.R. McMahon, editors. Biology of the land crabs. Cambridge University Press, New York.

Hervant, F., and D. Renault. 2002. Long-term fasting and realimentation in hypogean and epigean isopods: a proposed adaptive strategy for groundwater organisms. Journal of Experimental Biology 205:2079–2087.

Hervant, F., D. Garin, J. Mathieu, and A. Freminet. 1999. Lactate metabolism and glucose turnover in the subterranean crustacean *Niphargus virei* during post-hypoxic recovery. Journal of Experimental Biology 205:579–592.

Hill A.D., A.C. Taylor, and R.H.C. Strang. 1991. Physiological and metabolic responses of the shore crab *Carcinus maenas* (L.) during environmental anoxia and subsequent recovery. Journal of Experimental Marine Biology and Ecology 150:31–50.

Hochachka, P.W., and G.N. Somero. 2002. Biochemical adaptation—mechanism and process in physiological evolution. Oxford University Press, Oxford, U.K.

Holman, D., and S.C. Hand. 2009. Metabolic depression is delayed and mitochondrial impairment averted during prolonged anoxia in the ghost shrimp, *Lepidophthalmus louisianensis* (Schmitt, 1935). Journal of Experimental Marine Biology and Ecology 376:85–93.

Hoogewijs, D., N.B. Terwilliger, K.A. Webster, J.A. Powell-Coffman, S. Tokishita, H. Yamagata, T. Hankein, T. Burmester, K.T. Rytkönen, M. Nikinmaa, D. Abele, K. Heise, M. Lucassen, J. Fandrey, P.H. Maxwell, S. Påhlman, and T.A. Gorr. 2007. From critters to cancers: bridging comparative and clinical research on oxygen sensing, HIF signaling, and adaptations towards hypoxia. Integrative and Comparative Biology 47:552–577.

Houlihan, I.F., and A.J. Innes. 1984. The cost of walking in crabs: aerial and aquatic oxygen consumption during activity of two species of intertidal crab. Comparative Biochemistry and Physiology 77A:325–334.

Issartel, J., F. Hervant, Y. Voituron, D. Renault, and P. Vernon. 2005. Behavioural, ventilatory and respiratory responses of epigean and hypogean crustaceans to different temperatures. Comparative Biochemistry and Physiology 141A:1–7.

Jeckel, W.H., J.E.A. de Moreno, and V.J. Moreno. 1990. Changes in biochemical composition and lipids of the digestive gland in females of the shrimp *Pfeoticus muelleri* (Bate) during the molting cycle. Comparative Biochemistry and Physiology 96B:521–525.

Jiang, H., Y.M. Cai, L.Q. Chen, X.W. Zhang, S.N. Hu, and Q. Wang. 2009. Functional annotation and analysis of expressed sequence tags from the hepatopancreas of mitten crab (*Eriocheir sinensis*). Marine Biotechnology 11:317–326.

Jimenez, A.G., and W.A. Bennett. 2007. Metabolic responses of sand fiddler crabs, *Uca pugilator*, in northwest Florida to seasonal temperature change. Journal of Thermal Biology 32:308–313.

Jimenez, A.G., B.R. Locke, and S.T. Kinsey. 2008. The influence of oxygen and high-energy phosphate diffusion on metabolic scaling in three species of tail-flipping crustaceans. Journal of Experimental Biology 211:3214–3225.

Jimenez, A.G., S.K. Dasika, B.R. Locke, and S.T. Kinsey. 2011. An evaluation of muscle maintenance costs during fiber hypertrophy in the lobster, *Homarus americanus*: are larger muscle fibers cheaper to maintain? Journal of Experimental Biology 214:3688–3697.

Jimenez, A.G., R.M. Dillaman, and S.T. Kinsey. 2013. Large fiber size in skeletal muscle is metabolically advantageous. Nature Communications. 4:2150.

Johnson, L.K., R.M. Dillaman, D.M. Gay, J.E. Blum, and S.T. Kinsey. 2004. Metabolic influences of fiber size in aerobic and anaerobic locomotor muscle of the blue crab, *Callinectes sapidus*. Journal of Experimental Biology 207:4045–4056.

Kamp, G. 1989. Glycogenolysis during recovery from muscular work. Biological Chemistry Hoppe-Swyler 370:565–573.

Khayat, M., E. Lubzens, A. Tietz, and B. Funkenstein. 1994. Cell-free synthesis of vitellin in the shrimp *Penaeus semisulcatus* (de Haan). General Comparative Endocrinology 93:205–213.

Kinsey, S.T., and W.R. Ellington. 1996. ^{1}H- and ^{31}P-Nuclear magnetic resonance studies of L-lactate transport in isolated muscle fibers from the spiny lobster, *Panulirus argus*. Journal of Experimental Biology 199:2225–2234.

Kinsey, S.T., and B.C. Lee. 2003. The effects of rapid salinity change on in vivo arginine kinase flux in the juvenile blue crab, *Callinectes sapidus.* Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 135:521–531.

Kinsey, S.T., E. Buda, and J. Nordeen. 2003. Scaling of metabolic potential in gills of the blue crab, *Callinectes sapidus*, as a function of salinity. Physiological and Biochemical Zoology 76:105–114.

Kinsey, S.T., P. Pathi, K.M. Hardy, A. Jordan, and B.R. Locke. 2005. Does intracellular metabolite diffusion limit post-contractile recovery in burst locomotor muscle? Journal of Experimental Biology 208:2641–2652.

Kinsey, S.T., K.M. Hardy, and B.R. Locke. 2007. The long and winding road: influences of intracellular metabolite diffusion on cellular organization and metabolism in skeletal muscle. Journal of Experimental Biology 210:3505–3512.

Kinsey, S.T., B.R. Locke, and R.M. Dillaman. 2011. Molecules in motion: influences of diffusion on metabolic structure and function in skeletal muscle. Journal of Experimental Biology 214:263–274.

Kuballa, A.V., T.A. Holton, P. Paterson, and A. Elizur. 2011. Moult cycle specific differential gene expression profiling of the crab *Portunus pelagicus*. BMC Genomics. 12:147–165.

Kucharski, L.C.R., and R.S.M. Da Silva. 1991a. Effect of diet composition on the carbohydrate and lipid metabolism in an estuarine crab, *Chasmagnathus granulata* (Dana, 1851). Comparative Biochemistry and Physiology 99A:215–218.

Kucharski, L.C.R., and R.S.M. Da Silva. 1991b. Seasonal variation on the energy metabolism in an estuarine crab, *Chasmagnathus granulate* (Dana, 1851). Comparative Biochemistry and Physiology 100A:599–602.

Kuramoto, T. 1993. Cardiac activation and inhibition involved in molting behavior of a spiny lobster. Experientia 49:682–685.

Kushmerick, M. 1983. Energetics of muscle contraction. Pages 189–236 *in* L.D. Peachy, R.H. Adrian, and S.R. Geiger, editors. Handbook of muscle physiology—Skeletal muscle. American Physiological Society, Bethesda.

Lallier, F.H., and P.J. Walsh. 1991. Activities of uricase, xanthine oxidase and xanthine dehydrogenase in the hepatopancreas of aquatic and terrestrial crabs. Journal of Crustacean Biology 11:506–512.

Lallier F.H., and P.J. Walsh. 1992. Metabolism of isolated hepatopancreas cells from the blue crab (*Callinectes sapidus*) under simulated postexercise and hypoxic conditions. Physiological Zoology 65:712–723.

Lee, R.F., and A. Walker. 1995. Lipovitellin and lipid droplet accumulation in oocytes during ovarian maturation in the blue crab, *Callinectes sapidus*. Journal of Experimental Zoology 271:401–412.

Levin, L.A. 2003. Oxygen minimum zone benthos: adaptation and community response to hypoxia. Pages 1–45 *in* R.N. Gibson and R.J.A. Atkinson, editors. Oceanography and marine biology: an annual review. Taylor and Francis.

Li, T., and M. Brouwer. 2007. Hypoxia inducible factor, HIF, of the grass shrimp *Palaemonetes pugio*: molecular characterization and response to hypoxia. Comparative Biochemistry and Physiology Part B Biochemistry and Molecular Biology 147:11–19.

Lopez-Lopez, S., H. Nolasco, and F. Vega-Villasante. 2003. Characterization of digestive gland esterase-lipase activity of juvenile redclaw crayfish *Cherax quadricarinatus*. Comparative Biochemistry and Physiology 135B:337–347.

Luvizzotto-Santos, R., J.T. Lee, Z.P. Branco, A. Bianchini, and L.E.M. Nery. 2003. Lipids as energy source during salinity acclimation in the euryhaline crab *Chasmagnathus granula* Dana, 1851 (Crustacea-Grapsidae). Journal of Experimental Zoology 295A:200–205.

Maciel, F.E., M.A. Geihs, M.A. Vargas, B.P. Cruz, B.P. Ramos, O. Vakkuri, V.B. Meyer-Rochow, L.E.M. Nery, and S. Allodi. 2008. Daily variation of melatonin content in the optic lobes of the crab *Neohelice granulata*. Comparative and Biochemical Physiology 149A:162–166.

Mangum, C.P., B.R. McMahon, P.L. deFur, and M.G. Wheatly. 1985. Gas exchange, acid-base balance and the oxygen supply to tissue during a molt of the blue crab *Callinectes sapidus*. Journal of Crustacean Biology 5:188–206.

Mantel, L.H., and L.L. Farmer. 1983. Osmotic and ionic regulation. Pages 53–161 *in* L.H. Mantel, editor. The biology of Crustacea, Vol. 5. Academic Press, New York.

Martins, T.L., A.L. Chittó, C.L. Rossetti, C.K. Brondani, L.C. Kucharski, and R.S.M. Da Silva. 2011. Effects of hypo- or hyperosmotic stress on lipid synthesis and gluconeogenic activity in tissues of the crab *Neohelice granulata*. Comparative Biochemistry and Physiology A 158:400–405.

Marqueze, A., L.C. Kucharski, and R.M.S. Da Silva. 2006. Effects of anoxia and post-anoxia recovery on carbohydrate metabolism in the jaw muscle of the crab *Chasmagnathus granulatus* maintained on carbohydrate-rich or high-protein diets. Journal of Experimental Marine Biology and Ecology 332:198–205.

Massabuau, J.-C. 2001. From low arterial- to low tissue-oxygenation strategy. An evolutionary theory. Respiratory Physiology 128:249–261.

Matabos, M., V. Tunnicliffe, S.K. Juniper, and C. Dean. 2012. A year in hypoxia: epibenthic community responses to severe oxygen deficit at a subsea observatory in a coastal inlet. PLoS ONE 7:e45626.

McGaw, I.J., and B.R. McMahon. 2003. Balancing tissue perfusion demands: cardiovascular dynamics of *Cancer magister* during exposure to low salinity and hypoxia. Journal of Experimental Zoology 295A:57–70.

McGaw, I.J., and C.L. Reiber. 1998. Circulatory modification in the blue crab *Callinectes sapidus* during exposure and acclimation to low salinity. Comparative Biochemistry and Physiology 121A:67–76.

McMahon, B.R. 2001. Control of cardiovascular function and its evolution in Crustacea. Journal of Experimental Biology 204:923–932.

McNamara, J.C., and S.C. Faria. 2012. Evolution of osmoregulatory patterns and gill ion transport mechanisms in the decapod Crustacea: a review. Journal of Comparative Physiology B 182:997–1014.

Meyer, R.A. 1988. A linear model of muscle respiration explains monoexponential phosphocreatine changes. American Journal of Physiology 254:C548-C553.

Millamena, O.M., and F.P. Pascual. 1990. Tissue lipid content and fatty acid composition of *Penaeus monodon* Fabricius broodstock from the wild. Journal of the World Aquaculture Society 21:116–121.

Milligan, C.L., P.J. Walsh, C.E. Booth, and D.L. McDonald. 1989. Intracellular acid–base regulation during recovery from locomotor activity in the blue crab, *Callinectes sapidus.* Physiological Zoology 62:621–638.

Morris, S., and A.M. Adamczewska. 2002. Utilisation of glycogen, ATP, and arginine phosphate in exercise and recovery in terrestrial red crabs, *Gecarcoidea natalis*. Comparative Biochemistry and Physiology A 133:813–825.

Muhlia-Almazan, A., and F.L. Garcıa-Carreno. 2002. Influence of molting and starvation on the synthesis of proteolytic enzymes in the midgut of the white shrimp *Penaeus vannamei*. Comparative Biochemistry and Physiology 133:383–394.

Muriana, F.J.G., V. Ruiz-Gutiérrez, M.L. Gallardo-Guerrero, and M.I. Mınguez-Mosquera. 1993. A study of the lipids and carotenoproteins in the prawn, *Penaeus japonicus*. Journal of Biochemistry 114:223–229.

Mykles, D.L. 1999. Proteolytic processes underlying molt-induced claw muscle trophy in Decapod Crustaceans. Integrative and Comparative Biology 39:541–551.

Neiland, K.A., and B.T. Scheer. 1953. The influence of fasting and sinus gland removal on body composition of *Hemigrapsus nudus*. Part V of the hormonal regulation of metabolism in crustaceans. Physiological and Comparative Oecology 4:321–326.

Oliveira, G.T., and R.S.M. Da Silva. 1997. Gluconeogenesis in hepatopancreas of *Chasmagnathus granulata* crabs maintained on high-protein or carbohydrate-rich diets. Comparative Biochemistry and Physiology Part A 118:1429–1435.

Oliveira, G.T., and R.S.M. Da Silva. 2000. Hepatopancreas gluconeogenesis during hyposmotic stress in crabs *Chasmagnathus granulate* maintained on high-protein or carbohydrate-rich diets. Comparative Biochemistry and Physiology 127B:375–381.

Oliveira, G.T., I.C.C. Rossi, and R.S.M. Da Silva. 2001. Carbohydrate metabolism during anoxia and pos-anoxia recovery in *Chasmagnathus granulata* crabs maintained on high-protein or carbohydrate-rich diets. Marine Biology 139:335–342.

Oliveira, G.T., F.A. Fernandes, G. Bond-Buckup, A.A. Bueno, and R.S.M. Da Silva. 2003. Circadian and seasonal variations in the metabolism of carbohydrates in *Aegla ligulata* (Crustacea: Anomura: Aeglidae). Memoirs of Museum Victoria 60:59–62.

Oliveira, G.T., P. Eichler, I.C.C. Rossi, and R.S.M. Da Silva. 2004. Hepatopancreas gluconeogenesis during anoxia and post-anoxia recovery in *Chasmagnathus granulata* crabs maintained on high-protein or carbohydrate-rich diets. Journal of Experimental Zoology 301A:240–248.

Palacios, E., A.M. Ibarra, and I.S. Racotta. 2000. Tissue biochemical composition in relation to multiple spawning in wild and pond-reared *Penaeus vannamei* broodstock. Aquaculture 185:353–371.

Pellegrino, R., L.C. Kucharski, and R.S.M. Da Silva. 2008. Effect of fasting and refeeding on gluconeogenesis and glyconeogenesis in the muscle of the crab *Chasmagnathus granulatus* previously fed a protein- or carbohydrate-rich diet. Journal of Experimental Marine Biology and Ecology 358:144–150.

Penkoff, S.J., and F.P. Thurberg. 1982. Changes in oxygen consumption of the American lobster *Homarus americanus* during the molt cycle. Comparative Biochemistry and Physiology 72: 621–622.

Péqueux, A. 1995. Osmotic Regulation in Crustaceans. Journal of Crustacean Biology 15:1–60.

Piller, S.C., R.P. Henry, R.P., J.E. Doeller, and D.W. Kraus. 1995. A comparison of the gill physiology of two euryhaline crab species, *Callinectes sapidus* and *Callinectes similis*: energy production, transported related enzymes and osmoregulation as a function of acclimation salinity. Journal of Experimental Biology 198:349–358.

Pongsomboon, S., S. Udomlertpreecha, P. Amparyup, S. Wuthisuthimethavee, and A. Tassanakajon. 2009. Gene expression and activity of carbonic anhydrase in salinity stressed *Penaeus monodon*. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 152:225–233.

Pörtner, H.O., L.S. Peck, and T. Hirse. 2006. Hyperoxia alleviates thermal stress in the Antarctic bivalve *Laternula elliptica*: evidence for oxygen limited thermal tolerance. Polar Biology 29:688–693.

Quackenbush, L.S. 1994. Lobster reproduction: a review. Crustaceana 67:82–94.

Romano, N., and C. Zeng. 2012. Osmoregulation in decapod crustaceans: implications to aquaculture productivity, methods for potential improvement and interactions with elevated ammonia exposure. Aquaculture 334–337:12–23.

Rosa, R.A., and M.L. Nunes. 2003. Changes in organ indices and lipid dynamics during the reproductive cycle of *Aristeus antennatus, Parapenaeus longirostris* and *Nephrops norvegicus* (Crustacea: Decapoda) females from the south Portuguese coast. Crustaceana 75:1095–1105.

Rosas, C., G. Cuzon, L. Arena, L. Arena, P. Lemaire, C. Soyez, and A. VanWormhoudt. 2000. Influence of dietary carbohydrate on the metabolism of juvenile *Litopenaeus stylirostris*. Journal of Experimental Marine Biology and Ecology 249:181–198.

Ryan, J.A., and L.E. Hightower. 1994. Evaluation of heavy-metal ion toxicity in fish cells using a combined stress protein and cytotoxicity assay. Environmental Toxicological Chemistry 13:1231–1240.

Sánchez-Paz, A., L.F. Garcıa-Carreño, A. Muhlia-Almazán, N. Hernández-Saavedra, and G. Yepiz-Plascencia. 2003. Differential expression of trypsin mRNA in the white shrimp (*Penaeus vannamei*) midgut gland under starvation conditions. Journal of Experimental Marine Biology and Ecology 292:1–7.

Sánchez-Paz, A., F.L. García-Carreño, A. Muhlia-Almazán, A.B. Peregrino-Uriarte, J.Y. Hernández-López, and G. Yepiz-Plascencia. 2006. Usage of energy reserves in crustaceans during starvation: status and future directions. Insect Biochemistry and Molecular Biology 36:241–249.

Sánchez-Paz, A., F. García-Carreño, J. Hernández-López, A. Muhlia-Almazán, and G. Yepiz-Plascencia. 2007. Effect of short-term starvation on hepatopancreas and plasma energy reserves of the Pacific white shrimp (*Litopenaeus vannamei*). Journal of Experimental Marine Biology and Ecology 340:184–193.

Santos, E.A., and R. Keller. 1993. Effect of exposure to atmospheric air on blood glucose and lactate concentrations in two crustacean species: a role of the crustacean hyperglycemic hormone (CHH). Comparative Biochemistry and Physiology 106A:343–347.

Santos, E.A., L.E.M. Nery, R. Keller, and A.A. Gonçalves. 1997. Evidence for the involvement of the crustacean hyperglycemic hormone in the regulation of lipid metabolism. Physiological Zoology 70:415–420.

Schafer, H.J. 1968. The determination of some stages of the molting cycle of *Penaeus duorarum*, by microscopic examination of the setae of the endopodites of pleopods. Fisheries Reproduction 57:381–391.

Schein, V., A.L.F. Chittó, R. Etges, L.C. Kuscharski, A. Wormhoudt, and R.S.M. Da Silva. 2005. Effects of hypo- or hyperosmotic stress on gluconeogenesis, phosphoenolpyruvate carboxykinase activity, and gene expression in jaw muscle of the crab *Chasmagnathus granulata* seasonal differences. Journal of Experimental Marine Biology and Ecology 316:203–212.

Schmidt-Nielsen, K. 1997. Animal physiology (adaptation and environment), 5th ed. Cambridge Univ. Press, Cambridge, England.

Schmitt, A.S.C., and E.A. Santos. 1993. Lipid and carbohydrate metabolism of the interdial crab *Chasmagnathus granulata* Dana, 1851 (Crustacea: Decapoda) during emersion. Comparative Biochemistry and Physiology 106A:329–336.

Seear, P.J., G.A. Tarling, G. Burns, W.P. Goodall-Copestake, E. Gaten, Ö. Özkaya, and E. Rosato. 2010. Differential gene expression during the moult cycle of Antarctic krill (*Euphausia superba*). Journal of Experimental Marine Biology and Ecology 11:582–595.

Seibel, B.A. 2011. Critical oxygen levels and metabolic suppression in oceanic oxygen minimum zones. Journal of Experimental Biology 214:326–336.

Serrano, L., and R.P. Henry. 2008. Differential expression and induction of two carbonic anhydrase isoforms in the gills of the euryhaline green crab, *Carcinus maenas*, in response to low salinity. Comparative Biochemistry and Physiology D 3:186–193.

Shiau, S.Y., and C.Y. Peng. 1992. Utilization of different carbohydrates at different dietary protein levels in grass prawn, *Penaeus monodon*, reared in seawater. Aquaculture 101:241–250.

Silverman, H., Costello, W., and D.L. Mykles. 1987. Morphological fiber type correlates of physiological and biochemical properties in crustacean muscle. American Zoologist 27:l011–1019.

Somero, G.N. 2002. Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living. Integrative and Comparative Biology 42:780–789.

Soñanez-Organis, J.G., A.B. Peregrino-Uriarte, S. Goméz-Jiménez, A. López-Zavala, H.J. Forman, and G. Yepiz-Plascencia. 2009. Molecular characterization of hypoxia inducible factor-1 (HIF-1) from the white shrimp *Litopenaeus vannamei* and tissue specific expression under hypoxia. Comparative Biochemistry and Physiology C 150:395–405.

Soñanez-Organis, J.G., A.B. Peregrino-Uriarte, R.R. Sotelo-Mundo, H.J. Forman, and G. Yepiz-Plascencia. 2011. Hexokinase from white shrimp *Litopenaeus vannamei*: cDNA sequence, structural protein model and regulation via HIF-1 in response to hypoxia. Comparative Biochemistry and Physiology B 158:242–249.

Speed, S.R., J. Baldwin, R.J. Wong, and R.M.G. Wells. 2001. Metabolic characteristic of muscles in the spiny lobster, *Jasus edwardsii*, and responses to emersion during simulated live transport. Comparative Biochemistry and Physiology A 128:435–444.

Stillman, J.H., and G.N. Somero. 1996. Adaptation to temperature stress and aerial exposure in congeneric species of intertidal porcelain crabs (genus *Petrolisthes*): correlation of physiology, biochemistry and morphology with vertical distribution. Journal of Experimental Biology 199:1845–1855.

Stokes, D.R., and R.K. Josephson. 1992. Structural organization of two fast, rhythmically active crustacean muscles. Cell Tissue Research 267:571–582.

Sze Lo, T., Z. Cui, J.L.Y. Mong, Q.W.L. Wong, S.M. Chan, H.S. Kwan, and C.H. Chu. 2007. Molecular coordinated regulation of gene expression during ovarian development in the penaeid shrimp. Marine Biotechnology 9:459–468.

Taylor, H.H., and N. Leelapiyanart. 2001. Oxygen uptake by embryos and ovigerous females of 2 intertidal crabs, *Heterozius rotundifrons* (Belliidae) and *Cyclograpsus lavauxi* (Grapsidae): scaling and the metabolic costs of reproduction. Journal of Experimental Biology 204:1083–1097.

Teshima S., A. Kanazawa, and Y. Kakuta. 1986. Effects of dietary phospholipids on growth and body lipid composition of the juvenile prawn. Nippon Suisan Gakkaishi 52:155–158.

Thibodeaux, L.K., K.G. Burnett, and L.E. Burnett. 2009. Energy metabolism and metabolic depression during Exercise in *Callinectes sapidus*, the Atlantic blue crab: effects of the bacterial pathogen *Vibrio campbellii*. Journal of Experimental Biology 212:3428–3439.

Towle, D.W., and D. Weihrauch. 2001. Osmoregulation by gills of euryhaline crabs: molecular analysis of transporters. American Zoologist 41:770–780.

Tse, F.W., C.K. Govind, and H.L. Atwood. 1983. Diverse fiber composition of swimming muscles in the blue crab, *Callinectes sapidus*. Canadian Journal of Zoology 61:52–59.

Vernberg, W.B., and F.J. Vernberg. 1968. Physiological diversity in metabolism in marine and terrestrial Crustacea. American Zoologist 8:449–458.

Verri, T., A. Mandal, L. Zilli, D. Bossa, P.K. Mandal, L. Ingrosso, V. Zonno, S. Vilella, G.A. Ahearn, and C. Storelli. 2001. d-Glucose transport in decapod crustacean hepatopancreas. Comparative Biochemistry and Physiology—Part A: Molecular & Integrative Physiology 130:585–606.

Vetter, R.A.H. 1995. Ecophysiological studies on citrate synthase: (II) enzyme regulation of selected crustaceans with regard to life-style and the climatic zone. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 165:56–61.

Vetter, R.A.H., and F. Buchholz. 1997. Catalytic properties of two pyruvate kinase isoforms in Nordic krill, *Meganyctiphanes norvegica*, with respect to seasonal temperature adaptation. Comparative Biochemistry and Physiology 116A:1–10.

Vinagre, A.S., and R.S.M. Da Silva. 1992. Effects of starvation on the carbohydrate and lipid metabolism in crabs previously maintained on a high protein or carbohydrate-rich diet. Comparative Biochemistry and Physiology A 102:579–583.

Vinagre, A.S., and R.S.M. Da Silva. 2002. Effects of fasting and refeeding on metabolic processes in the crab *Chasmagnathus granulata* (Dana, 1851). Canadian Journal of Zoology 80:1413–1421.

Vinagre, A.S., A.P.N. Amaral, F.P. Ribarcki, E.F. Silveira, and E. Périco. 2007. Seasonal variation of energy metabolism in ghost crab *Ocypode quadrata* at Siriú Beach (Brazil). Comparative Biochemistry and Physiology A 146:514–519.

Walker, A., S. Ando, and R.F. Lee. 2003. Synthesis of a high-density lipoprotein in the developing blue crab (*Callinectes sapidus*). Biological Bulletin 204:50–56.

Weinstein, R.B. 2001. Terrestrial intermittent exercise: common issues for human athletics and comparative animal locomotion. American Zoologist 41:219–228.

Willsie, J.K., and J.S. Clegg. 2001. Nuclear p26, a small heat shock/alpha-crystallin protein, and its relationship to stress resistance in *Artemia franciscana* embryos. Journal of Experimental Biology 204:2339–2350.

White, A.Q., and C.P. Spirito. 1973. Anatomy and physiology of the swimming leg musculature in the blue crab, *Callinectes sapidus.* Marine Behavioral Physiology 2:141–153.

Wood, C.M., and D.J. Randall. 1981. Oxygen and carbon dioxide exchange during exercise in the land crab *Cardisoma carnifex.* Journal of Experimental Zoology 218:7–22.

Yancey, P.H. 2001. Protein, osmolytes and water stress. American Zoologist 41:699–709.

Yannicelli, B., and L. Castro. 2013. Ecophysiological constraints on the larvae of *Pleuroncodes monodon* and the implications for its reproductive strategy in poorly oxygenated waters of the Chile-Peru undercurrent. Journal of Plankton Research. 35:566–581.

Yannicelli, B., K. Paschke, R.R. González, and L.R. Castro. 2013. Metabolic responses of the squat lobster (*Pleuroncodes monodon*) larvae to low oxygen concentration. Marine Biology. 160:961–976.

Yehezkel, G., R. Chayoth, U. Abdu, I. Khalaila, and A. Sagi. 2000. High-density lipoprotein associated with secondary vitellogenesis in the hemolymph of the crayfish *Cherax quadricarinatus*. Comparative Biochemistry and Physiology B 127B:411–421.

Yepiz-Plascencia, G., F. Vargas-Albores, and I. Higuera-Ciapara. 2000. Penaeid shrimp hemolymph lipoproteins. Aquaculture 191:177–189.

Yepiz-Plascencia, G., F. Jimenez-Vega, M.G. Romo-Figueroa, R.R. Sotelo-Mundo, and F. Vargas-Albores. 2002. Molecular characterization of the bifunctional VHDL-CP from the hemolymph of the white shrimp *Penaeus vannamei.* Comparative Biochemistry and Physiology 132B:585–592.

Zainal, K.A.Y., A.C. Taylor, and R.J.A. Atkinson. 1992. The effect of temperature and hypoxia on the respiratory physiology of the squat lobsters, *Munida rugosa* and *Munida sarsi* (anomura, galatheidae). Comparative Biochemistry and Physiology Part A 101:557–567.

Zimmer, M. 2002. Nutrition in terrestrial isopods (Isopoda: Oniscidea): an evolutionary-ecological approach. Biological Reviews 77:455–493.

13

CRUSTACEAN GENOMICS AND FUNCTIONAL GENOMIC RESPONSES TO ENVIRONMENTAL STRESS AND INFECTION

Jonathon H. Stillman and David A. Hurt

Abstract

High-throughput DNA sequencing has facilitated genome-scale studies of many organisms and issues, including how crustaceans respond to environmental stresses, including temperature, viral and bacterial infection, metal and organic toxicants, hypoxia, and salinity. The work has been uneven; whereas economic drivers have spurred studies across a wide array of experimental conditions in shrimp and bioindicator taxa (e.g., Cladoceran crustaceans in the genus *Daphnia*) dominate studies of environmental pollutants, ecologically important crustaceans (isopods, amphipods, euphausids) have been neglected. From these studies have emerged genes that encode mitochondrial proteins, chaperonins, structural proteins, reproductive proteins, and extracellular receptor proteins and are responsive to multiple types of environmental stressors across a range of crustaceans. This work highlights aspects of crustacean cellular biology that elucidate how these diverse organisms respond to a small set of changing environmental stressors across many of Earth's habitats.

INTRODUCTION

Functional genomic, proteomic, and metabolomic analyses are powerful tools for examining the comprehensive set of responses that organisms make in response to environmental variation. Increasingly, these tools are applied to nonmodel organisms as advances in genomics and high-throughput screening technologies have lowered both the time and cost investment. Genomic-scale data have been generated for a small number of crustaceans (Fig. 13.1, Table 13.1); in this chapter, we review the literature on the application of functional genomic (transcriptomic), proteomic, and metabolomic approaches that have been applied to investigate how crustaceans respond to changes in their physical environment, to environmental toxicant exposure, and to infection by viral and bacterial pathogens (Table 13.2). Responses to those abiotic and biotic factors

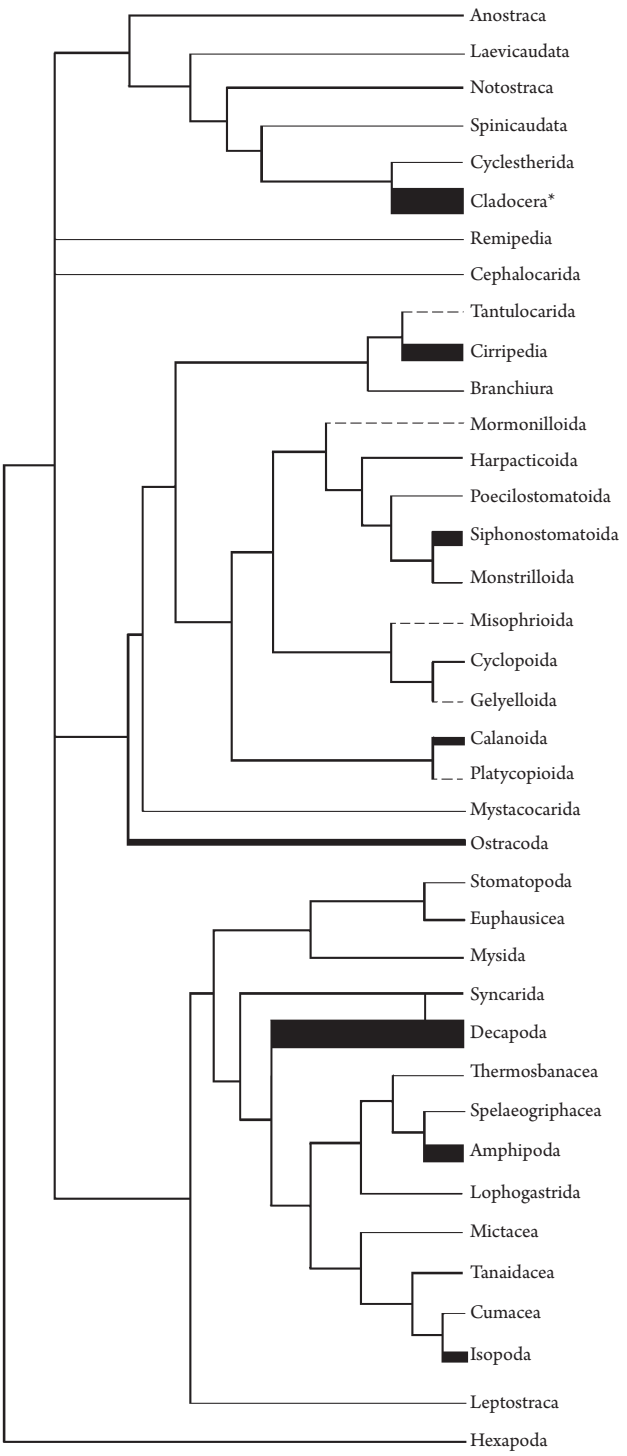


Fig. 13.1.
Phylogenetic distribution of GenBank nucleotide data for the Crustacea. Branch weight corresponds to the number of nucleotide entries as of May 2011. Dashed lines indicate no entries. Due to an unresolved crustacean phylogeny, this tree is a composite and is for illustrative purposes only (not evolutionary inference).* Genome has been sequenced for *Daphnia pulex*, and there are currently 14,177 entries in GenBank for this species.

Table 13.1. Genomic data available in public databases for the Crustacea.

Class (Subclass, Infraclass)	Order	Genus species	Genomic Data Types[1]	Archived Data (GenBank)		
				Nucleotide	ESTs	GEO Data Series
Branchiopoda (Sarsostraca)	Anostraca	*Artemia franciscana*	*M,E*	1,673	37,590	0
		Artemia parthenogenetica	*E*		695	0
		Artemia sinica	*E*		2	0
Branchiopoda (Phyllopoda)	Notostraca	*Triops cancriformis*	*M,E*	1,046	3,981	0
		Triops longicaudatus	*M*		0	0
Branchiopoda (Phyllopoda, Diplostraca)	Laevicaudata			252		
	Spinicaudata			875		
	Cyclestherida			14		
	Cladocera	*Daphnia carinata*	*E*	12,742	6,364	0
		Daphnia magna	*G,E*		13,517	11
		Daphnia pulex	*G,M,E*	200 Mbp	15,2659	11
Maxillopoda (Copepoda)	Calanoida	*Calanus finmarchicus*	*E*	4,697	11,461	0
	Cyclopoida	*Paracyclopina nana*	M	1,018	0	0
	Gelyelloida			0		
	Harpacticoida	*Tigriopus californicus*	*M,E*	1,380	4,801	0
		Tigriopus japonicus	*M*		0	0
	Misophrioida			0		
	Monstrilloida			15		
	Mormonilloida			0		
	Platycopioida			0		
	Poecilostomatoida			310		
	Siphonostomatoida	*Caligus clemensi*	*E*	7,674	14,806	0

		Caligus rogercresseyi	*E*		32,037	0
		Lepeophtheirus salmonis	*M,E*		129, 250	4
		Lernaeocera branchialis	*E*		14,927	0
Maxillopoda (Thecostraca, Cirripedia)	Pygophora			7		
	Apygophora			14		
	Kentrogonida	*Sacculina carcini*	*G*	220	0	0
	Akentrogonida			16		
	Pedunculata	*Capitulum mitella*	*M*	1,212	0	0
		Pollicipes pollicipes	*E*		4,191	0
	Sessilia	*Balanus amphitrite*	*E*	7,141	905	0
		Megabalanus volcano	*M*		0	0
		Tetraclita japonica	*M*		0	0
	Facetotecta			21		
	Ascothoracida			23		
Maxillopoda (Branchiura)	Arguloida	*Argulus americanus*	*M*	92	0	0
	Cyclida			0		
Maxillopoda (Pentastomida)	Cephalobaenida			8		
	Porocephalida	*Armillifer armillatus*	*M*	54	0	0
Maxillopoda (Mystacocarida)	Mystacocaridida			44		
Maxillopoda (Tantulocarida)	Basipodellidae			0		
	Deoterthridae			0		
	Doryphallophoridae			0		
	Microdajidae			0		
Ostracoda (Myodocopa)	Myodocopida	*Vargula hilgendorfii*	*M*	630	0	0
	Halocyprida			95		
Ostracoda (Podocopa)	Platycopida			5		

(*continued*)

Table 13.1. *(Continued)*

Class (Subclass, Infraclass)	Order	Genus species	Genomic Data Types[1]	Archived Data (GenBank)		
				Nucleotide	ESTs	GEO Data Series
	Podocopida			2,563		
Malacostraca (Eumalacostraca)	Bathynellacea			39		
	Anaspidacea			28		
	Spelaeogriphacea			1		
	Thermosbaenacea			4		
	Lophogastrida			17		
	Mysida			1,195		
	Mictacea			2		
	Amphipoda	*Gammarus pulex*	*E*	8,776	12,345	0
		Caprella mutica	*M*		0	0
		Caprella scaura	*M*		0	0
		Metacrangonyx longipes	*M*		0	0
		Onisimus nanseni	*M*		0	0
		Parhyale hawaiensis	*E*		55,663	
	Isopoda	*Eurydice pulchra*	*E*	5,301	1,026	0
		Eophreatoicus sp.	*M*		0	0
		Ligia oceanica	*M*		0	0
	Tanaidacea			98		
	Cumacea			153		
	Euphausiacea	*Euphausia superba*	*G,E*	1,193	6,142	0
	Amphionidacea			0		

	Decapoda	*Alpheus distinguendus*	*M*	39,985	0	0
		Bythograea thermydron	*G*		0	0
		Callinectes sapidus	*M,E*		10,563	0
		Carcinus maenas	*E*		15,558	0
		Celuca pugilator	*E*		3,646	0
		Charybdis japonica	*M*		0	0
		Cherax destructor	*M*		0	0
		Cherax quadricarinatus	*E*		120	2
		Eriocheir hepuensis	*M*		0	0
		Eriocheir japonica	*M*		0	0
		Eriocheir sinensis	*M,E*	70,985	16,987	0
		Exopalaemon carinicauda	*M*		0	0
		Farfantepenaeus californiensis	*M*		0	0
		Fenneropenaeus chinensis	*G,M,E*		10,446	1
		Fenneropenaeus indicus	*E*		714	0
		Fenneropenaeus merguiensis	*E*		11	0
		Gandalfus yunohana	*M,E*		310	0
		Gecarcoidea natalis	*E*		2,118	0
		Geothelphusa dehaani	*M*		0	0
		Halocaridina rubra	*M*		0	0
		Homarus americanus	*E*		29,957	2
		Ilyoplax pusilla	*E*		438	0
		Litopenaeus setiferus	*E*		1,042	0
		Litopenaeus stylirostris	*M,E*		416	0
		Litopenaeus vannamei	*G,M,E*	75,329	16,1248	4
		Macrobrachium lanchesteri	*M*		0	0

(*continued*)

Table 13.1. *(Continued)*

Class (Subclass, Infraclass)	Order	Genus species	Genomic Data Types[1]	Archived Data (GenBank)		
				Nucleotide	ESTs	GEO Data Series
		Macrobrachium nipponense	*M,E*	557	8,458	0
		Macrobrachium rosenbergii	*M,E*	602	4,427	0
		Marsupenaeus japonicus	*G,M,E*		3,156	0
		Metacarcinus magister	*E*		1,137	0
		Metapenaeus ensis	*E*		13	0
		Neocaridina denticulata	*E*		132	0
		Pacifastacus leniusculus	*E*		802	0
		Pagurus longicarpus	*M*		0	0
		Palaemonetes pugio	*E*		42	1
		Panulirus argus	*E*		16	0
		Panulirus japonicus	*M,E*		2,673	0
		Panulirus ornatus	*M*		0	0
		Panulirus stimpsoni	*M*		0	0
		Penaeus monodon	*G,M,E*		39,397	3
		Petrolisthes cinctipes	*E*		97,806	2
		Portunus trituberculatus	*M,E*		9,552	0
		Procambarus clarkii	*E*		42	0
		Pseudocarcinus gigas	*M*		0	0
		Scylla olivacea	*M*		0	0
		Scylla paramamosain	*M*		0	0
		Scylla serrata	*M*		0	0

		Scylla tranquebarica	*M*		0	0
		Shinkaia crosnieri	*M*		0	0
		Upogebia major	*M*		0	0
		Xenograpsus testudinatus	*M*		0	0
Malacostraca (Hoplocarida)	Stomatopoda	*Gonodactylus chiragra*	*M*	607	0	0
		Harpiosquilla harpax	*M*		0	0
		Lysiosquillina maculata	*M*		0	0
		Oratosquilla oratoria	*M*		0	0
		Squilla empusa	*M*		0	0
Malacostraca (Phyllocarida)	Leptostraca	*Squilla mantis*	*M*	242	0	0
Remipedia	Nectiopoda	*Speleonectes tulumensis*	*M,E*	102	981	0
Cephalocarida	Brachypoda	*Hutchinsoniella macracantha*	*M*	145	0	0

1. Sequence data types: G, genomic; M, mitochondrial; E, gene expression (cDNA / EST).

Table 13.2. Synopsis of genome-wide studies in the Crustacea.

Species	Taxonomy[1]	Tissue	'Omic Type[2]	Method	Experiment	Citation	Reviewed here
			Temperature				
Petrolisthes cinctipes	Malacostraca Decapoda, Anomura: Porcellanidae	Hepatopancreas	T	cDNA microarray	Kinetics of heat stress response	Teranishi and Stillman 2007	Yes
Petrolisthes cinctipes	Malacostraca Decapoda, Anomura: Porcellanidae	Heart	T	cDNA microarray	Acclimatization of heat and cold stress response	Stillman and Tagmount 2009	Yes
Petrolisthes cinctipes	Malacostraca Decapoda, Anomura: Porcellanidae	Heart	T	cDNA microarray	Thermal acclimation of extreme cold tolerance	Ronges et al. 2012	No
Rimicaris exoculata	Malacostraca Decapoda, Caridea: Bresiliidae	Abdomen with cuticle	T	SSH[2]	Heat stress	Cottin et al. 2010	Yes
Penaeus monodon	Malacostraca Decapoda, Penaeoidea: Penaeidae	Hemocytes	T	cDNA microarray	Heat stress	de la Vega et al. 2007	Yes
			Temperature + ecotoxicant mixed effects				
Calanus finmarchius	Maxillopoda Calanoida: Calanidae	Whole organism	T	SSH	Heat stress, MEA, WSFs, Cu	Hansen et al. 2007	Yes
			Oxygen				
Penaeus monodon	Malacostraca Decapoda, Penaeoidea: Penaeidae	Hemocytes	T	cDNA microarray	Hypoxia	de la Vega et al. 2007	Yes
Fenneropenaeus chinensis	Malacostraca Decapoda, Penaeoidea: Penaeidae	Hepatopancreas	P	2DE[3] + LC ESI-MS/MS[4]	Hypoxia	Jiang et al. 2009	Yes

Palaemonetes pugio	Malacostraca Decapoda, Caridea: Palaemoninae	Hepatopancreas	T	cDNA microarray	Hypoxia	Li and Brouwer 2009b	Yes
Palaemonetes pugio	Malacostraca Decapoda, Caridea: Palaemoninae	Hepatopancreas	T	SSH	Hypoxia	Li and Brouwer 2009a	Yes
			Infection				
Penaeus (Litopenaeus) vannamei	Malacostraca Decapoda, Penaeoidea: Penaeidae	Hemocytes	P	2DE + LC ESI-MS/MS	Taura Syndrome Virus (TSV)	Chongsatja et al. 2007	Yes
Litopenaeus vannamei	Malacostraca Decapoda, Penaeoidea: Penaeidae	Gills, Hemocytes, Hepatopancreas, Muscle	T	SSH, cDNA microarray	White spot syndrome virus (WSSV)	Robalino et al. 2007	Yes
Procambarus clarkii	Malacostraca Decapoda, Astacidea: Cambarinae	Hemocytes	T	SSH, cDNA microarray	WSSV	Zeng and Lu 2009	Yes
Litopenaeus stylirostris	Malacostraca Decapoda, Penaeoidea: Penaeidae	Hemocytes	T	SSH	*Vibrio penaeicida*	de Lorgeril et al. 2005	Yes
Penaeus monodon	Malacostraca Decapoda, Penaeoidea: Penaeidae	Hemocytes	P	2DE + LC ESI-MS/MS	*Vibrio harveyi*	Somboonwiwat et al. 2010	Yes
Homarus americanus	Malacostraca Decapoda, Astacidea: Nephropidae	Muscle, gill, heart, hepatopancreas, brain, branchiostegite, gonad	T	SSH	Epizootic shell disease	Tarrant et al. 2010	Yes

(*continued*)

Table 13.2. *(Continued)*

Species	Taxonomy[1]	Tissue	'Omic Type[2]	Method	Experiment	Citation	Reviewed here
Penaeus (Litopenaeus) stylirostris	Malacostraca Decapoda, Penaeoidea: Penaeidae	hepatopancreas	T	cDNA microarray	WSSV	Dhar et al. 2003	Yes
Litopenaeus vannamei	Malacostraca Decapoda, Penaeoidea: Penaeidae	Gill (proteome)	T (review) + P (new data)	SSH, cDNA microarray, dsRNA (RNAi), 2D LC-MS-MS	WSSV	Robalino et al. 2009	Yes
Marsupenaeus japonicus	Malacostraca Decapoda, Penaeoidea: Penaeidae	Hemocytes	T	cDNA Microarray	Peptidoglycan	Fagutao et al. 2008	Yes
Marsupenaeus japonicus	Malacostraca Decapoda, Penaeoidea: Penaeidae	Hemocytes	T	EST library	WSSV	Rojtinnakorn et al. 2002	Yes
Armadillidium vulgare	Malacostraca Isopoda, Ligiamorpha, Armadillididae	Hemocytes	P	2DE	Wolbachia	Herbiniere et al. 2008	Yes
Armadillidium vulgare	Malacostraca Isopoda, Ligiamorpha, Armadillididae	Hemocytes	T	cDNA library	Wolbachia	Chevalier et al. 2012	No
			Ecotoxicant: Organics				
Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal	T	cDNA microarray	Estrogens	Iguchi et al. 2006	Yes
Palaemonetes pugio	Malacostraca Decapoda, Caridea: Palaemoninae	Hepatopancreas	T	SSH EST library	Pyrene	Li and Brouwer 2009a	Yes

Diporeia spp.	Malacostraca Amphipoda, Gammaridea: Pontoporelidaae	Whole animal	M	GCxGC/ TOF-MS[6]	Atrazine	Ralston-Hooper et al. 2008	Yes
Penaeus monodon	Malacostraca Decapoda, Penaeoidea: Penaeidae	Hemolymph	P	2D-DIGE[7]	Enrofloxacin and furazolidone	Silvestre et al. 2010	Yes
Neocaridina denticulate	Decapoda, Caridea, Atyidae	Whole animal	T	SSH	Nonylphenol	Liu and Sung 2011	No
Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal	M	FT-ICR MS[8]	Fenvalerate, dinitrophenol, and propranolol	Taylor et al. 2010	Yes
Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal	T	cDNA microarray	Pyrene and fluoranthene	Vandenbrouck et al. 2010	Yes
Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal	T	cDNA microarray	Ibuprofen	Heckmann et al. 2008	Yes
Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal	T	SSH, cDNA microarray	Fenarimol	Soetaert et al. 2007	Yes
Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal	T	cDNA microarray	bNF	Watanabe et al. 2008	Yes
Procambarus clarkii	Malacostraca Decapoda, Astacidea: Cambarinae	Nervous tissue, digestive gland	P	2DE	Chlorpyrifos and carbaryl	Vioque-Fernandez et al. 2009	Yes

(*continued*)

Table 13.2. *(Continued)*

Species	Taxonomy[1]	Tissue	'Omic Type[2]	Method	Experiment	Citation	Reviewed here
			Ecotoxicant: Metals				
Litopenaeus vannamei	Malacostraca Decapoda, Penaeoidea: Penaeidae	Whole animal (larvae)	T	EST library	Cadmium (Cd)	Keating et al. 2007	Yes
Tigriopus japonicus	Branchiopoda Maxillopoda, Copepoda: Harpacticidae	Whole animal	T	EST library, Oligonucleotide microarray	Copper (Cu)	Ki et al. 2009	Yes
Palaemonetes pugio	Malacostraca Decapoda, Caridea: Palaemoninae	Hepatopancreas	T	SSH EST library	Copper (Cu)	Li and Brouwer 2009a	Yes
Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal	T	cDNA microarray	Cu, Cd, Zn	Poynton et al. 2008	Yes
Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal	T	cDNA microarray	$AgNO_3$, Ag nanoparticles	Poynton et al. 2012	Yes
Daphnia pulex	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal	T	cDNA microarray	Cd	Shaw et al. 2007	Yes
Eriocheir sinensis	Malacostraca Decapoda, Brachyura: Varunidae	Gills	P	2DE	Cd	Silvestre et al. 2006	Yes
Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal	M	FT-ICR MS	Cd	Taylor et al. 2010	Yes

Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal (adult and larvae)	M	FT-ICR MS	Cu	Taylor et al. 2009	Yes
Artemia sinica	Branchiopoda Anostraca, Artenmiina: Artemiidae	Whole animal (larvae)	P	2DE	Cu	Zhou et al. 2010	Yes
Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Hemolymph	M, T	FT-ICR MS, NMR, Oligonucleotide microarray	Cd	Poynton et al. 2011	Yes
Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal	T	cDNA microarray	Cu, Cd, Zn	Poynton et al. 2007	Yes
Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal	T	cDNA microarray	Ni, Ni+Pb. Ni+Cd	Vandenbrouck et al. 2009	Yes
			Salinity				
Penaeus monodon	Malacostraca Decapoda, Penaeoidea: Penaeidae	Hemocytes	T	cDNA microarray	Hypo-osmotic salinity	de la Vega et al. 2007	Yes
			Developmental Regulation				
Artemia franciscana	Branchiopoda Anostraca, Artenmiina: Artemiidae	Embryos:	T	EST library	Dehydrated and rehydrated cysts	Chen et al. 2009	No
Balanus amphitrite	Maxillopoda Sessilia, Balanoidea: Balanidae	Whole animals	P	2DE	Nauplius, the swimming cyprid, the attached cyprid, and the metamorphosed cyprid	Thiyagarajan and Qian 2008	No

(*continued*)

Table 13.2. *(Continued)*

Species	Taxonomy[1]	Tissue	'Omic Type[2]	Method	Experiment	Citation	Reviewed here
Balanus amphitrite	Maxillopoda Sessilia, Balanoidea: Balanidae	Whole animal	P	2DE	Cyprid larvae	Zhang et al. 2010	No
Balanus amphitrite	Maxillopoda Sessilia, Balanoidea: Balanidae	Whole animals	P, PhosphoP	2DE	Larvae	Thiyagarajan et al. 2009	No
Artemia franciscana	Branchiopoda Anostraca, Artenmiina: Artemiidae	Embryos:	P	2DE	Post-diapaused cysts	Wang et al. 2007	No
Lepeophtheirus salmonis	Maxillopoda Copepoda, Siphonostomatoida: Caligidae	Post molting maturation and egg production	T	EST library	Pre-adult and adult	Eichner et al. 2008	No
		Endocrine/Exocrine					
Daphnia pulex	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole Pre-vitellogenic females	T	cDNA microarray	Methyl farnesoate (MF)	Eads et al. 2008	No
Balanus amphitrite	Maxillopoda Sessilia, Balanoidea: Balanidae	Cyprid larvae	P	2DE	Biofilm, conspecific settlement factor	Thiyagarajan 2010	No
Cherax quadricarinatus	Malacostraca Decapoda, Astacidea: Parastacidae	Hepatopancreas	T	cDNA microarray	Ecdysteroid	Shechter et al. 2007	No
Cherax quadricarinatus	Malacostraca Decapoda, Astacidea: Parastacidae	Hypodermis, gastrolith disk	T	cDNA microarray	Ecdysone	Yudkovski et al. 2010	No
Daphnia magna	Branchiopoda Diplostraca, Cladocera: Daphniidae	Whole animal	T	SSH, cDNA microarray	20-hydroxyecdysone	Soetaert et al. 2007	No

Sexual Differentiation							
Echinogammarus marinus	Malacostraca Amphipoda, Gammaridae: Gammaridae	Whole animals	T	cDNA microarray	Normal and intersex populations	Ford and Thain 2008	No
Scylla serrata	Malacostraca Decapoda, Brachyura: Portunidae	Gonad?	T	EST	Gonad	Zou et al. 2009	No
Fenneropenaeus chinensis	Malacostraca Decapoda, Penaeoidea: Penaeidae	Ovary	T	SSH	Diploid vs. Triploid	Xie et al. 2010	No
Eriocheir sinensis	Malacostraca Decapoda, Brachyura: Varunidae	Testes	T	EST	Testes	Zhang et al. 2011	No
Molt Cycle/Exoskeleton Formation							
Portunus pelagicus	Malacostraca Decapoda, Brachyura: Portunidae	Various tissues and whole animals	T	EST, cDNA microarray	Molt, post-molt, intermolt, pre-molt	Kuballa et al. 2007	No
Portunus pelagicus	Malacostraca Decapoda, Brachyura: Portunidae	Various tissues and whole animals	T	EST, cDNA microarray	Molt, post-molt, intermolt, pre-molt	Kuballa and Elizur 2008	No
Euphausia superba	Malacostraca Euphausiacea: Euphausidae	Heads	T	EST, cDNA microarray	8 molt stages (A/B, C early, C, C late, D_0, D_1, D_2, D_3)	Seear et al. 2010	No
Portunus pelagicus	Malacostraca Decapoda, Brachyura: Portunidae	Various tissues and whole animals	T	EST, cDNA microarray	Molt, post-molt, intermolt, pre-molt	Kuballa et al. 2011	No
Callinectes sapidus	Malacostraca Decapoda, Brachyura: Portunidae	Gills, hypodermis	T	EST	Pre-molt D_2 and early post-molt	Shafer et al. 2006	No

(*continued*)

Table 13.2. *(Continued)*

Species	Taxonomy[1]	Tissue	'Omic Type[2]	Method	Experiment	Citation	Reviewed here
Gecarcinus lateralis	Malacostraca Decapoda, Brachyura: Gecarcinidae	Y-organs	P	2DE	Intermolt	Lee and Mykles 2006	No
			Olfaction				
Homarus americanus	Malacostraca Decapoda, Astacidea: Nephropidae	Olfactory organ mature zone	T	EST library, cDNA microarray	Olfactory genes	McClintock et al. 2006	No

1. Taxonomy: Class, Order, Infraorder or Superfamily: Family.
2. Omic Type: T, Transcriptome; P, Proteome; M, Metabolome.
3. SSH: EST libraries constructed from suppressive subtractive hybridization.
4. 2DE: Two-dimensional gel electrophoresis.
5. LC ESI-MS/MS: Liquid chromatography electrospray ionization tandem mass spectroscopy.
6. GCxGC/TOF-MS: Two-dimensional gas chromatography coupled with time of flight mass spectroscopy.
7. 2D-DIGE: Two-dimensional differential in gel electrophoresis.
8. FT-ICR MS: Fourier transform ion cyclotron resonance mass spectrometry.

vary across factor and taxonomic group, and interpreting the responses of commonly observed differentially expressed genes (Table 13.3) may lead to new fundamental understanding of crustacean physiology. Additional studies using these tools in crustaceans and involving other processes such as growth, development, and metamorphosis are listed in Table 13.2, but are not reviewed here. Because this field is one of rapid advancement and discovery fueled by growing access to advanced technology and increased expertise in the crustacean community, we fully expect future advancements in the field to eclipse what has been learned to date (Ou et al. 2012). Nevertheless, this review may provide a useful foundational resource.

TEMPERATURE

Crustaceans are found in just about every thermal habitat on Earth, from icy Antarctic waters, to highly variable marine intertidal zone habitats, to constantly warm tropical reefs, and extremely hot hydrothermal vent habitats. The literature is rich in examples of candidate gene approaches to studying thermal adaptation, acclimation/acclimatization, and thermal stress responses. In contrast, crustacean biologists are at the dawn of the genomics era with respect to thermal biology, and relatively few studies have specifically examined genome-wide responses to thermal habitat or thermal stress (Fig. 13.2).

Crustacean responses to thermal stress have been studied extensively in the porcelain crab *Petrolisthes cinctipes*, for which one of the largest collections of expressed sequence tags (ESTs) of any crustacean species presently exists (Table 13.1), comprising a large fraction of genomic data for the Decapoda (Fig. 13.1; see Stillman et al. 2006, Stillman et al. 2008, Tagmount et al. 2010). Transcriptomic analysis of responses to thermal stress have been conducted in hepatopancreas and cardiac tissues of *P. cinctipes* (Table 13.2) using 5 K unigene cDNA microarrays (5 K unigene is equal to ~5,000 spots, each representing a different gene; see Stillman et al. 2006, Teranishi and Stillman

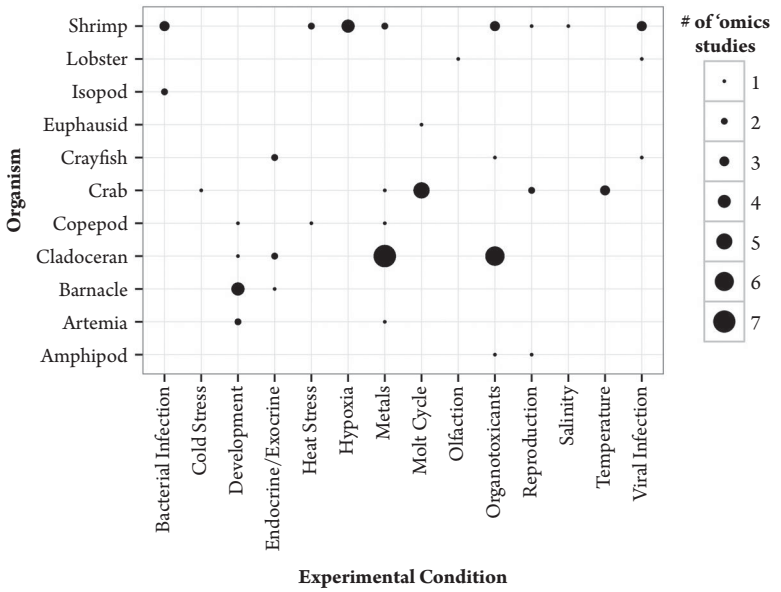


Fig. 13.2.
Graphical representation of the number of genomic and proteomic studies that have been conducted on taxonomic groups of crustaceans across a range of experimental conditions. Data obtained from Table 13.2.

Table 13.3. Genes and proteins that were observed as differentially expressed in response to multiple types of environmental stressors. Response to stressors indicated as up (upregulation), down (downregulation), and up & down (different expression patterns were observed in multiple studies).

Gene or Protein	Warm Acclimation	Cold Acclimation	Heat Shock	Viral Infection	Bacterial Infection	Organic Ecotoxicants	Heavy Metals	Hypoxia
ATP synthase		Up	Down		Down	Up		
Myosin		Up	Down	Down				Up
Actin		Up	Down	Up	Up			
β-tubulin	down	Up	Up & Down	Up				
Chaperonin				Up		Down	Up	
Thioredoxin				Up			Up	Down
Hsp70			Up				Up	Down
Lysozyme			Up & Down	Up	Up			
14-3-3-γ				Up	Down	Up		
cytochrome c oxidase		Up	Down					Up
Crustin		Up	Up		Up			
Hsp90			Up		Down	Up		
Extensin-like	down	Up	Up & Down					
Ribin	down	Up	Up & Down					
Vitellogenin						Down	Down	Up & Down
α-tubulin			Up					Down
Glutathione-S-transferase				Down				Down
Mannose receptor I			Up	Up				
Ubiquitin			Up	Up				
Serine protease				Up	Up & Down			
Ankyrin		Up	Down					
Cuticular proteins			Up			Down		

Cytochrome b	Up	Down	
Cytochrome c	Up	Down	
Isocitrate dehydrogenase	Up	Down	
NADH dehydrogenase	Up	Down	
Succinate dehydrogenase	Up	Down	
Titin	Up	Down	
Tropomyosin	Up	Down	
Troponin	Up	Down	
Hemocyanin		Up & Down	Up & Down

2007, Stillman and Tagmount 2009). In hepatopancreatic tissue, gene expression was monitored at nine time points from 30 min to 30 h of recovery following thermal stress (Teranishi and Stillman 2007). Genes involved with protein chaperone activity, including heat shock proteins (hsp), were strongly induced following heat stress (Teranishi and Stillman 2007). Heat shock protein (hsp) 20 and hsp70 genes had equally high induction at all time-points, whereas induction of hsp90 genes was strongest during the first 2 h of recovery and undetectable following 18 h of recovery (Teranishi and Stillman 2007). Ubiquitin, a tag for protein denaturation, was only strongly induced during the first 2 h following heat stress (Teranishi and Stillman 2007). Hsp20 and hsp70 induction beyond 2 h of recovery from heat stress suggests chaperone activity for newly synthesized proteins rather than the refolding of extant proteins. Ribosomal proteins were strongly induced for the first 2 h following heat stress but not at longer recovery times, thus suggesting that hepatopancreatic tissue responds to thermal stress by increasing protein synthesis. Relatively few genes were repressed throughout the recovery period. The mitochondrial ATP synthase was the most strongly repressed gene and was repressed for up to 24 h following heat stress. A number of other genes involved in oxidative metabolism were repressed immediately following heat stress. Hemocyanin expression was repressed between 12 and 24 h, but not during the first 12 h following heat stress (Teranishi and Stillman 2007).

In cardiac tissue, transcriptome profiles were monitored following both heat and cold stress in porcelain crabs acclimatized to winter or summer conditions at one latitude (June and December in Cape Arago, Oregon) or across latitude within one season (June in Monterey, California; Cape Arago, Oregon; and Bamfield, British Columbia; Stillman and Tagmount 2009; see Table 13.2). In both sets of comparisons, there was variation in both average temperature (summer warmer than winter) as well as the degree of thermal variability (summer more thermally variable than winter, high latitudes more thermally variable than low latitude due to difference in the timing of low tide; Stillman and Tagmount 2009). Specimens were given a heat stress, cold stress, or held at the collection temperature as a control, and the stress effect was calculated as the difference in gene expression between the heat- or cold-stressed individuals and the control individuals for each gene that significantly differed in expression (Stillman and Tagmount 2009). Overall, there were much stronger transcriptome responses to heat stress than to cold stress in all specimens, and only heat-stress responses varied among porcelain crabs acclimatized to different temperatures. Heat stress induced the expression of hsp20, hsp70, and some other proteins regardless of thermal history, but induction was much stronger (up to 32-fold induction) in crabs acclimatized to warmer and more thermally variable habitats (e.g., summer, high latitudes). In contrast, hsp90 was weakly induced following heat stress in summer or high-latitude acclimatized crabs and was not induced at all in winter or low-latitude acclimatized crabs.

Observed gene expression profiles, taken in aggregate, proved to be useful to discriminate across stress type as well as thermal acclimatization state. Principal components analysis of genes that were commonly differentially expressed in crabs from different latitudes and seasons that were exposed to heat stress (Fig. 13.3) or cold stress (Fig. 13.4) show that heat stress has a stronger impact on genome activity and that acclimatization is stronger with respect to heat stress than cold stress. The data also indicate that thermal acclimatization presents a stronger overall effect on transcriptome profiles than does heat stress because the first principal component largely separated seasonal and latitudinal groupings, whereas the second principal component separated control and heat-stressed crabs (Fig. 13.3). Crabs from cool, thermally constant habitats (December in Oregon, June in Monterey California) had similar shifts across the principal component that were distinct from warm, thermally variable habitats (June in Cape Arago, Oregon and Bamfield, British Columbia; Fig. 13.3). Although such groupings were also present in cold-stressed crabs (e.g., clustering of June in Monterey, California and December in Cape Arago, Oregon cold stress and stress effect crabs away from other groups), the patterns were not as strong (Fig. 13.4). These results prove that

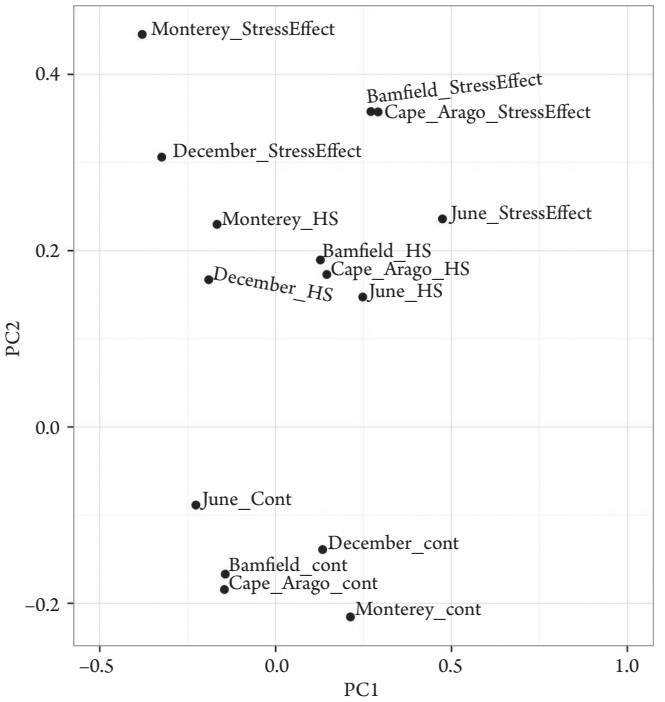


Fig. 13.3.
Graphical representation of the first two principal components from a reanalysis of data on the effect of heat stress across samples collected in two different seasons (December, June) at Cape Arago, Oregon, and at three different latitudes during summer (Monterey, California; Cape Arago, Oregon; and Bamfield, British Columbia). Microarray features used for principal components analysis were only those that commonly significantly differed in the seasonal and latitudinal study (total n = 40 features). Data from Stillman and Tagmount (2009).

transcriptome profiles are a reliable molecular-level biomarker for thermal acclimatization state and prove their utility in broader scale ecological studies.

The largest variation in cardiac transcriptome response to heat stress across crabs acclimatized to different conditions was in a small set of mostly uncharacterized genes that included β-tubulin, a 28s rRNA-encoded gene known as ribin, and genes whose only homology is to extensin-like proteins (Stillman and Tagmount 2009). These transcripts were constitutively repressed in crabs acclimatized to cool, low-variability temperatures but were strongly induced following heat stress (up to 16-fold induction). In contrast, these transcripts were constitutively induced in crabs acclimatized to warm, thermally variable habitats but strongly repressed following heat stress (up to 16-fold repression). Thus, heat stress caused 256-fold variation in the expression of these transcripts relative to control specimens, depending on thermal acclimatization state (Stillman and Tagmount 2009). The functional roles of the genes within this cluster are not known, although because α-tubulin had a very different expression profile from β-tubulin, microtubule formation is not likely one of those functions (Stillman and Tagmount 2009). β-tubulin, in addition to being a component of microtubules, has been shown to have chaperone activity in refolding heat denatured proteins (Guha et al. 1998) and restoring activity to unfolded enzymes (Manna et al. 2001), and thus it may be acting as a chaperonin in porcelain crab cardiac tissue.

Cardiac tissue of porcelain crabs acclimatized to cool, thermally constant habitats showed induction of a large number of genes involved in oxidative energy metabolism, including electron

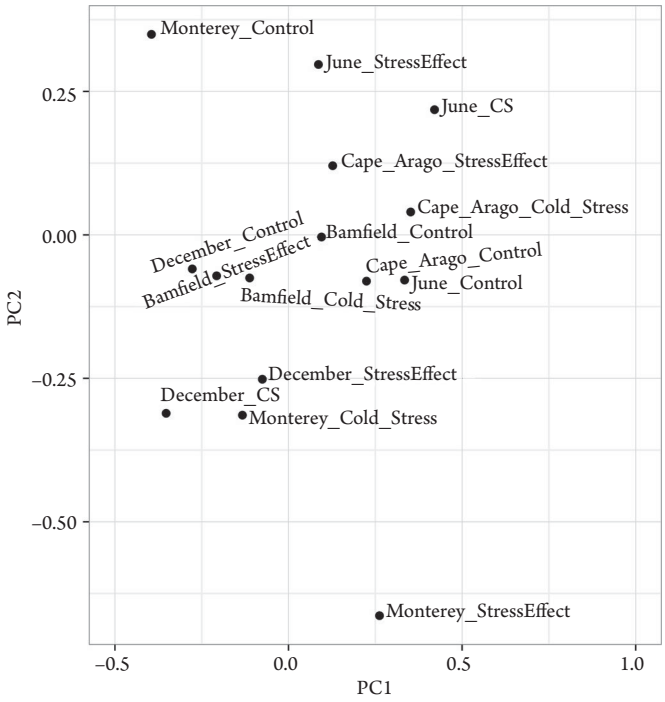


Fig. 13.4.
Graphical representation of the first two principal components from a reanalysis of data on the effect of cold stress across samples collected in two different seasons (December, June) at Cape Arago, Oregon, and at three different latitudes during summer (Monterey, California; Cape Arago, Oregon; and Bamfield, British Columbia). Microarray features used for principal components analysis were those that commonly significantly differed in the seasonal and latitudinal study (total n = 20 features). Data from Stillman and Tagmount (2009).

transport and citric acid cycle, and these genes were repressed following heat stress (Stillman and Tagmount 2009), similar to that observed in hepatopancreatic tissue (Teranishi and Stillman 2007). Cold acclimatization may induce greater expression of these genes to overcome Q_{10} effects and/or lower efficiency of mitochondrial function. Genes encoding structural and muscle proteins had acclimatization-dependent responses to heat stress, with induction following heat stress in warm acclimatized specimens and repression following heat stress in cool acclimatized specimens (Stillman and Tagmount 2009). Why thermal acclimatization should alter the thermal stress responses of these proteins is unknown, but ultrastructural analyses of muscle could be informative.

Immune responsive genes including antimicrobial peptides and complement were induced in porcelain crabs acclimatized to cooler temperatures but were not further induced by thermal stress (Stillman et al. 2006). Expression of the antimicrobial peptide carcinin has been shown to be more responsive to thermal stress than to infection load in *Carcinus maenas* (Brockton and Smith 2008), suggesting that immune-responsive genes may have temperature-related regulatory mechanisms. Additional efforts to dissect the role that temperature versus pathogen load has in the expression of immune responsive genes in crustaceans are warranted.

Variation in the transcriptome response to heat stress has been investigated in the hydrothermal vent shrimp *Rimicaris exoculata* (Cottin et al. 2010). These shrimp swarm near black smoker chimneys and may experience large variation in habitat temperature, including periods that exceed their long-term thermal maxima (Cottin et al. 2010). Thus, it was of interest as to whether they exhibited similar gene expression responses to heat stress as organisms living in more mesic and constant thermal environments. Shrimp exposed to a transient 20°C temperature spike, from 10°C to 30°C in 1 h and back to 10°C

for 2 h were used to identify differentially expressed genes using suppression subtractive hybridization cDNA libraries (SSH; Cottin et al. 2010). Of the 192 cloned differentially expressed cDNAs, approximately 47% of the induced clones and 62.5% of the repressed clones following heat stress matched known genes (Cottin et al. 2010). Genes identified as differentially expressed between control and heat-stressed *R. exoculata* specimens represented a wide range of functional processes: cellular stress response; antioxidant defense; immune response; energetics and metabolism; structural and cytoskeletal; ribosomal, transcription, and protein regulation; and calcium homeostasis (Cottin et al. 2010).

Based on the number of redundant clones sampled, the most abundant repressed transcripts encoded sarcoplasmic calcium-binding protein (SCP)-1 genes and structural and cytoskeletal genes, suggesting that processes involving muscle function and cellular organization were negatively impacted by heat stress (Cottin et al. 2010). The most abundant induced transcripts were hsp70, antioxidants, and ribosomal proteins. Quantitative real-time PCR (qPCR) was used to verify the differential expression of a select number of the transcripts identified by SSH. Of those, only two hsp70 genes and one hsp90 gene were observed to shift in expression greater than twofold (either induced or repressed) in heat-stressed versus control shrimp (Cottin et al. 2010).

In hemocytes of the black tiger shrimp *Penaeus monodon*, SSH and non-subtracted libraries were used to construct a 4 K cDNA microarray that was used to profile responses to multiple environmental stressors, including temperature (de la Vega et al. 2007). *P. monodon* were exposed to temperature increases of 6°C, from 29°C to 35.5°C (at a rate of 2°C/h), maintenance at 35.5°C for 24 h, and decrease to 29°C (at a rate of 1°C/h). Hemolymph samples were withdrawn for transcriptome profiling at four time points: (i) before the initial thermal ramp, (ii) after reaching 35.5°C, (iii) 24 h after returning to 29°C, and (iv) several days following return to 29°C (de la Vega et al. 2007). Sixty-eight features were significantly differentially expressed across the four sampling time points, and those did not include hsps. The fact that this study did not observe induction of hsps following thermal stress and that relatively few differentially expressed genes were observed could be due to the relatively low sample sizes (n = 2 for each time point) leading to low statistical power. Alternatively, the transcriptional responses of hemocytes to heat stress may be limited to just the few genes observed here and do not include induction of the cellular stress response. Finally, it is possible that 35.5°C is not a stressful temperature for these tropical shrimp and that there was little physiological requirement to induce any sort of response following a 1 day exposure to that temperature.

In the copepod *Calanus finmarchicus*, SSH was used to capture genes that were responsive to a mixed array of environmental stressors, including elevated temperature (Hansen et al. 2007). However, since there were no stressor-specific SSH libraries constructed, results can only be used to generate a generalized stress-responsive transcriptome profile. Genes observed to be induced in response to stress included common elements of the cellular stress response, β-tubulin, but not α-tubulin (Hansen et al. 2007), similar to what was observed in porcelain crabs (Stillman and Tagmount 2009).

INFECTION

The economic importance of crustaceans has motivated genomics-level studies of response to viral and bacterial pathogens commonly encountered in marine aquaculture and fisheries. These studies aim for enhanced detection of disease, as well as an understanding of disease mechanisms in order to allow the development of preventative measures.

Viral Infection

Penaeid shrimp are an important aquaculture species and are known to be highly susceptible to viral infections for which there are no drug treatments available. Genomic and proteomic screens thus afford the ability to identify target cellular responses that are repressed following infection

and present possible candidates for genetic engineering of shrimp variants that possess greater immunity. The response of the pacific whiteleg shrimp *Litopenaeus vannamei* hemocytes to Taura Syndrome Virus (TSV), a virus that can cause large-scale mortality in shrimp aquaculture, was monitored using a proteomic approach (Chongsatja et al. 2007). Using two-dimensional gel electrophoresis of infected and control specimens, a total of 32 protein spots were found to be differentially expressed between control and infected shrimp, with eight proteins strongly upregulated and five proteins strongly downregulated. Proteins strongly upregulated following infection functioned in signal transduction, carbohydrate metabolism, detoxification, and cellular structure and protein modifications potentially involved with stress responses. Proteins strongly downregulated following infection have functional roles in cellular defenses against oxidative stress. Hemocyanins that did not have infection-dependent expression were among the most highly abundant proteins. Specific TSV responses of hemocyanins were inferred from the fact that differentially expressed hemocyanins upregulated following TSV infection matched mainly the acidic C-terminus of the protein (average pI = 4.98), whereas those downregulated following infection matched the N-terminus (average pI = 5.23; Chongsatja et al. 2007).

Response to White Spot Syndrome Virus (WSSV) has been investigated in multiple studies utilizing genomics approaches (Fig. 13.4, Table 13.2), including transcriptomic studies in *L. vannamei* hepatopancreas, gill, hemocyte, and muscle tissues (Robalino et al. 2007, Robalino et al. 2009), transcriptomic studies of *Litopenaeus stylirostris* (Dhar et al. 2003), transcriptomic responses in *Procambarus clarkii* hemocytes (Zeng and Lu 2009), and proteomic responses of *L. vannamei* gill tissue (Robalino et al. 2009). *L. vannamei* were injected with active virus at two temperatures (27°C and 32°C; the higher of which inhibits virulence), and, as controls for general response to stimuli, shrimp were also injected with heat-killed bacteria and fungal spores (Robalino et al. 2007). ESTs (n = 7,021) captured in cDNA and SSH libraries assembled into 3 K unigenes, approximately 900 of which were represented by multiple ESTs. Immune response-related cellular responses including antimicrobial, antiviral, cell adhesion, cell death, oxidative stress, proteases, protease inhibitors, RNA interference, signal transduction, and transcriptional control were represented by 98 unigenes, many of which were differentially regulated with respect to infection. Among these, ESTs with high biological significance in response to viral infection included IκB kinase, a positive regulator of the NF-κB pathway, and signal transducer and activator of transcription (STAT), a core component in the interferon response in vertebrates and antiviral responses in arthropods (Robalino et al. 2007). Additional potential antiviral genes identified included three components of the RNAi pathway (Robalino et al. 2007). Following WSSV infection, a 2.5 K unigene cDNA microarray was used to examine differential expression in hepatopancreas tissues of *L. vannamei* (Robalino et al. 2007). WSSV induced genes involved in antimicrobial response, protein degradation, chromatin remodeling and energy metabolism. WSSV repressed genes involved xenobiotic response, transmembrane proteins and cell signaling, protein synthesis, and oxidative stress (Robalino et al. 2007). To what extent antimicrobial genes induced by viral infection represent secondary microbial infection versus general immune response pathways requires further study. Many of the putative immune-responsive genes identified in the SSH libraries were not observed to be differentially expressed following WSSV infection in hepatopancreas by cDNA microarray, a result that could be due to tissue-specific responses to WSSV.

Gill proteome response to WSSV infection in *L. vannamei* was examined using 2-dimensional liquid chromatography together with tandem mass spectrometry (2D LC-MS-MS) at 0 and 12 h post infection (Robalino et al. 2009). Only at 12 h post infection were any peptides determined to be upregulated by the infection, although this could be a result of endogenous rhythmicity of protein expression. Those peptides indicate that changes in chromatin structure, cytoskeleton, protein turnover, pigments, and possibly energy metabolism may result from WSSV infection in gill tissue (Robalino et al. 2009).

Transcriptomic responses of *L. stylirostris* hepatopancreas tissue to WSSV infection, monitored on a very small (n = 100 features) microarray made from cDNAs cloned from infected shrimp and

in differential display libraries, found strong induction of a serine protease and genes involved within cellular surface recognition proteins, thus suggesting strong antimicrobial response (Dhar et al. 2003). Repressed genes were difficult to functionally characterize (Dhar et al. 2003). Some of the unknown genes were cloned by differential display and thus may represent 3′ untranslated regions (UTRs) for the genes, requiring further DNA sequencing into the protein coding region of the gene before assessing gene homology (Dhar et al. 2003). These results indicate that antibacterial genes are induced following viral infection, similar to that observed by (Robalino et al. 2007).

In the crayfish *P. clarkii*, hemocytes of WSSV infected and noninfected specimens were used to make a cDNA microarray from cDNAs captured using SSH (Zeng and Lu 2009). A total of 255 cDNAs were induced, and 23 cDNAs were repressed following WSSV infection. Of the induced cDNAs, the strongest response (four- to fivefold induction) was an inhibitor of apoptosis (Zeng and Lu 2009). Additional genes induced by WSSV encoded proteins involved in protein homeostasis, the cytoskeleton, and energy metabolism (Zeng and Lu 2009). There was little overlap in the most strongly differentially expressed genes in shrimp and crayfish following WSSV infection, although potentially a different set of genes was examined.

Bacterial Infection

Transcriptomic and proteomic responses to bacterial infection have been examined in crustaceans infected with *Vibrio* (gram negative γ-proteobacteria), *Wolbachia* (α-proteobacteria), and unknown bacteria or bacterial assemblages that cause lesions in crustacean shells known as epizootic shell disease.

Response to *Vibrio penaeicida* infection was examined using SSH in hemocytes of *L. stylirostris* that survived to 96 h postinfection (de Lorgeril et al. 2005). Macroarrays were constructed from 320 randomly selected cloned cDNAs that assembled into 52 clusters and 158 singletons, for a total of 210 unigenes (de Lorgeril et al. 2005). Those cDNAs with homology to functionally annotated genes were involved with immune function (21% of identified cDNAs); cell proliferation (20%); metabolism (17%); DNA modification, gene expression, and protein synthesis (15%); cell signaling (12%); and cell structure (5%; de Lorgeril et al. 2005). There was relatively little differential expression of any of the SSH identified genes between infected and uninfected shrimp, but qPCR analysis indicated a greater than twofold upregulation of several immune function genes and a gene product involved in cell division and proliferation following infection (de Lorgeril et al. 2005).

Proteomic analysis of black tiger shrimp, *P. monodon*, hemocytes following *Vibrio harveyi* infection identified 27 protein spots by two-dimensional gel electrophoresis that differed between control and infected specimens (Somboonwiwat et al. 2010). Proteins upregulated by infection included prophenoloxidase 2 and actin 2, suggesting that those proteins have a specific role in response to infection (Somboonwiwat et al. 2010). Proteins upregulated in infected shrimp but also present in control specimens include hemocyanin, arginine kinase, twinstar, tubulin, serine protease, and transaldolase (Somboonwiwat et al. 2010). Proteins downregulated by infection included multiple immune-responsive proteins, including α2 macroglobulin, prophenoloxidase-1 and -2, serine proteases, hsp90, 14-3-3 protein epsilon, calmodulin, karyopherin, and ATP synthase (Somboonwiwat et al. 2010). It is likely that the differences in protein pools between infected and uninfected shrimp are reflections of post-translational modification rather than protein expression, and thus the prophenoloxidase observed in control shrimp may be the same gene product as the one observed in infected shrimp but with a different set of post-translational modifications involved with regulation of this immune-responsive enzyme (Somboonwiwat et al. 2010).

Cell wall peptidoglycans prepared from *Bifidobacterium thermopilum* were fed to the kuruma shrimp *Marsupenaeus japonicus* to examine hemocyte transcriptome responses to noninfectious bacterial components (Fagutao et al. 2008). cDNA microarrays containing 2 K unigenes

identified from WSSV-infected and -uninfected EST library construction projects in *M. japonicus* (Rojtinnakorn et al. 2002) and *P. monodon* (Supungul et al. 2002) were used to examine responses at 1, 7, and 14 days following peptidoglycan injections (Fagutao et al. 2008). The strongest gene expression response in transcripts induced or repressed by peptidoglycans occurred at 1 day postingestion. Induction of known antimicrobial, clotting, healing, and melanization genes was observed (Fagutao et al. 2008). Transcripts strongly induced for up to 2 weeks following peptidoglycan exposure were involved in protein synthesis, immune response, and other functions, suggesting that a strong and prolonged immune response can be initiated by noninfectious elements of bacterial cell walls (Fagutao et al. 2008).

Terrestrial arthropods are commonly infected by *Wolbachia* bacteria, and these infections can be passed to subsequent generations in the germ line (Herbiniere et al. 2008). To investigate the immune response of terrestrial isopod crustaceans to *Wolbachia* infection, proteomic analysis was performed on hemolymph samples using two-dimensional gel electrophoresis (Herbiniere et al. 2008). Of the 300 protein spots that were identified in all three of the replicate gels, about one-third were excised and characterized using Q-TOF-MS analysis, resulting in 56 identified proteins (Herbiniere et al. 2008). Of those proteins, a number were found to be involved with immune functioning, including non-self recognition and melanization of xenobiotics coagulation, detoxification, cell adhesion, cell communication, protease defense, stress response, and the cytoskeleton (Herbiniere et al. 2008). Identification and characterization of these proteins in a terrestrial isopod form a valuable tool for examination of infection in noninsect terrestrial arthropods.

Epizootic shell disease is manifested by characteristic lesions on the external surface of crustaceans. Although the bacteria responsible for causing these lesions are not well characterized, one hypothesis is that they are normal members of the marine microbial assemblage that cause disease when the infected animals are suffering from poor physiological condition. Variation in multiple tissues of American lobsters, *Homarus americanus*, that were either suffering from epizootic shell disease or were asymptomatic were used to determine whether general physiological condition plays a role in the susceptibility of lobsters to the bacteria that cause shell erosion. Differentially expressed transcripts were captured by SSH using two sets of symptomatic and asymptomatic lobsters (Tarrant et al. 2010). Approximately equal numbers of genes were induced (n = 73) as repressed (n = 66) in symptomatic lobsters. There was little concordance in the specific ESTs that were identified as induced or repressed in symptomatic lobsters between the two sets of SSH libraries. For example, in one SSH library, myosin, actin, and a mannose-binding protein were induced; whereas in the second SSH library, arginine kinase, cysteine protease, cytochrome b, cytochrome c, and keratinocyte-associated and mannose-binding proteins were induced, with the only overlap between the two libraries being the mannose-binding protein (Tarrant et al. 2010). However, to confuse matters, mannose-binding protein was also observed in both SSH libraries to be repressed in symptomatic lobsters, as were a number of the other genes identified as induced in the second SSH library in symptomatic lobsters (Tarrant et al. 2010). Muscle arginine kinase expression, quantified by qPCR, was repressed in symptomatic lobsters, a result that was interpreted as representing an energetic drain resulting from or enabling the infection (Tarrant et al. 2010). Hemocyanin expression in hepatopancreatic tissue was also repressed in symptomatic lobsters, suggesting that lower respiratory capacity is involved in epizootic shell disease (Tarrant et al. 2010). Ovarian levels of α2 macroglobulin were induced in symptomatic lobsters, suggesting that these lobsters are coping with higher levels of proteolytic activity, potentially due to infection, in ovarian tissues (Tarrant et al. 2010). Although this initial study of transcriptional response to epizootic shell disease was by no means comprehensive, nor did it identify a smoking gun, the study does lend some evidence that the shell disease is at least associated with changes across the entire organism and not just at the sites where lesions are located.

ENVIRONMENTAL TOXICANTS

Much of the "omics-based" work on crustacean responses to environmental stress has been in the area of environmental toxicology. Ecotoxicogenomics is a rapidly growing field wherein researchers are realizing the potential of genomic and other approaches for characterizing stressor-specific response profiles (Snape et al. 2004). The applied potential of ecotoxicogenomics for identifying biomarkers is great, and this approach has identified rapid biomarkers for reproductive effects, such as vitellogenin mRNA (Soetaert et al. 2006), and it has characterized novel metallothioneins (Shaw et al. 2007) in cladocerans, which are efficacious as markers of trace metal exposure (Amiard et al. 2006). Although there are many overlapping, common molecular stress response pathways induced by ecotoxicants in general, evidence is compelling for stressor-specific responses. These specific response profiles may be grouped broadly into two categories based on the chemical characteristics of toxicants: those induced by heavy metals and those induced by organic compounds.

The bulk of published work on ecotoxicogenomics in crustaceans has thus far focused on metals, specifically the trace metal cadmium, likely due to its effects on human health (Nordberg 2009). Other work has focused on responses to copper and organic compounds such as atrazine, monoethyl amine, propiconazole, pyrene, water-soluble fractions of oil, and organophosphates. For the most part, studies have focused on responses at the level of the transcriptome, but some proteomic (Silvestre et al. 2010) and metabolomics (Ralston-Hooper et al. 2008) approaches have been taken as well. Daphnids have been the main focus of ecotoxicogenomics efforts, due to the history of these species in toxicity testing. However, work has also been done on other crustacean taxa, including other groups within the Branchiopoda, Maxillopoda, and Malacostraca.

Organic Compounds

Crustacean sensitivity to insecticidal organics (e.g., organochlorines, organophosphates, and pyrethroids) is likely due to their shared ancestral lineage with the insect arthropods that those toxins target. Thus, establishing genomic response profiles of crustaceans to organic toxicants is of great utility to ecotoxicology. For these reasons, there is a growing body of work aimed at characterizing genome-wide induction by organics in crustaceans to develop biomarkers and elucidate basic cellular response mechanisms.

In *Daphnia magna*, functional genomics approaches have been employed to investigate molecular responses to the triazole fungicide propiconazole (Soetaert et al. 2006) and to polycyclic aromatic hydrocarbons (PAHs) fluoranthene and pyrene (Vandenbrouck et al. 2010). SSH was used to generate a cDNA library of differentially expressed genes in *D. magna* between adults and juveniles, enriched for reproduction-specific transcripts (Soetaert et al. 2006). This cDNA library has been utilized in multiple studies (Soetaert et al. 2006, Soetaert et al. 2007, Vandenbrouck et al. 2010), which illustrates the utility to the scientific community of constructing "omics-scale" resources.

The 1,189 unique reproduction-specific cDNA fragments generated for *D. magna* corresponded to transcripts involved in ribosomal RNA/proteins, cell cycle, molting, embryonic development, energy metabolism, nervous system development, and other cellular processes (Soetaert et al. 2006). The effect of propiconazole, a compound known to have detrimental effects on daphnid embryos, was measured via a cDNA microarray generated from the reproduction-specific library (Soetaert et al. 2006). Far more transcripts were repressed than induced, and these displayed a time- and concentration-dependent expression pattern because the majority of transcripts were most strongly repressed immediately following exposure (Soetaert et al. 2006). The most strongly repressed transcripts were vitellogenin, larval-specific gene, stromal cell-derived factor, and chaperonin, and the

most strongly induced transcripts were hsp90 and ATP synthase (Soetaert et al. 2006). Although multiple genes involved in cellular energy metabolism were represented by the array, ATP synthase was the only differentially expressed metabolic gene in response to propiconazole.

The same cDNA library was used to investigate the effects of the PAHs fluoranthene and pyrene on *D. magna* in an integrated approach incorporating metabolomics and energetics (Vandenbrouck et al. 2010). To investigate the effect of various mixtures of the similarly acting compounds and to potentially parse out the molecular differences in response, mixtures of varying toxic units, as well as just one compound or the other, were used as treatments (Vandenbrouck et al. 2010). Along with cDNA microarray experiments, validations were performed using qPCR, and metabolomics analyses were conducted using nuclear magnetic resonance spectroscopy (^{1}H NMR) and gas chromatography mass spectrometry (GC-MS). Similar to the response observed to propiconazole, more genes were repressed than induced: 34 in response to solely fluoranthene and 27 to solely pyrene (Vandenbrouck et al. 2010). Unlike propiconazole, gene expression changes elicited by fluoranthene and pyrene did not change in a concentration-dependent manner, and, based on a hierarchical clustering analysis on all differentially expressed genes, there was no difference in response to the two compounds (Vandenbrouck et al. 2010).

Vitellogenin was repressed in response to fluoranthene and pyrene in *D. magna*, as was also observed in response to propiconazole; however, there was no reported induction of hsp90 or any other canonical stress response proteins or significant induction of energy metabolism-related transcripts (Vandenbrouck et al. 2010). Although all differentially expressed genes were not significantly different between the two PAHs, there were observed differences between them at the single transcript level. For example, in response to pyrene, two carboxypeptidases and a chymotrypsin-like protease were induced, whereas fluoranthene elicited a repression of cuticular protein-related transcripts (Vandenbrouck et al. 2010), which appears to be a common response to metal contaminants, as discussed in upcoming paragraphs.

D. magna also responded differently to binary mixtures of fluoranthene and pyrene, exhibiting greater differential expression than the expected sum of the two parts when exposed to both compounds (Vandenbrouck et al. 2010). This finding supports a potential synergistic rather than additive interaction effect on gene expression. Thus, although commonalities exist among daphnid responses to the organics propiconazole, fluoranthene, and pyrene, there are also stressor-specific responses that can be valuable for establishing unique response profiles.

SSH libraries were generated for the copepod *C. finmarchicus* exposed to a sublethal mixture of environmental stressors (Hansen et al. 2007) and to diethanolamine (DEA; Hansen et al. 2010). The sublethal mixture was composed of monoethanolamine (MEA), water-soluble fractions of oil, copper, and elevated temperature, and it induced differential expression of genes such as hsp, antioxidants, and cytochrome P450 (Hansen et al. 2007). Although exposing organisms to a mixture of compounds may provide some qualitative insight into the inducible transcriptome, it is difficult to determine which changes are attributable to which compounds, and one mixture represents a minute fraction of the possible combinations of toxicants to which an organism may be exposed. Further, interactions of specific compounds make it difficult to characterize a response because a pair of toxicants may share an antagonistic interaction on gene expression. Pairing investigations of exposure to mixtures with single-compound exposures as a contrast will help to provide insight into potential antagonistic, additive, or synergistic effects on the expression of genes or proteins in the future.

A concentration of DEA corresponding to 25% of the lethal concentration for *C. finmarchicus* caused differential expression of 865 clones that were identifiable by basic local alignment search tool (BLAST; Hansen et al. 2010). Of the 865 clones, gene ontology (GO) terms could be used to annotate 194 of the induced transcripts and 511 of the repressed transcripts, the majority of which corresponded to molecular functions of binding, catalytic activity, structural molecule activity, and transcription regulator activity (Hansen et al. 2010). Interestingly, the proportion of enzymatic

transcripts that were repressed was 60% higher than the proportion induced and approximately four times in magnitude (202 transcripts repressed vs. 48 transcripts induced; Hansen et al. 2010), much like the aforementioned effects of propiconazole, fluoranthene, and pyrene on gene expression in *D. magna*.

Metabolomic analysis of *C. finmarchicus* exposed to DEA using a high-resolution magic angle spinning NMR (HR-MAS NMR) indicated choline deficiency (Hansen et al. 2010), which could have an effect on lipid metabolism (Barbee and Hartung 1979). Indeed, changes in transcripts for enzymes involved in lipid metabolism were found in the SSH library and confirmed using qPCR (Hansen et al. 2010).

Antibiotics and their effect on crustacean physiology is a growing concern due to their increasing inclusion as a common practice in aquaculture. The effect of the antibiotics enrofloxacin and furazolidone on *P. monodon* has been evaluated using a differential proteomic approach (Silvestre et al. 2010). *P. monodon* did not exhibit significantly different protein expression profiles from controls in response to the antibiotics except for one unidentified protein, which was significantly decreased in abundance in response to enrofloxacin (Silvestre et al. 2010). However, this study was considerably limited by statistical power due to experimental design (three replicates per treatment of six pooled individuals). The type of culture system used had a more significant effect on protein expression than did antibiotic treatment. Two systems were used: intensive culturing ponds and improved extensive culturing ponds (Silvestre et al. 2010). Intensive culture systems are characterized as being small and relatively deep, with high stocking densities and water exchange regulated by pumps and aeration systems, whereas the improved extensive systems are large, shallow, with low stocking densities and a tidal water exchange (Silvestre et al. 2010).

Nine proteins were found to be differentially expressed between *P. monodon* reared in intensive or improved extensive culturing systems, of which three were identified as hemocyanins and were highly expressed in the intensive system (Silvestre et al. 2010). Two proteins were identified as SCPs and were highly downregulated in the intensive system. These results suggest that perhaps hypoxic conditions in the intensive system led to increased hemocyanin production to bind oxygen more effectively and that these individuals were experiencing suppressed immune capacity because SCPs are involved in Ca^{2+} homeostasis and linked to immune function in *Drosophila* (Engstrom et al. 2004).

Crustacean responses to organic compounds at the omic level, although still quite understudied, are beginning to show some commonalities. For example, whether at the transcript- or protein-level, it remains consistent among studies, compounds, and organisms that repression far outweighs induction of gene expression, perhaps due to an energetics tradeoff necessary for cellular repair mechanisms or sustaining metabolic performance under stress. Evidence thus far is suggestive of a dose-dependent magnitude of expression change for some compounds. More studies targeted at gaining a quantitative understanding of expression responses to toxicants will be necessary to establish reliable indicators of exposure to organic ecotoxicants.

Metals

Almost without exception, omic studies of crustacean responses to metals have focused on cadmium (Cd), copper (Cu), or zinc (Zn). Many studies have focused on daphnids: eight involving *D. magna* and one *D. pulex*. Other taxa studied include the Chinese mitten crab *Eriocheir sinensis*, shrimp *L. vannamei*, brine shrimp *Artemia sinica*, copepod *Tigriopus japonicas*, and the grass shrimp *Palaemonetes pugio*. Within the daphnids, most published work is transcriptomic, although there have been some metabolomics studies. The *D. pulex* genome sequencing effort (Colbourne et al. 2011) will undoubtedly increase the output of toxicogenomics studies, and productivity in years to come will be prolific for daphnids and other crustacean taxa as a result.

The same cDNA libraries enriched for energy metabolism, molting, and life stage-specific processes (Soetaert et al. 2006) mentioned in the previous section on organics were used to establish a cDNA microarray for *D. magna* to measure transcriptome responses to 0, 10, 50, and 100 μg/L of Cd at 48 and 96 h exposures (Soetaert et al. 2007). Cd exposure resulted in differential expression (≥1.8-fold) of 112 nonredundant gene fragments that exhibited a very clear time- and concentration-dependent relationship (Soetaert et al. 2007). The differentially expressed fragments corresponded to genes involved in digestion, oxygen transport, acid–base balance, and immune response, based on GO annotations (Soetaert et al. 2007). A first-generation cDNA microarray was also established for *D. pulex* to measure expression changes resulting from Cd exposure, leading to the identification of novel metallothioneins (Shaw et al. 2007). Since these initial studies, a number of efforts have been made to further investigate the effects of Cd and other metals on daphnids—although mostly in *D. magna*, likely due to its history as a sentinel for aquatic ecosystem health.

D. magna exposed to nickel (96 h at either 0.125, 0.5, 1, or 2 mg/L), exhibited repression of hemoglobin-associated genes but induced genes potentially involved in early heme biosynthesis (Vandenbrouck et al. 2009). These conflicting results suggest that hemoglobin effects observed in daphnids exposed to metals could be due to disruption of heme biosynthesis and not hypoxia inducible factor-related transcriptional regulation of the hemoglobin protein (Vandenbrouck et al. 2009). By pairing microarray results with a time-course of available energy reserves, it was concluded that vitellogenin repression under heavy-metal stress might be due simply to a lack of energy reserves, which could preclude the use of vitellogenin as a biomarker for juvenile hormone exposure (Vandenbrouck et al. 2009) although targeted expression studies will be needed in the future.

A cDNA microarray consisting of 5 K randomly selected cDNA clones from the Daphnia Genome Consortium was used to generate distinct expression profiles for *D. magna* exposed to Cu, Cd, and Zn at 5% of the lethal concentration for 24 h (Poynton et al. 2007). Animals exposed to Cd exhibited the greatest differential expression, with the majority of transcripts being repressed (Poynton et al. 2007). In *D. magna* exposed to Zn and Cu, the proportion of repressed and induced transcripts was roughly equal, whereas Cd-exposed individuals had fourfold greater repressed genes (Poynton et al. 2007). There were only four differentially expressed genes common to all three metal exposures; all four were repressed and corresponded to digestion and nutrient absorption (Poynton et al. 2007). A larger 15 K oligonucleotide was used to demonstrate that in *D. magna* exposure to silver (Ag) complexed with nanoparticles caused physiological responses that were different from exposure to Ag alone (Poynton et al. 2012). Metal responsive and DNA damage repair genes were induced by Ag in nanoparticle complexes, but not $AgNO_3$, suggesting that we need to re-evaluate toxicity of metals when they are complexed in nanoparticles.

To establish biomarkers for metal exposure that extend in utility beyond a qualitative indicator, concentration- and metal-dependent gene expression profiles have been generated and field-validated for *D. magna* (Poynton et al. 2008). When exposed to concentrations of 5% of the effective concentration, 5% of the lethal concentration, and a nonobservable effect concentration of Cu, Cd, and Zn, *D. magna* gene expression, quantified using a previously developed cDNA microarray (Poynton et al. 2007), exhibited very specific profiles dependent on the concentration and type of metal (Poynton et al. 2008). Exposure to lower concentrations resulted in a more resolved and metal-specific expression profile for all three metals, whereas exposures of near-acute toxicity resulted in many more common stress response genes being expressed (Poynton et al. 2008). Thus, the utility of gene expression profiles for distinguishing between metal types may be limited to chronic sublethal exposures in daphnids, although a general inference of metal exposure may be gained under acutely toxic conditions. The feasibility of using microarray technology to identify a specific metal in a natural setting has been validated for *D. magna*. Animals exposed to field samples collected from Cu mines in California were used to create gene expression profiles and compared to previously generated profiles for Cu, Cd, and Zn (Poynton et al. 2008). Cu was

successfully identified as the primary pollutant based on clustering analysis and class prediction algorithms (Poynton et al. 2008), thus demonstrating the applied potential of daphnid ecotoxicogenomic assessments.

Although a number of studies have demonstrated that gene expression profiles are metal- and concentration-specific in daphnids, expression data without any indication of ecological significance are of little importance if one aims to evaluate ecosystem health. In *D. magna*, transcriptome profiles generated using a 14 K feature cDNA microarray were compared to population growth rate (Connon et al. 2008). As population growth rates declined with increasing Cd concentrations, increased differential expression of transcripts associated with cellular processes such as growth and molting, ion transport, and general stress responses was observed (Connon et al. 2008). Similarly, *D. magna* fed a Zn-enriched diet suffered decreases in population and delays in molt cycle relative to controls, and differential expression of transcripts involved in growth and molting was also observed (De Schamphelaere et al. 2008). The pairing of transcriptomics studies with higher level observations, such as decreased population growth rates and depleting energy reserves (Vandenbrouck et al. 2009), suggests that chronic effects observed in daphnids due to metal exposure may be due in part to a reduction in the bioavailability of resources to the organism and thus the ability to assimilate energy that would otherwise drive somatic growth.

There are limited metabolomics studies involving crustacean responses to metals. When combined with expression data from a 44 K oligonucleotide microarray, results from Fourier transform ion cyclotron resonance mass spectrometry and NMR spectroscopy analyses indicate that fatty acid metabolism and nutrient absorption may be primary avenues through which Cd reduces energy reserves in *D. magna* (Poynton et al. 2011). Four fatty acids decreased in *D. magna* hemolymph resulting from Cd exposure: lauric acid, myristic acid, palmitic acid, and steric acid, which are all part of the fatty acid biosynthesis pathway (Poynton et al. 2011). Furthermore, six of the seven amino acids that decreased in *D. magna* hemolymph after Cd exposure were essential, thus indicating the perturbation of nutrient absorption, a result supported by transcriptome changes of genes involved in protein and carbohydrate metabolism (Poynton et al. 2011). Metabolomic analysis has been used in *D. magna* to establish signatures of specific compounds with differing modes of action, such as Cd and various nonmetal compounds (Taylor et al. 2009, Taylor et al. 2010). Initial studies provide compelling evidence that metabolomics, especially when paired with transcriptomics and other approaches, will prove a valuable tool in elucidating mechanisms underlying toxicity in crustaceans.

Outside of the daphnids, omic studies of crustacean responses to metals are taxonomically limited. Most nondaphnid studies are on the decapods or copepods. Within the Decapoda, there have been two EST studies and one proteomic study. ESTs have been generated in response to Cd in postlarvae of the shrimp *L. vannamei* (Keating et al. 2007) and in Cu-exposed *P. pugio* (Li and Brouwer 2009a). A proteomic analysis of the anterior gill of the mitten crab *E. sinensis* under exposure to Cd has also been performed (Silvestre et al. 2006). In copepods, there has been one oligochip microarray study in *T. japonicas* exposed to Cu and an SSH library (Ki et al. 2009) prepared from *C. finmarchicus* exposed to a mixture of MEA, Cu, and water-soluble fractions of oil at elevated temperature (Hansen et al. 2007). One other proteomic study on the response of *A. sinica* larvae to Cu-sulfate has been performed (Zhou et al. 2010). There is a great necessity for future efforts to include functional genomics approaches to metal exposure in nondaphnid taxa because such studies will allow comparative approaches between these taxa and the well-studied *D. magna*.

In *E. sinensis*, acute and chronic Cd-exposure regimes express a very different gill proteome (Silvestre et al. 2006). *E. sinensis* expressed six two-dimensional gel electrophoresis protein spots differentially after acute (500 μg/L for 3 days) exposure and 31 spots after chronic (50 μg/L for 30 days) Cd exposure, which resulted in acclimation or increased resistance to subsequent acute exposure (Silvestre et al. 2006). The upregulation of several antioxidant proteins and chaperones

during chronic acclimation suggests that Cd induces toxicity primarily through oxidative stress and sulfhydryl-binding in *E. sinensis* (Silvestre et al. 2006). Similarly, in response to Cu-sulfate, *A. sinica* larvae upregulated chaperones as well as peroxiredoxin (Zhou et al. 2010). Although these results are highly suggestive of oxidative stress as a primary route of toxicity for these metals in both malacostracan and maxillopodan crustaceans, many more proteomic analyses in multiple species will be necessary to offer a better-resolved mechanism.

T. japonicas consistently induced and repressed 138 and 375 genes, respectively, when exposed to 10 μg/L Cu for 6, 12, and 24 h (Ki et al. 2009). Similar to the effects of propiconazole on daphnids (Soetaert et al. 2006), the greatest number of differentially expressed transcripts occurred at the earliest time point, indicating an acute effect on gene expression, and the majority of differentially expressed genes were repressed (Ki et al. 2009), an observed commonality in crustacean responses to both organics and metals. The majority of repressed genes were involved in growth and development, again suggesting an energetics tradeoff in transcription during stress. Contrary, however, to the common finding of repressed mRNA as well as protein-level responses to environmental toxicants, the copepod *C. finmarchicus* exhibited more induction (127 ESTs) than repression (54 ESTs) of identifiable transcripts in response to a combination of stressors (Hansen et al. 2007). Increased induction may be a product of limited annotation power, but it could also be a true result indicating an emergent property of gene expression under multiple stressors, and it highlights the confounding effects toxicant mixtures may have on crustacean biology.

HYPOXIA

Crustaceans are commonly found in hypoxic habitats, and induction of hemoglobins is well known in the Branchiopoda following hypoxic or anoxic exposure. However, relatively few studies have employed omics approaches to examine the responses of crustaceans to hypoxia.

Grass shrimp, *P. pugio*, exposed for 3 to 5 days to severe (1.5 mg/L O_2) and moderate (2.5 mg/L O_2) chronic hypoxia and to variable oxygen tensions between severe hypoxia and normoxia (1.5→7 mg/L) were used to construct hepatopancreas tissue SSH libraries in order to identify genes involved with response to environmental oxygen (Li and Brouwer 2009a). For each oxygen exposure, induced and repressed cDNAs were identified and sequenced. The three oxygen exposures resulted in three distinct sets of differentially expressed transcripts for which functional homology was ascribed, with only one transcript differing specifically between oxygen treatments (Li and Brouwer 2009a). Translation elongation factor 2 was induced in both chronic oxygen exposures, and cytochrome c oxidase subunit III was induced in both chronic and variable exposures where severe hypoxia was reached (Li and Brouwer 2009a). Cytochrome c oxidase subunit I was observed to be upregulated across all oxygen exposures (Li and Brouwer 2009a). Chronic severe hypoxia resulted in six induced transcripts, three of which were not observed in any of the other oxygen treatments (Li and Brouwer 2009a). In contrast, moderate chronic hypoxia and cyclical hypoxia resulted in 15 and 22 differentially induced genes, respectively. Both moderate and cyclical hypoxia induced genes involved with oxygen transport and gluconeogenesis when mean oxygen tensions were low but not severe (Li and Brouwer 2009a). Genes induced in chronic moderate hypoxia included several lipid-binding/modification genes and oxygen-binding proteins (Li and Brouwer 2009a). Genes expressed only in cyclical hypoxia included potential DNA modification proteins, as well as oxidative metabolism (Li and Brouwer 2009a). There was a larger magnitude response of genes repressed by chronic and cyclical hypoxia, and, like induced genes, most differentially repressed genes were expressed only in one of the three hypoxia treatments. Chronic severe hypoxia resulted in 21 repressed genes, one of which, vitellogenin, was also observed as repressed in the other two treatments. Moderate chronic hypoxia and cyclical hypoxia resulted in 47 and 58 repressed genes, respectively, with five transcripts repressed in both of those treatments (Li and Brouwer 2009a). Under chronic severe hypoxia, the repressed genes encoded some immune

responsive proteins, fermentative metabolism, lipid binding, and cell–cell communication (Li and Brouwer 2009a). Moderate chronic hypoxia repressed some structural protein genes, immune responsive genes, protein synthesis, and polyamine synthesis inhibition. Genes involved in sulfur redox and (homo)cysteine metabolism were all repressed in response to cyclic hypoxia. Additionally, cyclical hypoxia repressed genes that may be involved in vitamin metabolism and, potentially, pH regulation (Li and Brouwer 2009a).

The SSH libraries generated by Li and Brouwer (2009a) were used to construct a cDNA microarray to determine a higher resolution response to hypoxia in thorax and hepatopancreatic tissues of *P. pugio* at between 0 and 240 h after severe chronic hypoxia (Li and Brouwer 2009b). The first 5 days of hypoxia caused induction of genes that were involved in oxygen transport, cell surface recognition, and iron homeostasis but little in the way of gene repression. At 10 days of hypoxia, there was a shift in gene expression patterns, with induction of only a few genes but repression of a large number of genes involved in metabolism and, potentially, cell cycle regulation (Li and Brouwer 2009b). Overall, the results suggested that a limited set of transcripts were modulated by hypoxia but that these transcripts were both induced and repressed by hypoxia, depending on the hypoxia duration. Genes induced at 6, 24, and 120 h hypoxia tended to be repressed at 12, 48, and 240 h hypoxia (Li and Brouwer 2009b), potentially because of confounding effects of endogenous rhythms of gene expression.

Proteomic two-dimensional electrophoretic analysis of hepatopancreas response to hypoxia in the fleshy prawn *Fenneropenaeus chinensis* identified 67 protein spots that changed following 3.5 h at 45% oxygen (Jiang et al. 2009). Fifty-one of the 67 differentially expressed protein spots had homology to known proteins. Fifteen spots representing 11 proteins were upregulated, and 36 spots representing 23 proteins were downregulated during hypoxia. Proteins upregulated by hypoxia were involved in carbohydrate metabolism, immune response, chaperone proteins, and exoskeletal pigmentation (Jiang et al. 2009). Proteins downregulated by hypoxia were involved in energy metabolism, antioxidant response, chaperones, and cytoskeletal proteins (Jiang et al. 2009). The most strongly upregulated and among the most strongly downregulated proteins were the protein disulfide isomerase chaperonins (PDIs). Because PDIs were observed in spots both up- and downregulated following hypoxia, these spots likely represent different post-translational modifications that play a role in regulation of activity (Jiang et al. 2009).

In hemocytes of *P. monodon*, SSH and nonsubtracted libraries were used to construct a 4 K feature cDNA microarray that was used to profile responses to multiple environmental stressors, including hypoxia (de la Vega et al. 2007). *P. monodon* held at 29°C and salinity 35 were exposed to 1 part per million (ppm) oxygen saturation for 8 h and then returned to normoxia. Control specimens were maintained under normoxic conditions for the duration of the experiment. Hemolymph samples were withdrawn for transcriptome profiling at four time points: (i) before the hypoxia, (ii) after reaching 1 ppm O_2, (iii) 1 day after returning to normoxia, and (iv) several days following return to normoxia (de la Vega, Hall et al. 2007). Seventy-five features were significantly differentially expressed across the four sampling time points within the hypoxia-treated shrimp, only a few of which had putative functional homology. The strongest repressed genes during hypoxia encoded proteins involved in oxygen transport and the immune response (de la Vega et al. 2007). At time point (ii), comparison of hypoxia-treated and control specimens yielded 26 differentially expressed genes, including hemocyanin and crustin, both of which were repressed in response to hypoxia (de la Vega et al. 2007). This result suggests that hypoxia exposure could result in increased susceptibility to infection.

OSMOTIC STRESS

There exists little published work utilizing omics-based investigations of crustacean responses to osmotic stress. Many targeted expression and candidate gene approaches have been taken, and utilizing the power of omics technologies may increase the mechanistic implications of these studies.

de la Vega et al. (2007) constructed a cDNA microarray from both SSH and normal cDNA libraries from hemocytes of *P. monodon* (as previously mentioned) to evaluate transcriptomic responses to multiple environmental stressors. Genome-wide expression profile responses to hypo-osmotic conditions in *P. monodon* indicated differential expression of a number of cDNA clones; however, many did not correspond to sequences of known biological function (de la Vega et al. 2007). Genes induced following hyposmotic stress encoded respiratory and immune proteins. Repression of retrotransposons was observed immediately following exposure to hypo-osmotic conditions, which warrants further investigation into the role of these elements in the stress response because retrotransposons were also observed to be differentially expressed in response to temperature stress (de la Vega et al. 2007). When compared to controls, differentially expressed genes were almost all repressed immediately following osmotic stress except for hemocyanin, which was induced.

Although de la Vega et al. (2007) were able to identify few cDNA clones with putative biological function, there were 69 clones that exhibited temporal variation in expression through the course of exposure to osmotic stress and 45 clones that were differentially expressed relative to controls immediately following exposure, indicating that perhaps the largest magnitude of expression change occurs relatively quickly. However, the lack of experiments in this area makes it difficult to infer any general omic-scale responses in crustaceans to osmotic stress. Because of this, it is crucial that future studies be targeted at this area to better elucidate effects of salinity on crustacean gene and protein expression.

ACIDIFICATION

Carbon dioxide-driven acidification of seawater, a process termed *ocean acidification* (OA), has recently been the focus of much research in environmental physiology. Compared to studies on mollusks and echinoderms, relatively few studies have examined the effects of OA on crustaceans, and, of those, there have been only a small number involving genome-scale approaches. Studies to date suggest that there are moderate to small changes at the transcriptome or proteome level following exposure to OA conditions in crustaceans. For example, in a two-dimensional gel analysis of the barnacle *Balanus amphitrite* cyprid, only nine of 566 protein spots changed in response to acidification, four of which were upregulated and five of which were downregulated in individuals exposed to pH 7.6 versus the control pH 8.1 (Wong et al. 2011). Although ascribing cellular function shifts following such a small change in the proteome is tenuous at best, upregulated proteins were involved in protein synthesis, respiratory gas transport, and energy metabolism, whereas downregulated proteins encoded endocytosis and proteolytic proteins (Wong et al. 2011).

FUTURE DIRECTIONS

Genome-scale studies of crustacean responses to environmental stress that have been done to date have been focused on relatively few taxa (Fig. 13.1) and with unequal representation of stressors examined across taxa (Fig. 13.4). Future studies, aided by the reduced costs and ease of next-generation DNA sequencing for genomic and transcriptomic (i.e., RNA-seq) data collection and new approaches in LC-MS/MS-based proteomics that have hugely more resolving power than two-dimensional gel electrophoresis, are likely to be more numerous in crustaceans and have the potential to be conducted on a greater array of taxa, hence presenting a better balance of studies and taxa than seen now (Fig. 13.4). Data collected to date have begun to reveal some common cellular response pathways to an array of stressors (Table 13.3), and it is entirely likely that new fundamental understanding of crustacean physiology will be aided by comparative functional genomic and proteomic analyses in the years to come.

Of significance in understanding the ecological responses of crustaceans using a molecular physiology approach is the examination of responses to interactive or synergistic stressors (e.g., temperature and salinity; temperature and infection) in comparison to the responses to those stressors in isolation. The natural world is changing in a multifactorial and complex manner, and whereas molecular biomarkers such as transcriptome fingerprints have great potential for unraveling physiological differences in field-acclimatized specimens (Fig. 13.3), realizing that potential requires characterization of those molecular fingerprints under controlled conditions where environmental stressor combinations reflect the natural habitat conditions. Although such experiments are difficult to conduct and can be expensive, it is those studies that are likely to be highly informative in understanding the biology of crustaceans under future biotic and abiotic scenarios. Of particular interest should be resolving physiological responses under combinations of temperature, pH, hypoxia, infectious organisms, and ecotoxicants because all of those stressors are predicted to change in the future, and crustacean responses to those variables have not been studied in a multifactorial context.

CONCLUSIONS

We hope that this chapter provides a valuable benchmark for the state of studies employing genomic approaches in the analysis of crustacean responses to environmental stress and infection. We have aimed to provide a comprehensive review of the literature, inclusive of all germane publications, and apologize for any studies we neglected to include. An abundance of genomic, proteomic, and metabolomic studies in crustaceans also exist for studies of the endocrine system, developmental regulation, and the molt cycle, which were not included here due to space limitations.

Taxonomic breadth of coverage of crustacean omic-scale responses to environmental stress and infection is quite limited. However, the relatively few studies that have been conducted to date allow some early inferences to made into shared stress responses. For example, we begin to see that there are many genes that have expression responses, whether up or down, to multiple types of environmental stressors (Table 13.3). Based on the literature reviewed here, some common mechanisms shared between taxa can be gleaned. For example, it appears that in crustaceans there is a specific time course of hsp transcript expression in response to a stressor, be it temperature or a heavy-metal toxicant. Generally, it appears that hsp90 is expressed rapidly in response to an acute exposure, whereas onset of other chaperones of this family is slower and more drawn out (e.g., hsp70, hsp20). Due to the differential expression of oxidative stress genes by various stressors (e.g., infection, heavy metal; Table 13.3) it appears that this may be a common pathway through which a number of stressors operate to cause detrimental cellular and physiological effects across the crustacean phylogeny. However, more comparative work on the role of oxidative stress and the molecular constituents involved will be necessary to elucidate this process on a mechanistic level because oxidative stress genes are repressed under pathogen infection and induced under heavy-metal exposure. Vitellogenin is another gene that has been observed to respond to environmental stressors of numerous types across crustacean taxa. Although there is much mechanistic work targeted at the role of vitellogenin in various endocrine and other cellular processes, future efforts should be directed at reconciling the differences in expression observed in response to different stressors in different crustacean taxa (Table 13.3).

Overall, the use of transcriptomic and proteomic approaches to understand physiological processes occurring across environmental gradients, as well as in laboratory studies investigating physiological responses to abiotic and biotic stressors, is just beginning in crustaceans. The development of genomics information in crabs (Tagmount et al. 2010), shrimp (Jung et al. 2011, Ma et al. 2012), amphipods (Zeng et al. 2011), and *Daphnia* (Colbourne et al. 2011, Orsini et al. 2011), among

other crustaceans (Table 13.1), as well as the spectacular reduction in both cost and effort of new genomic data acquisition afforded by next-generation DNA sequencing technologies will likely accelerate the pace of such studies in the near future.

ACKNOWLEDGMENTS

This material is based on work supported by the National Science Foundation (grant no. 1041225 to J.H.S.; Graduate Research Fellowship to D.A.H.).

REFERENCES

Amiard, J.C., C. Amiard-Triquet, S. Barka, J. Pellerin, and P.S. Rainbow. 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. *Aquatic Toxicology (Amsterdam)* 76:160–202.

Barbee, S.J., and J. Hartung. 1979. The effect of diethanolamine on hepatic and renal phospholipid metabolism in the rat. *Toxicology and Applied Pharmacology* 47:421–430.

Brockton, V., and V.J. Smith. 2008. Crustin expression following bacterial injection and temperature change in the shore crab, *Carcinus maenas*. *Developmental and Comparative Immunology* 32:1027–1033.

Chen, W.-H., X. Ge, W. Wang, J. Yu, and S. Hu. 2009. A gene catalogue for post-diapause development of an anhydrobiotic arthropod *Artemia franciscana*. *BMC Genomics* 10:52.

Chevalier, F., J. Herbiniere-Gaboreau, D. Charif, G. Mitta, F. Gavory, P. Wincker, P. Greve, C. Braquart-Varnier, and D. Bouchon. 2012. Feminizing *Wolbachia*: a transcriptomics approach with insights on the immune response genes in *Armadillidium vulgare*. *BMC Microbiology* 12:S1.

Chongsatja, P.O., A. Bourchookarn, C.F. Lo, V. Thongboonkerd, and C. Krittanai. 2007. Proteomic analysis of differentially expressed proteins in *Penaeus vannamei* hemocytes upon Taura syndrome virus infection. *Proteomics* 7:3592–3601.

Colbourne, J.K., M.E. Pfrender, D. Gilbert, W.K. Thomas, A. Tucker, T.H. Oakley, S. Tokishita, A. Aerts, G.J. Arnold, M.K. Basu, D.J. Bauer, C.E. Cüceres, L. Carmel, C. Casola, J.-H. Choi, J.C. Detter, Q. Dong, S. Dusheyko, B.D. Eads, T. Fröhlich, K.A. Geiler-Samerotte, D. Gerlach, P. Hatcher, S. Jogdeo, J. Krijgsveld, E.V. Kriventseva, D. Kültz, C. Laforsch, E. Lindquist, J. Lopez, J.R. Manak, J. Muller, J. Pangilinan, R.P. Patwardhan, S. Pitluck, E.J. Pritham, A. Rechtsteiner, M. Rho, I.B. Rogozin, O. Sakarya, A. Salamov, S. Schaack, H. Shapiro, Y. Shiga, C. Skalitzky, Z. Smith, A. Souvorov, W. Sung, Z. Tang, D. Tsuchiya, H. Tu, H. Vos, M. Wang, Y.I. Wolf, H. Yamagata, T. Yamada, Y. Ye, J.R. Shaw, J. Andrews, T.J. Crease, H. Tang, S.M. Lucas, H.M. Robertson, P. Bork, E.V. Koonin, E.M. Zdobnov, I.V. Grigoriev, M. Lynch, and J.L. Boore. 2011. The ecoresponsive genome of *Daphnia pulex*. *Science* 331:555–561.

Connon, R., H.L. Hooper, R.M. Sibly, F.-L. Lim, L.-H. Heckmann, D.J. Moore, H. Watanabe, A. Soetaert, K. Cook, S.J. Maund, T.H. Hutchinson, J. Moggs, W. De Coen, T. Iguchi, and A. Callaghan. 2008. Linking molecular and population stress responses in *Daphnia magna* exposed to cadmium. *Environmental Science & Technology* 42:2181–2188.

Cottin, D., B. Shillito, T. Chertemps, A. Tanguy, N. Leger, and J. Ravaux. 2010. Identification of differentially expressed genes in the hydrothermal vent shrimp *Rimicaris exoculata* exposed to heat stress. *Marine Genomics* 3:71–78.

de la Vega, E., M.R. Hall, K.J. Wilson, A. Reverter, R.G. Woods, and B.M. Degnan. 2007. Stress-induced gene expression profiling in the black tiger shrimp *Penaeus monodon*. *Physiological Genomics* 31:126–138.

de Lorgeril, J., D. Saulnier, M.G. Janech, Y. Gueguen, and E. Bachere. 2005. Identification of genes that are differentially expressed in hemocytes of the Pacific blue shrimp (*Litopenaeus stylirostris*) surviving an infection with Vibrio penaeicida. *Physiological Genomics* 21:174–183.

De Schamphelaere, K.A.C., T. Vandenbrouck, B.T.A. Muyssen, A. Soetaert, R. Blust, W. De Coen, and C.R. Janssen. 2008. Integration of molecular with higher-level effects of dietary zinc exposure in *Daphnia magna*. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* 3:307–314.

Dhar, A.K., A. Dettori, M.M. Roux, K.R. Klimpel, and B. Read. 2003. Identification of differentially expressed genes in shrimp (*Penaeus stylirostris*) infected with White Spot Syndrome Virus by cDNA microarrays. *Archives of Virology* 148:2381–2396.

Eads, B.D., J. Andrews, and J.K. Colbourne. 2008. Ecological genomics in *Daphnia*: stress responses and environmental sex determination. *Heredity* 100:184–190.

Eichner, C., P. Frost, B. Dysvik, I. Jonassen, B. Kristiansen, and F. Nilsen. 2008. Salmon louse (*Lepeophtheirus salmonis*) transcriptomes during post molting maturation and egg production, revealed using EST-sequencing and microarray analysis. *BMC Genomics* 9:126.

Engstrom, Y., O. Loseva, and U. Theopold. 2004. Proteomics of the *Drosophila* immune response. *Trends in Biotechnology* 22:600–605.

Fagutao, F.F., M. Yasuike, C.M. Caipang, H. Kondo, I. Hirono, Y. Takahashi, and T. Aoki. 2008. Gene expression profile of hemocytes of kuruma shrimp, *Marsupenaeus japonicus* following peptidoglycan stimulation. *Marine Biotechnology* 10:731–740.

Ford, A.T., and S.S. Thain. 2008. Novel metabolomic fingerprinting of normal and intersex crustacea. *Marine Environmental Research* 66:177.

Guha, S., T.K. Manna, K.P. Das, and B. Bhattacharyya. 1998. Chaperone-like activity of tubulin. *Journal of Biological Chemistry* 273:30077–30080.

Hansen, B.H., D. Altin, T. Nordtug, and A.J. Olsen. 2007. Suppression subtractive hybridization library prepared from the copepod *Calanus finmarchicus* exposed to a sublethal mixture of environmental stressors. *Comparative Biochemistry and Physiology Part D Genomics & Proteomics* 2:250–256.

Hansen, B.H., D. Altin, A. Booth, S.-H. Vang, M. Frenzel, K.R. Sorheim, O.G. Brakstad, and T.R. Storseth. 2010. Molecular effects of diethanolamine exposure on *Calanus finmarchicus* (Crustacea: Copepoda). *Aquatic Toxicology (Amsterdam)* 99:212–222.

Heckmann, L.H., R.M. Sibly, R. Connon, H.L. Hooper, T.H. Hutchinson, S.J. Maund, C.J. Hill, A. Bouetard, and A. Callaghan. 2008. Systems biology meets stress ecology: linking molecular and organismal stress responses in *Daphnia magna*. *Genome Biology* 9:R40.

Herbiniere, J., P. Greve, J.-M. Strub, D. Thierse, M. Raimond, A. van Dorsselaer, G. Martin, and C. Braquart-Varnier. 2008. Protein profiling of hemocytes from the terrestrial crustacean *Armadillidium vulgare*. *Developmental & Comparative Immunology* 32:875–882.

Iguchi, T., H. Watanabe, and Y. Katsu. 2006. Application of ecotoxicogenomics for studying endocrine disruption in vertebrates and invertebrates. *Environmental Health Perspectives* 114:101–105.

Jiang, H., F. Li, Y. Xie, B. Huang, J. Zhang, J. Zhang, C. Zhang, S. Li, and J. Xiang. 2009. Comparative proteomic profiles of the hepatopancreas in *Fenneropenaeus chinensis* response to hypoxic stress. *Proteomics* 9:3353–3367.

Jung, H., R.E. Lyons, H. Dinh, D.A. Hurwood, S. McWilliam, and P.B. Mather. 2011. Transcriptomics of a giant freshwater prawn (*Macrobrachium rosenbergii*): de novo assembly, annotation and marker discovery. *PLoS One* 6:e27938.

Keating, J., M. Delaney, D. Meehan-Meola, W. Warren, A. Alcivar, and A. Alcivar-Warren. 2007. Histological findings, cadmium bioaccumulation, and isolation of expressed sequence tags (ESTs) in cadmium-exposed, specific pathogen-free shrimp, *Litopenaeus vannamei* postlarvae. *Journal of Shellfish Research* 26:1225–1237.

Ki, J.-S., S. Raisuddin, K.-W. Lee, D.-S. Hwang, J. Han, J.-S. Rhee, I.-C. Kim, H.G. Park, J.-C. Ryu, and J.-S. Lee. 2009. Gene expression profiling of copper-induced responses in the intertidal copepod *Tigriopus japonicus* using a 6K oligochip microarray. *Aquatic Toxicology (Amsterdam)* 93:177–187.

Kuballa, A.V., and A. Elizur. 2008. Differential expression profiling of components associated with exoskeletal hardening in crustaceans. *BMC Genomics* 9: Article No.: 575.

Kuballa, A.V., D.J. Merritt, and A. Elizur. 2007. Gene expression profiling of cuticular proteins across the moult cycle of the crab *Portunus pelagicus*. *BMC Biology* 5:45.

Kuballa, A.V., T.A. Holton, B. Paterson, and A. Elizur. 2011. Moult cycle specific differential gene expression profiling of the crab *Portunus pelagicus*. *BMC Genomics* 12:147.

Lee, S.G., and D.L. Mykles. 2006. Proteomics and signal transduction in the crustacean molting gland. *Integrative and Comparative Biology* 46:965–977.

Li, T., and M. Brouwer. 2009a. Bioinformatic analysis of expressed sequence tags from grass shrimp *Palaemonetes pugio* exposed to environmental stressors. *Comparative Biochemistry and Physiology Part D Genomics & Proteomics* 4:187–195.

Li, T., and M. Brouwer. 2009b. Gene expression profile of grass shrimp *Palaemonetes pugio* exposed to chronic hypoxia. *Comparative Biochemistry and Physiology Part D Genomics & Proteomics* 4:196–208.

Liu, C.L., and H.H. Sung. 2011. Genes are differentially expressed at transcriptional level of *Neocaridina denticulata* following short-term exposure to nonylphenol. *Bulletin of Environmental Contamination and Toxicology* 87:220–225.

Ma, K.Y., G.F. Qiu, J.B. Feng, and J.L. Li. 2012. Transcriptome analysis of the Oriental River Prawn, *Macrobrachium nipponense* using 454 pyrosequencing for discovery of genes and markers. *PLoS One* 7:e39727.

Manna, T., T. Sarkar, A. Poddar, M. Roychowdhury, K.P. Das, and B. Bhattacharyya. 2001. Chaperone-like activity of tubulin: binding and reactivation of unfolded substrate enzymes. *Journal of Biological Chemistry* 276:39742–39747.

McClintock, T.S., B.W. Ache, and C.D. Derby. 2006. Lobster olfactory genomics. *Integrative and Comparative Biology* 46:940–947.

Nordberg, G.F. 2009. Historical perspectives on cadmium toxicology. *Toxicology and Applied Pharmacology* 238:192–200.

Orsini, L., E. Decaestecker, L. De Meester, M.E. Pfrender, and J.K. Colbourne. 2011. Genomics in the ecological arena. *Biology Letters* 7:2–3.

Ou, J.T., Q.G. Meng, Y. Li, Y.J. Xiu, J. Du, W. Gu, T. Wu, W.J. Li, Z.F. Ding, and W. Wang. 2012. Identification and comparative analysis of the *Eriocheir sinensis* microRNA transcriptome response to *Spiroplasma eriocheiris* infection using a deep sequencing approach. *Fish & Shellfish Immunology* 32:345–352.

Poynton, H.C., J.R. Varshavsky, B. Chang, G. Cavigiolio, S. Chan, P.S. Holman, A.V. Loguinov, D.J. Bauer, K. Komachi, E.C. Theil, E.J. Perkins, O. Hughes, and C.D. Vulpe. 2007. *Daphnia magna* ecotoxicogenomics provides mechanistic insights into metal toxicity. *Environmental Science & Technology* 41:1044–1050.

Poynton, H.C., A.V. Loguinov, J.R. Varshavsky, S. Chan, E.I. Perkins, and C.D. Vulpe. 2008. Gene expression profiling in *Daphnia magna* part I: concentration-dependent profiles provide support for the no observed transcriptional effect level. *Environmental Science & Technology* 42:6250–6256.

Poynton, H.C., N.S. Taylor, J. Hicks, K. Colson, S.R. Chan, C. Clark, L. Scanlan, A.V. Loguinov, C. Vulpe, and M.R. Viant. 2011. Metabolomics of microliter hemolymph samples enables an improved understanding of the combined metabolic and transcriptional responses of *Daphnia magna* to cadmium. *Environmental Science & Technology* 45:3710–3717.

Poynton, H.C., J.M. Lazorchak, C.A. Impellitteri, B.J. Blalock, K. Rogers, H.J. Allen, A. Loguinov, J.L. Heckrnan, and S. Govindasmawy. 2012. Toxicogenomic responses of nanotoxicity in *Daphnia magna* exposed to silver nitrate and coated silver nanoparticles. *Environmental Science & Technology* 46:6288–6296.

Ralston-Hooper, K., A. Hopf, C. Oh, X. Zhang, J. Adamec, and M.S. Sepulveda. 2008. Development of GCxGC/TOF-MS metabolomics for use in ecotoxicological studies with invertebrates. *Aquatic Toxicology (Amsterdam)* 88:48–52.

Robalino, J., J.S. Almeida, D. McKillen, J. Colglazier, H.F. Trent, III, Y.A. Chen, M.E.T. Peck, C.L. Browdy, R.W. Chapman, G.W. Warr, and P.S. Gross. 2007. Insights into the immune transcriptome of the shrimp *Litopenaeus vannamei*: tissue-specific expression profiles and transcriptomic responses to immune challenge. *Physiological Genomics* 29:44–56.

Robalino, J., R.B. Carnegie, N. O'Leary, S.A. Ouvry-Patat, E. de la Vega, S. Prior, P.S. Gross, C.L. Browdy, R.W. Chapman, K.L. Schey, and G. Warr. 2009. Contributions of functional genomics and proteomics to the study of immune responses in the Pacific white leg shrimp *Litopenaeus vannamei*. *Veterinary Immunology and Immunopathology* 128:110–118.

Rojtinnakorn, J., I. Hirono, T. Itami, Y. Takahashi, and T. Aoki. 2002. Gene expression in haemocytes of kuruma prawn, *Penaeus japonicus*, in response to infection with WSSV by EST approach. *Fish and Shellfish Immunology* 13:69–83.

Ronges, D., J.P. Walsh, B.J. Sinclair, and J.H. Stillman. 2012. Changes in extreme cold tolerance, membrane composition and cardiac transcriptome during the first day of thermal acclimation in the porcelain crab *Petrolisthes cinctipes*. *Journal of Experimental Biology* 215:1824–1836.

Seear, P.J., G.A. Tarling, G. Burns, W.P. Goodall-Copestake, E. Gaten, O. Ozkaya, and E. Rosato. 2010. Differential gene expression during the moult cycle of Antarctic krill (*Euphausia superba*). *BMC Genomics* 11:582.

Shafer, T.H., M.A. McCartney, and L.M. Faircloth. 2006. Identifying exoskeleton proteins in the blue crab from an expressed sequence tag (EST) library. *Integrative and Comparative Biology* 46:978–990.

Shaw, J.R., J.K. Colbourne, J.C. Davey, S.P. Glaholt, T.H. Hampton, C.Y. Chen, C.L. Folt, and J.W. Hamilton. 2007. Gene response profiles for *Daphnia pulex* exposed to the environmental stressor cadmium reveals novel crustacean metallothioneins. *BMC Genomics* 8:477.

Shechter, A., M. Tom, Y. Yudkovski, S. Weil, S.A. Chang, E.S. Chang, V. Chalifa-Caspi, A. Berman, and A. Sagi. 2007. Search for hepatopancreatic ecdysteroid-responsive genes during the crayfish molt cycle: from a single gene to multigenicity. *Journal of Experimental Biology* 210:3525–3537.

Silvestre, F., J.-F. Dierick, V. Dumont, M. Dieu, M. Raes, and P. Devos. 2006. Differential protein expression profiles in anterior gills of *Eriocheir sinensis* during acclimation to cadmium. *Aquatic Toxicology (Amsterdam)* 76:46–58.

Silvestre, F., T. Huynh Thi, A. Bernard, J. Dorts, M. Dieu, M. Raes, P. Nguyen Thanh, and P. Kestemont. 2010. A differential proteomic approach to assess the effects of chemotherapeutics and production management strategy on giant tiger shrimp *Penaeus monodon*. *Comparative Biochemistry and Physiology Part D Genomics & Proteomics* 5:227–233.

Snape, J.R., S.J. Maund, D.B. Pickford, and T.H. Hutchinson. 2004. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *Aquatic Toxicology (Amsterdam)* 67:143–154.

Soetaert, A., L.N. Moens, K. Van der Ven, K. Van Leemput, B. Naudts, R. Blust, and W.M. De Coen. 2006. Molecular impact of propiconazole on *Daphnia magna* using a reproduction-related cDNA array. *Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology* 142:66–76.

Soetaert, A., K. van der Ven, L.N. Moens, T. Vandenbrouck, P. van Remortel, and W.M. De Coen. 2007. *Daphnia magna* and ecotoxicogenomics: gene expression profiles of the anti-ecdysteroidal fungicide fenarimol using energy-, molting- and life stage-related cDNA libraries. *Chemosphere* 67:60–71.

Soetaert, A., T. Vandenbrouck, K. van der Ven, M. Maras, P. van Remortel, R. Blust, and W.M. de Coen. 2007. Molecular responses during cadmium-induced stress in *Daphnia magna*: integration of differential gene expression with higher-level effects. *Aquatic Toxicology (Amsterdam)* 83:212–222.

Somboonwiwat, K., V. Chaikeeratisak, H.-C. Wang, C.F. Lo, and A. Tassanakajon. 2010. Proteomic analysis of differentially expressed proteins in *Penaeus monodon* hemocytes after *Vibrio harveyi* infection. *Proteome Science* 8: Article No.: 39.

Stillman, J.H., and A. Tagmount. 2009. Seasonal and latitudinal acclimatization of cardiac transcriptome responses to thermal stress in porcelain crabs, *Petrolisthes cinctipes*. *Molecular Ecology* 18:4206–4226.

Stillman, J.H., K.S. Teranishi, A. Tagmount, E.A. Lindquist, and P.B. Brokstein. 2006. Construction and characterization of EST libraries from the porcelain crab, *Petrolisthes cinctipes*. *Integrative and Comparative Biology* 46:919–930.

Stillman, J.H., J.K. Colbourne, C.E. Lee, N.H. Patel, M.R. Philips, D.W. Towle, B.D. Eads, G.W. Gelembuik, R.L. Henry, E.A. Johnson, M.E. Pfrender, and N.B. Terwilliger. 2008. Recent advances in crustacean genomics. *Integrative and Comparative Biology* 48:852–868.

Supungul, P., S. Klinbunga, R. Pichyangkura, S. Jitrapakdee, I. Hirono, T. Aoki, and A. Tassanakajon. 2002. Identification of immune-related genes in hemocytes of black tiger shrimp (*Penaeus monodon*). *Marine Biotechnology (New York)* 4:487–494.

Tagmount, A., M. Wang, E. Lindquist, Y. Tanaka, K.S. Teranishi, M. Wong, S. Sunagawa, and J.H. Stillman. 2010. The porcelain crab transcriptome and PCAD, the porcelain crab microarray and sequence database. *PLoS ONE* 5:e9327.

Tarrant, A.M., J.J. Stegeman, and T. Verslycke. 2010. Altered gene expression associated with epizootic shell disease in the American lobster, *Homarus americanus*. *Fish & Shellfish Immunology* 29:1003–1009.

Taylor, N.S., R.J.M. Weber, A.D. Southam, T.G. Payne, O. Hrydziuszko, T.N. Arvanitis, and M.R. Viant. 2009. A new approach to toxicity testing in *Daphnia magna*: application of high throughput FT-ICR mass spectrometry metabolomics. *Metabolomics* 5:44–58.

Taylor, N.S., R.J.M. Weber, T.A. White, and M.R. Viant. 2010. Discriminating between different acute chemical toxicities via changes in the daphnid metabolome. *Toxicological Sciences* 118:307–317.

Teranishi, K.S., and J.H. Stillman. 2007. A cDNA microarray analysis of the response to heat stress in hepatopancreas tissue of the porcelain crab *Petrolisthes cinctipes*. *Comparative Biochemistry and Physiology Part D Genomics & Proteomics* 2:53–62.

Thiyagarajan, V. 2010. A review on the role of chemical cues in habitat selection by barnacles: new insights from larval proteomics. *Journal of Experimental Marine Biology and Ecology* 392:22–36.

Thiyagarajan, V., and P.Y. Qian. 2008. Proteomic analysis of larvae during development, attachment, and metamorphosis in the fouling barnacle, *Balanus amphitrite*. *Proteomics* 8:3164–3172.

Thiyagarajan, V., T. Wong, and P.Y. Qian. 2009. 2D gel-based proteome and phosphoproteome analysis during larval metamorphosis in two major marine biofouling invertebrates. *Journal of Proteome Research* 8:2708–2719.

Vandenbrouck, T., A. Soetaert, K. van der Ven, R. Blust, and W. De Coen. 2009. Nickel and binary metal mixture responses in *Daphnia magna*: molecular fingerprints and (sub)organismal effects. *Aquatic Toxicology* 92:18–29.

Vandenbrouck, T., O.A.H. Jones, N. Dom, J.L. Griffin, and W. De Coen. 2010. Mixtures of similarly acting compounds in *Daphnia magna*: from gene to metabolite and beyond. *Environment International* 36:254–268.

Vioque-Fernandez, A., E.A. de Almeida, and J. Lopez-Barea. 2009. Biochemical and proteomic effects in *Procambarus clarkii* after chlorpyrifos or carbaryl exposure under sublethal conditions. *Biomarkers* 14:299–310.

Wang, W., B. Meng, W. Chen, X. Ge, S. Liu, and J. Yu. 2007. A proteomic study on postdiapaused embryonic development of brine shrimp (*Artemia franciscana*). *Proteomics* 7:3580–3591.

Watanabe, H., K. Kobayashi, Y. Kato, S. Oda, R. Abe, N. Tatarazako, and T. Iguchi. 2008. Transcriptome profiling in crustaceans as a tool for ecotoxicogenomics. *Cell Biology and Toxicology* 24:641–647.

Wong, K.K.W., A.C. Lane, P.T.Y. Leung, and V. Thiyagarajan. 2011. Response of larval barnacle proteome to CO_2-driven seawater acidification. *Comparative Biochemistry and Physiology D-Genomics & Proteomics* 6:310–321.

Xie, Y., F. Li, B. Wang, S. Li, D. Wang, H. Jiang, C. Zhang, K. Yu, and J. Xiang. 2010. Screening of genes related to ovary development in Chinese shrimp *Fenneropenaeus chinensis* by suppression subtractive hybridization. *Comparative Biochemistry and Physiology Part D Genomics & Proteomics* 5:98–104.

Yudkovski, Y., L. Glazer, A. Shechter, R. Reinhardt, V. Chalifa-Caspi, A. Sagi, and M. Tom. 2010. Multi-transcript expression patterns in the gastrolith disk and the hypodermis of the crayfish *Cherax quadricarinatus* at premolt. *Comparative Biochemistry and Physiology D-Genomics & Proteomics* 5:171–177.

Zeng, V., K.E. Villanueva, B.S. Ewen-Campen, F. Alwes, W.E. Browne, and C.G. Extavour. 2011. De novo assembly and characterization of a maternal and developmental transcriptome for the emerging model crustacean *Parhyale hawaiensis*. *BMC Genomics* 12:581.

Zeng, Y., and C.-P. Lu. 2009. Identification of differentially expressed genes in haemocytes of the crayfish (*Procambarus clarkii*) infected with white spot syndrome virus by suppression subtractive hybridization and cDNA microarrays. *Fish & Shellfish Immunology* 26:646–650.

Zhang, W., H. Wan, H. Jiang, Y. Zhao, X. Zhang, S. Hu, and Q. Wang. 2011. A transcriptome analysis of mitten crab testes (*Eriocheir sinensis*). *Genetics and Molecular Biology* 34:136–141.

Zhang, Y., Y. Xu, S.M. Arellano, K. Xiao, and P.-Y. Qian. 2010. Comparative proteome and phosphoproteome analyses during cyprid development of the barnacle *Balanus* (=*Amphibalanus*) *amphitrite*. *Journal of Proteome Research* 9:3146–3157.

Zhou, Q., C. Wu, B. Dong, F. Li, F. Liu, and J. Xiang. 2010. Proteomic analysis of acute responses to copper sulfate stress in larvae of the brine shrimp, *Artemia sinica*. *Chinese Journal of Oceanology and Limnology* 28:224–232.

Zou, Z., Z. Zhang, Y. Wang, J. Chen, X. Jia, S. Wang, and P. Lin. 2009. The construction of normalized cDNA library and preliminary EST analysis from the gonad development and sexual differentiation related organs of *Scylla serrata*. *Journal of Tropical Oceanography* 28:88–94.

14

ENDOCRINE-DISRUPTING CHEMICALS

Peter L. deFur and Laura E. Williams

Abstract

Chemicals that disrupt animal endocrine systems have been the topic of scientific investigations for some years and of public interest in recent years. Much of the attention has been focused on vertebrates, including the recent phenomena of sex reversal in male fish. However, two of the best examples of environmental endocrine disruption are from invertebrates: gastropod mollusks affected by tributyltin and insects affected by pesticides. Crustacean endocrine systems are known to be sensitive to several types of endocrine-disrupting chemicals, largely because of the similarity between insect and crustacean hormones. Crustacean endocrine systems, although not as comprehensively examined as vertebrate endocrine systems, utilize a range of compounds including steroids, terpenoids, amines, and peptides as neurohormones. Early investigations into crustacean endocrine disruption is derived from research on a few experimental models and field studies. Subsequent research extends these observations on endocrine disruption in crustaceans to terrestrial species and several other orders.

INTRODUCTION

The topic of endocrine disruption entered the lexicon of modern biological researchers in the 1990s based on laboratory and field investigations on vertebrates, although earlier work included a polyphyletic background (Gorbman and Davey 1991, deFur et al. 1999). Soto et al. (1991) reported that chemicals leaching from plastic culture dishes exhibited estrogenic properties in the MCF7 breast cancer cell line that is responsive only to estrogen. Soto et al. (1991) then screened other commonly used chemicals and found that many exhibited estrogenic activity. Based on the in vitro assay, a number of commonly used chemicals have the potential to activate a hormone pathway known to

be a cancer risk factor. The significance of these results is their applicability to other receptor-based hormones. A broader perspective and more complete picture of endocrine disruption resulted from a focused workshop comparing their effects across vertebrate species (including humans), physiological systems, and animal models (workshop proceedings published by Colborn and Clement 1992). It is clear from the results presented and reviewed in Colborn and Clement (1992) and in the subsequent review paper (Colborn et al. 1993) that basic physiological mechanisms could be affected by anthropogenic chemicals. The physiological systems of greatest interest in these reviews were endocrine and developmental.

The review by Colborn et al. (1993) across laboratories and species indicated that man-made compounds could interact with endocrine systems, including those of crustaceans, but no review of endocrine disruption had taken a truly multiphyletic approach. Indeed, reviews of the topic by the National Academy of Sciences (NRC 1999), Kendall et al. (1998), and the Environmental Protection Agency (US EPA 1997) only included a passing reference to invertebrates. deFur et al. (1999) was the first volume (proceedings of a workshop organized by the Society for Environmental Toxicology and Chemistry [SETAC]) to address invertebrate endocrine disruptors across phyla and end points. Among the conclusions by deFur et al. (1999) are that endocrine-disrupting chemicals can influence growth, reproduction, and sexual differentiation in various species, notably arthropods. Oberdorster and Cheek (2000) and more recent publications (summarized in this chapter) reached a similar conclusion in reviewing the phenomenon in marine crustaceans.

Invertebrates constitute about 95% of the known species on our planet and play a pivotal role in ecosystem dynamics (deFur et al. 1999), in addition to acting as biological indicators for other biological systems. Of the invertebrates, crustaceans are critical components of many ecosystems: zooplankton abound throughout the world's oceans, larger species are scavengers and predators in shallow marine and estuarine habitats, and many species are a food source for the human population. The evaluation of the effects of endocrine-disrupting chemicals on crustaceans is necessary to understanding the broader impacts of endocrine-disrupting chemicals.

Aspects of the crustacean endocrine system are described in detail in Webster's chapters (see Chapters 1 and 2 in this volume), and the reader is referred to them for a comprehensive treatment of the systems and hormones. Some important features need to be appreciated in a consideration of crustacean endocrine disruptors. Typically, endocrine systems function via cascades that are initiated by environmental or physiological cues and result in a terminal hormone and its action on a target organ. The cascade acts as a line of communication between the nervous system and the endocrine system and has been appreciably conserved over evolutionary time. The activity of all hormones is mediated by specific hormone receptors. Common across all hormone action is (i) the presence of specific, high-affinity protein receptors found within or on the target cell for a particular hormone, and (ii) the complex formed from binding of the particular hormone and its receptor results in biochemical activity in the target tissue. Invertebrate hormone systems generally, and crustacean systems specifically, rely to a great extent on neurohormones that are not steroidal; the ecdysteroids are more an exception to this generalization as an important steroid hormone class in Arthropoda.

A key feature of endocrine systems is the multistep aspect of the cascading response system. Such systems begin with hormone synthesis and proceed with intracellular transport, release into a body fluid, transport (possibly in a bound form) in the body, and recognition at the target tissue/cell site, followed by the response at the cellular level. Cellular responses include receptor binding, additional protein binding, nuclear transport (in the case of steroids), gene activation, and any subsequent metabolic synthesis, transformations, and secretion. The cascading sequence of events means that anthropogenic chemicals may interfere at any one of a large

number of steps in the long series of biochemical events of normal endocrine functioning that are present in all systems.

ENDOCRINE DISRUPTION

Endocrine disruption is a form of toxicological effect that targets endocrine systems and frequently causes changes that are not acutely lethal or even chronically debilitating. Definitions of endocrine-disrupting compounds (EDCs) abound in the open literature and in regulatory documents (US EPA 1997, US EPA 1998, deFur et al. 1999, NRC 1999); most of these sources describe an EDC as a chemical that interacts with a hormonal pathway and elicits an abnormal or harmful response, a generalized definition we adopt here. Weis's chapter (see Chapter 15 in this volume) discusses toxicology in the Crustacea, and the present chapter does not attempt to duplicate the basic toxicological information. Endocrine disruption deals with one specific mode of action in toxic chemicals, and, for this reason, the definition may provoke considerable controversy. Classifying a chemical as an EDC, with all the associated connotation and regulatory implications, would seem to require that the compound functionally interact with the endocrine system and not simply affect an endpoint under endocrine control. The subject itself deals with endocrinology, toxicology, physiology, and chemical regulatory policy.

The recent scientific and regulatory interest over endocrine-disrupting chemicals began with a focus on the specific mechanism of action of the receptor binding step in hormone function; some chemicals can bind to hormone receptors and modulate the activity of these receptors (McLachlan et al. 1992, Kelce et al. 1995). The estrogen system was an early focus of endocrine disruptor research. Some xenobiotics exhibit agonistic behavior by binding to a hormone receptor and functioning like that hormone (Arnold et al. 1996). Other xenobiotics exhibit antagonist behavior by binding to the hormone receptor without stimulating the activity of the receptor. This binding blocks the receptor from accepting the endogenous hormone for that receptor, thus competitively inhibiting its activity. The endocrine system, however, consists of much more than receptor binding, as noted earlier, and xenobiotics can also act as endocrine disruptors without interacting with hormone receptors. Instead, some chemicals can induce increases or decreases in levels of an endogenous hormone. For example, the fungicide ketoconazole can lower serum testosterone levels by inhibiting testosterone synthesis (Arnold et al. 1996).

A critically important result in the early investigation of endocrine disruption was the observation of altered secondary sexual characteristics in fish, first reported in the United Kingdom (Purdom et al. 1994, Sumpter and Jobling 1995). Several investigations in John Sumpter's lab revealed that male fish in waters downstream from sewage treatment facilities produced egg protein, vitellogenin, in response to estrogenic chemicals in the discharges. These results demonstrated that external exposure to at least estrogens and perhaps other endocrine disruptors could effectively initiate responses other than the expected responses, whether lethal or not. The significance for crustaceans is that many are aquatic and all are oviparous, as are fish, raising the possibility that aquatic crustaceans may be susceptible to similar exposures and effects as reported by Sumpter and Jobling (1995) in fish. Subsequent research supports the applicability to aquatic crustaceans (deFur et al. 1999), as described later (see Lye et al. 2005, Lye et al. 2008). Recent developments regarding the susceptibility of freshwater crustaceans to estrogenic substances provide evidence that gammaridian amphipods are likely susceptible to wastewater effluents (Schirling et al. 2005).

Another outcome of the work reported by Sumpter and co-workers in Great Britain was completion of similar investigations in the United States, reported first by Folmar et al. (1996). As a result, the US Geological Survey (USGS) initiated a more comprehensive survey of contaminants in surface waters of the United States to include chemicals that had not been previously assessed. The USGS termed these chemicals "emerging contaminants" and published the results of a survey of 139 locations across the continental United States (Kolpin et al. 2002). The results indicated the widespread occurrence of a range of chemicals in rivers, streams, lakes, and ponds. The chemicals include pharmaceuticals (ethinyl estradiol) and industrial chemicals (polychlorinated biphenyls) known to be hormonally active in cell-based or whole-animal bioassays (see Colborn et al. 1993 for early review on the classification). The significance of these results for crustaceans is that aquatic crustaceans in all types of habitats are likely exposed to some range of chemicals known to be hormonally active in animal systems.

Early indications of the sensitivity of aquatic crustaceans to EDCs is found in the investigations of such chemicals as chlordecone (trade name Kepone), spilled into the James River, Virginia, in the 1970s (Schimmel et al. 1979) and interfering with normal molting of blue crabs. Chlordecone is an estrogenic insecticide that was removed from the market following the Virginia incident. In addition, the early evidence that farnesol mimics juvenile hormone (JH) activity was accompanied by evidence that insect JH mimics also altered normal molting patterns in crustaceans (Laufer et al. 1998, Tuberty and McKenney 2005, Zou 2005).

VERTEBRATE RESEARCH INFORMS THE FIELD

We gain valuable perspectives and insights from understanding the research on EDCs in vertebrates, where the topic has been more extensively studied as a general and more public matter (NRC 1999). Colborn and Clement (1992), Colborn et al. (1993), and the NRC (1999) reviewed a number of cellular and biochemical mechanisms by which EDCs may act, but the most frequently investigated mechanism was estrogen receptor function. EDCs interfere with steroid hormones, such as estrogens, and have been associated with altered reproductive function in all vertebrate classes (Tyler et al. 1998). The vertebrate hormone receptor system is highly conserved and is better understood than that of the invertebrates. Receptor-binding activity of xenobiotics has been largely documented with steroid hormone receptors in vertebrate systems (deFur et al. 1999). One of the first indications of vertebrate exposure to EDCs was the presence of vitellogenin (an egg yolk protein) in male fish (Purdom et al. 1994). A decreased population of juvenile alligators in Lake Apopka, Florida (Jennings et al. 1988) led Guillette et al. (1994) to determine that the juvenile males had poorly organized testes and small phalli, and the females had multinucleated oocytes. Dicofol, DDT, and agricultural runoff were the active agents with hormonal activity reported by Guillette et al. (1994).

INVERTEBRATES

Endocrine disruption was documented in the invertebrates (deFur et al. 1999) long before the phenomenon was recognized in vertebrates in the past two decades. A number of insecticidal agents were intentionally formulated to interfere with growth and/or metamorphosis, and some exhibited serious effects on nontarget species of aquatic crustaceans (Touart 1989). Furthermore, the boat paint biocidal agent tributyltin has been known to alter normal

reproductive organ growth and development in marine snails since at least 1981 (LeBlanc and Bain 1997). Scientific understanding of endocrine disruption in the invertebrates generally has developed along quite a different pathway than in the vertebrates, as summarized earlier and as described in numerous volumes. Both the basic endocrinology and life history of those vertebrates investigated were reasonably well described, even if imperfectly in some species. But the same is not true for the vast majority of invertebrates (Fingerman 1997, deFur et al. 1999, Chang and Mykles 2011). The state of biological knowledge of most invertebrate endocrine systems is not sufficient to explore mechanistic aspects of EDCs.

Compared to the vertebrates, invertebrates have evolved a multitude of varying strategies to complete growth, development, and reproduction. Due to the high degree of evolutionary divergence found among the invertebrate phyla, regulation of invertebrate neuroendocrine systems is much more diverse than in vertebrates. Furthermore, regulation of invertebrate neuroendocrine systems is based on steroid, terpenoid, and peptide hormones, where peptide hormones are by far the most common (Gorbman and Davey 1991). One major difference among the invertebrate phyla is between the deuterostomes that use vertebrate-type sex steroids as terminal hormones and the protostomes that use neuropeptides much more commonly. Insects and crustaceans, however, challenge a simple dichotomous distinction and utilize both ecdysteroid (steroid) and terpenoids as terminal hormones within a neuroendocrine cascade. Invertebrate secretory structures more often consist of neurosecretory cells or organs instead of true glands, maintaining a close connection between nervous and endocrine tissues (deFur et al. 1999).

Arthropods

Insects as a Basis for Understanding Crustaceans

The insect endocrine system is the most well known of the invertebrates generally and the arthropods more specifically, owing to the importance of insects in agriculture and the manipulation of insect physiology to control pests; this system serves as the prototype for the other arthropods, including the crustaceans. Several of the hormones, notably molting hormones and JHs, are structurally similar or identical among arthropods. The insect system consists of neurosecretory cells found mostly in the central nervous system, three endocrine glands, the corpora allata, the corpora cardiaca and prothoracic gland, and the gonads. Neurosecretory cells of the insect release neuropeptides into the hemolymph, controlling growth, molting, and reproduction in response to external cues. Growth and development is accomplished through a series of molts that produce ever larger exoskeletons revealed after the shedding of the old cuticle (Nijhout 1994). An important neuropeptide in the regulation of molting is prothoracicotropic hormone (PTTH), which is released from neurosecretory cells in the brain and stimulates the prothoracic glands to secrete ecdysone, a steroid hormone also known as the molting hormone (generically named ecdysteroids). Ecdysone travels via hemolymph to other tissues that convert ecdysone to 20-hydroxyecdysone (20E), which acts on certain target cells. Ecdysone is involved in cell proliferation (Champlin and Truman 1998), and 20E is involved in cell differentiation important in the production of the new cuticle. Production of the new cuticle usually does not begin until ecdysone starts to decline. JH, a terpenoid hormone, is the secretory product of another neuroendocrine cascade and plays a role in regulating the action of 20E. JH is important for the maturation of an animal that molts to transition from juvenile to adult stages.

Ecdysteroids

Ecdysone was the first steroid hormone shown to have an action directly on DNA, summarized in Nijhout (1994). Clever and Karlson (1960) demonstrated that ecdysone applied to the salivary glands of the midge *Chironomus tentans* caused synthesis of mRNA and proteins (Clever 1964). Similar studies in *Drosophila* led to a model for ecdysteroid action (Ashburner et al. 1974) in which the biologically active 20E, coupled to the ecdysone receptor (EcR), differentially regulates several classes of target genes.

Metamorphosis and Development

The presence or absence of the sequiterpenoid hormone JH determines whether the end-product of the molt is a juvenile or an adult. In the absence of JH at the time of ecdysone release, metamorphosis will occur. Exposure of a pupa to JH prevents development to an adult and causes a second pupal molt. Timing of the JH release is critical. The presence of JH during the final larval stage produces excess larval molting, and the absence of JH from earlier larval instars can cause premature transformation to adulthood or precocious metamorphosis (Riddiford 1994). In contrast, the higher flies, such as *Drosophila melanogaster*, have a fixed number of instars, and most of the adult is formed from precursor cells that proliferate during larval life (Riddiford 1993). However, the presence of JH at the critical final instar can prevent metamorphosis of the nervous system (Restifo and Wilson 1998) and of the adult abdomen (Postlethwait 1974, Riddiford and Ashburner 1990). Exposure to JH during the JH-free stages of embryonic development can disrupt later embryonic development (Staal 1975, Riddiford 1994).

Reproduction

In adult female insects, JH regulates egg maturation (Wyatt and Davey 1996) and, in many insects, JH stimulates the production of vitellogenin, an egg protein. Vitellogenin is secreted from the fat body into the hemolymph, transported to the gonad, and taken up by specific receptors on the oocyte membrane. In *D. melanogaster*, both the fat body and the ovarian follicle cells make vitellogenin, and both JH and 20E are needed for yolk production and deposition (Riddiford 1993). In most insects, the female also produces a sex pheromone that attracts a mate. In some cockroaches and houseflies, pheromone biosynthesis is regulated by JH and by ecdysone, respectively (Blomquist et al. 1993). Male spermatogenesis is under the control of hormones responsible for metamorphosis and therefore begins when ecdysone is released in the absence of JH (Happ 1992). In some insects, ecdysteroids are produced in the testis and play a role in genital duct development (Adams 1997). Male accessory glands are often also under the control of JH (Happ 1992, Wyatt and Davey 1996, Wolfner 1997).

Endogenous Regulators of JH Production

The activity of the corpora allata appears to be under the control of the regulatory neuropeptides that either stimulate (allatotropins) or inhibit (allatostatins) the production of JH. The first allatotropin to be characterized came from *Manduca sexta* and stimulates the adult corpora allata (Kataoka et al. 1989), whereas allatostatins were first characterized from the brains of cockroaches *Diploptera punctata* (Woodhead et al. 1989). The distribution of allatostatin-like immunoreactivity within the nervous system of a range of parasitic worms, or helminths, has led to speculation that

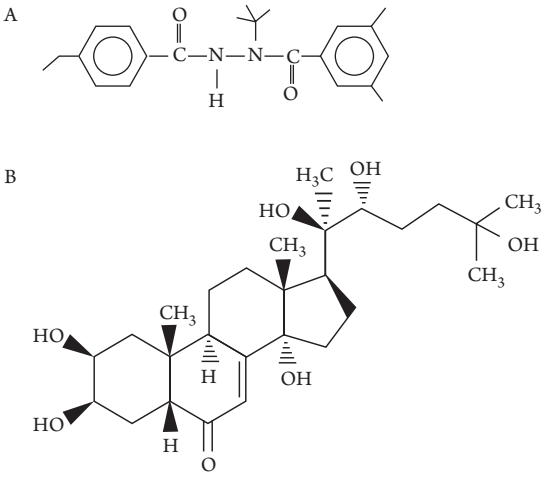


Fig. 14.1.
Structure of insecticide tebufenozide (A) compared with the molting hormone 20-hydroxyecdysone (B).

they may have neurotransmitter/neuromodulation functions with a role in locomotion, feeding, reproduction, and sensory perception (Smart et al. 1995).

Endocrine Disruption via Ecdysteroid-mediated Processes

Effects of the insect ecdysteroids can be interrupted by inhibition of synthesis and/or release from the prothoracic glands, interference with the peripheral metabolism of 20E, or by competition at its receptor level. The interaction of a compound at the ecdysone receptor can be either agonistic, causing persistent 20E, or antagonistic, causing low levels/absence of 20E. Because of differences in receptors, the activity of a chemical in one order of insects or other arthropods does not mean it will act similarly in another. There are few ecdysteroid-mimicking pesticides; tebufenozide is one that has been developed and marketed in recent years (Fig. 14.1).

Tebufenozide is formulated as a lepidopteran-specific ecdysteroid pesticide that acts as an agonist at the receptor level, increasing the rate of expression of upregulated genes. Retnakaran et al. (2001) found that ingestion of tebufenozide impacts the molting process of spruce budworm larvae and thereby causes premature death. Because of its persistence and ecdysteroid activity, tebufenozide also inhibits the expression of genes that are "downregulated" and "normally expressed in the absence of 20E" (Retnakaran et al. 2001).

Juvenile Hormone

The great majority of identified EDCs in insects are JH agonists or antagonists; the most commonly used commercial EDCs are agonists (Fig. 14.2). Methoprene is one such JH agonist or mimic that is used commonly to control insect pests, and a number of other JH agonists with similar chemical structures are registered for use in the United States.

Methoprene is a JH analog (JHA) that mimics the action of naturally occurring JHs. These analogs can be applied at specific life stages of an insect life cycle to complicate the molting process and ultimately cause death. The strategic application of JHAs as a pesticide introduces JHs during

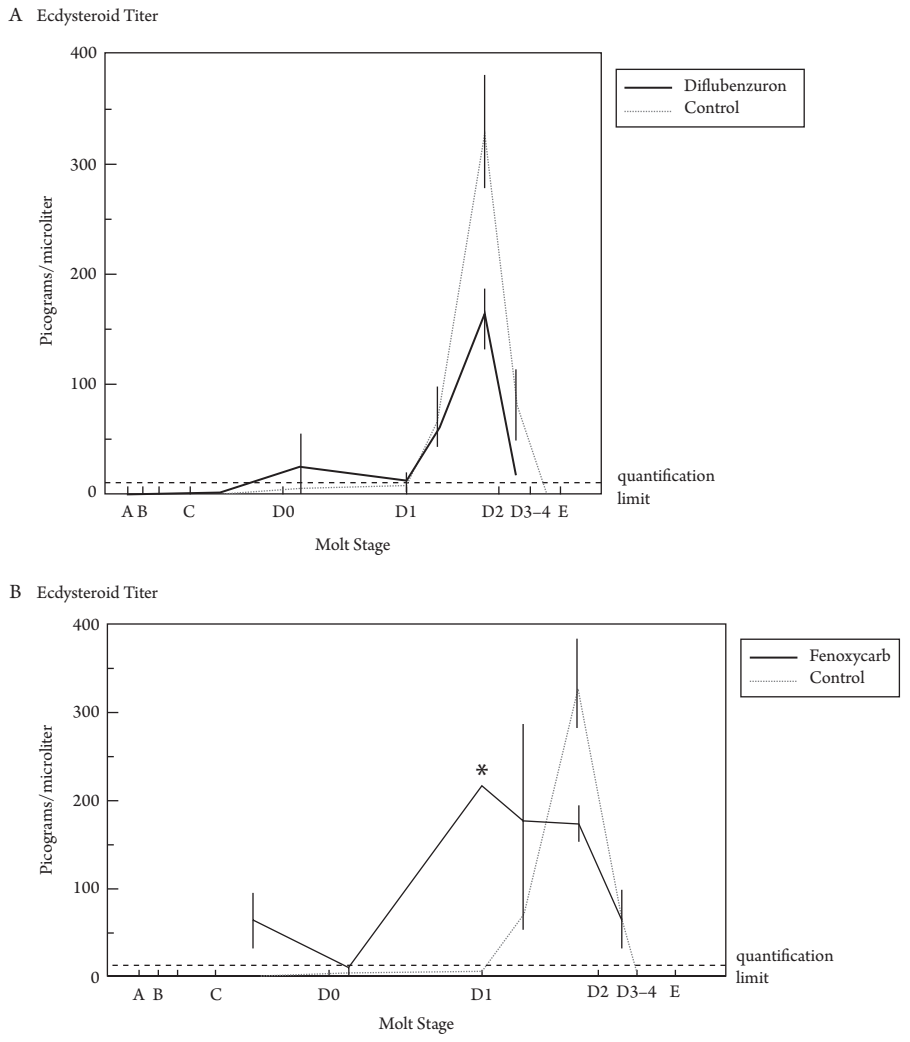


Fig. 14.2.
Circulating ecdysteroid levels in grass shrimp *Palaemonetes pugio* exposed to (A) diflubenzuron or (B) fenoxycarb compared with unexposed control shrimp. With permission from Touart (1989).

a period of growth when JH should be absent (Dhadialla et al. 1998). JHAs such as methoprene are commonly used in flea and/or tick control growth regulators (Young et al. 2004).

A range of commercial formulations of JHAs are in production and have been the subject of some research on the effects of JHAs on aquatic crustaceans (Touart 1989). As Touart (1989) reported, several JHAs alter normal growth and molting in grass shrimp *Palaemonetes pugio* at concentrations that may occur in surface waters.

Crustacea

The current understanding of EDCs in crustaceans is based on limited direct research prior to 2000 and a subsequent growing literature in the fields of endocrinology, toxicology, and environmental biology in recent years. Crustacean endocrinology has been investigated principally in larger

animals, crabs, lobster, shrimp, and crayfish, for various logistical and practical reasons (availability, blood volumes, economic importance). Modern methods have enabled measurements on smaller samples and thus smaller species, providing an important understanding of key processes and phenomena (i.e., the androgenic hormone of amphipods). Research on EDCs in crustaceans has employed both laboratory- and field-based approaches, combining the ability to manipulate and control experimental conditions with the knowledge of conditions in the animals' natural habitat. EDC research in crustaceans, as is the case with all invertebrates, has been hampered by a less than complete understanding of the underlying endocrinology and life history in some species, limited long-term funding, and difficulty of obtaining data from natural habitats. Notwithstanding these challenges, recent research is filling in some of these gaps and providing information on EDC effects in crustaceans, with some mechanistic insights.

A wide range of physiological processes and functions are known to be under endocrine control and often cued or influenced by external environmental stimuli (briefly reviewed in Fingerman 1997), including molting, sexual maturation, reproduction, sexual differentiation, limb regeneration, secondary limb regeneration, pigmentation, hemolymph glucose levels, water balance, ion transport, heart rate, blood pressure, and cardiovascular function. Indeed, continued research into crustacean endocrine function suggests that additional systems, such as the pigment control hormones, likely play a role in reproductive function, thus opening the possibility for other control pathways and the modulation thereof (Sarojini et al. 1995).

Several lines of investigation and monitoring have added greatly to the understanding of EDCs in crustaceans: (i) field investigations conducted on populations of crustaceans (deFur et al. 1999), (ii) laboratory toxicity studies using methods ranging from whole animals to isolated cell fractions and molecular identification (see LeBlanc 2007), and (iii) endocrinology research at multiple levels of organization (see Chapters 1 and 2 in this volume). These research efforts have added greatly to the current understanding of how anthropogenic chemicals alter endocrine function and the physiological process. The limiting factor in the current research is that specific mechanisms of action are known for only a few chemicals and endocrine pathways. Nonetheless, endocrine disruption in crustaceans has been demonstrated for three processes or pathways: ecdysteroid-regulated molting, juvenoid-controlled developmental maturation, and sexual maturation/determination; these are examined in the next sections.

Ecdysteroids and Molting

The crustacean ecdysteroids, isolated and characterized in the lobster *Jasus lalandei* (Hampshire and Horn 1966, Horn et al. 1966) are responsible for the principal biological events of molting (Chang and Mykles 2011). Ecdysteroids are, in turn, regulated or under the influence of molt-inhibiting hormone (MIH) and methyl farnesoate (MF; the equivalent of crustacean JH). Crustacean ecdysteroids function in a similar fashion as originally described in insects, the details of which are described in Chapter 1 of this volume.

Little experimental information exists on endocrine-disrupting artificial or synthetic ecdysteroids because pesticides targeting the molting hormone have not reached the market until quite recently. The insecticide tebufenozide, described earlier, is one of the few of these developed for the market, and research has yet to identify any consequences for nontarget crustaceans, either terrestrial or aquatic.

Feminization/Masculinization

Imposex, or feminization and masculinization, can occur as a result of exposure to several different contaminants, and the most studied case of imposex is in gastropod mollusks exposed to tributyltin (TBT; reviewed in deFur et al. 1999). Female snails grow penises and vas deferens after exposure to

just 1 ng/L of TBT (Matthiessen and Gibbs 1998). LeBlanc et al. (2005) offer evidence that TBT acts on testosterone esterification in snails, yet Oberdorster et al. (2005) suggest that TBT increases levels of the neurotransmitter APWGamide, a gastropod penile growth stimulator. Neither of these mechanisms would appear to be operative in crustaceans. The morphological phenomenon of feminization/masculinization has, however, been reported in crustaceans in several developmental contexts. Males

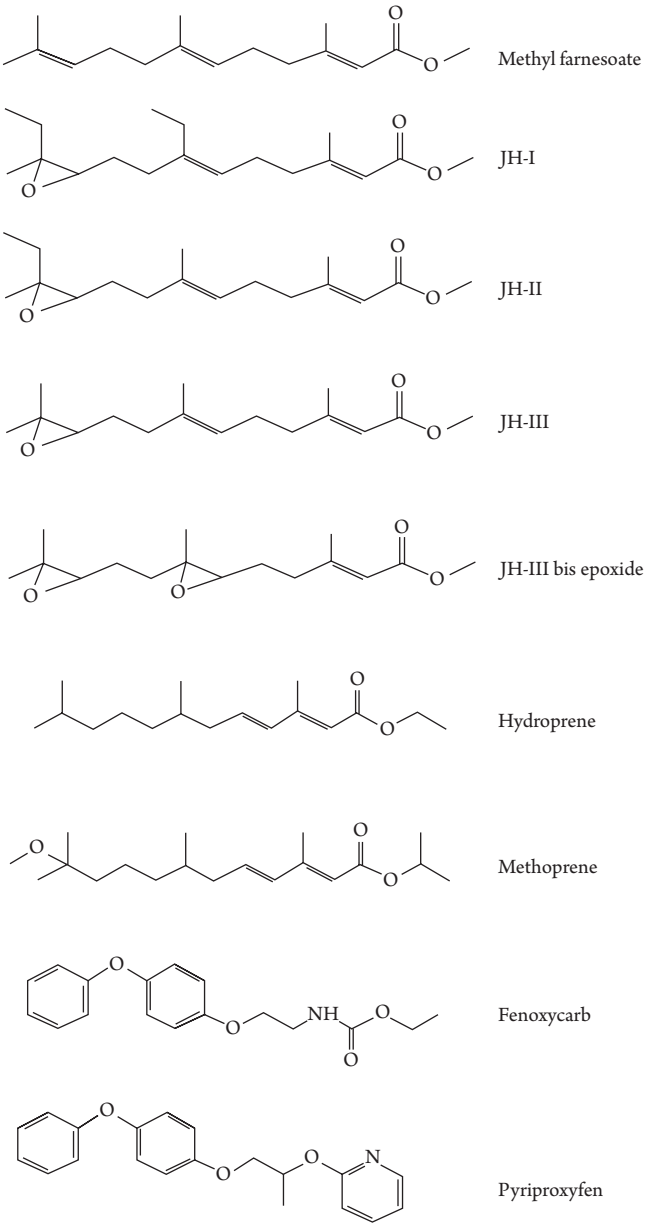


Fig. 14.3.
Structures of several commercially formulated juvenile hormone agonists that are used as insecticides, shown with methyl farnesoate and insect juvenile hormone for comparison.

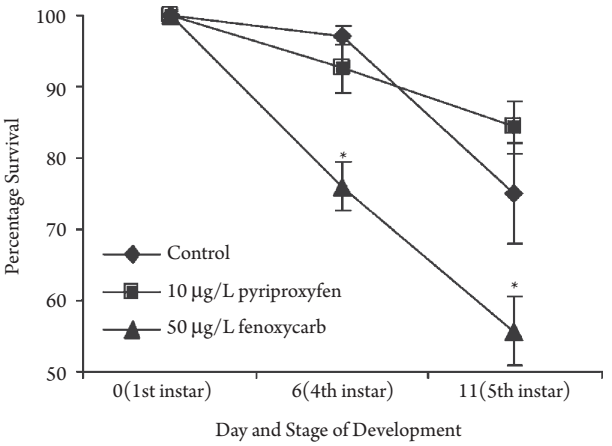


Fig. 14.4.
Mean survivorship of *Palaemonetes pugio* from day 0 to 11 of development. Asterisk (*) denotes significant ($P < 0.05$) difference from control. From Tuberty and McKenny (2005), with permission from Oxford University Press.

of the freshwater crab *Geothelphusa dehaani* in Japan have undergone feminization, demonstrating female genital (gonopore-like) openings at a proportion of 8–32%. This value increased with male growth and was absent in males collected from unpolluted waters (Ayaki et al. 2005). Takahashi et al. (2000) also found the presence of penis-like appendages on the female crab of this species in Japan. The mechanisms of action suspected to cause crustacean imposex are several, including inhibition of cytochrome P450, alteration of reductase activities, and conversion of testosterone to 17β-estradiol.

Juvenile Hormone and MF

The crustacean JH MF was first identified in crustaceans by Laufer et al. (1987); the same laboratory later reported the stimulatory effect of MF on ovarian maturation. In several reviews (deFur et al. 1999, Oberdorster and Cheek 2000), MF is described as the crustacean equivalent of insect JH, and this point is now accepted by an increasing number of investigators (LeBlanc 2007, Rodríguez et al. 2007). The several JH agonists being marketed as insect growth regulators (e.g., methoprene, pyriproxyfen, hydroprene; the structures shown in Fig. 14.3) are able to act on aquatic crustaceans as well as insects, impairing growth, metamorphosis, and survival (Touart 1989, McKenney 2005, Tuberty and McKenney 2005, LeBlanc 2007, Rodríguez et al. 2007).

Touart (1989) found that several different insecticides formulated as growth regulators and acting on the molting process in insects—largely through interfering with maturation at the molt—also impair normal molting in grass shrimp *P. pugio* (Fig. 14.2). Diflubenzuron and methoprene (not shown in Fig. 14.2) both depress circulating ecdysteroid titers in grass shrimp at the molt stage when ecdysteroid peaks (Fig. 14.2A). Fenoxycarb both depresses ecdysteroid titers and causes ecdysteroid levels to peak early, at stage D_1 instead of D_2 (Fig. 14.2B). These JH agonists alter the normal pattern of molting hormone and can result in increased larval mortality (Fig. 14.4). The adverse effects of JH or JHA have also been reported in a number of other aquatic crustaceans (McKenny 2005) including daphnids (Olmstead and LeBlanc 2003, LeBlanc 2007), crabs (Takahashi et al. 2000, Lye et al. 2008), grass shrimp (Touart 1989, Tuberty and McKenny 2005), and lobsters (Walker et al. 2005). Recent investigations have turned to other crustacean groups,

including gammarid amphipods and isopods, thus extending the observations of endocrine disruption to these two crustacean orders.

FUTURE DIRECTIONS

The current trends in research on crustacean endocrinology and endocrine disruption are encouraging with respect to identifying the occurrence of EDCs and elucidating their underlying mechanisms. Given the importance of crustaceans in aquatic ecosystems (e.g., amphipods in freshwaters, copepods in estuaries and coastal waters), in commercial open-water fishing (shrimp, crabs, lobster) where chemical pollution is a known risk factor, and in fish farming (shrimp, lobster), there is every reason to anticipate a continued need for further understanding of the topic. One example of research that extends scientific information on endocrine disruption in a new area is the ongoing work on terrestrial isopods in the lab of Lemos (Lemos et al. 2010a,b,c). Their work examines how two known endocrine disruptors, vinclozolin (antiandrogenic) and bisphenol A (estrogenic), may interfere with normal function via protein expression. The important ecological role of terrestrial isopods is clearly threatened by widespread and continued exposures to EDCs.

We expect to see elaboration of all aspects of the endocrine disruption in crustaceans, notably (i) the endocrine mechanisms in crustaceans generally; (ii) specific mechanistic information about MF (JH) and ecdysteroids, as well as crustacean hyperglycemic hormone (CHH) and the CHH complex of neuropeptides; (iii) the mechanism of action of various types of EDCs, specifically JHAs, ecdysteroids, and signaling pathway inhibitors; (iv) the occurrence of endocrine disruption in crustacean populations in nature (as predicted by the multiple authors in deFur et al. 1999); (v) the development of alternative research designs for assessing field-level effects that are not readily observable; (vi) investigations into the consequences of EDC effects at the population level, similar in scope and focus to the work on gastropod mollusks exposed to TBT; and (vii) interactions between or among known and suspected EDCs and other environmental perturbations, such as increased temperatures, hypoxia, altered water chemistry, and the like.

A few of these research areas warrant some additional discussion. First, the understanding of basic endocrinology is absolutely essential, especially for the crustaceans that utilize neuroendocrine control to a far greater extent than the vertebrates and in a fashion not found in the insects (the two groups that have traditionally been the focus of much endocrinological research). As noted by several recent investigators (Zou 2005, Rodríguez et al. 2007, Chang and Mykles 2011), the control of molting is under the direct or indirect influence of both environmental factors (deFur et al. 1999) and multiple hormones. Ecdysteroids directly influence the target tissues, but ecdysteroid synthesis and release is in turn regulated by other (neuro)hormones, and even those are or may be regulated by neuroendocrine secretions.

Field methods for detecting endocrine disruption in invertebrates are now extending to caged amphipods (Jubeaux et al. 2012, Besse et al. 2013), although the methods and confirmation of consistency with lab results are in process. We note that caged-animal studies by Sumpter and Jobling (1995) were instrumental in the early investigations of in situ endocrine disruption in fish (Bundschuh et al. 2011).

New biochemical methods of investigating crustacean endocrinology and toxicology have the capability of detecting and identifying changes in gene expression and protein regulation (Stillman et al. 2008). As these new approaches and methods provide information regarding changes in gene expression, scientists will be better able to inform managers about the types of biological responses to anthropogenic chemicals. These new methods will surely change the way in which ecotoxicology is practiced around the world.

REFERENCES

Adams, T.S. 1997. Arthropoda: insects. Pages 277–338 *in* K.G. Adiyoda and R.G. Adiyoda, editors. Reproductive biology of invertebrates. Wiley, Chichester, UK.

Arnold, S., B. Collins, M. Robinson, L. Guillette Jr., and J. McLachlan. 1996. Differential interaction of natural and synthetic estrogens with extracellular binding proteins in a yeast estrogen screen. Steroids 61:456–474.

Ashburner, M., C. Chihara, P. Meltzer, and G. Richards. 1974. On the temporal control of puffing activity in polytene chromosomes. Cold Spring Harbor Symposium on Quantitative Biology 38:655–662.

Ayaki, A., Y. Kawauchino, C. Nishinura, H. Ishibashi, and K. Arizono. 2005. Sexual disruption in the freshwater crab (*Geothelphusa dehaani*). Integrative and Comparative Biology 45:39–42.

Besse, J.P., M. Coquery, C. Lopes, A. Chaumot, H. Budzinski, P. Labadie, and O. Geffard. 2013. Caged *Gammarus fossarum* (Crustacea) as a robust tool for the characterization of bioavailable contamination levels in continental waters: towards the determination of threshold values. Water Research 47:650–660.

Blomquist, G., J. Tillman-Wall, L. Guo, D. Quiliar, P. Gu, and C. Schal. 1993. Hydrocarbon and hydrocarbon-derived sex pheromones in insects: biochemistry and endocrine regulation. Pages 317–335 *in* D.W. Stanley-Samuelson and D.R. Nelson, editors. Insect lipids: chemistry, biochemistry and biology. University of Nebraska Printing, Lincoln.

Bundschuh, M., J.P. Zubrod, and R. Schulz. 2011. The functional and physiological status of *Gammarus fossarum* (Crustacea; Amphipoda) exposed to secondary treated wastewater. Environmental Pollution 159:244–249.

Champlin, D.T., and J.W. Truman. 1998. Ecdysteroids govern two phases of eye development during metamorphosis of the moth, *Manduca sexta*. Development 125:2009–2018.

Chang, E.S., and D.L. Mykles. 2011. Regulation of crustacean molting: a review and our perspectives. General and Comparative Endocrinology 172:323–330.

Clever, U. 1964. Actinomyosin and puromycin: effects on sequential gene activation by ecdyson. Science 146: 794–795.

Clever, U. and P. Karlson. 1960. Induktion von puff-veranderungen in den speicheldrusen chromosomen von *Chironomus tentans* durch ecdyson. Experimental Cell Research 20:623–626.

Colborn, T., and C. Clement. 1992. Chemically-induced alteration in sexual and functional development: the wildlife/human connection. Advances in Modern Environmental Toxicology. Vol. 21. Princeton Scientific Publishing Co. Inc., Princeton, New Jersey.

Colborn, T., F. vom Saal, and A. Soto. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environmental Health Perspectives 101:378–384.

deFur, P., M. Crane, C. Ingersoll, and L. Tattersfield. 1999. Endocrine disruption in invertebrates: endocrinology, testing, and assessment. SETAC Press, Brussels.

Dhadialla, T.S., G.R. Carlson, and D.P. Le. 1998. New insecticides with ecdysteroidal and juvenile hormone activity. 1998. Annual Review of Entomology 43:545–569.

Fingerman, M. 1997. Crustacean endocrinology: a retrospective, prospective and introspective analysis. Physiological Zoology 70:257–269.

Folmar, L.C., N.D. Denslow, V. Rao, M. Chow, D.A. Crain, J. Enblom, J. Marcino, and L.J. Guillette Jr. 1996. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environmental Health Perspectives 104:1096–1101.

Gorbman, A., and K. Davey. 1991. Endocrines. Pages 693–754 *in* C.L. Prosser, editor. Neural and integrative animal physiology. Wiley-Liss, New York.

Guillette Jr., L., T. Gross, G. Masson, J. Matter, H. Percival, and A. Woodward. 1994. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environmental Health Perspectives 102:680–688.

Hampshire, R., and D. Horn. 1966. Structure of crustecdysone, a crustacean molting hormone. Chemical Communications 2:37–38.

Happ, G. 1992. Maturation of the male reproductive system and its endocrine regulation. Annual Review of Entomology 37:303–320.

Horn, D.H.S., E.J. Middleton, J.A. Wunderlich, and F. Hampshire. 1966. Identity of the molting hormones of insects and crustaceans. Chemical Communications 1966: 339–340.

Jennings, M., H. Percival, and A. Woodward. 1988. Evaluation of alligator hatchling and egg removal from three Florida lakes. Proceedings of the Annual Conference of the Southeastern Association of Fish and Wildlife Agencies 42:283–294.

Jubeaux, G., R. Simon, A. Salvador, C. Lopes, E. Lacaze, H. Quéau, A. Chaumot, and O. Geffard. 2012. Vitellogenin-like protein measurement in caged *Gammarus fossarum* males as a biomarker of endocrine disruptor exposure: inconclusive experience. Aquatic Toxicology 122–123:9–18.

Kataoka, H., A. Toshci, J. Li, R. Carney, D. Schooley, and S. Kramer. 1989. Identification of an allatotropin from adult *Manduca sexta*. Science 243:1481–1483.

Kendall, R., R. Dickerson, J. Giesy, and W. Suk. 1998. Principals and processes for evaluating endocrine disruption in wildlife. SETAC Press.

Kelce, R., C. Stone, C. Laws, J. Gray, J. Kemppainen, and E. Wilson. 1995. Persistent DDT metabolite p,p′-DDE is a potent androgen receptor antagonist. Nature 975:581–585.

Kolpin, D.W., E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, and H.T. Buxton. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. Environmental Science and Technology 36:1202–1211.

Laufer, H., D. Borst, F.C. Baker, C. Carrasco, M. Sinkus, C.C. Reuter, L.W. Tsai, and D.A. Schooley. 1987. Identification of a juvenile-hormone like compound in a crustacean. Science 235:202–205.

Laufer, H., W.J. Biggers, and J.S.B. Ahl. 1998. Stimulation of ovarian maturation in the crayfish *Procambarus clarkii* by methyl farnesoate. General and Comparative Endocrinology 111:113–118.

LeBlanc, G.A. 2007. Crustacean endocrine toxicology: a review. Ecotoxicology 16:61–81.

LeBlanc, G.A., and L.J. Bain. 1997. Chronic toxicity of environmental contaminants: sentinels and biomarkers. Environmental Health Perspectives 105:65–80.

LeBlanc G.A., Gooding M.P., and Sternberg, R.M. 2005. Testosterone fatty acid esterification: a unique target for the endocrine toxicity of tributyltin to gastropods. Integrative and Comp. Biology 45:81–87.

Lemos, M.F., A.C. Esteves, B. Samyn, I. Timperman, J. van Beeumen, A. Correia, C.A.M. van Gestel, and A.M.V.M. Soares. 2010a. Protein differential expression induced by endocrine disrupting compounds in a terrestrial isopod. Chemosphere 79:570–576.

Lemos, M.F., C.A.M. van Gestel, and A.M.V.M. Soares. 2010b. Developmental toxicity of endocrine disrupters bisphenol A and vinclozolin in a terrestrial isopod. Archives of Environmental Contamination and Toxicology 59:274–281.

Lemos, M.F., C.A.M. van Gestel, and A.M.V.M. Soares. 2010c. Reproductive toxicity of the endocrine disrupters vinclozolin and bisphenol A in the terrestrial isopod *Porcellio scaber* (Latreille, 1804). Chemosphere 78:907–913.

Lye, C.M., M.G. Bentley, A.S. Clare, and E.M. Sefton. 2005. Endocrine disruption in the shore crab *Carcinus maenas*—a biomarker for benthic marine invertebrates? Marine Ecology Progress Series 288:221–232.

Lye, C.M., M.G. Bentley, and T. Galloway. 2008. Effects of 4-nonylphenol on the endocrine system of the shore crab, *Carcinus maenas*. Environmental Toxicology 23:309–318.

Matthiessen, P., and P.E. Gibbs. 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environmental Toxicology and Chemistry 17:37–43.

McKenney, C.L. Jr. 2005. The influence of insect juvenile hormone agonists on metamorphosis and reproduction in estuarine crustaceans. Integrative and Comparative Biology 45:97–105.

McLachlan, J., R. Newbold, C. Teng, and K. Korach. 1992. Environmental estrogens: orphan receptors and genetic imprinting. Pages 107–112 *in* T. Colborn and C. Clement, editors. Chemically induced alterations in sexual and functional development: the wildlife/human connection. Princeton Scientific Publishing, Princeton, New Jersey.

National Research Council. 1999. Hormonally active agents in the environment. National Academy Press, Washington, DC.

Nijhout, H. 1994. Insect hormones. Princeton University Press, Princeton, New Jersey.

Oberdorster, E. and A.O. Cheek. 2000. Gender benders at the beach: endocrine disruption in marine and estuarine organisms. Environmental Toxicology and Chemistry 20: pp. 23–36.

Oberdorster, E., J. Romana, and P. McClellan-Green. 2005. The neuropeptide APGWamide as a penis morphogenic factor (PMF) in gastropod mollusks. Integrative and Comparative Biology 45:28–32.

Olmstead, A.W., and G.A. LeBlanc. 2003. Insecticidal juvenile hormone analogs stimulate the production of male offspring in the crustacean *Daphnia magna*. Environmental Health Perspectives 111:919–924.

Postlethwait, J. 1974. Juvenile hormone and the adult development of *Drosophila*. Biological Bulletin 147:119–135.

Purdom, C., P. Hardiman, V. Bye, N. Eno, C. Tyler, and J. Sumpter. 1994. Estrogenic effects of effluents from sewage treatment works. Chemistry and Ecology 8:275–285.

Restifo, L., and T. Wilson. 1998. A juvenile hormone agonist reveals distinct developmental pathways mediated by ecdysone-inducible broad complex transcription factors. Developmental Genetics 22:141–159.

Retnakaran, A., I. Gelbic, M. Sundaram, W. Tomkins, T. Ladd, M. Primavera, Q. Feng, B. Arif, R. Palli, and P. Krell. 2001. Mode of action of the ecdysone agonist tebufenozide (RH-5992), and an exclusion mechanism to explain resistance to it. Pest Management Science 57:951–957.

Riddiford, L. 1993. Hormones and *Drosophila* development. Pages 899–939 *in* M. Bate and A. Martinez-Arias, editors. The development of *Drosophila*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Riddiford, L. 1994. Cellular and molecular action of juvenile hormone. I. General considerations and premetamorphic actions. Advances in Insect Physiology 24:213–274.

Riddiford, L., and M. Ashburner. 1990. Role of juvenile hormone in larval development and metamorphosis in *Drosophila melanogaster*. General and Comparative Endocrinology 82:172–183.

Rodríguez, E.M., D.A. Medesani, and M. Fingerman. 2007. Endocrine disruption in crustaceans due to pollutants: a review. Comparative Biochemistry and Physiology Part A 146:661–671.

Sarojini, R., R. Nagabhushanam, and M. Fingerman. 1995. A neurotransmitter role for red-pigment-concentrating hormone in ovarian maturation in the red swamp crayfish *Procambarus clarkii*. Journal of Experimental Biology 198:1253–1257.

Schimmel, S.C., J.M. Patrick, Jr., L.F. Faas, J.L. Oglesby, and A.J. Wilson, Jr. 1979. Kepone: toxicity and bioaccumulation in blue crabs. Estuaries 2:9–15.

Schirling, M., D. Jungmann, V. Ladewig, R. Nagel, R. Triebskorn, and H.R. Köhler. 2005. Endocrine effects in *Gammarus fossarum* (Amphipoda): influence of wastewater effluents, temporal variability, and spatial aspects on natural populations. Archives of Environmental Contamination and Toxicology 49:53–61.

Smart, D., C. Johnson, A. Maule, D. Halton, G. Hrckova, C. Shaw, and K. Buhchanan. 1995. Localization of *Diploptera punctata* allatostatin-like immunoreactivity in helminthes: an immunocytochemical study. Parasitology 110:87–96.

Soto, A., H. Justicia, J. Wray, and C. Sonnenschein. 1991. p-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. Environmental Health Perspectives 92:167–173.

Staal, G. 1975. Insect growth regulators with juvenile hormone activity. Annual Review of Entomology 20:417–460.

Stillman, J.H., J.K. Colbourne, C.E. Lee, N.H. Patel, M.R. Phillips, D.W. Towle, B.D. Eads, G.W. Gelembuik, R.P. Henry, E.A. Johnson, M.E. Pfrender, and N.B. Terwilliger. 2008. Recent advances in crustacean genomics. Integrative and Comparative Biology 48:852–868.

Sumpter, J., and S. Jobling. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environmental Health Perspectives 103:173–178.

Takahashi, T., A. Araki, Y. Nomura, M. Koga, and K. Arizonoa. 2000. The occurrence of dual-gender imposex in Japanese freshwater crab. Journal of Health Science 46:376–379.

Tuberty, S.R., and C.L. McKenney, Jr. 2005. Ecdysteroid responses of estuarine crustaceans exposed through complete larval development to juvenile hormone agonist insecticides. Integrative and Comparative Biology 45:106–117.

Touart, L.W. 1989. Effects of selected insect growth regulators on ecdysteroid titers in the grass shrimp, *Palaemonetes pugio*. 161 pages. Ph.D. Dissertation. George Mason University, Fairfax, VA.

Tyler, C.R., S. Jobling, and J.P. Sumpter. 1998. Endocrine disruption in wildlife: a critical review of the evidence. Critical Reviews in Toxicology 28:319–361.

US Environmental Protection Agency. 1997. Special report on environmental endocrine disruption: an effects assessment and analysis prepared for the risk assessment forum 630/R-96/012. Technical Panel Office of Research and Development, Washington, D.C.

US Environmental Protection Agency. 1998. Endocrine disruptor screening and testing advisory committee (EDSTAC) final report. EPA Office of Chemical Safety and Pollution Prevention, Washington DC.

Walker, A.N., P. Bush, J. Puritz, T. Wilson, E.S. Chang, T. Miller, K. Holloway, and M.N. Horst. 2005. Bioaccumulation and metabolic effects of the endocrine disruptor methoprene in the lobster, *Homarus americanus*. Integrative and Comparative Biology 45:118–126.

Wolfner, M. 1997. Tokens of love: functions and regulation of *Drosophila* male accessory gland products. Insect Biochemistry and Molecular Biology 27:179–192.

Woodhead, A.P., B. Stay, S.L. Seidel, M.A. Kahn, and S.S. Tobe. 1989. Primary structure of four allostatins: neuropeptide inhibitors of juvenile hormone biosynthesis. Proceedings of the National Academy of Sciences U.S.A. 86:5997–6001.

Wyatt, G., and K. Davey. 1996. Cellular and molecular actions of juvenile hormone in adult insects. Advances in Insect Physiology 36:1–155.

Young, D.R., E.C. Jeannin, and A. Boeckh. 2004. Efficacy of fipronil/(S)-methoprene combination spot-on for dogs against shed eggs, emerging and existing adult cat fleas (Ctenocephalides felis, Bouché). Veterinary Parasitology 125:397–407.

Zou, E. 2005. Impacts of xenobiotics on crustacean molting: the invisible endocrine disruption. Integrative and Comparative Biology 45:33–38.

15

SOME PHYSIOLOGICAL RESPONSES OF CRUSTACEANS TO TOXICANTS

Judith S. Weis

Abstract

Investigating effects of pollutants on various aspects of crustacean physiology has a long history and an enormous literature. This chapter focuses on work on decapods, mysids, and amphipods and covers effects of metals, oil, and other organic contaminants on physiological functions including feeding and digestion, respiration, osmoregulation, excretion, regeneration, and molting. Physiological processes generally tend to be reduced or inhibited by contaminants, but in each case some chemicals or species are exceptions to this general tendency. Mechanisms of uptake, storage, and metabolism of metals and organic contaminants are also reviewed. Although most studies have been laboratory bioassays in which organisms are exposed to particular chemicals for a relatively short time, a number of studies compared animals collected from contaminated field sites with those from cleaner areas. These studies give greater insight into ecological effects resulting from physiological changes and compensatory mechanisms that may take place after long-term exposures.

INTRODUCTION

The other chapters in this volume review recent work on various aspects of crustacean physiology. It is unfortunate that many crustaceans today live in contaminated environments, and toxic contaminants may alter their physiology. Unless an entire book is devoted to this topic, it is impossible to do a thorough review of such a comprehensive topic, which has been the focus of intensive study for more than 40 years. Review articles have generally focused on a type of effect, a group of crustaceans, or a type of pollutant, for example, the recent review of Kouba et al. (2010) of crayfish responses to metals. Consequently, this chapter is only a general survey that touches on

the different types of responses (omitting reproduction and endocrine disruption) and focuses primarily on decapods. Also, there is an enormous literature on daphnids and copepods that is not covered.

Among the contaminants of greatest concern are those that are persistent in the environment, are toxic, and bioaccumulate in organisms; among the types of chemicals that have received the most attention are metals and organic chemicals including oil, pesticides, and industrial chemicals like polychlorinated biphenyls (PCBs). Some of these chemicals are no longer in use, but they remain in the environment because they are stable and persistent, especially in aquatic sediments where they can continue to affect crustaceans and other organisms. In contaminated environments, crustaceans at higher trophic levels may be exposed via their food, as well as through the water or sediments.

Generally, when an organism is exposed to contaminants, rates of physiological processes tend to be reduced, although there are cases in which rates are increased. Most of the research has utilized a laboratory bioassay approach in which organisms are exposed (generally through the water, but sometimes via food) to different concentrations of particular chemicals and the responses quantified. Initial work in environmental toxicology sought to establish concentrations that caused mortality (the LC_{50} approach), which is most useful in ranking chemicals in terms of their toxicity. Studies subsequently examined physiological responses, which are sublethal responses to lower concentrations of chemicals and are more likely to occur in nature. Fundamental work in this field was presented in a series of symposia on pollution and physiology of marine organisms in the 1970s and 1980s organized by John and Winona Vernberg, Fred Thurberg, and Anthony Calabrese. A number of studies from these symposia, although "old," are discussed in this review because they are basic to the field and might otherwise be lost to future researchers. Early studies generally used high concentrations; more recently, lower concentrations have been used that are closer to environmental levels. There have also been some studies in which organisms from polluted areas have been compared to ones from reference sites. These studies are ecologically more realistic, but because contaminated sites usually have multiple pollutants, it is difficult to attribute responses to any particular contaminant.

FEEDING AND DIGESTION

Reduced feeding and digestion are commonly observed responses to pollutants. Because crustaceans play a major role in the cycling of nutrients in estuarine and freshwater food webs, alterations in their feeding rates, nutrient assimilation, and energetics could impact not only their own population dynamics, but also could have community-wide repercussions.

Feeding

Reduced food consumption is an almost universal response in a wide variety of taxa to a wide variety of toxicants (Taylor et al. 1993, Maltby and Crane 1994, Blockwell and Taylor 1998, Wallace et al. 2000). However, increased feeding rates have occasionally been found, for example, in amphipods exposed to lindane (Blockwell and Taylor 1998). Pollutant-induced decreased feeding is not only a general response to contaminants, but also can result in a "positive feedback" situation, in that poor nutrition resulting from decreased feeding can in turn make animals more susceptible to contaminants (Dissanayake et al. 2008b). These authors advised that "ecotoxicological studies need to take into account the nutritional state of the test organism to achieve the full assessment of contaminant impact" (p. 40). However, it is also likely that reduced feeding will reduce further uptake of contaminants. This is particularly true for organisms that acquire much of their body burden of contaminants from their food.

Metals

Chronic exposure to copper (Cu; 85 and 212 μg/L) and zinc (Zn; 106, 212, and 525 μg/L) reduced growth of shrimp larvae (*Farfantepenaeus paulensis*) due to reduced feeding. Both metals reduced the number of *Artemia* captured by the shrimp larvae during 30 min. Oxygen consumption was reduced by about 30% in all concentrations (Santos et al. 2000). Similarly, gut fullness of the shrimp *Metapenaeus ensis* larvae feeding on *Chaetoceros gracilis* was reduced by a 2 h exposure to Cu at 0.25 mg/L. In contrast, gut fullness was not affected even after 24 h exposure to chromium (Cr), Cu, or nickel (Ni) at concentrations close to the 48 h LC_{50}. However, postlarval shrimp exposed for 24 h to those concentrations of Cr, Cu, or Ni consumed fewer *Artemia nauplii* (Wong et al. 1993).

Organic Contaminants

Reduced feeding activity of juvenile amphipod *Gammarus pulex* was a sensitive response to lindane and 3,4-dichloroaniline (3,4-DCA). Reduced feeding was detected after 96 h exposure at 8.4 μg/L lindane and 240 hours exposure at 918 μg/L 3,4-DCA (Blockwell and Taylor 1998). However, a significant increase was seen in feeding of those exposed for 240 h to 0.09 μg/L lindane. This may be evidence of a hormetic response. Jensen and Carroll (2010) examined feeding of copepods exposed to the water-soluble fraction (WSF) of crude oil. Feeding was inhibited in *Calanus finmarchicus* exposed to 0.4 μg/L of the WSF, showing that adults are sensitive to exposure to crude oil.

Impacts in Polluted Sites

A number of studies have assessed feeding rates or prey capture in animals living in contaminated sites. Perez and Wallace (2004) found that grass shrimp (*Palaemonetes pugio*) from a clean reference site captured brine shrimp about twice as fast as shrimp from more contaminated sites. Shrimp from the clean site that were maintained in the laboratory for 8 weeks with sediment and water from the contaminated site showed reduced prey capture ability, comparable to that of grass shrimp inhabiting that site, thus demonstrating that the difference was due to the environment. Videotape analysis indicated that the reduced prey capture was due to shrimp using a less efficient grab type of capture, rather than a lunge or pursuit type of attack. Khoury et al. (2009) compared feeding rates (number of scoops) of fiddler crabs (*Uca pugnax*) from a contaminated site and a reference site. Crabs from the reference site had twice the number of scoops (on the same sediment) as those from the contaminated site. Blue crabs (*Callinectes sapidus*) from a contaminated site captured fewer active prey (killifish or juvenile blue crabs) compared with crabs from cleaner environments, but ate comparable amounts of less active prey (fiddler crabs and mussels) suggesting that coordination was affected, rather than appetite (Reichmuth et al. 2009). Gut content analysis showed that crabs from the contaminated site ate much less fish or crab but much more detritus, algae, and sediment than did crabs from the clean site. These are not typical food items for this predatory species. Transplanting polluted crabs to the clean site or keeping them in the laboratory on food from the clean site allowed them to become better predators on juvenile blue crabs. Transplanting clean crabs to the polluted site or maintaining them in the lab on food from the polluted site caused them to become poor predators on juvenile blue crabs; this was correlated with the accumulation of mercury (Hg).

Cellular activity, immune function, cardiac activity, and foraging behavior were studied in green crabs, *Carcinus maenas*, collected from a polycyclic aromatic hydrocarbon (PAH)-contaminated site and two comparatively clean field sites and compared with responses of crabs exposed in the laboratory to the PAH pyrene (200 μg/L) for 28 days (Dissanayake et al. 2010). Impacts at the

cellular level were evaluated in hemocytes by assessing membrane integrity and immune function (phagocytosis), which were decreased by contaminant exposure in the laboratory. In the field study, no significant impacts were observed at the cellular or physiological level in crabs from the contaminated site, but, when brought into the laboratory, foraging behavior was significantly reduced, thus demonstrating that feeding behavior is a more sensitive response. Crabs from the contaminated estuary took significantly longer than other field-collected and laboratory-exposed crabs to approach a cockle and break into the shell, resulting in differences in overall prey handling time, with PAH-contaminated groups showing significantly longer handling times.

Digestion

Ingested pollutants can alter digestive physiology even before they are assimilated; when in the gut fluids, they can affect gut motility, enzyme activities, or absorption (De La Ruelle et al. 1992). This is termed "pre-assimilatory toxicity." Post-assimilatory toxicity occurs after the pollutant has been incorporated into tissues; this may damage gut tissues, interfere with enzyme synthesis or release, and interfere with absorption, transport, and assimilation of nutrients and thus impact energy reserves (Seebaugh 2010). Most studies, however, do not attempt to distinguish between pre- and post-assimilatory toxicity.

Metals

In general, digestive enzymes are inhibited by metal contaminants. Cadmium (Cd) exposure reduced amylase activity in gastric juice of the crayfish *Procambarus clarkii* (Reddy and Fingerman 1994). Activities of tryptase, pepsin, cellulase, amylase, and the metabolic enzymes, alkaline phosphatase, acid phosphatase, superoxide dismutase, and glutathione-S-transferase in the hepatopancreas of the prawn *Macrobrachium rosenbergii* were reduced after exposure to Cu^{2+} concentrations ranging from 0.01 to 0.5 mg/L (Li et al. 2008). Inhibition of digestive enzymes was observed, with the maximum inhibition in amylase. Acid and alkaline phosphatase were decreased, correlated with increased Cu^{2+} concentrations. Decreased glutathione-S-transferase activity was observed after exposure to 0.01 mg/L Cu^{2+}.

Digestive enzymes (cellulase, amylase, β-galactosidase, trypsin, and esterase) were studied in *Daphnia magna* after exposure to various metals (De Coen and Janssen 2007). Both Cd (0.8 μg/L) and Hg (1.8 μg/L) inhibited enzyme activities after 48 h exposure. However, after 96 hours, no inhibition was produced by Hg, and increased enzyme activity was seen with Cd. This increased enzyme activity was considered to reflect altered food assimilation efficiency to cope with reduced food uptake. Gaudy et al. (1991) also found that Cd exposure (0.05 mg/L) reduced assimilation efficiency and fecal pellet production in *Leptomysis*. The decreases in fecal pellet production and assimilation efficiency reflected a significant decrease in energy (about 43%), which authors felt would lead to an unbalanced energy budget and lower reproductive potential. Hydrolase activities increased initially in the presence of 0.2 mg/L Cd, but declined after 48 h, reaching very low values at 72 h. The unbalanced energy budget was considered a consequence of the inability to utilize food.

Cd body burdens in prey also altered assimilation efficiency of Cd in the grass shrimp *P. pugio* (Seebaugh and Wallace 2004). Cadmium assimilation was positively correlated with gut residence time in shrimp collected along a pollution gradient. Increased gut residence time can, in turn, influence (increase) pollutant assimilation. Ingestion of a pulse of Cd reduced protease activities and fecal elimination rate (Seebaugh 2010). Protease activities could have been influenced by pre-assimilatory interactions between Cd in the gut and enzyme-secreting cells, or they could have resulted from impacts on stored or circulating enzymes. Previous exposure to dietary metals

can induce changes in digestive physiology and affect digestive enzymes that may influence future digestion and assimilation.

Organic Contaminants

There have been few studies on effects of organic pollutants on digestion. Horst et al. (2007) found that exposure to the juvenile hormone analog methoprene (50 μg/L) caused upregulation of some genes in the hepatopancreas of the lobster *Homarus americanus*, including the enzymes betaine-homocysteine *S*-methyltransferase (BHMT) and other enzymes of the methionine cycle. Increased levels of enzymes associated with protein turnover, including trypsin, ubiquitin conjugating enzyme, and ubiquitin carboxyl terminal hydrolase were also observed.

Animals from Polluted Sites

Grass shrimp (*P. pugio*) from polluted sites had reduced digestive protease activity compared to shrimp from a reference site. Casein hydrolysis rates were negatively correlated with gut residence time and inversely related to assimilation efficiency of Cd (Seebaugh 2010), which would affect future assimilation of pollutants. However, carbon assimilation was not affected in these shrimp, suggesting that they can compensate for metal-induced post-assimilatory toxicity to maintain assimilation of nutrients (Seebaugh 2010). There was a trend of increasing gut residence time with increasing dietary Cd but not with Hg or C. Increased gut residence time can compensate for reduced digestive enzyme activities. Fecal elimination rate was not affected by field exposure, which also may be a compensatory response to impacts of pollutants. It appears that gut plasticity allows shrimp in contaminated sites to maintain adequate assimilation of essential nutrients but may increase the risk of dietary exposure to specific pollutants.

RESPIRATION/METABOLIC RATE

Most toxicants have been found to reduce metabolic rate and respiration of crustaceans and other organisms. Many studies have relied primarily on oxygen consumption to determine changes in metabolic rates. In some cases, lowered oxygen consumption can be attributed to reduced gill ventilation, and, in other cases, the mechanism is disruption of the enzymes of cellular respiration. Few studies have related effects on respiration to total carbon assimilation through measures of feeding and excretion or have examined effects on the total carbon, nitrogen, or energy budget of a crustacean.

Metals

Cadmium

Early studies showed that Cd reduced O_2 consumption in both larval and adult fiddler crabs, *Uca pugilator* (Vernberg et al. 1974) and grass shrimp (0.1 and 0.5 mg/L) (Hutcheson et al. 1985). Similarly, Barbieri (2007) found that exposure of pink shrimp (*F. paulensis*) to Zn (0.31 mg/L) or to Cd (0.18 mg/L) inhibited oxygen consumption. However, lobsters exposed to much lower concentrations of Cd (3 μg/L) had elevated gill oxygen consumption and increased ATPase activity (Thurberg et al. 1977). This may be an example of hormesis (the tendency for low doses of contaminants to have "positive" results, whereas at high doses processes are inhibited). Gaudy et al. (1991)

found that temperature affected the responses of the mysid *Leptomysis lingvura* to Cd. At 18°C, the respiration rate was affected only by concentrations greater than 0.05 mg/L Cd. Exposure to 0.1 mg/L Cd depressed the respiration rate more significantly at 20°C than at 10°C (Fig. 15.1). Because most studies are performed at only one temperature, this may help explain some of the differences observed in the literature in the direction of change of respiration.

Mercury

Oxygen consumption in adult fiddler crabs, *U. pugilator*, was reduced by exposure to 0.18 mg/L Hg (Vernberg and Vernberg 1972). St-Amand et al. (1999) exposed zoea larvae of the shrimp *Pandalus borealis* to inorganic Hg (0–160 µg/L) for 27 h and measured oxygen consumption, potential respiration (determined by respiratory electron transfer system activity [ETSA]), and swimming activity. ETSA was constant after 27 h exposure to 160 µg/L Hg, whereas both oxygen consumption and swimming decreased, showing that Hg disturbed part of the respiration process but not activity of the enzymes involved in the ETSA assay. In the crayfish *Astacus astacus*,

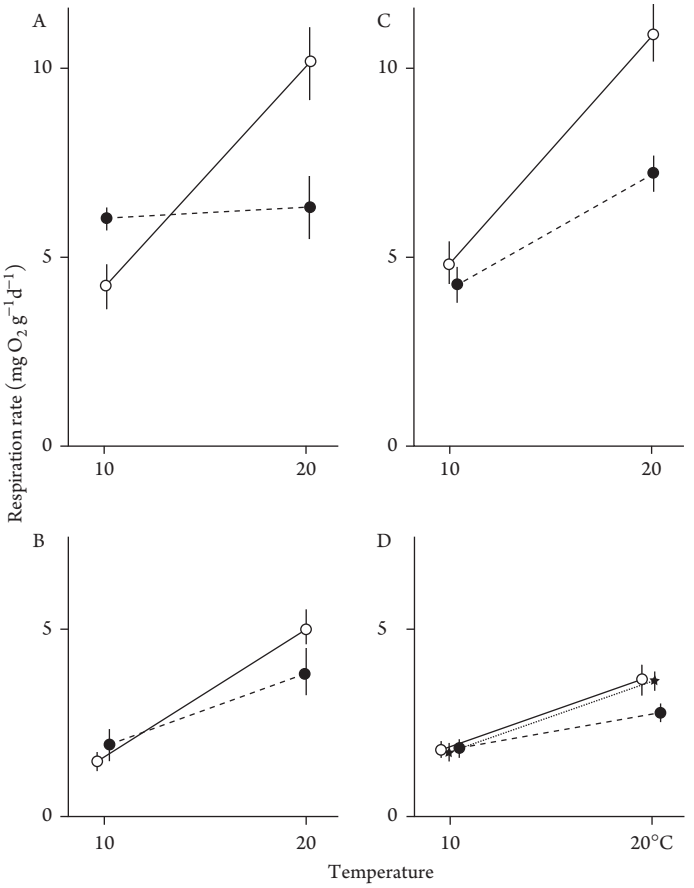


Fig. 15.1.
Respiration rate of *Leptomysis lingvura* at 10°C and 20°C in four experiments (A,B,C,D). Open circles are controls, closed circles are Cd-exposed at 0.1 mg/L, stars are Cd-exposed at 0.01 mg/L. Mean + SD. From Gaudy et al. (1991), with permission from Springer.

exposure to $HgCl_2$ (0.1 mg/L Hg) produced cardiac arrhythmia (Styrishave and Depledge 1996, Kouba et al. 2010), which may be related to metabolic disturbances.

Copper

Spicer and Weber (1991) found impairment of respiratory function of *Cancer pagurus* after 7 days of exposure to sublethal concentrations of Cu and Zn (0.4 mg/L), but only during hypoxic exposures. The Cu and Zn did not cause significant changes in ventilation or perfusion rates, although there was some indication that cardiac output may increase in respiratory-impaired individuals. The authors thought that respiratory impairment was due to an increase in diffusion barrier thickness at the gills and that this was reversible even during continued exposure. In a review, Spicer and Weber (1992) concluded that the essential metals Cu and Zn act on the respiratory system primarily by disrupting gill function, causing development of internal hypoxia, whereas the more toxic Hg and Cd interfere with the respiratory system at every level of organization, including cellular respiration itself.

Lead

Lead (Pb) decreased the respiration rate in crayfish *P. clarkii* (Torreblanca et al. 1987). Oxygen uptake of whole animals generally decreased with increasing Pb concentration, but was not statistically significant, whereas that of excised gills decreased significantly. Histology of the gill filaments of crayfish treated with 200 mg/L Pb indicated a general disorganization. Ahern and Morris (1999) found that exposure of the crayfish *Cherax destructor* to 100 or 0.5 mg/L Pb reduced oxygen consumption significantly, along with a decrease in heart rate, although ventilation rate was unchanged. There was also reduction of the oxygen transfer factor across the gills after 21 days. Despite reduced oxygen consumption and oxygen transfer factor, there was almost no change in acid–base status. Although part of the typical response of crustaceans to hypoxia is hyperventilation, Pb did not elicit an increase in ventilation and therefore no attempt was made to maintain normal oxygen levels. Metabolism was not supplemented by anaerobiosis, and thus overall energetic demand would have been lowered.

Organic Contaminants

Oil

Effects of oil and its constituent hydrocarbons vary considerably among crustaceans, with a number of studies showing increased metabolic rates in response to exposure. Studies have generally been done with individual hydrocarbons or with the WSF of oil, which lowered respiration in the shrimp *Crangon* (Edwards 1978). However, in adult shrimp *P. borealis*, energy balance (scope for growth [SFG]) declined after exposure to WSF (20–36 μg/L) due to reduced food intake, but remained positive at all oil concentrations (Stickle et al. 1987). The energy budget developed by these investigators measured costs of respiration and ammonia excretion and found that costs did not change significantly in response to differing levels of fuel oil, whereas the reduction in feeding rate was concentration dependent. In fact, at low concentrations, feeding rates increased, a possible case of hormesis, wherein low levels of contaminants have "beneficial" results, whereas at high doses feeding decreased. Because the metabolic costs remained relatively constant at all exposures, consumption alone determined energy production in this study. Nitrogen excretion accounted for only 10–20% of metabolic costs, whereas oxygen

metabolism accounted for 80–90% of the costs. Conversely, blue crabs (*C. sapidus*) exposed to WSF increased their energy expenditure and decreased their SFG in a dose-dependent manner, due primarily to reduced feeding. They reduced their energy intake without a reduction in maintenance costs, and thus had reduced growth and longer intermolt periods at 800 μg/L (Wang and Stickle 1987). In both these studies, metabolic costs such as respiration remained relatively constant while feeding rate changed in response to contamination. Lobster larvae (*H. americanus*) exposed to 0.25 mg/L South Louisiana crude oil showed a reduction in respiration rate and O:N ratio (Capuzzo and Lancaster 1981). Low O:N ratios suggest the organism is deriving energy from protein catabolism rather than carbohydrates or lipids. Energy metabolism did not return to control values after a week in clean water. *Cancer irroratus* incubated in ^{14}C-naphthalene-labeled oiled sea water accumulated the isotope into their hemolymph (Vandermeulen et al. 1980). Respiration was lowered in 11.0 mg/L and returned to control levels when crabs were returned to clean sea water. The O_2 binding potential and structural integrity of hemocyanin were unaltered, suggesting that disruption of hemocyanin-O_2 binding is not a mechanism of hydrocarbon toxicity. Whereas oxygen consumption increased in 8 mg/L naphthalene-treated mud crabs *Scylla serrata* (Vijayavel and Balasubramanian 2006), activity of the respiratory enzymes lactate dehydrogenase, isocitrate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, and α-ketoglutarate dehydrogenase decreased in the hepatopancreas, ovary, and gills for all the tested concentrations. Naphthalene at the much lower concentration of 0.2 mg/L also increased oxygen consumption in adult *Neomysis americana* (Smith and Hargreaves 1985). In another example of increased metabolic rate, Laughlin and Linden (1983) found that exposure to high levels of WSF of crude oil (200 and 1,000 μg/L) increased metabolic rate and ammonia excretion in the mysid *Neomysis integer*. Effects were influenced by temperature, with the greatest effect at 21.5°C, the highest temperature tested. This again suggests that temperature may be a partial explanation for the opposite results seen among the various studies.

Differences have been found in sensitivity of juvenile versus adult shore crabs (*C. maenas*) to oil (Dissanayake et al. 2008a). Seven days of exposure to 200 μg/L of pyrene reduced immunocompetence, elevated basal heart rate, and decreased respiration of juveniles but had no overall impact on adults. Juveniles were more susceptible than adults using various endpoints. Thus, basing "safe" concentrations on the tolerances of adults fails to protect more sensitive life stages, and all life stages need to be studied.

Pesticides and PCBs

Fenvalerate, a pyrethroid insecticide, reduced weight gain in *P. pugio* larvae and juveniles at 10 μg/kg due to altered energy metabolism. Affected larvae contained significantly less N than controls, whereas exposed postlarvae contained significantly less carbon and less energy (McKenney et al. 1998; Figs. 15.2 and 15.3). Thiobencarb, a carbamate insecticide, at 100 μg/L also stimulated the respiratory rate in *Mysidopsis bahia*, thus reducing the amount of energy available for growth. Higher O:N ratios suggested a greater reliance on energy-rich lipid substrates resulting in less lipid being available for gamete production (McKenney 1985). Energy metabolism of *M. bahia* was also altered by the pesticide fenthion. Juveniles responded with elevated respiration, which reduced the amount of energy available for growth and resulted in reduced growth (McKenney and Matthews 1990).

Verslycke et al. (2004) studied cellular respiratory responses of *N. integer*, including SFG and cellular energy allocation (CEA). Both assays are based on the concept that energy in excess of that required for normal maintenance will be available for growth and reproduction. Mysids were exposed to environmentally realistic concentrations of the organophosphate chlorpyrifos,

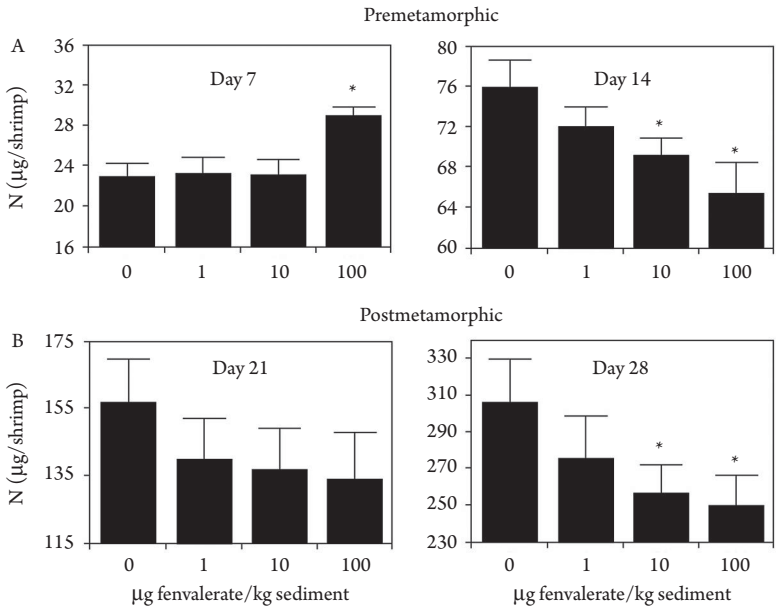


Fig. 15.2.
Nitrogen content (mean + SE) of *Palaemonetes pugio* larvae and postlarvae in different concentrations of fenvalerate in sediment. Asterisks indicate significant differences ($p < 0.05$) from control. From McKenney et al. (1998), with permission from Springer.

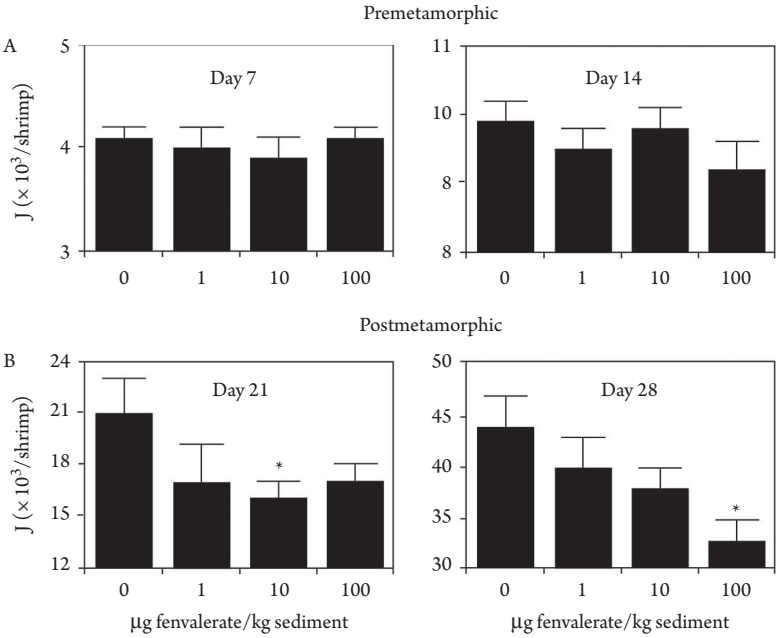


Fig. 15.3.
Energy content (in joules) (+ SE) of *Palaemonetes pugio* larvae and postlarvae in difference concentrations of fenvalerate in sediment. Asterisks denote significant differences ($p < 0.05$) from control. From McKenney et al. (1998), with permission from Springer.

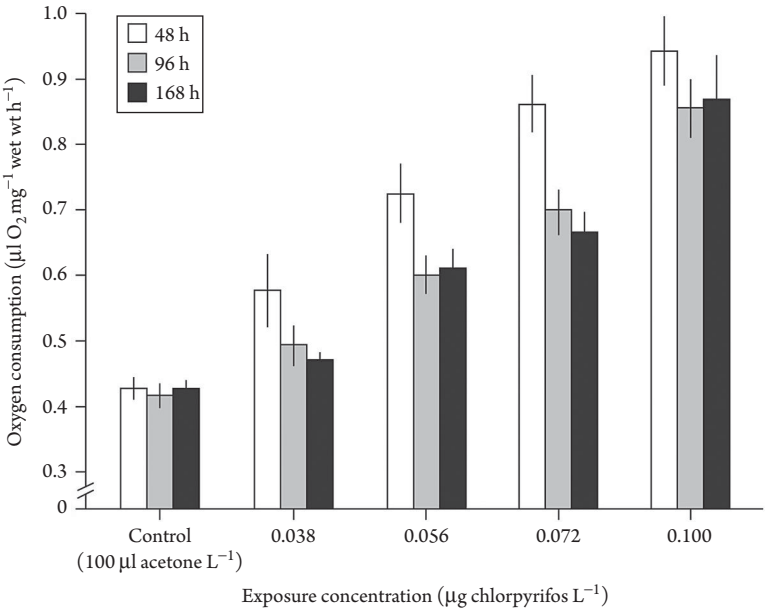


Fig. 15.4.
Oxygen consumption (+ SD) by *Neomysis integer* following exposure to chlorpyrifos at three different time periods. From Verslycke et al. (2004), with permission from Elsevier.

and assays were conducted. Results of both assays were correlated, and both were significantly affected (Fig. 15.4). CEA was more sensitive and reduced at lower concentrations (0.038 and 0.056 µg/L) than SFG. Effects of pentachlorophenol, a pesticide and wood preservative, on metabolic rate of grass shrimp depended on the molt cycle stage of the animal, with molting-stage animals much more sensitive than intermolt animals (Cantelmo et al. 1978). This study also found inhibition of several respiratory enzymes in blue crabs, including fumarase, succinate dehydrogenase, malate dehydrogenase, glucose-6-phosphate dehydrogenase, pyruvate kinase, and lactic dehydrogenase.

Effects of PCBs on *U. pugilator* respiration were variable: at some temperatures, exposure to 50 µg/L increased metabolic rate, and at other temperatures, it decreased the metabolic rate (Vernberg et al. 1978). Again, temperature may be responsible for some of the disparate results obtained in different studies.

OSMOREGULATION

The ability to maintain salt concentrations in the body regardless of the salt concentration of the environment is particularly important in animals living in estuaries, which may be exposed to variable salinity and pollution stress. Two enzymes play a major role in osmoregulation: Na^+/K^+-ATPase and carbonic anhydrase (CA). Na^+/K^+-ATPase in intestines and gills maintains gradients needed for salt movement and is related to Na^+ and Cl^- exchanges across tissues. CA is involved in the hydration of CO_2 to produce H^+ and HCO_3^-, playing a role in osmoregulation, as well as in gas exchange and acid–base balance (Lionetto et al. 2000). It should be noted that impaired osmoregulation, like feeding, may result in altered uptake of toxicants (by altered rates of pumping), which could then modify toxic effects.

Metals

The toxicity of many trace metals is higher in less saline water, in part because more of the metal is in free ion form and more bioavailable, but also because of physiological responses of organisms. Metal uptake may be reduced as salinity approaches the isosmotic point of a species because of reduced activity of ion exchange pumps. Effects of metals on estuarine animals were reviewed by Monserrat et al. (2007). A key mechanism of acute metal toxicity in many organisms has been reported to be osmoregulatory impairment associated with gill Na^+/K^+-ATPase inhibition. Copper, Ag, Cd, Pb, Zn, and Hg have all been found to impair osmoregulation related to inhibition of Na^+/K^+-ATPase in freshwater, brackish, and marine animals (Péqueux et al. 1996, Bianchini and Castilho 1999). Inhibitory effects of Ag (0.05–0.5 μM), Cd (0.05–0.5 μM or 1.25 mg/L), Cu (0.05–0.5 μM), and Zn (2–6 μM) have been also reported on CA in euryhaline crabs (Vitale et al. 1999, Skaggs and Henry 2002; Fig. 15.5).

Copper

Exposure of *C. maenas* to 1 mg/L Cu altered hemolymph osmolality and ion balance (Bjerregaard and Vislie 1986); disruption of Na^+/K^+-ATPase was considered the cause of the disturbance. Hansen et al. (1992) found that exposure to 10 mg/L Cu for 1 week reduced this enzyme by 50–60% and resulted in a major reduction in hemolymph Na^+ concentration. However, osmoregulation was less sensitive to Cu than respiration (Hebel et al. 1999). In this species, the anterior gills (numbers 1–6) are primarily respiratory in function, whereas the posterior gills (numbers 7–9) play an osmoregulatory role. Following exposure to sublethal concentrations of Cu, damage (epithelial hyperplasia and necrosis) initially occurred in respiratory gills at 100 μg/L Cu. No damage was seen in osmoregulatory gills at levels up to 300 μg/L Cu. In an investigation of Cu (0.78 μM) effects on *C. sapidus* at low (2 ppt) and high (30 ppt) salinity, Martins et al. (2011) found that crabs acclimated to dilute seawater showed inhibition of expression of mRNA of the genes for the Na^+/K^+-ATPase and the $Na^+/K^+/2Cl^-$ co-transporter, but Na^+/K^+-ATPase activity itself was not

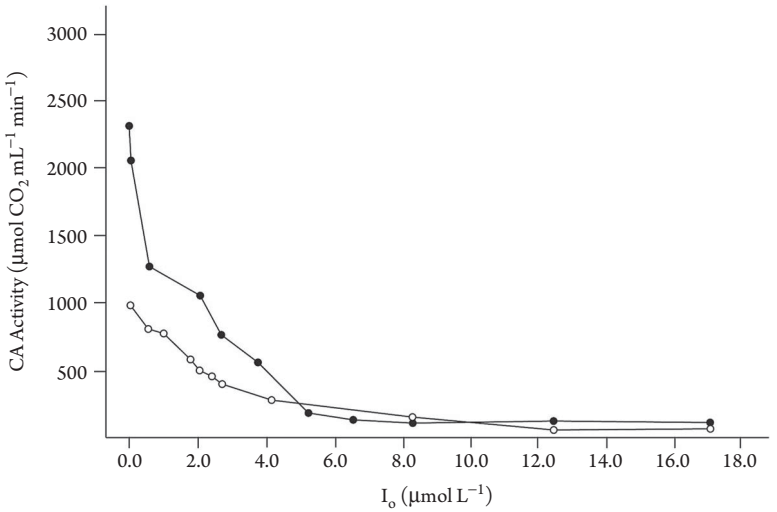


Fig. 15.5.
Inhibition of branchial carbonic anhydrase activity in *Callinectes sapidus* by Cu^{2+}. Open circles are 1 mM and solid circles are 2.5 mM CO_2 substrate. Modified from Skaggs and Henry (2002), with permission from Elsevier.

affected, indicating that the gene transcription is downregulated before significant inhibition of enzyme activity occurs. No effects were seen at high salinity, possibly because of lower bioavailability of the free ion. Bambang et al. (1995) found that concentrations of 500 (low), 1,000 (medium), and 1,500 (high) µg/L Cu altered both hypo- and hyper-osmoregulation in larval shrimp *Penaeus japonicus*. Hypo-osmoregulation was reduced after 4 days at low Cu and was suppressed at medium and high levels. Hyperosmoregulatory capacity was significantly reduced after 4 days exposure to low and medium concentrations. Only the shrimps exposed to low and medium concentrations of Cu recovered their hypo-osmoregulatory capacity after 7 days back in control seawater. Tolerance to Cu increased when nauplii became juveniles.

Mercury

Mercury (0.04 µM) inhibited Na and Ca influx in the freshwater isopod *Asellus aquaticus* (Wright and Welbourn 1991). Péqueux et al. (1996) investigated the possibility that toxicity results from interference with osmoregulatory mechanisms and that impairment of osmoregulation is greater in lower salinity. They examined effects on three crab species with various degrees of osmoregulatory ability: the strong regulator *Eriocheir sinensis*, the weak regulator *C. maenas* (both euryhaline), and the stenohaline osmoconformer *C. pagurus*. They found synergistic effects between salinity and $HgCl_2$ (0.1 mg/L) toxicity in the euryhaline species that are hyperregulators in dilute media (*E. sinensis* and *C. maenas*). In *E. sinensis*, Na^+ and Cl^- permeability of the gill epithelium was affected, as well as Na^+ and Cl^- active transport processes. They showed that Hg drastically disturbs the Na^+/K^+ pump and the Cl^- channels in the posterior gills.

Cadmium

Sublethal Cd (7.5 and 15 µg/L) exposure of the amphipod *G. pulex* caused a significant decrease of osmolality and hemolymph Ca^{2+} but not hemolymph Na^+ and Cl^- concentrations. However, Na^+/K^+-ATPase activity was significantly increased (Felten et al. 2008), contrary to the typical response. On the other hand, feeding rate and locomotor and ventilatory activities were significantly reduced in Cd-exposed organisms.

Organic Contaminants

There has been less work on effects of organic chemicals on osmoregulation.

Oil

Palaemon adspersus, a hyper- and hypo-osmoregulating shallow-water shrimp, was exposed to 20, 70, 100, and 200 µg/L WSF of North Sea crude oil (Baden 1982). The ability to maintain hyper-osmolality decreased after 1, 2, and 3 weeks exposure to 200, 70, and 100 µg/L, respectively, but no effect was observed at 20 µg/L.

Pesticides and PCBs

Neufeld and Pritchard (1979a) found that gill Na^+/K^+-ATPase was inhibited by both in vitro and in vivo exposure to DDT (1 µg/L) in *C. irroratus*. However, in *C. sapidus*, the in vivo response was transient and disappeared after return to clean water (Neufeld and Pritchard 1979b). The

authors felt it was likely that induction of new Na^+/K^+-ATPase in response to osmoregulatory stress protected the crabs from osmotic failure in response to DDT. Sublethal levels of lindane altered the ionic and osmoregulatory ability of the mud crab *Eurypanopeus depressus*. Chloride (Cl) ion regulation was disrupted at 0.70 µg/L, whereas hemolymph osmotic concentration was reduced at 1.45 µg/L (Shirley and McKenney 1987). In juvenile *P. japonicus* in seawater or diluted seawater, the insecticide fenitrothion decreased osmoregulatory capacity at both lethal and sublethal (low µg/L) concentrations (Lignot et al. 1997). The effect was time- and dose-dependent. In seawater, shrimp could recover in less than 48 when transferred to clean water. In dilute seawater, recovery at 48 h was possible only after exposure to the lowest concentration (Fig. 15.6). The PCB mixture Aroclor 1254 at 7.5 or 29 µg/L did not significantly alter hemolymph Cl and osmotic concentrations or Cl-exchange kinetics in adult *P. pugio* (Roesijadi et al. 1976). However, disruption of hemolymph Cl regulation was seen in juveniles and was associated with mortalities not seen in adults.

Shrimp (*P. japonicus*) tolerance to tributyltin increased with development from larvae to juveniles. Acute exposures (0.88 µg/L for nauplii to 708 µg/L for juveniles) decreased osmoregulatory capacity (difference between the hemolymph osmolality and the osmolality of the medium) of animals exposed to lethal and sublethal concentrations (Lignot et al. 1998). Pathology in gills increased with the concentration and was considered the cause of impaired osmoregulation. However, the ability to osmoregulate recovered after shrimp were put in water without TBTO for 48–120 h.

Animals from Polluted Sites

Animals from polluted sites have been studied in terms of their tolerance to contaminants. Effects of Zn were studied in two populations of the freshwater amphipod *G. pulex*. Animals from a relatively

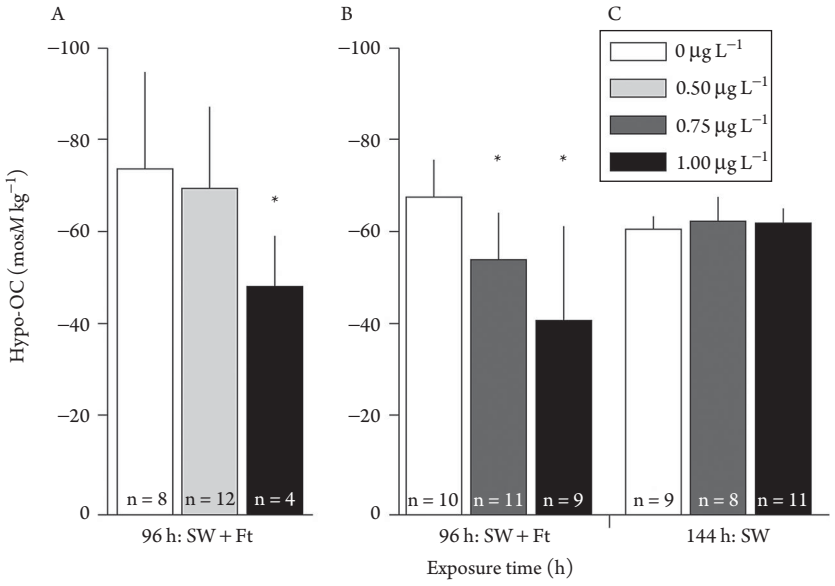


Fig. 15.6.
Hypo-osmoregulatory capacity of juvenile *Penaeus japonicus* after 96 h exposure to (A) control, 0.5 and 1 µg/L fenitrothion in seawater; (B) controls, 0.75 and 1 µg/L in seawater, and (C) controls, 0.75 and 1 µg/L fenitrothion followed by 48 h in clean water. Asterisks indicate significant ($p < 0.05$) differences. From Lignot et al. (1997), with permission from Springer.

clean site showed a marked hemoconcentration after 4 days at 37 μmol/L Zn or 5 days at 18.2 μmol/L Zn as shown by an increase in hemolymph osmotic pressure (OPh) and $[Na^+]$ and $[K^+]$. However, after 5 days at 37 μmol/L Zn, hemolymph exhibited an OPh significantly less than controls. Animals from a contaminated site showed a reduction in OPh (but not ions) only after 5 days at 76.2 μmol/L Zn, indicating they had a higher tolerance to Zn than the reference site animals (Spicer et al. 1998). This paper did not examine differences between controls from the different sites, however. The mangrove crabs *Ucides cordatus* and *Callinectes danae* were sampled from "polluted" mangrove areas and a reference site (Harris and Santos 2000). Individuals of both species from the polluted site showed greater ability to regulate blood osmotic concentrations at low salinity (9 ppt). However, *U. cordatus* showed reduced hyporegulatory ability in 34 ppt. *C. danae* from the polluted site had significantly higher Na^+/K^+-ATPase levels in posterior gills than did "unpolluted" crabs. These differences may reflect adaptive changes following long-term exposure to contamination.

EXCRETION

Although there has been much research on the excretion of metals and other contaminants, there have been relatively few studies on the physiological effects of contaminants on the process of excretion itself. There have been reports of both increases and decreases in ammonia excretion in different species, sometimes in response to the same toxicants. Reduced excretion rate can contribute to an increased body burden of contaminants.

Metals

Gaudy et al. (1991) found that Cd (0.05 mg/L) reduced ammonia excretion in *Leptomysis*. Exposed mysids had reduced ability to utilize food (see the section "Digestion"), so this could be a result of reduced protein. Ammonia excretion rate decreased with increasing concentrations of Zn (<0.2 mg/L) and Cu (<0.1 mg/L) in the freshwater shrimp *Macrobrachium carcinus* (Correa 1987). After exposure to Cd or Zn (1 mg/L of each), ammonium excretion in the white shrimp *Litopenaeus vannamei* was higher than in controls (Wu and Chen 2004)—the opposite effect from that observed by Correa (1987) and Gaudy et al. (1991) with much lower concentrations. Barbieri (2007) found similar results in *Litopenaeus schmitti*: 0.18–0.98 mg/L Cd and 0.31–1.64 mg/L Zn increased ammonium excretion (Fig. 15.7). Barbieri et al. (2005) found that Hg (0.045 mg/L) reduced oxygen consumption but increased ammonia excretion in larvae of *F. brasiliensis*, a commercial shrimp. However, postlarvae of *Penaeus indicus* showed a decrease in ammonia excretion with increasing concentrations of Pb up to 7 mg/L (Chinni et al. 2002).

Organic Contaminants

Laughlin and Linden (1983) found that exposure of the Baltic mysid *N. integer* to WSF at concentrations of 200–1,000 pg/L produced decreases in ammonia excretion that were strongly influenced by temperature, with the greatest effect at the highest temperature tested. Cypermethrin (0.01 and 0.1 μg/L) increased ammonia excretion in the freshwater crab *Trichodactylus borellianus* in a dose-dependent manner (Verónica and Collins 2003), suggesting increasing catabolism of amino acids. Montagna and Collins (2008) studied effects of chlorpyrifos and endosulfan on ammonia excretion of the freshwater crab *T. borellianus*, and found a significant increase of excretion at 150 and 300 μg/L of chlorpyrifos. The O:N ratio decreased with chlorpyrifos and with 2,500 μg/L endosulfan, indicating a shift toward protein metabolism.

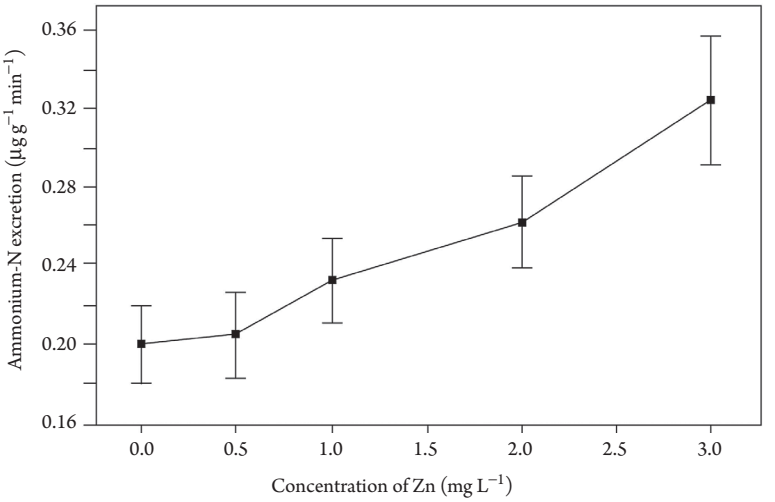


Fig. 15.7.
Shrimp (*Litopenaeus schmitti*) ammonium excretion in different Zn concentrations. From Barbieri (2007), reprinted with permission from Water Environment Research. Copyright © 2007 Water Environment Federation, Alexandria, Virginia.

REGENERATION AND MOLTING

Decapods can autotomize injured limbs—that is, break them off at a preformed breakage plane where there is a membrane, thus minimizing tissue damage. Autotomy is an effective antipredator response, leaving the predator with only a limb or claw while the intended prey escapes and can regenerate the lost appendage. Regeneration begins after a period of tissue reorganization and is first noticeable as a small bump at the autotomy plane. The limb buds grow within a thin covering of cuticle. In crabs, they grow folded and will unfold when the animal molts. In shrimp, they are not folded, but have joints nestled within one another so they also cannot expand until the animal molts. Thus, regeneration is closely tied with the molt cycle. Regeneration can be divided into two stages: basal growth, when tissue differentiation occurs and that is independent of the molt cycle, and proecdysial growth, in which rapid growth occurs and that is dependent on the presence of molting hormones. There may be a plateau phase when basal growth is completed and proecdysial growth has not yet started. Multiple autotomy, removal of many limbs at once, results in accelerated regeneration and molting.

Many chemicals have been found to alter the rate of limb regeneration and molting. In some cases, regeneration and molting may be affected independently, but in many studies it is not possible to distinguish effects on regeneration per se from those on the molt cycle because they are usually coupled, and both processes are simultaneously retarded. A number of toxicants produce morphological alterations in the regenerated limbs. These may be relatively minor, such as reduced number of pigment cells, setae, or tubercles in the regenerated limbs (these tubercles function in stridulatory sound production during courtship), or there may be more major deformities, such as abnormal bending in the limb or claw or defects in chitin formation in the exoskeleton.

Metals

The most common effect of metals is retardation of regeneration accompanied by a delay in ecdysis; in some cases, regeneration is affected without altering the timing of molting. A series of studies

on fiddler crabs was conducted in the 1970s and 1980s by Weis and colleagues; these are reviewed in Weis et al. (1992). Delayed regeneration and molting were observed in *U. pugilator* after exposure to $HgCl_2$, methyl mercury (MeHg), Cd, and Zn at concentrations of 0.5–1.0 mg/L. Retardation of regeneration was accompanied with a delay in ecdysis so that, at molt, limbs were fully formed. MeHg at 0.1 mg/L inhibited the development of melanin pigment in the regenerated limbs and reduced the number of tubercles on regenerated first walking legs of males. Although Hg and Cd individually retarded limb regeneration, the presence of MeHg reduced the particularly toxic effects of the Cd at low salinity. Zinc and Hg together were additive, whereas Zn and Cd interacted in an antagonistic manner. Pre-exposure to a lower concentration of Cd enhanced Cd tolerance; these crabs regenerated more rapidly in Cd than those that had not been pre-exposed, but molting still was delayed. *U. pugnax* from a contaminated site were less affected by MeHg than those from a relatively clean site (limb regeneration was less retarded) indicating that they had acquired tolerance to MeHg. However, short-term pre-exposure to low concentrations of MeHg did not enhance tolerance to higher concentrations. In *U. pugnax*, MeHg did not retard ecdysis, but did retard regeneration. Juvenile tiger shrimp, *Penaeus monodon*, experienced shortened time to the first molt and decreased molting frequency after exposure to 0.9 mg/L Cr (Chen and Lin 2001).

Organic Contaminants

Oil and Its Constituents

Exposure of juvenile blue crabs (*C. sapidus*) to 1 mg/L benzene or dimethylnaphthalene increased the length of the intermolt cycle, decreased the increment per molt, and retarded limb regeneration (Cantelmo et al. 1982). Affected crabs showed a longer plateau stage and a longer time for regenerated limbs to develop pigmentation. Wang and Stickle (1987) found that the WSF (1.5 or 2.5 mg/L) of South Louisiana crude oil inhibited growth and molting in blue crabs, reduced the increment at molt, and prolonged the intermolt period.

Pesticides, PCBs, and Dioxins

The PCB mixture Aroclor 1242 (8 mg/L) was found by Fingerman and Fingerman (1978) to inhibit limb regeneration in *U. pugilator*, with greater inhibition at high and low salinities than at intermediate salinities. PCBs retarded molting also in crabs that were not undergoing regeneration. Chlorophenols and dithiocarbamates at 0.1–1.0 mg/L retarded regeneration in *P. pugio* without affecting the timing of molting. Early stages of regeneration were more sensitive to pentachlorophenol than later phases (Rao et al. 1979). Other chlorophenols (2,3,4,5-tetrachlorophenol and 2,3,4,6-tetrachlorophenol) inhibited limb regeneration at 0.3 mg/L and 0.7 mg/L respectively) but did not alter the molt cycle, suggesting an effect on limb growth rather than on molting. (Rao et al. 1981). However, DDT (10 μg/L) accelerated limb regeneration in fiddler crabs (Weis and Mantel 1976). In the case of crabs with multiple autotomy, the time to ecdysis was shortened as well. These responses may have been due to heightened excitation of the nervous system and secretion of neuroendocrine factors promoting molting. Exposure to the antifouling agent tributyltin (TBT) retarded limb regeneration and molting in *U. pugilator* at concentrations of 0.5 μg/L and produced anatomical abnormalities in regenerated chelae of males, in which the regenerated dactyl curved upward, away from the pollex, instead of downward toward it (Weis et al. 1987a).

Current insect growth-regulating pesticides are very toxic to growth processes in crustaceans. The chitin synthesis inhibitor diflubenzuron (Dimilin) at levels of 0.5, 5.0, and 50 μg/L produced a dose-dependent retardation of regeneration in *U. pugilator*; crabs that molted in higher concentrations showed high mortality (Weis et al. 1987b). Regenerated limbs had blackened areas in which

the cuticle had not developed properly. Diflubenzuron at 0.11 μg/L also retarded the molt cycle of grass shrimp and caused dose-related inhibition of limb regeneration (Touart and Rao 1987). This suggests that, in addition to its effects on cuticle synthesis, it affects molting and inhibits mitosis and differentiation of limb buds. Stuekle et al. (2008) found that methoprene, a juvenile hormone mimic, retarded regeneration in *U. pugnax*, with greater effects on males, which took longer than females at 0.1 μg/L and exhibited greater frequency of abnormal limb formation at 1.0 μg/L. Abnormal limbs failed to regenerate; had bent or bulging merus, carpus, or propodus; or had a hook-shaped dactyl.

BIOACCUMULATION, STORAGE, DETOXIFICATION, METABOLISM OF CONTAMINANTS

Uptake of contaminants occurs via the skin, respiratory system, or food. For crustaceans with a chitinous exoskeleton, uptake is mostly via the gills or food, except perhaps directly after ecdysis when the exoskeleton is paper-thin.

Metals

Bioaccumulation

Crustaceans can take up metals from the water, the sediments, or their food. The accumulation pattern (distribution among tissues) of different metals varies considerably among taxa depending on the uptake rate, degree to which excretion plays a role, and where the metal is stored. The ionic form of most metals is the most bioavailable form, thus is generally taken up the most. The subsequent fate of the metal depends on the animal's physiology and whether or not the metal is essential. Essential metals tend to be regulated at optimum concentrations, above which excretory mechanisms come into play. Potentially toxic metals must either be excreted or sequestered in a nonavailable form if they are not to cause damage. Toxicity occurs when the concentration exceeds the amount that can be stored in nontoxic form or excreted. Metals tend to be stored in specific tissues such as the midgut gland (hepatopancreas; Rainbow 1988), which is generally the site with the highest accumulation of Cd, Zn, Cu, Pb, and Cr. However, significant concentrations may be found in muscle, which has implications for human consumption of edible species. For example, in many crustaceans including the edible crayfish and blue crab, Hg (the most toxic metal and one that has caused neurotoxic effects in humans from consuming contaminated fish) is accumulated largely in the muscle (Simon et al. 2000, Kouba et al. 2010, Reichmuth et al. 2010). However, crustaceans do not feed so high on the food chain as to accumulate high levels like those found in large predatory fishes.

Cadmium is taken up and accumulated from the water and food (Devi et al. 1996), and it is stored and detoxified mainly in the hepatopancreas (White and Rainbow 1986). The order of Zn accumulation in crayfish tissue was found by Bagatto and Alikhan (1987) to be hepatopancreas>exoskeleton>digestive tract>abdominal muscle. Marine crustaceans appear to have similar Zn accumulation patterns. Copper in decapod crustaceans is essential as part of the structure of the hemocyanin molecule. It is regulated to an approximately constant level until it exceeds a threshold and net accumulation begins (White and Rainbow 1982, Rainbow and White 1989). Excess levels of Cu and Zn, both of which are essential metals, can be rapidly depurated when the animals are returned to clean water (Kouba et al. 2010). Some species, such as *Palaemon elegans*, can match excretion to uptake of certain metals such as Zn (Rainbow 1993). Martins et al. (2010) showed that Cu influx into the gills of blue crabs was higher than into other tissues and that gills were important sites of

Cu accumulation at both high and low salinities. In vitro experiments with isolated perfused gills showed a positive relationship between Cu accumulation in both anterior and posterior gills and the metal's concentration in the incubation media.

Lead is neither essential nor beneficial and generally accumulates to the greatest extent in the hepatopancreas. After 10 weeks of exposure of *A. astacus* to 0.02 mg/L Pb, it accumulated to the highest concentration in the hepatopancreas, carapace, and gills (Meyer et al. 1991, cited in Kouba et al. 2010). However, the freshwater crab *Potamonautes perlatus* had the lowest concentration in the hepatopancreas and the highest concentration in the gonads (Reinecke et al. 2003).

Because crustaceans periodically molt their exoskeleton, depositing metals in the exoskeleton prior to molting is a potential way to depurate contaminants. However, when *P. pugio* were exposed to Cu, Zn, and Cd and then placed in clean water to molt, relatively low percentages of metals were actually depurated via ecdysis (<26% for Cd). It appeared that some of the Cu in the exoskeleton was reabsorbed prior to molting (Keteles and Fleeger 2001). In contrast, *U. pugnax*, particularly specimens from a contaminated site, moved considerable amounts of Pb and Hg from their soft tissues into their exoskeleton prior to molting while moving the essential Cu and Zn from their exoskeleton into the soft tissues (Bergey and Weis 2007). This was an effective method to depurate the more toxic metals and may be a mechanism of increased tolerance for the population in the contaminated site.

Subcellular Disposition/Detoxification

The cellular localization of metals can be critical to their toxicity. Metals associated with metal-sensitive intracellular components (e.g., organelles and enzymes) may impact cell functioning. Metals tend to bind to proteins and may prevent them from functioning normally. For example, metals can bind to active sites of enzymes, changing their configuration and inhibiting their activity. Khoury et al. (2009) found that fiddler crabs (*U. pugnax*) from a contaminated site had elevated metals in the heat-denatured proteins (HDP, enzymes) fraction. Other types of proteins can bind to and then detoxify the metals (to a degree). Thus, the metal may be toxic and available or may be detoxified, depending on what kind of protein it is bound to. In environments with high levels of available metals, animals tend to have evolved mechanisms to enhance detoxification.

Metallothioneins

Metallothioneins are low-molecular-weight heat-stable proteins that are rich in cysteine and capable of binding high amounts of Ag, Cu, Zn, Cd, and Hg. They normally play a role in regulating Cu and Zn. Blue crabs that had not been exposed to metals were examined for partitioning of Zn and Cu into different fractions during different phases of the molt cycle (Engel 1987). Copper and Zn in the hemolymph and digestive gland decreased during molt. Metallothioneins were highest during intermolt and premolt and lowest just after ecdysis, suggesting that they are naturally occurring and are involved in Cu regulation (perhaps hemocyanin synthesis) and Zn regulation. However, these proteins are also involved in detoxification of nonessential metals. Their synthesis, primarily in hepatopancreas and gills, is stimulated by metal exposure, and, by binding metals, they limit their toxicity (Engel and Brouwer 1989). Most of the Cd taken up by *P. elegans* binds to metallothioneins without being excreted (Rainbow and White 1989). Recently, it has been found that different isoforms of metallothionein are involved in Cu metabolism versus metal detoxification (Schlenk and Brouwer 1991, Monserrat et al. 2007).

Mechanisms involved in tolerance can be linked to trophic transfer of contaminants. Metals bound to metallothioneins tend to be available for trophic transfer and are therefore more available to predators than are metals associated with insoluble cellular constituents. Seebaugh et al.

(2005) fed *P. pugio* brine shrimp that had been exposed to Cd. Metals associated with metallothioneins in the brine shrimp were associated with enhanced trophic transfer of Cd to the grass shrimp. Trophically available metal (TAM) consists of the metals associated with the subcellular fractions containing metallothioneins, enzymes, and organelles. The overall amount of TAM may be a way to predict transfer to predators (Seebaugh and Wallace 2004). A direct relationship was observed between the partitioning of Cd and Zn to the TAM compartment of brine shrimp and the absorption of these metals by the grass shrimp. Similarly, when Cd-exposed amphipods (*Gammarus lawrencianus* exposed to 0.01–0.51 mg/L Cd) were fed to grass shrimp, the amount of TAM was related to the assimilation efficiency of grass shrimp for Cd. The TAM was considered to be the maximum bioavailable Cd in the prey (Seebaugh et al. 2006). Although the partitioning of metals to TAM can, in some cases, be a predictor of metal trophic transfer, it is clear from recent studies that ultimate absorption of TAM metals by predators is also linked to digestive processes in the predator (Goto and Wallace 2009).

Lysosomes

Lysosomes can sequester metals in many invertebrates and thereby play an important role in detoxification. Lysosomes may accumulate metals and trap them in lipofuscin granules, making them unavailable to the cell; this is a mechanism that leads to elimination of the metals through the kidneys. Nassiri et al. (2000) investigated the amphipod *Orchestia*, which uses lysosomes in the ventral caeca as a mechanism of metal detoxification. Animals from contaminated and reference sites were investigated. There was no difference in Zn uptake by the different populations when they were exposed to the same concentration of Zn. However, the contaminated population took up significantly less Cd than the reference population after exposure. Following laboratory exposure to Cu, Zn, and Cd, the lysosomes usually contained both Cu and Zn but not Cd. Although Cd was mostly associated with metallothioneins, the lysosomes in cells of the ventral caeca appeared to be a major detoxification pathway for Cu and Zn. Ahearn et al. (2010) summarized recent investigations of lysosomal function in lobster hepatopancreatic epithelia and described carrier-mediated transport processes on lysosomal membranes that accumulate metals from the cytoplasm. They discussed how metal transporters are linked with the uptake of anions that may then precipitate concretions within the lysosomes. The potential role of the organic anion transporter (OAT) in transporting glutathione with its associated metals from the cytoplasm into the lysosomal interior was described.

Insoluble Granules

Trace metals can be detoxified in the form of insoluble concretions or deposits, which prevents their toxicity because the metals are trapped in an insoluble form. Juvenile prawns, *P. monodon*, were exposed to Cu and Pb to investigate the formation and accumulation of metal granules as well as their excretion (Vogt and Quinitio 1994). Copper-containing granules accumulated primarily in the hepatopancreatic tubules. The amount and size of the granules increased along the tubules in accordance with the cell age; the granules were released by discharge of senescent cells into the intestine and were added to the feces. Lead-containing granules were found in the thoracic extensions of the antennal gland (kidney), where they were discharged into the gland lumen by secretion and then excreted with the urine. Although metals associated with metallothioneins are available to predators, metals in granules are less available for trophic transfer (Wallace and Lopez 1997) and can pass, unaltered, through the gut of a predator.

Stress Proteins

Stresses can bring about changes in protein conformation, and it is important to have mechanisms to maintain proteins in their functional conformation. Heat-shock proteins, originally found

in various organisms after exposure to elevated temperature, play a role in protein folding and assembly and can protect damaged cells from further damage. These proteins are also induced by contaminants and have general protective functions, so are more accurately termed "stress proteins." They confer increased tolerance to toxicants (Sanders 1993). The rate of survival and stress protein response were investigated in the amphipod *Gammarus fossarum* during a stress and recovery experiment by Schill et al. (2003). Lower Cd^{2+} concentrations led to induction of stress proteins whereas higher Cd^{2+} concentrations resulted in a reduced response, most likely due to pathological damage. Those amphipods that survived the exposure retained the ability to produce stress proteins during the recovery period. Eckwert et al. (1997) found that the expression of stress proteins in the terrestrial woodlouse *Oniscus asellus* was marginally increased by low concentrations of a variety of metals; at intermediate concentrations, a strong induction occurred, but high concentrations caused a decline of stress protein levels, which may reflect pathological damage. Combinations of metals increased the induction more intensely than comparable concentrations of individual metals.

Organic Contaminants

The uptake of foreign hydrocarbons presents organisms with the need to metabolize, store, and excrete them. Organic contaminants tend to be accumulated primarily in the hepatopancreas, where they may be transformed into metabolites that can be excreted via the gills and kidneys. Sometimes a metabolite may be more toxic than the original chemical. Synthesis of detoxification enzymes can be induced by chemical exposure.

Metabolism

Pathways of organic contaminant metabolism involve two phases. Phase I reactions typically reduce the contaminant's activity and render the molecule susceptible to phase II reactions, often by hydrolyzing or oxidizing the molecule to make it more polar or water soluble. Phase II reactions involve conjugation of the product of the phase I reaction with a substance that makes it less bioactive and more readily excreted. (Some compounds are directly conjugated without a phase I metabolism.) The most common phase I reaction is carbon oxidation. The enzymes responsible for oxidation of foreign compounds are termed mixed function oxidases (MFOs), and these include the highly studied cytochrome P-450 (CYP) system. CYPs, found in many tissues, are involved in oxidative metabolism of a wide range of organic compounds including PAHs, PCBs, pesticides, and other chemicals. The MFO system requires nicotinamide adenine dinucleotide phosphate (NADP or NADPH) plus molecular oxygen to convert nonpolar PAHs into polar hydroxy derivatives and arene oxides (some of which may be more toxic than the parent compound). In phase II reactions, another molecule (such as acetate, glucuronic acid, sulfate, glycine, or glutathione) is conjugated to a group on the xenobiotic, making it more readily excreted.

Rates of detoxification in crustaceans were initially found to be slow, which partially explains their sensitivity to oil pollution (Burns 1976). More recent studies demonstrate that crustaceans do have CYPs to metabolize organic compounds, and the activity of these systems varies among species. The shore crab *C. maenas* has a high capacity to metabolize PAHs with CYP enzymes. Expression of *CYP2*- and *CYP3*-like genes fluctuated over the molt cycle, with low expression during premolt and maximum expression during late postmolt, and expression was predominant in the hepatopancreas, whereas expression of *CYP4*-like genes was predominant in gills and epidermis (Dam et al. 2008). In addition, *CYP2*- and *CYP3*-related genes respond to ecdysteroid and xenobiotic treatment. The data suggest that premolt crabs with low gene expression would be more susceptible to organic pollutants than postmolt crabs.

PAHs can be conjugated and then excreted in the urine. *C. maenas* individuals were exposed to phenanthrene and pyrene (separately) at 0–200 μg/L. After 48 h, urine samples were taken and analyzed. Urinary levels were dose dependent for both compounds (Fillmann et al. 2004). In another study, *C. maenas* was exposed to waterborne pyrene for 48 h, and depuration was monitored. No unchanged pyrene was detected in samples from exposed crabs in this study, which had biotransformed it into conjugates that were then excreted in the urine (Watson et al. 2004). Urinary levels reached a maximum 2–4 days after exposure and then decreased.

FUTURE DIRECTIONS AND CONCLUSIONS

Viewing these physiological responses individually, they are, for the most part, deleterious. Although there are exceptions, there is a general response of reducing the rates of digestion, metabolism, excretion, osmoregulation, and molting. However, when looking at the "big picture," these effects collectively could be viewed as adaptive responses to conserve homeostasis. The cluster of effects of reduced respiration, digestion, osmoregulation (which requires energy expenditure), excretion, regeneration, and molting may all be associated with the almost-universal response of decreased food intake. With reduced food consumption and more energy devoted to detoxifying the contaminants, slowing down physiological processes is a way to compensate for reduced energy intake. Although productivity (growth and reproduction) would be expected to be lower in contaminated habitats, reduced productivity may not always be the case. Reduced respiration and excretion may in some cases be able to compensate for reduced food intake.

There are, however, cases in which physiological processes are increased after contaminant exposure in the laboratory. One would predict that when physiological functions are increased while energy intake is reduced, the most deleterious effects would occur because a negative energy balance could not be sustained over more than a short time. Future research should examine in greater detail those cases in which physiological processes are increased while food/energy intake is reduced to see whether these effects are transient and if the rate subsequently decreases to compensate for reduced energy intake. It may be that some of the observed cases of increased physiological function were really examples of hormesis, and higher concentrations of the toxicant would have the opposite effect. The role of temperature and other aspects of seasonality in modifying or even changing the direction of responses to toxicants is also in need of further investigation.

Future work is likely to continue in more biochemical, molecular, genomic, and proteomic directions in order to better understand mechanisms underlying physiological responses, and such work will also need to consider a suite of "emerging" contaminants such as flame retardants, pharmaceuticals and personal care products, nanoparticles, and the ubiquitous reduction of the ocean's pH (ocean acidification). For example, elevated P_{CO_2} levels in seawater, as predicted for the year 2300, reduce calcification, growth, and molting frequency in marine crustaceans. At these levels, embryonic development is impaired, and larvae and juveniles are affected when changes in P_{CO_2} are accompanied by rising temperatures (Whiteley 2011). Regarding nanoparticles, the nano form of metals is generally far more toxic than the ionic forms that have been studied previously (Karu and Dubourguier 2010).

There is also a great need for connecting effects observed in laboratory bioassays to the lives of animals living in the "real world." Additional studies on contaminated populations will be needed in order to understand ecological consequences of altered physiology, as well as the compensatory mechanisms that are used to survive in stressful environments. The various mechanisms that increase tolerance to contaminants (MTs, stress proteins, CYPs, etc.) all take energy. Environmental stress can reduce the assimilated energy available for maintenance and reproduction, but compensatory partitioning could be a common phenomenon to counteract deleterious effects of

pollutants. For example, effects on growth do not necessarily result in altered population dynamics if the effects are compensated for by increased reproduction. A reduction in resources allocated to respiration, for example, could allow additional resources to be allocated to growth or reproduction in order to maintain a population. More studies are needed of compensatory partitioning of energy resources in contaminated populations, which could be an important strategy to deal with anthropogenic stressors. Such populations may have been subjected to selection pressure and may have evolved enhanced tolerance to the contaminants. They may be more tolerant because of reduced uptake, altered inducibility of cytochrome P-450 systems, enhanced binding to molecules such as metallothioneins, or increased ability to depurate or store contaminants in intracellular structures that prevent damage. The inherent or induced tolerance of these populations enables them to persist. However, tolerance comes with energy and fitness costs, and it has frequently been noted that populations adapted to one set of environmental stressors have increased susceptibility to other stressors and may show tradeoffs among life history traits.

REFERENCES

Ahearn, G.A., M. Sterling, P.K. Mandal, and B. Roggenbeck. 2010. Heavy metal transport and detoxification by crustacean epithelial lysosomes. Pages 49–71 *in* G.A. Gerencser, editor. Epithelial transport physiology. Humana Press, New York.

Ahern, M.D., and S. Morris. 1999. Respiratory, acid–base and metabolic responses of the freshwater crayfish *Cherax destructor* to lead contamination. Comparative Biochemistry and Physiology Part A 124:105–111.

Baden, S.P. 1982. Impaired osmoregulation in the shrimp *Palaemon adspersus* exposed to crude oil extract. Marine Pollution Bulletin 13:208–210.

Bagatto, G., and M.A. Alikhan. 1987. Zinc, iron, manganese and magnesium accumulation in crayfish populations near copper-nickel smelters at Sudbury, Ontario, Canada. Bulletin of Environmental Contamination and Toxicology 38:1076–1081.

Bambang, Y., P. Thuet, M. Charmantier-Daures, J.-P. Trilles, and G. Charmantier. 1995. Effect of copper on survival and osmoregulation of various developmental stages of the shrimp *Penaeus japonicus* (Crustacea, Decapoda). Aquatic Toxicology 33:125–139.

Barbieri, E. 2007. Use of oxygen consumption and ammonium excretion to evaluate the sublethal toxicity of cadmium and zinc on *Litopenaeus schmitti* (Burkenroad, 1936, Crustacea). Water Environment Research 79:641–646.

Barbieri, E., E.A. Passos, and C.A. Garcia. 2005. Use of metabolism to evaluate the sublethal toxicity of mercury on *Farfantepenaeus brasiliensis* larvae (Latreille 1817). Journal of Shellfish Research 24:1229–1233.

Bianchini, A., and C. Castilho. 1999. Effects of zinc exposure on oxygen consumption and gill Na,K-ATPase of the estuarine crab *Chasmagnathus granulata* Dana, 1851 (Decapoda, Grapsidae). Bulletin of Environmental Contamination and Toxicology 62:63–69.

Bergey, L., and J.S. Weis. 2007. Molting as a mechanism of depuration of metals in the fiddler crab, *Uca pugnax*. Marine Environmental Research 64:556–562.

Bjerregaard, P., and T. Vislie. 1986. Effect of copper on ion- and osmoregulation in the shore crab *Carcinus maenas*. Marine Biology 91:69–76.

Blockwell, S.J., and E.J. Taylor. 1998. The influence of fresh water pollutants and interaction with Asellus aquaticus (L.) on the feeding activity of *Gammarus pulex* (L.). Archives of Environmental Contamination and Toxicology 34:41–47.

Burns, K.A. 1976. Hydrocarbon metabolism in the intertidal fiddler crab *Uca pugnax*. Marine Biology 36:5–11.

Cantelmo, A.C., P.J. Conklin, F.R. Fox, and K.R. Rao. 1978. Effects of sodium pentachlorophenate and 2,4-dinitrophenol on respiration in crustaceans. Pages 251–263 *in* K.R. Rao, editor. Pentachlorophenol: chemistry, pharmacology and environmental toxicology. Plenum Press, New York, U.S.A.

Cantelmo, A.C., L. Mantel, R. Lazell, F. Hospod, E. Flynn, S. Goldberg, and M. Katz. 1982. The effects of benzene and dimethylnaphthalene on physiological processes in juveniles of the blue crab, *Calinectes*

sapidus. Pages 349–389 *in* W. Vernberg, A. Calabrese, F.P. Thurberg, and F.J. Vernberg, editors. Physiological mechanisms of marine pollutant toxicity. Academic Press, New York.

Capuzzo, J.M., and B.A. Lancaster. 1981. Physiological effects of South Louisiana crude oil on larvae of the American lobster, *Homarus americanus*. Pages 405–423 *in* F.J. Vernberg, A. Calabrese, F.P. Thurberg, and W.B. Vernberg, editors. Biological monitoring of marine pollutants. Academic Press, New York.

Chen, J.-C., and C.-H. Lin. 2001. Toxicity of copper sulfate for survival, growth, molting and feeding of juveniles of the tiger shrimp *Penaeus monodon*. Aquaculture 192:55–65.

Chinni, S., R.N. Khan, and P.R. Yallapragada. 2002. Acute toxicity of lead on tolerance, oxygen consumption, ammonia-N excretion, and metal accumulation in *Penaeus indicus* postlarvae. Ecotoxicology and Environmental Safety 51:79–84.

Correa, M. 1987. Physiological effects of metal toxicity on the tropical freshwater shrimp *Microbrachium carcinus* (Linneo, 1758). Environmental Pollution 45:149–155.

Dam, E., K.F. Rewitz, B. Styrishave, and O. Andersen. 2008. Cytochrome P450 expression is moult stage specific and regulated by ecdysteroids and xenobiotics in the crab *Carcinus maenas*. Biochemical and Biophysical Research Communication 377:1135–1140.

De Coen, W.M., and C.R. Janssen. 2007. The use of biomarkers in *Daphnia magna* toxicity testing II. Digestive enzyme activity in *Daphnia magna* exposed to sublethal concentrations of cadmium, chromium and mercury. Chemosphere 35:1053–1067.

De La Ruelle, M., M. Hajjou, F. Van Herp, and Y. Le Gal. 1992. Aminopeptidase activity from the hepatopancreas of *Procambarus clarkii*. Biochemical and Systematic Ecology 20:331–337.

Devi, M., D.A. Thomas, J.T. Barber, and M. Fingerman. 1996. Accumulation and physiological and biochemical effects of cadmium in a simple aquatic food chain. Ecotoxicology and Environmental Safety 33:38–43.

Dissanayake, A., T.S. Galloway, and M.B. Jones. 2008a. Physiological responses of juvenile and adult shore crabs *Carcinus maenas* (Crustacea: Decapoda) to pyrene exposure. Marine Environmental Research 66:445–450.

Dissanayake, A., T.S. Galloway, and M.B. Jones. 2008b. Nutritional status of *Carcinus maenas* (Crustacea: Decapoda) influences susceptibility to contaminant exposure. Aquatic Toxicology 89:40–46.

Dissanayake, A., C. Piggott, C. Baldwin, and K A. Sloman. 2010. Elucidating cellular and behavioural effects of contaminant impact (polycyclic aromatic hydrocarbons, PAHs) in both laboratory-exposed and field-collected shore crabs, *Carcinus maenas* (Crustacea: Decapoda). Marine Environmental Research 70:368–373.

Eckwert, H., G. Alberti, and H.-R. Kohler. 1997. The induction of stress proteins (hsp) in *Oniscus asellus* (Isopoda) as a molecular marker of multiple heavy metal exposure: I. Principles and toxicological assessment. Ecotoxicology 6:249–262.

Edwards, R.R. 1978. Effects of water-soluble oil fractions on metabolism, growth and carbon budget of the shrimp *Crangon crangon*. Marine Biology 46:259–265.

Engel, D.W. 1987. Metal regulation and molting in the blue crab *Callinectes sapidus*: copper, zinc, and metallothionein. Biological Bulletin 172:69–82.

Engel, D.W., and M. Brouwer. 1989. Metallothionein and metallothionein-like proteins: physiological importance. Advances in Comparative and Environmental Physiology 5:53–75.

Felten, V., G. Charmantier, R. Mons, A. Geffard, P. Rousselle, M. Coquery, J. Garric, and O. Geffard. 2008. Physiological and behavioural responses of *Gammarus pulex* (Crustacea: Amphipoda) exposed to cadmium. Aquatic Toxicology 86:413–425.

Fillmann, J., G.M. Watson, M. Howsam, E. Francioni, M.H. Depledge, and J.W. Readman. 2004. Urinary PAH metabolites as biomarkers of exposure in aquatic environments. Environmental Science and Technology 38:2649–2656.

Fingerman, S., and M. Fingerman. 1978. Effects of two polychlorinated biphenyls (Aroclors® 1242 and 1254) on limb regeneration in the fiddler crab, *Uca pugilator*, at different times of the year. Vie Milieu 28–29:69–75.

Gaudy, R., J.-P. Guérin, and P. Kerambrun. 1991. Sublethal effects of cadmium on respiratory metabolism, nutrition, excretion and hydrolase activity in *Leptomysis lingvura* (Crustacea: Mysidacea). Marine Biology 109:493–501.

Goto, D., and W.G. Wallace. 2009. Influences of prey- and predator-dependent processes on cadmium and methylmercury trophic transfer to mummichogs (*Fundulus heteroclitus*). Canadian Journal of Fisheries and Aquatic Sciences 66:836–846.

Hansen, J.I., T. Mustafa, and M. Depledge. 1992. Mechanisms of copper toxicity in the shore crab *Carcinus maenas*. I. Effects on Na-K-ATPase activity, haemolymph electrolyte concentrations and tissue water contents. Marine Biology 114:253–257.

Harris, R.R., and M.C. Santos. 2000. Heavy metal contamination and physiological variability in the Brazilian mangrove crabs *Ucides cordatus* and *Callinectes danae* (Crustacea: Decapoda). Marine Biology 137:691–703.

Hebel, D.K., M.B. Jones, R.M. Moate, and M.H. Depledge. 1999. Differing sensitivities of respiratory and osmoregulatory gill tissue of *Carcinus maenas* (Crustacea: Decapoda) to water-borne copper. Marine Biology 133:675–681.

Horst, M.N., A.N. Walker, P. Bush, T. Wilson, E.S. Chang, T. Miller, and P. Larkin. 2007. Pesticide induced alterations in gene expression in the lobster, *Homarus americanus*. Comparative Biochemistry and Physiology D: Genomics and Proteomics 2:44–52.

Hutcheson, M., D.C. Miller, and A.Q. White. 1985. Respiratory and behavioral responses of the grass shrimp *Palaemonetes pugio* to cadmium and reduced dissolved oxygen. Marine Biology 88:59–66.

Jensen, L.K., and J. Carroll. 2010. Experimental studies of reproduction and feeding for two Arctic-dwelling *Calanus* species exposed to crude oil. Aquatic Biology 10:261–271.

Karu, A., and H.-C. Dubourguier. 2010. From ecotoxicology to nanoecotoxicology. Toxicology 269:105–119.

Keteles, K.A., and J.W. Fleeger. 2001. The contribution of ecdysis to the fate of copper, zinc, and cadmium in grass shrimp, *Palaemonetes pugio* Holthuis. Marine Pollution Bulletin 42:1397–1402.

Khoury, J., E. Powers, P. Patniak, and W. Wallace. 2009. Relating disparity in competitive foraging behavior between two populations of fiddler crabs to the subcellular partitioning of metals. Archives of Environmental Contamination and Toxicology 56:489–499.

Kouba, A., M. Buřič, and P. Kosák. 2010. Bioaccumulation and effects of heavy metals in crayfish: a review. Water, Air, and Soil Pollution 211:5–16.

Laughlin, R., and O. Linden. 1983. Oil pollution and Baltic mysids: acute and chronic effects of the water soluble fractions of light fuel oil on the mysid shrimp *Neomysis integer*. Marine Ecology Progress Series 12:29–41.

Li, N., Y. Zhao, and J. Yang. 2008. Effects of water-borne copper on digestive and metabolic enzymes of the giant freshwater prawn *Macrobrachium rosenbergii*. Archives of Environmental Contamination and Toxicology 55:86–93.

Lignot, J.H., J.-P. Trilles, and G. Charmantier. 1997. Effect of an organophosphorus insecticide, fenitrothion, on survival and osmoregulation of various developmental stages of the shrimp *Penaeus japonicus* (Crustacea: Decapoda). Marine Biology 128:307–316.

Lignot, J.H., F. Pannier, J.-P Trilles, and G. Charmantier. 1998. Effects of tributyltin oxide on survival and osmoregulation of the shrimp *Penaeus japonicus* (Crustacea, Decapoda). Aquatic Toxicology 41:277–299.

Lionetto, M.G., M.E. Gioradano, S. Villela, and T. Schettino. 2000. Inhibition of eel enzymatic activities by cadmium. Aquatic Toxicology 48:561–571.

Maltby, L., and M. Crane. 1994. Responses of *Gammarus pulex* (Amphipoda: Crustacea) to metalliferous effluents: identification of toxic components and importance of interpopulation variation. Environmental Pollution 84:45–52.

Martins, C., I.F. Barcarolli, E.J. de Menezes, M.M. Giacomin, C.M. Wood, and A. Bianchini. 2010. Acute toxicity, accumulation and tissue distribution of copper in the blue crab *Callinectes sapidus* acclimated to different salinities: in vivo and in vitro studies. Aquatic Toxicology 101:88–99.

Martins, C.G., D.V. Almeida, L.F. Marins, and A. Bianchini. 2011. mRNA expression and activity of ion-transporting proteins in gills of the blue crab *Callinectes sapidus*: effects of waterborne copper. Environmental Toxicology and Chemistry 30:206–211.

McKenney, C.L. 1985. Associations between physiological alterations and population changes in an estuarine mysid during chronic exposure to a pesticide. Pages 397–418 *in* F.J. Vernberg, F.P. Thurberg, A. Calabrese, and W.B. Vernberg, editors. Marine pollution and physiology: recent advances. University of South Carolina Press, Columbia.

McKenney, C.L., and E. Matthews. 1990. Alterations in the energy metabolism of an estuarine mysid (*Mysidopsis bahia*) as indicators of stress from chronic pesticide exposure. Marine Environmental Research 30:1–19.

McKenney, C.L., D.E. Weber, D.M. Celestial, and M.A. MacGregor. 1998. Altered growth and metabolism of an estuarine shrimp (*Palaemonetes pugio*) during and after metamorphosis onto fenvalerate-laden sediment. Archives of Environmental Contamination and Toxicology 35:464–471.

Meyer, W., M. Kretschmer, A. Hoffmann, and G. Harisch. 1991. Biochemical and histochemical observations on effects of low-level heavy metal load (lead, cadmium) in different organ systems of the freshwater crayfish, *Astacus astacus* L. (Crustacea: Decapoda). Ecotoxicology and Environmental Safety 21:137–156.

Monserrat, J.M., P.E. Martínez, L.A. Geracitano, L.L. Amado, C.M. Martins, G.L. Pinho, I.S. Chaves, M. Ferreira-Cravo, J. Ventura-Lima, and A. Bianchini. 2007. Pollution biomarkers in estuarine animals: critical review and new perspectives. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 146:221–234.

Montagna, M.C., and P.A. Collins. 2008. Oxygen consumption and ammonia excretion of the freshwater crab *Trichodactylus borellianus* exposed to chlorpyrifos and endosulfan insecticides. Pesticide Biochemistry and Physiology 92:150–155.

Nassiri, Y., P.S. Rainbow, C. Amiard-Triquet, F. Rainglet, and B.D. Smith. 2000. Trace-metal detoxification in the ventral caeca of *Orchestia gammarellus* (Crustacea : Amphipoda). Marine Biology 136:477–484.

Neufeld, G.J., and J.B. Pritchard. 1979a. Osmoregulation and gill Na, K-ATPase in the rock crab, *Cancer irroratus*: response to DDT. Comparative Biochemistry and Physiology Part C 62:165–172.

Neufeld, G.J., and J.B. Pritchard. 1979b. An assessment of DDT toxicity on osmoregulation and gill Na, K-ATPase activity in the blue crab. Pages 23–34 *in* L. Marking, and R. Kimerle, editors. Aquatic toxicology ASTM STP 667. American Society for Testing and Materials, Philadelphia.

Péqueux, A., A. Bianchini, and R. Gilles. 1996. Mercury and osmoregulation in the euryhaline crab, *Eriocheir sinensis*. Comparative Biochemistry and Physiology Part C 113:149–155.

Perez, M.H., and W.G. Wallace. 2004. Differences in prey capture in grass shrimp *Palaemonetes pugio*, collected along an environmental impact gradient. Archives of Environmental Contamination and Toxicology 46:81–89.

Rainbow, P.S. 1988. The significance of trace metal concentrations in decapods. Symposium of the Zoological Society of London 59:195–211.

Rainbow, P.S. 1993. The significance of trace metal concentrations in marine invertebrates. Pages 3–23 *in* R. Dallinger, and P.S. Rainbow, editors. Ecotoxicology of metals in invertebrates. Lewis Publishers, Boca Raton, Florida.

Rainbow, P.S., and S.L. White. 1989. Comparative strategies of heavy metal accumulation by crustaceans: zinc, copper and cadmium in decapod, an amphipod and a barnacle. Hydrobiologia 174:245–262.

Rao, K.R., F.R. Fox, P. Conklin, A.C. Cantelmo, and A. Brannon. 1979. Physiological and biochemical investigations of the toxicity of pentachlorophenol to crustaceans. Pages 307–340 *in* W.B. Vernberg, F.P. Thurberg, A. Calabrese, and F.J. Vernberg, editors. Marine pollution: functional responses. Academic Press, New York.

Rao, K.R., F.R. Fox, P. Conklin, and A. Cantelmo. 1981. Comparative toxicology and pharmacology of chlorophenols: studies on the grass shrimp, *Palaemonetes pugio*. Pages 37–72 *in* F.J. Vernberg, A. Calabrese, F.P. Thurberg, and W.B. Vernberg, editors. Biological Monitoring of Marine Pollutants. Academic Press, New York.

Reddy, P.S., and M. Fingerman. 1994. Effect of cadmium chloride on amylase activity in the red swamp crayfish *Procambarus clarkii*. Comparative Biochemistry and Physiology 109 C:309–314.

Reichmuth, J.M., R. Roudez, T. Glover, and J.S. Weis. 2009. Differences in prey capture behavior in populations of blue crab (*Callinectes sapidus* Rathbun) from contaminated and clean estuaries in New Jersey. Estuaries and Coasts 32:298–308.

Reichmuth, J.M., P. Weis, and J.S. Weis. 2010. Bioaccumulation and depuration of metals in blue crabs (*Callinectes sapidus* Rathbun) from a contaminated and clean estuary. Environmental Pollution 158:361–368.

Reinecke, A.J., R.G. Snyman, and J.A. Nel. 2003. Uptake and distribution of lead (Pb) and cadmium (Cd) in the freshwater crab, *Potamonautes perlatus* (Crustacea) in the Eerste River, South Africa. Water, Air, and Soil Pollution 145:395–408.

Roesijadi, G., J.W. Anderson, S.R. Petrocelli, and C.S. Giam. 1976. Osmoregulation of the grass shrimp *Palaemonetes pugio* exposed to polychlorinated biphenyls (PCBs). I. Effect on chloride and osmotic concentrations and chloride-and water-exchange kinetics. Marine Biology 38:343–355.

Sanders, B.M. 1993. Stress proteins in aquatic organisms: an environmental perspective. Critical Reviews in Toxicology 23:49–75.

Santos, M.H., N. da Cunha, and A. Bianchini. 2000. Effects of copper and zinc on growth, feeding and oxygen consumption of *Farfantepenaeus paulensis* postlarvae (Decapoda: Penaeidae). Journal of Experimental Marine Biology and Ecology 247:233–242.

Schill, R., H. Görlitz, and H.-R. Köhler. 2003. Laboratory simulation of a mining accident: acute toxicity, hsc/hsp70 response, and recovery from stress in *Gammarus fossarum* (Crustacea, Amphipoda) exposed to a pulse of cadmium. Biometals 16:391–401.

Schlenk, D., and M. Brouwer. 1991. Isolation of three copper metallothioneins isoforms from the blue crab (*Callinectes sapidus*). Aquatic Toxicology 20:25–34.

Seebaugh, D.R. 2010. Relationships between pollutant-induced digestive toxicity and the assimilation and subcellular partitioning of elements by grass shrimp *Palaemonetes pugio*. PhD Dissertation, City University of New York, New York.

Seebaugh, D.R., and W.G. Wallace. 2004. Importance of metal-binding proteins in the partitioning of Cd and Zn as trophically available metal (TAM) in the brine shrimp *Artemia franciscana*. Marine Ecology Progress Series 272:215–230.

Seebaugh, D.R., D. Goto, and W.G. Wallace. 2005. Bioenhancement of cadmium transfer along a multi-level food chain. Marine Environmental Research 59:473–491.

Seebaugh, D.R., A. Estephan, and W.G. Wallace. 2006. Relationship between cadmium assimilation by grass shrimp (*Palaemonetes pugio*) and trophically available cadmium in amphipod (*Gammarus lawrencianus*) prey. Bulletin of Environmental Contamination and Toxicology 76:16–23.

Shirley, M.A., and C.L. McKenney. 1987. Influence of lindane on survival and osmoregulatory/metabolic responses of the larvae and adults of the estuarine crab *Eurypanopeus depressus*. Pages 275–297 *in* W.B. Vernberg, A. Calabrese, F.P. Thurberg, and F.J. Vernberg, editors. Pollution physiology of estuarine organisms. University of South Carolina Press, Columbia.

Simon, O., F. Ribeyre, and A. Boudou. 2000. Comparative experimental study of cadmium and methylmercury trophic transfers between the Asiatic clam *Corbicula fluminea* and the crayfish *Astacus astacus*. Archives of Environmental Contamination and Toxicology 38:317–326.

Skaggs, H.S., and R.P. Henry. 2002. Inhibition of carbonic anhydrase in the gills of two euryhaline crabs, *Callinectes sapidus* and *Carcinus maenas*, by heavy metals. Comparative Biochemistry and Physiology Part C:133: 605–612.

Smith, R.L., and B.R. Hargreaves. 1985. Respiratory rate in the mysid *Neomysis americana*: effects of naphthalene, temperature and other factors. Pages 477–503 *in* F.J. Vernberg, F.P. Thurberg, A. Calabrese, and W.B. Vernberg, editors. Marine pollution and physiology: recent advances. University of South Carolina Press, Columbia.

Spicer, J.I., and R.E. Weber. 1991. Respiratory impairment in crustaceans and molluscs due to exposure to heavy metals. Comparative Biochemistry and Physiology C 100:339–342.

Spicer, J.I., and R.E. Weber. 1992. Respiratory impairment by water-borne copper and zinc in the edible crab *Cancer pagurus* (L.) (Crustacea: Decapoda) during hypoxic exposure. Marine Biology 112:429–435.

Spicer, J.I., D. Morritt, and L. Maltby. 1998. Effect of water-borne zinc on osmoregulation in the freshwater amphipod Gammarus pulex (L.) from populations that differ in their sensitivity to metal stress. Functional Ecology 12:242–247.

St-Amand, L., R. Gagnon, T.T. Packard, and C. Savenkoff. 1999. Effects of inorganic mercury on the respiration and the swimming activity of shrimp larvae, *Pandalus borealis*. Comparative Biochemistry and Physiology C 122:33–43.

Stickle, W.B., M.A. Kapper, T.C. Shirley, M.G. Carls, and S.D. Rice. 1987. Bioenergetics and tolerance of the pink shrimp (*Pandalus borealis*) during long-term exposure to the water soluble fraction and oiled sediment from Cook Inlet crude oil. Pages 87–106 *in* W.B. Vernberg, A. Calabrese, F.P. Thurberg, and F.J. Vernberg, editors. Pollution physiology of estuarine organisms. University of South Carolina Press, Columbia.

Stuekle, T., J. Likens, and C.M. Foran. 2008. Limb regeneration and molting processes under chronic methoprene exposure in the mud fiddler crab, *Uca pugnax*. Comparative Biochemistry and Physiology C 147:366–377.

Styrishave, B., and M.H. Depledge. 1996. Evaluation of mercury-induced changes in circadian heart rate rhythms in the freshwater crab, *Potamon potamios* and the crayfish, *Astacus astacus* as an early predictor of mortality. Comparative Biochemistry and Physiology Part A: Physiology 115:349–356.

Taylor, E.J., D.P. Jones, S.J. Maund, and D. Pascoe. 1993. A new method for measuring the feeding activity of *Gammarus pulex* (L.) Chemosphere 26:1375–1381.

Thurberg, F.P., A Calabrese, E. Gould, R.A. Grieg, M.A. Dawson, and R.K. Tucker. 1977. Response of the lobster *Homarus americanus*, to sublethal levels of cadmium and mercury. Pages 185–197 *in* F.J. Vernberg, A. Calabrese, F.P. Thurberg, and W.B. Vernberg, editors. Physiological responses of marine biota to pollutants. Academic Press, New York.

Torreblanca, A., J. Diaz-Mayans, J. Del Ramo, and A. Núñez. 1987. Oxygen uptake and gill morphological alterations in *Procambarus clarkii* (Girard) after sublethal exposure to lead. Comparative Biochemistry and Physiology C, 86:219–224.

Touart, L.W., and K.R. Rao. 1987. Influence of diflubenzuron on survival, molting, and limb regeneration in the grass shrimp *Palaemonetes pugio*. Pages 333–349 *in* W.B. Vernberg, A. Calabrese, F.P. Thurberg, and F.J. Vernberg. Pollution physiology of estuarine organisms. University of South Carolina Press, Columbia, South Carolina.

Vandermeulen, J.H., J. Hanrahan, and T. Hemsworth. 1980. Respiratory changes and stability of haemocyanin—O_2 binding capacity in the crab *Cancer irroratus* exposed to Kuwait crude oil in sea water. Marine Environmental Research 3:161–170.

Vernberg, F.J., M.S. Guram, and M.A. Savory. 1978. Metabolic response to thermal changes of the adult fiddler crab *Uca pugilator* and the effect of PCBs. Marine Biology 48:135–141.

Vernberg, W.B., and F.J. Vernberg. 1972. The synergistic effects of temperature, salinity and mercury on survival and metabolism of adult fiddler crabs, *Uca pugilator*. Fishery Bulletin 70:415–420.

Vernberg, W.B., P.J. DeCoursey, and J. O'Hara. 1974. Multiple environmental factor effects on physiology and behavior of the fiddler crab *Uca pugilator*. Pages 381–425 *in* F.J. Vernberg, and W.B. Vernberg, editors. Pollution and physiology of marine organisms. Academic Press, New York.

Verónica, W., and P.A. Collins. 2003. Effects of cypermethrin on the freshwater crab *Trichodactylus borellianus* (Crustacea: Decapoda: Braquiura). Bulletin of Environmental Contamination and Toxicology 71:106–113.

Verslycke, T., S.D. Roast, J. Widdows, M.B. Jones, and C.R. Janssen. 2004. Cellular energy allocation and scope for growth in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) following chlorpyrifos exposure: a method comparison. Journal of Experimental Marine Biology and Ecology 306:1–16.

Vijayavel, K., and M.P. Balasubramanian. 2006. Changes in oxygen consumption and respiratory enzymes as stress indicators in an estuarine edible crab *Scylla serrata* exposed to naphthalene. Chemosphere 63:1523–1531.

Vitale, A.M., J.M. Monserrat, P.C. Castilho, and E.M. Rodríguez. 1999. Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulate* (Decapoda, Grapsidae). Comparative Biochemistry and Physiology C 122:121–129.

Vogt, G., and E.T. Quinitio. 1994. Accumulation and excretion of metal granules in the prawn, *Penaeus monodon*, exposed to water-borne copper, lead, iron and calcium. Aquatic Toxicology 28:223–241.

Wallace, W.G., and G.R. Lopez. 1997. Bioavailability of biologically sequestered cadmium and the implications of metal detoxification. Marine Ecology Progress Series 147:149–157.

Wallace, W.G., T. Hoexum Brouwer, M. Brouwer, and G.R. Lopez. 2000. Alterations in prey capture and induction of metallothioneins in grass shrimp fed cadmium-contaminated prey. Environmental Toxicology and Chemistry 19:962–971.

Wang, S.Y., and W.B. Stickle. 1987. Bioenergetics, growth and molting of the blue crab, *Callinectes sapidus*, exposed to the water-soluble fraction of Couth Louisiana crude oil. Pages 107–126 *in* W.B. Vernberg, A. Calabrese, F.P. Thurberg, and F.J. Vernberg, editors. Pollution physiology of estuarine organisms. University of South Carolina Press, Columbia.

Watson, G.M., O.-K. Andersen, T.S. Galloway, and M.H. Depledge. 2004. Rapid assessment of polycyclic aromatic hydrocarbon (PAH) exposure in decapod crustaceans by fluorimetric analysis of urine and haemolymph. Aquatic Toxicology 67:127–142.

Weis, J.S., and L. Mantel. 1976. DDT as an accelerator of regeneration and molting in fiddler crabs. Estuarine and Coastal Marine Science 4:461–466.

Weis, J.S., F. Gottlieb, and J. Kwiatkowski. 1987a. Tributyltin retards regeneration and produces deformities of limbs in the fiddler crab *Uca pugilator*. Archives of Environmental Contamination and Toxicology 16:321–326.

Weis, J.S., R. Cohen, and J. Kwiatkowski. 1987b. Effects of diflubenzuron on limb regeneration and molting in the fiddler crab *Uca pugilator*. Aquatic Toxicology 10:279–290.

Weis, J.S., A. Cristini, and K.R. Rao. 1992. Effects of pollutants on molting and regeneration in crustacea. American Zoologist 32:495–500.

White, S.L., and P.S. Rainbow. 1982. Regulation and accumulation of copper, zinc and cadmium by the shrimp *Palaemon elegans*. Marine Ecology Progress Series 8:95–101.

White, S.L., and P.S. Rainbow. 1986. Accumulation of cadmium by *Palaemon elegans* (Crustacea: Decapoda). Marine Ecology Progress Series 32:17–25.

Whiteley, N.M. 2011. Physiological and ecological responses of crustaceans to ocean acidification. Marine Ecology Progress Series 430:257–271.

Wong, C.K., K.H. Chu, K.W. Tang, T.W. Tam, and L.J. Wong. 1993. Effects of chromium, copper and nickel on survival and feeding behaviour of *Metapenaeus ensis* larvae and postlarvae (Decapoda: Penaeidae). Marine Environmental Research 36:63–78.

Wright, D.A., and P.M. Welbourn. 1991. Effect of mercury on unidirectional sodium and calcium influx in *Asellus aquaticus*. Archives of Environmental Contamination and Toxicology 21:567–570.

Wu, J.P., and H.-C. Chen. 2004. Effects of cadmium and zinc on oxygen consumption, ammonium excretion, and osmoregulation of white shrimp (*Litopenaeus vannamei*) Chemosphere 57:1591–1598.

INDEX