Volume 1

Physiology of Molluscs

A Collection of Selected Reviews

Editors Saber Saleuddin | Spencer Mukai

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Edited by

Saber Saleuddin, PhD
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ABOUT THE EDITORS

Saber Saleuddin, PhD

Saber Saleuddin, PhD, is a University Professor Emeritus of the Department of Biology at York University in Toronto, Ontario, Canada. Dr. Saleuddin received his early education in Bangladesh. He received his doctorate in molluscan zoology from the University of Reading in the UK. After an NRC Research Fellowship at the University of Alberta, studying biomineralization in molluscs, he continued his research on biomineralisation in the laboratory of Karl Wilbur at Duke University. Though offered a position at Duke, he accepted a faculty appointment at York University in Canada, where he taught for 37 years. The university recognized his outstanding contributions to research, teaching, and administration by honoring him as a University Professor. He has published more than a hundred papers in international journals and has co-edited three books on molluscan physiology. He served as co-editor of the *Canadian Journal of Zoology* for 18 years and was president of the Canadian Society of Zoologists, from whom he was awarded the Distinguished Service Medal.

Spencer Mukai, PhD

Spencer Mukai, PhD, is currently an instructor and technician at York University's Glendon College campus (Toronto, Ontario, Canada), where he is facilitating the implementation of a new biology undergraduate teaching laboratory. Dr. Mukai's research interests are in the neuroendocrine regulation of reproduction, growth, and osmoregulation in molluscs. He has published in and served as reviewer for national and international journals. After receiving his BSc and PhD from the Department of Biology, York University, Dr. Mukai has spent time as a postdoctoral fellow and research associate as well as an instructor at York University's Keele campus. He has demonstrated labs in invertebrate physiology and zoology for many years and has taught a variety of courses, including invertebrate physiology and endocrinology, animal physiology, environmental physiology, histology, human physiology, parasitology, introductory biology, ecology, and conservation biology.

DEDICATION TO OUR MENTORS

Professor Alastair Graham, FRS
1906–2000

Professor Alastair Graham, born in Edinburgh, was one of the most distinguished molluscan biologists. Professor Graham started his teaching career at Sheffield University. After 20 years of teaching and administrative duties, including the Chair of Zoology and Dean of the Faculty at Birkbeck College in London, he took up the Chair of Zoology at the University of Reading in 1952 and later served as Deputy Vice Chancellor. During his career he published many papers in collaboration with Dr. Vera Fretter. One of their most important publications is the classic *British Prosobranch Molluscs.* He received many accolades for his scholarship, including a DSc and a Fellowship of the Royal Society. Professor Graham was also the editor of *The Journal of Molluscan Studies* for many years.

Duke Professor Karl M. Wilbur
1912-1994

Professor Wilbur was born in New York, and following his doctoral degree at the University of Pennsylvania, he joined the Zoology Department of Duke University in 1946. He became a James B. Duke Professor in 1961. His major interest in research was the physiology of mineralization, primarily in molluscs. Professor Wilbur was an eminent cell physiologist. In addition to many scientific papers, Professor Wilbur is best remembered for coediting the classical volume *The Physiology of Mollusca* and being Editor-in-Chief of the series *The Mollusca,* published by then Academic Press. One of Professor Wilbur's long-time collaborators was Professor Norimitsu Watabe of the University of South Carolina. Together they published many articles that made a significant advancement to the field of biomineralization.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ABO	accessory boring organ
AMPs	antimicrobial peptides
BaPH	BaP-hydroxylase activity
DEB	dynamic energy budget
DOPA	3,4-dihydroxyphenylalanine
ECOD	ethoxycoumarin-O-deethylase activity
EDTA	ethylenediamine tetracetic acid
EPH	epoxide hydrolases
ER	endoplasmic reticulum
FREPs	fibrinogen-related proteins
G6PDH	glucose-6-phosphate dehydrogenase
GPX	glutathione peroxidase
GPX	glutathione peroxidase
LAAOs	l-amino acid oxidases
LPO	lipid peroxidation
MAPKs	mitogen-activated protein kinases
MRP	multidrug resistance-associated protein
MTs	metallothioneins
NMO	menadione reductase
OCLTT	capacity-limited thermal tolerance
PDH	pyruvate dehydrogenase
PKC	protein kinase C
ROS	reactive oxygen species
SOD	superoxide dismutase
TMP-1	thread matrix protein-1
UBF	upstream binding factor
XRD	X-ray diffraction

PREFACE

The first comprehensive treatment on the physiology of molluscs was published in two volumes, edited by K. M. Wilbur and C. M. Yonge in 1964 and 1966. Almost 20 years later, a landmark compendium in multiple edited volumes on the biology of molluscs was published between 1983 and 1988. This series dedicated two volumes (volumes 4 and 5) to review papers on molluscan physiology. K. M. Wilbur was the editor-in-chief of this important series. The volumes in 1964 and 1966 and those in the 1980s were all published by then Academic Press.

The only review series on selected aspects of molluscan physiology since the 1980s was a special volume of the *Canadian Journal of Zoology*, published in 2013, which was edited by Saber Saleuddin. As luck would have it, we were approached by Apple Academic Press in 2014 to edit another volume dedicated to molluscan physiology, which we enthusiastically agreed to undertake.

With the rapid development of cutting-edge proteomic, molecular biological, and cellular imaging techniques, our understanding of molluscan physiology, specifically in the areas of neurobiology, reproductive biology, and shell formation, has increased exponentially over the last several years. Therefore, we felt that compiling an edited volume of review papers was warranted, and we hope that this book will serve as an important resource for researchers, professors, and students.

Editing a review series is a daunting task. The major challenge of such an endeavor is not what areas we could cover but how to deal with topics where we were unable to find excellent contributors. Thus, the titles and areas of research included in this book are our personal choices based on availability of contributors and their willingness to write within the allotted time frame. Furthermore, in certain fields of physiology, such as osmoregulation and defense mechanisms, we felt that the fields have not advanced significantly enough to warrant reviews. To partially compensate for not covering certain fields, we have included two papers previously published in the *Canadian Journal of Zoology.* The only instructions we gave to contributing authors is that the coverage be comprehensive, with a brief introduction, present knowledge highlighting the significant recent findings, and finally, provide suggestions about future directions in the context of recent developments.

We are indebted to friends and colleagues around the globe who have kindly contributed to this volume. During the months of writing, rewriting, and editing, the authors have been unfailingly cooperative in all we have requested them to do. We gratefully thank the appraisers who provided an immense service by providing critical appraisal and evaluation of each paper. Each revised paper was so much better following the evaluation reports. The fact that this service is given freely attests to the generosity of our colleagues.

We had expected that a single volume should suffice, but as the project developed it became apparent we needed two volumes. In grouping papers for the two volumes, we tried to ensure that the majority of papers in each volume complemented each other and were aimed at specific readers. Thus, Volume 1 is on shell structure, mineralization, the dynamics of calcium transport, shell drilling, byssus proteins, locomotion, and reproduction. Volume 2 includes reviews on the neural mechanisms of learning, reproductive behavior, responses to environmental stress and hormones, and neurotransmitters. We believe that the reviews included in these two volumes make a significant contribution to our understanding not only of molluscan physiology but also the physiology of animals in general.

We are grateful to Sandra Jones Sickels, Ashish Kumar, and Rakesh Kumar of Apple Academic Press for their invaluable guidance and support not only at the planning stages, but also during the editing and printing processes. Finally, we are grateful to the Canadian Science Publishing of Ottawa for allowing us to reprint two papers from the *Canadian Journal of Zoology.*

CHAPTER 1

DEVELOPING PERSPECTIVES ON MOLLUSCAN SHELLS, PART 1: INTRODUCTION AND MOLECULAR BIOLOGY

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ABSTRACT

Molluscs (snails, slugs, clams, squid, chitons, etc.) are renowned for their highly complex and robust shells. Shell formation involves the controlled deposition of calcium carbonate within a framework of macromolecules that are secreted by the outer epithelium of a specialized organ called the mantle. Molluscan shells display remarkable morphological diversity, structure, and ornamentation; however, the physiological mechanisms underlying the evolution and formation of the shell are just beginning to be understood. Examination of genes expressed in the mantle and proteins incorporated into the shell suggests that the genetic program underlying shell fabrication is rapidly evolving. This includes lineage-specific integration of conserved, ancient gene families into the mantle gene regulatory network and the evolution of genes encoding proteins with novel repetitive motifs and domain combinations, which results in the expression of markedly different shell matrix protein repertoires in even closely-related molluscs. Here, we review the molecular physiology of shell formation with emphasis on the protein components that are particularly rapidly evolving. Nonprotein components such as chitin, other polysaccharides, and lipids are also reviewed. The high degree of novelty in molluscan biomineralized structures is discussed with emphasis on topics of recent interest including the image-forming aragonitic eye lenses of chiton shells and shell pigments. Finally, unanswered questions including some dealing with basic concepts such as the homology of the nacreous shell layers of gastropods and bivalves are discussed.

1.1 INTRODUCTION

Biomineralization is the process by which living organisms convert ions in solution into solid minerals (Simkiss & Wilbur, 1989). The great success of molluscs can be attributed in part to their ability to secrete calcareous skeletal structures with evidence for molluscan biomineralization extending back to the late Precambrian (Runnegar, 1996). All eight major lineages of Mollusca produce calcified exoskeletons, in the form of shells (such as those produced by bivalves, gastropods, and *Nautilus*) or sclerites (spines, scales, etc. produced by chitons and aplacophorans). However, secondary reduction or loss of the shell has occurred in several lineages (e.g., Kröger et al., 2011; Wägele & Klussmann-Kolb, 2005). In this chapter, we begin the discussion of molluscan biomineralization physiology with an emphasis on recent insights on the molecular biology of shell formation from studies

using evolutionary developmental, comparative genomic/transcriptomic, and proteomic approaches. We highlight the importance of comparative studies in understanding the principles of biomineralization and a need for more such studies that include representatives from all lineages of Mollusca.

1.1.1 DIVERSITY AND STRUCTURE OF MOLLUSCAN EXOSKELETONS

With forms as disparate as the familiar garden snail, "headless" filter feeding bivalves, tiny meiofaunal worms, and giant squid, there is extreme variation in morphology among the eight major lineages of Mollusca (Haszprunar et al., 2008). Figure 1.1 shows the current consensus of molluscan phylogeny based on recent studies (Kocot et al., 2011; Smith et al., 2011; Vinther et al., 2012) with an exemplar of each major lineage. These are Polyplacophora (chitons), Caudofoveata (=Chaetodermomorpha), Solenogastres (=Neomeniomorpha), Monoplacophora, Gastropoda (snails and slugs), Bivalvia (clams, scallops, oysters, etc.), Cephalopoda (octopuses, squids, and *Nautilus*), and Scaphopoda (tusk shells). Despite the disparity in morphology among the major lineages of Mollusca, the majority of species rely on mineralized exoskeletons in the form of a shell and/or sclerites. Molluscan exoskeletons provide physical defense, support, and, in some species, desiccation resistance (Carefoot & Donovan, 1995; Fishlyn & Phillips, 1980; reviewed by Furuhashi et al., 2009). Examination of the diversity of form and structure of molluscan exoskeletons quickly reveals the great diversity that has evolved (Fig. 1.2).

Exoskeletons of extant molluscs are layered structures that contain calcium carbonate, proteins, glycoproteins, polysaccharides, and lipids. In many shelled molluscs, the mineralized layers are often covered by an entirely organic outer layer (the cuticle or periostracum). Mineralized layers are composed predominantly of calcium carbonate (as aragonite, calcite, or rarely vaterite) with a small fraction of protein and polysaccharides (reviewed by Furuhashi et al., 2009; Marin et al., 2013). A number of different shell microstructures may occur in mineralized layers of molluscan shells (Chateigner et al., 2000). These are generally classified as (1) prismatic microstructures with mutually parallel, adjacent prism-shaped crystals that do not strongly interdigitate along their mutual boundaries, (2) nacreous microstructures with laminar polygonal to rounded tablets arranged in broad sheets, (3) crossed or crossed lamellar microstructures with sheets of thin, parallel rods, and (4) homogeneous microstructures with aggregations of

irregularly shaped crystallites with a granular appearance (Chateigner et al., 2000; see Bandel, 1990; Carter & Clark, 1985 for detailed discussions of shell microstructure). Of these, the prismatic and nacreous microstructures are the best studied. The prismatic layer is resistant to crack propagation and puncture (Eichhorn et al., 2005; Li & Nardi, 2004; Su et al., 2004), whereas the nacreous layer is best known for being more ductile and fracture resistant (Chateigner et al., 2000; Li et al., 2006). We refer the reader to Chateigner et al. (2000) for high-quality scanning electron micrographs of each of these different microstructure types.

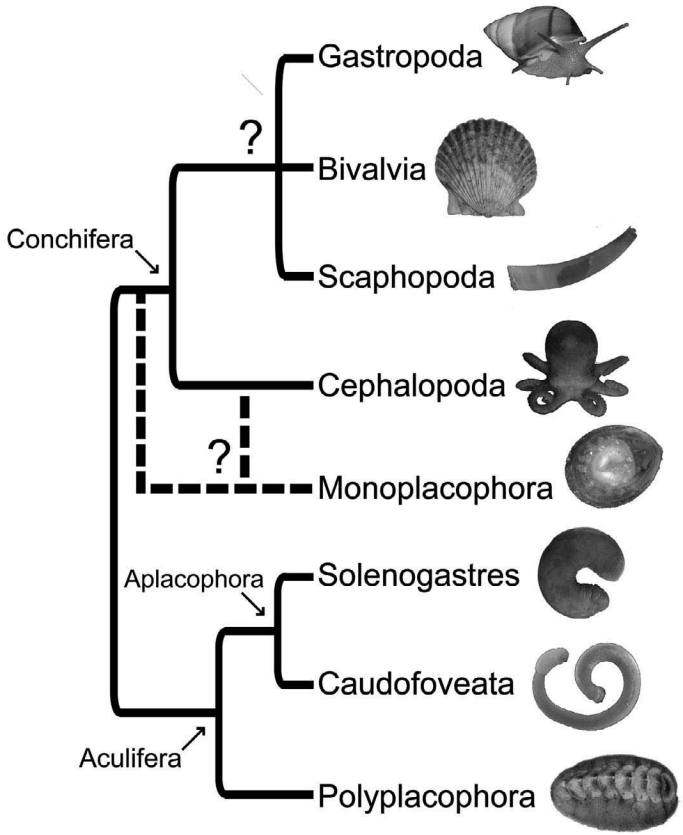

FIGURE 1.1 Current consensus of evolutionary relationships among the major lineages of Mollusca as inferred by Kocot et al. (2011), Smith et al. (2011), and Vinther et al. (2012). Photos are not to scale. Photo of *Argopecten* (Bivalvia) by Dan Speiser. Photo of *Chaetoderma* (Caudofoveata) by Christiane Todt. Photo of *Laevipilina* (Monoplacophora) by Greg Rouse and Nerida Wilson. (Used with permission.)

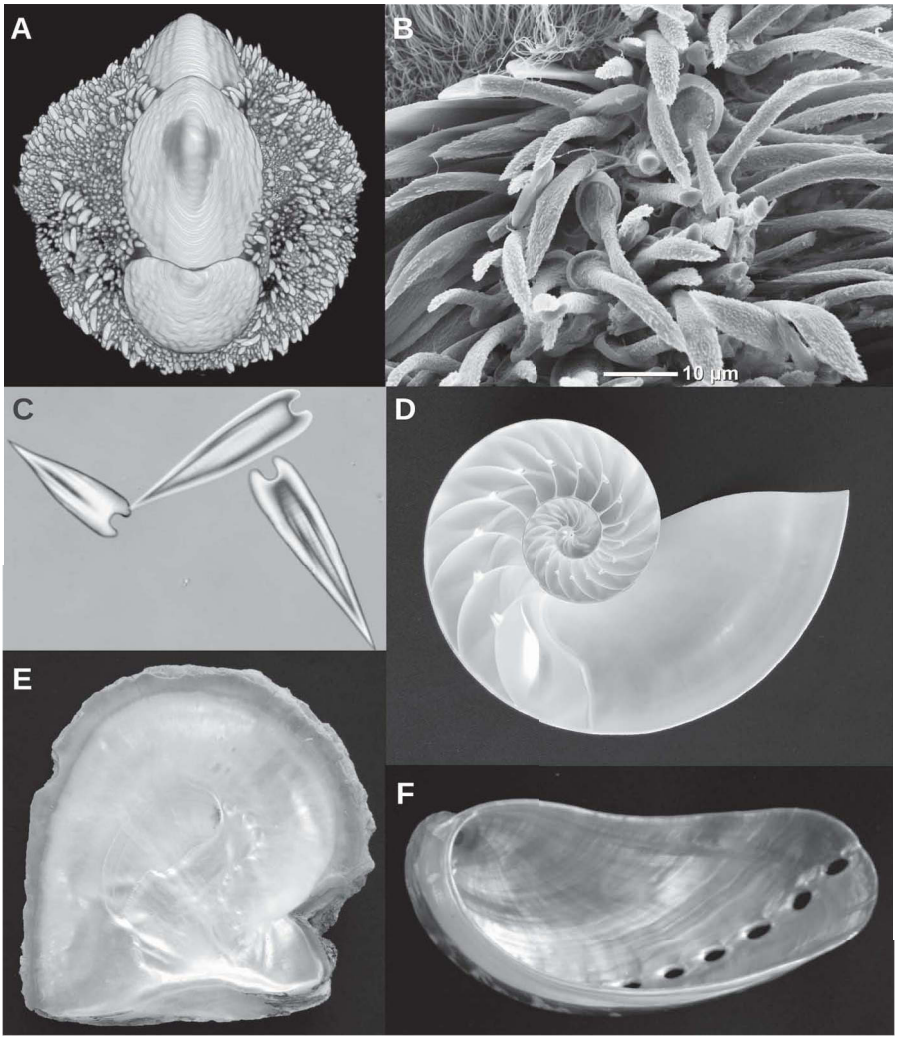

FIGURE 1.2 Diversity of mineralized structures fabricated by extant molluscan lineages. A. Micro CT scan of a juvenile specimen of *Cryptoplax larvaeformis* (Polyplacophora) showing anterior shell valves and sclerites. Specimen is approximately 1-cm wide. Photo by Jeremy Shaw. B. Scanning electron micrograph (SEM) of sclerites of *Macellomenia schanderi* (Solenogastres). C. Micrograph of sclerites of an undescribed species of *Falcidens* (Caudofoveata) from New Zealand illuminated with polarized light. Smallest sclerite is approximately 100 µm in length. D. Laterally bisected shell of *Nautilus* (Cephalopoda). E. Shell of the pearl oyster *Pinctada maxima* (Bivalvia). F. Shell of the abalone *Haliotis asinina* (Gastropoda).

Polyplacophora is a clade of slug-like molluscs that are dorsally protected by eight serially arranged shells (=valves) and a thick, fleshy girdle bearing calcareous sclerites. The shells of polyplacophorans, or chitons as they are commonly called, typically consist of four layers (Haas, 1972, 1976, 1981; summarized by Kaas & Van Belle, 1985; Fig. 1.3). The outermost layer is the cuticle, which is sometimes called the periostracum or "properiostracum," as it differs from conchiferan periostracum in composition (reviewed by Haas, 1981; Saleuddin & Petit, 1983). This thin, transparent layer covers the tegmentum, which is the dorsally visible part of the shell. The tegmentum of chiton shells is quite different from that of shell layers observed in conchiferan shells as it contains calcium carbonate as well as substantial amounts of organic material (mostly polysaccharides). Calcified layers of conchiferan shells typically have some, but relatively very little organic material (see below; reviewed by Eernisse & Reynolds, 1994). The tegmentum is typically sculptured, and may be pigmented (e.g., Sigwart & Sirenko, 2012). Below the tegmentum is the articulamentum. This shell layer contains less organic material and is "somewhat nacreous" (Haas, 1981). In most chitons (but not the basal Lepidopleurida), the articulamentum forms insertion plates, which project into the surrounding leathery girdle to anchor the shells in place. The hypostracum, which is also a predominantly calcareous layer, underlies the articulamentum. This layer differs from the articulamentum by having significantly less organic material and a different microstructure (see below). Finally, the myostracum, which lies below the hypostracum, is a modified hypostracum that serves for attachment of muscles. The girdle or mantle, which surrounds the shells, is covered with the same glycoproteinaceous cuticle material that covers the shells (Beedham & Trueman, 1968; Kniprath, 1981) and bears many calcareous sclerites. Chiton sclerites vary in morphology from fine, scale-like structures to large pronounced spines (Haas, 1981). The calcareous layers of chiton shells and sclerites are composed of aragonite (Carter & Hall, 1990; Haas, 1981; Treves et al., 2003). The crystalline structure of chiton shells has been explored in relatively few taxa. In those that have been studied, the tegmentum is formed by rods of spherulitic sectors. The hypostracum is composed of crossed lamellae with bundles of crystals. Unlike conchiferans (see below), the hypostracum crystallographic c-axis coincides with the bisectrix of these crossing fibers (Haas, 1981). Because of the unique microstructure of the hypostracum and unusual composition and structure of the chiton tegmentum, it has been hypothesized that chiton shells are not strictly homologous to the shells of other conchiferans (Eernisse & Reynolds, 1994; Furuhashi et al., 2009; Haas, 1981; Scheltema, 1993; reviewed by Kocot, 2013).

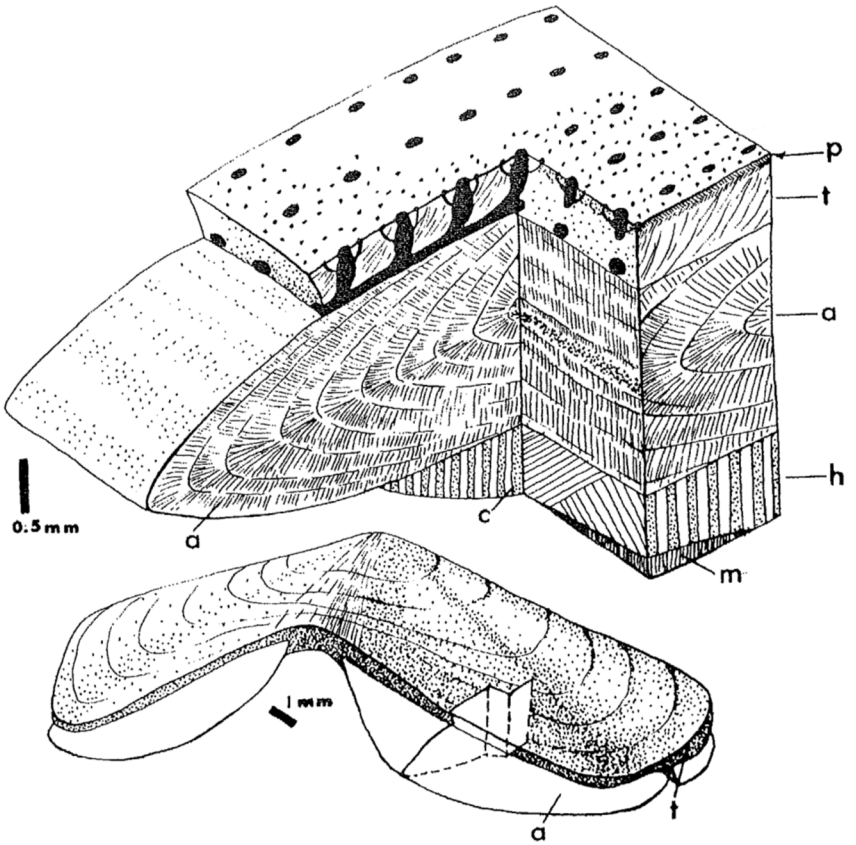

FIGURE 1.3 Structure of a chiton-shell valve. Above: Whole shell valve with cut-out region corresponding to enlargement below. Below: Enlargement showing shell layers. Abbreviations: a, articulamentum; c, crossed lamellar structure of hypostracum; h, hypostracum; m, myostracum; pp, properiostracum (cuticle); t, tegmentum. Modified from Haas (1976).

Caudofoveata (=Chaetodermomorpha) and Solenogastres (=Neomeniomorpha), collectively called Aplacophora, are worm-shaped, shell-less molluscs (reviewed by Todt et al., 2008; Todt, 2013). Although traditionally viewed as basal, plesiomorphic molluscs (see Salvini-Plawen & Steiner, 2014 and references therein), recent molecular phylogenetic studies (Kocot et al., 2011; Smith et al., 2011; Vinther et al., 2012) have grouped Aplacophora + Polyplacophora in a clade called Aculifera (Scheltema, 1993; Fig. 1.1). Examination of fossil paleoloricate "chitons" (Sutton & Sigwart, 2012; Sutton et al., 2012) has led to the interpretation that aplacophorans are derived from chiton-like ancestors that secondarily lost their shells (Sutton

& Sigwart, 2012; Sutton et al., 2012; Vinther et al., 2012; Vinther, 2014, 2015). Developmental studies have also been cited as evidence for a chiton-like ancestor of Aplacophora (Scheltema & Ivanov, 2002). Although extant aplacophorans lack shells, most of the body surface is covered with a glycoproteinaceous cuticle and a dense coat of calcareous sclerites. Although the sclerites of the burrowing caudofoveates are relatively uniform, there is great variation in the morphology of solenogaster sclerites. Solenogaster sclerites may be solid or hollow and can exhibit a variety of shapes, such as needles, scales, hooks, and paddles, just to name a few (García-Álvarez & Salvini-Plawen, 2007). Presence of scale-like sclerites in the putatively early branching solenogaster order Pholidoskepia (Salvini-Plawen, 2003) and observation of scale-like sclerites in larvae and early juvenile solenogaster species that later develop hollow needles (Okusu, 2002; Todt & Kocot, 2014) suggests that scale-like sclerites (as also found in Caudofoveata) are plesiomorphic for Aplacophora (Salvini-Plawen, 2003). Aplacophoran spicules are composed of aragonite (Rieger and Sterrer 1975; Scheltema & Ivanov, 2002, 2004), with the long axis of the crystals aligned with the long axis of the spicules (reviewed by Ehrlich, 2010).

Monoplacophora is a small group of around 30 described species of single-shelled molluscs that mostly live in the deep sea (reviewed by Haszprunar & Ruthensteiner, 2013; Haszprunar, 2008; Lindberg, 2009). Some authors prefer the more specific name Tryblidia for the extant Monoplacophora because several extinct "monoplacophorans" are of uncertain phylogenetic affinity. Most monoplacophorans have a thin outer periostracum, a prismatic shell layer with large quadrangular or hexagonal prisms, and an inner nacreous layer (Erben et al., 1968; Hedegaard & Wenk, 1998; Meenakshi et al., 1970; Wingstrand, 1985). However, in *Veleropilina*, *Rokopella*, and *Micropilina*, the prismatic layer is apparently absent (see Haszprunar & Ruthensteiner, 2013 for discussion) and the outer shell layer is composed of smooth or granular material with unknown microstructure (presumably homogeneous; Checa et al., 2009; Cruz et al., 2003; Marshall, 2006; Warén & Hain, 1992).

Scaphopods are marine burrowing microcarnivores with a conical shell that is open at both ends. The shell grows from the anterior end and is removed at the posterior end to allow for increased water flow into the mantle cavity as the animal grows (de Paula & Silveira, 2009). Some species produce "tubes" or "pipes" from the posterior mantle margin (Hebert, 1986; Shimek, 1989). The shell may bear longitudinal or, rarely, annular ribs. Generally, scaphopods have a trilayered shell organization similar to that of gastropods and bivalves. The organic periostracum may be thick but

typically it is very thin or completely eroded in adult animals, probably due to their sand burrowing activity. An outer, very thin crystalline prismatic layer with tightly packed crystals is present in the majority of species of the order Gadilida giving these species a polished appearance. The inner-most shell layer is a complex, crossed-lamellar layer, which may have a regular or irregular structure (Steiner, 1995; Reynolds & Okusu, 1999). The shell is composed of aragonite (Bøggild, 1930).

Cephalopoda includes the extant nautiloids, octopods, vampyropods, and decabrachians (cuttlefish, squid, and *Spirula*) as well as a rich diversity of fossil forms (reviewed by Kröger et al., 2011; Young et al., 1998). Among the living cephalopods, only members of Nautiloidea have retained an external shell as adults, whereas others have reduced or (more-or-less) completely lost their shell. Cephalopod shell structure and the general mechanisms of shell formation in this group were reviewed by Bandel (1990) and Budelmann et al. (1991). In Nautiloidea, the most plesiomorphic extant cephalopod lineage, the thick, external shell is aragonitic with prismatic, spherulitic, and nacreous configurations. In *Nautilus*, internal chambers of the shell are used for buoyancy control; an osmotic gradient is established by active transport of salts to the space between the mantle tissue and the shell. This allows for the extraction of liquid from the hollow chamber and inward diffusion of gas (reviewed in detail by Budelmann et al., 1991). Most of the extant diversity of Cephalopoda is dominated by taxa with internalized and usually highly reduced shells (Birchall & Thomas, 1983; Hunt and El Sherief, 1990; Sousa Reis & Fernandes, 2002). The pelagic cephalopod *Spirula* has a calcified internal shell similar to that of *Nautilus*, which is also used for buoyancy control. Cuttlefish (e.g., *Sepia*) also use their internal shell for this function. Here, the shell is not coiled with relatively few large chambers, but contains small chambers with many flat, subdivided chambers subdivided by serially arranged organic membranes. Most other cephalopods (e.g., octopus and squid) have completely uncalcified, chitinous vestiges of the shell.

The filter- or deposit-feeding bivalves are easily recognized by their characteristic hinged shell. Shell structure and mineralogy within the group are highly variable (Kobayashi & Samata, 2006). The Lower-Middle Cambrian protobranch bivalve *Pojetaia runnegari* is the oldest known bivalve fossil. It seems to have had a single-layer shell with a prismatic microstructure that was deposited onto an organic periostracum (Runnegar & Pojeta, 1985). The pearl oysters (Pterioida) are perhaps the best-studied bivalve molluscs with respect to biomineralization, due to their economic importance. Pearl oysters exhibit the condition observed in most bivalves; they have a shell

with an inner nacreous layer, a middle prismatic layer, and an outer proteinaceous layer.

Gastropoda is the most species-rich class of Mollusca. There is a great diversity of shell organization and microstructures within this clade. In the well-studied vetigastropod *Haliotis*, the shell consists of three layers: an outer organic periostracum (that is often eroded in adults), a prismatic layer made up of needle-shaped crystals enveloped by an organic sheath, and a nacreous layer consisting of aragonitic tablets surrounded and perfused by thin organic matrix (summarized by Marie et al., 2010). Adult patellogastropods such as *Lottia* have a shell consisting of five layers (Mann et al., 2012; Marie et al., 2013; Suzuki et al., 2010). The outer-most layer is primarily calcite with a mosaic organization whereas the remaining layers are composed of prismatically arranged crystals of aragonite (Marie et al., 2013; Suzuki et al., 2010). Crossed lamellar shell microstructure is widespread in other gastropods (Dauphin & Denis, 2000).

1.1.2 MANTLE TISSUE

Mantle tissue (=pallial tissue; Fig. 1.4) is responsible for the secretion of molluscan shells and sclerites. The mantle forms and isolates a chamber from the external environment (see Simkiss Chapter 2 of this volume) and secretes an organic matrix of polysaccharides (e.g., chitin) and protein, which is presumed to be the site of calcium carbonate crystal nucleation (reviewed by Addadi et al., 2006; Furuhashi et al., 2009; Wilbur & Saleuddin, 1983; Wilbur, 1972). Mantle tissue morphology and the process of shell formation in general are most well-known in bivalves and gastropods. In these taxa, there are conserved cellular and morphogenetic movements that initiate larval shell secretion. Larval shell formation begins at the end of gastrulation, with the differentiation and local thickening of a group of ectodermal cells in the post-trochal dorsal region (the shell gland or shell field). These cells elongate and then invaginate transitorily to form the shell gland, which is analogous to the adult mantle and responsible for the secretion of larval shell. The periphery of the shell gland produces an extracellular lamella—the organic periostracum—that will serve as the site of calcium carbonate deposition (Bielefeld & Becker, 1991; Cather, 1967; Hohagen & Jackson, 2013; Kniprath, 1981). Later, the shell gland flattens and grows into the more recognizable adult mantle epithelium (Jackson et al., 2007; Kniprath, 1977, 1980, 1981).

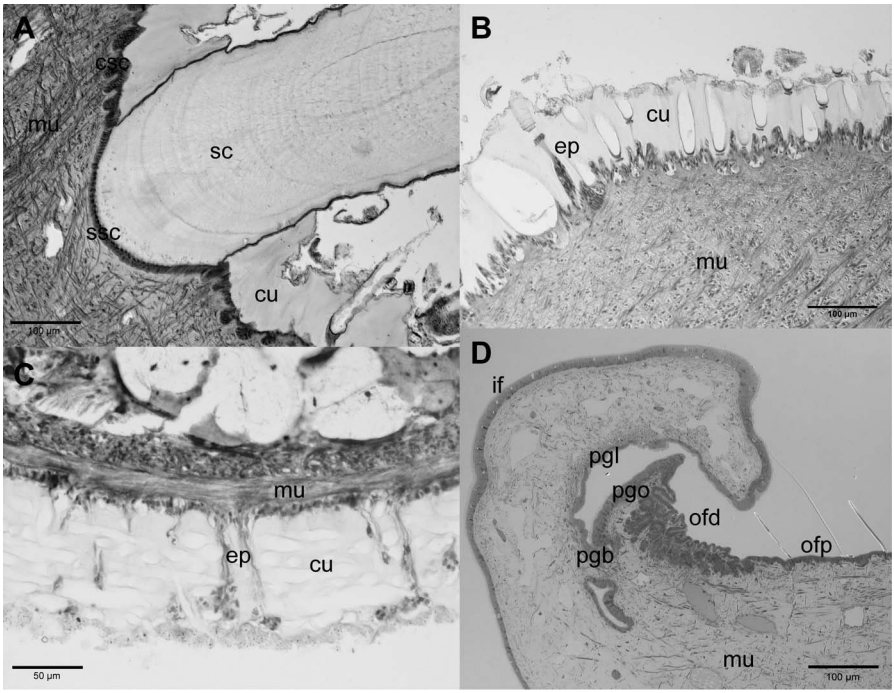

FIGURE 1.4 Histological sections of molluscan mantle tissues. A. Sclerite secretion in *Acanthopleura gemmata* (Polyplacophora). B**.** Various stages of sclerite secretion and lifting through cuticle in *Cryptoplax larvaeformis*. C. Epidermal papillae, cuticle, and voids from decalcified sclerites in the thick cuticle of *Proneomenia custodiens* (Solenogastres). D. Mantle tissue of *Haliotis asinina* (Gastropoda). Specimen prepared by Kathryn Green. Abbreviations: csc, cuticle secreting cells; cu, cuticle; ep, epidermal papillae; inner fold of mantle; mu, muscle; ofd, distal part of outer fold; ofp, proximal part of outer fold; pgb, base of the periostracal groove ; pgl, periostracal groove; pgo, outer fold of the periostracal groove; sc, sclerite; ssc, sclerite secreting cells.

Larval conchiferans (e.g., gastroods, bivalves, scaphopods) typically have a discreet shell gland that secretes the periostracum at its distal edge. The mantle tissue and the periostracum form the crystallization chamber where calcium is deposited adjacent to the periostracum. In contrast to conchiferans, chiton shells are secreted underneath a thin layer of cuticle (the same material that covers the entire dorsum; "properiostracum" *sensu* Haas, 1981) by a broad "plate field" (Kniprath, 1980; reviewed by Eernisse & Reynolds, 1994). This dramatic difference in shell formation mode has led some workers to question the homology of chiton shells to those of conchiferans (reviewed by Kocot, 2013; see below).

A number of studies have examined the anatomy of bivalve (reviewed by Morse and Zardus, 1997; see also Acosta-Salmón & Southgate, 2006; Checa, 2000; Fang et al., 2008) and gastropod (e.g., Fleury et al., 2008; Jackson et al., 2006; Jolly et al., 2004; Kapur & Gibson, 1967; McDougall et al., 2011; Sud et al., 2002; Werner et al., 2013; Zylstra et al., 1978) mantle tissue. Bivalve mantle differs from that of gastropods in some key ways. Most notably, the mantle margin, the active site of shell formation, in bivalves has three folds or grooves whereas gastropods generally only have two (Kniprath, 1978; Zylstra et al., 1978). However, this may be an over-generalization as the keyhole limpet *Diodora* sp. mantle margin has three folds (Budd et al., 2014). In adult bivalves, a ridge between the outer and median fold defines the periostracal groove, which secretes the periostracum. This outer organic shell layer is secreted from basal cells with a greatly infolded apical cell membrane or, in the case of *Crassostrea*, a specialized "periostracum gland" (Morrison, 1993). The outer epithelium of the mantle (i.e., the surface of the mantle facing the shell) secretes the calcified layers of the shell. Here, different zones of cells secrete different types of layers. In bivalves with a typical three-layered shell consisting of periostracum, prismatic, and nacreous layers, the epithelial cells that secrete the prismatic shell layer are columnar (Carriker, 1992) and distal to the those that secrete nacre, which are cuboidal (Fang et al., 2008; Sudo et al., 1997).

The sclerite-bearing epidermis of chitons (Haas, 1976; Kniprath, 1981) and aplacophorans (Kingsley et al., 2012; Woodland, 1907) contains calcium carbonate-secreting cells, cuticle-secreting cells, and papillae (reviewed by Ehrlich, 2010). In most chitons, an epithelium of columnar cells secretes calcium carbonate portion of the sclerite while marginal cells containing many vesicles secrete the cuticular covering of the sclerite (Haas, 1981, Fig. 1.4A). Sclerite secretion in the chiton *Cryptoplax* (Fig. 1.4B) is similar but, because this species has a relatively thick cuticle, sclerites must be pushed up through the cuticle. This appears to be achieved by growth of mantle cells (possibly papillae) that subsequently "retreat." This process is similar to what has been observed in proneomeniid (and other) solenogaster aplacophorans (e.g., Woodland, 1907), which also have a thick cuticle (Fig. 1.4C). Sclerite secretion in the solenogaster aplacophoran *Helicoradomenia* is similar to that of chitons except just one cell secretes the calcareous portion of the sclerite (as is the case in *Proneomenia*) and no special cell elongation is needed to push the sclerite through the relatively thin cuticle of this species (Kingsley et al., 2012).

1.2 INSIGHTS FROM GENOMICS, TRANSCRIPTOMICS, AND PROTEOMICS

At the time of writing this chapter, well-annotated genomes were publicly available from only three molluscs: *Lottia gigantea* (Simakov et al., 2013), *Pinctada fucata* (Takeuchi et al., 2012), and *Crassostrea gigas* (Zhang et al., 2012). However, advances in high-throughput sequencing (reviewed by Metzker, 2010) have made it possible for researchers to deeply sequence the transcriptomes of biological samples as small as a single cell (e.g., Hashimshony et al., 2012). Studies applying such an approach to the study of molluscan mantle tissue have provided new insight into the genes expressed in mantle and their interactions. Recent phylogenomic studies addressing molluscan evolutionary relationships have also contributed a significant amount of transcriptome data (González et al., 2015; Kocot et al., 2011; Smith et al., 2011; Zapata et al., 2014). Similarly, proteomic tools make it possible to identify the proteins and peptides incorporated into mineralized structures (e.g., Mann & Edsinger-Gonzales, 2014; Mann & Jackson, 2014; Mann et al., 2012). Here, we summarize recent studies that have employed such approaches to improve understanding of the molecular physiology of molluscan biomineralization.

1.2.1 DIFFERENT GENE REPERTOIRES

Several studies have used transcriptomic approaches to identify the biomineralization gene repertoires of bivalves including *Pinctada* (pearl oysters; Fang et al., 2011; Gardner et al., 2011; Huang et al., 2013; Jackson et al., 2010; Jones et al., 2014; Joubert et al., 2010; Kinoshita et al., 2011; McGinty et al., 2012; Shi et al., 2013; Zhao et al., 2012), *Mytilus* (mussels; Freer et al., 2014; Hüning et al., 2013), *Pecten* (Artigaud et al., 2014), *Hyriopsis* (Bai et al., 2010, 2013), and *Laternula* (Clark et al., 2010; Sleight et al., 2015) and gastropods including *Haliotis* (abalone; Jackson et al., 2006, 2007, 2010), *Patella* (Werner et al., 2013) *Cepaea* (Mann & Jackson, 2014). However, relatively few comparative studies have been performed (Jackson et al., 2010). By directly comparing the transcriptome of nacre-forming cells in a bivalve (*Pinctada* maxima) and gastropod (*Haliotis asinina*), Jackson et al. (2010) found tremendous differences in these two mantle transcriptomes, with less than 10% of the genes expressed in the nacre-secreting cells having significant similarity. Of these, most could be identified as being involved in processes other than biomineralization. Notably, *P. maxima* had high

representation of genes annotated with lyase activity due to the abundant expression of two alpha carbonic anhydrase (CA) genes. Alpha CAs have previously been shown to be involved in biomineralization in various metazoan taxa (Horne et al., 2002; Jackson et al., 2007; Miyamoto et al., 1996; Moya et al., 2008; Wilbur & Saleuddin, 1983).

In order to focus on genes likely involved in the patterning of the nacreous layer of these animals' shells, Jackson et al. (2010) identified gene products that possessed a signal peptide (indicating an extracellular [secreted] protein) from each gene set. From *H. asinina* they identified 129 sequences and from *P. maxima* they identified 125 sequences that bear a signal peptide. When these "secretomes" were searched against each other and a variety of databases, the authors found that the majority were unique; 95 (74%) and 71 (57%) of the putative secreted proteins in *H. asinina* and *P. maxima*, respectively, shared no similarity with sequences in GenBank's nonredundant protein database or EST databases, or the genome of the patellogastropod *Lottia gigantea*. Of the 54 *P. maxima*-secreted products that shared similarity with a previously described sequence, 12 of these were previously identified as bivalve-specific biomineralization proteins (McDougall et al., 2013; Yano et al., 2006; Zhang et al., 2006; Aguilera et al., 2014 manuscript in preparation). Interestingly, only six novel *H. asinina* proteins and one novel *P. maxima* secreted protein shared similarity with proteins encoded by the *Lottia* genome, suggesting rapid evolution of lineage-specific biomineralization gene repertoires.

Proteomic studies have also shed light on differences among molluscan lineages in the molecular physiology of biomineralization (e.g., Joubert et al., 2010; Liao et al., 2015; Mann & Jackson, 2014; Mann et al., 2012; Marie et al., 2011; Marie et al., 2013; Pavat et al., 2012). Marie et al. (2011) observed that the shell protein repertoire of the mussel *Mytilus edulis* is partly similar to that of other bivalves (i.e., *Pinctada*), but also shares few similarities with that of the gastropod *Haliotis*. Also, Marie et al. (2013) examined the proteins incorporated into the shell of the patellogastropod *Lottia gigantea*. Similar to the results of Jackson et al. (2010), who used a transcriptomic approach, the shell matrix protein (SMP) repertoire of *Lottia* was found to be more similar to that of the bivalve *Pinctada* than to that of the vetigastropod *Haliotis*. Given the fundamental crystallographic differences between the limpet and abalone shells (e.g., presence/absence of nacre and crossed lamellae), these results might suggest that the secretome of the abalone mantle is relatively derived. These works highlight the importance of comparative studies for elucidating the evolution of the molluscan biomineralization toolkit.

To this end, Mann and Jackson (2014) characterized the transcriptome and shell matrix proteome of another gastropod, the common grove snail *Cepaea nemoralis*. Interestingly, the shell proteome was dominated by novel proteins with no known protein domains. Specifically, 31 out of the 59 identified shell proteins (52.5%) were completely unknown. Comparison of the *C. nemoralis* shell proteome to shell proteomes of five molluscan species (*Crassostrea gigas*, *L. gigantea*, *H. asinina*, *P. maxima*, and *P. margaritifera*) revealed 28 of 59 *C. nemoralis* proteins (47.5%) that shared similarity with one or more proteins in shell proteomes of the other species. Interestingly, only one *C. nemoralis* protein had high similarity to one of the 94 proteins in the shell of *H. asinina* and only 34 were similar to proteins (631 in total) in the *L. gigantea* shell proteome. Taken together, these studies indicate that the SMPs directing shell formation in bivalves and gastropods (and even among lineages of gastropods) are markedly different.

1.2.2 COMMON PRINCIPLES

Recent comparative studies have revealed a surprising diversity in the genetic toolkits used in shell secretion by different molluscs. However, there are underlying common principles. All shell- and/or sclerite-forming molluscs use specialized cellular machinery located in the mantle tissue to actively concentrate and secrete calcium carbonate into a closed-off space formed by the mantle and an organic matrix. The shell matrix, which consists of proteins, glycoproteins, chitin, and other polysaccharides, has been shown to be very important in determining the structure of the resulting shell (reviewed by Furuhashi et al., 2009; Marin et al., 2008, 2013).

1.2.2.1 STRUCTURAL PROTEINS

Earlier hypotheses of mollusc shell formation focused on the presence of an extrapallial fluid (e.g., Wilbur & Saleuddin, 1983). However, most contemporary views of biomineralization refer to a protein–polysaccharide gel rather than a fluid (Addadi et al., 2006; Marin et al., 2013) and view certain SMPs in this gel as the site of nucleation (Evans, 2008). Marin et al. (2008, 2013) and Evans (2008) reviewed the structure, function, and evolution of molluscan shell proteins. Structural proteins are by far the best-known component of the molluscan shell matrix. These proteins appear to function in promoting (Kim et al., 2004, 2006) or inhibiting (Kim et al., 2006; Mann et al., 2007;

Michenfelder et al., 2003) crystallization of aragonite or calcite and modulating the morphology of the structures that are produced (Evans, 2008).

1.2.2.1.1 Acidic Shell Proteins

Highly acidic proteins have been implicated in the biomineralization of many organisms, and molluscs are no exception. The organic matrix of bivalve, gastropod, and polyplacophoran shells contains a high proportion of acidic amino acids – particularly aspartate, one of two amino acids that possess a negative charge (the other acidic amino acid, glutamate, is much less common; Hare, 1963; Piez, 1961; Simkiss, 1965). This amino acid bias is reflected in a number of notably acidic characterized SMPs, including MSP1 (pI 3.2; Sarashina & Endo, 2001), Aspein (pI 1.45; Tsukamoto et al., 2004), Caspartin (Marin et al., 2005), Calprismin (Marin et al., 2005), MPP1 (pI 1.21; Samata et al., 2008), Pif (which is cleaved to produce two acidic peptides with pI's of 4.99 and 4.65; Suzuki et al., 2013), and the Asprich family (pI 3.1; Gotliv et al., 2005). Additionally, many other SMPs contain short acidic domains, such as N16/Pearlin (Samata et al., 1999), AP7 and AP24 (Michenfelder et al., 2003), some Shematrin proteins (Yano et al., 2006), and Silkmapin (Liu et al., 2015). Recent transcriptomic and proteomic studies have confirmed that the presence of acidic proteins is a common theme in molluscan shells, and have indicated that many more proteins of this nature await characterization (e.g., Jackson et al., 2010; Mann & Jackson, 2014; Marie et al., 2013).

That acidic proteins directly interact with positively charged calcium ions is well-accepted, but their true function within the shell matrix is not completely understood. In the context of in-vitro assays, acidic peptides have been demonstrated to trigger crystal nucleation via the concentration of calcium ions (Hare, 1963), or to control polymorph selection by interacting with and restricting growing crystal step-edges (Michenfelder et al., 2003). The first characterized acidic matrix proteins were isolated from calcitic layers and caused the precipitation of calcite in vitro (Falini et al., 1996; Marin et al., 2005; Takeuchi et al., 2008), prompting speculation that they were involved in the selection of this particular crystal polymorph. Subsequently, acidic proteins were also identified from aragonitic shell layers (Fu et al., 2005; Suzuki et al., 2009) indicating that the role of these proteins is not restricted to a particular $CaCO_3$ polymorph. Recent research has found that acidic proteins (or mimics thereof) can trigger the formation and stabilization of amorphous calcium carbonate (Politi et al., 2007; Smeets

et al., 2015), which is thought to be the initial phase of biomineralization in molluscan and other systems (reviewed by Marin et al., 2008; Weiner & Addadi, 2011).

Marie et al. (2007) examined the physical properties of the SMP repertoire of the (freshwater) unionid bivalve *Unio pictorum* using trifluoromethanesulfonic acid-induced deglycosylation. Two-dimensional (2D) gel electrophoresis analysis of the SMPs before and after deglycosylation showed that the SMPs are heavily glycosylated. Glycosylation imparts an acidic pH to SMPs. The sulfated sugar moiety bound to these proteins (Crenshaw & Ristedt, 1976; Marxen & Becker, 1997; Simkiss, 1965) appears to impart a calcium-binding activity, which is weakened by deglycosylation (Marie et al., 2007). A similar calcium-binding activity has been observed in a vertebrate calcified tissue-associated glycoprotein (Ganss & Hoffman, 1993). Calcium-binding activity imparted by saccharides is also known in echinoderms (Farach-Carson et al., 1989) and has been suspected among mollusc shell components (Samata, 1990) previously.

1.2.2.1.2 Basic Shell Proteins

While the acidic protein fraction has been included in models of biomineralization as a major element (e.g., Addadi et al., 2006), the role of basic proteins has generally been overlooked. Basic proteins (or proteins with basic domains) have the potential to interact either directly with carbonate ions, or with other acidic macromolecules within the organic matrix. The existence of basic proteins has been revealed via 2D gel electrophoresis of SMPs from a number of taxa (Furuhashi et al., 2010; Marie et al., 2007; Marie et al., 2009; Pavat et al., 2012), and a growing number of proteins with a predicted basic pI have been characterized, including Lustrin A (Shen et al., 1997), Prisilkin (Kong et al., 2009), PFMG3 (Wang et al., 2011), Periostracin (Waite et al., 1979), Perlucin (Weiss et al., 2000), Perlustrin (Weiss et al., 2000), Perlwapin (Treccani et al., 2006), and Perlinhibin (Mann et al., 2007). In pearl oysters, two gene families encoding basic proteins, the lysine (K)-rich mantle proteins (KRMPs; McDougall et al., 2013; Zhang et al., 2006) and Shematrins (McDougall et al., 2013; Yano et al., 2006), are among the most highly expressed genes in the mantle (Jackson et al., 2010; Kinoshita et al., 2011) and are major components of the shell matrix, particularly within the prismatic layer (Marie et al., 2012). The level of expression of these proteins indicates that they may function in providing the framework of the organic matrix via interactions mediated by basic domains.

1.2.2.1.3 Silk Proteins and Other Repetitive Low-complexity Domain-containing Proteins

A particularly striking feature of SMPs is the preponderance of repetitive, low-complexity domains found within them. For example, of 39 proteins identified in the *Lottia* shell matrix identified by Marie et al. (2013), 13 were repetitive low complexity domain-containing (RLCD) proteins; likewise, 4 out of 14 and 23 out of 83 proteins from abalone shells (Marie et al., 2010) and pearl oyster shells (Marie et al., 2012), respectively, were found to possess RLCDs.

In many cases these RLCD domains contain a high proportion of glycine and alanine residues (e.g., McDougall et al., 2013), explaining why these amino acids were found to be highly abundant in amino acid analyses of shell matrices (Hare, 1963; Piez, 1961; Simkiss, 1965). This particular amino acid composition and the detection of an X-ray diffraction pattern suggestive of a beta-sheet structure drew researchers to liken this component of SMPs to spider silk fibroins, which have similar characteristics (Weiner & Hood, 1975; Weiner & Traub, 1980), and silk-like proteins became a central tenet of the model proposed for molluscan biomineralization (Weiner and Traub, 1984). Subsequent research demonstrated that the beta-sheet diffraction pattern probably originated from chitin within the matrix rather than the silk-like proteins themselves, which are likely to exist in a disordered state and form a hydrogel-like structure (Addadi et al., 2006; Falini et al., 2003; Levi-Kalisman et al., 2001). Interestingly, spider silk fibroins exist in a disordered state within silk glands prior to being extruded in a fibrous form (Hijirida et al., 1996).

Structural disorder of matrix proteins is rapidly becoming a widely recognized feature of biomineralized structures in many taxa and, interestingly, is associated with biased amino acid compositions and protein repetitiveness (Kalmar et al., 2012). Therefore, the presence of RLCDs in biomineralization-associated proteins may reflect their tendency to adopt an intrinsically disordered conformation. A survey of 39 molluscan aragonite-associated proteins revealed that all possessed a disordered region and that many were associated with aggregation motifs (Evans, 2012). Proteins of this type are likely responsible for assembling the framework of shell organic matrices.

Interestingly, RLCD-containing protein-encoding genes seem to be fast-evolving. For example, the pearl oyster shematrin gene family includes at least eight orthology groups that differ by the gain, loss, and shuffling of motifs (McDougall et al., 2013; Fig. 1.5). This high rate of evolution is likely due to the instability of repetitive sequences (Sezutsu & Yukuhiro, 2000).

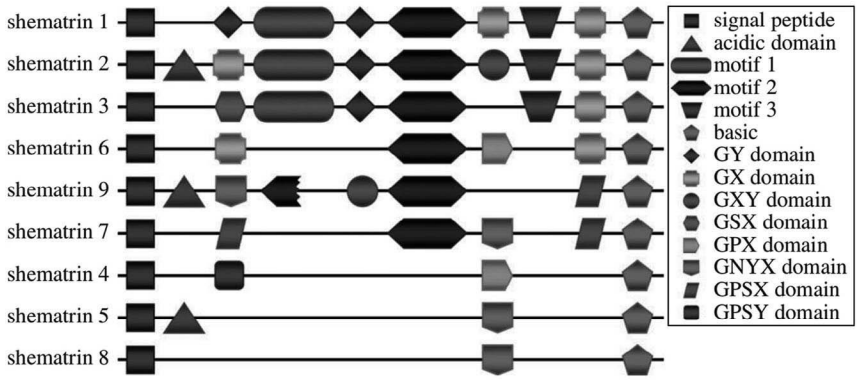

FIGURE 1.5 Schematic representation of sequence motifs in shematrin genes from pearl oysters. Modified from McDougall et al. (2013).

1.2.2.1.4 Modularity

Many SMPs exhibit a modular architecture with each module (i.e., protein domain) having distinct functionality. The most well documented examples of modular SMPs correspond to nacrein and Lustrin A. Nacrein contains a CA domain that is interrupted by the insertion of a RLCD rich in Gly and Asn. CAs have previously been shown to be involved in biomineralization in various metazoans (Horne et al., 2002; Jackson et al., 2007; Miyamoto et al., 1996; Moya et al., 2008). This RLCD region has been proposed to regulate the activity of the CA domain, acting as an inhibitor of the precipitation of calcium carbonate (Miyamoto et al., 2005). Lustrin A is the most complex multimodular SMP discovered so far and is characterized by numerous proline-, cysteine-, and GS-domains. The C-terminus domain of lustrin A exhibits high similarity with several protease inhibitors (Shen et al., 1997; Gaume et al., 2014). Although most SMPs do not exhibit sequence similarity with known proteins, many proteins contain, in addition to RLCDs, enzymatic domains such as peroxidase, CA, tyrosinase, or glycosidase domains. For example, *Lottia gigantea* CA-2 contains Asp- and Glu-rich domains in its C-terminus (Marie et al., 2013).

1.2.2.2 CHITIN AND OTHER POLYSACCHARIDES

Currently, understanding of chitin and other polysaccharides and their function in molluscan shells lag behind that of proteins. Proteins have been found

in every type of molluscan shell analyzed so far, but whether chitin and/or other polysaccharides are present in all molluscan shells/sclerites is unclear. Furuhashi et al. (2009) provided a detailed review on the understanding of chitin and its role in molluscan shells. The few analyses of the polysaccharides in molluscan shells performed so far suggest that molluscs exhibit different sugar signatures (Marie et al., 2007 2009; Pavat et al., 2012). Chitin has been reliably identified in the shells of at least some bivalves, gastropods, and cephalopods but details on the structure and polymorphism (α- vs. β-chitin) are wanting. A number of different approaches have been used to detect chitin in molluscan shells, but these tests may also produce false positives or provide inaccurate pictures of chitin structure in the presence of other molecules. For example, Calcofluor White binds to chitin as well as certain acidic proteins (Albani et al., 1999, 2000). Inferences with respect to chitin network structure may be inaccurate due to nonspecific binding of such stains to molecules other than chitin. Infrared spectroscopy has also been used to test for chitin presence but insoluble proteins may confound results from this approach. Furuhashi et al. (2009) advocate the use of fluorescence probes with chitin-binding proteins (e.g., GFP-tagged chitin binding protein) and infrared spectroscopy before and after treatment with chitinase more specific tools for detection of chitin than stains such as Calcofluor White. Using the latter approach, they demonstrated the presence of both neutral polysaccharides and chitin in the cuticle of an unidentified solenogaster, the shell plates and sclerites of the chiton *Acanthopleura japonica*, the shells of the bivalves *Pinctada fucata* and *Atrina japonica*, the gastropod *Haliotis discus*, and the cephalopod *Nautilus* sp.

Much of our knowledge on chitin in mollusc shells is thanks to transcriptomic and proteomic approaches. In an attempt to understand the molecular basis underlying shell formation, Aguilera (2014) analyzed the mantle transcriptome of eight bivalve and three gastropod species. This study found over-representation of proteins with polysaccharide-binding domains within the mantle transcriptomes. These include chitin-binding Periotrophin-A, chitin-binding domain, chitinases II, chitinase-insertion domain, polysaccharide deacetylase, and galactose-binding domain-like, among others. In addition, Mann and Jackson (2014) described several *C. nemoralis* shell proteins that have high similarity with other molluscan shell-forming proteins. These include two chitin-binding domain-containing proteins. Further, they also found a protein with a chitin-binding Periotrophin-A domain and a chitinase in most of the sampled gastropods and bivalves. This emphasizes the importance of chitin in shell formation in at least these taxa (Falini and Fermani, 2004; Weiss et al., 2006).

1.2.2.3 LIPIDS

Lipids have long been known to be a minor constituent of the organic molecules found in mollusc shells (Wilbur & Simkiss, 1968). Cobabe and Pratt (1995) investigated the lipid content of the shells of *Arca zebra*, a heterotrophic bivalve, *Codakia orbicularis*, a bivalve that hosts chemoautotrophic bacteria, as well as several fossil bivalves (1.4 myo). They found that lipids comprise between 300 and 700 ppm of the total shell weight and did not vary with trophic strategy. This shell–lipid suite is dominated by cholesterol, fatty acids (recovered as fatty acid methyl esters), ketones, phytadienes, and, in some cases, alkanes. Samata and Ogura (1997) showed that lipids are present in the nacreous layer of *Pinctada fucata* and Rousseau et al. (2006) showed that lipids are present in the nacreous layer of *Pinctada margaritifera*. More recently, Farre and Dauphin (2009) examined in detail the lipid composition of the pteriomorph bivalves *Pinctada margaritifera* and *Pinna nobilis*. The shells of these bivalves contain polar lipids (phospholipids), sterols (cholesterol), triglycerides (triolein), fatty acids (oleic acid), steroids (stearyl oleate), and waxes. In the nacreous layer, the most abundant lipid components are apolar waxes, free fatty acids, and very polar lipids. Steroids and sterols are represented in lesser amounts and there are only traces of triglycerides. The situation is similar in the prismatic layer except fatty acids are lacking whereas triolein is more abundant in the prismatic layer than the nacreous layer. The physiological function of lipids in molluscan biomineralization is unclear. Extracted phospholipids have the ability to bind calcium ions (Isa & Okazaki, 1987), so they may be involved in calcification.

1.3 NOVELTY IN MOLLUSCAN BIOMINERALIZATION

Perhaps the most fascinating aspect of molluscan biomineralization is the degree of novelty it encompasses at all levels of organization: from the genes and proteins controlling the process, to the diversity of microarchitectures represented, through to the myriad of structures generated. This highly evolvable system is reflected in some astonishing innovations within the molluscan phylum, such as image-forming aragonite lenses found in chiton-shell plates (Speiser et al., 2011, 2014) and the exquisite paper-thin brood chambers of argonauts, which are often mistaken to be true shells but are, in fact, secreted from specialized webs at the tips of the arms of the female and held on to via suckers (Finn, 2013). Novelty can also be generated by the loss or reduction of structures, as seen in many cephalopods and opisthobranchs.

Structure aside, incredible diversity can also be seen in the coloration incorporated into molluscan shells and in the minerals from which the structures are composed. Some of these phenomena are explored further below.

1.3.1 REDUCTION OR LOSS OF THE SHELL

Many gastropods, particularly terrestrial and marine slugs, have reduced, internalized, or completely lost the shell. Why would these animals give up the safety afforded to them by the shell? In the terrestrial realm, loss of the shell is likely an evolutionary response to calcium limitation (Solem, 1974, 1978; South, 1992). In the marine realm, this secondary reduction or loss of the shell usually coincides with the sequestration or production of toxic chemical compounds that make these animals noxious or toxic (Derby et al., 2007; Wägele & Klussmann-Kolb, 2005). For example, the shell-less nudibranch *Glossodoris quadricolor* feeds on the sponge *Latrunculia magnifica and sequesters from it the* ichthyotoxic substance latrunculin B. It is thought that this compound then protects it from predation by fish (Mebs, 1985).

Interestingly, in some sea slugs that have secondarily lost shells, subdermal calcareous sclerites are produced (e.g., Brenzinger et al., 2013; Jörger et al., 2010; Schrödl & Neusser, 2010). Subepidermal, calcareous spicules are present in the meiofaunal gastropod taxa Acochlidia, Rhodopemorpha, and potentially *Platyhedyle* (Saccoglossa). Here, they are considered as an adaptation to the interstitial habitat, probably serving to stabilize certain body parts during movements through the interstices (Jörger et al., 2008). Many larger sea slugs such as nudibranchs also have internalized calcareous spicules. Here, these structures are often spiny and are thought to serve a defensive purpose (Penney, 2006; Thompson, 1960). Whether the production of these spicules is governed by a similar process to that in the Aculifera is unknown.

Further, the shelled deep-sea scaly foot gastropod (Neomphalida) has a foot covered in sclerites, which are noncalcified but contain iron as pyrite and greigite (Chen et al., 2015; Warén et al., 2003). Little is known about the physiology underlying the formation of these structures.

1.3.2 COLORATION OF MOLLUSCAN SHELLS

The natural beauty of seashells never fails to attract the attentions of beachgoers, young and old alike, and has done so since early human history

(d'Errico et al., 2005). Part of this attraction stems from the stunning array of shapes that molluscan shells exhibit, and part from the often bright or ornately patterned coloration that they possess. The role of coloration in molluscan shells is not well understood; in some cases, the patterning quite effectively camouflages the organism against their habitat; however, in many molluscs this is not the case. Given that many molluscs with colored shells do not have image-forming eyes, reproduce via broadcast spawning, or remain buried in sediment for the extent of the life of the organism, the extravagant patterns are unlikely to serve as a signal to conspecifics (Bauchau, 2001). The fundamental role of coloration has been hypothesized to be as a means to dispose of waste products of metabolism (Comfort, 1951), to increase shell strength (Cain, 1988), or as a means to provide positional information to the mantle (Bauchau, 2001); however, support for all three of these theories is lacking.

The mechanisms underlying the production of color in molluscan shells are diverse (Aguilera et al., 2014; Barnard & De Waal, 2006; Comfort, 1951; Hedegaard et al., 2006). Pigments can be found within the proteinaceous periostracum that covers the outer surface of the shell, and also within the calcified layers themselves (Budd et al., 2014; Needham, 1975). Numerous types of pigments have been identified from molluscan shells, including pyrroles (bilins and porphyrins), polyenes (including carotenoids), and melanins (Barnard & De Waal, 2006; Comfort, 1951; Hedegaard et al., 2006). In some cases, these pigments appear closely associated with protein shell components; however, other species do not appear to use protein-associated pigmentation mechanisms (Mann & Jackson, 2014). Some molluscs do not use pigments to create their coloration at all—they have evolved shell microstructures which produce structural color, that is, color generated through the interference of reflected wavelengths of light from thin films. The most notable example of structural color in molluscs is mother-of-pearl; the architecture of nacre tablets in species such as pearl oysters and abalone results in a stunning display of reflected colors (Rayleigh, 1923; Snow et al., 2004; Webster & Anderson, 1983) that is likely to be the byproduct of an architecture that has been optimized for shell strength. However, there are examples of structural color that have clearly evolved to serve a function in their own right, such as the striking iridescent blue lines found on the shell of the limpet *Patella pellucida*. In this species, the shell ultrastructure maximizes the intensity of blue reflection from the stripes, possibly to mimic the bright blue coloration of toxic nudibranchs found in the same habitat (Li et al., 2015).

Very little is known about how shell coloration and patterning is controlled at the molecular level. A number of studies have demonstrated that

pigmentation in species displaying intraspecific variation follows Mendelian patterns of inheritance (Evans et al., 2009; Gantsevich et al., 2005; Liu et al., 2009; Luttikhuizen & Drent, 2008), indicating a genetic basis for pigmentation in these molluscs. Evidence for a genetic basis also comes from studies on the juvenile abalone, *Haliotis asinina*. In this species, the expression of the *sometsuke* gene maps precisely with areas of red pigmentation on the shell, and the corresponding protein has been isolated from the shell itself (Jackson et al., 2006; Marie et al., 2010). *Sometsuke* has not been identified in any other molluscan shell proteome, indicating that it may be restricted to abalone.

It appears that the incredible diversity of coloration seen within molluscan shells is reflected in the complexity underlying it. The coloration can be generated by a diversity of pigments (or by no pigment at all!), can fulfill a broad range of functions, and is likely controlled by a number of different molecular processes. The lack of common principles indicates that shell coloration, like many aspects of biomineralization, likely evolved many times independently across the phylum.

1.3.3 CHITON SHELL EYES

In most chitons, the tegmentum is permeated by sensory structures called esthetes, which have a variety of sensory and possibly secretory functions (e.g., Eernisse & Reynolds, 1994; Speiser et al., 2011). In Schizochitonidae and Chitonidae esthetes may be capped with an ocellus that includes a lens (reviewed by Eernisse & Reynolds, 1994). Speiser et al. (2011) recently used electron probe X-ray microanalysis and X-ray diffraction to show that the chiton *Acanthopleura granulata* has shell eyes with the first aragonite lenses ever discovered. These eyes appear to be used to sense shadows produced by a would-be predator passing over the animal. Further, it appears that the eye structure results in two different refractive indices that are hypothesized to be optimal for function when the animal is submersed in water at high tide and exposed to air at low tide, respectively.

1.4 CONCLUSIONS AND OPEN QUESTIONS

1.4.1 MORE COMPARATIVE STUDIES NEEDED

Numerous recent studies have employed high throughput sequencing and proteomic approaches to improve our understanding of the process of

biomineralization in molluscs. However, the vast majority of these studies have focused on economically important gastropods and bivalves. Currently, high quality genomes are available only from gastropods and bivalves (Simakov et al., 2013; Takeuchi et al., 2012; Zhang et al., 2012), although comparable data from cephalopods are forthcoming (Albertin et al., 2012). For obvious reasons, high quality genomic resources from other lineages of Mollusca would be highly beneficial toward understanding the evolution of the physiological mechanisms responsible for biomineralization.

Scaphopods are of particular interest because of their apparent close relationship to gastropods and bivalves (Kocot et al., 2011; Smith et al., 2011; Vinther et al., 2012). Because gastropods and bivalves are economically and ecologically important and well-studied with respect to biomineralization, comparative work in Scaphopoda has important bearing on studies in these two groups. In particular, given the apparent differences in biomineralization between gastropods and bivalves (e.g., Jackson et al., 2010), data from Scaphopoda would help clarify if either gastropods or bivalves are derived with respect to biomineralization or if the process is as highly variable across Mollusca in general (as suspected). Some very detailed studies have addressed scaphopod development (Wanninger & Haszprunar, 2001, 2002, 2003), but little is known about their biomineralization and limited genomic resources are available (Kocot et al., 2011; Smith et al., 2011).

Although relatively hard to obtain (but see Wilson et al., 2009), Monoplacophora would be another very interesting group to study due to its antiquity (Haszprunar, 2008; Haszprunar & Ruthensteiner, 2013; Lindberg, 2009). Genome sequencing of the monoplacophoran *Laevipilina antarctica* is currently underway (M. Schrödl, personal communication).

Because Aculifera (Aplacophora + Polyplacophora) is sister to all other extant molluscs (Kocot et al., 2011; Smith et al., 2011), studies of this group would provide important evolutionary context for molluscan biomineralization. Although many aplacophorans live in deep and/or polar habitats, some species are relatively easily accessible and have been successfully spawned in the laboratory (Okusu, 2002; Todt & Wanninger, 2010). In particular, solenogaster aplacophorans produce a phenomenal array of diverse sclerite types (reviewed by García-Álvarez & Salvini-Plawen, 2007). How these structures are achieved is a mystery, but their morphology is likely regulated by the same type of organic matrix found in shelled molluscs. Deeper and wider taxon sampling in comparative studies of biomineralization will help to understand the essential requirements for the production of mineralized structures in the Mollusca.

1.4.2 ARE SHELLS AND SCLERITES PRODUCED BY DIFFERENT MOLLUSCAN LINEAGES HOMOLOGOUS?

Although aculiferan (chiton + aplacophoran) sclerites, chiton valves, and conchiferan shells are all extracellular calcareous secretions of the mantle, structural, and developmental differences suggest that these features are not strictly homologous (Eernisse & Reynolds, 1994; Furuhashi et al., 2009; Haas, 1981; Scheltema, 1993; reviewed by Kocot, 2013). Specifically, the lack of a true periostracum, periostracal groove, and a differentiated larval shell-secreting epithelium (shell gland) in chitons distinguishes their shell structure and formation from that of the conchiferans. Further, developmental studies have shown that chiton shells are secreted by postrochal (2d) cells (Heath, 1899; Henry et al., 2004) during development. These cells (Conklin, 1897; Lillie, 1895), but sometimes also other micromere lineages (2a, 2b, 2c, and sometimes 3c), form the conchiferan shell gland (Damen & Dictus, 1994; Render, 1997). Interestingly, chiton sclerite-secreting cells arise from postrochal (2a, 2c, 3c, and 3d) as well as pretrochal cells (1a and 1d), suggesting that chiton sclerites are not strictly homologous to chiton or conchiferan shells (no cell lineage studies have been conducted in aplacophorans). Hence, the gene regulatory networks and physiological mechanisms that produce these structures may differ significantly.

There is also some question regarding the homology of shell layers within the Conchifera. The debate centers on nacre, which is found in bivalve, gastropod, cephalopod, and monoplacophoran lineages (Chateigner et al., 2000). Although generally similar, there are fundamental differences in mineralogy between the taxa; bivalves and monoplacophorans possess "sheet nacre' (tablets arranged in a brick-like pattern) with alignment of all three axes of the aragonite tablets (bivalves) or a randomly oriented *a* axis (monoplacophorans), gastropods and cephalopods possess "columnar nacre" (tablets stacked upon each other), with the *c*-axis of the tablet perpendicular to the surface of the shell and the *a* and *b* axes aligned within a stack (gastropods) or alignment of all three axes (cephalopods) (Chateigner et al., 2000; Meldrum & Cölfen, 2008). These differences, and the strikingly different nacre building gene sets that underlie them (Jackson et al., 2010), call in to question the assumption of homology of nacre in conchiferan taxa and has bearing on our understanding of the evolution of biomineralization in molluscs. Whether the other shell layers are similarly divergent between molluscan classes remains to be investigated.

1.4.3 WHAT DOES IT ALL MEAN?

Even with high quality genomic resources spanning the diversity of Mollusca, more data does not mean more understanding. However, genomic resources will continue to provide profound insight into physiological processes such as biomineralization. There is a growing need for implementation of advanced analytical techniques looking at gene family evolution (Aguilera, 2014; De Bie et al., 2006; Domazet-Lošo et al., 2007) and gene networks (Shannon et al., 2003; Smoot et al., 2011). Further, more "traditional" techniques with a much longer history of use in the field of physiology (see Simkiss Chapter 2 of this volume) should not be forgotten in the "-omics" era. Such comparative studies will undoubtedly continue to improve understanding of the complex physiological process of molluscan biomineralization.

KEYWORDS

- **biomineralization**
- **shell**
- **periostracum**
- **mantle**
- **silk**
- **RLCD**

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CHAPTER 2

DEVELOPING PERSPECTIVES ON MOLLUSCAN SHELLS, PART 2: CELLULAR ASPECTS

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CONTENTS

ABSTRACT

The biological components involved in shell formation are considered in relation to the anatomical compartments, the extrapallial fluid, crystal nucleation, and mineral/matrix interactions. Four main theories of shell formation are considered in relation to calcium transport, organic frameworks and amorphous minerals, facilitated diffusion, exosomes, and molluscan calcium cells. The approach is to review the standard models and to indicate where alternative developments are required.

2.1 INTRODUCTION AND APPROACH

Collecting, studying, and admiring molluscan shells is a major study for many biologists and some hobbyists. Most natural history museums have their own collections, and many libraries have some books on the subject. Put the word conchology into any search system or booksellers catalog and you will be given access to a fund of information in books costing anywhere from £5 to £20,000 (Sowerby's 1812 edition). If your interests are wider and extend to the biology of molluscs, the word to explore is malacology and there you will also find a wealth of information on the Internet.

So, why write reviews to describe the models and concepts that are now in the literature databases? Greenhalgh's (2010) booklet "How to read a paper" suggests that a scientific review should be centered on the formulation of a problem, require a search for "evidence-based" studies, involve a test of hypotheses, and only accept good methodology that reflects the design of the research. These are clearly the basics of science, but those who work on molluscan shells and those who review their findings only rarely put them in the clear context of raising questions and testing hypotheses.

In an attempt to bring some order into a variety of examples of biomineralization as diverse as corals, sponges, molluscs, and crabs, Karl Wilbur (1980) identified three basic levels of complexity. These were (1) mineral formation that occurred intracellularly within cells as in coccolithophorids, (2) extracellular mineralization induced by single cells such as skeleton deposition in sea urchin larvae, and (3) mineralization by a sheet of cells as in the epithelia of molluscs. This classification presented an interesting proposal because it completely ignored phylogeny and emphasized some sort of "systems analysis." Wilbur was, however, not alone in this approach of searching for some basic components in what might support a fundamental scheme. A number of scientists have always used what is known as

the "August Krogh Principle" which suggests that for many biological problems there may be an organism on which it can be most conveniently studied (Krebs 1975). Many biologists would respond to this approach with the retort "but only if you believe in comparative physiology," while the chemists would probably issue a warning that "it shows you are at last concerned with basic processes."

It must be acknowledged that molluscs are not always the most convenient organisms on which to investigate a process. The shell is a remarkably good defense against many types of investigation, and gastropods and bivalves often do seem to work very slowly. There are, therefore, some aspects of biomineralization where it may be helpful to compare molluscan activities with some other examples such as eggshell formation in birds to fill this need. The average rate of shell formation in a mollusc involves a calcium flux of about 10^{-6} mol/cm^2/h, whereas the formation of the avian eggshell involves a calcium flux of about 10^{-5} mol/cm^2/h. These examples are toward the two extremes of the rates of calcification, but both illustrate what Wilbur would call epithelial biomineralization. Both systems are also capable of inducing the formation of crystals of calcium carbonate. Both do this in an enclosed space using an organic substrate and both types of organism seem to be able to control the mineralogy of the deposits (i.e., whether it is calcite and/or aragonite based). Both examples of mineral formation will stop if you add inhibitors of the enzyme carbonic anhydrase and both use cellular control systems to turn the mineralization systems on and off.

2.2 HOW TO BEGIN?

There are eight main classes of mollusc ranging from chitons to squids, but this review will only concentrate on the gastropods and bivalves. They live in three types of habitat (marine, freshwater, and terrestrial) and produce shells of one or two main minerals (calcite and/or aragonite). Even so, many of the experiments that have been performed would have been easier to discuss if the systems had involved less types of test organisms from fewer and more stable environments.

Most scientists will go through some sort of check list before they design their experiments. This is because it is almost impossible to interpret experiments if there are too many variables and that makes it very difficult to claim to be testing major hypotheses without making major mistakes. There are about 85,000 species of molluscs in the world today that have had

roughly 545 million years of evolutionary divergence. Compare that with one surviving species of human and only 2 million years of change.

2.3 IDENTIFYING THE BIOLOGICAL COMPONENTS

The requirements for shell formation in molluscs involve an enclosed space associated with cells that can secrete and accumulate specific ions within those spaces. These ions are then moved from the liquid to the solid phase and converted into inorganic crystalline minerals that are initiated, or nucleated, and held together in organic matrices.

Figure 2.1 is a generalized drawing of a bivalve shell. It is not drawn to scale and is intended to be used to identify the biological components with which it may be involved. In a slightly provocative way, it answers Greenhalgh's first requirement. It has been drawn to help the formulation of at least five problems. So now we need to make it evidence-based.

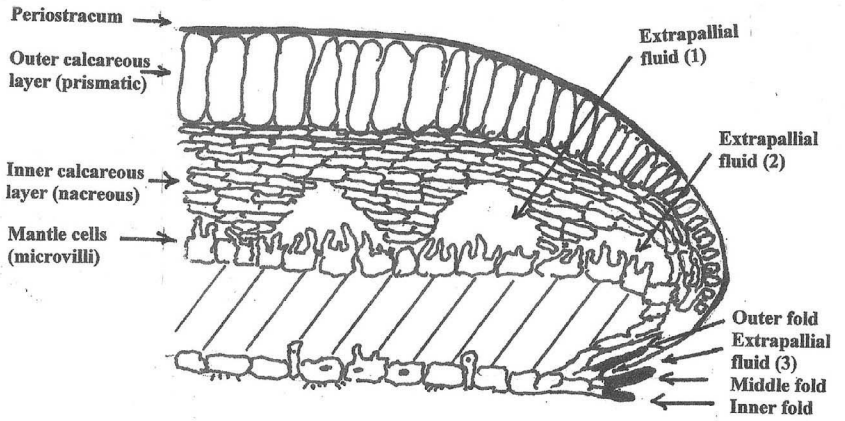

FIGURE 2.1 A vertical section through a bivalve shell and its mantle tissue. There are three folds in the mantle tissue edge. The space between the outer and middle folds form the periostracal gland that secretes the periostracum as a sheet that covers the shell. Note that the inner extrapallial fluid (1) is further away from the environmental water and contains most of the fluid while the outer extrapallial fluid (2) is smaller and in closer contact with the microvillus cells. The space in the periostracal gland also contains extrapallial fluid, and it should be noted that there is a specific basal cell at the base of the gland (3). All three fluids are involved in secreting and maintaining the shell. (The illustration is not to scale nor based on any species.)

On the outside of the shell is an impermeable sheet of denatured protein called the periostracum. This covers and protects the layers of crystals of

calcium carbonate (calcite and/or aragonite) that are embedded in thin layers of soluble and insoluble matrix that form the shell. On the inside of the shell layer is a small gap called the extrapallial space. This is filled with an extrapallial fluid that is secreted by the epidermal sheet of mantle cells. From this it is possible to derive five questions. These are first, where are the anatomical compartments where the minerals are deposited? Second, what are the extrapallial fluids? Third, how do you nucleate and grow crystals? Fourth, what do shell proteins do? And finally, are there any theories about how this all fits together?

2.3.1 QUESTION 1: WHERE ARE THE ANATOMICAL COMPARTMENTS?

Molluscs have a remarkably versatile epidermis that covers their body. It is referred to as the mantle or pallium where it covers the viscera. This layer of "skin" can be used for functions as diverse as locomotion, respiration, and osmoregulation but, unlike the human skin, it is not covered in a layer of dead cells. There are basically three types of cell in the molluscan epidermis, namely, "mucus" cells, ciliated cells, and cells bearing microvilli on their apical surface (Simkiss, 1988). There is considerable diversification in the function and distribution of these cell types over the body but there is one particular region, the mantle, that is involved in generating the shell on the outside of the molluscs body.

The shell-forming epithelium of the mantle has been described in detail for a number of bivalve molluscs (McElwain & Bullard, 2014) and gastropods (Zylstra et al., 1978; McDougall et al., 2011). Where there are two different layers of the mineralized shell, the outermost region is usually made up of long crystals of prismatic calcite that are embedded in a sclerotized organic matrix and covered on the outside by a sheet of protein called the periostracum. Beneath the prismatic layer is the inner layer often composed of aragonite arranged like a stack of plates to form the nacreous layer. The whole shell is impregnated with a matrix of carbohydrates, lipids, and proteins that are trapped both within (i.e., intracrystalline) and between (i.e., intercrystalline) layers of inorganic minerals.

There has been considerable interest in the way that these layers of organic matrix might initiate the nucleation of the inorganic crystals. There are two theories involved, that is, the heteroepitaxial system where crystals form at selected sites on the matrix or the mineral bridge model where superimposed holes in the organic sheets permit a single crystal to pass through

a number of stacked layers. This bridge system would result in one crystal carrying a continual mineral lattice through a number of matrix layers (Nudelman, 2015). There are some difficulties with this theory if different minerals occur where the nacreous and prismatic layers meet. There are, however, spacial differences in gene expression in some mantle tissues (Gardener et al., 2011) where different secretory processes are associated with prism and nacre deposition (Marie et al., 2012).

The organic matter of the shell that holds the crystals together is generally credited with at least three other functions. First, it may determine the type of mineral that is deposited in the shell; second, when bound together with the mineral layer, it produces a composite material of increased strength; and third, it may be involved with the organization of the mineral layers that are variously described as homogenous, prismatic, foliated, crossed lamellar, or other complex structural forms (Watabe, 1988). Clearly, the physical properties of the shell are influenced by both their composite form and their spatial arrangements, so that the strength of the shell differs significantly from its individual components. This is reflected physically in terms of the modulus of tensile strength, compression, bending strength, and hardness (Table 2.1). The mineralogy and the composite structure have contributed important physical properties that have undoubtedly influenced the types of protection that the shell has evolved in different kinds of molluscs (Currey, 1988, 1999; Vincent, 1982).

TABLE 2.1 Strength of Molluscan Shell Materials (Currey, 1988; Vincent, 1982).

Structure	Tension (MPa)	Compression (MPa)	Bending (MPa)	Hardness (kg mm^{-2})
Prismatic	60	250	140	162
Nacreous	80	380	220	168
Crossed lamella	40	250	100	250
Homogeneous	30	250	80	–

The shell of the adult mollusc is covered on its outer surface by a sheet of protein (the periostracum) and on the inner surface by the mantle epithelium. At the outer edge of this layer the mantle becomes folded, typically into two or three ridges that are referred to as the outer (nearest to the shell), middle, and inner mantle folds. It is the space between the outer and middle folds that secretes the periostracum, and this region is then referred to as the periostracal groove and by attaching to the outer region of the shell it

converts the extrapallial space into a closed compartment (Fig. 2.1). This seal may occasionally be broken, particularly if a gastropod retracts into its shell (Nakahara, 1991). A number of attempts have been made with dyes and other fluids to confirm that there is a functional seal, keeping the extrapallial fluid away from the external environment, and for the purposes of this review, it will be considered normally to be intact. As the mollusc grows, the periostracum continues to be secreted and it is toughened by a denaturing of the proteins with a quinone "tanning" type of process (Gordon & Carriker, 1980; Waite, 1983). The structure and functions of the periostracum are so surprising that they deserve a small section on their own.

The structure of the periostracal groove in the primitive "living fossil" *Neotrigonia* has recently been described by Checa et al. (2014). As in all bivalves, there is a basal cell or group of cells at the bottom of this groove and it is these cells that secrete the protein sheet or pellicle that rapidly darkens as it becomes cross linked or tanned into a harder secretion. This is the periostracum, and as it continues to be formed, the outer surface becomes coated in a glycoprotein and starts to develop calcified nodules or "bosses" of calcite until it eventually emerges from the groove. The remarkable feature of *Neotrigonia* is that all the normal activities of shell formation, that is, secretion of protein, tanning, coating with glycoprotein and synthesis of crystals have already started while it is still in this simple groove of the epidermis. Within that space the epidermal cells also extend their microvilli by a distance of about 100 nm, and the whole process continues as the periostracum develops the layer of prismatic crystals that will eventually cover the mantle. Descriptions of some of the variants of these processes in other molluscs have been explored in detail by Saleuddin and Petit (1983) and are discussed in detail by Kocot et al. (Chapter 1, this volume).

So there are now three possible compartments that might be involved in shell formation, namely the larger inner extrapallial space between the shell and the mantle cells; the smaller outer extrapallial region that is so much closer to the microvilli of the mantle cells that they may contact the crystal nuclei; and the space between the outer and middle folds of the mantle edge where the periostracum is formed in what is then referred to as the periostracal groove.

2.3.2 QUESTION 2: WHAT IS EXTRAPALLIAL FLUID?

The extrapallial fluid is the film of liquid that receives all the secreted products of the mantle cells that are required to form and maintain the shell. It is

present in all three of the compartments so the question arises as to whether there are one, two, or three varieties or states of this fluid.

There are relatively few analyses of the inorganic ions in these fluids as they lie beneath the shell and form only a thin layer of fluid (Table 2.2). The composition of this solution in marine bivalves shows a striking similarity to sea water whereas the fluid from freshwater molluscs is more dilute and clearly secreted from the cells of an osmoregulating animal. What one might have expected is that the fluids in contact with the calcareous shell would have been in equilibrium with the solubility of calcium carbonate or, if the shell was still being formed, it might be slightly supersaturated. The solubility of calcium carbonate minerals is normally expressed as the product of the concentrations of calcium and carbonate ions and is referred to as the solubility product or K_{sp}. This value differs for the different forms of calcium carbonate. Thus calcite, the least soluble of these crystals has a log K_{sp} value of −8.42, aragonite has a value of −8.22, vaterite −7.60 with amorphous calcium carbonate being very variable but the most soluble. Unfortunately, the value of K_{sp} is not a particularly useful concept in biological situations because it tends to vary with both the size of the crystals and the time taken for them to equilibrate with the solution (Williams, 1976). Even more important, it depends on the ionized rather than the total concentrations of the inorganic components in the fluids. Thus, when Misogianes and Chasteen (1979) looked at the concentration of calcium in the extrapallial fluid of the mussel *Mytilus edulis* they recorded a total level of 9.8 mM, but that included calcium bound to five proteins so that 84.7% of the calcium was complexed to small molecules and only 15.3% of the calcium was represented as the free ion.

TABLE 2.2 Concentrations of Ions in Extrapallial Blood or Sea Water Samples (Crenshaw, 1972; Moura et al., 2003).

Species	Fluid	Na (mM)	K (mM)	Mg (mM)	Ca (mM)	Cl (mM)
	Fresh water					
Anodonta cygnea	Extrapallial	15.5	0.46	4.3	3.14	17.1
Anodonta cygnea	Blood	14.8	0.36	4.4	2.95	17.1
	Sea water	427	18.0	53.0	9.2	49.6
Mytilus edulis	Extrapallial	442	9.5	58.0	10.7	477
Crassostrea virginica	Extrapallial	441	9.4	57.0	10.5	480

When these experiments were originally performed the main object of the analyses was to determine if the extrapallial fluid was supersaturated with calcium carbonate as this would suggest that the shell might be forming crystals out of this fluid. The results are, however, of limited value unless a specific mineral is formed. For example, the amorphous form of calcium carbonate has no fixed lattice and is only found when nucleation fails and growth of the solid phase becomes less organized. If these conditions persist the first solid material is likely to be amorphous, but that might then slowly transform into the more insoluble states of vaterite, aragonite, and finally calcite. This somewhat empirical situation is referred to as the Ostwald Lussac law of ripening (Williams, 1989) and it implies that the most soluble form of a polymorphic material may be the first solid phase to emerge. Given this situation, it is difficult to interpret the results of what an extrapallial fluid should contain, although there are always other details in a good experiment. When Crenshaw (1972) collected some extrapallial fluid samples, he used an indwelling catheter. He then noticed that if the bivalve closed its valves it was followed, after about 15 min, by a rise in the calcium level and a fall in the pH of the extrapallial fluid. This appeared to be due to a decline in the oxygen available to the bivalve and led to the accumulation of succinic acid. This situation persisted until the oyster reopened its valves and it suggested that the calcareous minerals of the molluscan shell were buffering the extrapallial fluid during the period of hypoxia (Crenshaw & Neff, 1969).

Most inorganic shells of molluscs are composed of the calcium salts of the main inorganic buffers that are found in the body, that is, the phosphates and carbonates. The fundamental equation for forming such calcareous crystals is $Ca^{2+} + HCO_3^- = CaCO_3 + H^+$. It is a proton releasing reaction and typically results in acidosis that should also be detectable at the site of shell formation unless these ions are being involved in other processes that displace the equilibrium.

It may be concluded that the composition of the extrapallial fluid may vary with ongoing activities so that chemical concepts, such as the solubility products of various crystal deposits, are difficult to apply in biological situations. As a result a different approach for assessing the activities of the ions in the extrapallial fluid(s) was attempted by using an *in vitro* preparation to investigate ion movements by measuring the electrical potential across this tissue (Istin & Kirschner, 1968). That work detected a large difference of about 50 mV across the tissue, but it was the mantle surface facing the shell that was positively charged. If this persisted *in vivo*, it would make it more difficult to accumulate calcium onto the shell, and it unfortunately appeared to be chloride ions rather than calcium ions that were crossing the

epithelium. The possible effect of bicarbonate ions appeared to have been largely neglected. Subsequent work on mantle tissues from both freshwater and marine clams gave similar electrical potential differences with the shell side still positively charged. What had been expected was that the existence of an electrical potential across the mantle tissue would provide evidence for the transfer of ions onto or into the shell. It was subsequently discovered that the results were mainly due to the diffusion of calcium ions and chloride ions along concentration gradients and that any charge effects were actually mediated through an effect on other ions (Sorenson et al., 1980). This was an interesting but not very helpful contribution in trying to understand the movement of charged ions across a membrane that might have been secreting calcium and carbonate ions. Perhaps the most detailed study of the electrophysiology of the mantle epithelium was subsequently undertaken by Coimbra et al. (1988) using the freshwater bivalve *Anodonta cygnea*. The approach used both intact as well as stripped epidermal cells. Radioisotopes were used to track particular ions, while microelectrodes measured specific ion activities and millivolt potentials that could be modified with short circuit currents or enzyme inhibitors. This detailed work showed that as long as there were identical solutions on the two sides of the epithelium there was virtually no potential, but if the concentration of calcium ions was reduced on the shell-facing side a positive charge developed. There was no evidence for involving a calcium ion pump but the shell side of the epithelium had a large permeability for calcium ions, that is, roughly 10 times that of sodium, potassium, or chloride. They suggested a sodium/potassium ATPase system linked to bicarbonate produced by carbonic anhydrase might be one part of a transport system providing calcium and carbonate for shell formation. The epithelium was found to be clearly sensitive to carbon dioxide levels leading to speculations about the acid/base balance of the tissue (Moura et al., 2003). This aspect of biomineralization will be considered again in Section 2.4.1.

This now seems to be one of the situations when it might be helpful, to know the type of membrane potentials that exist in other organisms that form mineralized shells. Such measurements by Hurwitz et al. (1970) were taken from electrodes across the shell gland of a laying bird. The mucosal surface was about 10 mV negative, relative to the serosal layer, that is, the opposite orientation to that of the mollusc mantle.

So far, this review has considered four frustrating experiments. The search for a compartment that contained the ions necessary to form a shell revealed three possible compartments, and it was not certain that the extrapallial fluid that they contained was supersaturated enough to produce a calcium carbonate mineral. The mantle epithelium that produced the fluid

also did not seem to be actively pumping calcium ions into the extrapallial space. There are a number of possible explanations for these results. The most obvious is that shell formation may be an intermittent event in the mollusc or it may be easily shut down by the experimental procedures. A second set of suggestions would be that the initial hypotheses were wrong. The electron micrographs of the mantle of the bivalve *Pinctada radiata* showed that the epithelial cells were within less than 1 μm of the aragonite shell (Nakahara, 1991). Any fluid in this area would probably not be the same as the bulk extrapallial fluid that has been extracted for analysis and it therefore represents a separate functioning compartment. In addition, some samples of extrapallial fluid in *Mercenaria mercenaria* appeared to contain blood proteins suggesting that there may be leakage between the extrapallial fluid and the blood under experimental conditions (Yin et al., 2005). The design of the research in this work and the methodologies that were used forestalled the initial test of hypotheses about these extrapallial fluid(s).

Clearly, there are more details to be studied in interpreting the many functions in which the extrapallial fluid may be involved. Thus, for example, Hattan et al. (2001) purified a calcium-binding protein that represented over 50% of the organic matter in the extrapallial fluid of *M. edulis* and that appeared to regulate the growth of calcium carbonate crystals.

2.3.3 IDENTIFYING THE CHEMICAL COMPONENTS

Many of the approaches to understanding chemical reactions have been formulated by simplifying or controlling the conditions under which the studies have been undertaken. As such, they are often difficult to apply in a quantitative way to biological situations that are full of interfering components and very variable time scales.

2.3.4 QUESTION 3: HOW DO MOLLUSCS NUCLEATE AND GROW CRYSTALS?

A saturated solution is one that has dissolved a solid, such as a crystalized mineral, until it is unable to dissolve any more. A supersaturated solution is one that contains that little bit more. It is apparent straight away that there is a problem in those definitions, and if you wait long enough, the solution will revert to being a saturated one. The situation has a time and energy requirement and biological systems manipulate them both.

Calcium pumps are present in most cells and they control the cytoplasmic content of this ion at a very low level. The enzyme carbonic anhydrase is also present either in solution or attached to a variety of proteins. It is, therefore, relatively easy to envisage a fluid that becomes supersaturated with calcium and carbonate ions that may persist for some time in that state as there are a number of energy barriers involved in the formation of crystal lattice structures from hydrated ions.

In the absence of a preexisting surface, the formation of a new phase (such as a crystal of calcite) from another phase (such as an extrapallial fluid) would be called homogeneous nucleation. It is what many biologists hope to observe but most chemists doubt if it ever occurs. The problem is that very small clusters of ions have extremely large surfaces in relation to their volume so that they tend to dissolve again before they can grow. Perhaps even more important is the fact that when an ion passes from solution into a lattice structure, it may have to shed some bound water so that the free energy of the new phase is less than that of the solvated phase. One of the ways to facilitate this is to provide a preexisting surface. This is known as heterogeneous nucleation and it occurs extensively in biological systems that incorporate either preexisting crystal lattices, or organic molecules with charged sites. Unfortunately, it is extremely difficult to relate model systems to experiments with biological matrices. As an example, it is possible to extract water soluble proteins from many biological shells but it is not clear whether this is a protein that is derived from within crystals or from between crystals. There is similarly an insoluble matrix. Is this the same as a soluble matrix except that it has been attached to a structural protein or is it entirely different? Do the extraction methods using acids or water define the properties of subsequent tests? Is the system hydrophobic or hydrophilic before or after extraction and are the subsequent tests based on isolated groups or flexible molecules. Do calcium or carbonate ions influence the reactive properties of the matrix proteins before, during or after mineralization, that is, do the ions fold the proteins? There is a tendency in molluscan research to regard the mineralized matrix as a single entity whereas sections of a decalcified matrix may show a separate nucleation matrix followed by a spherulite-containing matrix followed by a columnar matrix as a temporal sequence from the initiation to the completion of the mineralized structures. Searching for nucleation sites from within shells is clearly a very complex system in biological materials but there are some excellent reviews of some of these very demanding experiments (Mann, 1989, 2001; Wheeler and Sikes 1989).

There is one other aspect of mineral formation that should be noted. If the level of supersaturation of the saline continues to rise, and there are only

poorly organized or sparse nucleation sites then amorphous, rather than crystalline, minerals are likely to form. In that situation, as has already been explained, the calcium carbonate minerals will be produced in the sequence amorphous > vaterite > aragonite > calcite (Ostwald Lussac's law), whereas the series of insolubility is calcite > aragonite > vaterite > amorphous (Mann, 1986; da Silva & Williams, 1991).

It is apparent that these two series contain the possibility of a paradox but it all depends on the local conditions that dictate how and when calcium carbonate forms. And that directs you back to the aqueous conditions that exist around and within molluscan cells.

2.3.5 QUESTION 4: WHAT DO SHELL PROTEINS DO?

An important review of the main matrix proteins from the outer layer of the molluscan shell was published by Zhang and Zhang (2006). These proteins had been isolated and identified, together with some comments on their structure and suggestions as to their role in shell formation. They reported results for 13 of the main proteins in the nacreous layers of 2 species of *Haliotis*, 2 species of *Pinctada*, 2 species of *Biomphalarisa*, and 1 of *Pinna*, together with results for 10 proteins from the calcite layer of *Mytilus*, *Pinctada*, *Crassostrea*, *Painopecten*, *Adamussium*, *Atrina*, and *Pinna.* There were also eight proteins that occurred in both layers of shell. For the moment, it is the potential properties that were attributed to these proteins. These included some structural features, some sites that might block the various crystal faces of particular minerals, some that favored particular planes, some that induced step functions that might encourage crystal growth, some with strongly acidic groups, and some with enzyme sequences similar to carbonic anhydrase (i.e., nacreins) that can induce or act as negative regulators. It is obvious that with the right properties in the right places the shell matrix proteins could interact with the shell minerals in a wide range of ways.

This was a great start but a further major change in the study of matrix proteins occurred in 2011 when the first whole genome of a mollusc, the limpet *Lottia gigantea* was released into the public domain. This made it possible to produce a combination of proteomic and transcriptomic studies of the acid soluble and acid insoluble matrix proteins. Thus, for example, the *Lottia* shell matrix contained three proteins with strong similarities to nacreins, one of which also contained a secretion signal. The presence of a chitin framework with silk-like and strongly acidic proteins also suggested

a key structural component for shell formation (Mann et al., 2012). The subsequent revelations of molecular biology that have been exposed as the genomes and protein repertoires of various molluscs have been discussed by Kocot et al. (Chapter 1, this volume).

A recent analysis of gene expression in different mantle areas and especially in the periostracal groove have confirmed and extended the structural continuity between the three folds at the mantle edge and two regions of shell formation, that is, the columnar and nacreous layers. The outer fold of the mantle is confirmed as being responsible for much of the secretory activities of the periostracum groove while the middle fold is a sensory organelle and the inner fold is involved with muscular activities (Gardener et al., 2011). One of the recurrent problems in understanding the biomineralization processes of the prismatic and nacreous layers of the shell was also studied by Marie et al. (2012) who were able to track "prismatic proteins" to the mantle edge and "nacreous proteins" to the central mantle pallium. This confirmed the finding that different secretory repertoires were producing prismatic calcite or nacreous aragonite presumably by transcriptional regulation of a single genome. This would similarly explain how mantle cells were able to transiently change the type of mineral they induced when repairing a damaged shell. What came as a major surprise, however, was the disparity between the genes in the nacre forming cells in the mantle of a bivalve (*Pinctada maxima*) and a gastropod (*Haliotis asininae*) that had so little in common that Jackson et al. (2010) concluded that they must be an example of convergent evolution rather than a closely related genome.

There are several large groups pursuing these molecular approaches in order to get an understanding of the evolution of the proteins in the molluscan shell (Jackson et al., 2006; Marin et al., 2008), for influencing studies on the of deposition of prism and nacre in the pearl oyster shell (Marie et al., 2012) for identifying the role of the calcifying mantle and shell of the pearl oyster (Joubert et al., 2010) and transcriptomes for the mantle tissue of *Mytilus edulis* (Freer et al. 2014). These samples have been used to identify which genes are turned on and off and which proteins are released at various times and at different sites. There are clearly some very effective interactions between switching on certain genes to form matrix proteins and inducing specific crystal forms (Belcher et al., 1996). Most molluscan shells are, however, composed of mainly calcium-based minerals, and, like the genes and proteins, these inorganic components will also be controlled by cells.

2.3.6 QUESTION 5: HOW IS CALCIUM TRANSPORTED TO FORM THE SHELL?

In 1971, E. W. Sutherland won the Nobel Prize for his discovery of the second messenger. It was soon to become a major component of cell biology as it explained how primary signals such as nerve impulses or water soluble hormones could pass across the cell membrane into the cytoplasm and trigger a controlled response (Clapham, 2007). One of the most common of these second signals involves the release of calcium ions inside the cell so that Berridge et al. (2000) would write about "the versatility and universality of calcium signalling." It soon became apparent, however, that intracellular levels of ionic calcium were maintained at about 100 nM whereas extracellular calcium was around 1 mM. There is a gradient of roughly 10,000 times between the high calcium activity levels outside and the extremely low levels inside a cell whereas even a small tenfold increase of ionic calcium inside a cell could be lethal. This implies that it would be very difficult to transport calcium ions through the cells of an epithelium such as the mantle tissue of a mollusc. There seemed to be only three ways around this difficulty, namely, to move the calcium around the cells, to encapsulate the calcium in a membrane bound vesicle, or to transport it in a non-ionic form.

2.3.6.1 THE PARACELLULAR ROUTE

If calcium ions are transported around the cells of an epithelium they require spaces between the cells together with some form of control over the types of materials that could use them. These intercellular channels provide what is termed the paracellular route. A variety of "tight" and "gap" junctions between cells are known but in terms of restricting access and controlling the passage of ions they do not seem to be very selective. These spaces are, however, strongly advocated as providing the route taken by calcium ions in a number of vertebrate studies (Hoenderop et al., 2005) including the intestinal absorption of calcium in humans (Khanal & Nemere, 2008). This route was also suggested by Neff (1972) whose ultrastructural study of the mantle of the clam exposed intercellular deposits of calcium between the mantle cells. These results were extended by Bleher and Machado (2004) who added some data on the rate of passage of ions along these intercellular routes.

2.3.6.2 THE VESICULAR ROUTE

There are a variety of vesicles in the cytoplasm of many cells, some of which are formed by an infolding of the plasma membrane (endocytosis) and some that are derived from intracellular organelles such as multivesicular bodies, lysosomes, Golgi complex, and endoplasmic reticulum systems (Simkiss, 2015). Many of these vesicles may be associated with microtubules and deliver their contents by exocytosis out of the cell, thereby providing the possibility of a vesicular route for calcium transport. By these means, a large number of different types of vesicle may be involved with the movement of inclusions into, out of, or around the cell. In an interesting calculation, Addadi et al. (2006) found that it would require 10^5 times the volume of a saturated calcium carbonate solution to move the same mass of solid aragonite through a cell. Clearly, transporting some materials in the solid state could have a lot of benefits over handling very large volumes of solution.

2.3.6.3 CALCIUM BINDING

The third suggestion for moving calcium ions through a cell without raising the calcium level of the cytoplasm would be to bind the ions to a protein. The recent proteome reports on molluscan proteins have revealed a large number of calcium-binding proteins in both shells and mantle samples. At the present time, there is only limited information on the strength of the binding of calcium by these proteins or on their relationships to other groups of proteins.

2.4 FOUR THEORIES

This section poses the final Greenhalgh (2010) question. Is it possible from the information in this review to propose a coherent theory that provides some testable hypotheses on how shells are formed? Remember that the motivations for much of the current work on molluscan shells were initiated by objectives that ranged from measuring the environmental impact of global warming to understanding evolution, and from fundamental science to commercial exploitation. They have exploited new technologies such as proteomes, transcriptomes, biocomposites, amorphous minerals, the moulding of crystal products, and even the promise of *in vitro* pearl culture. Despite that, it is possible to derive four possible attempts to explain molluscan shell formation from the viewpoint of cell physiology.

The first approach is based on the electrophysiological data obtained from the freshwater bivalve *Anodonta cygnea*. The second provides a basis for converting amorphous calcium carbonate into an organized crystalline structure. This theory does not provide explanations for the cellular accumulation of the ions or their transport to the mineralization sites. The third theory deals largely with calcium transport across epithelia and the acid–base consequences of mineral formation. It does not involve itself with nucleation, crystal growth, or matrix interactions. The fourth theory involves the genetic and transcriptome approaches to a variety of stress adaptations in the oyster and compares shell formation with shell repair. All four theories are shown in an abbreviated form in Figure 2.2.

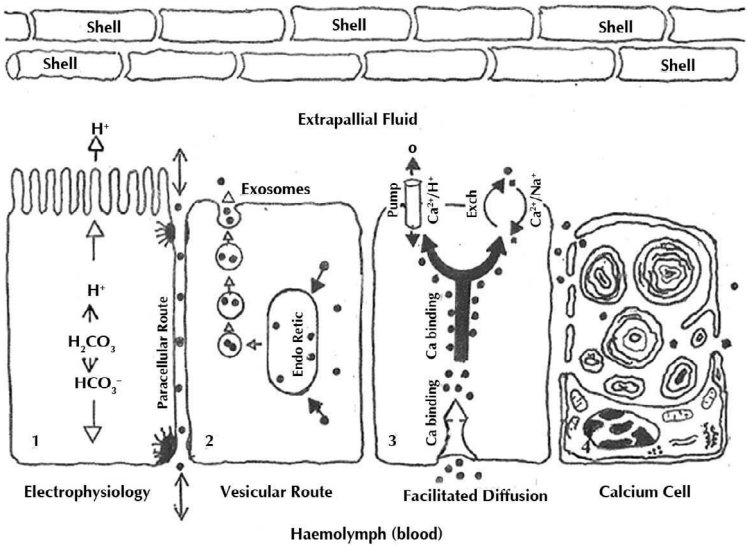

FIGURE 2.2 Diagram illustrating four possible systems involving the movement of ions at sites of shell formation (calcium ions are shown as black circles). (1) The paracellular route between two epithelial cells permits the diffusion of calcium ions into the extracellular fluid (Bleher & Machado, 2004). Intracellular ion movements produce an acid/base response resulting in the acidification of the extracellular fluid and possible release of calcium deposits (Machado et al., 1990). (2) The vesicular route delivering amorphous calcium carbonate to the extrapallial fluid (Addadi et al., 2006; Addadi & Weiner, 2014). The theory has been extended to imply that calcium leakage into the cell results in its transport into the endoplasmic reticulum and the production of vesicles of amorphous calcium carbonate. This route may also release exosomes from cell debris (Zang et al., 2012). (3) Facilitated diffusion of calcium through the epithelium based on the interpretation of avian shell formation (Jonchere et al., 2012). Calcium ions bound onto calbindin are delivered to calcium pumps and ion exchangers. (4) Molluscan calcium cells form intracellular deposits that may subsequently release calcium and bicarbonate ions through membrane pores (Sminia et al., 1977; Watabe & Blackwelder, 1980).

2.4.1 THEORY 1: ELECTROPHYSIOLOGY AND PROTONS

As explained in Section 2.3.2, this approach has utilized some of the best techniques to track the movement of ions across the bivalve mantle (Istin & Kirschner, 1968; Coimbra et al., 1988). The short circuit data indicate, however, that there is an acidification of the extrapallial fluid that bathes the shell (Machado et al., 1990). This is balanced across the outer mantle epithelium by a Cl^{-}/HCO_3^{-} exchange but it is a counter intuitive result in that the shell would be expected to dissolve in the presence of increasing levels of protons. The ability to deposit calcium carbonate on the shell is also complicated as no calcium pump can be detected, and it has been suggested that calcium moves across the mantle through the paracellular route (Bleher & Machado, 2004). Such a system usually invokes diffusion. A possible explanation of shell formation in the freshwater mussel has been related to a study throughout a year since there are seasonal variations in the composition of both the hemolymph and the extrapallial fluids that may explain some of these anomalies. Environmental acidosis may also have considerable effects on the calcareous deposits that are formed and resorbed by the mussel during an annual cycle (Moura et al., 2003).

2.4.2 THEORY 2: FRAMEWORKS, AMORPHOUS MINERALS, AND VESICLES

In the early 1980s, Bevelander and Nakahara (1969, 1980) observed organic compartments that they interpreted as preformed structures for nacre formation. Shortly afterward, Wiener et al. (1983) proposed an organic framework with silk fibroid and acidic protein linings. These two concepts were to become a new approach for an understanding of the molluscan matrix and its relation to aragonite nacre.

According to the theory of Addadi et al. (2006) and Addadi and Weiner (2014), the formation of the nacreous layer of the molluscan shell involves the creation of a structural framework of chitin onto which hydrophobic silk gel and acid-rich proteins are adsorbed to form a compartment for the growth of aragonite tablets. There has been some discussion as to whether these compartments precede the mineralization process or whether the growth of the aragonite crystals contributes to the organic phase and its dehydration. A detailed model of the components of this structural framework and their relation to shell formation has been discussed by Furuhashi et al. (2009). It is suggested that once the structural framework has been

developed, an amorphous form of calcium carbonate is transported in vesicles from the epithelial cells of the mantle into these organic structures to nucleate the formation of aragonite crystals. This raises the question as to whether or not the compartments are influenced as a result of mineral formation, but once the process has started, there may be further interactions between the organic proteins and the growth of the inorganic crystals. It is an extensive theory in that it encompasses a large database of experimental work including Weiner's demonstration of amorphous calcium carbonates at the sites of biomineralization in many different invertebrate shells (Weiss et al., 1991).

An intracellular pathway of vesicles carrying ions in an amorphous state to the sites of biomineralization in molluscs was originally proposed by Abolins-Krogis (1965, 1970) and reviewed for a variety of invertebrates by Simkiss (1976). Such vesicular routes are involved in a whole range of functions but mineralization is a particularly challenging one as the concentration of calcium ions in the blood is so much higher than its level in the cytoplasm of the cell.

The properties of vesicles that contain amorphous calcium salts have been studied using both the very soluble calcium carbonate-based granules of *Helix aspersa* (Greaves et al., 1984; Taylor et al., 1986) and the highly insoluble amorphous phosphate/pyrophosphate granules of the same organism (Taylor & Simkiss, 1989). As can be seen (Table 2.3), the amorphous calcium carbonate is very soluble and could probably drive the nucleation and formation of calcareous minerals. The structural and analytical properties of the amorphous minerals have been investigated using a random network model of amorphous calcium carbonate to explore their potential involvement in biomineralization (Simkiss, 1991). There is clear evidence in the abalone *Haliotis discus hannai* for an amorphous calcium carbonate-binding protein that has been isolated by Huang et al. (2009) and this could provide an insight into how such an unstable molecule could be controlled (Su et al., 2013). Further evidence for such a system was provided by Jacob et al. (2011) who discovered an extremely thin layer of amorphous calcium carbonate between the periostracum and the prismatic layers of the shells of *Hyriopsis cumingii* and *Diplodon chilensis*; the implication being perhaps, that this indicates where the first nucleation of calcium carbonate crystals occurs. There are hints that some amorphous materials may already contain "ghosts" of the various minerals into which they transform and the concept has now reached the stage where there is a question of "how many amorphous calcium carbonates are there?" (Cartwright et al., 2012).

TABLE 2.3 Composition and Solubility of Amorphous Calcium Carbonate and Amorphous Calcium Phosphate Granules Obtained from Different Organs of the Snail *Helix aspersa* (Simkiss, 1976).

	Composition of granule (molar ratios)				Solubility in Krebs saline (mM)			Solubility in snail saline (mM)			
Type of granule	Ca^{2+}	Mg^{2-}	PO_4^{3-}	CO_3^{2-}	Ca^{2-}	PO_4^{3-}	pH	Ca^{2+}	Mg^{2+}	PO_4^{3-}	pH
None (saline)	–				2.3	1.3	7.6	4.2	4.8	0.0	7.9
Foot	1	0.06	0.06	1.04	9.4	0.6	7.7	12.5	4.5	0.2	7.8
Hepatopancreas	1	0.96	1.35	0.53	2.1	1.5	7.7	5.3	8.7	0.3	7.8

The theory as presented does not seem to make many suggestions as to the origin of prismatic layers in those molluscs such as *Mytilus edulis* that have an outer calcite layer of this mineral, although Bevelander and Nakahara (1980) have described envelopes and compartments in detail in the prisms of *Pinctada radiatae.* They have also suggested that there may be considerable similarities between the protein components of the two types of compartments that house calcite and aragonite minerals. These possibilities have been advanced by Nudelman et al. (2007) who found clear evidence for acidic proteins in both prismatic and nacreous layers but with no direct evidence for the presence of amorphous minerals at either of those sites.

It is not clear as to whether the carbonate ions are contained in the "delivery vesicles" or whether carbonic anhydrase (Nielsen & Frieden, 1972) or nacrein-related proteins (Norizuki & Samata, 2008) provide the anion during crystallization. This may not be as straightforward as expected as Miyamoto et al. (2005) have found that nacrein can also act as a negative regulator of calcification in *Pinctada jucata*, although binding it to other proteins may help to regulate the formation of the aragonite crystal.

2.4.3 THEORY 3: FACILITATED DIFFUSION

This is an alternative approach to the "framework, gel, vesicle, and amorphous mineral theory" of biomineralization in that it contains none of those components. It is a cell-based approach derived from vertebrate studies rather than invertebrates so it could be ignored here for having little experimental relevance to molluscan shells. The interest in calcium ions as a crucial component of the "second messenger" signaling system has shown that the cytoplasm has to be maintained at the very low concentration of around

1 μM. That concentration would appear to rule out any direct involvement in transporting the ion through the cytoplasm but it has also stimulated a lot of interest in calcium movements across epithelia (Hoenderop et al., 2005).

The theory to explain calcium movements across epithelia was initially developed by Wasserman (Feher et al., 1992) and is referred to as "facilitated diffusion." Calcium enters the cell passively through a specific ion channel in the serosal surface of the cell and is then buffered by the protein calbindin that also facilitates its diffusion through the cytoplasm (Lambers et al., 2006). Free calcium is then released by calcium ATPase into crossing the apical membrane to where it is involved in extracellular shell formation. Carbon dioxide similarly diffuses into the cells and is hydrated via carbonic anhydrase to form bicarbonate ions that are pumped by a variety of ion exchange systems to produce carbonate ions.

A worked example of this system has compared proteomic and transcriptomic analyses of the shell gland of the fowl in laying or resting states. The results have been interpreted using the facilitated diffusion model for intracellular calcium transport (Jonchere et al., 2012). It is abbreviated in Figure 2.2 simply to show that the initial concern that transcellular calcium movements would be potentially lethal to cells were no longer applicable. Calcium ions pass through an open calcium channel that is closed if intracellular calcium ions accumulate near the channel exit. The calcium-binding protein calbindin plays a dual role in that it removes these ions, so as to keep the channel open, and binds them so as to buffer the cytoplasm. The calbindin calcium is then delivered to a calcium ATPase pump at the basolateral membrane and transported into the lumen of the shell gland where it reacts with carbonate to form a calcite shell. A number of features should be emphasized. The calcium pump is a calcium/proton exchanger together with a calcium/sodium exchanger. There is a proton flux away from the shell and a large potassium movement toward the mineralizing surface. This might help to explain the potassium current in the electric potential observed in the molluscan mantle. Overall, there are a total of 37 highly expressed ion transport genes balancing the fluid movements associated with avian shell formation.

At the present time, there is limited data on the molluscan mantle that could be used to test the application of the facilitated diffusion model although the molluscan genome appears to contain a rich selection of interesting genes. Evidence exists for gated calcium channels with calcium dependent inactivation (Kits and Mansveider 1999). There is a plasma membrane calcium ATPase (Lopes-Lima et al., 2008) with calconectin (Duplat et al., 2006), some calmodulin (Fang et al., 2008), and more importantly calbindin (Jackson et al., 2007) as calcium-binding proteins together

with inositol triphosphate sensitivity (Fink et al., 1988). Clearly, the data are not sufficient for a definitive study and equally clearly it need not be in conflict with the Weiner/Addadi studies that could be interpreted as an endoplasmic reticulum safeguard removing free calcium ions.

2.4.4 THEORY 4: MOLLUSCAN "CALCIUM CELLS" AND EXOSOMES

Virtually, every study on shell formation in molluscs eventually ends up discovering intracellular deposits of calcium in some isolated cells. The cells are sometimes in connective tissue, sometimes in the mantle and other organs, and occasionally free in the blood. They are variously described as a subgroup of amoebocyte as granulocytes, as interstitial cells, as calcium cells, or as hemocytes (Watabe et al., 1976). Any attempt to quantify the distribution of calcium stores in the molluscs usually identifies a significant quantity variously positioned around the animal (Greenaway 1971; Sminia et al., 1977). Recently, Mount et al. (2004) described these granulocyte cells as having macrophage-like functions and moving through the outer mantle of the "oyster" to deliver crystals of calcium carbonate at sites of shell damage. It was a well-documented example of cell-mediated biomineralization.

A few years later a consortium of roughly 80 researchers published an analysis of the genome of the oyster *Crassostrea gigas* (Zhang et al., 2012) commenting on the stress responses of the organism and the complexity of its shell formation. They identified 259 shell proteins including fibronectin but found no evidence of silk-like material. Genes coding for fibronectins and integrins suggested that hemocytes and other active cells might be the source of exosomes near the sites of biomineralization.

Exosomes were discovered about 30 years ago but have only been treated as a major development in cell biology in the past decade. They are small (c. 100 nm diameter) microvesicles that are shed by many cell lines and are found in virtually all body fluids (blood, seminal fluid, urine, etc.) of many vertebrates and invertebrates. They variously carry packages of enzymes, hormones, and nucleic acids and fuse with other cells around the body (Keller et al., 2006). Currently, exosomes and other microvesicles are seen as organelles that can form intercellular communications systems capable of distributing molecules such as mRNA, DNA and various enzymes so as to modify the activities of other cells (Thery, 2011; Raposo & Stoorvogel, 2013). These activities have attracted a great deal of attention in both the medical and pharmaceutical sciences. The vesicles occur in almost all the

body fluids at concentrations of up to 10^{10}/ml and they have recently been classified into two groups by Cocucci and Meldolesi (2015). The vesicles are formed in the endosome systems of cells with the exosomes arising from the multivesicular bodies.

The second group of vesicles arise directly beneath the plasma membranes and are referred to as ectosomes. The discovery, therefore of exosomes around the sites of oyster shell damage could have potentially great significance. Were they involved as part of a defense system or a repair mechanism? Alternatively, suppose that what was proposed as the "electrophysiology theory of shell formation" (Section 2.4.1) is actually interconnected by exosome interactions to what was suggested as the "calcium cell theory" (Section 2.4.3). "Dream on?" Perhaps but if the 80-odd researchers of the oyster genome project ever got together some must have had such thoughts. Certainly, 10 of the original participants extended the results of the genome analysis of the oyster to question whether the "chitin, silk and acidic protein" model of shell formation really reflected the activity of the mantle tissue or whether there should be a reconsideration of the model for shell formation (Wang et al., 2013). What this group had discovered was that when the shell was damaged many of the proteins that had previously been identified in the oyster shell as secretory products of the oyster mantle had actually been produced by a wide range of other organs around the body of the oyster. The repair of the damaged shell involved significant activity by other cells such as those in the digestive gland, the gills, and gonads with the transport of their products to the site of usage. Is biomineralization a whole body experience? Exosomes in action by intercellular signaling?

2.5 CONCLUSIONS

The simplistic view of shell formation in molluscs is that it consists of three components. The first is the involvement with the movement of calcium cations (usually out of the cell), the second is the production of carbonate anions (probably via carbonic anhydrase), and the third is the regulation of the mineral products by organic molecules (almost certainly involving proteins). These are all basic properties of aerobic cells so the literature is full of the search for the genetic assembly of a "biomineralization tool kit." That concept would be severely damaged if it turned out not to exist as a single system but to involve convergent evolution (Jackson et al., 2010). The following information provides an exemplar example.

Watabe and Wilbur (1960) demonstrated that the decalcified matrix of a molluscan aragonite shell would deposit calcite crystals if it was remineralized in a calcite-forming snail. It was a clear demonstration that the organic composition of the shell matrix determined the mineral form of the inorganic crystals. Roughly 20 years later, Wheeler and Sikes (1984, 1989) showed that it was possible to separate the matrix components to form crystal-facilitating and crystal-inhibiting fractions. The question that was then raised was "why was there an inhibitory factor in shell matrices" and one of the answers was that "life originated in a pre-Cambrian sea that had a very high calcium content" and the invertebrates used mucins to inhibit being smothered by calcium deposits that crystallized out of the sea water. As the calcium level of the post-Cambrian seas fell the situation changed and the mucins evolved into a shell binding system (Marin et al., 2000). What originated as a calcium-inhibiting product evolved into a calcium-mineralizing system.

There is now a medical approach that considers that the vertebrate bone system is too sensitive, and it has a tendency to produce ectopic pathological calcification (Jahnen-Dechent, 2004). The soft tissues of the body would mineralize unless they are continually exposed to inhibitory proteins such as Fetuin-A (Jahnen-Dechent et al., 2011). In the absence of such inhibitory molecules, ectopic biomineralization occurs in the cardiovascular system and smooth muscle tissues (Kapustin et al., 2015; Leopold, 2015) The implication is that biomineralization is a potentially dangerous system and that we should expect the physiological process to be regulated by an inhibitory counter activity. That would require a major rethinking of biomineralization processes.

The second novel reconsideration of calcification processes is the suggestion that they may involve the cellular derived systems of exosome/ectosome vesicles (Section 2.4.4). A frequently cited theory for bone formation in vertebrates is that the process is initiated by cells that release vesicles and nucleate mineral deposition (Anderson et al., 2005; Golub, 2011; Shapiro et al., 2015). A possible molluscan form of this theory is as follows. Molluscs, as with all other studied species, have cells that release exosomes into their body fluids. The cells contain cytoplasmic endosomal systems that form the multivesicular bodies, and lysosomal structures with the potential for controlling ion fluxes (Scott & Gruenberg, 2010). The endosome system produces exosomes and ectosomes along two somewhat different routes and pass the membrane vesicles into the blood system. There are two studies where the exosomes are found in oysters that are remineralizing damaged shells. The first study found that oyster shell proteins were released from most of the organs in the recovering mollusc. The response appeared to be associated

with exosomal activity (Wang et al., 2013) The second study traced hemocyte cells, with exosomes containing calcite particles, being transported to the site of shell damage (Johnstone et al., 2014). A possible interpretation of these studies is shown in Figure 2.3.

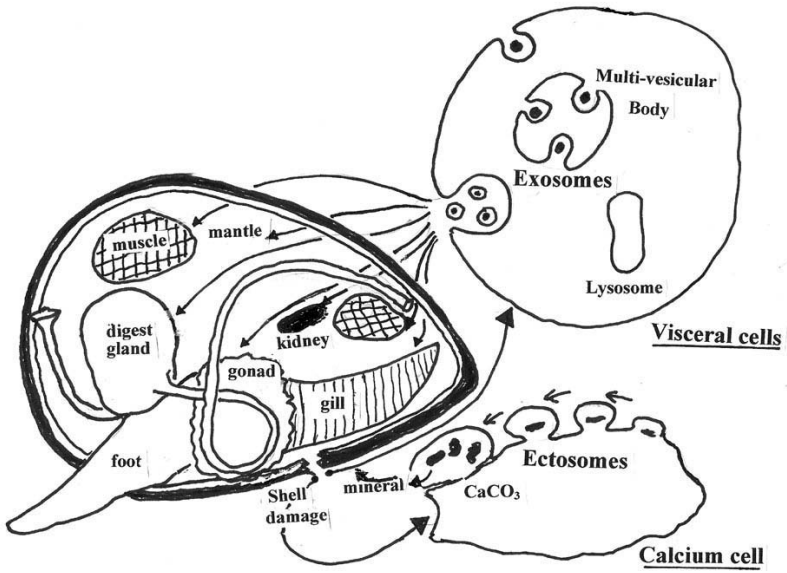

FIGURE 2.3 Illustration of how damage to the shell could be repaired by the exosomes and ectosomes of a bivalve. The cells of the viscera produce exosomes from the multivesicular bodies causing the release of shell proteins from most of the molluscs organs (data derived from Wang et al, 2013). The mineralization of the shell proteins is facilitated by the release of calcium carbonate deposits formed by ectosomes in calcium cells (data derived from Johnstone et al., 2014). Much more data would be necessary to support these concepts although analagous work on bones provides some support.

Finally the history of an absence of good control over the breeding of oysters and the problems of diseases have led to a change in the technology away from the traditional use of oyster farms and mantle implants (Simkiss & Wada, 1980) to the study of isolated cells and tissue culture. The aim is to produce pearls *in vitro* by using cell cultures. It seems quite likely that the responses of isolated mantle cells probably involve different cellular responses from the pathways that were involved in the forming the original shell. Despite this, the general observation on the use of primary cultures of mantle epithelial cells to grow pearls in tissue culture would seem to indicate that the distinction between the biomineralization products of epithelial and

isolated cells may not be very large (Barik et al., 2004) and that the *in vivo* and *in vitro* activities will show many new similarities (Awaji & Machhii, 2011; McGinty et al. 2012). On the technology side, however, there is a question as to the extent that organs and cells can be studied in isolation. The study of pearl sacs and isolated cell cultures could involve some very fundamental experiments in biomineralization.

The studies that will benefit from these approaches should stimulate the somewhat isolated areas of enzymology, endocrinology, hormonal specificity, and the implications of secretory signals. This information is necessary in many experiments where much could be learnt if one could block or stimulate one variable and then measure the response.

As far as the aspirations made in Section 2.2 of this review are concerned, one accepts the reality of mineralizing compartments and the revelations of the amorphous phase but the benefits of a "systems approach" are not there yet. There is relatively little in terms of a full testable theory for how shell formation occurs although it doesn't seem to be a particularly difficult system. But, if there are 85,000 species of molluscs that indulge in convergent evolution we will have to decide which few should be considered in detail. Perhaps as Freer et al. (2014) commented on completion of their transcriptome study of mussel mantle tissue, what is now required is "the careful application of bioinformatics with an essential hands-on approach and where researchers use their savvy to derive the best from multiple data sets." To that one would simply add two sets of comments. First, where in the physiology of shell formation does calcium meet the carbonate ion? What conditions determine whether carbonic anhydrase is intracellular or extracellular? Are we dealing with the diffusion of a neutral gas or the pumping of a bicarbonate anion? Where is the proton sink? What is the calcium pathway? Where do the nacreins fit in and what are the properties of proteins that dictate the structure of crystals?" Second, and more briefly, is the molluscan mantle a manufacturing or a packaging station? "Bring on the bioinformatics."

KEYWORDS

- **molluscan**
- **biomineralization**
- **extrapallial space**

- **extrapallial fluid**
- **calcium fluxes**
- **ectosomes**
- **exosomes crystal formation**
- **gene expression**
- **matrix inhibitors**

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CHAPTER 3

DRILLING INTO HARD SUBSTRATE BY NATICID AND MURICID GASTROPODS: A CHEMO-MECHANICAL PROCESS INVOLVED IN FEEDING

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CONTENTS

ABSTRACT

Evidence of the drilling muricid and naticid gastropods, shells with precise holes, are evident on marine coastlines the world over. Although there are other groups that bore into hard substrates substrates, including other gastropods, snails of the Naticidae (moon snails) and Muricidae (whelks and drills) are the most widespread and diverse, as well the most well studied groups. Drilling in these two families is convergent, the process involves alternating phases of mechanical rasping with a radula, and chemical dissolution with acid-produced by the accessory boring organ. Although some of the specifics vary, this gives them acces to their prey directly through the shell. The ABO is a complex organ that uses carbonic anhydrase and V-ATPase pumps to control acid production, in a process similar to mitochondria rich cells in other groups. The boreholes produced are diagnostic, allowing for a rare case of direct evidence of behavior and ecological interactions both in recent and fossil populations. Modern techniques have opened the door to newly feasible studies on the drilling process at a cellular and molecular level, and much is still not known about how drilling works, especially in taxa other than muricids and naticids.

3.1 INTRODUCTION

Traverse a marine coastline virtually anywhere in the world, and one is certain to find evidence that predatory gastropods have been at work. Most obvious on the shells of bivalves or snails, but also in other groups, is often a precisely drilled hole. This indicates that these animals were consumed by a drilling predator. The most familiar of these "boring" predators are the whelks and drills (Muricidae) and the moon snails (Naticidae), groups who almost exclusively drill the shells of their prey (Carriker, 1961). Boring in these groups is now known to result from a chemo-mechanical process: alternating mechanical rasping using the radula with chemical dissolution using acidic secretions from the accessory boring organ (ABO). Eventually piercing the shell, the proboscis is then inserted into the hole, and feeding begins. Naticids prey mostly on infaunal molluscs, usually boring in with a characteristic countersunk round hole, while muricids feed on more diverse prey, leaving a roughly circular hole.

3.2 DRILLING IN OTHER GROUPS

A number of diverse groups are capable of dissolving carbonates, generally as epibionts or endolithic burrowers: bacteria, algae, and fungi; sponges, bryozoans, turbellarians, phoronids, polychaetes, sipunculids, barnacles, and bivalves (Carriker, 1981; Carriker & Gruber, 1999; Carriker & Smith, 1969; Carriker & Yochelson, 1968; Katz et al.,2010). This is substrate boring, where the shell or other substrate is the target, rather than the tissue within (Carriker & Yochelson, 1968). Shell boring is a relatively rare predatory strategy; it has appeared only a few times in Gastropoda, Octopoda, Nematoda, and Turbellaria (Bromley, 1981; Kowalewski, 2002; Matsukuma, 1978; Sohl, 1969).

Within the Gastropoda, drilling is most well studied in the Naticidae and the Muricidae. There are several other gastropod families that include boring members, but these are generally poorly documented, with our understanding of their drilling mechanism, prey specificity, and borehole morphology varying widely (Kowalewski, 2002). The only known opisthobranch borer is *Vayssierea elegans*, a dorid nudibranch (family Okaidaiidae) that bores into spirorbid polychaetes by simultaneously applying the radula for mechanical abrasion and stomodeal secretions for chemical dissolustion in periodic bouts (Young, 1969). Within the pulmonates, three families are known to bore into other land snails. The oleacinid genus *Poiretia* scrape away the sides of the shell, leaving irregular holes (Helwerda & Schilthuizen, 2014; Schilthuizen et al., 1994; Wächtler, 1927). Similar large irregular holes are produced by zonitid snails such as *Aegopinella nitens* (Barker & Efford, 2004; Mordan, 1977). Rathouissid slugs in the genus *Atopos* have been observed drilling small circular holes into their microsnail prey (Schilthuizen & Liew, 2008; Schilthuizen et al., 2006).

There are a number of marine snails that drill. Some species of capulids (cap snails), such as *Capulus danieli*, are kleptoparasties. Some bore holes in their scallop hosts, while others simply notch the shell, allowing them to steal food, while not damaging their host (Matsukuma, 1978; Orr, 1962). These holes are oval or tear shaped, and may be surrounded by a telltale "home scar" or growth deformations. In the Nassaridae, newly settled *Nassarius festivus* bored cannibalistically into conspecifics when starved, although adults show no evidence of boring behavior (Chiu et al., 2010). The resulting borings left evidence of both mechanical and chemical action (Morton & Chan, 1997). There are other claims of nassarid drilling, but they are unsubstantiated (Morton & Chan, 1997). Two species of *Austroginella* are the only reported marginellids to bore bivalve prey. The shape of

the crystals in the borehole suggests chemical dissolution, with no sign of radula marks. The holes produced are wide and circular at the outset, with a very small, irregularly shaped inner edge; this inner hole is smaller than the proboscis and is probably used for toxin injection rather than feeding (Ponder & Taylor, 1992). In the Buccinidae, two species of *Cominella* were reported to drill thin bivalves. Boreholes were described as indistinguishable from muricid drill holes, but no figures were presented (Peterson & Black, 1995).

A few families bore echinoderms, whether as parasites or predators (Kowalewski & Nebelsick, 2003). There is evidence that some platyceratids in the Paleozoic were boring parasites on blastoid and crinoids echinoderms, possibly using the drill hole to steal food from its host (Baumiller, 1990, 1993, 1996). Some Eulimidae, though all may be parasites and predators of echinoderms, actively penetrate the test of their echinoderm hosts (Crossland et al., 1991). Snails in the genus *Hypermastus* produce pit-shaped holes with a small terminal hole penetrating to the tissue. *Thyca* are obligate parasites on starfish of the genus *Linckia*, and leave a distinct trace made up of a circular groove surrounding a small hole (Neumann & Wisshak, 2009). Due to the shape of the hole, and the fact that they lack a radula, they are thought to chemically dissolve the holes (Warén, 1983). Several species of Cassidae drill into echinoderms. Drilling is done with a taenioglossate radula and sulfuric acid in buccal secretions (Hughes & Hughes, 1971, 1981). Rather than drilling a countersunk borehole, a circular groove is cut, and then punched out, leaving a circular to ragged outline on the test, with parallel walls (Nebelsick & Kowalewski, 1999). Some cymatid and tonnid gastropods also reportedly drill prey, but no details have been provided (Day, 1969; Morton & Miller, 1973).

3.3 THE DRILLING OF MECHANISM OF MURICID AND NATICID GASTROPODS

3.3.1 HISTORY OF RESOLVING THE DRILLING MECHANISM

Some 2300 years ago, Aristotle made the first surviving record that predatory marine snails were able to drill holes in the shells of bivalves to feed (Carriker, 1981; Jensen, 1951). Since boreholes made by muricids and naticids are characteristically cylindrical and smooth inside, Réaumur (1709) first concluded that their drilling was perhaps a chemical process, while in naticids, Schiemenz (1891) provided the first serious evidence for chemical

drilling. He argued that the radula was too soft, and the proboscis insufficiently mobile, to bore holes through hard substrate. When he noticed that the secretion from the "boring gland," a hemispherical boss that underlies the ventral lip of the proboscis, had reddened litmus paper, he hypothesized that an acid secretion was responsible for producing the hole (Fretter & Graham, 1962; Schiemenz, 1891). Additional support for a chemical drilling mechanism came from observations that the diameter of an engorged boring gland and that of the borehole were the same (Hirsch, 1915), and a demonstration that secretions obtained from the boring gland of *Natica* could remove the gloss from a polished shell (Ankel, 1937). In later studies, observing the etching effect of ABO on shell was hit or miss; Ankel (1938) proposed that "calcase," a compound active under only certain conditions, was responsible for the chemical dissolution the shell. Other investigators around this time argued that only the rasping action of the radula on the substrate (a mechanical model) was necessary to drill a hole. The naticid boring gland was first described in the mid-nineteenth century by Troschel (1854), who believed it was simply a muscular sucker used to hold the prey during the drilling process, but did remark on the acidic secretions produced by salivary glands (Carriker & Gruber, 1999). The muscular nature of the structure and a failure to color litmus paper (Fischer, 1922) did not support a chemical role for the boring gland. This conclusion was supported by the investigations of Pelseneer (1925) and Loppens (1926) and later studies (Jensen, 1951; Ziegelmeier, 1954), who concluded that drilling must be solely mechanical in nature. This view was held until the 1960s, when ultrastructural analysis of the naticid boring gland (now termed the ABO) showed similarities to the ABO of muricids (Bernard & Bagshaw, 1969).

The mechanism underlying muricid boring was also contested. As with naticids, a mechanical process that involved only the rasping action of the radula on the substrate was favored by a number of researchers (Graham, 1941; Jensen, 1951; Pelseneer, 1925). The muricid ABO, first described in 1941 (Fretter), was also initially believed to act as a sucker to hold the snail in place during drilling (Fretter & Graham, 1962). This makes sense as, during the drilling process, the ABO everts and swells into a fungiform structure with a diameter about equal to that of the proboscis (Carriker, 1981; Carriker & Gruber, 1999; Fretter & Graham, 1962; Webb & Saleuddin, 1977). The role of the ABO in the drilling process was demonstrated through amputation experiments in *Urosalpinx* and *Eupleura*, where only those animals possessing both an ABO and proboscis were able to bore, and furthermore, both the ABO and the proboscis of these muricids regenerated rapidly following amputation (Carriker, 1959). Subsequent studies in 1972 further confirmed that both the

proboscis and ABO are essential when drilling hard substrate (Carriker et al., 1972; Carriker & Van Zandt, 1972a). Carriker was a proponent of the chemo-mechanical model of boring, whereby secretions from the ABO containing acid, enzymes, and chelators are used to soften the calcareous substrate, which is then rasped away by the radula. Thus, the borehole is drilled by an alternation of chemical dissolution and mechanical rasping until the hole is complete (Carriker et al., 1963; Fretter & Graham, 1962). Following the removal of the periostracum by radular rasping, Carriker and colleagues recorded periods of apparent inactivity lasting from a few minutes in naticids to approaching an hour in muricids, which were followed by brief periods of rasping. Periods of inactivity and rasping were alternated until a hole was either completed or abandoned (Carriker et al., 1963). Carriker interpreted these extensive periods of inactivity as when chemical dissolution was occurring and subsequent studies confirmed that secretions from the ABO contain enzymes, chelators, and acid (reviewed in Carriker, 1978; Carriker, 1981; Carriker & Gruber, 1999). The acidic nature of the secretions from the ABO were clearly demonstrated by Carriker et al., (1967) using a glass-shell model and pH-sensitive glass electrodes (a new development at the time). They determined that the secretions from the ABO of *Urosalpinx* could fall as low as pH 3.8. Moreover, etchings in shells and artificial substrates produced by ABO secretions were similar to those produced by exposure to HCl and EDTA (Carriker & Williams, 1978). Enzymatic secretions from the ABO have also been documented to include chelators (Bernard & Bagshaw, 1969; Person et al., 1967). (See reviews by Carriker, 1981; Carriker & Gruber, 1999; Carriker & Williams, 1978; Kabat, 1990.)

The most recent advance in our understanding comes from the identification of V-ATPases in the secretory epithelium of the muricid ABO (Clelland & Saleuddin, 2000). This, in addition to various anatomical and biochemical features of the ABO, including the long-established presence of carbonic anhydrase (CA) in the ABO of both muricid and naticid gastropods (Chétail & Fournié, 1969; Smarsh, 1969; Webb & Saleuddin, 1977), established a means of HCl production. This chemo-mechanical model of drilling is now widely accepted.

3.3.2 SHAPE OF BOREHOLES

Boreholes can have a characteristic size and shape based on the properties of the boring mechanism and the size of the borer. Both the chemical weakening and radular abrasion of the prey shell affect borehole characteristics

(Kabat, 1990). The diameter of the borehole is generally indicative of the size of the snail; large holes having been drilled by large predators, small holes by small predators (Kabat, 1990). Boreholes left by muricids tend to be uniformly cylindrical, with virtually straight edges; the diameter of the hole matching the size of the ABO of the drilling animal. Boreholes produced by naticids are shallower and appear to be countersunk due to the noticeably beveled edges. Moreover, the shape of the hole conforms to that of the ABO, to the extent that damage to an ABO (due to surgical manipulation or natural causes) can be identified in the borehole (Carriker & van Zandt, 1972b).

Should the thickness of the shell exceed the drilling limits of the predator, or something interrupts the predator, the borehole will be unsuccessful. Incomplete boreholes of muricids have a smooth bottom, whereas those of naticids have a shallower bowl and usually have a raised boss in the center (Fretter & Graham, 1962; Kabat, 1990). These characteristics can be used to differentiate the boreholes of muricids and naticids (and indeed those of other borers, such as octopus) from each other, for both recent and fossilized events (Sohl, 1969). The shell thickness that can be drilled by a muricid is effectively a function of the depth to which the ABO can be extended. For naticids, it has long been held that in order for the diameter of the inner hole to be sufficiently large to permit passage of the proboscis, the ratio of the diameter at the bottom of the borehole to that of the top cannot be less than 0.5 (Grey et al., 2005; Kitchell et al., 1981). Since the top diameter is a function of the diameter of the ABO, larger naticids, with correspondingly larger ABOs, can drill deeper holes than smaller ones. This metric has been used in paleobiological studies to make inferences about the putative size of naticid predators and was assumed to be relevant for all species. However, a species-specific component to borehole geometry, including the ratio of inner to outer borehole diameters, has been demonstrated for both extant and extinct naticid species. The inner-to-outer borehole ratios varied from 0.78 (*Euspira heros*) to 0.53 (*Euspira lewisii*) (Grey et al., 2005). Thus, a given borehole could be produced by either a smaller *E. lewisii* or a larger *E. heros*, demonstrating that caution must be taken when assigning the identity of a predator, based solely on the metrics of a particular borehole. Interestingly, in the case of muricids, a larger animal could potentially enlarge an existing borehole made by a smaller animal in an unsuccessful attempt to breach the shell of the prey, since incomplete boreholes may be detected and used as points of entry (Hughes & de B. Dunkin, 1984). In the case of naticids, boreholes are almost always started fresh, regardless of the presence of incomplete holes, and any reoccupation of holes appears to occur only by chance, due to stereotypic prey handling behavior (Kitchell et al., 1981).

3.3.3 THE MECHANICAL PROCESS

Muricid boreholes are produced mainly by the chemical dissolution of the shell by the secretions of the ABO, combined with relatively infrequent rasping of the radula. Removal of chemically softened and dissolved shell is completed by the rasping action of the radula. During the rasping process, the ABO is retracted and the proboscis is inserted into the borehole at the anterior edge of the foot, through a channel that is formed by an upward folding of the propodium. The posterior part of the foot remains firmly attached to the shell, holding the predator in position (Carriker, 1981). It is the central rachite teeth of the radula that is responsible for the rasping of the shell, as evidenced by the wear they suffer during the boring process. Rasping is completed in a posterior direction, resulting in the front teeth wearing away fastest. Teeth are replaced at the posterior of the odontophore and move anteriorly in a continuous conveyor like fashion. In muricids, the odontophore is able to rotate through at least 180° to the left or to the right, such that the bottom of the hole is rasped in two complete hemispheres, which results in the smooth-bottomed borehole, characteristic of incomplete muricid boreholes. The marginal teeth of the muricids are sickle shaped and are believed to be used for the tearing of flesh rather than the rasping of shell (Carriker, 1969, 1981). Shell material that has been removed by rasping is swallowed and eliminated via the feces (Carriker, 1977; Carriker et al., 1963). The construction of a shell-glass model permitted Carriker et al. (1967) to view these processes, which otherwise are hidden from sight by the foot.

The mechanical boring process of the naticids was thoroughly described by Ziegelmeier (1954). This was made much more difficult due to the infaunal nature of the naticids; drilling is normally completed within the sediment out of sight, and the fact that they often envelop their prey with their large foot, further obscuring the boring process (Fretter & Graham, 1962). As with muricids, drilling is initiated by removal of periostracum, or other organic layers, on the shell of the prey by radular rasping. The ABO, which in naticids is found on the proboscis, is then applied to begin softening of the shell using acidic secretions. The borehole cavity is sealed by mucous secreted from glandular cells which surround the central stroma of the naticid ABO (Bernard & Bagshaw, 1969). After a period of softening, shell fragments are rasped away and swallowed. Unlike muricids, the naticid odontophore can only rotate 90° in either direction, with rasping progressing

in a posterior fashion. Rasping is always initiated from the centre of the borehole, and once a 90° sector is rasped, the proboscis is lifted. The ABO is reapplied to soften more shell, after which the proboscis is reinserted, but twisted to rasp a different 90° sector (often the opposite direction). Mechanical rasping is alternated with chemical softening in the described fashion, until the hole is completed or abandoned. The boss that is characteristic of incomplete naticid boreholes results from the fact that rasping is always initiated from the inside to the outside, leaving more unrasped material in the center compared to the periphery. Drilling is completed as a series of overlapping quadrant-sized sweeps of the odontophore, alternating left and right inside the larger circle formed by the central disk area of the ABO, which continue in a progressive fashion (Fretter & Graham, 1962).

3.3.4 ANATOMY OF THE ABO

The ABO of muricid gastropods is located in the anterior mid-ventral region of the foot whereas that of naticids is located on the underside of the proboscis, at the anterior-ventral lip (Fig. 3.1). In males, the muricid ABO is located in a vestibule (crypt) situated behind the transverse furrow (Fig.3.1A), while in females, it is located atop the ventral pedal gland (Fig.3.1B) or in a vestibule situated between the transverse furrow and the ventral pedal gland (Fig.3.1C; Carriker, 1981). The ABO in these two families are remarkably similar, but for their location on the foot and proboscis, respectively. They must have arisen independently, representing a “striking case of convergent evolution” (Carriker & Gruber, 1999; Kabat, 1990). Viewed in cross section, the muricid ABO is mushroom shaped, with a cap and a long stalk (Fig. 3.2). The ABO is everted through muscular contraction of foot around the vestibule, which squeezes blood from the contiguous blood sinus of the stalk into the cap, extruding the ABO from the sole of the foot. When the foot relaxes, the ABO is retracted via longitudinal muscles in the stalk, pulling it back into the crypt and out of sight. The cap of the ABO is composed of a single layer of long epithelial cells with a prominent brush border. Mucous glands are present in the short epithelial cells of the foot tissue surrounding the ABO, rather than being in the ABO proper. Secretions from these mucous glands form a seal around the borehole when the ABO is everted, preventing seawater from diluting the secretions of the gland (Clelland & Saleuddin, 2000).

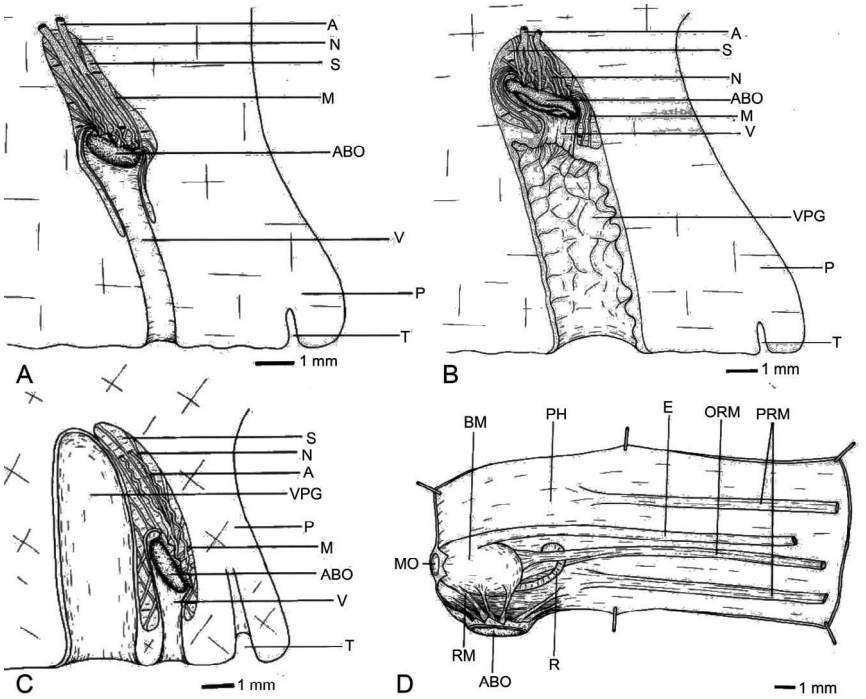

FIGURE 3.1 Muricid and naticid ABO anatomy. (A–C) Drawing of sagittal section of the anterior part of muricid ABO. (A) Foot of a male *Rapana thomasiana*. (B) Foot of female *R. thomsiana*. The ABO is located atop the ventral pedal gland and passes through the lumen of the gland when everted. (C) Foot of a female *Urosalpinx cinerea follyensis*. Arteries, A; nerves, N; muscles, M; propodium, P; transverse furrow, T; S, ABO sinus containing arteries (A), nerves (N) and muscles (M) passing to the back of the ABO; V, ABO vestibule through which ABO is extended to the borehole; ventral pedal gland, VPG. (D) Drawing of the left side of proboscis of the naticid *Polinices duplicatus*, opened laterally. Buccal mass, BM; esophagus, E; mouth, MO; odontophoral retractor mussel, ORM; proboscidial hemocoel, PH; radular sac, R; retractor muscle, RM. (Modified from Carriker, M.R. *Malacologia* **1981,**20, 405–406 with permission.)

In contrast, the naticid ABO appears as a shapeless mass of tissue attached to the lower lip when flaccid, but reveals a fungiform pad with a diameter equal that of the proboscis when engorged with blood (Fig.3.1D). Viewed in cross section (Fig.3.3), the gland is connected to the ventral side of the distal tip of the proboscis via a short stalk. A blood sinus contiguous with the proboscis connects to the ABO. The central epithelium of the naticid ABO is composed of long epithelial cells similar to those of the muricid ABO, but this central disk is surrounded by a ring of short mucous secreting cells that are separated from the central disk by a narrow band of lateral epithelium.

Secretions from the mucous cells of the ABO serve the same function as those in the foot of the muricids; they seal the central region of the ABO against the borehole, preventing dilution of secretions (Bernard & Bagshaw, 1969). The difference in the anatomy of the two ABOs is a major cause of the differences in the resulting borehole shape (Bernard & Bagshaw, 1969; Clelland & Saleuddin, 2000; Fretter & Graham, 1962; Kabat, 1990). The diameter of the ABO and the mean width of the radula (at the drilling position) are loosely related to the size of the individual and to the species, although the correlation is not strong. In a comprehensive listing of boring gastropods (Carriker & Gruber, 1999), the size of the ABOs ranged from 0.9 to 4.4 mm (mean diameter) and the radula varied from 0.12 to 1.4 mm (mean width), while shell height (as an index of size) varied from 12.1 to 115.0 mm in mean diameter.

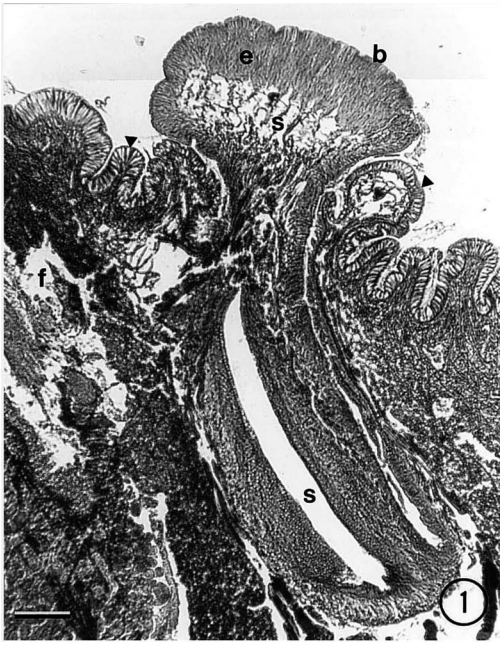

FIGURE 3.2 Section of an everted ABO of *Nucella lamellosa*, stained with Mallory–Heidenhain quick stain. Note the mushroom shape of the organ. The cap is composed of a single layer of epithelial cells (e) surrounding a central sinus (s), which is contiguous with the sinus in the stalk. The stalk invaginates into the foot (f). The epithelial cells of the ABO cap are long (200–300μm), with a prominent brush border (b). By contrast, the epithelial cells of the surrounding foot tissue (arrow heads) are short (40μm). Muscle is seen in the left-hand side of the stalk and is presumably used to retract the ABO into its crypt (vestibule). Scale bar: 200μm. (From Clelland, E.S.;Saleuddin, A.S.M. *Biol. Bull*.**2000,***198*, 276,with permission.)

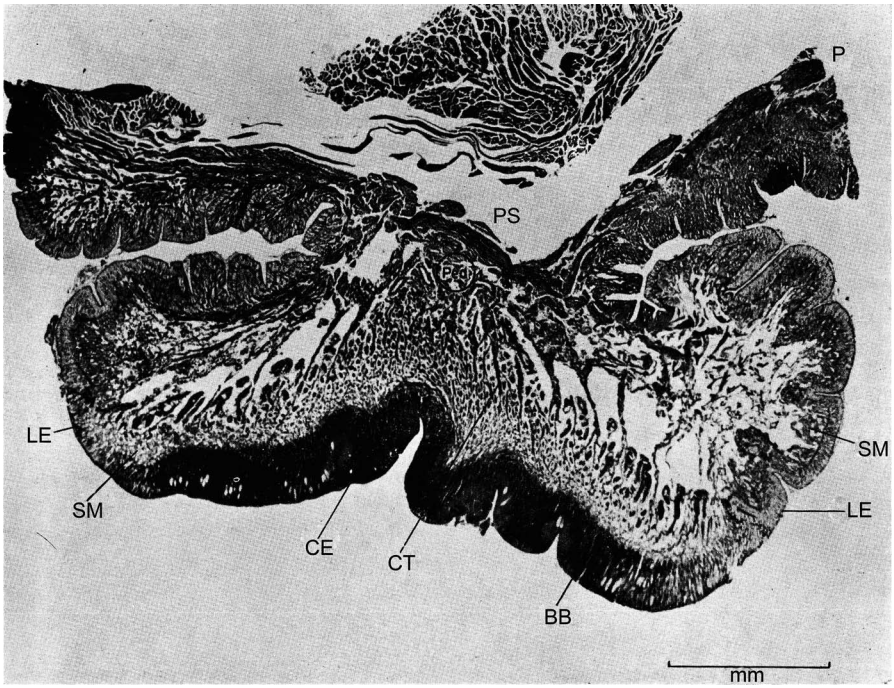

FIGURE 3.3 Median sagittal section of the ABO of *Polinices lewisi*. Stained by Lillie's Allochrome. Brush border, BB; central epithelium, CE; connective tissue, CT; lateral epithelium, LE; proboscis, P; proboscidal sinus, PS; subdermal mucocytes, SM. Scale bar = 1 mm. (From Bernard, F.R.; Bagshaw, J.W. J. *Fish. Res. Bd. Canada* **1969,** *26*, plate 1, © Canadian Science Publishing or its licensors. (Used with permission.)

3.3.5 FINE STRUCTURE OF THE ABO

To our knowledge, only one study has been conducted on the fine structure of a naticid ABO, *Polinices lewisi* (Bernard & Bagshaw, 1969); thus, details are primarily from investigations of muricid ABOs. The cap of the muricid ABO and the central disc of the naticid ABO are formed by a single layer of tall secretory cells (Fig. 3.4). These cells have three regions; apically they possess prominent microvilli and numerous mitochondria, the intermediate zone contains relatively few organelles, and the basal zone contains the nucleus, Golgi complex, and endoplasmic reticulum (Bernard & Bagshaw, 1969; Carriker & Gruber, 1999; Derer, 1975; Nylen et al., 1969; Webb & Saleuddin, 1977). The cells are arranged in compact groups that form a continuum over the surface, but are separated basally by blood-filled interstitial spaces. An elaborate network of blood vessels, nerves, and muscle

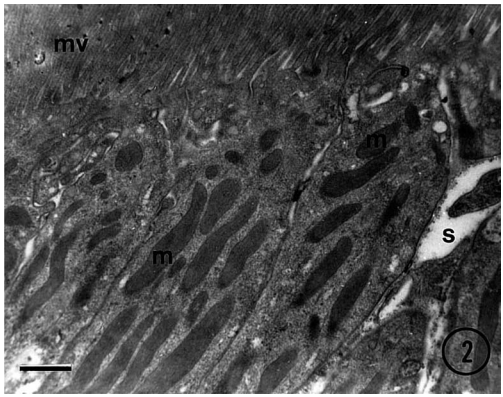

FIGURE 3.4 TEM of the apical region of the ABO cap epithelium. Note the long microvilli (mv) of the brush border, the numerous mitochondria (m), and the interstitial spaces (s) between the cells. Dense granules seen in the cells may contain degradative enzymes. Scale bar = 1.5 mm. (From Clelland, E.S.; Saleuddin, A.S.M. *Biol. Bull*. **2000,** *198*, 281,with permission.)

pass through the ABO sinus to the apex of the epithelium. Blood is drained across the basolateral membranes into the interstitial spaces then into the open sinus. The basal membranes of the cells are highly infolded, increasing surface area, and ensuring efficient transport of gas, nutrients, and metabolites. Pools of glycogen are present in the basal regions of the cells and accumulate during inactive periods, providing energy for cellular functions when the ABO is active (Carriker & Gruber, 1999; Webb & Saleuddin, 1977). Apically, the cells are joined together by various junction complexes, including gap junctions, which serve to coordinate the action of the cells (Clelland & Saleuddin, 2000; Nylen et al., 1969). Secretion granules are produced in the basal region and are believed to travel apically via star-shaped interstitial ducts to the base of the microvilli (Carriker & Gruber, 1999), where they discharge from the ABO (Nylen et al., 1969). Synthesis and discharge of secretory products is highest during periods of active boring (Carriker & Gruber, 1999). Uniform 3 nm particles, lining the inner side of the microvillar membranes, noted in *Nucella lapillus*, were believed to indicate the presence of proton pumps (Derer, 1975), and subsequent identification of V-ATPase in the microvilli of *Nucella lamellosa* (Clelland & Saleuddin, 2000; Fig. 3.5) substantiates this claim. Active ABOs are conspicuously different in appearance than inactive ABOs, with glandular cells that are taller, have longer microvilli, and contain more secretory granules, endoplasmic reticulum, and lysosomes than those of inactive ABOs (Carriker

& Gruber, 1999). Active ABOs also contain relatively little glycogen when compared to inactive ABOs (Chétail et al., 1968) and the interstitial sinuses of active ABOs also have higher concentrations of hemocyanin (Carriker & Gruber, 1999; Provenza et al., 1966).

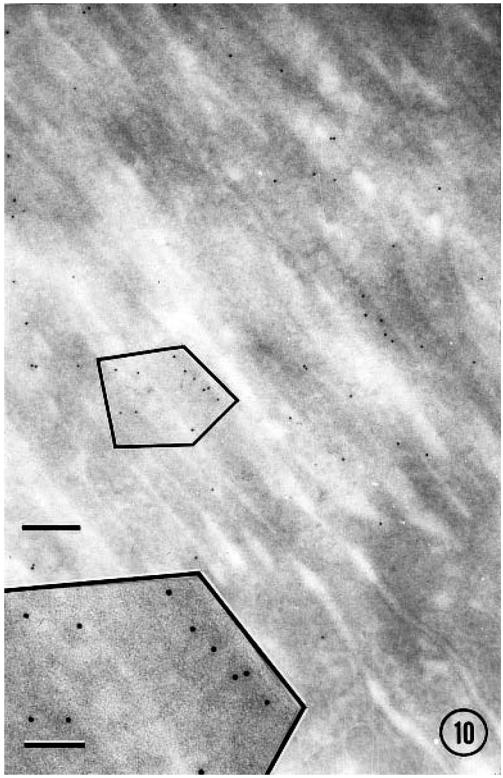

FIGURE 3.5 TEM of the microvilli (mv) that form the brush border of the ABO of *Nucella lamellosa.* Black spots are gold nanoparticles immunoconjugated to V-ATPase (39-kDa d subunit) showing that the immunoreactive sites for V-ATPases are located in the microvillar membrane. Scale bar = 200 nm. Inset: Gold particles lie close to the plasma membranes of the microvilli. Scale bar = 50 nm. (From Clelland, E.S.; Saleuddin, A.S.M. *Biol. Bull*. **2000,** 198, 281, with permission.)

3.3.6 PHYSIOLOGY OF THE ABO

Although the physiology of the ABO has been investigated in only a small number of primarily muricids (Carriker & Gruber, 1999; Clelland & Saleuddin, 2000), it is believed to be similar for all ABOs. The physiology

of the ABO varies depending on whether the animals are fed or unfed, and if the ABO is active and everted (Carriker & Gruber, 1999; Franchini et al., 1983). The ABO produces a number of enzymes such as cytochrome oxidase, succinate dehydrogenase, lactate dehydrogenase, lipase alkaline, and acid phosphatases. The ABO is also rich in CA (Carriker & Gruber, 1999 and references therein). ABOs of starved individuals are poor in various secretory granules and vesicles, relative to those of satiated controls, but this situation is quickly remedied after feeding. It is believed that ABO activity and protein synthesis are under nervous control, as part of the overall regulation of drilling behavior (Carriker, 1981; Franchini et al., 1983). Changes in synthesis rates of various enzymes associated with activation of the ABO are difficult to quantify objectively due to differences in ABO isolation techniques, treatment and fixation protocols, assay procedures, and inconsistencies in the literature regarding the state of the ABO at the time of examination (Carriker & Gruber, 1999). However, it is generally accepted that synthesis increases in active ABOs, as aerobic processes are enhanced through increased blood flow into the ABO and by consumption of glycogen reserves built up during inactive periods (Nylen et al., 1969; Person et al., 1967).

The most prominent enzyme of the ABO is CA (Carriker & Gruber, 1999), where it is localized in the secretory cells of the ABO in concentration levels much higher that surrounding pedal tissues (Smarsh, 1969). Smarsh (1969) also found CA in the brush border, whereas Webb and Saleuddin (1977) did not. Similarly, many investigators have noted that CA is about equally abundant in active (boring) and inactive (resting) ABOs, while others have noted an increase in active ABOs (Carriker & Gruber, 1999). CA is associated with secretory cells known for active transport of hydrogen ions, bicarbonate ions, and carbon dioxide (Webb & Saleuddin, 1977) and is a hallmark of the mitochondria rich (MR) cells known to utilize V-ATPase proton pumps (Brown & Breton, 1996; Clelland & Saleuddin, 2000; Harvey et al., 1998; Wieczorek et al., 1999). Here, they generate free protons and bicarbonate ions by catalyzing the reaction of water and carbon dioxide through a carbonic acid intermediary (Fig.3.6); the free protons being extruded from the cytoplasm by V-ATPases at the expense of ATP. While it has long been held (Carriker & Chauncey, 1973) that CA does not act directly as a demineralization agent, it is known to be vitally important in shell dissolution during boring, since application of specific CA inhibitors such as Diamox have been shown to impair the drilling process (Carriker & Gruber, 1999).

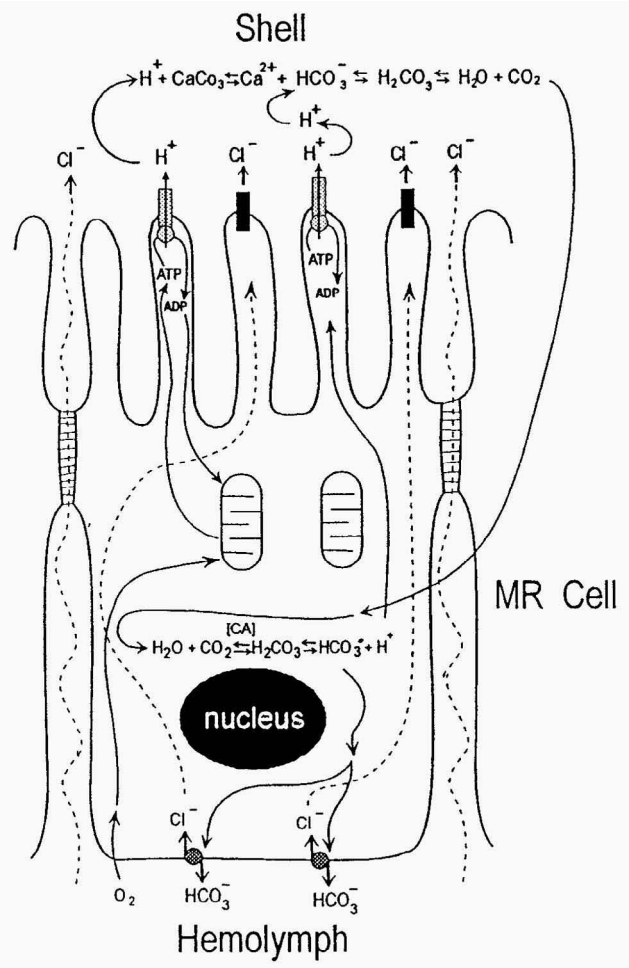

FIGURE 3.6 Model of the mechanism of proton transport in the muricid ABO. Carbonic anhydrase (CA) catalyzes the production of H^+ and HCO_3^-via the carbonic acid pathway. Bicarbonate is removed from the mitochondria-rich (MR) epithelial cell via basal HCO_3^-/Cl^-antiporters, while protons are extruded from the cell into the bore hole by V-ATPase pumps located in the microvilli. Mitochondria generate ATP to power the extrusion process, generate metabolic CO_2 for the carbonic acid reaction, and provide a reducing environment to stabilize V-ATPase molecules. Chloride ions exit the cell via apical ion channels, and possibly by paracellular routes. The protons and chloride ions (HCl) act to dissolve the mineralized component ($CaCO_3$) of the shell, while degradative enzymes also present in the secretions of the ABO break down the organic matrix. Carbon dioxide liberated from the dissolving shell may diffuse into the cell to enhance the carbonic acid reaction. The presence of HCO_3^-/Cl^- antiporters and, as speculated here, of chloride ion channels is based on comparable studies of MR cells in other animal epithelia. (From Clelland, E.S.;Saleuddin, A.S.M. *Biol. Bull*. 2000, *198*, 281,with permission.)

The secretion of the active ABO that is released onto the surface is granular and viscous, and generally insoluble in sea water. It contains membrane-bound vesicles and granules in a thick mucus, limiting its dispersion during boring (Carriker, 1981; Carriker & Gruber, 1999). It accumulates on the surface of the ABO during the rasping phase and is introduced into the borehole on the next dissolution phase (Carriker et al., 1978). Application of excised live ABOs to polished mollusc shells etches the surface, and is inhibited by co-application with papain, or heat treatment of the ABOs to 80°C prior to application (Carriker et al., 1978), suggesting the inactivation of an enzymatic component (Carriker & Gruber, 1999). The presence of protein in the ABO secretion has been also been demonstrated (Evans, 1980). Blots of the secretions obtained from active, everted ABOs using a "valve model for secretion collection" have been found to contain 1–2 μg of water-absorbent material after drying, but as much as an estimated one-third of the initial secretion is composed of volatile components which evaporate (Carriker et al., 1978). Furthermore, using this model, the authors were able to confirm the acidic nature of the ABO secretion (shown previously by Carriker et al. (1967), with measures ranging from pH 3.8 to pH 4.0, and to demonstrate that while the ABO was everted into the bore hole, the Cl^- concentration increased in a stepwise fashion, with maximal levels ranging from 0.79 to 1.71M; much higher than the 0.5M Cl^- content of the surrounding seawater.

When the ABO is engorged, increased blood flow infuses oxygen into the secretory cells, which stimulates aerobic processes within the cells. Aerobic synthesis is upregulated and acid production is initiated as mitochondrial activity and overall mitochondrial number increases, leading to the generation of additional ATP to drive various catalytic and transport processes (Carriker & Gruber, 1999; Carriker & Williams, 1978; Webb & Saleuddin, 1977). It is well established that the V_0 (transmembrane) and V_1 (catalytic) domains of V-ATPases can reversibly assemble and that the catalytic domain can activate, depending upon on the redox potential inside a cell or organelle (Merzendorfer et al., 1997). V-ATPases in the apical brush border of the ABO secretory cells are activated by changes in the redox potential when the organ is engorged with blood. Energy liberated by the catalytic conversion of ATP to ADP is utilized by these pumps to expel protons across the apical membrane and onto the substrate (Clelland & Saleuddin, 2000). As modeled by Webb and Saleuddin (1977) and Clelland and Saleuddin (2000), CA within the secretory cells catalyzes the production of $NaHCO_3$ and free protons. The free protons are pumped into the cavity of the borehole by the apical proton pumps, while the HCO_3^- is exchanged for Cl^- at the basolateral membrane. The chloride ions pass through apical membrane channels and

perhaps via paracellular routes into the borehole, thus maintaining the acid–base balance of the secretory cells, and upon binding with the protons in the borehole cavity, produce HCl (Fig.3.4).

3.3.7 CONVERGENCE OF THE ABO, MR CELLS, AND V-ATPASES

In a 1999 review, Carriker and Gruber describe the ABO of drilling gastropods as "unique among the organs of invertebrates." This compact fungiform organ has twice evolved physiological and biochemical mechanisms that permit penetration of the $CaCO_3$ external shells of their prey. As an organ, the gastropod ABO is unquestionably unique, albeit having evolved separately in muricids and naticids. However, the localization of V-ATPase proton pumps in the ABO of *Nucella lamellosa*, as well as a number of other characteristics common to the ABO cells of all gastropods thus far examined, suggests that the mechanism for decalcification of calcareous substrates is actually conserved (Clelland & Saleuddin, 2000).

A similar process has been shown to occur in bivalve mantle epithelium, where free protons and Cl^- are expelled by mitochondrial-rich (MR) cells against the shell surface to alleviate acidosis when the shells are closed (da Costa et al., 1999; Hudson, 1992; Hudson, 1993) and by MR osteoclast cells of vertebrate bone, during the processes of bone resorption and remodeling (Brown & Breton, 1996). Interestingly, as reviewed by Ehrlichet al. (2008), aquatic vertebrates such as turtles (shell), crocodilians (osteoderms), and amphibian (femur) also utilize the buffering capacity of bone to alleviate systemic acidosis by extruding HCl from MR cells against boney substrate. More recently, a bone resorption process has also been described for the polychaete boneworm, *Osedax*, where the MR cells of their dorsal root are involved in the dissolution of whale bone in which they reside (Tresguerres et al., 2013). In some epithelia, V-ATPases are known to change polarity (i.e., move between the apical and basolateral membranes of the cell), depending upon the requirements at a given time. For example, extrusion of HCl against the cuticle from apically positioned V-ATPases can aid moulting in the terrestrial isopod *Porcellio scaber* through the demineralization of calcium carbonate, whereas assembly in the basolateral membrane can drive processes leading to remineralization of the cuticle through uptake and accumulation of calcium (Ziegler, 2004). V-ATPase containing MR cells are found in the transport epithelia of fish gills (Sullivan et al., 1995), frog skin (Ehrenfeld & Klein, 1997), toad and turtle bladder (Brown et al., 1987),

insect Malpighian tubules (al-Fifi et al., 1998; Maddrell & O'Donnell, 1992), kidneys (Brown et al., 1988; Sallman et al., 1986), seminiferous tubules (Brown et al., 1997), etc. V-ATPase pumps are utilized to extrude protons at the expense of ATP, either to directly acidify specific compartments or to utilize the ensuing electrochemical gradient to drive the movement of ions or other molecules across cell membranes (Ehrlich et al., 2008; Ehrlich et al., 2009; Harvey et al., 1998; Merzendorfer et al., 1997; Wieczorek et al., 1999). In the tissues of developing pond snails, *Lymnaea stagnalis*, proton extrusion is linked to calcium uptake for shell deposition (Ebanks et al., 2010), while in insect Malpighian tubules, protons extruded from the apical membrane of the epithelial cells into the lumen of the tubule, return to the cell via cation antiporters in exchange for potassium or sodium, leading to the alkalinization of the tubule (Maddrell & O'Donnell, 1992). Thus, while the nature of these various epithelia (ABO, mantle, gill, Malpighian, root, osteoclast, etc.) are quite different, the fundamental machinery and function of the MR cells is similar; all possess abundant levels of CA to catalyze production of protons and bicarbonate, all have numerous mitochondria to produce ATP as fuel, and all possess electrogenic V-ATPase proton pumps. Subsequent studies in decapods (Ziegler, 2004), pulmonates (Ebanks et al., 2010), and polychaetes (Katz et al., 2010; Tresguerres et al., 2013), among others have provided further evidence in support of a conserved mechanism for decalcification *in vivo*. Reviews on the principles of demineralization (Ehrlich et al., 2008; Ehrlich et al., 2009) have included observations of the gastropod boring mechanism and the structure and function of the gastropod ABO amongst some of the most significant events in the history of demineralization in the past 500 years. These authors have also remarked upon the conservative nature of decalcification *in vivo*, and the involvement of V-ATPases in MR cells.

3.4 FEEDING BEHAVIOR AND ENVIRONMENTAL EFFECTS

Drilling gastropods are known to consume a variety of prey, although the majority of muricids eat bivalves, gastropods (including smaller individuals of the same species), and barnacles (Carriker, 1981; Kabat, 1990). Much of what is known about feeding behavior comes from observations of muricid snails, which are common intertidally and thus are relatively easy to study. For example, *Nucella lapillus* feeds primarily on barnacles and mussels (and occasionally other molluscs) in post juvenile stages (Hughes & de B. Dunkin, 1984a), and *Thais melones* feeds on a variety of bivalves, limpets,

and polychaetes (West, 1988). Muricids are also known to eat carrion, bryozoans, crabs, and although less so in the present, brachiopods (Kelley & Hansen, 2003; Leighton, 2003). Some juvenile muricids feed on ostracods (Reyment & Elewa, 2003). Strangely, there is almost no evidence of muricids drilling chitons; there is a single fossil drill hole attributed to muricids found on a chiton in the Late Pleistocene of Uruguay (Rojas et al., 2014), with almost no recent descriptions (Taylor & Morton, 1996).

Studies of naticids are more difficult because much of the activity occurs out of sight beneath the substrate (Kabat, 1990). Naticids are generally thought to be more restricted in their diets than muricids, eating primarily live bivalves (Carriker, 1981), in particular, infaunal species. Naticids are known to feed on other, mainly soft-substrate gastropods, and occasionally scaphopods. *Neverita duplicata* is the sole naticid that has been observed feeding on polychaetes (*Owenia fusiformis*) (Paine, 1963), although another naticid is implicated in the drill holes of *Ditrupa arietina*, another polychaete (Morton and Salvador, 2009). One species, *Conuber sordidus* has been observed feeding on Blue soldier crabs (*Mictyris*) and hermit crabs (Huelsken, 2011). There are also reports based on stereotypical drill holes of naticids drilling of egg capsules, of other gastropods and elasmobranchs, as well as brachiopods (Ansell, 1961; Cox et al., 1999; Kabat, 1990; Leighton, 2003). Incidents of scavenging and feeding on fish that seem to be limited to aquarium situations, but field observations have been made (Kabat, 1990; Kelley & Hansen, 2003). Juvenile naticids are even thought to prey on ostracods (Reyment & Elewa, 2003) and foraminiferans (Arnold et al., 1985; Culver & Lipps, 2003).

From a distance, prey is located principally by the osphradium (chemoreception), where chemical odors carried on the current direct the predators toward their source (Carriker, 1981; Morgan, 1972; Rittschof et al., 1983). These odors are primarily composed of peptides and amino acids produced by metabolic processes such as digestion (Rittschof, 1980a; Rittschof, 1980b; Rittschof et al., 1983). Upon reaching the prey, tactile cues allow the predator to further assess the quality of the prey (Carriker & Gruber, 1999; Hughes & de B. Dunkin, 1984a; Kabat, 1990). Predators are able to discriminate amongst these cues to identify specific types of prey and can often develop preferences for particular foods (Carriker, 1981; Wood, 1968); with time and experience, they can learn to select the optimum sized prey and attack methodology (Hughes & de B. Dunkin, 1984a; Hughes & Drewett, 1985; Rovero et al., 1999). *Urosalpinx* can discriminate between starved and satiated oysters, preferentially feeding on the latter (Carriker, 1981). Preference for particular prey is not genetically fixed, and laboratory experiments

demonstrate that predators prefer effluents from prey species they have recently fed upon (ingestive conditioning) over those of other prey species (Carriker, 1981). Field studies have also demonstrated that there may be wide intraspecific variation in prey selection (West, 1986). *Nucella lapillus*, for example, may develop preferences for either mussels or barnacles (Hughes & de B. Dunkin, 1984b; Hughes & Drewett, 1985) and learns to how to best manipulate its prey to minimize handling time (Rocha-Barreira et al., 2004; Rovero et al., 1999). Similarly, *N. emarginata* are selective in the size and nature of their bivalve and barnacle prey, depending on their size and experience (Palmer, 1990).

In addition to chemoreception, Naticids also likely detect vibrations produced by their prey, a method that is better suited to their infaunal lifestyle than the hard substrate preferred by many muricids (Kitching & Pearson, 1981). Naticids have also been observed foraging with the siphon extended to the surface, where directionality and strength of chemical cues are less likely to be perturbed or diffused by the substrate. They display stereotypical feeding behaviors involving detection of prey, followed by evaluation and seizure. Prey is enveloped in thick pedal mucous, which may contain an anesthetic, wrapped in the foot, dragged some distance away, and then carried deeper into the sand where boring begins (Carriker, 1981; Kabat, 1990). Naticids are usually absent from high-energy, wave-disturbed beaches where olfactory and vibrational cues tend to be diffused. An interesting exception, is the moon snail *Polinices incei*, native to exposed beaches of Queensland, Australia, which has evolved a surfing behavior. They float upside down via an inflated foot to get caught in the wash and swept up the beach. There they attack juvenile surf clams (*Donax deltoides*). *P. incei* hunts infaunally or by galloping on the surface of the sand to catch their leaping clam prey (Morton, 2008). Once seized, prey is enveloped by the foot and the snail rolls down the beach in the surf, presumably to less turbulent areas for drilling and consumption (Morton, 2008).

Drilling is not always limited to calcareous exoskeletons, which helped confound the debate on the nature of the drilling process (Bernard & Bagshaw, 1969). Penetration of soft tissues of egg cases of gastropods (Jensen, 1951) and elasmobranchs (Ansell, 1961) was likely completed by mechanical rasping alone, and no evidence of this would remain even if there was participation from the ABO. Furthermore, drilling is often unnecessary in instances where there is ready access to soft tissues. For example, the proboscis can be inserted between the operculum plates of barnacles, through the valve openings (gape) of bivalves, or via the aperture of gastropods, to feed without drilling. Field and laboratory studies have shown that

with age (i.e., size) and experience, predators are more likely to minimize or forego drilling whenever possible (Kabat, 1990; Morgan, 1972; Palmer, 1990; Rocha-Barreira et al., 2004; Rovero et al., 1999). For example, under laboratory conditions, *Thais haemastoma floridana*, consumed about 31% of feed oysters by forcing the proboscis through the valves, and of those drilled, virtually all holes were found along of adjacent to the shell margins (Rocha-Barreira et al., 2004). Some muricids can penetrate a barnacle or mussel by ramming the margin with a labial tooth, a downward protrusion from the aperture (MacGinitie & MacGinitie, 1968; Perry, 1985; Spight & Lyons, 1974). Some tropical naticid species are also known to preferentially drill bivalve prey at the edge of the valves, where the shell is thinner and easier to bore (and holes are often overlooked) and they may forego drilling entirely if there is sufficient gape for insertion of the proboscis. Still other species have been found to smother their prey, although recent work suggests this may be an artifact of lab conditions (Visaggi et al., 2013). In the case of gastropod prey, it is possible to pry open the operculum of the prey to permit access to the soft tissues via the aperture (Kabat, 1990). The presence of narcotizing or toxic agents in the saliva to paralyze the prey are also in the repertoire of some muricid and naticids (Andrews et al., 1991; Gordillo, 2013; Rovero et al., 1999) and can remove the need for drilling.

The foraging and feeding behaviors of predatory gastropods are complex, involving factors ranging from ingestive conditioning and prey experience to abiotic factors such as temperature or salinity. Especially for intertidal species, variation in the mortality risks associated with feeding and foraging can alter feeding patterns, and when environmental conditions are severe, these may almost entirely determine the timing of foraging bouts and resting periods (Burrows & Hughes, 1991). Furthermore, extended periods of unfavorable foraging conditions tend to synchronize foraging and resting periods in a population, since every individual begins the first subsequent foraging bout with an empty stomach (Burrows & Hughes, 1991). Selection of drilling site is another confounding factor. *Thais haemastoma* can immediately perceive the presence of prey oysters under laboratory conditions and preferentially drills near the shell border to minimize drilling time and forgoes drilling when possible (Rocha-Barreira et al., 2004). Pharmacologically active compounds present in the saliva of predatory gastropods (e.g., *Nucella lapillus*, *Thais floridanum*, etc.) favor edge drilling or gape access via valves. Only small openings are required to introduce paralytic compounds into the tissues, to relaxing adductor muscles, and allowing for rapid access to the tissues (Andrews et al., 1991). The hypobranchial and accessory salivary glands of many muricids contain active compounds, including the potent

paralytic urocanylcholine and various other choline esters (Andrews et al., 1991; Keyl et al., 1957; Whittaker, 1960). These are the compounds responsible for the intense color of Tyrian purple, long harvested from Mediterranean muricids for dye (Keyl et al., 1957). Despite the advantages of drilling in areas of the shell where it is the thinnest, the location of the borehole (if present) is often simply a consequence of which part of the shell is accessible for drilling during prey manipulation (Kabat, 1990). Environmental factors also impinge on feeding behavior. Temperature has a major effect on temperate and boreal species such as the naticids *Neverita duplicata* and *Euspira heros* which do not feed at low temperatures (2 and 5°C, respectively), nor at salinities below 10‰ (Kabat, 1990). Adult *Nucella lapillus* are subject to torpor below 5°C, but have their highest growth efficiencies at moderate temperatures (between 10 and 15°C). Temperatures above 20°C and below 10°C have a negative impact on growth (Stickle & Bayne, 1987). For juvenile *N. lapillus*, the drilling time is roughly constant between 10 and 20°C, while the ingestion time drops from approximately 45to 15 min (i.e., by ~2/3) across this range (Miller, 2013). Warm water species such as the Southern oyster drill, *Stramonita haemastoma*, are also affected by temperature and salinity, with optimal growth rates in the 25–35°C range, suboptimal growth occurring at 20°C, and 50% lethality at 37.5°C. Temperatures in excess of 40°C are 100% fatal. These animals limited to salinities greater than 15‰ (Brown & Stickle, 2001). Aerial exposure also becomes an issue for these snails at high temperatures, most likely due to the elevated metabolic requirements at higher temperatures.

Drilling rates have been published over the years for various muricid and naticid species, for example 0.3–0.5 mm per day for *Urosalpinx cinerea* (Carriker & Williams, 1978), 0.36 mm per day for *Nucella lapillus* (Hughes & de B. Dunkin, 1984a) and for naticids, 0.6 mm per day for *Euspira nitida* (Ziegelmeier, 1954), ~0.54 mm per day for *Neverita duplicata* (Kitchell et al., 1981), and so on (Fretter & Graham, 1962; Kabat, 1990). Environmental effects such as temperature and salinity are now known to affect the feeding rate; prey investigation time, drilling time, and consumption time (Kabat, 1990), so published rates should be viewed only as approximations. They are useful, however, for estimating the quantity of prey consumed under various environmental conditions (Kabat, 1990; Miller, 2013).

Once the shell of a prey animal is pierced, additional predators and scavengers are quickly attracted to the site due to the increased release of odors via enzymatic digestion of the prey tissue (Rittschof, 1980b). These include hermit crabs, which may be attracted as scavengers or as opportunists waiting for an empty shell. These competitors may compete with the original borer

for possession of the prey or in the case of larger bivalve prey, may simply wait until the prey is agape, then take advantage of the opportunity to feed at a different site feed alongside at little cost to themselves.

Another interesting facet of the behavior of predatory gastropods involves notions of cannibalism. Cannibalism is relatively common in the animal world (Kelley & Hansen, 2007) and examples of intraspecific predation (true cannibalism) and interspecific predation of gastropods can be found in the literature for both extinct and extant species (Gordillo, 2013). Muricid cannibalism likely occurs in situations where there are too many predators and insufficient prey, a view supported by observations of the fossil record for Halocene and living specimens of *Trophan geversianus* from South Africa (Gordillo, 2013). Muricid cannibalism has been recorded in the field for *Murex trunculus* and in the laboratory for *Triplofusus giganteus* (Kelley & Hansen, 2007). Evidence of naticid cannibalism dates back to the Cretaceous Period (Gordillo, 2013; Kelley & Hansen, 2007); examples from the Miocene St. Marys Formation in Maryland including cannibalism in two common species *E.heros* and *N. duplicata* (Kelley & Hansen, 2007) and in Pliocene naticids from southern France and Antarctica (Gordillo, 2013). Energetically, there is a benefit to cannibalism in naticids; high success rates of penetration due to thin shells and the opportunity for prising, coupled with the high energy richness of the prey's flesh, yield a high energy gain per foraging unit in cost–benefit analyses (Gordillo, 2013; Kelley & Hansen, 2007; Kitchell et al., 1981).

3.5 PALEONTOLOGICAL HISTORY OF DRILLING PREDATION

The drilling predation of gastropods is of great interest to paleontologists. This behavior is one of the few that leaves a direct record of predation through trace fossils (ichnofossils). Paleontological studies are limited in their ability to determine actual ecological interactions. In the case of predation, most evidence is indirect and can be difficult to interpret. Direct evidence comes from traces of predation, such as drill holes or breakage scars. Other types of direct evidence are rare: coprolites, stomach contents, or exceptional preservation events showing an actual interaction preserved (Kowalewski, 2002). There are some clear limitations, however, and steps must be taken to ensure holes are infact due to drilling by a predator (Carriker & Yochelson, 1968; Kelley & Hansen, 2003). Some marks can be made abiotically, or could be made biotically for other purposes such as parasites or epibionts. Drilling offers further advantages over other evidence such as breakage scars as they

are easier to recognize and differentiate from abiotic damage (Harper et al., 1998). Numerous authors have listed possible criteria to distinguish drilling from other holes: holes should be circular to oval, perpendicular, drilled from outside to in, regular placement or size, and usually not more than one complete hole (Kelley and Hansen, 2003), and references therein). Furthermore, there are a number of assumptions that must be made about predator and prey behavior and taphonomy that can bias the data, and must be taken into account (Kelley & Hansen, 2003; Kowalewski, 2004; Leighton, 2001).

Fossil traces of predation, including drill holes, offer a number of benefits. These traces are common in a broad range of environments, taxa, and times, and are easily preserved in the hard prey skeletons. This allows for quantification of predation events across time and space on a variety of scales (Kowalewski, 2002; Kowalewski, 2004; Kowalewski et al., 1998). A primary direction of inquiry relates to how predation affects evolution, through coevolution and/or escalation, and how the magnitude of these interactions compares to abiotic selective pressures (Vermeij, 1987; Vermeij, 1994).

Fossil drilling data are commonly used to determine predation frequency, but can also be used to look into other aspects. Predation efficiency can be estimated by comparing complete and incomplete drill holes, although this is more problematic for muricids who can restart previous holes, eat without completing a drill hole, and some drill cooperatively (Kowalewski, 2004; Taylor & Morton, 1996). Size relationships can be determined as the size of the boring is related to the size of the predator, although as previously mentioned, care must be taken if assigning the identity of specific predators, due to uncertainty in the borehole ratio parameters (Grey et al., 2005). Selectivity of predators can be examined by determining the distribution of drill holes in prey, and prey choice is demonstrated by which species show similar drillings (Kelley & Hansen, 2003). Kelley and Hansen (2003) compiled a summary of this body of research, and concluded that the situation is complicated and requires more work; however predator–prey interactions do have important effects on evolution on a large scale, and support hypotheses of escalation. There is evidence for increased selectivity on drilling position, increased size selectivity, increased conforming to optimal foraging strategy, and increased prey defensive capabilities over time.

The first drill holes appear before the Cambrian, in the tubes of *Claudina* (550 mA), and predatory boring is found throughout the fossil record to the present day (Bengtson & Zhao, 1992; Hua et al., 2003; Kelley & Hansen, 2003; Kowalewski et al., 1998). Except during the Cenozoic, most drill holes do not have a hypothesized driller; unknown gastropods are most likely,

although octopods, nematodes, or flatworms are also possible (Kabat, 1990; Sohl, 1969). There is a reasonable record of Paleozoic drillers with a peak in the Devonian, where most holes were found in brachiopods and echinoderms (Kelley & Hansen, 2003; Kowalewski et al., 1998; Leighton, 2003). Platyceratid gastropods have been found *in situ* parasitizing echinoderms, identifying at least one Paleozoic driller, though they go extinct before the Mesozoic (Baumiller, 1990; Baumiller, 1993; Baumiller, 1996). Evidence of drilling is quite weak throughout most of the Mesozoic. A sharp increase in drilling occurs in the Late Cretaceous, probably corresponding to the evolution and diversification of modern drillers (Kowalewski et al., 1998).

The origin of the Naticidae and their drilling is not well understood; the evidence of possible naticid boring is from the Late Triassic. This is followed by a large 120 million year gap in the record of drill holes, where there are few reported drillings. Some of these Triassic borings are associated with the Ampullospirinae, whose relationship with the naticids is in unclear (Kabat, 1990; Sohl, 1969). This group has even been suggested to be herbivorous, but this interpretation is not well supported (Aronowsky & Leighton, 2003; Kase & Ishikawa, 2003a; Kase & Ishikawa, 2003b). If these earlier holes were not made by naticids, it leaves in doubt the driller, even after naticids have clearly evolved (Kabat, 1990). Naticid shells and drill holes are generally accepted as present starting with a few records in the late Cretaceous, and then diversifying into the modern fauna (Aronowsky & Leighton, 2003; Harper et al., 1998; Kabat, 1990; Kowalewski et al., 1998; Sohl, 1969).

The origin of the Muricidae is less muddy, there is at least one site with undisputed muricid shells seen in the Late Cretaceous, and these are accompanied by muricid-type boreholes (Harper et al., 1998; Merle et al., 2011). There is evidence of muricid-like boreholes earlier in the Mesozoic, but these are generally attributed to some unknown, convergent, culprit (Harper et al., 1998). The family radiated from there to the current diversity of extant muricids (Kowalewski et al., 1998).

3.6 FUTURE DIRECTIONS

Research conducted over the past 100 years, primarily from the 1940s onward, as led to a solid understanding of the mechanism of drilling utilized by muricids and naticids. The fundamental physiology of the ABO is well understood and the mechanism for production of acidic secretions has been established. This said, there are still some gaps that need to be filled. Almost nothing is known about the other gastropod groups that drill prey, and

observations and experiments similar to those previously done on muricids and naticids would broaden our understanding of drilling predation and its evolution. Considering the behavior of so many gastropods is still unknown, it seems likely other groups will continue to be added to this list, whether as confirmation of previous claims or as new groups (Sohl, 1969).

The presence of V-ATPase in the apical epithelia of the ABO secretory cells should be confirmed in naticids, as well as additional muricid species; the comprehensive listing of boring gastropod species provided by Carriker and Gruber (1999) could serve as a guide for these studies. Technology has advanced such that these studies would be much simpler. Whereas Clelland and Saleuddin (2000) utilized noncommercial V-ATPase antibodies, commercially produced antibodies are now available for virtually all of the ~13 V-ATPase subunits. Alternatively, utilization of molecular biological techniques (mRNA isolation, ssDNA synthesis, sequence alignment, etc.) to determine conserved epitopes within target subunits for the production of antibodies (Tresguerres et al., 2013) targeted against V-ATPase peptides of predatory gastropods, should now be a relatively straightforward undertaking. Such antibodies could be employed in microscopic and peptide expression analyzes (Clelland & Saleuddin, 2000) using more modern techniques, such as immunofluorescence imaging and chemiluminescence. *In situ* hybridization analyzes of mRNA expression might also be informative. Along the same vein, although the cationic pathway is well established, questions still need to be answered regarding the chloride ion component. Identification of basolateral and apical anion exchange molecules (exchangers, channels, etc.) would help clarify the chloride pathway(s) in the ABO. Judicious application of antibodies to fixed specimens of inactive and active ABOs may also shed light on the activation of V-ATPases. If activation results from the assembly of V_0 and V_1 complexes as the ABO is engorged with blood, it should be possible to probe for V_1 specific subunits and compare their localization in active and inactive ABOs.

From a physiological perspective, the advent of relatively inexpensive versions of Bafilomycin, a specific inhibitor of V-ATPases (Clelland & Saleuddin, 2000) presents opportunities to conduct activity assays for V-ATPase in a grander scale. One can also envision use of this inhibitor to directly monitor the effects of its application on proton and chloride fluxes from live ABOs in Carriker's shell-glass model (discussed above). Scanning Ion-selective Electrode Technique (SIET) has been utilized to study such fluxes in most recent studies (Ebanks et al., 2010; Jonusaite et al., 2011; Nguyen & Donini, 2010) and could be adapted to the shell-glass arrangement. SIET provides exquisitely precise measures of ion flux. Effects of other

pharmacologicals (CA inhibitors, channel blockers, etc.) on ion flux could also be determined using this technique. Additional study is needed to identify other components of the ABO secretions, most specifically, the as yet unknown calcium chelator (Carriker & Gruber, 1999).

The recent paper by Miller (2013) poses some interesting questions regarding the effect of climate change on the activity of predatory gastropods. Temperature changes could have the effect of prolonging the length of the feeding period in cold water species, but may well extirpate warm water species should temperatures rise to lethal threshold levels (Brown & Stickle, 2001). Effects of increasing ocean acidification may also play an important role. Increased temperature and lower pH are likely to place stress on larval and juvenile snails (and those of their prey) which may impinge on their viability. In adults, shells of both predators and prey may become thinner, which will have an impact on drilling rates, the ability to survive in active surge zones, and an increase overall metabolic rate. From the perspective of drilling, increased temperature and lower pH would be expected to speed up the drilling process.

3.7 CONCLUSIONS

The chemo-mechanical process utilized by naticid and muricid gastropods to drill into hard substrate is a fascinating process. The development of specialized glands, the ABOs, for the production of acids, enzymes, and chelators used to dissolve calcareous substrate is unique, as is the drilling process, where chemical softening of substrate is alternated with mechanical rasping, until a hole is bored. The cells of the ABO themselves, however, fall within a broader grouping of similar MR cells characterized by high levels of CA, V-ATPase proton pumps, chloride transport molecules, etc. which are used by animals of a number of phyla for the dissolution calcified substrates. Whereas the drilling process is well understood in the naticids and muricids, there remains numerous aspects of the process that await further investigation (e.g., the elucidation of the anionic components of ABO physiology) and confirmation of the process as modeled, as well as its conservation or diversity in other groups. We look forward to reading such reports in the future.

KEYWORDS

- **moon snails**
- **molluscs**
- **muricids**
- **naticids**
- **accessory boring organ**
- **drilling predation**

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CHAPTER 4

THE ROLE OF METAL IONS IN THE MUSSEL BYSSUS

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CONTENTS

ABSTRACT

Marine mussels (Mytilidae) are sessile bivalve mollusks that populate the highest wave-impact regions of rocky seashore habitats. A key to their evolutionary success in the intertidal environment is the byssus—a protein-based fibrous anchor that is fabricated by the mussel. The byssus is made up of numerous byssal threads, which are without hyperbole, the lifeline of the organism. Each byssal thread can be subdivided into three distinct functional elements—the core, the cuticle, and the plaque, which function as a self-healing shock-absorbing tether, an abrasion resistant coating, and a versatile underwater glue, respectively. A critical feature of each of these three elements is the presence of protein–metal coordination interactions that influence to a large degree the material properties. In particular, interactions between histidine and Zn/Cu contribute significantly to the deformation and self-healing behavior of the thread core, while interactions between 3, 4-dihydroxyphenylalanine and Fe enhance the mechanical function of the cuticle, as well as the formation and adhesive properties of the plaque. In the present chapter, we will provide a comprehensive overview of the state-of-the-art understanding of the role of protein–metal interactions in the material properties and formation of the byssus, while highlighting open questions.

4.1 INTRODUCTION

4.1.1 THE MUSSEL BYSSUS

With the right equipment, survival is possible under even the most extreme circumstances. Just as mountain climbers scaling Everest must rely on the proper gear, organisms inhabiting extreme environments have evolved distinctive adaptations to not only survive, but to thrive under extremely unfavorable conditions. While humans are not generally accustomed to thinking of the seaside intertidal zone as an extreme environment, the animals inhabiting this nutrient-rich niche face tremendous daily challenges to survival. These include exposure to temperature extremes, solar radiation, predation, sandblasting, and most notably, intense and incessant forces from crashing waves (Denny et al., 1998; Denny and Gaylord, 2010; Harrington & Waite, 2008a) (Fig. 4.1A). Waves provide a primary selective pressure in the marine intertidal zone, capable of producing enormous lift and drag forces (Denny and Gaylord, 2002) (Fig. 4.1B). Not coincidentally, the organisms residing

in the high-intertidal zone often possess remarkable strategies and materials for producing a secure attachment to the rocky substrates characteristic of seashore environments. Examples of successful anchoring strategies include the cement of barnacles (Kamino, 2010), the tube feet of sea stars (Hennebert et al., 2014) and the topic of the current chapter, the mussel byssus.

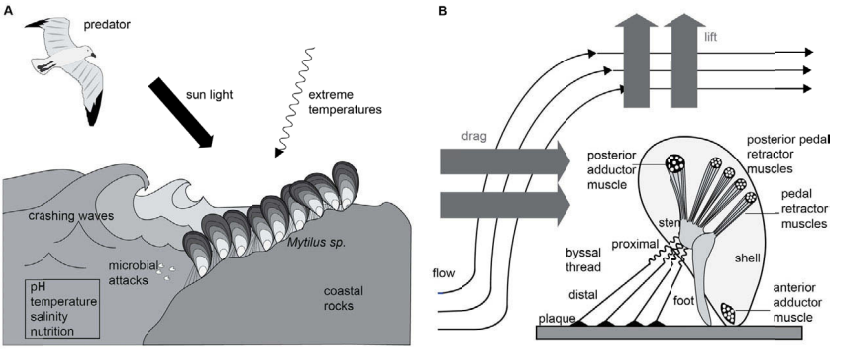

FIGURE 4.1 Habitat of marine mussels (*Mytilus sp.*). (A) Mytilid mussels inhabit rocky seashore environments where they face a number of challenges to survival. (B) Mussels attach to hard substrates with byssal threads, which prevent dislodgement from lift and drag forces caused by crashing waves. The inner anatomy of a mussel is shown in schematic emphasizing the musculature and the foot, which is the organ that forms the byssal threads. Figure adapted from source details (Source: Denny & Garland, 2010; illustration by Antje Reinecke.)

Marine mussels from the species *Mytilus* are bivalve mollusks that typically reside in sprawling beds along rocky coastal regions between high and low tides. The dominance of the organism in this habitat makes it vital part of the complex intertidal ecosystem (Denny and Gaylord, 2010). For the majority of their lives, besides early-larval and postlarval stages, the mytilid mussels maintain an entirely sessile way of life (Yonge, 1962) in which they sustain themselves on ample nutrient sources by filter feeding. Due to their sessile lifestyle, however, mussels cannot escape the often physically challenging conditions defining their environment. In particular, the enormous lift and drag forces associated with incessant wave action carries the constant threat of dislodging mussels from their attachment point or shattering their hard shells against the rocks (Fig. 4.1B) (Denny and Gaylord, 2010). Mussels overcome this dominant challenge to survival by fabricating a protein-based attachment holdfast called the byssus (Harrington & Waite, 2008a; Yonge, 1962).

The byssus is a nearly universal feature of almost all bivalves in the postlarval stage, used for securing attachment to various substrates during

settlement. However, only a few bivalve groups retain the byssus into adulthood, including marine mussels from the family Mytilidae (Brazee & Carrington, 2006; Yonge, 1962). The byssus of mussels from *Mytilus* species are composed of numerous extracellular protein-based fibrous byssal threads, each of which is several centimeters long, possessing an ellipsoidal cross-section ranging from approximately 100–200 µm along the long axis (Fig. 4.2A) (Harrington & Waite, 2008a). In the present chapter, the byssal threads of *Mytilus* species *Mytilus californianus*, *Mytilus galloprovincialis*, and *Mytilus edulis* will provide the main focus, as they are the most thoroughly characterized.

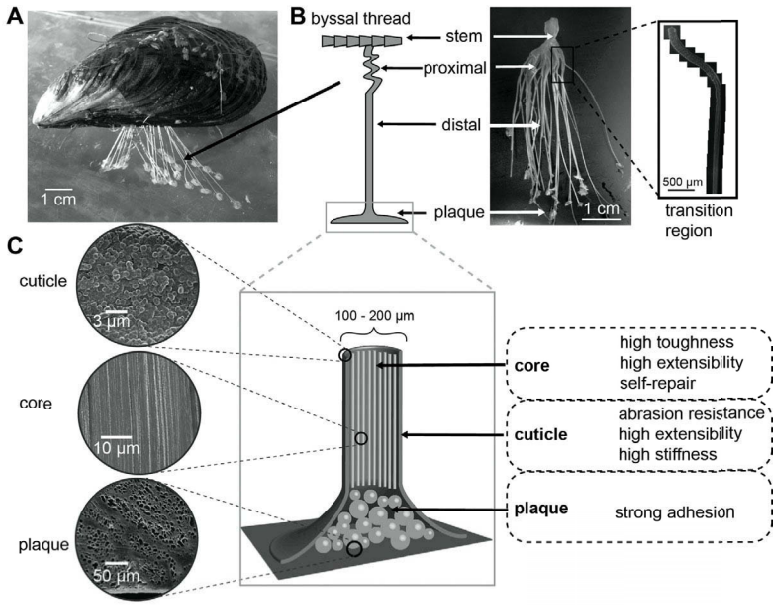

FIGURE 4.2 Byssal thread morphology. (A) Image of a mussel with its byssus. (B) Byssus removed from the mussel with a schematic showing important morphological features. SEM image of the transition region highlights the gradient between the distal and proximal regions of the thread. (C) Schematic of the core, cuticle, and plaque regions of the thread. SEM images highlight the micron-scale structural features.

Mussel byssal threads not only provide a means of anchoring mussels to the hard substratum, but they also function collectively as a robust, shock-absorbing holdfast (Qin & Buehler, 2013). Each individual thread possesses remarkable mechanical features, such as high extensibility, high stiffness, high toughness, abrasion-resistance, self-healing behavior, and underwater

adhesion (Carrington & Gosline, 2004). Along the length of a single thread, it can be divided into distinct regions (Fig. 4.2B, Table 4.1). At the most distal point (i.e., at the byssus-surface interface), each thread is glued to the hard substratum by an adhesive plaque, which functions as a strong anchoring point to a range of different surface types. The ability of the plaque to create strong adhesive interfaces under harsh seawater conditions has made it a primary target of biomimetic research in recent years (Lee et al., 2011) (Fig. 4.2B and C, Table 4.2). The other end of the thread, a region known as the stem, is inserted into the living mussel tissue anchoring the thread to the organism (Fig. 4.2A).

TABLE 4.1 Overview of Metal-dependent Performance in the Byssus.

	Thread core	**Cuticle**	**Plaque**
Function	Holdfast fiber	Protective coating	Attachment
Metal-dependent material properties	Mechanical energy dissipation Self-healing Extensibility Stiffness	Abrasion resistance Hardness Self-healing Extensibility	Underwater adhesion
Metal–ligand	His–Zn (Broomell et al., 2008) His–Cu (Broomell et al., 2008) His–Ni?	DOPA–Fe (Sun & Waite, 2005)	DOPA–Fe (Hwang et al., 2010)
Protein with metal–ligand	PreCol, PTMP-1, TMP-1	Mfp-1	Mfp-2–6
Ligand content (mol % per AA)	2% His total, 20% in His-rich domains (Harrington & Waite, 2007; Waite et al., 1998)	10–15% DOPA (Lin et al., 2007; Sun & Waite, 2005)	2% (Mfp-4) (Zhao & Waite, 2006b)–28% (Mfp-5) (Waite & Qin, 2011) DOPA 11 Cys in Mfp-6 (Yu et al., 2011b; Zhao & Waite, 2006a)

Between the plaque and stem, the fiber-like thread, which can reach 3–4 cm in length, can be subdivided into the distal and the proximal regions, which exhibit distinctive morphological and mechanical properties (Harrington & Waite, 2008a). The proximal region of the thread is relatively thick and corrugated with a crimped surface and comprises between 20% and 44% of the total thread length of *M. californianus* and *M. edulis*, respectively (Carrington, 2002; Mascolo & Waite, 1986; Qin & Buehler, 2013). It is

TABLE 4.2 Overview of Byssal Thread Proteins and Their Properties.

Protein	Location	Mass (kDa)	pI	Amino acids with mechanical function	Interactions with metal ions	Potential function	
Mfp-1	Cuticle	90–108	10.5	Hyp 15 mol% DOPA	Cross-linking of mfp-1 by DOPA–Fe^{3+}	Abrasion resistance & extensibility of cuticle	Lee et al. (2011)
Mfp-2	Interior of plaque	45	9.5	Cross-linked Cys 5 mol% DOPA	Ca^{2+}-binding, DOPA–Fe^{3+} Cross-linking of mfp-2 by DOPA–Fe^{3+} and with Ca^{2+}	Stability of foam-like structure of plaque	Hwang et al. (2010), Inoue et al. (1995), Rzepecki et al. (1992)
Mfp-3	Plaque–substratum interface	5–7		10–20 mol% DOPA		Adhesion	Carrington et al. (2015), Papov et al. (1995), Wei et al. (2013)
Mfp-4	Plaque–thread core interface	93		2 mol% DOPA	Ca^{2+}-binding motif might bind to plaque proteins, His-rich N-terminal might bind to his-rich domains of preCols (by Cu^{2+}-binding)	Interconnection of preCols and plaque proteins (especially mfp-2)	Lee et al. (2011), Zhao and Waite (2006b)
Mfp-5	Plaque–substratum interface	9	9	28 mol% DOPA	Ca^{2+}/Mg^{2+}-binding	Adhesion	Lee et al. (2011), Waite and Qin (2011)
Mfp-6	Plaque–substratum interface	11	9.5	3 mol% DOPA High amount of Cys		Anti-oxidant for DOPA in mfp-3 and mfp-5 Crosslinking with other mfps by cysteinyl–DOPA crosslinks	Yu et al. (2011b), Zhao and Waite (2006a)

TABLE 4.2 *(Continued)*

Protein	Location	Mass (kDa)	pI	Amino acids with mechanical function	Interactions with metal ions	Potential function	
PreCol-D	Thread core (more distal)	240	10.5	< 1 mol% DOPA Hyp	His–Zn^{2+} His–Cu^{2+}	Toughness, self-healing behavior, extensibility of thread core	Qin et al. (1997)
PreCol-P	Thread core (more proximal)	250		~20 mol% His in his-rich domains	His–Ni^{2+}		Carrington et al. (2015), Coyne et al. (1997)
PreCol-NG	Thread core	230	7.5				Qin and Waite (1998)
TMP-1		57	9.5	3–5 mol% DOPA			Carrington et al. (2015), Sagert and Waite (2009)
PTMP-1	Proximal region of thread core	250		3 mol% DOPA Disulfide bonds	Zn^{2+}/Cu^{2+}	Integrity of collagen/matrix Interconnection of preCols and other byssal proteins by metal coordination	Lucas et al. (2002), Suhre et al. (2014), Sun et al. (2002)

The protein in the light gray row is in the cuticle, proteins in the normal gray and dark gray rows are located in the plaque and in the thread core, respectively.

highly extensible (ε_{ult} > 200%) and exhibits a relatively low stiffness (E ~20 MPa) (Carrington & Gosline, 2004). In contrast, the distal region is more fibrous and exhibits a much higher stiffness (E ~500–800 MPa depending on the species), high toughness, and the ability to self-heal following damage during cyclic loading (Carrington & Gosline, 2004; Mascolo & Waite, 1986).

Surrounding the core of the *Mytilus* byssal thread is a thin outer cuticle (thickness = 5–10 μm) that possesses a characteristic granular morphology (Fig. 4.2C, Table 4.1). Based on a combination of high hardness and high extensibility, the cuticle is proposed to function as a protective coating for the softer thread core (Holten-Andersen et al., 2007). Mussels fabricate byssal threads one-by-one as an orchestrated secretion of an assortment of at least 11 distinct proteins into the byssus-forming organ, known as the foot (Hagenau et al., 2014; Silverman & Roberto, 2010) (Fig. 4.1B). During secretion, the proteins self-assemble and cross-link into a fully functional byssal thread that is attached to the existing byssus (Silverman & Roberto, 2010). Despite the wealth of descriptive data and molecular information of the comprising protein components, presently the details of the assembly process are poorly understood. The final section of this chapter will be dedicated to this topic.

Considering that the byssus is an acellular proteinaceous material that functions extracorporeally (i.e., outside the living organism), its material properties must arise from specific features of the material itself, programed through intrinsic physicochemical properties of the biomolecular protein building blocks and their self-assembly. Thus, insights into the origin of the remarkable (and industrially attractive) material properties of the byssus (e.g., self-healing, abrasion resistance, underwater adhesion—Fig. 4.2C, Table 4.1) can be ascertained by elucidating the underlying material design principles. Over the last 30 years, intensive biochemical and structural–mechanical investigations of the mussel byssal threads from Mytilid species have led to an impressive understanding of the structure–function relationships that define this material (Carrington et al., 2015). A particularly exciting aspect of byssal thread design that has emerged in recent years is the role of protein–metal interactions as mechanical cross-links that contribute to the material performance of the fibrous core, cuticle, and adhesive plaque (Degtyar et al., 2014). In fact, based on these findings, in the last 5 years, there has been a surge in the production of "mussel-inspired" metallopolymers that harness protein–metal chemistry of the byssus for applications in technical and biomedical settings (Li et al., 2015). Thus, the mussel byssus is attracting a broad interest in diverse fields ranging from evolution and biochemistry to ecology and materials science.

In the present chapter, we will discuss the structure–function relationships that define the material function of the mussel byssus, with a particular focus on the essential role of metal coordination cross-links in the self-healing fibrous core, the abrasion-resistant extensible cuticle, and the versatile adhesive plaque. The next section will provide a general overview of the role of metal-coordination in biological systems and the growing understanding of protein–metal cross-links in biological materials, specifically the byssus.

4.1.2 METAL IONS AS MECHANICAL CROSS-LINKING AGENTS

Metal acquisition and metal homeostasis are vital functions of nearly every living organism (Outten et al., 2007). In excessive amounts metals can be dangerous cellular toxins; however, in small doses, particular metals are essential for proper cellular function. For example, countless proteins require metal ions such as Zn, Cu, Fe, Ca, and Mg as cofactors in physiologically important functions and essential biochemical pathways including gas transport, respiration, nitrogen fixation, and photosynthesis (Outten et al., 2007). On top of these roles, metal coordination bonds in proteins also serve to increase protein stability (Glusker, 1991; Holm et al., 1996). Protein-bound metal ions act as a bridge between multiple amino acid side chains to form a coordination complex in which each amino acid side chain donates a pair of electrons to empty orbitals in the outer electron shell of the metal ion. Amino acid side chains such as histidine, cysteine, aspartate, and glutamate are common ligands for a range of metal ions found bound to proteins (Glusker, 1991).

From a mechanical perspective, metal coordination bonds are intermediate to covalent and non-covalent bonds in bond strength and lability. This simply means that they exhibit breaking forces significantly higher than hydrogen bonds, while maintaining the transient ability to reform on biologically relevant timescales once ruptured (Lee et al., 2006). The mechanical properties of biologically relevant protein–metal coordination bonds have been tested using single-molecule force spectroscopy techniques such as atomic force microscopy (AFM), which confirm, for example, that histidine coordination interactions with Zn, Cu, and Ni are both strong and reversible (Schmitt et al., 2000). Furthermore, it has also been demonstrated that bioengineering histidine–metal-binding sites into proteins can lead to an increase in both the thermodynamic (Kellis et al., 1991) and mechanical (Cao et al., 2008) stability of the folded protein.

Based on some of the same features that make metal ions attractive as cofactors for physiologically relevant functions (e.g., redox activity, low

kinetic lability, high bond strength), certain protein-based biological materials have evolved the capacity to harness metal ions as essential cross-linking agents that enhance material performance. (See recent review by Degtyar et al., 2014.) For example, the hard biting parts and stingers of arachnids such as spiders and scorpions, the mandibles of insects such as termites, and the piercing jaws of particular marine worms (*Nereis* sp. and *Glycera* sp.) utilize histidine-rich proteins coordinated to metal ions such as Cu^{2+} and Zn^{2+} in order to harden and stiffen the protein-based scaffolds as a lightweight alternative to mineralization (Broomell et al., 2006; Degtyar et al., 2014; Politi et al., 2008; Schofield et al., 2002). Protein–metal interactions in materials, however, are quite versatile and can be used for more than just hardening and stiffening organic structures. As will become clear in this chapter, metal coordination chemistry can be harnessed and tailored to achieve a broad range of mechanical functions from self-healing, adhesion, abrasion resistance, and triggered self-assembly—all of which are exploited in the mussel byssus (Degtyar et al., 2014) (Fig. 4.2, Table 4.1).

4.1.3 METAL IONS IN THE MUSSEL BYSSUS

The presence of metals in the mussel byssus was first highlighted in the 1970s as researchers searched for a reliable method for determining pollution levels in ocean waters around the world (Coombs & Keller, 1981; Phillips, 1976a, b). As filter feeders, mussels are constantly sampling the local water column, and it was found that they incorporate various metals from the surrounding environment into their shells, soft tissues, and their byssus. Due to the mussels ability to concentrate an expansive range of trace elements existing in the seawater into their byssus (e.g., Ag, Cd, Cr, Pb, Ti, Fe, Zn, Cu, and Ni) (Coombs & Keller, 1981), byssal threads have become a standard and reliable environmental biomarker for harmful heavy metals in polluted coastal and estuarine habitats (Szefer et al., 2006). For example, based on their propensity for concentrating V and Ni ions, mussels can be used to assess pollution levels following oil spills (Amiard et al., 2004), as well as radioactive pollution levels in sources of nuclear effluents through the uptake of radionuclides such as uranium, plutonium, and polonium (Hamilton, 1980; Hodge et al., 1979). As a consequence, large efforts have been dedicated to elucidating the complex mechanisms underlying metal uptake, bioaccumulation, transport, and release of metal ions in mussels. Here, we provide only a brief overview of these processes, as this topic

has been exhaustively covered in several earlier reviews (Marigomez et al., 2002; Viarengo, 1989; Viarengo & Nott, 1993) (Fig. 4.3).

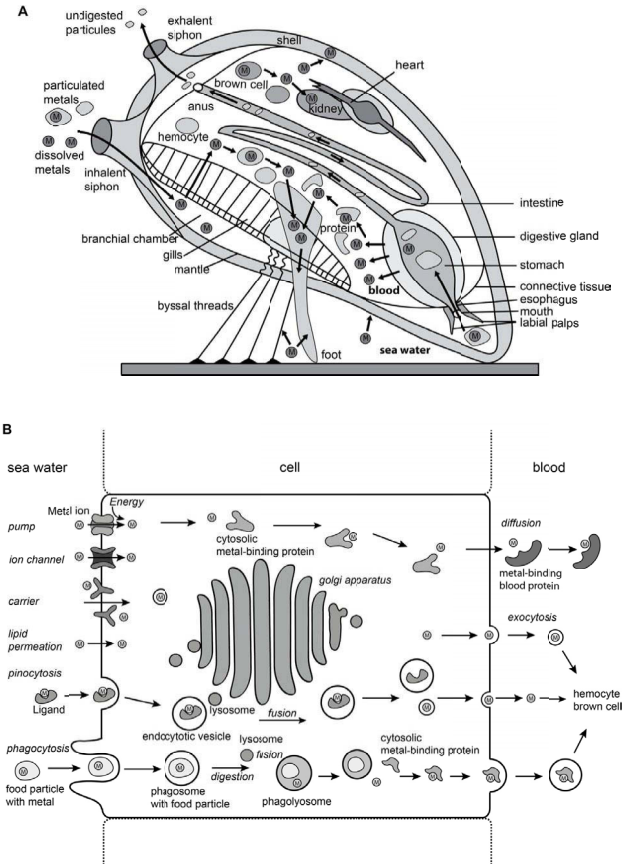

FIGURE 4.3 Possible mechanisms of metal uptake, transport, and accumulation in the byssus. (A) Schematic illustrating proposed movement of dissolved and particle-bound metal ions into and through the mussel tissue. Dissolved metal ions and metals bound to food particles are inhaled through the ingoing siphon, transported via the mouth to the esophagus and stomach where digestion begins. Metals are taken up by cells of the digestive glands and released into the blood. Dissolved metals can also enter the cells of the gills, to be released into the blood. Metals are transported by proteins of the blood plasma or in blood cells (hemocytes/brown cells) to target tissues, for example, to detoxification organs or to the foot for byssus formation. (B) Possible cellular pathways of metal ions from the inhaled sea water into the blood (Simkiss & Taylor, 1989). Dissolved metal ions can be taken up by ion pumps, ion channels, carriers, or lipid permeation and bound to metal-binding proteins inside the cell. Particulated metals can be engulfed by endocytotic pathways. The resulting vesicle can fuse with lysosomes for hydrolysis. On the other side of the cell, metal ions are released into the blood plasma or are incorporated into blood cells after exocytosis.

The bioavailability of metals in seawater strongly depends on several biotic and abiotic factors. For example, metal uptake by mussels is influenced by the age of the organism, as well as by annual growth cycles (Langston & Spence, 1995; Wang & Fisher, 1997). Abiotic factors, on the other hand, such as seawater salinity, temperature, pH, oxygen concentration, redox conditions, and the presence of other trace elements or binding partners may also affect an organisms ability to uptake metals (Abbe & Sanders, 1990; Bjerregaard & Depledge, 1994). Furthermore, metals in seawater exist in many different forms such as dissolved free ions, complexes with various ligands or bound to particles (Abbe & Sanders, 1990), each of which requires different strategies for acquisition.

Mussels, as well as other bivalves, regulate metal uptake, trafficking, accumulation, and removal through complex cellular processes and organ systems (Marigomez et al., 2002) (Fig. 4.3). Specific pathways, for example, exist in the mussel for the acquisition of dissolved vis-à-vis particle-bound metal ions. In both cases, metal uptake commences when seawater containing suspended food particles is inhaled through the incurrent siphon, transported into the branchial chamber and released through the exhalent siphon (Fig. 4.3A). While resident within the confines of the mussel shell, dissolved metal ions in the inhaled seawater are taken up primarily through the gills via passive diffusion across the cell membrane, through ion-channels or via pinocytosis (Carpene & George, 1981; Marigomez et al., 2002) (Fig. 4.3A and B). Once they have entered the epithelial cells of the gills, metal ions can be bound to metal-binding proteins such as metallothioneins, engulfed into lysosomes and transported in vesicles across the cell membrane to be released into the blood plasma or incorporated into blood cells for transport to other tissues and destinations (Fig. 4.3A and B) (Marigomez et al., 2002; Mason, 1983). Metallothioneins are a class of low molecular weight cysteine-rich proteins that possess a high affinity for essential metals such as Zn and Cu, as well as for toxic metal such as Cd, Ag, and Hg (Viarengo & Nott, 1993). In the former case, Zn and Cu binding by metallothioneins is thought to function as a means of storing these metals for later use in enzymes or possibly, in the byssus. Notably, it was shown that lysosomes of marine mussels contain byssus-relevant metal ions including Cu, Zn, and Fe (Mason et al., 1984).

In contrast to dissolved metal ions which pass through the gills, particle-bound metal ions are primarily taken up by the digestive system of the mussel (Viarengo, 1989) (Fig. 4.3). In this case, the labial palps, located near the mouth, move particulate food via movement of cilia toward the esophageal opening and into the stomach where digestion begins (Fig. 4.3A). Surrounding the stomach is the digestive gland, which functions, among

other things, to facilitate the acquisition of metal ions from the digested food particles (Marigomez et al., 2002). To achieve this, one possible way is that digestive cells first take up metal-containing particles by phagocytosis into vesicles, which fuse with lysosomes where the particle is further hydrolyzed (Marigomez et al., 2002) (Fig. 4.3B). Similar to acquisition of dissolved ions through the gill epithelial cells, the specialized epithelial cells of the digestive gland (i.e., the digestive and basophilic cells) possess a range of metal-binding proteins, including metallothioneins, which present ligands that have affinities for a range of metal ions. Metal selectivity is primarily based on whether the ions prefer to complex with ligands possessing oxygen donor atoms (e.g., carbonate, phosphate, and sulfate ligands) or non-oxygen donor atoms (e.g., sulfur, nitrogen ligands) (Marigomez et al., 2002). However, there are some biomolecules, such as ferritin, that specifically bind only certain metal ions, in this case iron (Taylor, 1995). This differentiation in metal specificity leads to the non-homogenous distribution of metal ions between various cell types (Marigomez et al., 2002), which are then available for use in various physiological functions or alternatively, are destined for removal from the organism.

Essential metal ions (i.e., those destined for specific physiological roles) are transported to target tissues either bound to blood plasma-binding proteins or by cell-mediated means in hemocytes or brown cells, migratory phagocytic cells specialized for metal transport (Haszprunar, 1996) (Fig. 4.3A and B). Presently, however, the pathway by which the huge range of metals found in the byssus are specifically incorporated into byssal threads is not well understood. The best-characterized case is that of iron incorporation. It was previously demonstrated by raising mussels in seawater spiked with radiolabeled Fe ions that the iron present in the byssus is largely acquired through filter feeding, rather than passive diffusion into the threads (George et al., 1976). In this study, radiolabeled Fe was shown to first accumulate in the visceral tissue and then only during a period of approximately a week were radiolabeled Fe ions incorporated into byssal threads. Autoradiography studies on mussel foot sections have suggested that Fe might accumulate in localized micron-sized "hot spots" in the foot tissue; however, this was never further corroborated (Pentreath, 1973). In addition to this evidence for the "active" incorporation of metal ions into threads, metal ions can also clearly be integrated passively into the threads under certain conditions (Harrington et al., 2010). Thus, at this point, the mechanism of incorporation of metal ions into the byssus remains an open question.

While the puzzle of how metal ions are integrated into the byssus is still not entirely obvious, the question of what they are doing there is beginning

to become more clear. Based on the sheer diversity of metals found in the byssus, it was originally assumed that the byssus serves as a waste depository for excess and poisonous metal ions that had accumulated in the mussel soft tissue (Coombs & Keller, 1981; George et al., 1976). However, it was later proposed that at least some of the accumulated metal ions might instead be fulfilling a functional role in the material based on the high concentration of specific amino acid residues prevalent in byssal thread proteins known for their metal-binding prowess (Taylor et al., 1994; Waite et al., 1998). This includes histidine residues that were found to be concentrated in highly conserved patches at the end of proteins in the thread core (Harrington & Waite, 2007) (Fig. 4.4) and an elevated presence of a relatively rare post-translational modification of tyrosine called 3, 4-dihydroxyphenylalanine (DOPA), which is known for forming very stable complexes with a number of metal ions (Sever & Wilker, 2004) (Figs. 4.5 and 4.6).

A growing body of evidence now supports a critical role of protein–metal coordination bonds in byssal thread assembly and material performance between histidine–Zn/Cu and DOPA–Fe as summarized in Table 4.1. The next three sections will describe the state-of-the-art understanding of the relationship between material behavior and structure of byssal threads with the focus on the various proposed functions of metal coordination bonds, in the material performance of the self-healing core, abrasion-resistant cuticle, and adhesive plaque of byssal threads. The final section will provide an overview of the current understanding of byssal thread assembly, emphasizing the proposed role of protein metal interactions.

4.2 SACRIFICIAL METAL COORDINATION BONDS IN THE BYSSAL THREAD CORE

Living organisms invest ample resources and energy into building materials such as skeletal elements, soft tissues, and the extracellular matrix. Thus, inherent in the design of many biological materials are features to enhance the durability, toughness, and damage tolerance (Chen et al., 2012). Along these lines, a defining feature of many living organisms is the ability to heal damage. Normally, this is achieved by cellular-dependent processes as observed in bone mending (Fratzl & Weinkamer, 2007), wound healing (Werner & Grose, 2003), and even full limb regeneration (Nacu & Tanaka, 2011). Less common, however, is the ability of acellular materials to exhibit self-healing behaviors, as exhibited by the mussel byssus (Carrington & Gosline, 2004). In the absence of cellular intervention, damage tolerance

and self-healing in byssal threads must be programmed into the material itself through the biochemical features of the protein building blocks and their hierarchical organization.

The functionality and efficacy of the byssal attachment system is due in part to the ability of threads to combine the features of high stiffness, high strength, and high extensibility (Coyne et al., 1997). As previously mentioned, the byssal thread is divided into two regions, the proximal and distal region, each of which possesses different mechanical properties (Fig. 4.4A). The proximal region possesses a stiffness of ~20 MPa (Gosline et al., 2002) and is extensible up to 200% of its initial length, which is comparable to elastin and resilin (Bell & Gosline, 1996). In contrast, the distal region is less extensible (~100% strain), but stiffer (up to 800 MPa) and stronger than the proximal region (Fig. 4.4A). In particular, mechanical tests performed on the distal region exhibit a stress–strain curve with three phases during loading: (1) a linear region at low strain, (2) a yield point with a post-yield plateau, and (3) a post-yield stiffening before breakage (Fig. 4.4A). The combination of high extensibility and high strength provides the material with a high toughness. At low cyclic extensions (<10% strain), the distal portion of the thread deforms elastically and returns to its initial length when the applied stress is removed (Carrington & Gosline, 2004); however, when strained beyond the yield point, the material exhibits significant mechanical hysteresis during cyclic loading (up to 70%) (Fig. 4.4B).

While material yield provides an important means of dissipating energy from crashing waves, it also results in an apparent damage to the material that can be observed during subsequent loading cycles (Carrington & Gosline, 2004) (Fig. 4.4B). For example, when the distal thread is cyclically loaded to 35% strain, a second cycle following the first shows a reduction of ~65% in the stiffness and the energy dissipated (Harrington et al., 2009). Notably, however, the byssal thread possesses the remarkable ability to self-heal. Here, self-healing is simply defined as the time-dependent recovery of initial material properties following yield-induced pseudo-damage (Carrington & Gosline, 2004) (Fig. 4.4B). Times required to recover a significant proportion of initial properties are on the order of several hours, which is consistent with the low-tide periods in which mussels may be given a brief reprieve from the onslaught of crashing waves. However, at this point, there have been no studies specifically examining the role of self-healing in the natural environment. Nonetheless, it is noteworthy that byssal thread self-healing behavior occurs in the absence of an active metabolism and that threads regain functionality after damage in a completely acellular manner.

Therefore, the origin of this behavior must be intrinsic to the composition and structure of byssal threads.

The thread core consists of more than 95% protein by dry weight (Waite et al., 2002) as well as a small amount of metal ions (<1% by dry weight) (Coombs & Keller, 1981), particularly Zn^{2+} and Cu^{2+} (Fig. 4.4D, Table 4.2). X-ray diffraction studies performed as early as the 1950s suggested that the fibrous core of byssal threads is a fibrillar tendon-like collagen-based material (Mercer, 1952; Rudall, 1955). Biochemical confirmation of this hypothesis was hindered for quite some time due to the intractability of protein extraction; however, Waite and colleagues were eventually able to extract partial sequence of a collagen-like protein by pepsin digestion of threads (Benedict & Waite, 1986; Waite et al., 1998). Subsequent acquisition of the full-length sequences from cDNA revealed three variants of collagen-like proteins that were named preCol (for "pre-pepsizined" collagen) (Waite et al., 1998) (Fig. 4.4D, Table 4.2). All three variants, denoted preCol-D, -NG, and -P, possess a dominant central collagen domain with a typical $[Gly\text{-}X\text{-}Y]_n$ repeat sequence. However, at the N- and C-terminal ends, of all three proteins are non-collagenous domains, that unlike the pro-domains of type I collagen are not cleaved off and remain in the mature form of the protein (Waite et al., 1998). The non-collagen domains consist of the so-called flanking domains and the histidine-rich domains, which are described in the next two paragraphs (Fig. 4.4D).

The preCol flanking domains surround the central collagen domain at both ends and contain sequences that vary between the three variants. PreCol-D flanking domains exhibit multiple runs of polyalanine reminiscent of beta-sheet forming sequence in spider silk, whereas preCol-P flanking domains are proline-rich and have been shown to have sequence similarity to elastic proteins such as elastin and flagelliform silk protein (Harrington & Waite, 2007). PreCol-NG possesses flanking domains with sequences that combine features of both preCol-D and -P flanking domains (Harrington & Waite, 2007; Qin & Waite, 1998). Recent NMR- and FT-IR-based studies on byssal threads support the hypothesis that the flanking domain of both preCol-D and -NG form beta-sheet secondary structures (Arnold et al., 2013; Hagenau et al., 2011). PreCol-NG (*N*on-*G*raded) exists uniformly along the length of the byssal thread, while preCol-D (Distal) and -P (*P*roximal) exist in a complementary gradient with preCol-P more prevalent at the extensible proximal end of the thread and preCol-D more prevalent at the distal end. It is believed that this molecular gradient plays a key role in the mechanical gradient existing along the thread axis (Harrington & Waite, 2009; Waite et al., 2004) (Fig. 4.2D).

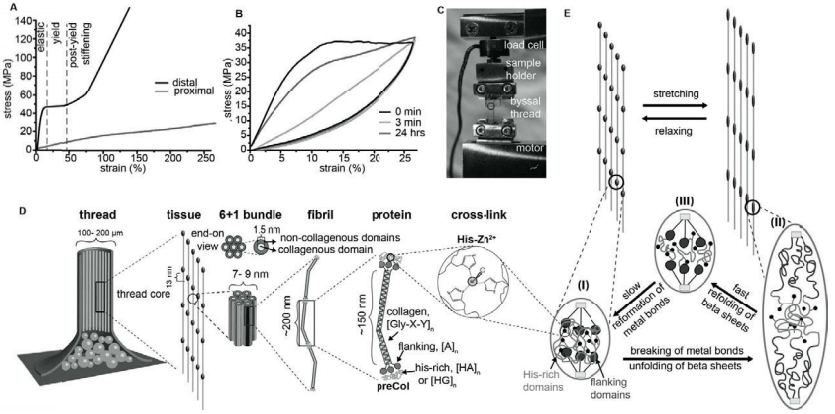

FIGURE 4.4 Structural hierarchy of the byssal thread core. (A) Comparative tensile stress–strain curves of the distal and proximal region of mussel byssal threads. (B) Self-healing behavior of the distal region during cyclic loading. (C) Mechanical tester used for tensile loading experiments. (D) The core of the byssal thread is composed primarily of a family of proteins known as preCols. PreCols possess several domains including a large central collagen domain, beta-sheet forming flanking domains and histidine-rich domains. PreCols form triple helices, which further assemble into 6 + 1 hexagonal bundles. PreCol bundles are arranged end-to-end in series with a 13 nm stagger between adjacent bundles, forming a highly organized semicrystalline framework. PreCol ends interact via the His-rich domains through metal coordination cross-links. (E) One proposed molecular level model of stretching, relaxing, and healing in mussel byssal threads. Stretching beyond the yield point results in the rupture of His–Zn^{2+} cross-links and unfolding of flanking domain beta-sheet structure. When the thread is relaxed, the beta-sheet domains are expected to refold immediately; however, reformation of broken metal coordination cross-links into a stable topology likely requires more time and provides a rate-limiting step in mechanical healing.

At the N- and C-terminal end of all preCols are the His-rich domains (Fig. 4.4D). The His-rich domains contain variable sequences, which are between 21 and 126 amino acids in length and invariably contain at least 20 mol% of histidine, which is ~10-fold higher than found in average protein sequences (Harrington & Waite, 2007; Lucas et al., 2002; Waite et al., 1998). The spacer residues between histidines are typically Ala and Gly, but also occasionally other residues such as Ser and Val (Harrington & Waite, 2007). The preCol sequences of three species are now known, and while interspecies variation exists in the spacer residues and surrounding sequences of the His-rich domains, the His residues themselves are almost completely conserved—strongly suggesting an important functional role (Harrington & Waite, 2007). In particular, the well-known metal-binding affinity of His for metal ions such as Zn and Cu, known to be in the thread, led to the hypothesis that they might contribute as cross-linking ligands (Fig. 4.4D and E) (Waite et al., 1998).

Based on the presence of a large collagen domain, the preCol monomers assemble into preCol triple helices, which further self-organize to hexagonal 6 + 1 bundles (Krauss et al., 2013) (Fig. 4.4D). X-ray diffraction (XRD) (Harrington et al., 2009; Krauss et al., 2013) and AFM (Hassenkam et al., 2004) investigations on the thread core have revealed that preCols are arranged in a highly organized semicrystalline framework in which preCol bundles are aligned end-to-end in series and organized laterally in a quasi-hexagonal array (Fig. 4.4D). End-to-end alignment of preCols indicates that His-rich domains from consecutive preCol bundles are in contact, and it has been proposed that the formation of coordination bonds between His-residues and transition metal ions bridge the ends of adjacent preCols (Harrington & Waite, 2007) (Fig. 4.4D).

In addition to the preCols, two other proteins have been localized to the byssal thread core—namely, thread matrix protein-1 (TMP-1) and proximal thread matrix protein-1 (PTMP-1) (Sun et al., 2002) (Table 4.2). As the names imply, these proteins are believed to be present in the matrix material surrounding the preCols, with PTMP-1 present primarily in the proximal region of the thread (Sun et al., 2002) and TMP-1 present in both the distal and proximal region (Sagert & Waite, 2009). The proximal region contains a higher content of matrix proteins, whereas the distal region exhibits much lower matrix content (2% or less by dry weight) (Sagert & Waite, 2009). TMP-1 has the distinctive property of undergoing spontaneous deamidation of numerous asparagine residues over time to form aspartate and isoaspartate residues, effectively lowering the isoelectric point of the protein (Sagert & Waite, 2009); however, it is unclear what the functional role of this might be. Recent work by the group of Scheibel has demonstrated an effect of PTMP-1 on collagen assembly *in vitro*, supporting an important role of the matrix proteins on assembly and function (Suhre et al., 2014). Furthermore, it was shown that PTMP-1 contains motifs consistent with metal-binding geometries found in other proteins and that the collagen binding of PTMP-1 is slightly enhanced in the presence of Zn^{2+} ions (Suhre et al., 2014). In general, the thread matrix proteins are proposed to separate and lubricate preCol fibrils; however, their specific role is still under discussion (Sagert & Waite, 2009; Suhre et al., 2014).

The understanding of the structure–function relationships defining the byssal thread core has been advanced in recent years by the use of spectroscopic and X-ray diffraction techniques in combination with *in situ* mechanical testing (Fig. 4.4E). For example, wide angle X-ray diffraction studies combined with *in situ* tensile tests have revealed that the collagenous domain strains by a mere 2%, even when the thread is strained to more

than 70% of its initial length (Harrington et al., 2009; Krauss et al., 2013). Because the preCols are aligned in series, this implies that the high extensibility of the thread must arise from the unfolding of folded protein structure (i.e., hidden length) of the non-collagenous domains of preCols (Fig. 4.4E) (Harrington et al., 2009; Krauss et al., 2013). In particular, the unfolding of the predicted beta-sheet forming flanking domains of preCol-D and possibly -NG is a prime candidate to provide extensibility as suggested by FT-IR and NMR-based studies (Arnold et al., 2013; Hagenau et al., 2011). Notably, however, small-angle X-ray diffraction studies suggest that the unfolded protein structure refolds without delay when the thread is relaxed (Fig. 4.4E) (Krauss et al., 2013). Considering that the mechanical properties recover only on a much longer timescale (Carrington & Gosline, 2004; Harrington et al., 2009), it was proposed that byssal thread healing proceeds by a two-step process consisting of a fast recovery of the folded protein length (presumably by refolding of unfolded beta-sheet structure) and a slower recovery of mechanical properties by reformation of a sacrificial bonding network (Krauss et al., 2013).

Sacrificial bonding is a strategy for enhancing the toughness that has been identified in a broad range of biological materials including wood, bone, and silk (Becker et al., 2003; Fantner et al., 2004; Keckes et al., 2003; Smith et al., 1999). The basic concept underlying sacrificial bonding is that weaker non-covalent bonds are strategically positioned to rupture prior to covalent bonds, dissipating applied mechanical energy and avoiding catastrophic rupture of the biomolecular backbone. Often, rupture of sacrificial bonds in protein-based biological materials results in the unfolding of folded protein length—so-called hidden length—which provides an effective means of increasing the extensibility and toughness of the material (Smith et al., 1999). Furthermore, if the "hidden length" can refold when unloaded and the sacrificial bonds are reversible (i.e., can reform on biologically relevant timescales), this can also lead to self-healing behavior.

Waite and colleagues first proposed that metal coordination bonds between histidine residues in the His-rich domains might be functioning as sacrificial bonds in the byssal thread core (Coyne et al., 1997). Since then, several pieces of important evidence have emerged that support this hypothesis (Harrington & Waite, 2007; Holten-Andersen et al., 2009a; Vaccaro & Waite, 2001). First, removal of Zn and Cu ions from byssal threads following their incubation with ethylenediamine tetracetic acid (EDTA), a metal-chelation agent, resulted in a significant decrease in thread stiffness and the loss of the yield point (Vaccaro & Waite, 2001). Second, byssal thread stiffness can be reduced by treatment at low pH, where histidine is

positively charged and unable to bind metal ions (Harrington & Waite, 2007) (p*K*a ~6.5). Furthermore, plotting thread stiffness as a function of pH reveals a sigmoidal curve that almost perfectly overlaps with the titration curve of histidine (Harrington & Waite, 2007). Third, it was demonstrated that the ability of byssal threads to heal is completely eradicated when threads were incubated at pH 4 (Harrington et al., 2009). Finally, synthetic peptides based on sequences of the histidine-rich domains of preCols were shown to exhibit reversible metal-dependent interactions *in vitro* (Schmidt et al., 2014), adding further support to potential use of histidine–metal cross-links as sacrificial bonds.

Based on these and other findings, a molecular-level model has been developed to explain the complex deformation and self-healing properties of byssal threads (Fig. 4.4E). His–metal bonds in byssal threads are believed to break at the yield point functioning as reversible sacrificial bonds. The rupture of sacrificial bonds leads to the unfolding of the flanking domain beta-sheet structure, providing the thread with increased extensibility and energy dissipation. Refolding of unfolded protein length is believed to occur immediately upon unloading (Krauss et al., 2013); however, as already mentioned, mechanical recovery requires longer rest periods. Therefore, based on the evidence provided in the previous paragraph, the reformation of a network of stable protein–metal coordination bonds seems a likely candidate for the slow step in the self-healing process. However, further research is undoubtedly needed to investigate the mechanical roles of the beta-sheet forming flanking domains and the metal-cross linking His-rich domains.

It is worth noting that the compositional and structural properties and thus, the mechanical behavior of byssus fibers vary between *Mytilus* species (Harrington & Waite, 2007). For example, *M. californianus* produces threads which are about twice as stiff as threads from *M. galloprovincialis* (Bell & Gosline, 1996). Furthermore, the mechanical properties of byssal threads produced by individual mussels can be influenced by external factors including water quality, predation, temperature, nutrition, sea current, density of the mussel bed, and quality of substratum (Bell & Gosline, 1997; Carrington, 2002; Carrington et al., 2008; Côté, 1995; Garner & Litvaitis, 2013; Lachance et al., 2008; Moeser & Carrington, 2006). For example, one study demonstrated that during fall and summer months *M. edulis* mussels on the northeastern seaboard of North America synthesized threads that were half as strong and extensible as those formed in the winter and spring (Moeser & Carrington, 2006). This reduced mechanical performance has been proposed to contribute to higher incidence of dislodgement events

during these seasons (Carrington et al., 2015; Denny and Gaylord, 2010). Additionally, it was found that threads grown under conditions mimicking forecasted ocean acidification exhibited weaker adhesive attachment than native threads (O'Donnell et al., 2013), while warmer waters were found to weaken the proximal region of the thread (Carrington et al., 2015). Presently, the molecular-level mechanisms underlying these mechanical modifications have not yet been elucidated; however, it is tempting to posit that they may reflect fundamental changes in the composition and structure of the byssal thread.

4.3 A STRETCHY AND ABRASION-RESISTANT COATING REINFORCED BY DOPA–FE^{3+}

Abrasion resistant coatings in technical applications are typically very hard materials with low strain limits. In contrast, the byssal thread cuticle, which is proposed to protect the thread against abrasion, degradation due to solar radiation and microbial attacks, is able to exhibit high stiffness and hardness values comparable to modern engineering epoxies, while still remaining surprisingly flexible and extensible (Holten-Andersen et al., 2007). These materials properties—that is, high hardness and high extensibility—are traditionally diametrically opposed and are almost never found in the same material in man-made polymers or composites. Thus, the byssal thread cuticle has emerged as an exciting biological archetype for the design of man-made coatings for a variety of possible biomedical and technical applications (Holten-Andersen & Waite, 2008).

The byssal thread cuticle is a thin (5–10 μm) coating that completely surrounds the fibrous core (Fig. 4.2C and Fig. 4.5A, Table 4.1). It has been shown that the cuticle of *Mytilus* species exhibits hardness and stiffness values that are about fivefold higher than that of the fibrous core, but still stays intact up to tensile strains of 100% in certain species (Holten-Andersen et al., 2007, 2009b; Lee et al., 2011) (Fig. 4.5A). The extraordinary combination of mechanical properties exhibited by the byssus cuticle is believed to arise mainly as a result of its composite-like structure in which micron-sized granular inclusions are embedded in an amorphous homogenous matrix (Fig. 4.2C and Fig. 4.5A and D). While the granules were observed to deform at low strain (<30%), they behave as stiff reinforcing elements at higher strains and microcracking is observed in the matrix between granules (Holten-Andersen et al., 2007) (Fig. 4.5D). Microcracking has been proposed as an effective toughening mechanism that distributes damage over a larger

volume, preventing the propagation of larger catastrophic cracks through the material (Holten-Andersen et al., 2007). In support of this hypothesis, it was shown that species with smaller granules, and thus, higher granular surface area, exhibit higher extensibilities than those with larger granules (Holten-Andersen et al., 2009b). Mussels living in subtidal habitats, such as *Perna canaliculus*, on the other hand possess cuticles without granular microarchitecture and exhibit fracture strains of ~30% (vs. up to 100% strain for *M. californianus*), suggesting that extensible granular cuticles might be an adaptation to high wave-exposure of mytilids (Holten-Andersen et al., 2007).

Presently, the only protein confirmed to be present in the cuticle is a DOPA-rich protein known as *m*ussel *f*oot *p*rotein-1 (mfp-1) (Table 4.2). Additionally, a small amount of Fe^{3+} and Ca^{2+} have been co-localized in the cuticle (Holten-Andersen et al., 2009a). As already mentioned, DOPA is known to form extremely stable coordination complexes with Fe^{3+} (Fig. 4.5C), leading the proposal that such interactions might be present in the cuticle stabilizing mfp-1 (Taylor et al., 1994). Recently, confocal Raman spectroscopic imaging has confirmed the presence of tris–DOPA–Fe^{3+} cross-links in byssal threads from *M. galloprovincialis* and *M. californianus*, which are specifically localized in the cuticle (Harrington et al., 2010) (Fig. 4.5B).

The mechanical importance of the DOPA–Fe^{3+} cross-links in the cuticle was shown by the fact that EDTA-mediated removal of Fe^{3+} ions resulted in a reduction of the DOPA–Fe Raman signal and in a 85% loss in hardness (Harrington et al., 2010; Holten-Andersen et al., 2009a; Schmitt et al., 2015). DOPA–metal cross-links have the advantage that they are at the same time strong and reversible, as demonstrated by the work of Messersmith and colleagues in AFM-based single molecule force experiments of a single DOPA side chain and a TiO_2 surface (Lee et al., 2006). In this study, it was demonstrated that a single DOPA–Ti interaction possesses a breaking force of approximately half that of a typical covalent bond, while being able to break and reform reversibly over hundreds of times (Lee et al., 2006). Furthermore, surface force apparatus (SFA) experiments have enabled the measurement of the adhesion forces between films of mfp-1 proteins in the presence and absence of Fe^{3+}. In this technique, the attractive and repulsive forces are measured between two mica surfaces coated with mfp-1, which are first brought together and then pulled apart (Israelachvili et al., 2010). Using SFA, the adhesion energy of the interaction is determined based on interaction forces and contact areas measured. In the absence of Fe^{3+}, layers coated with mfp-1 were not bridged (e.g., did not interact); however, in the

presence of Fe^{3+}, adhesion energies of up to 5 mJ/m² were calculated indicating very stable, but reversibly breakable cohesive interactions between proteins (Holten-Andersen & Waite, 2008; Lin et al., 2007; Zeng et al., 2010).

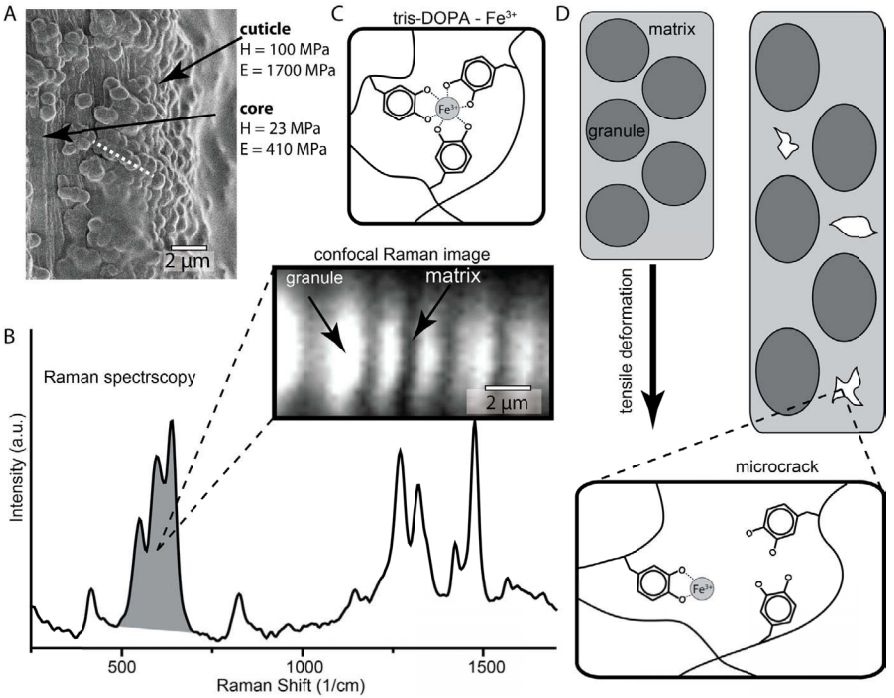

FIGURE 4.5 DOPA–Fe^{3+} cross-links in the byssal thread cuticle. (A) SEM image showing the granular morphology of the cuticle and the fibrous morphology of the core. Hardness (H) and stiffness (E) values are included for both. (B) Raman spectrum and confocal Raman image showing the presence and distribution of tris–DOPA–Fe cross-links (C) in the cuticle. Granules contain elevated cross-link density compared to the matrix. (D) Schematic model of microcrack formation in the cuticle. When stretched, the more heavily cross-linked granules behave stiffly. In the less cross-linked matrix, DOPA–Fe^{3+} complexes break sacrificially, allowing the formation of microcracks between granules.

The link between the unusual DOPA–Fe^{3+} cross-linking strategy and the hard, yet extensible behavior of the byssal thread cuticle was further elucidated by confocal Raman spectroscopic imaging of thin sections of the cuticle (Harrington et al., 2010) (Fig. 4.5B). This investigation demonstrated that the tris–DOPA–Fe^{3+} cross-links are more concentrated in the granular regions of the cuticle than in the surrounding matrix (Harrington et al., 2010;

Taylor et al., 1996). This is consistent with the more stiff behavior of the granules under tensile loading (Holten-Andersen et al., 2007) and suggests that the granules and matrix fulfill different mechanical functions (Fig. 4.5D). More specifically, the granules, owing to their elevated DOPA–Fe^{3+} cross-link density were proposed to provide hardness under compressive loading, as the granules are forced together during abrasion in marine environments (Harrington et al., 2010). The less cross-linked matrix material on the other hand, is proposed to provide extensibility, via rupture of sacrificial tris–DOPA–Fe^{3+} complexes at high strains (i.e., >30%), leading to formation of microcracks between the granules (Harrington et al., 2010; Holten-Andersen et al., 2007). Based on the ability of DOPA–metal bonds to break and reform reversibly, it was proposed that microcracks may self-heal once the cuticle is relaxed following extension; however, this is yet to be demonstrated experimentally. As already mentioned, elevated concentrations of Ca^{2+} are also co-localized with mfp-1 and Fe in the cuticle (Holten-Andersen et al., 2009a) yet presently, they have no clear function. DOPA is not known for its tendency to bind Ca^{2+} and furthermore, mfp-1 carries a strong net positive charge at seawater pH due to its high pI value (~10.5), making electrostatic interactions equally unlikely (Lee et al., 2011) (Table 4.2). It has been proposed that negatively charged fatty acids might also be present in the cuticle, which interact with Ca^{2+}; however, this has not yet been corroborated (Holten-Andersen et al., 2009a). It is, of course, possible that there is an as-of-yet unidentified cuticle component that might interact with Ca^{2+}, but presently no suitable candidates have been revealed.

4.4 ROLE OF PROTEIN–METAL INTERACTIONS IN THE ADHESIVE PLAQUE

Strong adhesion between two surfaces is dependent on the ability to establish strong physical interactions at the molecular interface (Stewart et al., 2011). On a dry clean surface, this is relatively straightforward; however, in seawater, this can become exceedingly tricky. The major impediment to establishing adhesion in marine environments is infiltrating the layer of ions and organic macromolecules that is fixed firmly to exposed surfaces in the ocean (Stewart et al., 2011). While man-made adhesives still struggle to overcome this fundamental challenge, the mussel rapidly and reliably fabricates a versatile glue, the byssal thread plaque, that is, compatible with an astonishing range of surface chemistries in marine environments. Based on its impressive performance, a substantial amount of work has been invested

in the last 30 years in order to unlock the secrets of mussel byssal thread plaque.

Biochemical investigations have identified at least five proteins that are localized in the byssus adhesive plaque—namely, mfp-2, -3, -4, -5, and -6 (Table 4.2). Like the cuticle protein mfp-1, these five proteins are characterized by a high isoelectric point and the presence of DOPA (Lee et al., 2011) (Table 4.2). Many years of work have localized each of the mfp proteins to specific regions of the byssal thread plaque as summarized in Figure 4.6. More recently, surface force apparatus (SFA—described in the previous section) experiments performed on extracted and purified mfp proteins by the groups of Waite and Israelachvili have begun to shed light on the functional roles of various mfp protein in the plaque. The following section provides an overview of the current understanding; however, we direct the reader to a recent and more thorough review specifically focused on the byssal adhesive plaque (Lee et al., 2011).

MALDI–TOF mass spectrometry performed on the adhesive residue remaining after plaque removal was able to provide strong evidence that the adhesive interface of the plaque consists primarily of mfp-3 and -5 (Zhao et al., 2006; Zhao & Waite, 2006a) (Fig. 4.6D). The adhesive function of mfp-3 and -5 was further supported by SFA measurements demonstrating that these two proteins display the highest adhesion energies of all the mfps. The fact that mfp-3 and -5 also have the highest DOPA content led to the hypothesis that DOPA is the primary adhesive agent. Contrary to initial speculations that DOPA–metal interactions, specifically, were the primary bonds controlling plaque adhesion, very high adhesive energies were recorded even in the absence of metal ions on surfaces such as mica where hydrogen bonding likely dominates (Lee et al., 2006; Lin et al., 2007; Yu et al., 2013a) (Fig. 4.6E). Nevertheless, mfp-3 was shown to create strong adhesion with TiO_2-coated mica surfaces via coordination with Ti at neutral to basic pH (Yu et al., 2013b), suggesting that plaque adhesion in nature likely occurs through an assortment of different physical interactions (Fig. 4.6E).

While these investigations indicate that DOPA residues in mfp-3 and -5 are able to mediate strong adhesion to a variety of surface chemistries, DOPA is nonetheless highly susceptible to oxidation into the quinone form of DOPA, which has a significantly reduced affinity for adhering to surfaces and a propensity for further cross-linking reactions (Lee et al., 2006; Yu et al., 2011a). Interestingly, however, the mussel has apparently evolved a mechanism for counteracting the oxidation of DOPA in mfp-3 and -5 at the plaque adhesive interface (Nicklisch et al., 2013; Yu et al., 2011b). In this

case, mfp-6, a cysteine-rich protein that is also localized near the plaque–surface interface (Fig. 4.6D) is proposed to function as a reducing agent that counteracts DOPA oxidation and enables more robust adhesion by mfp-3 and -5 (Yu et al., 2011b). In the proposed mechanism, free thiolates in mfp-6 are oxidized at the plaque–surface interface in a reaction coupled with the reduction of DOPA–quinone back to DOPA.

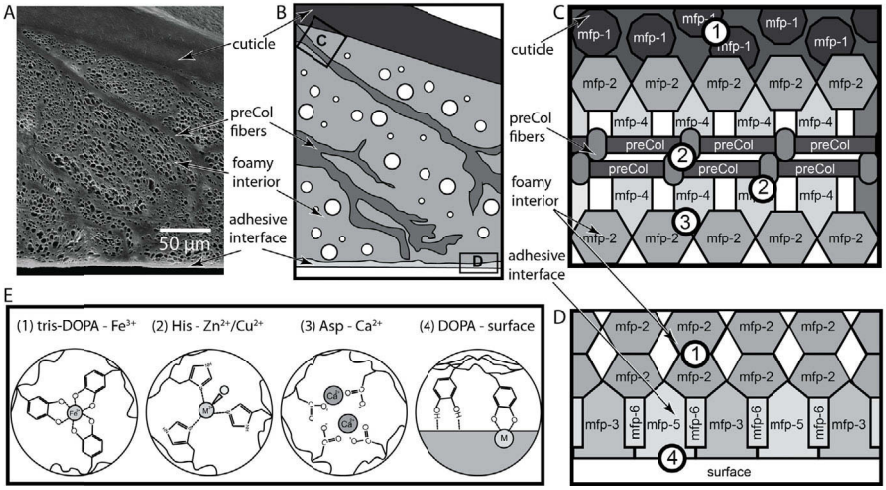

FIGURE 4.6 Metal interactions in the byssal thread adhesive plaque. (A) An SEM image of the adhesive plaque and (B) a schematic representation, which highlights specific morphological features (e.g., cuticle, preCol fibers, foamy interior, and adhesive interface). (C) and (D) Schematic models indicating the different protein variants found in the plaque in the region of the foamy interior near the cuticle (C) and at the adhesive interface (D) as indicated in panel (B). (E) Overview of different proposed metal-based interactions between the various protein components in the plaque. Numbers correspond to specific interactions identified in panels (C) and (D).

In contrast to mfp-3 and -5, the adhesive energy measured with SFA for mfp-2 (~5 mol% DOPA) on mica is almost negligible (Hwang et al., 2010). However, the cohesive forces between two symmetric layers of mfp-2 in the presence of Fe^{3+} ions were shown to be quite high, suggesting a nonadhesive role for this protein in the byssal plaque. *In situ* Raman microscopic imaging of the plaque confirmed that mfp-2, makes up the foamy interior and furthermore, that it forms tris–DOPA–Fe^{3+} interactions that are proposed to provide mechanical stability to the structure (Hwang et al., 2010) (Fig. 4.6C–E). Interestingly, it was also shown that Ca^{2+} could mediate weak adhesion between layers of mfp-2 in SFA measurements, possibly by electrostatic interactions

with a putative calcium-binding consensus sequence in the protein (Hwang et al., 2010) (Fig. 4.6D and E).

The protein mfp-4 is believed to be localized at the interface between the plaque and the distal region of the thread core and is therefore proposed to mediate the interaction between the preCols and the plaque proteins (Zhao & Waite, 2006b) (Fig. 4.6C). Unlike the other mfp proteins, mfp-4 is enriched in histidine residues (~20 mol%), which are arranged in numerous tandem decapeptide repeats and has been shown to have affinity for binding Cu^{2+}, similar to His-rich domains of the preCols (Zhao & Waite, 2006b). Additionally, mfp-4 also contains tandem repeats of aspartate-rich motifs that possess an affinity for binding Ca^{2+} similar to the calcium-binding domains of mfp-2 (Zhao & Waite, 2006b). Thus, it was proposed that mfp-4 might be a molecular bridge at the interface between the preCol fibers that interpenetrate into the foamy core of the plaque, mediated primarily through metal binding (Zhao & Waite, 2006b) (Fig. 4.6C and E).

To sum up, the byssal thread plaque is a complex underwater adhesive composed of at least five different protein variants localized to particular regions of the plaque where they are proposed to serve specific functions (Fig. 4.6, Table 4.2). Interactions between the different protein variants are vital to their synergistic function and are primarily mediated by molecular bridging and cross-linking via metal ions (Fig. 4.6E). In this respect, the example of the plaque truly underscores the extent to which the structure and biochemistry of the mussel byssus has been tuned and refined through evolution, and especially highlights the fact that not all protein metal bonds are created equal. It is of course, not completely understood why particular combinations of amino acid and metal ions are found in specific regions of the byssal thread and not others; however, it is tempting to speculate that the specific physicochemical properties of the different interactions (i.e., His-based vs. DOPA-based cross-linking) offer specific advantages during the function or formation of the material. Although there is much that still needs to be understood about the byssal thread plaque, the biochemical design principles extracted through their study have already led to the development of bioinspired materials for medical and technical applications. In particular, DOPA has been engineered into polymer chains to generate surgical glues, underwater adhesives, as well as, antifouling coatings in aquatic environments (Lee et al., 2011).

4.5 THE ROLE OF METAL IONS IN MUSSEL BYSSAL THREAD ASSEMBLY

It should be clear at this point that the mussel byssus is a multifaceted and structurally complex material. Thus, it is all the more surprising that in contrast to similarly complex biological materials, such as vertebrate tendon that forms over extended time periods, each byssal thread is formed in only a few minutes time. Byssal thread formation involves a choreographed secretion of the byssal protein precursor molecules, which then rapidly self-assemble into the specific nano-architectured structures that define the core, cuticle, and plaque (Pujol, 1967) (Fig. 4.2C and Fig. 4.7). As has been shown, quite a lot is now understood about the structure–function relationships that define these materials; however, comparatively little is understood about how byssal threads are fabricated by the mussel. This section will review the most current understanding of byssal thread assembly, highlighting the importance of protein–metal interactions.

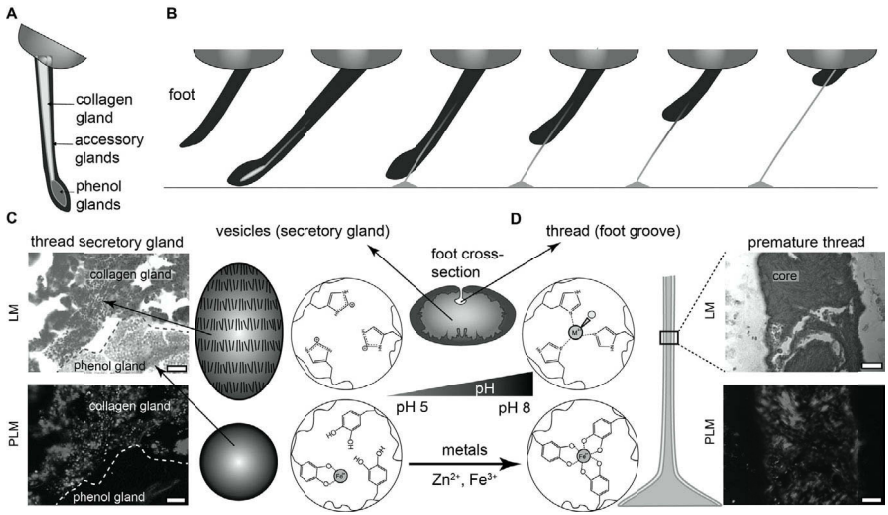

FIGURE 4.7 Byssal thread formation process and the role of metal ions. (A) Schematic view of the mussel foot indicating locations of the various secretory glands. (B) Schematic indicating the thread formation process. The mussel foot reaches out of the shell to find a spot on the surface to form a thread. Proteins are secreted into a groove in the foot, where they self-assemble and cross-link in a matter of minutes. (C) Thread proteins are stored in secretory vesicles as indicated in the light microscopy (LM) image, showing stained histological sections of the collagen gland (core) and phenol gland (plaque) in the a mussel foot. The polarized light microscopy (PLM) image indicates that the contents of the collagen gland are birefringent, suggesting liquid crystalline storage of preCols prior to thread formation.

Proteins are stored at low pH (pH < 5) in the vesicles, which prevents premature formation of metal coordination complexes by DOPA and histidine. (D) Thread formation involves the secretion of the proteins into the foot groove, which has a pH ~ 8, allowing DOPA and histidine to form complexes with available metal ions. LM and PLM images show early stages of thread core formation and alignment. All scale bars represent 10 μm.

The organ responsible for byssal thread formation is known as the foot. The foot is an extendable tongue-like appendage, which the mussel can extend outside of its shell (Fig. 4.7). Within the mussel foot are specialized secretory glands in which all the protein precursor molecules that comprise the thread are synthesized, packaged into secretory vesicles and stored (Fig. 4.7A and C). Extensive TEM studies of the mussel foot secretory glands were able to identify three different glands that produce three different vesicle types (Tamarin & Keller, 1972; Zuccarello, 1980, 1981)—namely, the phenol gland, the enzyme gland, and the collagen gland, which produce the plaque, cuticle, and thread core, respectively (Fig. 4.7A and C). The seemingly random gland nomenclature predates the discovery of the individual proteins that compose the threads.

Thread formation begins when the mussel extends its foot out of the protective confines of the shell and onto the surrounding surface (Fig. 4.7B). The mussel first searches the surface briefly until it settles on an appropriate spot to form a thread. At this point, the contents of the secretory vesicles are released into a groove running along one side of the mussel foot, which is sealed to the seawater (Fig. 4.7B and C). The end of the groove furthest from the mussel, known as the distal depression, is the site of plaque formation and is pressed tightly against the surface creating a water-tight groove into which the mfp-2, -3, -4, -5, and -6 are secreted (Hwang et al., 2010; Tamarin et al., 1976). Along the length of the foot groove, the mussel first secretes the collagen gland contents, forming the core, which is then followed by secretion of the cuticle-forming vesicles containing mfp-1. When the secretion process is over, the foot groove opens its seal and pulls away, leaving a white-golden byssal thread that will immediately be loaded by the next set of crashing waves (Fig. 4.7B).

The intricate details of the secretion process occurring within the foot are hidden from the prying eyes of researchers; thus, most of our knowledge comes from invasive techniques such as TEM or *in vitro* experiments on purified byssal thread proteins. For example, the ellipsoidal secretory vesicles of the collagen gland, which encapsulate the preCol molecules that will form the byssal thread core, exhibit a characteristic fibrous banding pattern in TEM imaging (Fig. 4.7C). Based on similarities between the spacing of

these fibrillar contents and those in the native thread (Hassenkam et al., 2004; Krauss et al., 2013), it was proposed that the preCols are pre-organized in the vesicles into a liquid crystalline-like phase with smectic structure (i.e., the preCols are aligned in the same direction with the collagen domains packed like books on a shelf) (Hassenkam et al., 2004) (Fig. 4.7C). A liquid crystalline phase would be advantageous during formation by allowing rapid assembly of the preCols into a highly ordered state reminiscent of a solid crystal, while still allowing the vesicle contents to flow like a fluid. Once the structure is set, however, the order must be locked in rapidly before the thread can assume its load-bearing role.

Several studies have suggested that pH-triggered His–metal cross-linking may contribute to the locking mechanism during byssal thread formation (Fig. 4.7C). The terminal ends of adjacent preCol bundles along the length of the thread core interact via their His-rich domains. As already mentioned, histidine, which possesses a typical p*K*a value of ~6.5, is highly sensitive to the pH of the local environment. The thread secretion in the region of the plaque has been measured to be quite acidic (pH < 5), suggesting that the preCols may also be stored under acidic conditions (Fullenkamp et al., 2014; Yu et al., 2011b). Assuming an average p*K*a of 6.5, the overwhelming majority of histidine residues would be positively charged under these acidic conditions and unable to bind metal ions (Fig. 4.7C). Within the secretory vesicles, this would provide a reliable means of preventing premature assembly of preCols, while still permitting their liquid crystalline alignment. Upon secretion into seawater (pH 8.2), the vast majority of histidine residues would be deprotonated, removing the charge repulsion and allowing coordination of divalent metal ions such as Zn^{2+} and Cu^{2+} (Fig. 4.7D).

Support for this hypothesis of thread core assembly was provided from *in vitro* assembly studies on purified preCol proteins, in which it was shown that preCol fibers were only formed at pH values above the p*K*a of histidine (Harrington & Waite, 2008b). Furthermore, it was possible to reduce the metal-dependent interaction energy between two layers of peptides based on preCol His-rich domain sequences by a factor of six when the pH of the surrounding medium was reduced from 8.0 to 4.8 (Schmidt et al., 2014). In spite of the growing support for this hypothesis, it may only be one contributing factor to the fast locking of preCol structure. For example, highly conserved tyrosine residues in the preCol His-rich domains are believed to be posttranslationally converted to DOPA, which could also contribute to cross-linking between neighboring preCol bundles during thread formation (Harrington & Waite, 2007).

After a new byssal core is assembled in the foot groove, the cuticle-forming vesicles are released into the groove where they are thought to merge and coalesce around the thread to form the protective cuticle (Holten-Andersen et al., 2011; Holten-Andersen & Waite, 2008; Zuccarello, 1980). As described above, the hard, yet extensible performance of the cuticle is highly dependent on the formation of a network of DOPA–Fe^{3+} cross-links with mfp-1, which are localized into dense cross-link centers (granules) in a less densely cross-linked milieu (matrix) (Harrington et al., 2010) (Fig. 4.5). It has been proposed that, similar to the formation of His–metal interactions in the thread core, the formation of the DOPA–Fe complexes in the cuticle is also triggered by a pH jump from acidic to basic conditions (Holten-Andersen et al., 2011) (Fig. 4.7C and D). This hypothesis is supported by *in vitro* experiments on DOPA–Fe^{3+} cross-linked polymer gels, which undergo a transition from the mono (1 DOPA:1 Fe^{3+}) to bis (2 DOPA:1 Fe^{3+}) to tris (3 DOPA:1 Fe^{3+}) forms of the DOPA–Fe complex as the pH was increased stepwise (Holten-Andersen et al., 2011). Transitions in DOPA coordination are accompanied by a concomitant transition in mechanical performance, going from a fluid-like behavior at low pH to a viscoelastic hydrogel at high pH (Holten-Andersen et al., 2011).

A key structural feature of the cuticle is its granular morphology; however, it is still an open question as to how this forms. Based on the fact that higher amounts of DOPA–Fe^{3+} cross-links are present in the granules than in the matrix (Harrington et al., 2010), two alternative hypotheses were proposed to account for this: (1) the granular protein is more highly condensed than the matrix or (2) the protein in the granules contains a higher content of DOPA. In support of the former hypothesis, the cuticle forming secretory vesicles undergo a maturation process in which the contents apparently undergo a condensation process to form the granule and matrix material within a single vesicle (Zuccarello, 1981). In support of the latter hypothesis, there have been two variants of mfp-1 identified, which different only in the degree to which the Tyr residues are converted to DOPA (Harrington et al., 2010; Sun & Waite, 2005). Presently, however, these hypotheses await further testing. Interestingly, a recent study suggests that subtle sequence differences in mfp-1 between *M. californianus* and *M. edulis* might contribute to the observed differences in granule morphology and mechanical performance of cuticles between the species (Das et al., 2015).

Like the plaque structure itself (Fig. 4.6), the plaque formation process appears to be quite complex. MALDI–TOF analysis of the forming plaque at various time points revealed a progression of different proteins being

secreted successively into the distal depression of the foot where the adhesive forms (Yu et al., 2011b). It was observed that variants of the adhesive protein mfp-3 are first secreted, followed shortly thereafter by the Cys-rich protein, mfp-6. As already mentioned, the authors of this study proposed that the adhesion of mfp-3 is enhanced on surfaces because oxidation to DOPA–quinone in mfp-3 is counteracted by the reducing thiolate groups of mfp-6. A recent investigation in which plaques were formed by mussels on surfaces coated with pH-sensitive dyes indicated that the initial adhesive secretion may have a pH as low as 2–3. These highly acidic conditions would be favorable for creating adhesion because they additionally hinder DOPA oxidation (Martinez Rodriguez et al., 2015). Furthermore, another recent study has suggested that under pH and ionic strength conditions similar to those expected during plaque secretion, mfp-3 will self-coacervate *in vitro*, which means that it forms a dense fluid phase of the protein separated from bulk water (Wei et al., 2014). The mfp-3 coacervate was found to have low interfacial energy, which may help it to spread over surfaces, enhancing adhesion mediated by DOPA. Based on the importance of DOPA–Fe^{3+} as stabilizing cross-links in the mfp-2 proteins comprising the foamy interior of the plaque (Hwang et al., 2010), it is not a stretch of the imagination to posit that a similar pH-triggered metal-dependent cross-linking mechanism might occur as proposed for the thread cuticle (Holten-Andersen et al., 2011) (Fig. 4.7C and D). However, at this point, it is still not clear how the complex foamy architecture is formed. Interestingly, recent *in vitro* results suggest that it would not be a clever strategy to store Fe and DOPA together at low pH. In this study, mixing purified mfp-1 and Fe^{3+} at low pH was shown to result in the oxidation of DOPA residues leading to formation of covalent diDOPA cross-links, which would be a highly unfavorable result prior to secretion (Fullenkamp et al., 2014).

To summarize, the rapid formation of byssal threads appears to be a highly orchestrated process, about which very little is known at present. Preliminary insights into this process reveal several common themes, which are worth repeating. First, the precursor molecules are stored in secretory vesicles in which they are pre-organized to facilitate rapid acquisition of the desired structure upon secretion (Fig. 4.7C). Second, the pH jump going from the acidic secretory vesicles (pH < 5) to the basic seawater (pH ~ 8) environment may play an important role in quickly locking the thread structure immediately after secretion of the precursors (Fig. 4.7C and D). Based on the sensitivity of both histidine and DOPA to pH in this physiologically relevant range (pH 5–8), protein–metal cross-linking has been highlighted as a likely candidate for the initial locking mechanism. While these insights are

exciting and already leading to the development of mussel inspired hydrogels (Fullenkamp et al., 2013; Holten-Andersen et al., 2011), there is still a great deal to be understood about byssal thread assembly.

4.6 SUMMARY

Byssal threads are remarkable protein-based fibers crucial to the evolutionary success of mussels. This is in no small part due to their impressive material properties—for example, self-healing, abrasion resistance, and wet adhesion. A key design feature contributing to these properties is the clever use of metal–protein interactions as critical cross-links. Enormous efforts over the last 30 years have advanced our understanding of the structure–function relationships that define this material to the point that mussel-inspired synthetic polymers are being developed and even applied in clinical settings (Haller et al., 2011). In spite of the rapid progress in our understanding of the mussel byssus, however, there are many exciting questions left to be answered that will provide even further insights into the formation and behavior of this extraordinary material.

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KEYWORDS

- **marine mussels**
- **byssal threads**
- **histidine**
- **DOPA**
- **metal coordination**

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CHAPTER 5

PHYSIOLOGY OF ENVENOMATION BY CONOIDEAN GASTROPODS

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CONTENTS

5.1 INTRODUCTION

This chapter focuses on the physiology of envenomation by gastropods in the superfamily Conoidea (Puillandre et al., 2011; Tucker & Tenorio, 2009); the best known of these are the cone snails (Röckel et al., 1995). This may be the most species-rich superfamily in the phylum Mollusca (it is estimated that there are over 12, 000 extant conoidean species) (Bouchet et al., 2009; Olivera et al., 2014). The vast majority of all conoideans are venomous, although a small minority of lineages have secondarily lost their venom apparatus (Holford et al., 2009). Significant advances have been made in elucidating physiological mechanisms that underlie the prey capture strategy of a few fish-hunting cone snail species.

In contrast to other predatory lineages of molluscs, except for their venom apparatus, conoidean snails are notably lacking in specialized morphological adaptations that facilitate prey capture. Among venomous animals, conoidean gastropods are at one extreme end of a continuous spectrum, where venom is used in conjunction with other types of weaponry for prey capture. Molluscs such as cephalopods use a combination of speed and anatomical adaptations such as tentacles for prey capture, but the slow moving conoidean snails are generally totally dependent on venom for capturing their prey. Not surprisingly, their evolution has resulted in highly complex and sophisticated venoms (Olivera, 2002).

The superfamily Conoidea is one of the major groups of predatory marine snails that have undergone a major adaptive radiation since the cretaceous extinction in the Order Neogastropoda and are prominent components of present-day shallow-water tropical marine communities. As a group, neogastropods have undergone an enormous anatomical diversification of foregut structures, with many neogastropod lineages evolving specialized glands for the biosynthesis of secretions that aid these predators in capturing their prey. The investigation of the chemical strategies of neogastropods is in its infancy, and the analyses of their biochemistry and molecular biology has been carried out on very few species. A recent pioneering study has been on the colubrarid snails, which are the vampires of the ocean that suck blood from fish (see Modica et al., 2015). However, in this chapter, the focus will be restricted to the superfamily Conoidea, which secrete the gene products for interacting with their prey, predators, and competitors in a venom synthesized characteristic and defining anatomical structure, the venom duct (also called the venom gland). At present, there are more biochemical/molecular data available for the superfamily Conoidea than for all other neogastropod lineages combined.

Conoideans have been the subject of several phylogenetic studies (see Puillandre et al., 2011); classically, three large divisions within the superfamily, cone snails, terebrids (or auger snails), and turrids were recognized, but it has become clear that the last group, the turrids is not monophyletic (see Taylor et al., 1993). In the taxonomic treatment for Conoidea by Puillandre et al. (2011), 14 family groups are proposed (instead of the 3 classically recognized in earlier work). Some of the larger groups comprise hundreds and even thousands of species; these appear to fall into two major clades; one that includes the family Conidae, with the Terebridae and Turridae (sensu stricto), as family groups in the other major clade.

Traditionally, all ~800 species of cone snails were assigned to a single genus, *Conus*. The molecular phylogeny that has recently been carried out (Puillandre et al., 2014) has led to the recognition that three groups of cone snails are quite distant phylogenetically from most *Conus* species, and these have been assigned to other genera. In this scheme, the family Conidae now comprises four genera, *Conus*, *Conasprella*, *Profundiconus*, and *Californiconus*. The redefined genus *Conus* encompasses the vast majority of species (>500). Two of the other lineages, *Profundiconus* and *Conasprella* are primarily deep-water groups (except in the Western Atlantic and Panamic regions where the latter is represented by a handful of shallow-water forms). Surprisingly, little is known regarding the physiology of envenomation of these groups. The available evidence from aquarium observations and gut-content analysis is that *Conasprella* species prey on polychaetes (Costa, 1994; Kohn, 2014). The genus *Californiconus* is a monospecific lineage, with *Californiconus californicus* being the only known species (Duda et al., 2001; Espiritu et al., 2001; Puillandre et al., 2015).

Within the impressive biodiversity of venomous conoideans, the documented knowledge regarding envenomation physiology is highly skewed. For a few species, physiological mechanisms that underlie prey capture are understood in exquisite mechanistic detail, to the point where detailed molecular interactions can be defined. In contrast, however, for most conoidean gastropods, virtually nothing is known about envenomation, and in some cases, even the major prey of entire major lineages in the superfamily have not been identified. Consequently, this chapter is somewhat schizoid: although case studies are presented where understanding of envenomation physiology is highly sophisticated, for most species in Conoidea, only fragmentary descriptive observations relevant to envenomation are available.

We begin by describing envenomation for two species of conoidean molluscs for which an intensive investigation has been carried out of both the pharmacology of the venom components and the physiology of prey capture. These two species are both cone snails (genus *Conus*). *Conus geographus*, the geography cone, is the first case study; this conoidean species is generally regarded as the most dangerous to man, having caused over a dozen human fatalities (Fegan & Andresen, 1997; Kohn, 1958) (which is why it has been intensively investigated). A second species, the purple cone *Conus purpurascens* has an entirely different strategy for prey capture. The contrast between the two species will illustrate how even though both have the same prey (fish), strikingly different physiological mechanisms have evolved in these congeners, and there is compelling evidence for distinct evolutionary pathways to fish hunting for these cone snails (Olivera et al., 2014, 2015).

5.2 PHYSIOLOGY OF ENVENOMATION: FISH-HUNTING *CONUS*

5.2.1 OVERVIEW OF ENVENOMATION

What emerges from the best-studied examples of conoidean envenomation is that multiple components of a conoidean venom acting coordinately are required for prey capture (Olivera, 1997, 2002). Each individual venom component functions as a potent pharmacological agent that specifically targets a particular molecular site in potential prey; when a venom component acts at its pharmacologically relevant site, it clearly elicits a downstream physiological consequence. The typical scenario is for multiple venom components to act together on a targeted circuitry, thus efficiently achieving a desired physiological endpoint relevant to prey capture.

Because the target sites can be functionally related to each other (and often are on signaling proteins that act sequentially within a physiological circuit), by targeting multiple sites simultaneously, the venom potently alters circuit function. One specific example is the circuitry responsible for neuromuscular transmission. The group of toxins that target distinct pharmacological sites on the same physiological circuitry is called a "cabal," with reference to the secret societies that are out to overthrow existing authority. Each cabal comprises groups of peptides that act in synergy to achieve a specific end point. The set of individual molecular targets that are functionally linked are referred to as a "constellation" of signaling components, required for the proper functioning of that circuit. In effect, conoidean

gastropods have evolved a highly sophisticated form of combination drug therapy that we shall refer to as "constellation pharmacology;" their strategy is to target functionally linked constellations coordinately, not just one individual molecular target at a time. The detailed physiology of prey capture (and how constellation pharmacology is applied) will be discussed using the two specific case studies that follow.

5.2.2 CASE STUDY 1: CONUS GEOGRAPHUS

C. geographus, the geography cone is the most deadly of all *Conus* species, responsible for most documented human fatalities from cone snail stings. In the absence of medical intervention, the fatality rate is ~70% (Yoshiba, 1984). Because of the considerable interest in the venom components from this species, it is possible to reconstruct in unprecedented detail the physiology of envenomation that this cone snail uses to capture its prey. There are probably more mechanistic insights for venom components from *C. geographus* than for any other species in the superfamily Conoidea.

The geography cone is found in the Indo-Pacific region, from the Western Pacific through the Indian Ocean to East Africa. *C. geographus* appears to be a highly specialized predator that can potentially capture a whole school of small fish hiding in reef crevices at night (presumably to avoid predation by sharks). For prey capture this snail has two distinctive suites of toxins, with each resulting in a different physiological end point.

One group of toxins, the "nirvana cabal, " is released into the water as the snail approaches a school of fish. The physiological endpoint for these venom components is to disorient the fish, largely by jamming sensory circuitry and to deter their escape, primarily by making the fish severely hypoglycemic and placid. The peptides of the "nirvana cabal" would render all of the fish in a school easier for the snail to engulf using its large distensible false mouth (rostrum) (Fig. 5.1 shows how the snail approaches prey with its distended rostrum).

Among the components of the nirvana cabal is a specialized, posttranslationally modified insulin (Safavi-Hemami et al., 2015), smaller than any other insulin peptide identified so far. When taken up through the gills, this triggers a fall in blood-glucose levels, leading to hypoglycemic shock, thereby deterring any attempt by the fish to escape. Other venom components of the nirvana cabal include a subtype-selective NMDA receptor antagonist (Donevan & McCabe, 2000), a neurotensin agonist (Craig et al., 1999b), a 5HT3 receptor antagonist (England et al., 1998) and an α9 α10

nicotinic receptor antagonist (S. Christensen et al., in preparation); together, these are thought to inhibit the sensory circuitry of the fish, particularly the lateral line system. As a result, an entire school of fish can be rendered both hypoglycemic and sensory deprived, and when the snail opens its cavernous false mouth (rostrum), the school can be engulfed. In effect, through the nirvana cabal, the school is primed for capture using the snail's "net." This prey capture strategy has been referred to as the "net" or "net engulfment" strategy (Olivera et al., 2015).

FIGURE 5.1 *Conus geographus*, foraging. As the snail approaches a fish, it opens its rostrum (false mouth) releasing the nirvana cabal components of the venom (see text).

The snail then injects venom into each captured fish, uses a hollow, needle-like radular tooth (see below); this delivers a group of paralytic toxins referred to as the "motor cabal." One inhibits the presynaptic Ca channels that control neurotransmitter release (Adams et al., 1993), another antagonizes the postsynaptic nicotinic acetylcholine receptor that is essential for depolarizing the muscle end plate (Gray et al., 1981), and finally, a

third toxin blocks the voltage-gated Na channels (Nav1.4) that underlie the muscle action potential (Cruz et al., 1985). Together, these cause an irreversible paralysis, by antagonizing neuromuscular transmission at multiple pharmacological sites. These individual venom components comprise the "motor cabal;" a cartoon representation of the molecular physiology is shown in Figure 5.2. Thus, by using two combinations of highly specific pharmacological agents, the snail can successfully capture multiple fish at one time.

Cases of human envenomation are clearly due to defensive stings by the snail; it is likely that it is the motor cabal components that cause human fatality. When hunting for prey, it appears that *C. geographus* can control the amount of venom injected. After being engulfed, each fish is injected with only a small amount of venom. However, when delivering a defensive sting to a human, the snail probably injects all available venom in a desperate bid to escape the predator. Thus, one factor likely to contribute to the high rate of human fatality is that when *C. geographus* injects all the venom in the duct, this exceeds a lethal dose for humans; the inhibitor of the nicotinic acetylcholine receptor and of voltage-gated Na channels are particularly potent peptides, and their activity is consistent with the symptomatology in clinical reports of human envenomation cases (i.e., paralysis of the diaphragm, leading to respiratory distress and death).

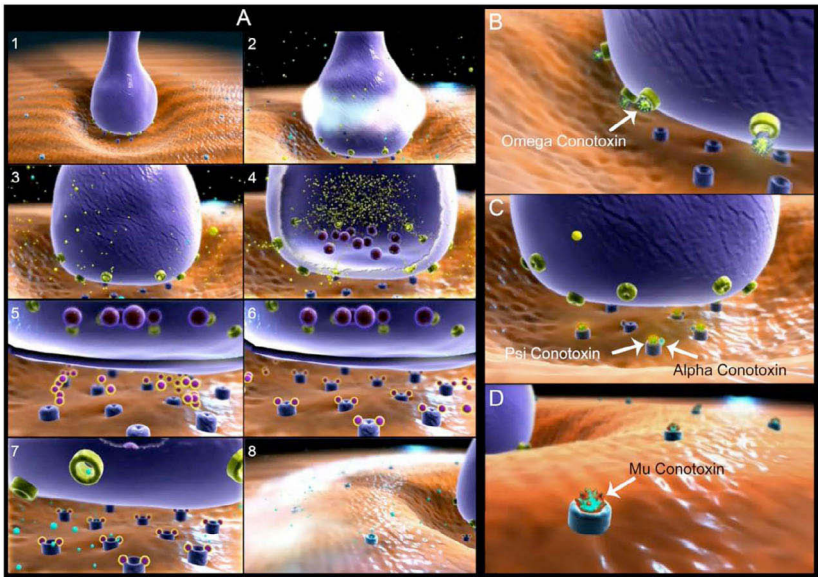

FIGURE 5.2 The Motor Cabal. Panel (A) Normal neurotransmission across the neuromuscular synapse. This panel illustrates the molecular events that occur between the arrival of an action potential at the presynaptic terminus (A1 and A2) to the generation of

FIGURE 5.2 *(Caption continued)*
the action potential on the postsynaptic muscle membrane (A8). Motor axon depolarization results in opening of voltage-gated Ca channels and the entry of calcium into the presynaptic terminus (A3 and A4). The elevated calcium triggers neurotransmitter release; the acetylcholine released then binds and activates the nicotinic acetylcholine receptor (A5 and A6), and channel opening causes depolarization of the postsynaptic terminus, which activates voltage-gated Na channels and triggers the muscle action potential (A7 and A8). Panels (B), (C), and (D) show block by various components of the motor cabal. Panel (B) shows the block by ω-conotoxin, which targets the voltage-gated Ca channels at the presynaptic terminus. Panel (C) shows block of the postsynaptic nicotinic acetylcholine receptor by α-conotoxins and psi-conotoxins; α-conotoxins are competitive antagonists and psi-conotoxins are channel blockers. Panel (D) shows the activity of μ-conotoxins—by blocking voltage-gated Na channels, the action potential on the postsynaptic side is inhibited. Cone snails use all of the inhibitory mechanisms in Panels (B), (C), and (D) in a combination to potently block neuromuscular transmission.

5.2.3 CASE STUDY 2: CONUS PURPURASCENS

C. purpurascens, the purple cone, is found in the Panamic biogeographic province, from the Galapagos to the Sea of Cortez. It is the only fish-hunting *Conus* in the Panamic region; its only close relative is *Conus ermineus*, which is the only fish-hunting species known in the Caribbean and Tropical Atlantic. A comprehensive analysis of the venom of *C. purpurascens* has been carried out (Hopkins et al., 1995; Shon et al., 1995; Terlau et al., 1996). As is the case described above for *C. geographus*, there are two distinctive suites of toxins in the venom that act to achieve different physiological end points that are essential for prey capture (these are referred to as the "lightning-strike cabal" and the motor cabal). The lightning-strike cabal causes an extremely rapid tetanic immobilization of the envenomated fish. In contrast, the motor cabal results in an irreversible block of neuromuscular transmission. This latter group has the same targeted end point as the motor cabal discussed above for *C. geographus*.

C. purpurascens has a highly distensible proboscis, which is colored bright red (see Fig. 5.3). When approaching potential fish prey, the proboscis is extended far out of the rostrum, and once the tip of the proboscis touches the skin of a fish, a disposable radular tooth jets out of the proboscis—this functions both as a hypodermic needle for venom injection, but also as a harpoon to tether the prey. The snail grasps the basal end of the harpoon with a powerful muscular sphincter at the tip of the proboscis. As the radular tooth pierces the skin of the fish, venom is injected, and the snail typically pulls back the proboscis. Since the radular tooth is highly barbed with an

accessory process (see Section 5.3.2), the fish becomes tethered through the radular tooth and as the proboscis is reeled back into the rostrum of the snail, the fish is completely engulfed by the rostrum.

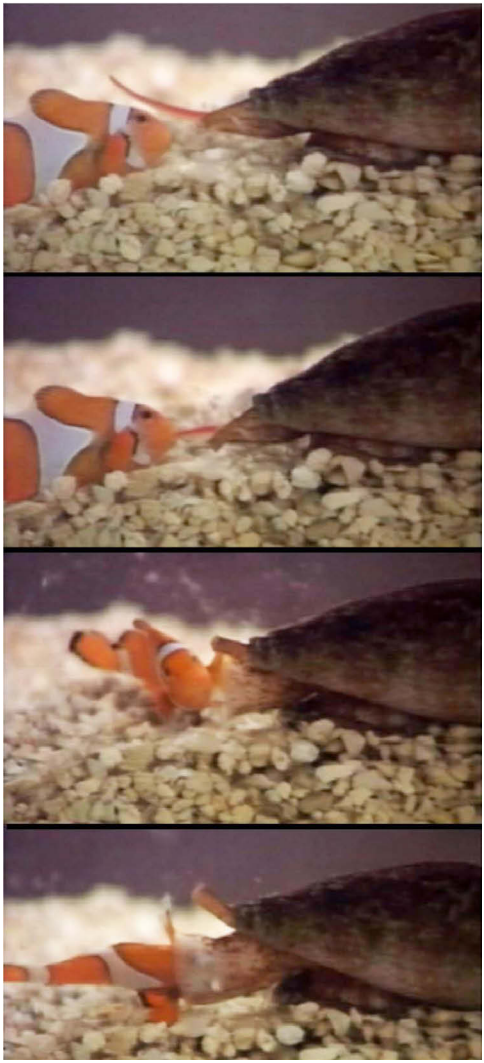

FIGURE 5.3 *Conus purpurascens*, envenomation sequence. Upon detecting a fish, the snail extends its bright red proboscis (top). After the fish is struck, the snail retracts its proboscis toward the rostrum, and the immobilized fish is engulfed by the "false mouth" and predigested. The scales and bones of the fish, and the radular tooth used to inject the venom are regurgitated, and the soft parts of the prey move further into the gut of the snail.

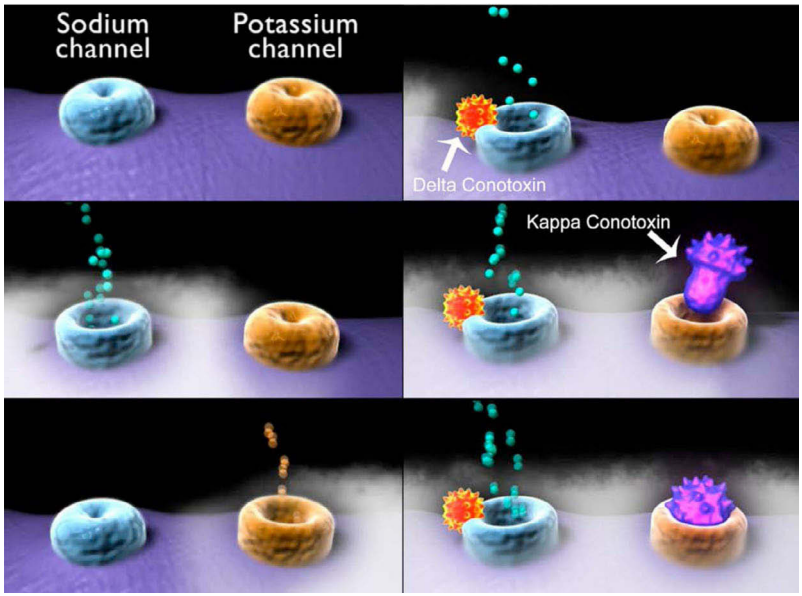

FIGURE 5.4 The effects of the "lightning-strike cabal" on action potentials of axons. Shown on the left panel is the normal progression of an action potential. As the membrane potential is depolarized, sodium channels open and rapidly inactivate (middle and lower left panels). This inactivation of sodium channels and the delayed opening of voltage-gated K channels together repolarize the cell membrane. Thus, these key channel properties, acting coordinately, are responsible for making an action potential transient. On the right panel is an illustration of how a cone snail targets these channels and modulates basic mechanisms of action potential generation. A delta toxin binds to voltage-gated sodium channels (top right) and when the channel opens, the delta toxin inhibits inactivation. This results in the channel remaining open and continuously depolarizing the surrounding membrane. The inhibition of fast inactivation of Na channels, when coupled to the binding of a kappa conotoxin to the voltage-gated K channels (right middle and lower panel) means that both molecular mechanisms for repolarizing the cell membrane are inhibited. As a consequence, the two toxins acting in concert cause a sustained depolarization of affected axonal membranes. In effect, this is the same as applying a powerful taser to a particular site. This is thus a key mechanism of the "taser and tether" strategy for prey capture.

would presumably be rendered largely quiescent, leading to sensory deprivation of the envenomated animal. Thus in many ways, the physiological endpoints are diametrically opposite: hyperstimulation of neuronal circuitry by the lightning-strike cabal, depression of the activity of neuronal circuitry by the nirvana cabal.

Both species of *Conus*, however, have a motor cabal, and, therefore, the broad physiological goals of these venom components are parallel: the complete suppression of neuromuscular transmission, ultimately resulting

The first suite of toxins, the lightning-strike cabal (see Fig. 5.4), has as key components, a δ-conotoxin (in *C. purpurascens*, the specific peptide is δ-conotoxin PVIA) (Terlau et al., 1996) and κ-conotoxins (κ-conotoxin PVIIA) (Shon et al., 1998). δ-Conotoxin PVIA acts by inhibiting fast inactivation of axonal voltage-gated Na channels. Thus, Na channels remain in an open state, and when this is combined with the action of κ-conotoxins, which block voltage-gated K channels on the same axon, all of the mechanisms for terminating an action potential are inhibited. Thus, near the venom injection site, all of the axons become massively depolarized; this is equivalent to applying a powerful electric shock, such as a taser. This generates an electrical storm in the nervous system of the fish, resulting in the tetanic paralysis of the fish within seconds.

This prey capture strategy has been called "taser and tether." The synergy between the two peptides, δ-conotoxin PVIA and κ-conotoxin PVIIA is very striking (Shon et al., 1995). Although δ-conotoxin PVIA will eventually cause a tetanic paralysis of the fish, it has a relatively long onset; κ-conotoxin PVIIA by itself has rather subtle behavioral effects on the fish. However, when the two are injected together, tetanic paralysis occurs in seconds, thereby recapitulating what is observed when the whole venom is injected into the fish by the snail. It is the joint action of the two peptides that is key to the extremely rapid immobilization of the fish prey.

In addition to the lightning-strike cabal of venom components, *C. purpurascens* also has a motor cabal that blocks neuromuscular transmission. The similarities and differences between the motor cabals of *C. purpurascens* and *C. geographus* will be discussed in the next section.

5.2.4 ENVENOMATION BY CONUS PURPURASCENS AND CONUS GEOGRAPHUS: SIMILARITIES AND DIFFERENCES

The most striking contrast between the physiological strategy of *C. geographus* versus *C. purpurascens* are the effects of the lightning-strike cabal of *C. purpurascens* versus the nirvana cabal of *C. geographus*. The *C. purpurascens* lightning-strike cabal causes nervous system circuitry to become hyperstimulated, presumably triggering the generation of trains of action potentials that ultimately cause tetanic paralysis. In contrast, venom components that comprise the *C. geographus* nirvana cabal quiet down the targeted neuronal circuitry, which appears to be largely sensory. Individual components of the *C. geographus* nirvana cabal inhibit molecular targets necessary for activity in the targeted circuitry, and by antagonizing these, the circuitry

in paralysis. However, analysis of detailed physiological mechanisms shows both similarities and differences at the molecular level. This is most clearly illustrated by the venom peptides targeted to the postsynaptic receptor. In *C. geographus*, the nicotinic receptor antagonist, α-conotoxin G1 (McManus et al., 1981) is a competitive blocker of acetylcholine binding to its ligand site. Similarly, in *C. purpurascens*, αA-conotoxin PIVA (Hopkins et al., 1995) is a competitive antagonist at the same site. Thus, although biochemically these peptides differ significantly from each other, they are genetically related, and physiologically homologous in their mechanism of activity. What is striking, however, is that *C. purpurascens* has a second peptide that is highly expressed in the venom that inhibits the postsynaptic nicotinic acetylcholine receptor, ψ-conotoxin PIIIE (Shon et al., 1997). This peptide is also an antagonist of the nicotinic acetylcholine receptor, but binds an entirely different pharmacological site on the receptor complex: instead of being a competitive antagonist at the ligand-binding site for acetylcholine, it appears to be a channel blocker. Thus, by having two peptides acting at two different pharmacological sites, presumably, there will be synergy making *C. purpurascens* venom extremely effective in antagonizing this key molecular target that is critical for neuromuscular transmission.

The other components of the motor cabal show both striking similarities and differences between the two species. Both *C. geographus* and *C. purpurascens* have μ-conotoxins that target the muscle subtype of voltage-gated Na channels (Nav1.4) (Mahdavi & Kuyucak, 2014); these peptides, μ-conotoxin GIIIA (Cruz et al., 1989) from *C. geographus* and μ-conotoxin PIIIA (Shon et al., 1998) from *C. purpurascens*, belong to the same gene superfamily (the M-superfamily) and are structurally similar, although diverge considerably in their AA sequences.

In contrast, *C. geographus* venom has, as a major component, conotoxins that block the voltage-gated Ca channels that control neurotransmitter release from the presynaptic terminus of the neuromuscular synapse (i.e., ω-conotoxin GVIA [Olivera et al., 1984] that targets the Cav2.2 [Yarotskyy & Elmslie, 2009] channel). These are not a major component of *C. purpurascens* venom. Thus, there is convergence in the physiological end points achieved, but considerable divergence in the specific molecular mechanisms used.

5.2.5 MOLECULAR PHYLOGENETICS OF FISH-HUNTING CONE SNAILS

There are over 100 species of *Conus* that are believed to primarily hunt fish. The present molecular phylogenetic evidence, summarized in Figure 5.5,

demonstrates the relevant branch of the *Conus* phylogenetic tree that has the clades that are primarily fish hunting. The number of species tentatively assigned to each clade is shown in the figure. It should be noted that there is a considerable disparity in the diversity of each lineage, with two (*Phasmoconus* and *Pionoconus*) being significantly more species-rich than the other piscivorous lineages. These branches of the phylogenetic tree were assigned a subgeneric rank (Puillandre et al., 2015). Thus, *C. geographus* belongs to the subgenus *Gastridium*, while *C. purpurascens* is in *Chelyconus*.

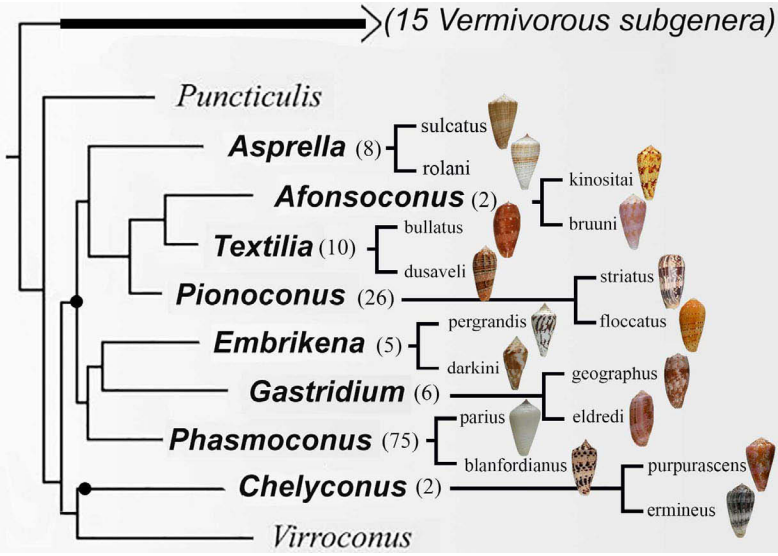

FIGURE 5.5 Molecular phylogeny of fish-hunting *Conus*. Shown are a section of the phylogenetic tree for the Conidae The eight potential piscivorous subgenera, the clades of *Conus* that are believed to be primarily fish hunting are shown in bold, and the number of species that have been assigned to each subgenus is indicated in parentheses (assignments are by Puillandre et al. (2015), except for the subgenus *Asprella*, where newer data (M. Watkins and B. Olivera, unpublished) suggests a larger number of species). Two examples are shown for each fish-hunting clade. The solid dots indicate ancestral nodes where it has been proposed that a fish-hunting ancestor evolved from a worm hunter (Olivera et al., 2015). Note that in this scheme, the subgenus *Chelyconus*, the only clade found in the New World, is postulated to have evolved fish hunting independently of the seven piscivorous Indo-Pacific subgenera.

The observations that have been made on the physiology of prey capture, when combined with the molecular phylogeny shown in Figure 5.5 suggest that fish-hunting arose independently from worm hunting at least twice. The specific molecular evidence that supports this hypothesis has recently been discussed (Aman et al., 2005; Olivera et al., 2015). However, the

possibility that some of the present day worm-hunting clades re-evolved from a piscivorous clade cannot be rigorously excluded. This hypothesis is consistent with some earlier studies that have demonstrated that the fish-hunting *Conus* species do not form a single monophyletic group (Puillandre et al., 2014). Although two of the putative subgenera shown in Figure 5.5, *Chelyconus* and *Pionoconus*, have the same general physiological strategies for capturing fish, they diverge in the molecular genetics and biochemistry of the venom components that have evolved in each subgenus for parallel physiological purposes.

The subgenus *Chelyconus*, which is restricted to the new world, has only two species, *C. purpurascens*, the purple cone, which is found in the Panamic region and *C. ermineus*, which is found in the entire tropical Atlantic, from the Gulf of Mexico to West Africa. There are no other known fish-hunting species in these marine biogeographic provinces. It is thought that the two species are very closely related and arose as a result of geographic isolation when the Central American isthmus created a barrier between the Panamic marine province and the Caribbean Sea (Puillandre et al., 2014).

In contrast, the *Pionoconus* clade is strictly Indo-Pacific, and is much more species rich. Both *Pionoconus* and *Chelyconus* (Shon et al., 1998) have a lightning-strike cabal and a motor cabal as described in detail above for *C. purpurascens*. The difference is that the gene superfamilies that have been recruited for similar physiological roles are entirely different in the two lineages. An example are the K-channel blockers that are an essential component of the lightning-strike cabal: these belong to the O-superfamily in *Chelyconus*, but in *Pionoconus*, a family of kunitz-domain containing polypeptides called conkunitzins (Bayrhuber et al., 2005) has been recruited as K-channel blockers in all of the species of *Pionoconus* examined so far.

5.2.6 FISH-HUNTING CONE SNAILS: ECOLOGY AS A DETERMINANT OF PHYSIOLOGY AND A DRIVER OF SPECIATION

For cone snails that primarily prey on fish, a key determinant for the success of an individual species is solving the problem of how to get close enough to a fish to be able to strike or engulf the potential prey. There are multiple solutions, but these are primarily dependent on the ecological setting: different habitats present different opportunities for approaching prey that is clearly equipped to quickly escape, once the predator is detected.

Species such as *C. geographus* that use a net strategy are primarily found in the tropical Indo-Pacific, in habitats where schools of small fish might hide close to where the snails may approach and engulf them. Thus, *C. geographus* has some unusual features; it has an extremely light shell; this allows the snail to be unusually active and agile. Furthermore, *C. geographus* can secrete a thick mucous thread that allows it to levitate itself from the edge of a rock, much like a spider lowering itself using a silk thread. It is a remarkable sight to see a large snail such as *C. geographus* lowering itself from an edge to which it attaches the mucous thread and secreting more of the thread as it lowers itself slowly downward; presumably this is an adaptation to allow it to approach a school of fish hiding in crevices—as it releases the nirvana cabal components of the venom, disorienting the school and making the fish hypoglycemic, it has a much higher probability of feasting on such a school. The other species that is known to use a net strategy, *Conus tulipa* is smaller with a proportionally heavier shell; however, the edges of its rostrum have cilia, the function of which is unknown. It has been speculated by Alan Kohn (personal communication) that the snail can use these to make itself resemble a sea anemone when it extends its rostrum, with the cilia looking like anemone tentacles—this could, therefore, presumably attract clownfish, but there is no field data to provide support for this speculation, and the true function of the unique cilia is unknown.

Probably a far more common approach is to ambush fish as they hide at night and sleep. Many snails have a very long, transparent proboscis that can be many times the length of the shell, and therefore as teleost fish hide from sharks, they are detected by the snails presumably through their potent chemosensory receptor, the osphradium. Thus, the hiding fish can be ambushed by the proboscis that would be almost invisible at night, and after the sting, the lightning-strike cabal of venom components would instantly immobilize the fish. Once tethered, upon retraction of the proboscis, the snail would then deliver the fish to its rostrum for predigestion. Typically, immediately after the snails engulf the tethered fish in their rostrum, they bury themselves, escaping predators of their own.

There appear to be many variations for approaching fish and successfully ambushing them. One species, *Conus obscurus* is reported to be collected under flat coral slabs in about 15–20 m off Hawaii. Presumably, fish hide under these flat coral slabs and the snail can, therefore, ambush fish from on top as they enter the cave-like compartment under the flat coral slab. Other species, such as *Conus stercusmuscarum* are active at night at low tide in pools created by the receding water. Often, many fish are trapped in such tide pools, and the very long proboscis of *Conus stercusmuscarum* allows it to

strike at fish that may come close in the shallow water that has become dense with potential fish prey (personal observations). Some other species may actively attract fish toward them. The bright red proboscis of *C. purpurascens* may serve as a lure, since when a fish is present, this species extends its brightly colored proboscis above the sand and writhes it enticingly, presumably making it look like an attractive worm for some unsuspecting fish (Kerstitch, 1979). There have even been reports by divers (P. Poppe, personal communication) that some species such as *Conus striatus* seem to attract certain species of fish toward them—these cone snails may secrete some attractant for particular species of potential fish prey. Another strategy is to remain cryptic: thus, *Conus monachus* has a deep black proboscis that resembles the muddy background in which it typically thrives; when it extends its proboscis, this is completely cryptic and can strike any fish that happens to be close by (personal observations).

5.3 ANATOMY AND BIOCHEMISTRY OF CONOIDEANS

5.3.1 STRUCTURE AND SYNTHESIS OF CONOTOXINS

Almost all known toxins produced in conoidean venom glands share some key structural features: these are short peptides (mostly 12–46 amino acids in length that usually have a high frequency of cysteine residues (Olivera, 2006; Norton & Olivera, 2006), which form disulphide cross-links that stabilize the secondary structure of the conopeptide and define its conformation. The number of cysteine residues and their arrangement (the so called Cys pattern or Cys framework) are one determinant of a conopeptide's physiological activity; one classification of the conopeptides is based on their Cys patterns. While the arrangement of cysteine residues in conotoxins is highly conserved, the rest of the venom peptide may vary considerably in amino acid sequence; the accelerated evolution of the genes encoding conopeptides makes them exceptionally diverse and evolutionarily flexible.

A high frequency of posttranslational modification is another unusual feature of many conopeptides (Craig et al., 1999a), which also contributes to their chemical diversity. Some of these modifications are well known (e.g., hydroxyproline, *O*-glycosylated serine or threonine [Craig et al., 1998]), while others are rare and/or unusual (6-bromotryptophan [Jimenez et al., 1997], [gamma]-carboxyglutamate [Bandyopadhyay et al., 2002], sulfotyrosine [Loughnan et al., 1998]). It was also found that some conopeptides contain D-amino acids (Buczek et al., 2008; Jimenez et al., 1996).

The venom components are synthesized in the tubular convoluted venom gland, opening at the proboscis base. The synthesis of all known *Conus* toxins in the venom gland epithelial cells follows a rather conserved scenario. All conopeptide's mRNAs are translated on ribosomes to generate a peptide precursor, its general structure is shared among most known conopeptides. This includes an N-terminal signal sequence (pre-site), followed by the pro-peptide region and a mature toxin region on the C-terminal end of the precursor; the latter is always present in a single copy (in contrast to many neuropeptide precursors that generate multiple mature peptides after posttranslational processing). All conopeptide precursors undergo proteolytic cleavage in the course of maturation with the removal of the signal sequence and pro-peptide region (Puillandre et al., 2010; Terlau & Olivera, 2004).

It was found that the sequence of the signal region is highly conserved across structurally similar conopeptides (Terlau & Olivera, 2004). The study of cDNA clones revealed that even the third codon position is highly conserved in the mRNAs, which encode the signal region of conotoxins (Woodward et al., 1990), although the signal sequences of some conoidean lineages may not be as stringently conserved as in the family Conidae (M. Watkins, unpublished results). The peptides sharing a common signal sequence are encoded by related genes; the conserved signal sequences provide a genetic basis for the classification of conopeptides, defining the gene superfamilies to which individual conopeptides belong.

The study of conopeptide expression levels in different portions of the venom gland revealed notably different expression profiles in distal, medial, and proximal portions (Garrett et al., 2005; Safavi-Hemami et al., 2014). In *Conus textile*, the conotoxins of A-, M-, P-, and T-superfamilies were preferentially expressed in the proximal ½–⅔ of the venom gland, whereas the levels of mRNAs corresponding to these toxins were significantly lower in the distal quarter of the gland. Conversely the conotoxins of the O-superfamily showed highest expression levels in the distal part of the venom gland. The most characteristic peaks, obtained in HPLC of the *C. textile* crude venom, corresponded to the group of μ-conotoxins produced in the proximal half of the venom gland and [delta]-conotoxin TxVIA in the medial part of the gland (Garrett et al., 2005).

Some more recent studies on the spatial differentiation of toxins expression have been carried out (Dutertre et al., 2014). It was suggested that the predatory/defensive functions of *Conus* venoms employed different complements of toxins that are produced in different portions of the venom gland. How the venom cocktail is actually deployed may be more subtle

and sophisticated than the two alternatives suggested, since the behavior of species such as *C. geographus* suggests a more complex strategy in capturing free-swimming prey (Olivera et al., 2015) than can be reconstituted under rather rigid and artificial experimental conditions in the laboratory.

5.3.2 BIOMECHANICS OF ENVENOMATION: MORPHOLOGICAL ADAPTATIONS FOR EFFICIENT VENOM DELIVERY

The sophisticated conoidean feeding mechanism based on envenomation of the prey was enabled by several unique morphological adaptations of the anterior foregut. In gastropod molluscs, the radular apparatus generally consists of a radular ribbon with numerous transverse rows of teeth (radula per se) and odontophore—a massive organ, consisting of several subradular cartilages and muscles, providing its movement. The radular apparatus is situated in the buccal cavity in close proximity to the mouth and can be partially everted through the mouth opening. The radula serves as an integrated organ for rasping or gripping food objects. An unusual peculiarity of conoidean foregut anatomy is that the buccal cavity together with radular apparatus is situated at the proboscis base (Fig. 5.6A and C). Consequently, the radula cannot be protruded through the mouth and used for grabbing and rasping the prey.

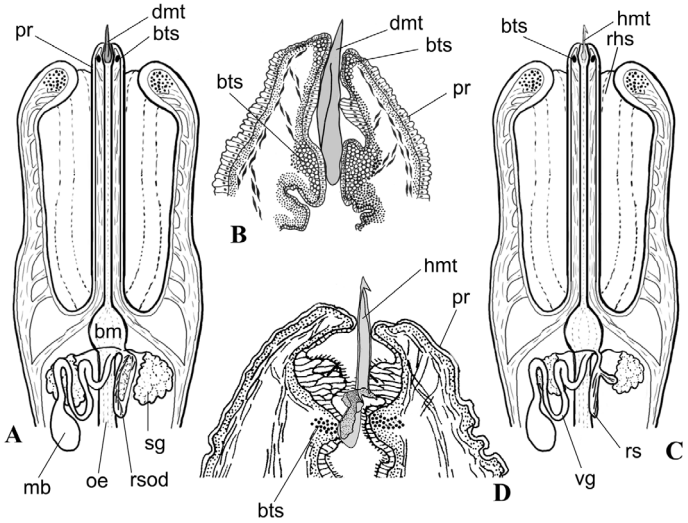

FIGURE 5.6 Diagrammatic sections through the anterior foregut of Conoidea. (A) Anterior foregut of the Conoidea with nonhypodermic marginal radular teeth and odontophore (generalized representative of the clade B in Fig. 5.8). A duplex marginal tooth detached from

FIGURE 5.6 *(Caption continued)*

the subradular membrane is used at the proboscis tip for stabbing and envenomating the prey. (B) Section of the tip of the proboscis with the duplex marginal tooth held by sphincters of the buccal tube (actual specimen of *Aforia kupriyanovi* Sysoev & Kantor, 1988—Cochlespiridae). (B) Anterior foregut of the Conoidea with hypodermic marginal radular teeth and lacking odontophore (generalized representative of clade A in Fig. 5.8). A hypodermic marginal tooth detached from the subradular membrane is used at the proboscis tip. (D) Section of the tip of the proboscis with the hypodermic marginal tooth held by a sphincter of the buccal tube (actual specimen of *Phymorhynchus wareni* Sysoev & Kantor, 1995—Raphitomidae). Abbreviations: bts—buccal tube sphincter, holding the base of the tooth at proboscis tip; dmt—duplex (nonhypodermic) marginal tooth at the proboscis tip; hmt—hypodermic marginal tooth at the proboscis tip; mb—muscular bulb of the venom gland; oe—esophagus; pr—proboscis; rhs—rhynchostome, or false mouth, through which the proboscis is everted; rs—radular sac without odontophore; rsod—radular sac with odontophore; sg—salivary gland; vg—venom gland.

What is essentially unique about the envenomation by conoideans is the use of individual radular teeth at the proboscis tip for stabbing and injecting venom into prey. This was long known for *Conus* spp. that possess elongate, barbed, harpoon-like, hollow marginal teeth (Kohn et al., 1999; Kohn, 1956, 1990; Olivera et al., 1990) (Fig. 5.7A), through which venom is injected into the prey. Recently, it was demonstrated that in the fish-hunting species *Conus catus* the tooth is propelled by a high-speed ballistic mechanism after the proboscis tip makes contact with the fish skin (Schulz et al., 2004) and then gripped by the proboscis tip to retain control of the stung fish prey while the proboscis is retracting. Within 50 ms, the onset of the tetanic immobilization elicited by the lightning-strike cabal toxins in the venom can be observed.

The tubular convoluted venom gland opens at the proboscis base and terminates in a large muscular bulb. The latter propels the venom through proboscis and the tooth cavity by contraction of muscles of the bulb walls. The proboscis of conoideans thus performs different functions: holding the tooth and bringing it in close proximity to the prey; functioning as a channel for venom to flow from the venom gland to the mouth (proboscis tip) and through the tooth (in the case of hypodermic teeth); and finally swallowing the prey. *Conus* species and other Conoidea implement a muscular hydrostat mechanism to enable the rapid (for some *Conus* species, a 7–8-ms long) strike of the proboscis. The muscular hydrostat is based on the fact that liquids are not compressed, and is realized through the coordinated contraction of the cephalic hemocoel muscles, causing the massive flow of the hemolymph into the proboscis. This results in the rapid protraction of the latter. The prey

cannot be rasped or fragmented, since the radula does not function as integral organ, and therefore it is swallowed whole. Observations on mollusc-hunting cone snails showed that mouth opening can expand more than 50 times in diameter to allow swallowing the prey, which can be comparable in size to the predator (Kantor, 2007).

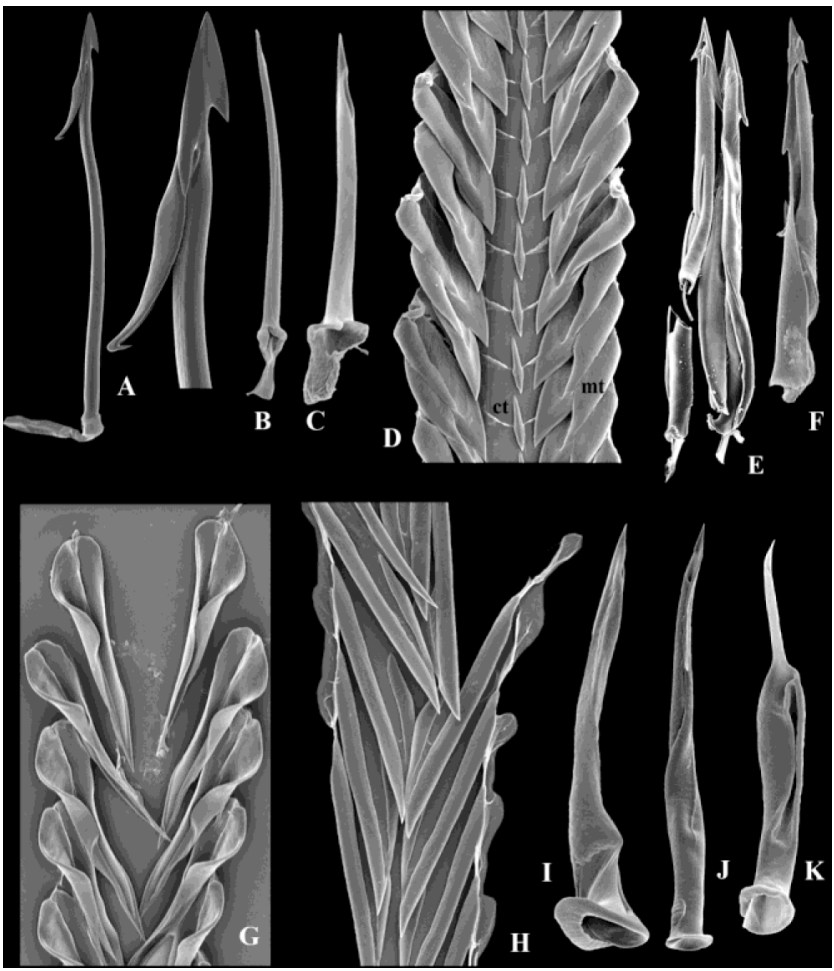

FIGURE 5.7 Variability of radulae of Conoidea. (A)–(C) Hypodermic marginal teeth of clade A in Fig. 5.8 of Conoidea, including only the species with hypodermic teeth and without odontophore. (A) Highly barbed tooth of fish-hunting *Conus striatus* (family Conidae) and enlarged tip of the tooth. (B) *Bathytoma neocaledonica* (family Borsoniidae). (C) *Toxicochlespira pagoda* (family Mangeliidae). (D)–(K) Radulae of clade B in Fig. 5.8 of Conoidea, which includes species with odontophore. (D) Typical radula with duplex marginal teeth and central tooth, *Turridrupa jubata* (Turridae). (E) Semienrolled, nearly

FIGURE 5.7 *(Caption continued)*

hypodermic marginal teeth, *Toxiclionella tumida* (family Clavatulidae). (F) Semienrolled, nearly hypodermic marginal tooth, *Cruziturricula arcuata* (family Drilliidae). (G) Radula with semienrolled, trough-shaped marginal teeth, *Ptychobela suturalis* (family Pseudomelatomidae). (H)–(K) Different radulae of Terebridae. (H) Primitive radula with duplex marginal teeth, *Clathroterebra poppei*. Next three figures depict hypodermic marginal teeth in Terebridae, originated independently in three clades, identified by molecular phylogeny: (I) *Terebra cingulifera*; (J) *Hastula lanceata*; (K) *Myurella kilburni*. Abbreviations in Figure 5.7D: ct—central tooth; mt—marginal tooth.

According to the latest molecular phylogeny of Conoidea (Puillandre et al., 2011), the group is split in two major branches (Fig. 5.8, clades A and B). One branch (clade A) includes *Conus* (belonging to family Conidae) and a number of related conoideans (currently assigned to nine families [Kantor et al., 2012b]). In this group the radular apparatus has undergone a profound transformation compared to the typical gastropod design, and the odontophore has completely disappeared (Taylor et al., 1993) (Fig. 5.6C). In conoideans of this branch, the radula comprises only a pair of hollow (hypodermic) marginal teeth in each transverse row and the radular membrane is greatly reduced (Fig. 5.7A–C). Sometimes, the teeth are rather simplified and form a trough rather than a tube.

Conversely, in most conoideans that fall within the second major branch (Fig. 5.8, clade B), the radular apparatus includes a well-developed subradular membrane and a fully functional odontophore with muscles, suggesting that the radula still has some (although maybe limited) function as an integral organ. These conoideans, classified in eight families (Bouchet et al., 2011; Puillandre et al., 2011), also have the radula and odontophore situated at the proboscis base and it normally cannot be protruded through the mouth (Fig. 5.6A). The radula in these conoidean families has very diverse morphology both in number of teeth in the transverse row (from 5 to 2) and in the morphology of the teeth themselves. In molluscs of this branch, the separate marginal tooth was very often (in most preserved specimens examined) found held at the proboscis tip gripped by special sphincter(s) (Kantor & Taylor, 1991; Sysoev & Kantor, 1987, 1989) (Fig. 5.6B). Therefore, it can be supposed that conoideans of this branch also use radular tooth for envenomation, but not via venom injection through the tooth (i.e., a hypodermic needle mechanism), but rather by dagger-style laceration of the prey's integument and releasing the toxic liquid through mouth. Very few direct observations on feeding of the conoideans of this second branch (including the example reported herein, the feeding of *Turridrupa*) are congruent with

the anatomical observations made on preserved specimens. In particular, the marginal teeth of *Turridrupa* are non-hypodermic, and belong to so-called duplex type (Kantor & Taylor, 2000) (Fig. 5.7D).

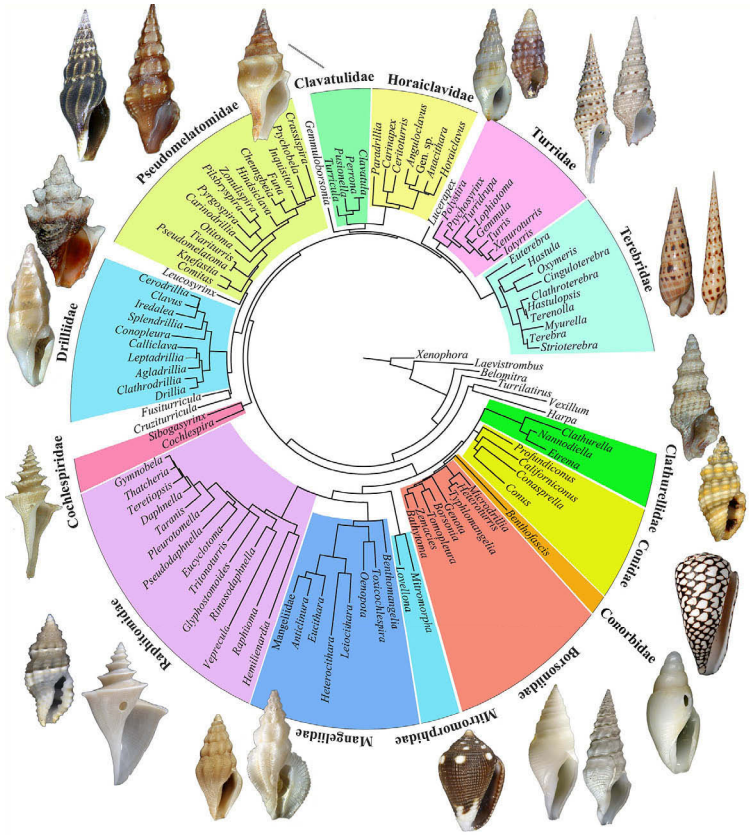

FIGURE 5.8 A phylogenetic tree of the superfamily Conoidea. The phylogenetic tree shows all major families included in the superfamily of Conoidea and is derived from the analysis of Puillandre et al. (2012). The section of the tree with fish-hunting cone snails comprises a very minor subset of the sector of the tree labeled Conidae. In this chapter, most of the physiology of envenomation discussed has focused on a species in the family Conidae, with the diversity of strategies for prey capture in the family Terebridae, a secondary focus. These are the most prominent shallow-water groups with relatively large species accessible by routine collection methods. Two major divisions of Conoidea are marked and referred to in the text as clades A and B.

It is remarkable that within conoideans of the second branch numerous cases of independent origin of hypodermic-like marginal teeth, resembling harpoons of *Conus*, were confirmed based on molecular phylogeny of

conoideans (Kantor & Puillandre, 2012; Taylor et al., 1993). In some cases, they remarkably resemble those "true" hypodermic teeth of the first branch (Fig. 5.7E–F, I–K); in other cases they are more simple, trough-like ("semi enrolled" [Kantor & Taylor, 2000]). The semienrolled teeth were found in at least three independent lineages—in the families Drilliidae (genera *Cruziturricula* Marks, 1951 and *Imaclava* Bartsch, 1944), Pseudomelatomidae (genera *Zonulispira* Bartsch, 1950, *Ptychobela* Thiele, 1925, others). The hypodermic teeth were recorded in the families Clavatulidae, Terebridae, and Drilliidae. It was also demonstrated based on extensive molecular phylogeny that within the single family Terebridae, hypodermic teeth originated at least thrice (Castelin et al., 2012) (Fig. 5.6, Radula I–K).

Reconstruction of the major morphological transformations of the radular apparatus in Conoidea was recently conducted based on the molecular phylogeny (Kantor & Puillandre, 2012). It revealed that the use of separate (individual) marginal teeth at proboscis tip was a key synapomorphy of Conoidea that appeared prior to the divergence of the two major branches.

A very remarkable and unexpected tendency in Conoidea is the complete loss of the radula and venom apparatus that is recorded in several unrelated lineages. This is most common in the families Raphitomidae and Terebridae belonging to two different major branches, clades A and B (Fig. 5.8). In both families, it occurred multiple times and is usually associated with the development of rhynchostomal introvert, or labial tube—greatly extended invertible extension of the head, similar to the one in *C. geographus* (Kantor & Sysoev, 1989; Taylor et al., 1993). The introvert is obviously used in the prey capture, as was demonstrated in live observation on feeding of different Terebridae (Miller, 1975). The latter family is one of the best studied of Conoidea in terms of molecular phylogeny, and it was convincingly demonstrated that the venom apparatus was lost eight times within terebrids (Castelin et al., 2012). Its worth mentioning that species without a venom apparatus were among most abundant Tereibridae (Kantor et al., 2012a), suggesting that toxin production is a high energy-consuming process, leaving less resources for reproduction. In addition to the mentioned families, some species lacking the venom apparatus were found also among Horaiclavidae and Borsoniidae (Fedosov & Kantor, 2008).

Data on feeding and diet of Conoidea are still very limited. *Conus* species can be separated into three major groups in relation to their diet: worm-, mollusc-, and fish-hunting. With the exception of *Conus*, information on feeding is available for fewer than 50 species (e.g., Taylor, 1980, 1986), and these reports involved much less direct observation (e.g., Heralde et al., 2010). Most of the conoideans (other than *Conus*) feed on sedentary

and errant polychaetes, although feeding on other worms (sipunculans and nemerteans), enteropneusts, and even molluscs has been recorded (see e.g., Miller, 1975, 1979). Both rather specialized and generalist species are known. For example, *Turricula nelliae spurius* in Hong Kong consumes at least 16 species of errant and sedentary polychaetes, while the sympatric species *Lophiotoma leucotropis* appeared to be more specialized, consuming mostly single species of polychaete worm (Taylor, 1980). This information is derived mainly from gut content analysis.

5.4 OTHER CONOIDEA

In the first sections of this chapter, the primary focus was on fish-hunting cone snails. Far more has been elucidated about the underlying physiological mechanisms for this miniscule fraction of all Conoidean species (<1% of the total) than for all of the other conoideans combined. Here, we provide a somewhat sketchy overview for the rest of the superfamily Conoidea, other than the fish-hunting cone snails. These range from other members of the family Conidae to major groups of Conoidea that clearly branched off from Conidae early in the evolution of the superfamily. As a representative of the latter, we will primarily focus our discussion on the auger snails (family Terebridae).

5.4.1 MOLLUSCIVOROUS AND VERMIVOROUS CONE SNAILS

Of the 750 named species of Conidae, over 600 are likely *not* fish hunting, with the vast majority believed to be vermivorous. It is generally assumed that the ancestral forms of Conidae were vermivorous, with polychaetes being the primary prey. Most of the rest of the superfamily Conoidea is widely assumed to be vermivorous, but there are very few documented observations in the literature.

Although there has been significantly less work on molluscivorous *Conus* species than on the fish-hunting clades, at the transcriptome level, there is an accelerating pace of elucidating venom components from several molluscivorous species. Many of these cone snails are easily accessible, larger, shallow-water species (such as *C. textile* and *Conus marmoreus*), so they are relatively straightforward to study at a biochemical and genomic level.

It seems clear that some of the results of the physiological studies carried out on fish-hunting *Conus* apply to molluscivorous *Conus* as well. Thus, a key mechanism used in the envenomation of fish prey is the "lightning-strike

cabal" strategy, to overstimulate neuronal circuitry by inhibiting the inactivation of voltage-gated Na channels and concomitantly blocking voltage-gated K channels in the same circuitry. At least some of the molluscivorous *Conus*, such as *C. textile* and *Conus gloriamaris* probably use an analogous strategy when envenomating their snail prey. However, while the purpose of the lightning-strike cabal in fish envenomation is to elicit an almost instant tetanic paralysis, the primary physiological purpose of the homologous toxins in snail-hunting cone snails is probably to guarantee that prey will not retract into its shell after the predator's initial strike.

By hyperstimulating the neuromuscular circuitry of the prey snail, the effect of envenomation is uncoordinated seizure-like motor activity: after the first venom injection, the envenomated snail is typically observed to be alternately contracting and relaxing its musculature, progressively extending further and further out of the shell. This guarantees that the prey does not retreat deep into its shell where it would be inaccessible. As the prey is moving seizure like outside the shell, the predator typically continues to inject additional venom, and it carefully examines where it envenomates the now helpless and uncoordinated prey. These species of molluscivorous *Conus* inject their prey multiple times (in contrast to fish-hunting cone snails that only envenomate once). What is remarkable is that after the predator has made a meal of the envenomated snail, literally nothing is left except for the shell. This must mean that the columellar muscles have been totally relaxed, making it possible for the predator to recover even the hepatopancreas, which is usually tightly coiled deep within the gastropod shell, and easily broken off.

Molluscivorous cone snails appear to be susceptible to their own venom, leading to some unusual facets of envenomation by molluscivorous *Conus*. When a colony of *C. textile* were maintained in an aquarium, and were not fed, the snails were quiescent, but otherwise did not attack each other. However, in one aquarium after an extended starvation period, some of the larger individuals began to envenomate smaller conspecifics and consume them; thus, it appears that some molluscivorous species will practice cannibalism, albeit reluctantly.

Another unusual feature of the physiology of envenomation is the competitive interactions. *C. marmoreus* individuals compete for prey; in one specific case observed by the authors, a smaller individual reached the potential prey snail first and envenomated it. When a larger *C. marmoreus* approached, it stung the small conspecific, apparently not with enough venom to kill it, but the smaller individual appeared to be stunned and was pushed aside and away from the prey. The larger snail then began to consume the prey, and

by the next day the smaller individual had recovered completely. Thus, this species can use venom for intraspecific competitive interactions.

Similar competitive interactions have been observed by Dylan Taylor (unpublished observations) for some vermivorous *Conus* species. Two individuals of *Conus lividus* will compete for the same polychaete worm, and both snails may begin to devour the worm from opposite ends. The snails then go into a competitive sucking match, with one individual ultimately being able to suck out the entire worm from the rostrum of the other snail. This was an observed case of competition between conspecifics for a single worm prey. However, cross-species competition was also observed by authors among worm-hunting *Conus*. One interaction in the aquarium is competition that would not occur naturally, since the two relevant *Conus* species do not overlap in their native geographic ranges. When a polychaete worm was introduced into the aquarium, *C. californicus*, a species found off the coast of Southern and Baja California, immediately began to attack the worm, and as is characteristic of this species, multiple individuals were observed feeding on the same worm, which was larger than any of the snails. Ultimately, a specimen of *Conus quercinus* (which does not occur in California, but rather throughout the Indo-Pacific, which is not a locality for *C. californicus*), approached the same worm and injected venom. What occurred was an immediate withdrawal by all of the *C. californicus*, implying that there was a strong deterrent to competitors in the venom of *C. quercinus*.

Some *Conus* species have been observed in the field to envenomate extremely large marine worms; a diver once observed *Conus betulinus* devouring an enormous worm (ca. 1-m long) and was able to watch this for over 20 min, at the end of which the snail had not completely engulfed its prey. These circumstances must attract potential competitors. Thus, making the worm unpalatable to anyone else would provide a clear selective advantage.

5.4.2 OTHER CONIDAE

The species *C. californicus* is believed to have been isolated from all other cone snails since the Miocene (Stanton, 1966) and is ecologically notable in that it lives in a temperate habitat and no other cone snails overlap with it throughout most of its geographic range (from the Baja California Pacific coast, north to Monterey Bay). This is the one species in the family Conidae outside the genus *Conus*, for which multiple observations have been recorded regarding its prey capture strategy. The overall impression gleaned from these is that the divergent phylogeny in fact reflects a corresponding divergent biology of prey envenomation.

Most cone snails are highly specialized with regard to the range of prey that they envenomate; *C. californicus* is a notable exception; it has been observed to attempt to envenomate prey in four different phyla (polychaetes, molluscs, shrimp, and fish) (Biggs et al., 2010; Kohn & Waters, 1966; Saunders & Wolfson, 1961; Stewart & Gilly, 2005). The primary prey is likely to be polychaete worms, but aquarium observations have been reported of this species attacking prey from other phyla, and an analysis of gut contents suggests that they successfully capture other prey. One notable difference between this species and all other *Conus* is that they will hunt as a pack to bring down larger prey. There is no record of any other species in the family Conidae that carries outgroup hunting behavior (although this is consistent with the hunting behavior of some Turridae). The piscivory of *C. californicus* has been intensively investigated and recorded (Stewart & Gilly, 2005). The strategy of *C. californicus* for capturing fish differs notably from those of the specialist fish-hunting *Conus* described above. Not only do multiple individuals prey on a single fish, but *C. californicus* will also routinely sting the prey multiple times in order to capture a large fish; the number of stings is directly correlated with the size of the fish, and up to seven stinging events were observed on a single fish before it was captured. No specialist piscivorous *Conus* has ever been observed to sting fish more than once. In part, it appears that a single envenomation event is insufficient to subdue the fish, so that it can be successfully engulfed by the rostrum of the snail. In effect, the multiple stings are a reflection of the increased dose of venom necessary to completely subjugate a larger fish prey.

There is a corresponding divergence in the conotoxins used for prey capture (Biggs et al., 2010; Gilly et al., 2011). Thus, while fish-hunting *Conus* snails use μ-conotoxins in the M-superfamily to block voltage-gated sodium channels, Gilly et al. (2011) have demonstrated that *C. californicus* uses an unusual family of peptides with four disulfide cross-links, more closely related to the O-superfamily. Similarly, the radular tooth of *C. californicus* is highly distinctive and unusually complex.

5.4.3 ENVENOMATION BY TEREBRIDS AND TURRIDS

All conoideans outside the family Conidae are conventionally referred to as either terebrids or turrids. The terebrids are a relatively small and distinctive group (417 species listed in WoRMS), but turrids comprise the major biodiversity of the superfamily and are now divided into many different families. The nominative genus, *Turris*, is assigned to the family Turridae, which is

much reduced compared to its former phylogenetic scope, a reduction that is thoroughly justified by the most recent available molecular phylogenetic data (Bouchet et al., 2011; Puillandre et al., 2011).

There are three terebrid species shown in Figure 5.9, and these are representative of divergent strategies of envenomation within the family Terebridae. A large set of terebrids, such as *Terebra* (*Oxymeris*) *maculata* have secondarily lost their venom apparatus, and do not envenomate their prey. The feeding behavior of three species of *Terebra* that lack venom suggests that they use a long pseudoproboscis for prey capture. The pseudoproboscis can be everted and used to simply suck in the prey without the need for using venom. Species that use this strategy will either feed on capitellid polychaetes that live in loosely compacted sand or on hemichordate worms.

However, two groups of terebrid species envemomate their prey, and these use quite different strategies. One group, including *Hastula strigilata* (shown in Fig. 5.9) might be referred to as the "surfboarding conoideans." The snail apparently detects their worm prey by chemoreception and uses its large foot as a surfboard to propel quickly through the surf zone. These species inject venom into their polychaete prey and typically attack worms that are exposed by wave action. Prey capture is completed between the passage of two successive ocean waves (Bratcher & Cernohorsky, 1987; Miller, 1970).

The third group of auger snails belong to the genus *Terebra* s.s. and are represented in Figure 5.9 by *Terebra triseriata*. These species have a small foot, live in deeper calm areas, and immobilize their prey to prevent it from retracting into its burrow. In contrast to *Hastula strigilata*, which burrows into the sand after having ingested prey, these species feed on small tube-dwelling polychaetes and do not burrow into the sand during feeding.

Thus, although all auger snails are specialized for sandy habitats, it is clear that there are a diversity of strategies used for capturing their prey and that there should be a corresponding diversity in the venom composition of the different types of auger snails.

Any discussion of conoidean physiology needs to address an obvious disparity between the distribution of biodiversity within the superfamily and our present knowledge base. The turrids, broadly defined as conoideans that are not cone snails or terebrids, comprise the vast majority of the diversity in the superfamily, but are also the least investigated group. The major reason for this disparity is that cone snails and terebrids are well represented in shallow-water marine environments, and some of the larger species found in shallow water are abundant and can be collected in great numbers. In

FIGURE 5.9 Diverse branches of Conoidea. The superfamily Conoidea, encompassing all of the known venomous gastropods was traditionally divided into three families: Conidae, Terebridae, and Turridae. Representatives of these three groups are shown in the figure. However, the family Turridae was clearly polyphyletic and the modern phylogeny of the superfamily is shown in Figure 5.8. These species in the figure, however, typify the diversity in the superfamily. On the upper left-hand corner are three species in Conidae, *Conus consors* (far left), a fish-hunting cone snail; *Conus tessulatus* (center), a worm-hunting cone snail; and *C. marmoreus* (right), a mollusc-hunting cone snail. On the right-hand side of the figure are three species in the family Terebridae. It is believed that most Terebridae eat polychaete worms and that their elongate shape is an adaptation for a sand-dwelling lifestyle. However, it is known that the far-right species, *Terebra triseriata* is venomous and lives offshore in deeper water; in contrast, *Terebra maculata* (top left), the largest terebrid species has secondarily lost its venom apparatus and apparently simply sucks up its polychaete prey. In contrast, the species shown on the lower left of the group is *Hastula strigilata*, a group of "surfer conoideans" that live near breaking waves, can use their large foot as a sail to essentially surf from one locality to another, and also allows them to, therefore, attack prey that might be exposed by the breaking waves. On the lower left are three species that represent the old family Turridae, but each is now placed in a separate family group. Far left is the wonder shell, *Thatcheria mirabilis*, which is in the family Raphitomidae. In the center is *Crassispira cerithina*, in the family Pseudomelatonidae and on the far right is *Turris grandis*, in the family Turridae. All of these species are venomous, but are not at all closely related to each other. These different groups are discussed in the text.

contrast, turrids are primarily a deeper water group, with very few species readily accessible in large numbers. This is why in this chapter, though the turrids represent greater than 95% of diversity, they are barely represented in the overview we are presenting. It should be noted that this situation has the potential to change very rapidly; deep-water collection methods, primarily developed by commercial fishermen in the Philippines, such as gill nets or *Lumun lumun* (Seronay et al., 2010) nets have made live specimens of turrids increasingly available. A continuing problem is that many turrid species are

FIGURE 5.10 Envenomation by a *Turridrupa*. Shown is the first recorded observation of a *Turridrupa* species envenomating prey. The *Turridrupa* extended its proboscis and envenomated the prey out of the field of view; the end of the worm that had been stung turned very dark, as if the worm were bleeding internally. This species was formerly identified as *Turridrupa bijubata*, but recent molecular evidence has demonstrated that it is distinct from that species. The genus *Turridrupa* is phylogenetically distant from cone snails and is a monophyletic group in the family Turridae.

extremely small, and therefore applying some of the more conventional methods for elucidating their physiology tend to be a challenge.

An example of how these new methods have made species available is shown in Figure 5.10; a tiny turrid previously unknown to science, *Turridrupa* sp. (still undescribed), has now been filmed envenomating its prey. The figure shows for the first time a turrid attacking its polychaete worm prey. Curiously, once envenomation occurs, there is an abrupt change in the color of the hemolymph of the worm. This raises the possibility that the venom of this species, instead of targeting molecules in the nervous system, which has certainly been the overall picture gained from this study of fish-hunting cone snails, may be targeting the circulatory system of the prey. Thus, the situation may be analogous to venomous snakes—cobra-like snakes have neurotoxins, while rattlesnakes and their relatives have potent factors that affect blood and the circulatory system. It should be possible to elucidate novel mechanisms that have evolved in the different lineages of turrids, now that these can be collected and observed. At present, however, very little is known about the underlying physiological mechanisms that lead to prey capture.

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- **Gastropoda**
- **Mollusca**
- ***Conus***
- **peptides**
- **cone snails**
- **turrids**
- **venom gland**
- **radula**

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CHAPTER 6

ESCAPE RESPONSES BY JET PROPULSION IN SCALLOPS[1]

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CONTENTS

[1]This review is one of a series dealing with trends in the biology of the phylum Mollusca.

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ABSTRACT

The impressive swimming escape response of scallops uses a simple locomotor system that facilitates analysis of the functional relationships between its primary components. One large adductor muscle, two valves, the muscular mantle, and the rubbery hinge ligament are the basic elements allowing swimming by jet propulsion. Although these basic functional elements are shared among scallop species, the exact nature of the escape response varies considerably within and among species. Valve shape and density have opposing influences upon the capacity for swimming and the ease of attack by predators once captured. Patterns of muscle use can partly overcome the constraints imposed by shell characteristics. The depletion of muscle reserves during gametogenesis leads to a trade-off between escape response performance and reproductive investment. However, changes in muscle energetic status influence repeat performance more than initial escape performance. Escape response performance is influenced by habitat temperature and mariculture techniques. During scallop ontogeny, changes in susceptibility to predation and in reproductive investment may influence escape response capacities. These ontogenetic patterns are likely to vary with the longevity and maximal size of each species. Although the basic elements allowing swimming by jet propulsion are common to scallops, their exact use varies considerably among species.

6.1 INTRODUCTION

Many benthic bivalves and gastropods have impressive responses to their predators, ranging from the jumping motions of clams to the elaborate twisting motions of gastropods such as the common whelk (*Buccinum undatum* L., 1758). None of these responses achieves the displacement and, in our opinion, the grace of the swimming response of scallops. This jet-propelled motion raises the animal in the water column and allows them to swim for considerable horizontal distances. The champion swimmers, such as the saucer scallop (*Amusium balloti* [Bernardi, 1861]), have been recorded to swim 30 m in a single swimming bout (Joll, 1989). The intensity of the scallop swimming response finds its closest parallel in vertebrates in the burst flight of pheasants and other gallinaceous birds. As the scallop locomotor system is composed of few functional components, a more complete evaluation of the impact of changes in muscle characteristics upon escape response behavior should be possible than in multifaceted musculoskeletal systems,

such as those of vertebrates. In the following review, we will examine the basic components and physiology of the scallop locomotor system and then evaluate the impact of environmental conditions, physiological status, and size upon escape response performance. The latter portion of the review will focus upon studies of the Iceland scallop (*Chlamys islandica* [O. F. Müller, 1776]), sea scallop (*Placopecten magellanicus* [Gmelin, 1791]), and Peruvian scallop (*Argopecten purpuratus* [Lamarck, 1819]).

6.2 BARE BONES LOCOMOTOR SYSTEM

Scallops swim using two valves, one muscle, and a rubbery hinge ligament. The phasic adductor muscle produces the power required for swimming, by rapidly closing the valves and expelling water through small lateral openings that prevent complete valve closure. The muscular mantle controls the size of the jets sent out through the lateral openings. This creates the jet propulsion that moves the scallop forward. When the adductor muscle relaxes, decompression of the hinge ligament opens the valves. The most complete model of this dynamic system focuses upon *P. magellanicus*. The model integrates properties of the hinge ligament, fluid movement around the valves, fluid pressure within the mantle cavity, contraction of the phasic adductor, and valve inertia (Cheng et al., 1996). The authors separated the locomotor system into two parts: a jet producing pressure pump and an oscillator involving the hinge ligament and the outer fluid. Their careful modeling shows that the pressure pump uses most of the mechanical energy produced by muscle contraction. Biomechanical analyses of scallop swimming compare the shells to airfoils and describe scallop swimming as flight through water. For a given scallop species, size, swimming angle, and current speed and direction set lift production and swimming capacity (Gruffydd, 1976; Millward and Whyte, 1992; Thorburn and Gruffydd, 1979). Although repetitive cycles of valve opening and closing allow forward or upward motion, scallops can also use their muscular mantle for fine motor control. This allows them to use adductions to create depressions and to bury themselves, or to move sideways. The precise nature of these motions speaks to integration of information obtained by the many eyes and tentacles on the fringed edge of the mantle.

The capacity for swimming differentiates pectinids from other bivalves. Whereas most bivalves respond to predators by closing their valves for long periods, burying themselves in the substrate through the action of a muscular foot, or using their foot for jumping, scallops can swim to escape

their predators. The capacity for jet propulsion is thought to have evolved during colonization of turbid habitats by ancestral scallops. Morphological changes of the valves, together with reduction or loss of the anterior adductor muscle, reduction of byssal attachment, and opening of the mantle (to allow increased flow for filtration) are thought to have permitted ancestral scallops to exploit deeper and more turbid habitats (Yonge, 1936). On the other hand, as the loss of a siphon made it more difficult to evacuate particle loads imposed by turbid waters, two other morphological changes occurred. First, the performance of the ciliary tract was improved. Second, the striated adductor muscle increased markedly in size, enhancing the speed and force of valve closure (Wilkens, 2006; Yonge, 1936). This permits a type of coughing that forcibly evacuates fluid from the mantle cavity (Wilkens, 2006). Furthermore, the infolding of the mantle was enlarged to form openings for the entry and exit of water from the mantle cavity (Wilkens, 2006). In summary, swimming is thought to have arisen in ancestral pectinids that had colonized turbid waters and had gained a capacity for forcible evacuation of water from the mantle cavity. Yonge (1936) suggests that the modifications of the structure of the shell, the mantle, and the adductor muscle are derived adaptations in the monomyairian bivalves.

6.3 SHELL CHARACTERISTICS AND THEIR INFLUENCE UPON SWIMMING CAPACITIES

Their great variety of shell shapes and colors has made scallops favorites of shell collectors around the world. Although the outline of the valves has a characteristic "scalloped" shape, the depth, symmetry, density, and surface characteristics of the valves vary considerably among the more than 300 species of pectinids. A wide range of swimming capacities parallels this structural variety. Minchin (2003) and Alejandrino et al. (2011) classify scallops into 5–6 major groups according to shell morphology, swimming capacities, and life habit. These groups range from the highly active *Amusium* species to the cemented rock scallops, with intermediate groups showing different degrees of byssal attachment. The critical shell characteristics that set swimming capacity include shell density, aspect ratio, and the shape of the shell's leading edge during movement (Dadswell and Weihs, 1990; Gould, 1971; Morton, 1980). Basically, scallops must produce lift to overcome gravity and thrust to overcome drag. Lift and drag are influenced by the angle and speed of swimming, as well as by shell shape. The impact of gravity is set primarily by the mass of the shell. When shell shape and

characteristics are unfavorable for swimming, thrust may also be used to produce lift as for species with plano-convex shells such as those in the genus *Pecten* (Millward and Whyte, 1992). Scallops with good swimming abilities generally have shells with a high aspect ratio, an upper valve that is more convex than the lower valve, and light valves with smooth surfaces (Gould, 1971; Soemodihardjo, 1974).

Besides being critical for swimming, shell characteristics also protect against predation. A thick and heavy shell can prevent predation by crabs and tightly sealing valves can hinder predation by starfish. However, heavy shells and reduced openings between the valves reduce swimming capacity. Clearly, characteristics that facilitate swimming are unlikely to protect against predation once a scallop is captured, so a trade-off between swimming capacities and mechanical defense against predation is likely. Furthermore, during growth, shells not only increase their size, but also often increase their thickness (and mass) and may change their surface characteristics as in the extreme case of cementing rock scallops. Overall, shell smoothness, mass, and shape provide considerable information about the probable swimming capacities of a scallop species. A smooth and light shell with a gentle curvature is likely to facilitate swimming, but as it would provide little protection against predation once captured, scallops with such shells are banking upon a strong swimming capacity to avoid predation. The relationship between shell characteristics and escape response capacities is likely to reflect the properties of the scallop's ecosystem. For example, *A. balloti* coexists with a variety of rapid crustacean predators against which a strong swimming capacity is useful (Himmelman et al., 2009). The similarly shaped Antarctic scallop (*Adamussium colbecki* [E. A. Smith, 1902]) remains byssally attached through its adult life and only swims short distances, perhaps due to ice scouring and the lack of crustacean predators in its habitat (Ansell et al., 1998).

Many types of predators attack scallops, with starfish, crustaceans, gastropods, and fish being common predators for most scallop species. Legault and Himmelman (1993) showed that scallops, as other benthic invertebrates, show the strongest escape responses to the predators, in a given guild, that present the greatest predation risk. However, even though crustaceans can present a strong predation risk (Grefsrud et al., 2003; Nadeau et al., 2009), few scallops respond to crustaceans with an escape response. *A. balloti* is an exception to this pattern in that it responds to various crustaceans, including the slipper or flathead lobster (*Thenus orientalis* [Lund, 1793]) and the blue swimming crab (*Portunus pelagicus* [L., 1758]), with intense swimming activity (Himmelman et al., 2009). *A. balloti* does not show a generalized

response to crustaceans, as portunid crabs common in its habitat did not elicit a swimming response (Himmelman et al., 2009). *P. magellanicus* generally responds to crustaceans by closing its valves (Barbeau and Scheibling, 1994), but has been reported to move away from crabs (Wong and Barbeau, 2003). Two major starfish predators of *P. magellanicus* differ in their effectiveness, with the common sea star (*Asterias vulgaris* Verrill, 1866 = *Asterias rubens* L., 1758) preying at higher rates than the polar six-rayed star (*Lepasterias polaris* [Müller and Troschel, 1842]) (Nadeau et al., 2009) but both elicit a strong escape response (H. E. Guderley, unpublished data). The giant Atlantic scallop (*Pecten maximus* [L., 1758]) responds differently to the species of predatory starfish it encounters (Thomas and Gruffydd, 1971). Although escape responses can protect against predation, they are evolved responses to predators encountered in the scallops' habitat. Exotic predators may not elicit escape responses by scallops (Hutson et al., 2005), increasing the potential for disruption of ecosystem structure. The specificity of the reactions of scallops to encounters with potential predators suggests that they use precise chemical and visual cues to activate the intense contraction of the phasic adductor muscle required for swimming or jumping.

6.4 STRUCTURE AND ROLES OF THE SCALLOP ADDUCTOR MUSCLE

The phasic and tonic portions of the scallop adductor differ in structure and function (Chantler, 2006). The phasic adductor contracts and fatigues rapidly, with times to peak tension and relaxation similar to those of vertebrate fast fibers (Marsh et al., 1992; Olson and Marsh, 1993; Pérez et al., 2009a; Rall, 1981). The phasic adductor carries out the rapid valve closures needed for scallop swimming (Millman and Bennett, 1976; Nunzi and Franzini-Armstrong, 1981; Olson and Marsh, 1993). In most scallops, the phasic adductor is considerably larger than the tonic adductor (Fig. 6.1); for example, in *P. magellanicus*, the phasic muscle accounts for 80% of the adductor muscle mass (de Zwaan et al., 1980). The tonic adductor muscle is composed of smooth muscle fibers that contract slowly and sustain their contraction for prolonged periods at low metabolic cost (Watabe and Hartshorne, 1990), using a catch mechanism similar to that of the anterior adductor muscle in mytilid bivalves (Nunzi and Franzini-Armstrong, 1981; Chantler, 2006). The tonic muscle is presumably used for maintenance of constant valve openings during filtration by undisturbed scallops, as well as for prolonged valve closure.

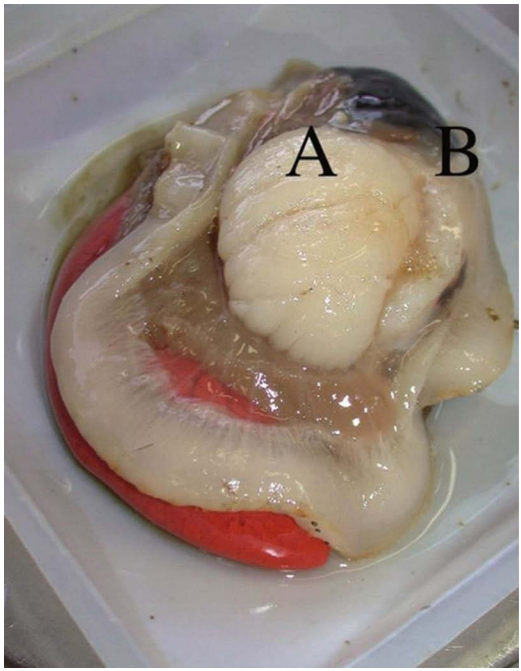

FIGURE 6.1 The two sections of the scallop adductor muscle in a sea scallop (*Placopecten magellanicus*): (A) phasic portion and (B) the tonic portion.

Scallops initiate jet propulsion by a wide gape that increases the volume of water in the mantle cavity. Rapid contraction of the phasic adductor then forces jets of water through focused openings in the mantle, moving the animal forward while closing the valves and compressing the hinge ligament that will open the valves once the muscle relaxes. The moment of force applied to the ligament varies with the distance between the adductor muscle and the ligament (Trueman, 1953; Gould, 1971). In analogy with vertebrate-striated muscles, power production by the phasic adductor should be optimal at intermediate speed and force of contraction. In muscles composed of a single fiber type, force increases with cross-sectional area, whereas speed of contraction rises with fiber length. The large size of the scallop phasic adductor (relative to valve area) clearly enhances force production, whereas the oblique attachment of the phasic adductor increases its length. Effectively, the phasic muscle insertion is closer to the hinge on the right than on the left valve and is generally wider on the left than on the right valve (Thayer, 1972; Soemodihardjo, 1974). This arrangement increases the obliqueness and hence the length of the phasic adductor (Thayer, 1972). In a

comparison of several monomyarian bivalves, Thayer (1972) found that the obliqueness of the phasic adductor (in a plane perpendicular to the hinge) increases with swimming ability. In contrast, the tonic adductor is generally attached in a perpendicular position between the two valves, showing much less obliqueness than the phasic adductor (Thayer, 1972; Soemodihardjo, 1974). Thus, the insertions of the tonic and phasic muscles can be separated, particularly on the right valves (Soemodihardjo, 1974).

6.5 METABOLIC SUPPORT OF SWIMMING IN SCALLOPS

As scallops fatigue rapidly during swimming bouts, their swims can be classified as sprints. Vertebrate sprints initially use creatine phosphate followed by anaerobic breakdown of glycogen (Hochachka and Somero, 2002). The limits of vertebrate sprint activity are reached when lactate, the anaerobic breakdown product of glycogen, accumulates to levels that perturb acid–base balance. In pectinids, escape responses also use a phosphagen (arginine phosphate), while anaerobic glycogen breakdown provides a secondary source of ATP. In the species that have been examined most extensively (i.e., *P. magellanicus*, pilgrim's scallop, *Pecten jacobeaeus* [L., 1758]), queen scallop (*Chlamys opercularis* [L., 1758] = *Aequipecten opercularis* [L., 1758]), bay scallop (*Argopecten irradians concentricus* [Say, 1822]), *P. maximus*, and *A. colbecki*), the majority (approximately 70%) of the contractile activity is supported by arginine phosphate breakdown with its rapid generation of 1 mol of ATP per mole (Grieshaber and Gäde, 1977; Grieshaber, 1978; Thompson et al., 1980; de Zwaan et al., 1980; Chih and Ellington, 1983; Bailey et al., 2003). Only the final 30% of rapid valve closures use ATP generated from glycogen breakdown (Grieshaber and Gäde, 1977; Gäde et al., 1978). Instead of accumulating lactate, scallops accumulate octopine, which is the condensation product of pyruvate and arginine. In contrast with vertebrates that primarily produce lactate during exercise, scallops continue to produce octopine after exercise, specifically during the valve closure (Grieshaber, 1978) that typically follows exhaustion. During valve closure produced by tonic contraction, the phasic adductor uses anaerobic glycolysis to partially recuperate adenylate levels (Livingstone et al., 1981; Pérez et al., 2008a). Full metabolic recovery from exhaustive escape responses requires aerobic metabolism (Livingstone et al., 1981), with muscle arginine phosphate levels returning to resting values within 12–24 h of valve opening in *P. magellanicus* (Livingstone et al., 1981; Pérez et al., 2008a), but within 2 h in *A. opercularis* (Grieshaber, 1978). In *A. colbecki*, *A. opercularis*, and *P.*

maximus, 50% recuperation of arginine phosphate levels occurs within 3–5 h (Bailey et al., 2003).

Although scallop muscles are poorly perfused and their hemolymph does not contain a respiratory pigment, oxygen uptake rises markedly during recovery from exhaustive exercise (Mackay and Shumway, 1980). In juvenile *C. islandica*, oxygen uptake rises to approximately 12-fold resting rates during recovery from exhaustive exercise (Tremblay et al., 2006). Oxygen uptake also rises after swimming in spear scallop (*Chlamys hastata* [G. B. Sowerby II, 1842]), the scallop *Chlamys delicatula* (Hutton, 1873) = *Zygochlamys delicatula* (Hutton, 1873), and *P. magellanicus* (Mackay and Shumway, 1980; Thompson et al., 1980; Donovan et al., 2003; Kraffe et al., 2008). This speaks to the participation of mitochondrial ATP production in the metabolic recovery of the phasic adductor muscle. After opening their valves, scallops recuperate their escape response capacity within minutes (tropical scallops) or hours (temperate-zone scallops). Despite their proximity, no metabolic exchanges appear to occur between the phasic and tonic adductor muscles (Livingstone et al., 1981), such that recovery from exhaustive exercise is accomplished by the mitochondria within phasic adductor fibers.

6.6 THE HINGE LIGAMENT

This critical structure is composed of two portions. The external flexible hinge ligament connects the two valves along the entire hinge. The external ligament contains parallel stratifications (Trueman, 1953). The internal portion of the hinge ligament is made of an elastic material that acts as a compression spring and opens the valves when the adductor muscles relax (Alexander, 1966). This pyramidally shaped section is attached to both valves. The ventral portion of the ligament is curved when the valves are closed. The internal ligament contains a central, noncalcareous rubbery part and two lateral calcified regions that anchor the ligament to the valves. The resilience of the hinge ligament is higher in scallops than in burrowing or sessile bivalves (Kahler et al., 1976). The resilience increases with glycine content and decreases with cysteine and CaCO3 contents (Kahler et al., 1976). The hysteresis loops of the hinge ligaments of the scallops *P. maximus* and *A. opercularis* are considerably tighter than those of sessile or burrowing bivalves (Trueman, 1953), indicating that less energy is lost during cycles of valve opening and closing in scallops than in other bivalves. The gape allowed by the hinge ligament is considerably larger in scallops than in other

bivalves (Trueman, 1953). Thus, as with the other major components of the scallop locomotor system, the hinge ligament shows properties that facilitate swimming by jet propulsion.

6.7 STUDIES OF ESCAPE RESPONSE PERFORMANCE

Contraction of the phasic adductor can be easily observed in intact animals, as it leads to dramatic valve closures, often described as "claps" or snaps. Understandably, studies of escape response performance have focused upon these strong valve closures. Although some studies have examined how scallops respond to disturbance or to predators in their natural habitat, the scallop escape response has mainly been studied in the laboratory. Laboratory studies range from observations of unrestrained scallops responding to contact with predators (Brokordt et al., 2000a, 2000b), to high-speed imaging of freely moving scallops (Bailey and Johnston, 2005a, 2005b; Cheng et al., 1996), to measurements of power production during swimming in response to contact with predators by cannulated scallops fitted with piezoelectric transducers (Marsh et al., 1992), and to examination of metabolic parameters within the contracting adductor muscle by magnetic resonance spectroscopy (Bailey et al., 2003). The range of techniques applied to swimming by the lowly scallop is impressive! Although each of these approaches has its strengths and limitations, none of these techniques reveals the integration of contractions of the phasic and tonic adductor muscle.

To better quantify the activity of the phasic and tonic adductor muscle during escape responses, we developed a simple technique that monitors force development in intact scallops (Fleury et al., 2005). The lower valve of the scallops is clamped to the bottom of an aquarium, while the upper valve is free to move. An extension of the force gauge is inserted under the upper valve at its margin. A test stand allows precise adjustment of the distance between the valves, which we set so that the scallop cannot close its valves more than the distance used during normal ventilation (Fig. 6.2). A computer records any contact of the upper shell with the extension of the force gauge. Rapid peaks reflect contraction of the phasic adductor muscle, whereas sustained force development reflects the contraction of the tonic adductor muscle (Fig. 6.3). In response to having their mantle touched by a predator (e.g., starfish arm), scallops generally increase the gape between the valves and then make a strong phasic contraction. The timing and type of contraction are easily obtained from these recordings (Fig. 6.3). Since the margin of the valve contacts the force gauge, the force recorded is

proportional (but not equivalent) to that produced by the muscle, given that the muscle is attached closer to the center of the valve (Pérez et al., 2009a). As this method prevents scallops from swimming or closing their valves, it cannot represent the dynamics of swimming and is best suited to showing the timing of contractions by the phasic and tonic adductor muscles. The relatively noninvasive nature of the force gauge method has allowed us to compare the response of individual scallops before and after a dietary treatment (Guderley et al., 2011), to test individuals during the act of spawning to see if their escape responses change with the effort of spawning (Pérez et al., 2009b), and to examine the metabolic status of individuals sampled in specific activity states (Pérez et al., 2008a, 2008b).

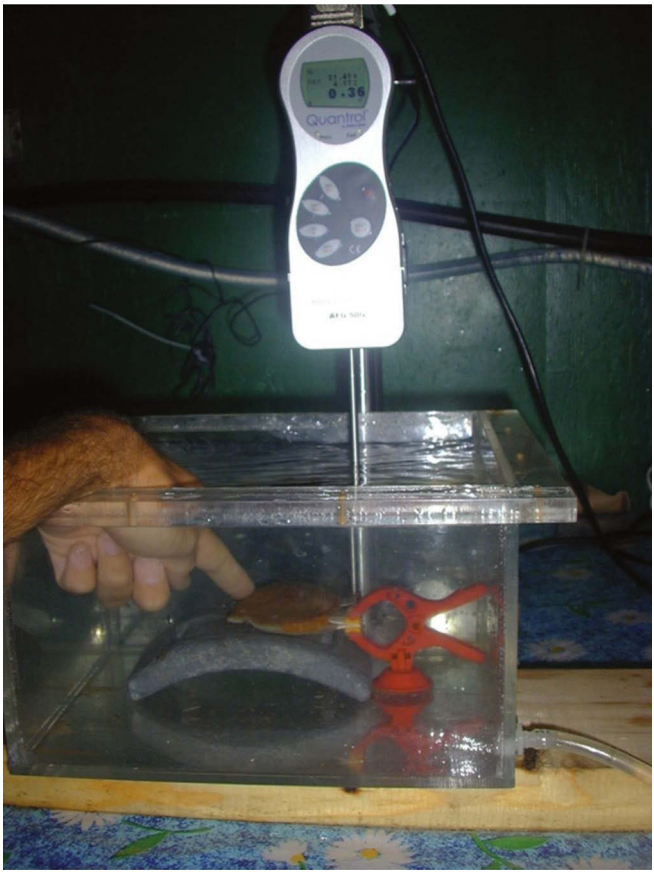

FIGURE 6.2 Force gauge for monitoring muscle activity in intact sea scallops (*Placopecten magellanicus*).

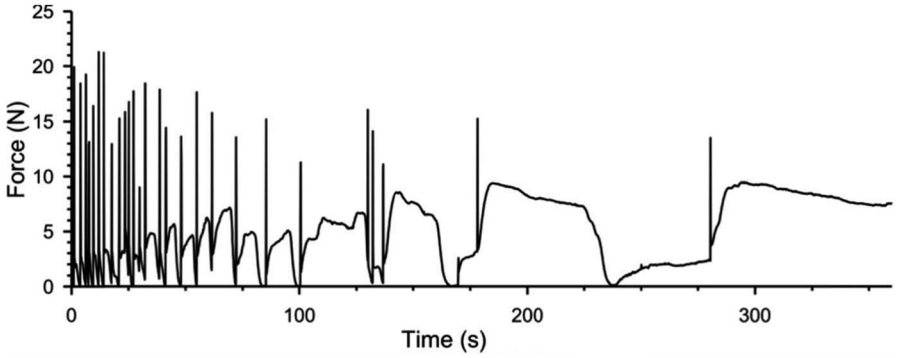

FIGURE 6.3 Typical recording of force production during an escape response by sea scallops (*Placopecten magellanicus*) following Fleury et al. (2005). Sharp peaks indicate phasic contractions, whereas sustained shoulders reflect contraction of the tonic adductor muscle.

Although there is considerable inter- and intraspecific variability in escape response performance, adult scallops typically carry out between 20 and 50 phasic contractions before ceasing to respond to stimulation (Tables 6.1 and 6.2). Generally, the initial response is the most intense, with a flurry of claps occurring at the start of stimulation followed by gradually diminishing numbers of phasic contractions. The frequency of phasic contractions (inverse of minimal interval between claps) varies considerably among species. Among a series of scallops that we compared, the southern scallop (*Pecten fumatus* Reeve, 1852), with its unfavorably shaped valves, made the most rapid succession of phasic contractions (Table 6.2) (Tremblay et al., 2012), followed closely by *A. balloti*. The timing of tonic contractions varies considerably among species, with some species, for example, the doughboy scallop (*Mimachlamys asperrima* [Lamarck, 1819]), starting their response to the predator with a tonic contraction, the strong swimmer, *P. magellanicus*, making many short tonic contractions throughout its escape response, and the best swimmer, *A. balloti*, delaying the onset of tonic contractions until well into its escape response (Table 6.2) (Tremblay et al., 2012). Once scallops cease to respond, most species close their valves and remain shut for several minutes (warm temperate zone scallops) or hours (polar and temperate zone species). The tropical zigzag scallop (*Euvola ziczac* [L., 1758]) (Brokordt et al., 2000a) and some juvenile *P. magellanicus* (H. E. Guderley, personal observation) do not close their valves after exhaustion.

TABLE 6.1 Mean Number of Phasic Contractions (Claps) and Clapping Rate During an Initial Escape Response, as well as the Percent Recuperation of Initial Claps in Adults of Different Scallop Species.

	No. of claps	Clapping rate (no. of claps/min)	Percent recuperation	Reference
Iceland scallop, *Chlamys islandica*	26±1	13±1	95.2±6.3	Brokordt et al. (2000a)
Zigzag scallop, *Euvola ziczac*	52±1	21±2	91.2±2.8	Brokordt et al. (2000b)
Peruvian scallop, *Argopecten purpuratus*				
Cultured	42±2	14±2	78±5	Brokordt et al. (2006)
Wild	33±1	19.5±2	72±3	Brokordt et al. (2006)
Wild sea scallop, *Placopecten magellanicus*	48±3	8.5±3	75.2±9.8	Kraffe et al. (2008)

Note: After visual evaluation of their initial escape response, scallops were given a standard recovery period (4 h for *C. islandica*, 20 min for *E. ziczac*, 20 min for *A. purpuratus*, and 30 min for *P. magellanicus*). These durations were chosen such that recovery was well advanced but not completed. Other details are given in the cited publications. Values are shown as mean ± SE. The data are given for scallops in the gametogenic phase of their reproductive cycle.

TABLE 6.2 Adductor Muscle Performance During Force Recordings of Escape Responses in Scallops with a Wide Range of Shell Morphologies.

	Total phasic contractions	Minimum interval between claps (s)	Time at first tonic (s)	Percent time in tonics
Saucer scallop, *Amusium balloti*	40.9±1.37	0.38±0.036	8.1±2.60	67±3.5
Cultured sea scallop, *Placopecten magellanicus*	27.5±1.65	1.12±0.144	1.1±0.50	82±3.2
Southern scallop, *Pecten fumatus*	32.7±2.86	0.32±0.042	31.7±4.37	65±6.1
Doughboy scallop, *Mimachlamys asperrima*	18.0±1.61	0.65±0.123	0.1±0.14	83±1.6
Giant rock scallop, *Crassadoma gigantea*	1.8±0.84	–	0.0±0.00	93±5.2

Note: Scallops were fixed to the bottom of an aquarium and force development was monitored during their escape responses from their predators, as described in the text. Phasic contractions were identified as sharp peaks in force production, whereas tonic contractions were continuous shoulders of force production. Escape responses were monitored for 355 s (Tremblay et al., 2012).

6.8 PHYSIOLOGICAL STATUS AND ESCAPE RESPONSE CAPACITIES

As the glycogen stores in the adductor muscle are a major energetic reserve, escape response performance could change with the energetic and physiological status of scallops. Seasonal changes in food availability and reproductive investment could modify escape response performance. A reduction in escape response performance by reproductive investment suggests that reproduction could reduce survival in the presence of predators. The impact of temperature and size upon physiological capacities could also modify escape response capacities, as could the stress caused by handling during fishing or culture operations. In the following, we will consider how physiological status modifies escape response performance (as monitored by visual examination of responses to predators) and examine the mechanisms underlying these changes.

Our first inkling that physiological status might modify escape response performance came from studies of mitochondrial physiology in *E. ziczac*. The oxidative capacity of muscle mitochondria changed seasonally, with decreased capacities observed during periods of greatest reproductive investment (Boadas et al., 1997). This suggested a trade-off between reproduction and escape response performance, or more specifically between reproduction and recuperation from exhaustive exercise. We hypothesized that the capacity for repeat escape response performance would be decreased in scallops sampled after gametogenesis and spawning.

K. B. Brokordt validated this hypothesis in two scallop species, *C. islandica* and *E. ziczac*, using visual observations of escape response behaviors of unrestrained scallops. For both species, recuperation from exhaustive escape response performance occurred much more slowly in individuals sampled after gametogenesis and(or) spawning than in individuals with immature gonads (Brokordt et al., 2000a, 2000b). It is well established that gametogenesis depletes macromolecular reserves from muscle and digestive gland in scallops, typically reducing muscle glycogen and even protein levels (Barber and Blake, 1991; Brokordt and Guderley, 2004a). *Chlamys islandica* and *E. ziczac* were no exception to this pattern and demonstrated marked changes in muscle glycogen levels with the reproductive cycle. In parallel, muscle activities of glycolytic and mitochondrial enzymes decreased, potentially due to the decreased availability of glycogen as a matrix for the binding of these enzymes (Brokordt and Guderley, 2004b). Mitochondrial oxidative capacities also decreased as reproductive investment increased.

6.9 REPRODUCTIVE INVESTMENT AND ESCAPE RESPONSE PERFORMANCE

The biochemical changes in muscle physiology during the reproductive cycle suggest that swimming performance could be affected by two, not mutually exclusive, mechanisms. Loss of metabolic and contractile elements in muscle could decrease swimming performance or a reduced aerobic capacity could impede maintenance of muscle status and recuperation from exhaustive exercise. Aerobic capacity could fall through loss of maximal aerobic capacity, or by an increase in routine metabolic requirements, in either case, metabolic recovery would take longer.

$\dot{V}o_{2max}$ were reduced or if routine metabolic requirements were increased by reproductive investment, aerobic scope would fall, slowing metabolic recuperation and reducing repeat performance. As reproductive investment requires synthesis of macromolecules and transfer of materials between tissues, routine metabolic requirements are likely to rise during gametogenesis and preparation for spawning. Mobilization of macromolecules from muscle could impair muscle metabolic capacities and reduce maximal rates of oxygen uptake.

To examine this question, we examined the metabolic capacities of *P. magellanicus* from their natural beds at gonadal maturity, after spawning, and during reproductive quiescence (Kraffe et al., 2008). Neither $\dot{V}o_{2max}$ nor mitochondrial oxidative capacities changed with reproductive status. In contrast, standard metabolic rate (SMR) was 60% of $\dot{V}o_{2max}$ in spawned scallops but only 30–40% of $\dot{V}o_{2max}$ in scallops with mature gonads or in reproductive quiescence (Fig. 6.4). Thus, reproductive investment is likely to reduce repeat performance of *P. magellanicus* by increasing maintenance costs and reducing aerobic scope. In other scallops, such as *C. islandica* and *E. ziczac*, as muscle mitochondrial capacities are reduced by reproductive investment (Brokordt et al., 2000a, 2000b). $\dot{V}o_{2max}$ could fall with these reduced mitochondrial capacities, slowing recuperation from exhaustive exercise by a second mechanism.

Examination of escape response behavior during the reproductive cycle of several scallop species indicates that the initial response to contact with a predator is quite constant. In *C. islandica*, the number and rate of phasic contractions changed little with gonadal status (Brokordt et al., 2000a). For the tropical scallop *E. ziczac*, reproductive investment did not change the number of phasic contractions, although their rate was slightly higher in spawned than in immature scallops (Brokordt et al., 2000b). The impact of reproductive investment upon escape response performance in *A. purpuratus*

differed between wild and cultured scallops, with wild scallops maintaining a constant number and rate of phasic contractions throughout their reproductive cycle, whereas cultured scallops lost performance as reproductive investment increased (Brokordt et al., 2006). Spawned *P. magellanicus* were not able to perform as many phasic contractions as scallops with mature gonads (Kraffe et al., 2008). In these three studies of wild populations, temperature changed little between the sampling times, indicating that changes in performance were due to intrinsic changes in the scallops. Finally, we established that the act of spawning, with its associated valve movements, did not significantly modify the escape response capacity (as measured with the force gauge method) of *A. purpuratus* (Pérez et al., 2009b). Overall, initial escape response performance changed little with reproductive investment, presumably limiting the compromise between survival and reproduction.

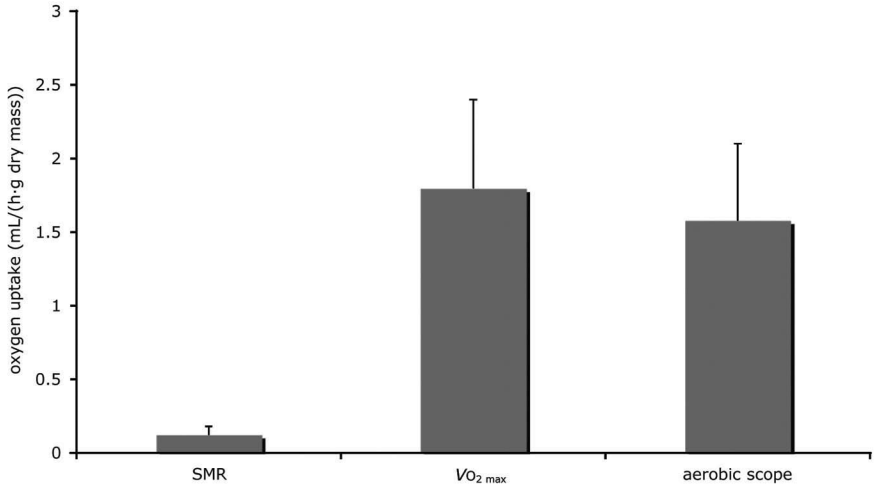

FIGURE 6.4 Changes in rates of oxygen uptake with activity in juvenile Iceland scallops (*Chlamys islandica*). Standard metabolic rates (SMR) were obtained in resting individuals that had fasted for 48 h. To determine the $\dot{V}o_{2max}$, each scallop performed two escape response tests separated by a 45 min recuperation period. The highest oxygen consumption measured for each individual was considered the $\dot{V}o_{2max}$ (Tremblay et al., 2006). Aerobic scope was calculated by subtracting the SMR from the $\dot{V}o_{2max}$.

In contrast to initial performance, repeat performance or recuperation from exhaustive exercise is considerably more sensitive to reproductive investment. *Chlamys islandica* with immature gonads can repeat their escape response performance within 4 h, whereas mature, prespawned, and spawned scallops needed 12–18 h for full recuperation (Brokordt et al., 2000a). For *E.*

ziczac, scallops with immature gonads could repeat their initial performance after 20 min of aerobic recuperation, but the time required for recovery increased markedly with reproductive investment (Brokordt et al., 2000b). Reproductive investment also decreased repeat performance in *A. purpuratus* and *P. magellanicus* (Brokordt et al., 2006; Kraffe et al., 2008).

The differing responses of the initial and repeat escape response performance presumably reflect the fact that the initial response is fuelled by anaerobic metabolism, whereas repeat performance requires aerobic recuperation. Effectively, scallops show their maximal rates of oxygen uptake ($\dot{V}o_{2max}$) after exhaustive escape responses (Mackay and Shumway, 1980; Tremblay et al., 2006), with oxygen uptake increasing up to 12-fold (Fig. 6.5).

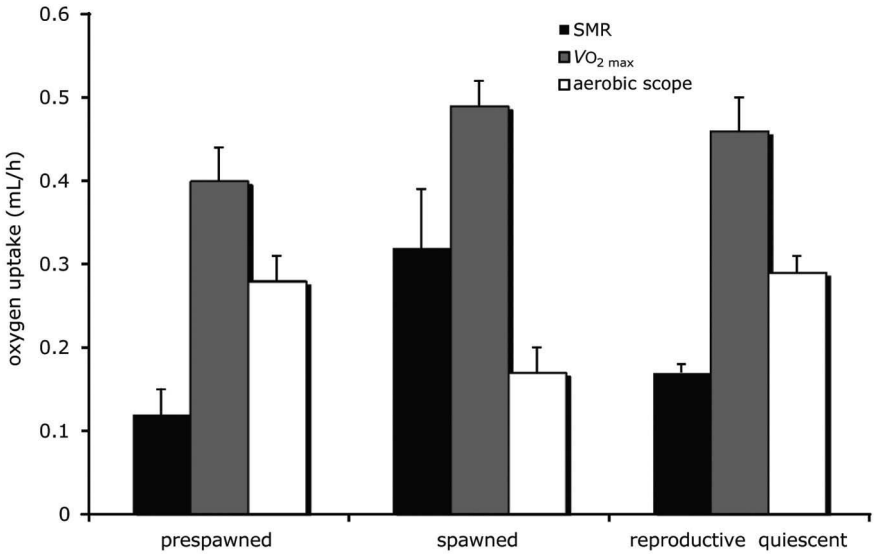

FIGURE 6.5 Oxygen uptake as a function of the reproductive state in adult sea scallops (*Placopecten magellanicus*). Metabolic rates were determined at habitat temperature (6.3–7.2°C) as described in Kraffe et al. (2008). Standard metabolic rate (SMR) was assessed in scallops that had been held in filtered water for 3 days, whereas $\dot{V}o_{2max}$ was measured directly after exhaustive escape responses. Aerobic scope was calculated by subtracting SMR from the $\dot{V}o_{2max}$. Oxygen uptake rates are expressed for a standard animal (50 g wet soft tissue mass).

A compromise between reproduction and locomotor capacity may also arise due to the macromolecular requirements for gametogenesis. When reproductive investment occurs during periods of low food availability, muscle glycogen and even protein may fall. Mobilization of muscle glycogen

in support of gonadal growth is common in scallops (Barber and Blake, 1981). In *C. islandica*, the loss of intramuscular glycogen reduces binding of glycogen phosphorylase and octopine dehydrogenase, thereby potentially destabilizing the intracellular organization of muscle enzymes (Brokordt and Guderley, 2004b). This loss of binding parallels the decrease in enzyme activity, potentially owing to accelerated turnover of "unbound" enzymes. In keeping with a role of muscle glycogen in maintaining muscle metabolic capacity, reproductive investment by *E. ziczac* leads to greater decreases of muscle metabolic capacity and repeat performance when reproductive cycles occurred during periods of low food availability (Brokordt et al., 2000b).

It is remarkable that initial escape response behavior remains relatively constant during scallop reproductive cycles, despite the marked changes in muscle metabolic status. For *E. ziczac*, *C. islandica*, and *A. purpuratus* that were sampled in their natural habitats, initial escape response performance changed little with reproductive status (Brokordt et al., 2000a, 2000b, 2006). The impact of reproductive investment upon initial escape response performance is much weaker than that upon repeat performance (Brokordt et al., 2006; Kraffe et al., 2008). This pattern emphasizes the selective importance of an effective initial escape from predators (Barbeau and Scheibling, 1994). As repeat escape performance is unlikely to provide much additional protection from predation, the gain in fitness obtained by increasing reproductive investment would seem to easily offset the loss in survival.

6.10 SIZE AND ESCAPE RESPONSE PERFORMANCE

Biomechanical considerations predict that the swimming capacity of scallops changes with size. The power required for swimming increases markedly with size, given the exponential rise in shell mass with size. Changes in shell shape with size will also influence swimming capacity. Although each scallop species has its own ontogenetic changes in shell characteristics, some generalizations are possible. The most extensive literature concerning the impact of size on swimming performance concerns *P. magellanicus* (Gould, 1971; Dadswell and Weihs, 1990; Manuel and Dadswell, 1991, 1993). Diving observations by Caddy (1968, 1972) separated its life cycle into a sessile phase (1–30 mm in shell height), a mobile stage (30–100 mm), and a more sedentary stage (>100 mm). The swimming style of *P. magellanicus* also varies with body size. Small scallops tend to rise in the water column and rarely succeed in swimming horizontally. Scallops of intermediate size rise in a rectilinear motion and can swim steadily for several meters (Caddy,

1968; Dadswell and Weihs, 1990; Manuel and Dadswell, 1993). Larger scallops only swim short distances given the mass of their shells. *Placopecten magellanicus* of intermediate size (40–80 mm) are the fastest swimmers and possess the greatest hydrodynamic efficiency (Dadswell and Weihs, 1990). Ontogenetic changes in swimming capacity are apparent in other scallop species. In contrast to most scallop species, *A. balloti* increases its swimming capacity with size (Joll, 1989). In *A. opercularis*, small scallops (<60 mm) swim more and close their shells more often than larger scallops that tend to jump (Schmidt et al., 2008). The most extreme ontogenetic changes occur in the rock scallop (*Crassadoma gigantea* [J. E. Gray, 1825]), which swims while small (<40 mm) and then cements to the substrate when larger (Yonge, 1951; Lauzier and Bourne, 2006).

In *P. magellanicus*, the impact of size upon muscle metabolic capacities and patterns of muscle use parallels its impact upon swimming performance (Labrecque and Guderley, 2011). Much as swimming performance peaks between 40 and 80 mm shell height, biochemical characteristics increase with size to peak near 60 mm and decrease at greater shell heights. Clearly, these functional characteristics do not follow the simple allometric dependence of metabolic capacity shown in interspecific comparisons of vertebrates (Hochachka and Somero, 2002). The increasing reproductive investment of *P. magellanicus* with size combined with its longevity may underlie this pattern.

As the major role of scallop swimming is predator avoidance (Legault and Himmelman, 1993), the suite of predators in the scallop's habitat must be considered in interpreting the size dependence of escape response performance. Large scallops with thick and heavy shells may have reached a size refuge, relaxing the requirements for high muscle metabolic capacities to power escape response performance. The major predators of *P. magellanicus* are sea stars (*A. vulgaris*), rock crabs (*Cancer irroratus* Say, 1871), American lobsters (*Homarus americanus* H. Milne-Edwards, 1837), and some species of fish (Barbeau and Scheibling, 1994; Elner and Jamieson, 1979; Naidu and Meron, 1986). The preference of sea stars for small *P. magellanicus* may underlie the strong escape responses of small *P. magellanicus* (Barbeau and Scheibling, 1994). Elner and Jamieson (1979) observed that adult rock crabs do not feed on *P. magellanicus* over 72 mm shell height. The stomach contents of American plaice (*Hippoglossoides platessoides* [Fabricius, 1780]) indicate that only small (<25 mm) *P. magellanicus* are consumed (Naidu and Meron, 1986). The simultaneous decline of hydrodynamic efficiency and behavioral and physiological capacities of *P. magellanicus* at shell heights greater than 65 mm suggest that they have reached

a size refuge. As *P. magellanicus* is a long-lived species that reaches sizes above 150-mm shell height (Naidu and Robert, 2006), this decline is unlikely to be due to senescence. It seems more likely that changes in predation pressure with size simultaneously affect shell morphology, swimming performance, and the physiological properties of the adductor muscle.

Although more is known about the allometry of escape response performance and its underlying physiology in *P. magellanicus* than in other scallop species, other scallops have similar attributes. Ontogenetic changes in the habitat occupied by *C. islandica* are linked to susceptibility to predation by sea stars and crabs (Arsenault and Himmelman, 1996). Accordingly, small *C. islandica* make more phasic contractions, but remain closed less long after exhaustion than large scallops (Tremblay et al., 2006). *Aequipecten opercularis* changes from swimming to jumping as it increases in size, and shows a marked decline in markers of aerobic metabolism and an increase in indicators of oxidative damage (Philipp et al., 2008). The authors suggest that senescence, with the gradual accumulation of damaged, atrophied tissues, take its toll in older *A. opercularis*.

6.11 TEMPERATURE AND ESCAPE RESPONSE PERFORMANCE

Temperature affects rates of physiological processes in ectotherms, with rates rising to an optimum and then stabilizing and eventually falling at higher temperatures (Hochachka and Somero, 2002). Gill-breathing organisms are particularly tied to environmental temperature, as the simple act of respiring requires intimate contact with water. This is doubly true for filter-feeding animals such as scallops. Thermal effects will arise directly from kinetic effects upon physiological processes and indirectly through changes in oxygen contents of the water and in cardiovascular capacities for oxygen delivery.

Although the thermal sensitivity of scallop escape response performance and of the contractile properties of adductor muscle fibers vary with thermal habitat, generally performance improves with temperature, up to an optimum, and then declines. Valve contraction rate of *P. magellanicus* increases with habitat temperature (Manuel and Dadswell, 1991). Time-related contractile properties (response latency, time to peak tension, and relaxation time) in Atlantic bay scallop (*Argopecten irradians* [Lamarck, 1819]) and *P. magellanicus* decrease with rising temperature, whereas force production changes little (Olson and Marsh, 1993; Pérez et al., 2009a). Perhaps the narrowest thermal optimum for swimming is that of the *A. colbecki*, as it ceases to

respond at 2°C (Peck et al., 2004). Interestingly, the abductin in the hinge ligament of *A. colbecki* has a greater resilience than that of temperate zone scallops, partially compensating for the effects of cold temperature on swimming performance (Denny and Miller, 2006). Transferring *P. magellanicus* from 18 to 8°C markedly decreased rates of phasic contraction and slowed recuperation from exhausting exercise for 156 h after the transfer (Lafrance et al., 2002). To examine the thermal sensitivity of swimming performance in *A. opercularis*, without the effects of acute thermal change, Bailey and Johnston (2005b) evaluated performance at acclimation temperatures of 5, 10, and 15°C. Contractile properties were more sensitive to temperature than the total duration of the activity cycle (opening and closing of the valves), presumably because the performance of the hinge ligament is less dependent on temperature than that of the muscle. Thermal acclimation only slightly modifies swimming speed of *A. opercularis*, despite considerable increases in power production with temperature (Bailey and Johnston, 2005a). The thermal sensitivity of peak acceleration is higher in winter- than autumn-acclimatized *A. opercularis* (Bailey and Johnston, 2005b). When escape response performance of *P. magellanicus* was monitored at habitat temperature during spring, summer, and fall, phasic force, the number of phasic contractions, and the minimal interval between phasic contractions remained stable, suggesting that scallops compensate for seasonal changes in temperature (Guderley et al., 2008).

The thermal sensitivity of escape response behavior is influenced not only by the intrinsic thermal sensitivity of the underlying contractile processes, but also by neurosensory integration. The season at which a scallop experiences a given temperature may modify its thermal sensitivities, through direct thermal effects or indirect seasonal effects. Indirect effects include perception of changes in day length and acclimation to prevailing seasonal temperatures and food availabilities. To evaluate the impact of a scallop's thermal history upon the thermal sensitivity of its escape response performance, we compared the responses of scallops sampled in May and September when habitat temperature was 12°C. In May, scallops performed better at 6 than at 12 or 18°C, whereas in September, performance was better at 6 and 12°C than at 19°C (Guderley et al., 2009). Much as with isolated muscle fibers (Olson and Marsh, 1993; Pérez et al., 2009a), mean and maximal force production was not affected by temperature, whereas the duration of phasic contractions fell as temperature rose (Guderley et al., 2009). Clearly, predictions of how temperature will affect scallop escape response performance require knowledge of their thermal history and their typical thermal environment.

6.12 ESCAPE RESPONSE PERFORMANCE AND CULTURE METHODS

As the strength of the scallop escape response is sensitive to their physiological status, we reasoned that escape response performance could be used to assess the impact of handling practices used during scallop culture. Particularly during transfers between the different culture phases, scallops are removed from one site, manipulated, and then brought to the next phase of their culture. Handling is often mechanized and can be quite rough. Virtually all aspects of escape response performance, as well as muscle levels of arginine phosphate and adenylate energy charge, are drastically reduced after juvenile *P. magellanicus* are exposed to a standardized handling stress modeled upon culture techniques (Guderley et al., 2008; Pérez et al., 2008b). Three hours recuperation allows metabolic parameters and escape response performance to return to control values (Pérez et al., 2008b). It is technically much simpler to measure escape response performance than the biochemical status of muscle, suggesting that measurements of escape responses could be used to refine culture practices. Greater reductions in escape performance occurred when the handling stress was applied under summer than fall conditions (Guderleyet al., 2008). The lower temperatures in the fall presumably reduced the impact of handling stress upon physiological status. Thus, when at all possible, culture manipulations should be carried out under cool conditions.

By its very nature, culture selects for rapid growth and may reduce exposure to the predators typically present in the cultured organism's habitat (but see work by J. H. Himmelman and L. Freites). These changing selection pressures may lead cultured organisms to differ from their wild counterparts in their performance characteristics. In one comparison, we found that wild *P. magellanicus* had stronger shells and higher clapping rates but were in weaker energetic condition than equivalently sized cultured scallops (Lafrance et al., 2003). Cultured *A. purpuratus* responded less rapidly to their starfish predator than their wild counterparts (Brokordt et al., 2006). The reduced activity of scallops during culture could affect their swimming capacities. Effectively, as frequent swimming by *P. magellanicus* increases adductor muscle size (Kleinman et al., 1996), culture under confined conditions could lead scallops to become sedentary, reduce muscle size, and swimming performance.

6.13 CONCLUSION

Rapid contractions by the striated adductor muscle allow scallops to use jet propulsion to escape their predators. In scallops with strong swimming capacities, the position and size of the muscle, characteristics of the hinge ligament, and the structure and shape of the valves facilitate swimming. However, species with unfavorable shell morphologies can start escape responses with an intense series of phasic contractions to partially overcome constraints imposed by the shape of their valves. Scallops differ considerably in how they use their adductor muscles, with tonic contractions used much more by some species than others. Even strong swimmers, such as *P. magellanicus*, make extensive use of tonic contractions, although the function of these numerous short tonic contractions is unclear. The behavioral differences suggest that the functional characteristics of the adductor muscle and hinge ligament vary among scallop species. Within scallop species, ontogenetic changes in susceptibility to predation, adjustments to environmental conditions, and reproductive investment can modify escape response performance. However, initial escape responses change less than repeat performance, presumably due to the selective importance of an effective escape response. The functional components required for an escape response are likely to evolve in a coordinated fashion. Modifications in shell morphology that lead to more effective valve closure could reduce the need (and capacity) for effective escape responses, relax requirements for muscle performance, and potentially increase reproductive success. Further analysis of the pectinid locomotor system is likely to reveal such fundamental tradeoffs between major fitness functions.

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KEYWORDS

- **scallop**
- **Pectinidae**
- **locomotion**
- **swimming**
- **adductor muscle**
- **escape response capacity**

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CHAPTER 7

LOCOMOTION OF COLEOID CEPHALOPODS

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CONTENTS

7.1 INTRODUCTION

Locomotion can be broadly defined as movement through one's environment (DeMont et al., 2005; Dickinson et al., 2000). It is important for accomplishing various goals such as finding food and mates, escaping predators, and migrating to find resources. From a physical point of view, locomotion can simply be thought of as a force exerted by an organism on its environment that, in obedience to Newton's laws, produces movement of the organism in the opposite direction. However, we see that the distribution of forces is anything but simple when we compare the diversity of locomotor abilities exhibited by animals (both vertebrates and invertebrates) in a suite of environments (e.g., water, substrate, and air). Locomotor design can be a key driver in the evolutionary history of animals that rely on movement. It can produce changes in morphology and physiology that affect their speed, acceleration, efficiency, endurance, and agility when moving in different environments (Dickinson et al., 2000). This is particularly true for the group of animals that will be discussed in this chapter, the cephalopods, which exhibit a wide array of locomotor types in their mostly water environment (there is one case of air-borne locomotion).

Cephalopods are a bilaterally symmetrical class of invertebrates within the phylum Mollusca that are often considered different from the rest of the group in many ways. They include two extant groups, the small one of nautiloids, which has an external shell, and the larger one of coleoids, which does not. The coleoids, which will be the focus of this chapter, include cuttlefish, squid, octopods (both the cirrates which have fins and the incirrates which do not), and the vampire squid (see Fig. 7.1). Some basic molluscan features that are expressed differently in the cephalopods include the muscular foot, the protective shell, and the mantle cavity (Trueman & Clarke, 1988). These are embodied in molluscan *bauplans* ("a combination of the most significant features of the phylum"; Haszprunar & Wanninger, 2012). The most typical *bauplan*, though others have been proposed (see "urmollusc" in Haszprunar & Wanninger, 2012; see Fig. 2 in Haszprunar, 1992), is the Hypothetical Ancestral Mollusc, or the HAM. The HAM most resembles gastropods with a shell, head, foot, mantle cavity, and visceral mass. Cephalopods have undergone changes in all three features: modifying the foot into funnel and arms, internalizing, reducing, or losing the shell entirely, and co-opting the mantle–funnel for movement (i.e., jet propulsion) as well as respiration of a closed circulatory system (Clarke & Trueman, 1988; Wells, 1994) (see anatomy by group in Figs. 2.1–2.3). These modifications produced fast moving, high metabolic rate animals from a slow moving, low metabolic

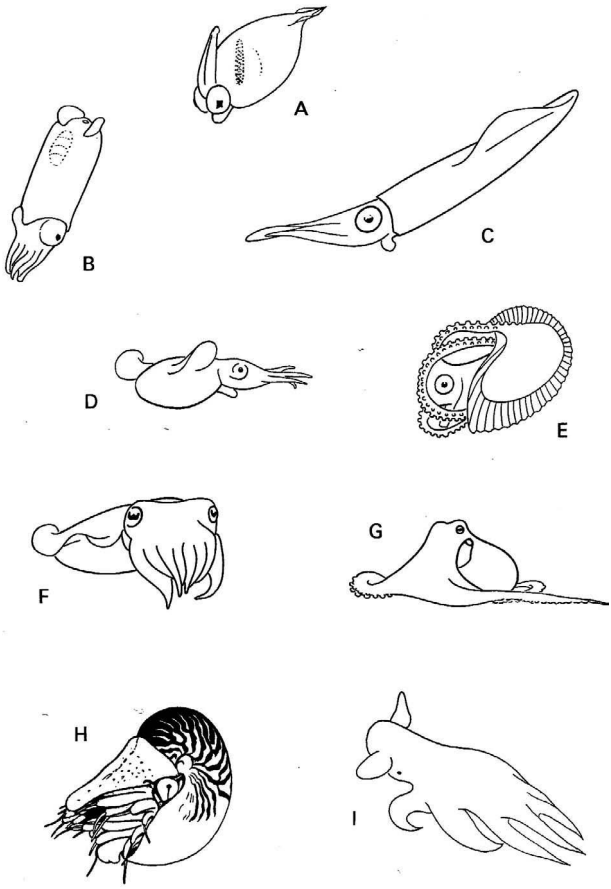

FIGURE 7.1 Representative drawings of extant cephalopods. (A) *Teuthowenia*, a teuthoid squid of the family Cranchiidae, hovering in mid-water with raised arms and tentacles. (Drawn after a photograph of Vecchione and Roper, 1991.) (B) *Spirula*, a sepiolid that can achieve vertical migrations over several hundred meters with a calcified, coiled, and chambered inner shell. (C) A loliginid squid hovering with dynamic lift provided by undulating fin movements and funnel jets. (D) A bobtail squid of the family Sepiolidae with a very small uncalcified internal shell swimming with undulating fins. (E) A female *Argonauta*, a pelagic incirrate octopod, with a calcitic pseudoconch used to brood eggs and maintain buoyancy. (F) *Sepia*, a cuttlefish with a calcified chambered shell and undulating fins for maneuvering. (G) A benthic incirrate octopus of the family Octopodidae crawling. (H) *Nautilus* with a coiled external, heavily calcified chambered shell giving the animal neutral buoyancy. (Drawn after a photograph published by the Japanese research program JECOL, 1977.) (I) A finned cirrate octopod moving with a partly retracted web. (Drawn after a video recording of the French research program CALSUB, 1989.) (From Budelmann, B. U.; Schipp, R.; Boletzky, S. von Cephalopoda. In *Microscopic Anatomy of Invertebrates*; Harrison, F. W., Kohn, A., Eds.; Wiley-Liss: New York, 1997; Vol. 6A, Mollusc II, pp. 119–414. Copyright © (2015) by John Wiley &Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.)

rate molluscan ancestor. They also had implications for other features such as increased brain and sensory development, appearance and use of fins in all groups but the incirrate octopods, a muscle-based structural support system, and an efficient fuel delivery system, resulting in more effective locomotor activities (Wells, 1994).

Unlike their molluscan relatives that rely primarily on their muscular foot to move in their environment (Trueman, 1983), cephalopods utilize different muscle groups, sometimes in concert, to achieve different modes of locomotion adapted to habitat differences. Most cephalopods use their mantle–funnel system to swim via jet propulsion in the open ocean. Cuttlefish and squid can also hover with the assistance of undulating fins (Clarke, 1988; Wells & O'Dor, 1991). In some cases, swimming is exclusively achieved by the fins, as in deep sea, low-metabolizing cirrate octopods, like *Cirrothauma* and *Grimpoteuthis* (Aldred et al., 1983; Clarke, 1988; Collins & Villaneuva, 2006; Vecchione, 1995;; Vecchione & Roper, 1991; Vecchione & Young, 1997). Some cirrate octopods like *Opisthoteuthis* and *Stauroteuthis* also use the web between their arms to eject water for medusoid swimming (Collins & Villaneuva, 2006; Vecchione & Roper, 1991; Vecchione & Young, 1997). *Vampyroteuthis infernalis* undergoes a "gait-transition" during development, using more medusoid swimming when it is juvenile and more fin swimming when it is an adult (Seibel et al., 1998). Some benthic cephalopods will also bury themselves using their arms, in the case of octopods, or mantle–funnel jets as seen in cuttlefish and bobtail squids (sepiolids) (Anderson et al., 2004; Boletzky, 1996; Hochberg et al., 2006; Mather, 1986). Octopods in both deep and shallow water have the ability, expressed in their predominantly benthic lifestyle, to crawl using a combination of their arms and suckers to push and pull against a substrate (Huffard, 2006; Mather, 1998; Villanueva et al., 1997a). An important influence on locomotion in the water that all cephalopods, nautiloids included, deal with is buoyancy. Although locomotion of nautlioids will not be discussed here, a detailed comprehensive literature is available (Baldwin, 2010; Chamberlain et al., 2010; Chamberlain, 1988, 1991; Jordan et al., 1988; Jacobs & Landman, 1993; Neumeister & Budelmann, 1997; O'Dor et al., 1990; Packard et al., 1980; Redmond et al., 1978). The trend for fast locomotion in the coleoid cephalopods is hypothesized to be an evolutionary response to the diversification of predatory paired-fin fish with their superior swimming abilities (Packard, 1972). The locomotion and subsequent design of fossil cephalopods, from which present ones evolved, are not covered in this chapter, but some key references are highlighted here (Chamberlain, 1980, 1981; Doguzhaeva et al., 1996; Ebel, 1999; Engeser,

1988; Jacobs et al., 1996; Jacobs, 1992; Klug & Korn, 2004; Kröger et al., 2011; Kröger, 2002; Ritterbush et al., 2014).

Muscles are a major driver of locomotion in Molluscs. Three major systems have been classified: The body wall consisting of circular, medial, diagonal, and longitudinal muscles; a pair of longitudinal muscles on either side of the foot; and the major dorsoventral muscles that move the entire foot (Haszprunar, 1992). Molluscs exhibit two types of skeletal structures, a hard shell or a hydrostatic skeleton. Lacking a hard exoskeleton, coleoids depend on hydrostatic skeletons for both structural support and movement. Hydrostatic skeletons can generate movement either by 1) muscles contracting around a fluid-filled (usually blood) body cavity held at constant volume, resulting in movement of the fluid to another region of the body, or by 2) muscle contractions antagonizing a three-dimensional arrangement of muscle fibers to produce actions (Kier, 1988). Coleoid cephalopods are primary examples of organisms that use muscular hydrostats to achieve movement. Examples of such hydrostats include the mantle–funnel complex in all coleoids to produce jet propulsion and respiration; lateral fins of squid used for swimming; squid arms, tentacles, and octopod arms that allow for extension and mobility, cephalopod suckers, and the tentacles of *Nautilus* (Kier, 1988). It is important to note that although the basic hydrostatic skeletons were thought to be unique generators of movement, many examples in the Mollusca show that muscular hydrostats are widely used. This includes columellar muscles in some gastropods that allow them to extend from the shell, tightly packed muscles in the foot of limpets and chitons that produce crawling waves, the siphons of some bivalves used in deposit feeding, and the tentacles of gastropods, such as abalone (*Haliotis tuberculata*), for sensory perception (Kier, 1988).

Many recent advances in the physiology of cephalopods, including neuronal (Bartol et al., 2008, 2009; Burford et al., 2014; Flash & Hochner, 2005; Hochner, 2012; Laan et al., 2014; Sumbre et al., 2001), developmental (Poirier et al., 2004; Robin et al., 2014; Seibel et al., 1998; Thompson & Kier, 2006; Villanueva et al., 1997b), and genetic (Albertin et al., 2012; Navet et al., 2010), have opened the field for studying many other aspects of locomotion. More information about cephalopod navigation (Alves et al., 2008; Jozet-Alves et al., 2014) and migration, both horizontal (across bodies of water) and vertical (up and down the water column) (Gilly, 2006; Hoving et al., 2014; Rosa & Seibel, 2010b), have led to better understanding of how locomotion affects foraging, reproduction, and other behaviors. Recent field observations of octopods have resulted in more knowledge about flexibility

in the muscular hydrostat of the arm (Huffard et al., 2005; Huffard, 2006). In particular, more work in how multiple degrees of freedom give rise to multiple gait patterns is being done (Hochner, 2012; 2013; Huffard et al., 2005; Levy et al., 2015). Better techniques to study cephalopod morphology for taxonomic as well as potential mechanistic purposes are being used (Margheri et al., 2009; Xavier et al., 2014). All these aspects have resulted in a better understanding of cephalopod locomotion and have allowed others to take advantage of that knowledge to develop the growing field of soft robotics. This includes investigations in kinematics (Crimaldi et al., 2002; Kang et al., 2011; Kier & Leeuwen, 1997; Yekutieli, Sagiv-Zohar, Aharonov, Engel, Hochner, & Flash, 2005; Yekutieli, Sagiv-Zohar, Hochner, & Flash, 2005; Zelman et al., 2013), biomaterials (Hou et al., 2011, 2012), sensorimotor control (Flash et al., 2012; Li et al., 2012; McMahan & Jones, 2011; Sfakiotakis et al., 2013b), and dynamical modeling (Calisti et al., 2011, 2012, 2014; Kang et al., 2012; Laschi et al., 2009; Mazzolai et al., 2007; Renda et al., 2014; Sfakiotakis et al., 2013a; Zheng et al., 2013), particularly in octopods though some in cuttlefish (Wang et al., 2008; Willy & Low, 2005).

Cephalopods move differently from most other molluscs, though the muscles that are involved in these movements are essentially the same as those found in other members of the phylum. The goal of this chapter is to provide a comprehensive understanding of the cephalopod muscular hydrostat and its production of different types of locomotion as well as the structures used in the physiological processes of this system.

7.2 FOUNDATIONS

Cephalopods are highly mobile animals that rely on a combination of systems, namely their structural (muscles and connective tissues), nervous, respiratory, and circulatory ones, to adapt to their active lifestyle. The following sections will highlight the basic components of these systems—the muscles, connective tissue, and nervous system—and go over how they work in concert to result in movement. Muscles and muscular structures are the primary effectors of locomotion in cephalopods and will be featured in most of the underlying discussion. There are four major muscular structures of interest, and we will discuss how the physiology of each one is arranged. More details have become available as a result of new technology and the innovative use of tools to visualize many of these structures *in vivo* (King et al., 2005; Margheri et al., 2010).

7.2.2 BASIC STRUCTURES

7.2.2.1 MUSCLES

All forms of locomotion in cephalopods are powered by muscle. The muscle can be studied on multiple levels and the organization within each level can affect the speed and distance of movement. The levels on which this section focuses are the arrangement of muscle-cell components (the ultrastructure), the orientation of muscle fibers (gross arrangement), and the grouping of these fibers into whole muscle structures. The protein filaments that make up the contractile elements of muscle are key to the ultrastructure of muscle cells. These filaments shorten and generate force for movement (DeMont et al., 2005). Most invertebrates have two kinds of filament arrangements, cross-striated or obliquely striated muscles (DeMont et al., 2005). Cross-striated muscles are made up of protein filaments that are highly organized in bands that run transversely. Obliquely striated muscles have protein filaments that are organized in bands that are staggered and at an angle relative to the long axis of the fiber. Like that of most molluscs, cephalopod musculature is dominated by obliquely striated muscles (Kier, 1985). Cross-striated muscles have been identified in the transverse and circular muscles of squid tentacles, the appendages they use for prey capture. The arrangement and dimensions of these protein filaments are key to their differential functions. Protein filaments of cross-striated fibers are much shorter (0.5–0.9 μm) than the protein filaments from obliquely striated fibers (2.8 μm or longer), thus contractions of the cross-striated fibers in the tentacle lead to faster shortening speeds, while obliquely striated muscle fibers in the arm are able to exert more force over a longer distance for bending movements (Kier, 1985, 1991). However, recent studies of obliquely striated fibers in the mantle of the squid indicate that there may be more variation in the function of these muscles depending on the species and even on their structure (Thompson et al., 2014).

At the level of gross arrangement, muscle fibers are tightly packed in bundles and arranged in mutually perpendicular directions (Kier, 1988, 1991; Trueman, 1983). In a basic muscular hydrostat that assumes a cylindrical or tubular shape, the fibers are oriented such that they are circular, transverse, or radial to longitudinal muscle fibers that are parallel to the body axis. Additionally, there are oblique fibers that are oriented in a spiral around the body (Kier & Thompson, 2003). When the muscle fibers of one orientation contract, they are antagonized by fibers oriented in the other directions. Many of the structures to be discussed in cephalopods are more complex shapes than the simple cylinder, but the same muscular hydrostat principles apply.

Bundles of differently oriented muscle fibers are grouped together to form overall muscle structures. These structures include the mantle/funnel (see Fig. 7.2), head/eyes, arms, tentacles, and fins (Boyle & Rodhouse, 2005a). These are surrounded by the skin, which contain structures important for color production (Hanlon & Messenger, 1996). All of these are known to be controlled from centers in the suboesophageal brain. Of the structures listed, the mantle (Ward & Wainwright, 1972), fins of cuttlefish and squid (Kier, 1989), arms and tentacles of squid and octopods, and their associated suckers (Kier, 1985; Kier & Stella, 2007; Van Leeuwen & Kier, 1997) are examples of the muscular hydrostats that are essential to cephalopod locomotion. Most recently, the skin was also identified to be a muscular hydrostat (Allen et al., 2013), but its role is primarily in body patterning and so it will not be discussed further.

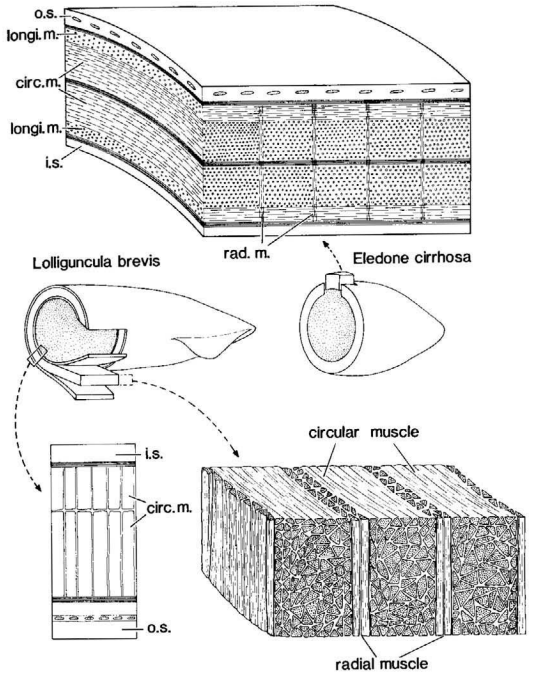

FIGURE 7.2 Mantle musculature cross section of octopus *Eledone cirrhosa* (above) and squid *Lolliguncula brevis* (below). Connective tissue divides layers of circular muscle (circ. m.) and makes up the surrounding tunic. Found in both species: outer skin (o. s.), longitudinal muscles (longi. m.), i. s. (inner skin), rad. m. (radial muscles). (Reprinted from Boyle, P. R. Neural Control of Cephalopod Behavior. In *The Mollusca: Neurobiology and Behavior Part 2*; Wilbur, K. M., Willows, A. O. D., Eds.; Academic Press: London, UK, 1986; Vol 9, pp 1–99. Copyright © (2015), with permission from Elsevier.)

One muscular structure that is physiologically important to locomotion is the heart. To accommodate their fast lifestyle, cephalopods have three hearts, one muscular systemic heart and two branchial hearts, one at the base of each of the two well-vascularized gills in the mantle cavity of dibranchiate coleoids. These hearts drive circulation and pump oxygenated blood through their closed circulatory system. The systemic heart of the octopod *Eledone cirrhosa* is composed of four layers of muscle that are cross-striated (Kier, 1985; Wells, 1983), exhibiting more musculature than the branchial hearts. Contraction of the systemic heart produces high pressure to circulate blood through a network of variously sized vessels. The branchial hearts are composed of only two thin outer layers of striated muscle. The layers run perpendicular to each other with the inside layer parallel to the long axis (Wells, 1983). The branchial hearts on either side of each gill are recruited to maintain circulation of well-oxygenated blood during locomotor activities (Wells, 1983, 1992).

7.2.2.2 CONNECTIVE TISSUES

Connective tissues are important structural components that resist changes in length from muscular contractions. These fibrous tissues also provide an important role in locomotion as they "transmit the force of muscular contraction, control shape change, and store elastic energy to reduce costs of locomotion, movement, or adhesion" (Kier & Thompson, 2003). Shape changes in particular are influenced by the arrangements of cross-linking fibers. These fibers wrap around the body in helices at an angle relative to the long axis, allowing for elongation and shortening (Kier, 2012). Collagen is one of the tensile fibers which resist extension in the direction of the fiber when stretched, but will readily deform when compressed by forces perpendicular to the fibers orientation (Wainwright et al., 1982). Collagen is present in layers of helically arranged fibers around the mantle of cephalopods. This arrangement resists increase in length along the mantle but provides more expansion in width (Wainwright et al., 1982).

Connective tissue fibers can also be embedded within layers of muscle fiber, as seen in the squid mantle and fins (Kier, 1992). Their flexible and resistant properties help restore contracted muscle fibers to their original length. Cartilage is a pliant material made up of flexible amorphous polymers that resist compression and bending forces to act like a rigid structure (Wainwright et al., 1982). It was thought to be vertebrate specific, but similar forms were found in the mouth parts of various molluscs to help control the

radula (Bairati, 1985) as well as in the head of octopods to provide structural support (Bairati et al., 1995). Cephalopods are the only invertebrates that have cartilage similar to the transparent cartilage found in vertebrates, known as hyaline (Cole & Hall, 2009). Roper and Lu (1990) systematically described the presence of cartilage and other connective tissues in oceanic squids (see Table 1 summary in Roper & Lu, 1990). In coleoids, the function of cartilage can vary from buoyancy (as in *Cranchia scabra* and *Galiteuthis glachialis*) to a pseudoskeleton to support muscles (e.g., *Histioteuthis meleagroteuthis* and *Liocranchia reinhardti*) and reducing drag (like *Tetronychoteuthis massyae*) (Roper & Lu, 1990). Cartilage is also important as an attachment site for major muscle groups and develops early in cuttlefish and other coleoid hatchlings with a benthic life history (Cole & Hall, 2009).

7.2.2.3 NERVOUS SYSTEM

The cephalopod nervous system is the most developed and complex one in the molluscs. This complexity results partly because different parts together control movement of the cephalopod body. The parts to consider are the central brain organized in lobes, the peripheral nervous system arranged in localized ganglia, sensory receptors and organs that gather external information for the central and peripheral systems and neurotransmitters which excite various nerve endings (Boyle & Rodhouse, 2005a). This section will provide an overview of these major components, but their detailed mechanisms for achieving different modes of locomotion will be discussed in subsequent sections. A summary table of all components of the nervous system based on the octopus can be found in Table III of Boyle (1986) and detailed diagrams can be seen in Young (1988) and Budelmann (1995a).

7.2.2.3.1 Central Brain

The coleoid central brain is composed of masses of nerve cells that are organized into lobes. It consists of a cerebral, brachial, pedal, and paired optic lobes, and it is situated between the eyes and around the esophagus (Boyle, 1986; Hanlon & Messenger, 1996). Sets of lobes above (supraesophageal) and below (subesophageal) the esophagus are central to the brain and connected by the magnocellular lobes. All of these lobes control different

systemic functions that are discussed in greater detail in the following literature: Boyle (1986); Nixon and Young (2003); in cuttlefish (*Sepia*) Boycott (1961); in squid (*Loligo*) Young (1974, 1976, 1977, 1979); Messenger (1979); and in *Octopus* Young (1971). They are not limited to a single function as many connections between multiple lobes allow for coordination.

Several lobes in the sub- and supraesophageal mass are important in the control of locomotion. The subesophageal mass consists of a pair of magnocellular lobes between the cerebral and optic lobes dorsally and the pedal and pallioviseral lobes ventrally. The anterior lobe of the middle subesophageal area coordinates the arms. Different parts of the pedal lobe control eye movement, attack initiation, and funnel movement. The pallio-visceral and magnocellular lobes help control the mantle in its respiratory and locomotor functions, particularly the fast escape jet. All coleoid cephalopods except the incirrate octopods (those that do not have fins on their mantle) also have fin lobes connected to the pallioviseral and pedal lobes, which all together help coordinate fin movement. In octopus, the pedal and magnocellular lobes are also involved in motor control of the arms (Budelmann, 1995a; Young, 1988).

The supraesophageal mass consists of the basal lobes and the frontal/vertical lobe system that are involved in numerous interdependent functions. The basal lobes, that include higher motor centers like the peduncle lobe, receive sensory information and regulate output motor activity. This includes motor control of swimming, respiration, and muscles in the skin (Budelmann, 1995a). The peduncle lobe in *Octopus* is part of the highly interconnected visual-motor system which includes the eyes, optic lobe, and basal lobes (Messenger, 1967a, 1967b). Visual cues guide many octopus movement patterns and activities (Gutnick et al., 2011). The peduncle lobe receives the visual information from the optic lobe and uses it to regulate motor actions (Messenger, 1967b). The frontal and vertical lobes are particularly important for vision in squid and cuttlefish (Young, 1988). The frontal and buccal lobes are also concerned with chemotactile information from the arms of octopods (Young, 1983). Chemoreception also involves feedback between the olfactory and basal lobes (Messenger, 1971). The suckers and olfactory pit (an organ close to the eye) of coleoid cephalopods are lined with ciliated chemoreceptors. Octopus suckers have nearly 100 times more chemoreceptors than cuttlefish suckers, which is likely a result of the octopuses' benthic lifestyle, exploring the bottom and requiring extraction of chemical cues from the environment (Budelmann, 1995b). These senses

provide input to the nervous system that can result in motor activity (Budelmann, 1995a; Young, 1988). The central nervous system lobes and their functional organization are summarized in Table 1 of Wild et al. (2014).

7.2.2.3.2 Peripheral Nervous System

The brain and lobes of the central nervous system initiate motor patterns which are carried out locally by the peripheral nervous system (Grasso, 2014). This system is made up of masses of nerve cells called ganglia that provide local control to the organs they are associated with (Boyle & Rodhouse, 2005a). The main ganglia are gastric, cardiac, branchial, buccal, and sub-radial (see Fig. 7.3A). Of particular interest to movement are the stellate ganglia, which control mantle contractions in all coleoid cephalopods (Young, 1972). The stellate ganglia are divided into two parts, ventral and dorsal, which control muscles in the mantle important for respiration and locomotion (Young, 1972). In *Octopus*, the interbrachial commissure connecting the eight arms in a ring and the nerve cords (brachial ganglia and sucker ganglia) that extend in each arm (Grasso, 2014) are key to coordination of arm movements. The mechanisms by which these ganglia control movement will be discussed in the following sections on the individual muscular structures.

The giant fiber system of squid is important for fast commands to the mantle musculature. Motor control is provided to the stellate ganglion by the brain through connections with the pallial nerve. The axons of motor cells fuse and create 1-mm diameter fibers (Young, 1938, 1939). The fiber does not need a myelinated sheath because the large diameter of the giant fiber itself results in fast transmission of nerve impulses. The giant fiber system is important for initiation of the escape-jet in squid and cuttlefish. The giant fiber is not found in octopuses, however, conduction speed to different areas in the body depend on the network of fibers (diameter and number) in the peripheral nervous system. Neurons in the arms are controlled directly from the brain by few but large fibers, whereas there are many small connections to the localized brachial and sucker ganglia (Young, 1965) (see Fig. 7.3B).

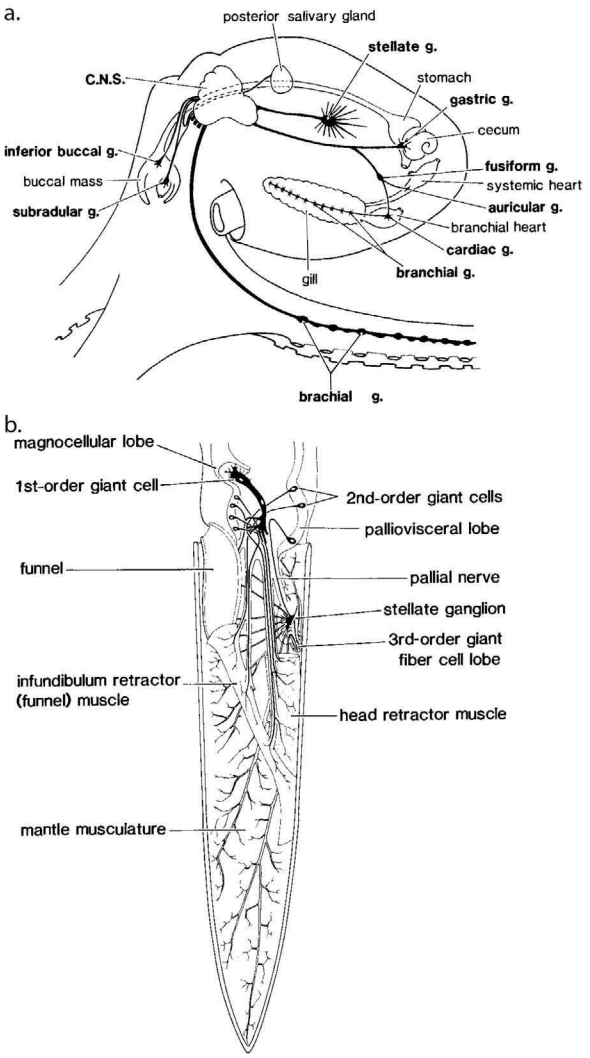

FIGURE 7.3 Illustrations of the central nervous system of two cephalopods. (A) The major ganglionic (g.) masses of the octopus *Eledone cirrhosa* in bold, and the rest of the central nervous system (CNS). Internal structures surrounding the ganglia are also listed in non-bold. (B) The giant nerve fiber system of the squid *Loligo pealei* including the three orders of giant fiber cells and various lobes, nerves, and muscles. (Reprinted from Boyle, P. R. Neural Control of Cephalopod Behavior. In *The Mollusca: Neurobiology and Behavior Part 2*; Wilbur, K. M., Willows, A. O. D., Eds.; Academic Press: London, UK, 1986; Vol 9, pp 1–99. Copyright © (2015), with permission from Elsevier.)

7.2.2.3.3 Sensory Receptors and Organs

A summary of cephalopod sensory systems is beyond the scope of this chapter. However extensive reviews of the different receptors and sense organs can be found in the following sources: Graziadei and Gagne (1976), Williamson (1991), Budelmann (1995b), Hanlon and Messenger (1996). Sensory systems that are of particular importance to locomotion, besides the eyes of these highly visual animals, include mechano-, chemo-, and proprioceptors (those that monitor muscle stretch) in the arms and suckers, especially in *Octopus* (Graziadei, 1962, 1965; Graziadei & Gagne, 1976). These provide tactile, chemical, and positional feedback to the nervous system. The octopus mantle (Boyle, 1976) and the squid and cuttlefish dorsal neck (Budelmann, 1995b; Preuss & Budelmann, 1995) also containss proprioceptors that provide feedback after mantle contractions in the former and control head-to-body movements in the latter. Found in all groups of coleoid cephalopods, paired statocysts serve as the equilibrium receptor system. The organ is composed of polarized hair cells that detect mechanical disturbance in surrounding fluid. These hair cells are divided into two different receptor systems, the macula/statolith/statoconia system which detects gravity and the crista/cupula system which detects angular acceleration (Budelmann, 1990, 1995b; Williamson & Budelmann, 1985; Young, 1984, 1989).

7.2.2.3.4 Neurotransmitters

Communication between neurons in the central and peripheral nervous systems is provided by chemicals known as neurotransmitters. Messenger (1996) and Tansey (1979) published the most comprehensive reviews of neurotransmitters in cephalopods. Initially, four neurotransmitters—acetylcholine, dopamine, noradrenaline, and 5-hydroxytryptamine (or serotonin)—were found in the cephalopod brain (Tansey, 1979). Other pharmacologically active chemicals were also located in different parts of the body of cephalopods (Boyle, 1986). For example, L-glutamate is in the mantle and fins of three squid and cuttlefish species—*Loligo*, *Alloteuthis*, and *Sepia* (Bone & Howarth, 1980; Boyle, 1986) and acetylcholine innervate the nerves in the octopus arm (Nesher et al., 2012). Interestingly, a recent study by Nesher et al. (2014) identified the role chemicals play in the skin in motor control, preventing octopus arms from attaching to one's self. Messenger (1996) added to this many more classes of neuroactive

substances, some of which are important to locomotor activity such as L-glutamate, the excitatory neurotransmitter for mantle contraction in the squid giant synapse. Since these reviews, little more has been found about the distribution and functions of these small molecules in the brain. Recently a protocol was published to detect the spatial distribution of different neuroactive substances in the cephalopod nervous system, the best studied so far being acetylcholine, dopamine, serotonin, GABA, and glutamate (Ponte & Fiorito, 2015).

7.2.3 EFFECTOR UNITS

Three key muscular hydrostats: the mantle–funnel complex, the fins, and the arms, including the web and suckers, are important for producing movement on a small scale as well as whole-body locomotion. All of these structures will be discussed in depth, including the various physiological processes involved in regulating each. Before that, it is important to understand how muscular hydrostats work, so a brief introduction to principles of hydrostatic skeletons will be given.

Hydrostats are generally composed of three main parts: a fluid, a cavity or container to hold the fluid, and muscles supported by connective tissue to change the shape of the container. Muscular hydrostats differ in that the fluid is replaced by dense muscle fibers oriented in different directions. The key principle that governs all hydrostats, whether based on muscles or on fluid, is that the volume of the container is virtually constant because muscles and fluid are fairly incompressible. When muscle fibers contract, they decrease the extension of the container in one direction. This results in increased pressure within the container and subsequently an increase in extension in another direction. This is the basis for movement in hydrostats (Kier, 1992, 2012; Kier & Smith, 1985).

In a basic muscular hydrostat that assumes a cylindrical shape, there are three muscle fiber orientations to the long axis of the body: perpendicular (arranged in a transverse, radial, or circular pattern), parallel (longitudinal bundles), and helical or oblique. Contraction in these different fiber orientations results in four types of movement. Elongation is achieved by decreasing the cross-sectional area and increasing the length of the body. This is done by contracting transverse, circular, or radial fibers, ones which are oriented perpendicular to the long axis. This activity is used for protrusion, such as in the quick elongation of squid tentacles to capture prey (Kier, 1985). Shortening is done by decreasing the length of the body with a resultant increase

in the cross-sectional area. This is done by contracting longitudinal muscle fibers. This shows the antagonistic effects of longitudinal fibers to those perpendicularly oriented to them. The last two movement patterns are due to localized contractions that require resistance in remaining parts of the body. Bending is due to contraction of longitudinal muscles on one side of the body while the animal still maintains a constant diameter by contracting perpendicular muscles. With this mechanism, the body can vary in how much and in what direction it can bend. Lastly, torsion leads to twisting of the body itself along its long axis. This is accomplished by contraction of the helical or oblique muscles. Helical muscles are also involved in shortening and elongation, depending on the angle of arrangement of the fibers with respect to the long axis (Kier & Smith, 1985). Longitudinal and oblique muscle fibers are present in all cephalopod groups, transverse fibers are only found in octopus and squid arms and squid tentacles, and circular muscles are also found in squid tentacles (Kier, 2012). (See Table 1 in Kier, 2012 for more detailed representation of the diversity of muscular hydrostats.)

The following sections will discuss how these different muscle fiber orientations and principles of hydrostats are organized into different muscular structures. For each structure, arrangements of the muscles, connective tissues, and nervous system will be reviewed with respect to the modes of locomotion the structure performs. Table 7.1 summarizes the muscular structures that are most commonly used for locomotion for the major groups of cephalopods.

TABLE 7.1 Locomotor Effector Units used in Cephalopods.

Group	Octopods		Squid	Cuttlefish	Vampyromorpha
	Cirrate	Incirrate			
Arms	xx	xx	x	x	x
Fins	x	–	xx	xx	xx
Tentacles	–	–	x	x	x
Web	xx	x	–	–	xx
Mantle–funnel	x	xx	xx	xx	x

x = occasional xx = common.

7.2.3.1 MANTLE–FUNNEL COMPLEX

The mantle–funnel complex of coleoids is essential for driving many physiological and locomotor activities. It is responsible for respiration

and swimming by jet propulsion in all cephalopods, as well as digging or burying in bottom-dwelling cuttlefish (Boletzky, 1996; Mather, 1986). The structure of squid mantles have been studied the most, but variation between the different coleoid groups is minimal.

7.2.3.1.1 Muscle and Connective Tissue

In squid (*Loligo pealei* and *Lolliguncula brevis*), and cuttlefish, the mantle is divided into three layers, a central one of closely packed muscle sandwiched between two layers of connective tissue called the inner and outer tunics (see Fig. 7.2). Muscle fibers in the central layer are oriented circularly around the body as well as radially attaching between the inner and outer tunics. These two types are obliquely striated and arranged in alternating rings along the body. Connective tissues are present not only in the tunics but in the muscle layer also (intramuscular connective fibers). Three intramuscular connective tissue fibers are identified in cuttlefish and squid mantles (Ward & Wainwright, 1972). These are oriented in different directions, with one in the inner and outer tunics that is straight or curved, another that is also in the tunics localized to radial muscle bands, and a third that is parallel to the circular muscle fibers not attached to the tunics (Kier & Thompson, 2003). Collagenous fibers that are tightly cross-linked make up the bulk of the outer tunic (Ward & Wainwright, 1972). The squid mantle also contains a chitinous skeletal structure called the pen. The pen is rigid and resists lateral bending and lengthening of the mantle. The connective tissues, and to some degree the pen, help maintain the squid's shape. The tunics limit length changes and store elastic energy while providing increases in the width or circumference of the body (Ward & Wainwright, 1972).

In the incirrate octopuses (e.g., *Octopus vulgaris* and *E. cirrhosa*), there are two layers of circular muscles, divided by a layer of connective tissue, stellar nerves, and blood vessels. Unlike squid, octopods have two thin layers of continuous longitudinal muscle that bound tightly packed radial and circular muscles (Boyle, 1986; Boyle & Rodhouse, 2005a). A similar organization of mantle musculature is seen in the cirrate octopus species *Cirrothauma murrayi* (Aldred et al., 1983). Some incirrate species (e.g., *Octopus bimaculatus*) do not have the defined inner and outer tunic layers of connective tissue seen in squid. They have collagen fibers arranged in different orientations in a fibrous array surrounding the mantle. They also have connective tissue fibers in the longitudinal muscle layers, oriented

parallel to the body axis (Kier & Thompson, 2003). Octopuses have far less mantle musculature than decapods.

A different arrangement in mantle musculature is present in gelatinous and deep water species of squids and octopuses. In these cephalopods, the gelatinous layer is surrounded by two thin layers of circular muscle fibers and a set of radial muscles (Kier & Thompson, 2003). Contraction of radial muscles thins the wall and subsequently expands the mantle (Clarke, 1988). Sheets of connective tissue reinforce the gelatinous layer. Some pelagic squids (Octopoteuthidae, Cycloteuthidae, and Lepidoteuthidae) have one layer of longitudinal muscle fibers, two layers of circular muscle fibers, and radial muscle fibers. These squid do not have well-defined inner and outer tunics of connective tissue. Some of the deep sea squids (Mastigoteuthidae, Chiroteuthidae, Histioteuthidae, and Batoteuthidae) have a similar muscular organization, except they lack longitudinal muscle fibers. These squid have well defined layers of connective tissue (Kier & Thompson, 2003). The reduction of dense musculature exhibited in the mantle of gelatinous species results in the mantle acting like a closed, fluid-filled hydrostatic skeleton rather than a muscular hydrostatic skeleton. The muscle fibers that surround the gelatinous fluid contract and create movement, similar to the hydrostatic skeleton of the gastropod foot. The arrangement of connective tissues in these species also results in the mantle storing less elastic energy than in the loliginid squid.

The funnel is an important component of jet propulsion, as it directs water flow from the mantle cavity. The funnel is highly muscularized with the ability to bend in any direction and to vary the opening of the tip to regulate jetting speed and acceleration. Its muscle fibers are obliquely striated. In squid, the muscle fibers are oriented in in three directions: longitudinal ones on the outer surface, circular ones in the majority of the funnel, and thin radial fibers that run across and attach to the inner and outer funnel walls. This is all enclosed by thin inner and outer connective tissue fiber layers (Kier & Thompson, 2003).

7.2.3.1.2 Nervous Control of Mantle–Funnel

Nervous control of mantle musculature involves the pallioviseral lobe of the brain, which sends messages through the pallial nerve to a pair of stellate ganglia, one on each side of the body on the inner mantle surface, branching into stellar nerves in the muscle (Young, 1972; Boyle, 1986). In squid, the tightly packed circular muscle layer contains a plane of branching stellar

nerves that runs horizontal to this muscle layer (Ward & Wainwright, 1972). In octopus, the two layers of circular muscles are divided by branches of the stellar nerve and multipolar nerve cells. The multipolar nerve cells are thought to be receptors in the proprioceptive feedback system of the mantle mediating its contractions (Boyle, 1976).

In squid (*L. pealei*), the giant fiber system is important for control of escape jetting. This is done by an all-or-nothing contraction system, whereby the muscles, particularly the circular fibers, of the mantle and those holding the funnel and head contract at the same time to obtain maximum velocity of water expulsion (Young, 1938). The giant fiber system consists of three orders of giant fibers, increasing in diameter from the brain to the mantle muscles. The first order fibers come from the magnocellular lobe of the brain, the second order fibers innervate the funnel, head retractor muscles, and stellate ganglia, and the third order fibers, which are also the longest and exhibit the largest diameters, connect to mantle musculature (Boyle & Rodhouse, 2005a; Wells, 1988; Young, 1938). The arrangement of the giant fiber system is an important innovation that allows squid to produce a very quick reaction from a simple trigger, based on the gradation of fibers that increase the muscles response amplitude and synchronizes contraction of mantle musculature (Boyle, 1986; Budelmann, 1995b). Cephalopods also have a small fiber system that regulates respiration and slower movements (Young, 1938; Wilson, 1960). Graded contractions of the circular muscles in the mantle are produced by stimulation of smaller fibers in the stellar nerve of *L. pealei* (Young, 1938), *Octopus* (Wilson, 1960), and *Sepia* (Packard & Trueman, 1974) presumed for respiration.

7.2.3.1.3 Biomechanics of Mantle–Funnel

During both jet propulsion and respiration, water is drawn into the mantle cavity through a pair of inhalant openings and expelled out of a single exhalant funnel. To produce thrust, a large amount of water passes through the funnel at high speeds due to whole mantle contraction initiated in the posterior. This is different from respiration, where contractions are confined to the anterior half of the mantle so a small amount of water is passed along the gills at slow speeds to maximize oxygen extraction (Packard & Trueman, 1974; Wells, 1988). One way squid deal with these dual incompatible actions of the mantle is through differentiation of their musculature. Their mantle is composed of anaerobic and aerobic muscle types. The anaerobic type, used for fast locomotion, is made of protein filaments with few mitochondria. The

aerobic muscle fiber type, used for respiration and steady swimming, is made up of fibers that have many mitochondria and a large supply of oxygenated blood (Bone et al., 1981; Wells, 1988). Also as a consequence of the different functions of the mantle, different groups of coleoids specialize in one of the functions. Squid rely on jet-propelled locomotion and thus their mantle has a more elongated streamlined form and their cavity accommodates larger volumes for greater range in oxygen extraction (Wells, 1990). Octopuses and the Sepioidea use other modes of locomotion more frequently than jet propulsion, and thus their mantle is smaller and more round, to specialize in respiration (Wells & O'Dor, 1991).

In squid (*Loligo opalescens*), three phases of muscular contraction occur during the escape jet (see Fig. 7.4). First, the radial muscles contract, thinning the mantle wall and resulting in hyperinflation of the cavity. Second, the circular muscles contract, decreasing the mantle diameter and increasing the wall thickness to power the jet. Third, the mantle refills and expands,

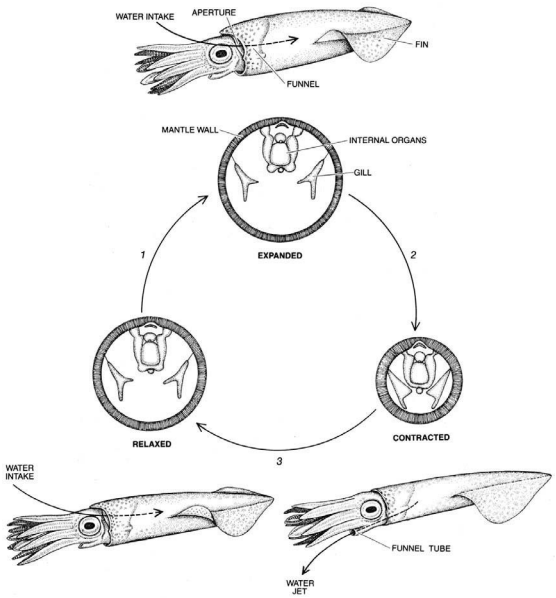

FIGURE 7.4 Escape-jet cycle of the squid *Loligo opalescens*. The cycle starts with hyperinflation of the mantle (1) where the diameter increases as water flows into the mantle cavity. After maximal expansion, the mantle muscles contract (2), pressure inside the mantle cavity increases and water is forced out the funnel, creating a jet. The mantle then refills to its relaxed state (3). (From Gosline, J. M.; DeMont, M. E. Jet-Propelled Swimming in Squids. *Scientific American.* 1985, *256*, pp. 96–103. With permission from illustrator Patricia J. Wynne.)

assisted by the stored elastic energy of the collagen fibers and slight contraction of the radial muscles (Gosline et al., 1983; Shadwick, 1994) (see Fig. 7.4). Two modes of respiration were seen in *L. opalescens*. The first depends on contractions of radial muscles antagonized by the elasticity of connective tissues. The second depends on contractions of the circular muscles, also antagonized by elasticity of the connective tissues. A procedure by which both muscle fibers contract and antagonize each other is possible, but is not observed frequently (Gosline et al., 1983).

Ventilation events in octopus and cuttlefish are similar to muscle actions produced during jet propulsion in squid. Detailed descriptions of water circulation patterns inside the mantle cavity are provided by Wells and Wells (1982) for *Sepia* and *Octopus*. During inhalation in octopuses (e.g., *O. vulgaris*), water is drawn into the mantle cavity as the dorsolateral mantle edge begins to thin out, the funnel tip folds up to close off water, and prebranchial spaces open up. Radial muscles in the rest of the mantle contract to expand the cavity briefly and draw in water which is then passed over the gills into the postbranchial cavity. During exhalation, water is expelled for a longer duration and out the funnel as a result of contraction of circular muscles in the lateral anterior mantle edge and closing of lateral flaps of the funnel over mantle edges (Wells & Smith, 1985; Wells & Wells, 1982). During inhalation in the cuttlefish, *Sepia officinalis*, the locking collar opens and the radial muscles contract to expand the mantle cavity and draw in water. Contraction of the collar flap muscles closes the mantle system and channels water out the funnel during exhalation. Elastic energy stored in the connective tissue network reestablishes the radial fibers to their initial state. Contraction of circular muscle fibers in high-pressure mantles is another mechanism used by some cephalopods during exhalation (Bone et al., 1994).

7.2.3.2 FINS

Cuttlefish and squid use their fins for various forms of swimming and hovering, as well as maneuvering their body in all directions (Bidder & Boycott, 1956; Hoar et al., 1994; Kier & Thompson, 2003; O'Dor & Webber, 1986; Russell & Steven, 1930). The fins of cirrate octopus and *Vampyroteuthis* are also important effectors for swimming movement, as these deep sea species do not rely only on rapid jet propulsion for locomotion (Collins & Villaneuva, 2006).

7.2.3.2.1 Muscles and Connective Tissue

Cuttlefish (*S. officinalis*) and some squid (*Loligo forbesi* and *Sepioteuthis sepiodea*) have similar fin musculature arrangements (Kier & Thompson, 2003). The lateral fins of *S. officinalis* and *S. sepiodea* extend the entire length of the mantle, tapering away from the base where the fin is thickest. The fin of *L. forbesi* is similar, except that it does not extend the entire length of the mantle and is triangular in shape (Kier, 1989). The fins are composed of a dorsal and ventral portion of musculature separated by a median connective tissue layer (a fascia). Two other layers of connective tissue, the dorsal and ventral fascias, which are made up of a meshwork of obliquely oriented fibers, are present. Additional connective tissues in the fin include the flattened cartilage at the base of the fin and crossed connective tissue fibers embedded in the musculature. In both the dorsal and ventral portions of the fin, obliquely striated muscle fibers are oriented in three mutually perpendicular directions: transverse, dorsoventral, and longitudinal. Transverse muscle fibers orient parallel to the fin axis, running laterally from the base to the fin margin. Sheets of dorsoventral muscle fibers separate bundles of transverse muscle. These sheets originate at the median fascia and extend either dorsally or ventrally on each side of the median. In the dorsal portion of the fin, dorsoventral muscle fibers connect to the dorsal fascia and vice versa for the ventral portion of the fin. Layers of longitudinal muscles are oriented parallel and adjacent to the median fascia on both the ventral and dorsal sides. Only transverse muscle fibers in both the dorsal and ventral surfaces have a zone with larger mitochondrial cores. These zones are close to the dorsal and ventral fascia. These muscle fibers are similar to the aerobic muscle present in the mantle and suggest that muscle fibers in these zones have higher aerobic capacity than those in the rest of the fin (see Fig. 1 in Kier, 1989 and Fig. 4 in Kier & Thompson, 2003).

Swimming by fin movement is one of the key modes of locomotion among cirrate octopods and *Vampyroteuthis*. Similar to the lateral fins of squid, the fins of cirrate octopods like *Cirrothauma* are tapered from a dense muscular base supporting the fin toward a thinner leading edge that is more gelatinous and transparent. Transparent dorsal cartilage also provides support for the powerful movements of these fins, forming attachment points for fin musculature. A large fin lobe in the brain provides control of the fins (Aldred et al., 1983). Fin shape and size varies between the different groups of cirrate octopods (see Fig. 12.10 in Boyle and Rodhouse (2005b). *Cirroctopus*, *Cirroteuthis*, *Cirrothauma* have large fins; *Grimpoteuthis*, *Luteuthis*, *Stauroteuthis*

have moderately sized fins; and *Cryptoteuthis* and *Opisthoteuthis* have small fins (Collins & Villaneuva, 2006). The cirrate octopod fin is separated into proximal and distal regions, both covered by a thin sheet of muscle. The core of the proximal region is occupied by flattened fin cartilage which provides skeletal support and attachment for muscle fibers. The cartilaginous core is surrounded by bundles of muscle fibers running parallel to the fin. The distal region has dorsal and ventral layers of transversely oriented muscle fibers that are similar to the squid and cuttlefish fins (Vecchione & Young, 1997).

Vampyroteuthis also uses swimming by its broad fins for locomotion. The fins attach to a broad gladius (similar to fin cartilage but chitinous in structure) by a well-developed anterior muscular band (Seibel et al., 1998). Like that of cirrate octopods, the proximal half of the fin contains a cartilaginous core. Early in development, *Vampyroteuthis* develops two pairs of fins. The first juvenile pair becomes resorbed and the second adult fin pair enlarges at the anterior region (Young & Vecchione, 1996).

7.2.3.2.2 Nervous Control of Fins

Little is known about the nervous control of fin movement in cephalopods. Electromyographic recordings indicate that gentle fin movements are produced by oxidative muscle fibers and supported by the crossed oblique connective tissues. Short bursts of vigorous fin movements are produced by the anaerobic muscle fibers which make up the bulk of the fin musculature (Kier et al., 1989). Fin nerves with both afferent and efferent connections are located along the plane of the median fascia (Kier et al., 1989). Mechanoreceptors are also likely found in the fin, which provide position information that help control coordination of the fins (Kier & Thompson, 2003).

7.2.3.2.3 Biomechanics of Fins

Swimming and hovering in cuttlefish and squid is a result of undulatory waves of the fin. Undulatory waves are a result of sequential bending of the dorsal and ventral portions of the fin. Contraction of transverse muscle bundles on one side of the fin results in lateral compression toward that side (either dorsal or ventral). However, bending will only occur if it is resisted on the opposite side of the fin. This can be done by contraction of longitudinal muscles supported by fin cartilage to resist length change, contraction of dorsoventral muscles to resist increased thickness, or stored elastic support

by intramuscular connective tissues. The mechanism employed depends on the type of movement produced. During hovering and gentle swimming, low-amplitude undulatory waves are produced by contraction of the layer of aerobic transverse muscle bundles and resisted by intramuscular connective tissue. During short bursts of vigorous movements, high-amplitude undulatory waves are produced by contraction of anaerobic transverse muscle bundles and resisted by contraction of dorsoventral muscles and sometimes longitudinal muscles on the opposite side of the fin. These vigorous movements are produced during prey capture, fin beating in agonistic encounters, and maneuvering (Johnsen & Kier, 1993; Kier, 1989; Kier & Thompson, 2003).

During fin swimming, the fins of cirrate octopods move symmetrically to produce backward motion of the body. Their fins move through a cycle of upstrokes and downstrokes, starting with the posterior margins of the fins pointed dorsally. The fins are then pushed ventrally during the downstroke and dorsally during the upstroke. Frequency of the cycle can vary from 4 to 30 strokes per minute (Collins & Villaneuva, 2006).

7.2.3.3 ARMS AND TENTACLES

The arms of octopods are able to do a variety of tasks involving a wide range of movements. Arms are particularly important to incirrate octopuses, which rely on crawling more than jet propulsion to move in their environment. Some species of benthic octopus also use arms for digging into the substrate. In squid and cuttlefish, the arms are used in postures as displays, swimming, and steering. The pair of tentacles in squid is specialized for quick elongation during prey capture, for example *L. pealei* extends its tentacles by 80% within 20–40 ms (Kier & Leeuwen, 1997). Muscular structures associated with these limbs, such as the suckers, their stalks and the interbrachial web between the arms, will also be discussed.

7.2.3.3.1 Muscles and Connective Tissue

All coleoid cephalopod arms consist of dense musculature that surrounds a central axial nerve cord and artery. The musculature is made up of obliquely striated fibers which are oriented in three directions, transverse muscles running perpendicular to the long axis of the arm, bundles of longitudinal

muscles running parallel to the long axis, and oblique muscles oriented in helices around the arm. Crossed-fiber connective tissue layers surround both the oral (side facing the mouth, with suckers) and aboral (outward facing, no suckers) sides of the arm and are the insertion points of the oblique muscle fibers. The fiber angle of the connective tissues and the oblique muscles are the same relative to the arm's long axis (Kier & Thompson, 2003). An additional sheet of connective tissue surrounds the octopus axial nerve cord. In octopuses (*O. bimaculoides*, *O. briareus*, and *O. digueti*) arms, the aboral side also has a layer of thin circular muscles wrapping the arm. An additional internal oblique muscle layer is found between transverse and longitudinal muscles of the octopus arms and this is all surrounded by an outer layer of circumferential muscles (Kier & Stella, 2007).

Squid (*L. pealei*) tentacle musculature is similar to that of the arm, with a few differences. One key difference is the cross rather than obliquely striated fiber composition of the transverse and circular muscles of the tentacles that give them their fast contractile properties (Kier, 1985; Kier & Curtin, 2002). Differentiation of the transverse muscle from obliquely to cross-striated fibers has been shown during development of the squid *Sepioteuthis lessoniana* (Kier, 1996). The transverse muscles of squid tentacles are continuous with a layer of circular muscles. Transverse muscle fibers turn and either become part of the circular muscles or insert at the connective tissue layer that encircles the circular muscle layer. Two layers of oblique muscles, oriented in opposing directions, surround the circular muscle layer, which in turn is surrounded by a layer of longitudinal muscle fibers (Kier, 1985; Kier & Thompson, 2003). Despite the presence of an axial nerve cord, presumably supplying efferent commands to the tentacle, little is known about the nervous system dynamics that control its extension (Kier & Leeuwen, 1997).

7.2.3.3.2 Nervous Control of Octopus Arms

The control of octopus arms and their associated structures (web and suckers discussed in the following section) is complex and its investigation is ongoing. It is complicated by the dual centralized and localized control of octopus motor action (Grasso, 2014). Centralized control is executed by their large bilaterally symmetrical brain, which Grasso (2014) refers to as the cerebral ganglia, made up of 35 lobes (Grasso & Basil, 2009; Young, 1971). Results from microstimulation experiments

suggest that arm movements are represented by overlapping pathways in higher motor centers of the lobes and that different movement components are not somatotopically represented, as the same movement patterns could be elicited by stimulation of different lobes (Zullo et al., 2009). The large brachial lobes (Maddock & Young, 1987), along with several others in the subesophageal mass (Budelmann & Young, 1985), send information from the brain down the brachial nerves to a series of brachial ganglia in each of the arms.

Localized control of the arms is at the brachial ganglia, where 3.5×10^8 of the total 5×10^8 neurons in the entire nervous system are located (Young, 1971). A series of brachial ganglia extends down each arm, making up the axial nerve cord. The eight nerve cords are connected at the base of the arms by the ring of the interbrachial commissure. This system is referred to as the brachial plexus. Four smaller intramuscular nerve cords run down the periphery of each arm and link to the brachial ganglia. Muscle fibers are innervated by motor neurons in the brachial ganglia and through coordination with the intramuscular nerves, which produces the diversity of movement patterns seen in the arm (Graziadei, 1971; Rowell, 1966). Each individual sucker is also associated with a sucker ganglion that is located at its base, just outside of the muscle. These sucker ganglia are also connected to corresponding brachial ganglia in the arm. Each sucker contains chemo- and mechanosensory receptors on the rim that provide sensory feedback to the sucker ganglion (Graziadei, 1971). Thus an arm can be divided into individual units of neural control called "local brachial modules" which consist of paired brachial and sucker ganglia (see Fig. 7.5). Efforts to understand the coordination of localized sets of suckers have been made (Grasso, 2008) but more information is yet to be learned about how these local brachial modules are coordinated in whole arm movements. The distributed neural control we see in octopus arms is one reason why they have a great deal of flexibility and is a good example of self-organization, which allows for greater adaptability of movement to different environmental and physiological situations (Hochner, 2013).

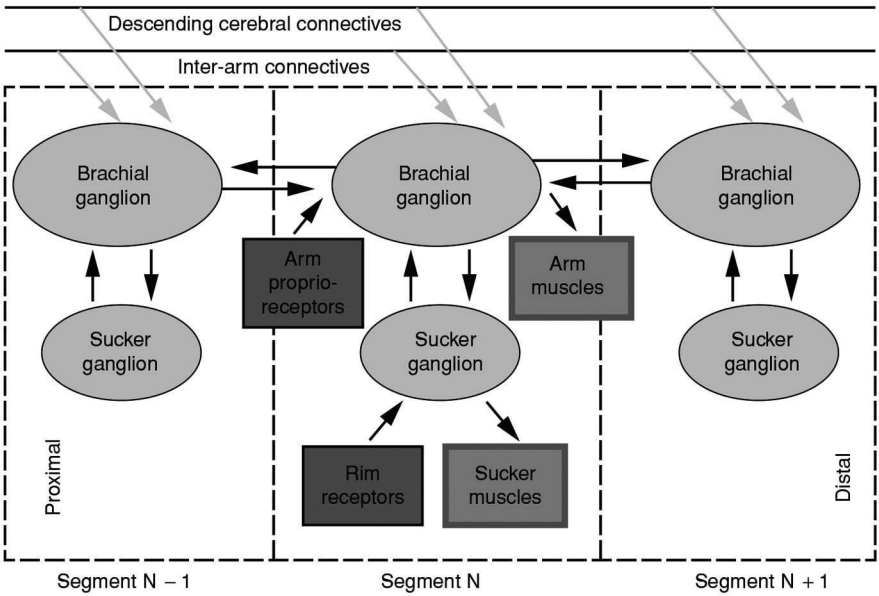

FIGURE 7.5 Organization of the peripheral system in the octopus arm. Repeating units of neural and muscular structures centered around each sucker run down the length of the arm. Segment N in the center illustrates a complete unit including sensory inputs (arm proprioceptors and rim receptors) to the ganglia (brachial and sucker). The brachial ganglion also receives inputs from descending cerebral connectives, connectives from other arms, neighboring brachial ganglia and the sucker ganglion in its segment. The brachial and sucker ganglia process motor commands to arm and sucker muscles in its segment. (From Grasso, F. W. The Octopus with Two Brains: How Are Distributed and Central Representations Integrated in the Octopus Central Nervous System? In *Cephalopod Cognition*; Darmaillacq, A.-S.; Dickel, L., Mather, J., Eds.; Cambridge University Press: Cambridge, UK, 2014; pp 94–124. With permission from Cambridge University Press.)

7.2.3.3.3 Biomechanics of Arms and Tentacles

The arrangement of muscle fibers in these limbs yields a variety of movement patterns based on the principles of muscular hydrostats (Kier, 1992). Elongation of the arm and tentacles is a result of transverse muscle contraction while shortening is a result of longitudinal muscle contraction. Extension of the tentacles occurred in 20–40 ms with maximum extension velocities of the tentacle stalk over 2 m/s (Kier & Leeuwen, 1997). Torsion or twisting of the arm is a result of contraction of the oblique muscle layers. Bending in the arm of squid and octopuses involve antagonistic actions of the longitudinal and transverse muscle fibers. Contraction of the longitudinal

muscle bundles on one side of the arm results in shortening. This is resisted by the simultaneous contraction of transverse muscle fibers, resulting in bending toward the side of longitudinal muscle contractions. Concurrent contractions of the transverse and longitudinal muscle increases the flexural stiffness (resistance while undergoing bending) of octopus arms and simultaneous contraction of oblique muscle layers oriented in both right- and left-handed directions results in increased torsional stiffness (Kier & Smith, 2002; Kier & Stella, 2007; Kier & Thompson, 2003) (see Figs. 5–7 in Kier & Thompson, 2003).

7.2.3.4 SUCKERS AND WEB

The arms of coleoid cephalopods contain suckers that are used during locomotion, particularly during crawling in octopuses. Most investigations of morphology have been on octopus suckers, though there are some noticeable differences from squid and cuttlefish ones. Like the arm, the sucker is composed of a dense array of muscle fibers oriented in three directions in the sucker wall: radial muscles that cross the wall, circular muscles that are arranged circumferentially around the wall, and meridional muscles that run perpendicular to the radial and circular muscles. An inner and outer layer of fibrous connective tissue surrounds the sucker and crossed connective tissue fibers are embedded in the muscle (Kier & Smith, 1990, 2002) (see Fig. 3 in Kier & Smith, 1990). The suckers of squid and cuttlefish differ from those of octopuses in that they have a muscular stalk which connects the sucker cup to the arm or tentacle. The stalk is composed of longitudinal and transverse muscle fibers along with connective tissue that result in elongation, shortening, and bending to manipulate placement of the sucker cup. These actions are carried out by similar muscle contraction patterns to those used for arms. Additionally, squid cups are lined with chitin and tooth-like projections. The roof of the sucker also contains muscles arranged like pistons so that when tension is applied to the stalk, it pulls on these muscles, creating more effective attachment to a substrate (Kier & Thompson, 2003).

Suckers attach to a substrate by decreasing pressure within the sucker cavity. When radial muscles contract in a sucker that is sealed to the substrate, the walls thin and the cohesiveness of water resists volume expansion of the sucker. This results in decreased pressure inside the sucker cavity. The circular and meridional muscles antagonize the radial muscles, resulting in a smaller sucker circumference and thicker walls. Elastic energy stored in the connective tissue fibers helps to maintain sucker attachment

for a prolonged period (Kier & Smith, 1990, 2002). Pressure differentials of octopus, squid, and cuttlefish suckers are similar at sea level, but at greater depths, squid and cuttlefish exhibit greater sucker strength than octopods (Smith, 1996).

Another component of the arms is the interbrachial membrane or web, which is a muscular fold of skin that extends between each of the arms. This is seen in *Vampyroteuthis* and all species of octopods but is most pronounced among the cirrate octopods. The arrangement of fiber trajectories in the web has been described by Guerin (1908), but since then little is known about the neural mechanisms or pathways that control this muscular structure. Activation of localized muscle fibers in the web can result in localized arm movements and rotations of the base aborally, orally, and laterally (Kier & Stella, 2007). The web can also contract at the base of the arm and produce swimming movements in the cirrate octopods by ejecting interbrachial water but the exact neural mechanism is unknown (Roper and Brundage, 1972).

7.2.4 TYPES OF MOTION

The diversity in physiological control of musculature in coleoid cephalopods underlies the various modes of locomotion that the different groups use. They produce three basic modes of movement: swimming, crawling, and burying. However, several variants of each occur between and among the coleoid cephalopod groups, depending on what muscular structure(s) are the main effectors of motion. Swimming can involve the mantle–funnel complex alone or in combination with fin undulations to produce rapid jet propulsion. It can also involve only the fins or, in the octopods and *Vampyroteuthis*, only the web. Crawling involves the use of one or more limbs and, in the case of coleoids, is almost exclusive to arms in octopods. Benthic cuttlefish sometimes use their arms to walk or crawl (see *Metasepia* in Roper & Hochberg, 1988). Benthic coleoids that hide in their surrounding substrate will bury or dig with a combination of all muscular structures, from the arms in octopus (Boletzky, 1996; Guerra et al., 2006) to arms and mantle–funnel exhalations in cuttlefish (Mather, 1986; Anderson et al., 2004). All types of locomotion are affected by the same principles and depend on what muscular structure and environmental factors are involved. Discussion of these movements will be made according to types—jet propulsion of the mantle or the web and limb-based movement of the arms and fins.

7.2.4.1 JET PROPULSION

Jet propulsion is movement that is achieved by the expulsion of water in one direction, creating an equal and opposite reaction that propels the animal in the opposite direction. The greater the volume of water expelled, the greater the distance that is traveled. A deformable body with elastic capabilities for expansion is required to be able to expel large amounts of fluid to power this mode of locomotion (Trueman, 1980, 1983). Cephalopods are prime examples of how jet-propelled swimming has allowed them to adapt to their environment and compete with fish in the same habitat (Packard, 1972). All cephalopods are able to swim by jet propulsion using their mantle–funnel complex, though squid are the best studied group. Jets from the mantle–funnel complex can also be used by some groups in burying. In addition, cirrate octopods can use another structure, their extensive web, to expel a large volume of water between the arms and propel them posterior first.

7.2.4.1.1 Mantle–Funnel

Jet propulsion by the mantle–funnel complex is well-studied in coleoid cephalopods. The mantle contracts to expel a large amount of water as well as expands and takes in water through the funnel to build high hydrostatic pressure. Studies have investigated cephalopod, predominantly squid, jet propulsion from various perspectives: swimming energetics (Bartol, Patterson, & Mann, 2001; O'Dor, 1982, 2002; O'Dor & Webber, 1986, 1991; O'Dor et al., 1994; Wells & O'Dor, 1991), hydrodynamics and mechanics (Anderson & DeMont, 2000; Anderson & Grosenbaugh, 2005; Anderson et al., 2001; Bartol, Patterson, & Mann, 2001; Bartol et al., 2009; O'Dor, 1988), respiratory and swimming dynamics (Aitken & O'Dor, 2004; Bartol, Mann, & Patterson, 2001; Payne et al., 2011), kinematics (O'Dor, 1982; Thompson & Kier, 2001, 2002), metabolics (Baldwin, 1982; Finke et al., 1996; O'Dor & Webber, 1986; O'Dor et al., 1994; Pörtner et al., 1993, 1996; Rosa & Seibel, 2010a; Trueblood & Seibel, 2014), swimming dynamics and efficiency throughout development (Bartol et al., 2008, 2009; O'Dor & Hoar, 2000; Thompson & Kier, 2001, 2002; York & Bartol, 2016), and physiological and environmental constraints (O'Dor & Webber, 1986, 1991; O'Dor et al., 2002; Pörtner & Zielinski, 1998). A recent review of jet propulsion and squid swimming and flying highlight current advancements especially for improving technologies in swim tunnel respirometry in the lab

and three-dimensional accelerometer chips and radio-acoustic positioning telemetry for tagging and tracking squid in the field.

Compared to other forms of locomotion, jetting is an inefficient way of moving (Alexander, 2003). The energetic costs of jet propulsion in squid are greater than the costs of swimming in fish, based on pressure sensor and acoustic telemetery studies of *L. forbesi* (O'Dor et al., 1994). Fishes undulate their body to push large volumes of water at low speeds for thrust. Cephalopods must accelerate water at high speeds through their funnel to produce the same thrust, but are limited in water volume by the capacity of their mantle cavity (O'Dor, 1988; O'Dor & Webber, 1986, 1991; Webber et al., 2000; Wells & O'Dor, 1991). This response (the rapid escape jet) is best used as an antipredatory response, but is energetically wasteful if used for normal swimming. Normal swimming in *Loligo* occurs with deeper respiratory pulses, where a large volume of water is expelled at low-amplitude jet pulses. During normal swimming, *Loligo vulgaris* generates one-tenth of the maximum pulse pressure in its mantle cavity (Trueman, 1980, 1983). Squid have elastic components (i.e., collagen fibers) in their muscular mantle–funnel propulsion system to help refill the mantle cavity (Gosline & DeMont, 1985). The flexibility of the cephalopod funnel provides maneuverability in controlling movement in any direction and speed (Anderson & DeMont, 2000; Bartol, Man, & Patterson, 2001; O'Dor, 1988). The fins also help compensate for inefficiencies by maintaining control and orientation during locomotion (O'Dor & Webber, 1986).

Jetting speed is influenced by how much musculature is present in the mantle and the diameter of the funnel, as the velocity of the jet is inversely proportional to the funnel's cross-sectional area. Faster and more forceful jetters, such as Loligonidae and Ommastrephidae squid, have muscular mantles that contract to force water out of a small funnel aperture (O'Dor & Webber, 1986). In contrast, the deep-sea squid *Taonis* and the pelagic octopod *Japatella* have wider funnels and slower jet propulsions. *Japatella* produces two additional jets as a result of passing water through the sides of the head via inhalant openings (Clarke, 1988). Larger mantle capacities and powerful mantle muscles are also found in octopods like *Eledone* which rely more on jet propulsion than benthic octopods that have smaller mantle capacities, less developed musculature, which result in short, low-amplitude jet pulses (Trueman & Packard, 1968). Several other factors influence the efficiency of jetting, including the mass of the animal, the mass of water in the cavity, how fast water is expelled, the magnitude of drag force from water motion, and the size of the funnel aperture. Recently, Staaf et al. (2014) generated a theoretical model of the effects of funnel aperture on jet propulsion of squid,

covering its range of sizes. Generally, squid of all sizes decrease funnel size during mantle contraction to increase efficiency; however, ecological pressures and the effects of size may lead to fine-tuned changes in contraction speed and funnel diameter.

Different gaits or swimming variants have been identified thought not well investigated, predominantly from swim tunnel studies, for various species of squid. Squid can use a fast escape jet, with hyperinflation of the mantle cavity, or a slow swim, with much less expansion and contraction, and any speed in between (Gosline & DeMont, 1985; O'Dor & Webber, 1986). A combination of slow funnel water pulses with variable use of the fins provides other variants of swimming movement (Hoar et al., 1994; Anderson & Grosenbaugh, 2005). Multiple gait patterns at different speeds, from posterior-first swimming in *L. opalescens* (O'Dor, 1988) and *L. brevis* (Bartol, Patterson, & Mann, 2001) have been identified as depending on variable contractions of several interacting muscle structures. Cruising locomotion, like gliding, soaring, blimping, and climb-and-glide, using jet propulsion and fins have also been observed to take advantage of environmental factors, like upwelling and currents, to reduce energy costs associated with transport and maintaining horizontal position (Gilly et al., 2012; O'Dor, 1988, 2002; O'Dor & Webber, 1986).

Jet propulsion is not only important for swimming, but is also used as the main effector for burying in bottom-living coleoid cephalopods. This action has been referred to as burying, digging, sand-digging, and sand-covering, but all involve an animal covering itself with the substrate, either sand or mud, until it cannot be seen (Hanlon & Messenger, 1996). The behavior pattern is well described in cuttlefish (*S. officinalis*) as consisting of three actions: "blow forward" where the mantle–funnel directs a jet of water anteriorly, "blow backward" which directs the jet of water posteriorly beneath the body, and "wiggle" where the dorsal mantle contracts in a series of small side-to-side movements (Mather, 1986). This stereotyped behavior exhibits some variation, particularly during the "wiggle" phase. Sepiolid squids bury in two phases (Boletzky, 1996). The first phase is similar to the behavioral pattern described for *Sepia*. The funnel directs a series of water jets forward and backward to create a depression for the animal to settle into. Fin undulations help resist the upward component of jetting. The second phase involves the second pair of arms which stretch, enclose, and sweep sediment over the body and head until they are completely covered. The funnel protrudes laterally at the surface between the head and mantle and respiration is maintained in a funnel pouch rather than the mantle. This pouch is a result of pulling

back a skirt found at the base of the funnel (Boletzky & Boletzky, 1970). This fixed sequence of behaviors during burying has been seen in *Rossia pacifica* (Anderson et al., 2004), *Euprymna scolopes* (Anderson et al., 1999), and *Sepiola atlantica* (Rodrigues et al., 2010) with variations in behaviors and body patterning due to environmental differences such as substrate type. Benthic octopuses have also been observed digging or burying into mud or sand (Guerra et al., 2006), however mantle–funnel jets are not involved in moving sediment in the process (Boletzky, 1996). The arms are the main effectors in octopus burying, so this action will be considered in the following section concerning arms.

7.2.4.1.2 Web-based

Another way coleoid cephalopods, mainly the deep sea cirrates (Opisthoteuthidae, Cirroteuthidae, Grimpoteuthidae, and Cirroctopodidae), can swim is by using the web to eject water when the arms come together. Seibel et al. (2000) suggested that these animals do not need high-speed locomotion for predator/prey visual detection in their low light environment. These slower modes of movement, based on the web as well as the fins, are more energetically efficient and less costly than jet propulsion (Seibel et al., 1997, 2000).

There are many modes of swimming used by the different cirrate groups. Roper & Brundage (1972) were among the first to describe some of these modes (water ejection, pulsating, and umbrella style) as access for observation of these deep sea species is difficult. With the availability of submersible techniques in more recent years, a suite of swimming patterns involving the web, in some cases assisted by the fins, has been seen *in situ*. "Pumping" produces slow propulsion in Cirroteuthids by alternating expansion and expulsion of water via peristaltic waves in the web (Villanueva et al., 1997a). The "Ballooning-response" was first described by Boletzky (1992) in *Cirroteuthis magna* as similar to pumping during filling, but in this case the water is retained in the web when the distal edges are contracted. All eight sections of the web are expanded simultaneously and filled with water (Villanueva et al., 1997a; Collins & Villaneuva, 2006). This posture may be used as a transition between different modes of locomotion and has also been seen in *Stauroteuthis syrtensis* (Vecchione & Young, 1997; Johnsen et al., 1999) *C. magna* (Villanueva et al., 1997a), and *Opisthoteuthis massyae* (Villanueva, 2000). "Take-off" consists of a single contraction by the web, sometimes proceeded by flapping of the fins. It has been seen as an escape response in *Cirroteuthis* and *Grimpoteuthis* (Villanueva et al., 1997a; Collins

& Villaneuva, 2006). "Umbrella-style drifting" is a passive mode of locomotion that makes use of the animal's neutral buoyancy and bottom currents to maintain position in the water column. As seen in *Cirroteuthis*, the web and arms are spread out with the fins folded in, similar to an open umbrella (Villanueva et al., 1997a). This is also present in *S. syrtensis* (Johnsen et al., 1999; Collins & Villaneuva, 2006). "Medusoid" was first described by Roper & Brundage (1972) in *Vampyroteuthis* to be an escape reaction that consists of rapid movement. The rate of web contractions can be as fast as 1.2/s with 2–3 fin strokes for every pulse of the web (Collins & Villaneuva, 2006). Also known as arm-web contraction, this action consists of pulsations of the round, bell-shape inflated web and is the primary mode of locomotion for *Opisthoteuthis* (Vecchione & Roper, 1991). It has also been seen in *S. syrtensis* and juvenile *Grimpoteuthis* (Vecchione & Young, 1997) which use fin flapping (also referred to as sculling) with medusoid pulsations partly to help steer the body (Johnsen et al., 1999; Collins & Villaneuva, 2006). As has been mentioned in some of the examples, the fins may also play a part in locomotion of these cirrate octopods. The web is inflated in a bell-like shape with the arms separated. Fins on either side of the mantle flap simultaneously for fin swimming (Collins & Villaneuva, 2006; Johnsen et al., 1999; Vecchione & Young, 1997).

Cirrate octopods generally exhibit web-based propulsion, fin swimming, or a combination of the two for fast burst escape responses (Seibel et al., 1998). Opisthoteuthids have oval shaped bodies, small fins, and a thick web with low protein and lipid content in the muscles. These characteristics reflect its predominant use of slow fin swimming. Cirroteuthids, with their long bodies, large fins, and well-developed web, use both the fins and web for swimming. Grimpoteuthidae and Cirroctopodidae have intermediate morphologies to Opisthoteuthidae and Cirroteuthidae and also use both web and fin swimming (Collins & Villaneuva, 2006; Seibel et al., 1998).

7.2.4.2 LIMB MOVEMENT

Both the fins (cirrate octopods, squid, and cuttlefish) and arms (octopods) of cephalopods can be used as effectors for locomotion. For many species of squid and cuttlefish, the fin is used to stabilize body position during jet propulsion. However, in cirrate octopods the fins assist in web-based swimming (discussed in the following section) or may be the primary means of locomotion. Different types of fin movement can result from variation in fin sizes and shape as well as depend on ontogenetic changes of the fins and

cephalopod lifestyle. Similarly, different gait patterns can result from variations in flexibility and coordination of arms in octopus. Squid and cuttlefish arms do not exhibit the same diversity of movements or dependence on arms for locomotion as seen in octopuses, with the exception of burying as previously described in Sepiolids and walking ("ambling with arms") exhibited by sequential shuffling of the ventral arms and ambulatory flaps along the bottom in flamboyant cuttlefish, *Metasepia pfefferi* (Roper & Hochberg, 1988).

7.2.4.2.1 Fin

Unlike jet propulsion, locomotion by fins is not well investigated, though there are a few studies (Anderson & DeMont, 2005; Bartol, Patterson, & Mann, 2001; O'Dor, 1988; Hoar et al., 1994; Stewart et al., 2010). Fins are limited by hydrodynamic constraints and scaled to the animal's body during development (Hoar et al., 1994; O'Dor & Hoar, 2000). Clarke (1988) identified nine types of fins that vary in shape and size for different species of finned cephalopods (see Fig. 7.6 for fin and body form examples from Packard, 1972). First, fringing fins, as seen in *Sepia* cuttlefish, produce waves with small amplitude that are used for maneuvering. Second, elongated flapping fins generate large-amplitude waves used for hovering or gentle swimming. Mather et al. (2010) described the different fin positions in *S. sepiodea*. These fins also produce powerful flaps that aid in jet action, which is exemplified in the squid *Thysanoteuthis rhombus*. Dorsoventral stiffness is provided by the squid pen. Hunt et al. (2000) described the locomotor and postural components of behavior for *L. opalescens*. Third, posterior broad triangular fins with a leading edge perpendicular to the sagittal plane of the body are used for fast muscular swimming in negatively buoyant squid. These fins make long undulatory waves that provide stability and produce hovering. There is a parallel variation in the fin structures of batoid fishes (Rosenberger, 2001). Individuals with this fin type usually have narrow pens to help with greater directional control and can interchange between backward and forward hovering. Examples include the Ommastrephids, like shortfin squid (*Illex illecebrosus*) and the Humboldt squid (*Dosidicus gigas*), as well as the Onychoteuthids, hooked squid. Harrop et al. (2014) describe locomotor and postural components of *in situ* behavior of *Illex*. Fourth, medially placed broad rounded fins give cephalopods, like the short-bodied Sepiolidae (bobtail squids), more turning mobility. Fifth, short round fins in species with no buoyancy or a center of buoyancy that is more toward the

posterior provide orientation control but do not affect backward or forward movement. Cephalopod examples include the Idiosepiidae, Cranchiidae, and Histioteuthidae. Sixth, oval fins produce large amplitude waves in squids that use ammonia-mediated buoyancy. These fins, which are seen in Architeuthids, Chiroteuthids, and Mastigoteuthids, provide control of orientation and more economical backward movement. Seventh, long circular fins along the mantle produce backward movement by fin beating. Squid with this fin type have robust thick cartilaginous pens. Squid species with this type of fin include *Discoteuthis*, *Ancistrocheirus*, and *Octopoteuthis*. Different postural and locomotor components have been described for *Octopoteuthis deletron* (Bush et al., 2009). Eighth, juvenile members of Grimalditeuthidae and larval Chiroteuthidae have secondary fins which are smaller, develop posterior to the primary fins and whose functions are unknown. Lastly, broad fins with highly muscular attachments to the body generate powerful fin beats for propulsion in Vampyromorpha and the cirrate octopods (Clarke, 1988).

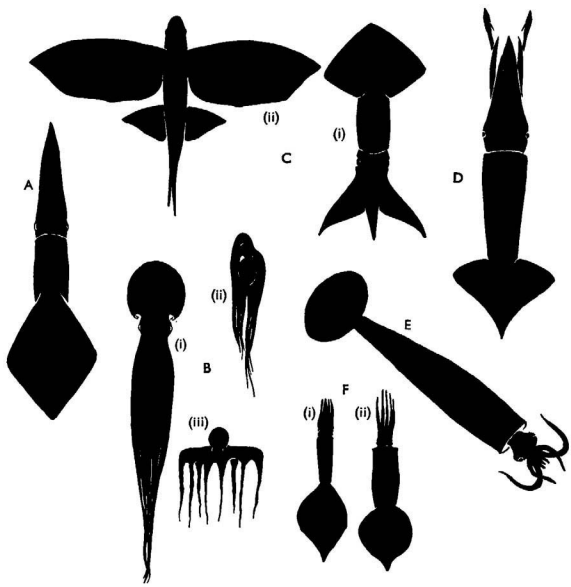

FIGURE 7.6 Different body forms during locomotion, with examples of various forms of cephalopod fins. (A) Adult *Loligo vulgaris*; (B) (i) and (ii) adult *Octopus vulgaris*, (iii) adult *Octopus dofleini*; C. (i) flying ommastrephid squid, (ii) flying fish; (D) adult gonatid squid *Gonatus*; (E) cranchid squid *Leachia*, F. (i) and (ii) different developmental stages of squid *Lepidoteuthis*. (From Packard, A. Cephalopods and Fish: the Limits of Convergence. *Biol. Rev*. 1972, 47, 241–307. Copyright © (2015) by John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.)

Fins produce two types of movement, either undulations (passing multiple waves down the fin) or fin beating or flapping (oscillatory) (Hoar et al., 1994). Parallel variation in the fin structures of batoid fishes show a continuum between undulatory and oscillatory locomotion (Rosenberger, 2001). Fin flapping predominates in cirrate octopods and Vampyromorpha, which have broad muscular fins. Fin swimming in cirrate octopods consists of beating their pairs of fins synchronously, either in combination with web-based propulsion (discussed in a future section) or alone. This results in mantle-first movement with the arms trailing behind. This is has been observed in *C. murrayi* (Aldred et al., 1983), *Cirrothauma magana* (Villanueva et al., 1997a), and *Grimpoteuthis* (Vecchione & Young, 1997). *V. infernalis* exhibits an ontogenetic change in locomotion based on changes in fin morphology with maturation. Juveniles primarily use jet propulsion, assisted by their small paddle-shaped fins for stability. As they become adults, the fins increase in size and vary in shape such that lift-based propulsion becomes more important and fin swimming is the primary mode of locomotion (Seibel et al., 1998). Other ontogenetic gait changes in locomotion have been seen in *L. brevis* (Bartol et al., 2009) and the effects of scaling of squid fins on locomotion types are discussed in Hoar et al. (1994). The lateral fins of *Sepia* primarily move by undulations to help the animal hover, but several squid patterns of swimming moderated by different fin morphologies fall between the two ends of the continuum. In the long-finned *L. pealei*, the fin is more undulatory at low speeds and more flap-like at high speeds (Anderson & DeMont, 2005). Shallow-water brief squid *L. brevis* also uses a range of fin movements at different speeds (Bartol, Patterson, & Mann, 2001) and recent studies of fin-only hydrodynamics confirm that the fins produce lift and thrust (Stewart et al., 2010). Fins also facilitate "flight" by jet propulsion out of the water into the air, assisted by fin flaps in several squid species (Maciá et al., 2004; O'Dor et al., 2013). These are usually seen in squid with broad fins and spread arm postures.

7.2.4.2.2 Arms

The arms in octopuses are powerful effectors of movement. When the musculature of the mantle and the arms are compared during swimming versus holding, five arms holding onto a side tank can produce enough tension to hold 30–100 times the octopuses' own body weight while tension from swim pulling can only sustain half the body weight (Trueman & Packard, 1968). Arrangements of the muscles also allows arms to have a large number of

degrees of freedom: bending, elongating, and twisting in an almost infinite number of directions. Efforts to understand the general principles that organize movement in a single arm, such as "reaching" (Gutfreund et al., 1996) and "fetching" (Sumbre et al., 2006) have been described, along with dynamic modeling of the biomechanics and neural control of the arm "reaching" pattern (Yekutieli, Sagiv-Zohar, Aharonov, Engel, Hochner, & Flash, 2005; Yekutieli, Sagiv-Zohar, Hochner, & Flash, 2005).

Descriptions for crawling are not as frequent as those for jet propulsion in cephalopods. However, benthic octopuses spend most of their time on the bottom using their arms as primary way to move around their environment (Huffard, 2006). Crawling patterns have been described for *Grimpoteuthis* (Villanueva et al., 1997a), *Amphioctopus marginatus* (Huffard & Godfrey-Smith, 2010; Sreeja & Bijukumar, 2013), and *Abdopus aculeatus* (Huffard, 2006) but also mentioned in behavioral and taxonomic accounts of other species (Hanlon & Wolterding, 1989; Hanlon et al., 1999; Mather, 1998; Mather & Mather, 1994; Norman & Finn, 2001; Packard & Sanders, 1971; Roper & Hochberg, 1988; Wells et al., 1983). Observations of arm movement across the substrate in the deep sea octopods *Graneledone*, *Benthoctopus*, and *Vulcanoctopus* have been made, but with no description of specific gait patterns such as crawling (Voight, 2008). "Crawling" has sometimes been used interchangeably with "walking" in the literature. Crawling can generally be described as actions by the arms and their suckers, with the arms spread on the bottom, pushing and pulling with the more proximal and medial suckers on the substrate (Mather, 1998). Multiple points of contact can be made along the arms with the substrate and in the direction of movement. The posterior and lateral pairs of arms are more often used during crawling, a preference that has been seen *O. vulgaris* with anterior arm preference to explore and forage (Byrne et al., 2006). Levy et al. (2015) recently described crawling in octopus and found that they lack a stereotyped pattern of arm coordination and that crawling direction can be determined by a force vector resulting from a combination of the pushing arms. They also found that instead of rotating the body, choosing to use a certain set of arms and using their vector combination will change the octopus's crawling direction. This suggests that motor control system of crawling consists of relatively stereotyped movements (i.e., a series of arm elongations and sucker adherence) produced by the peripheral system with the coordination of the arms determined by the central brain. Postures may vary within and between species during crawling (see Fig. 1 in Huffard, 2006).

Other gaits of arm-mediated locomotion have been described for a few species of octopus. Bipedal walking in *A. marginatus* and *A. aculeatus* uses

two arms (usually the posterior pair) to move backward using a rolling action of the arms (Huffard et al., 2005). The octopus pushes or rolls from the tip of the arm with its suckers against the substrate, alternating arms. Postures used during bipedal walking varied between *A. marginatus*, which typically draws in all six arms closer to the body, and *A. aculeatus*, which raises its two dorsal arms in a "flamboyant display" (Packard & Sanders, 1971). Another variant on arm position that involves multitasking is "stilt walking" and is only seen in *A. marginatus*. It involves bipedal walking in combination with holding an object with the other arms (Finn et al., 2009). "Tiptoe," which is a variant of crawling, involves adhesion, swinging, and release by a subset of suckers on all eight arms on proximal areas resulting in slow gliding movement (Mather, 1998). Locomotion speeds relative to body lengths were compared between different modes in *A. aculeatus* and although crawling (1.94 body lengths per second) is used predominantly, it is not as fast as jet-propelled movement (3.29 body lengths per second), but bipedal locomotion (2.25 body lengths per second) comes close (Huffard, 2006).

Octopuses can use their arms for various tasks such as "grooming" and "exploring", which do not have a set sequence of arm positions (Mather, 1998). Several other tasks have been named and qualitatively described (Borrelli et al., 2006; Huffard, 2006; Mather, 1998; Mather & Alupay, 2016; Packard & Sanders, 1971). One of the more often noted tasks is digging. Burying behavior in sand or mud has been observed in *Octopus burryi* (Hanlon & Hixon, 1980), *Octopus cyanea* (Roper & Hochberg, 1988), *Eledone moschata* (Boletzky, 1996; Mangold, 1983), and *E. cirrhosa* (Rodrigues et al., 2010). Observations in the field also show that *Macrotritopus defilippi* (Hanlon et al., 2010), *Thaumoctopus mimicus*, and *Wunderpus photogenicus* (Hanlon et al., 2007) also rapidly disappear into their sand environment, but the burying mechanism is unknown. There are very few descriptions of what exact actions occur in the arm during digging. The arms likely twist and the suckers sweep awaylarger grains of sediment (Boletzky, 1996). In *O. cyanea*, sediment is pushed to the side by ventral arms to form a cavity where the octopus can settle (Roper & Hochberg, 1988). It was noted that no water jets from the funnel were involved during the process. During sand-covering in *E. cirrhosa*, the arms push up small grained sediment around the body by twisting of the proximal and distal parts of the arm (Guerra et al., 2006). After making a hole, the octopus sinks in and the arms form a circle to throw out sediment from underneath its body, so the mantle and head sink deeper. In octopuses, digging is not only used for concealment,

but also to build a burrow for shelter (Guerra et al., 2006). The sequence of behaviors for the arms or other potential effectors is not known, so unlike in the Sepiolids (Boletzky & Boletzky, 1970), sequences of action that might be species-typical are not known.

7.2.5 CONTRIBUTION OF BUOYANCY

An important aspect to consider for all modes of locomotion in water is buoyancy. Having lost an external shell which might hold gas, the coleoid cephalopods have developed four general methods to maintain their vertical position in the water column (see summary Fig. 15 in Packard, 1972). The first uses variable gas-filled spaces in an internalized chambered shell, as seen in the enclosed *Nautilus*-like shell of *Spirula* (Denton et al., 1967) and the flattened cuttlebones of *Sepia* (Birchall & Thomas, 1983; Denton & Gilpin-Brown, 1961). The volume of gas in these spaces is regulated by pumping salts from the fluid filling the spaces of the cuttlebone to maintain an osmotic pressure that withstands hydrostatic pressure from water flowing into the shell (Boyle & Rodhouse, 2005a). The superfamily of pelagic octopuses, Argonautoidea, is an interesting example of how gas-mediated buoyancy has evolved in recent coleoids (Bello, 2012). The females of *Argonauta* have an external brittle shell whose primary role is to hold and brood eggs. The shell also controls buoyancy by retaining air "gulped" at the sea surface before the octopus descends deeper (Finn & Norman, 2010). *Ocythoe tubercuclata* females have gas-filled swim bladders (Packard & Wurtz, 1994) and octopod relatives *Tremoctopus* and *Haliphron* have homologous structures whose position in the mantle suggest they originate from the digestive system (Bello, 2012).

The three remaining methods use materials that are less dense than seawater to maintain neutral buoyancy. The most common method among squids is to use ammonium to replace sodium in lowering the density of internal fluids (Boucher-Rodoni & Mangold, 1995; Clarke et al., 1979; Clarke, 1988; Voight et al., 1995). Ammonia is a product of nitrogen metabolism present in all cephalopods. This fluid is stored in vacuoles within the muscle, but in one family, the Cranchiidae, it is stored in a specialized coelom (Clarke et al., 1979; Clarke, 1988; Denton & Shaw, 1969). A second method is to store lipids or oils in a digestive gland such as the liver, as seen in the Gonatid squid (Clarke, 1988; Seibel et al., 2004). The third method, exhibited in pelagic octopods like *Japatella*, is to have layers of low-density gelatinous tissue surrounding the musculature, which decrease the number of dense ions like sulfate and chlorine in the body (Clarke et al., 1979; Denton & Shaw, 1961; Denton et al., 1967).

7.3 LOOKING FORWARD

It is evident from this overview that the muscular hydrostat plays an important role in the types of locomotion used by coleoid cephalopods. The basic principles of how hydrostatic skeletons move and detailed descriptions of the structures involved were pioneered by Kier (1992, 2012) and Kier and Smith (1985). They found that the same leverage principles of agonist–antagonist muscles used in hard skeletal bodies also applies to the soft bodies of cephalopods and other muscular hydrostat-based animals. In fact, despite the great diversity of movement behaviors used by animals with different structures and environmental pressures, there are underlying similarities on multiple biological levels. One of the areas under investigation in hydrostat systems is understanding the fundamental mathematical basis for their movement. Some progress has been made in mathematically modeling body pattern formation on cephalopod skin (Mather et al., 2014), models that are being used by the same authors to also explain common dynamical principles in hydrostat systems besides cephalopods, like the nematode, *Caenorhabditis elegans*, and the human tongue.. In addition, recent developments in visualization techniques using ultrasound (King et al., 2005; Margheri et al., 2011) and magnetic resonance imaging (Xavier et al., 2014) provide a method for studying muscle dynamics *in situ* for these varieties of soft bodied organisms.

Despite the abundant information about the cephalopod musculature, control of these structures to produce different movement patterns is still not as well known. Detailed information about the neural structures and some of the pathways (namely the giant fiber system [Young, 1938, 1939, 1965]) has been described, but considering the diversity of locomotor modes, we still know very little about how movement is regulated. Coordination of mantle–funnel jets with different fin undulation and flapping patterns in squid and cirrate octopods still need better understanding. Even more complex is the coordination of arm movement used for crawling and other modes of locomotion in benthic octopods. Grasso (2014) outlined the center–periphery problem in coordinating the arm and sucker units. One approach to studying this is through the idea of embodiment (Hochner, 2012, 2013) where the central nervous system and peripheral programs of the arms and suckers interact with each other and are modulated by external information from the sensory and mechanical systems, and the environments. This interaction and feedback, though complex, is thought to be what underlies the combinations of many behaviors, including movement. Motor primitives (Flash & Hochner, 2005) are the simple units of behavior that in combination can lead to more complex locomotor programs. The

previously described "reaching" pattern is one such example of a unit. However, recent studies show that octopus arm movements may be more flexible than originally thought with respect to stereotyped movement patterns, as different individuals use different motor programs in the same situation (Richter et al., 2015).

The detailed descriptions of movement patterns from different cephalopod groups can be informative to other fields, including bio-inspired soft robotics. Several models for soft robotics have been based on the octopus arm because of its dexterity, flexibility, and variable stiffness, all controlled by its muscle arrangements (Cianchetti et al., 2011; Laschi et al., 2009). Furthermore, jet-propelled robots based on the cephalopod mantle (Renda et al., 2014) and web (Sfakiotakis et al., 2014) as well as undulations of the fin (Liu et al., 2012; Willy & Low, 2005) have all been mimicked for soft robotics. These models are often specialized to be efficient in only a few aspects and in isolation. By taking into account the diversity of movements by a combination of effectors, we may better obtain models for soft robotics. For example, the recent incorporation of compliant webbing in swimming movements of a multi-armed robot improved its velocity, thrust, and efficiency than having arms alone (Sfakiotakis et al., 2014).

7.4 CONCLUSIONS

Cephalopods are predominantly studied for their exceptional jet propulsion abilities and the principles behind their movement are thought to be based largely on the mantle–funnel complex. This review of coleoid cephalopod locomotion highlights the role of muscular hydrostats and the actions of multiple effector units—mantle–funnel complex, fin, arms, suckers, and the web in giving us a diversity of locomotor types. These structures, alone and in combination, produce several variations on swimming, crawling, and burying behavior. Using this overview as a basis, we can examine in more detail how the muscle structures adapt to these many modes of locomotion and better understand the general principles that underlie locomotion with muscular hydrostats.

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KEYWORDS

- **locomotion**
- **cephalopods**
- **mantle-funnel**
- **muscular hydrostat**
- **fin**
- **jet propulsion**
- **multiple arms**

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CHAPTER 8

KEY MOLECULAR REGULATORS OF METABOLIC RATE DEPRESSION IN THE ESTIVATING SNAIL *OTALA LACTEA*

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CONTENTS

ABSTRACT

Estivation is a state of aerobic dormancy used by the desert land snail, *Otala lactea*, to endure harsh environmental conditions. Paramount to survival in the estivating state is the sustained and profound depression of metabolic rate, which facilitates survival from limited endogenous energy stores for extended periods of time. Metabolic rate depression requires coordinated suppression of ATP-generating and ATP-consuming cellular functions by stable regulatory mechanisms. One such mechanism that has been well-studied in this estivating species is reversible protein phosphorylation. Studies in *O. lactea* have established that protein phosphorylation has far-reaching regulatory capacity in facilitating the biochemical transition between active and estivating conditions. This mechanism plays a role in modifying the activities of rate-limiting enzymes of carbohydrate metabolism, enhancing intracellular tolerance to oxidative stress, suppressing ATP-intensive processes such as global protein turnover and ion pumping, and activating specific arms of the AMP-activated protein kinase (AMPK) signaling and the insulin signaling cascades. This review chapter will document the evidence suggesting that differential protein phosphorylation, brought about by specific protein kinases and protein phosphatases, is essential to regulating biochemical adaptations in *O. lactea* that are critical to survival.

8.1 INTRODUCTION

When food or water is limiting, the land snail *Otala lactea* enters a state of aerobic dormancy termed estivation. A number of behavioral, physiological, and biochemical adaptations are associated with this survival strategy. One particularly remarkable aspect of estivation is that estivating animals can profoundly suppress their metabolic rate, and this metabolic rate depression can be sustained for extensive periods of time. This adaptation facilitates energy conservation that permits the snails to live off finite energy stores for prolonged periods of time, which in turn facilitates survival in unfavorable living conditions for many months. The metabolic rate depression characteristic of estivation is common to other survival strategies that have evolved to help cope with difficult environmental conditions and has been observed and characterized to varying degrees in hibernating, freeze-tolerant, and anoxia-tolerant species (Storey, 2002; Storey & Storey, 2004). While different aspects of estivation have been characterized in several species of snails, the molecular biochemistry underlying the depression of metabolic rate has

been particularly well-defined in *O. lactea*, especially in the recent decade. This chapter will discuss recent research highlights in the field of estivation, with a focus on biochemical markers that have been implicated as key regulators during the transition to the estivating state.

8.2 LACK OF WATER AND ELEVATED CARBON DIOXIDE ARE TRIGGERS FOR ESTIVATION

While *O. lactea* snails are native to the seasonally arid Mediterranean region, these animals have been introduced around the world to countries with seasonally challenging climates, including Canada. In fact, the studies described in this chapter involved *O. lactea* animals imported from Morocco and purchased from a local retailer in Ottawa, Ontario. *O. lactea* are typically active for only a few months of the year, particularly in challenging environments. During this time, these snails must accumulate body-fuel reserves that are sufficient for survival throughout many months in dormancy. Like many estivating animals, dormancy is triggered in *O. lactea* by sensing lack of food and water in the environment. When this occurs, these snails typically seek out sheltered sites that limit their exposure both to the elements and to predators. At the onset of estivation, snails secrete a calcified mucous membrane, called an epiphragm, which effectively limits evaporative water loss (Barnhart, 1983). Estivators may also elevate the solute concentrations of their body fluids, thereby utilizing the colligative properties of dissolved solutes such as urea to aid water retention (Withers & Guppy, 1996). Consistent with this, metabolic enzymes in estivating animals are frequently much more resistant to the denaturing effects of urea, when compared to enzymes in non-estivators (Cowan & Storey, 2002). Many animals (typically but not exclusively desert-dwelling organisms) also convert nitrogenous wastes into uric acid, since excretion and storage of nitrogen in this insoluble form requires minimal water (Dejours, 1989). The kidney has a relatively limited capacity for uric acid storage in several normal and estivating gastropods (e.g., Athawale & Reddy, 2002), where uric acid accumulates in specialized extrarenal cells and tissues (Giraud-Billoud et al., 2008; Vega et al., 2007). Extrarenal uric acid can protect cells and tissues against the oxidative stress associated with arousal from estivation (Giraud-Billoud et al., 2011, 2013). However, a role for uric acid in *O. lactea* has not yet been studied.

Evaporative water loss is also minimized by employing discontinuous breathing, as the snail's pneumostome, the specialized orifice leading to the lung, is open for brief periods, with long, irregular intervals between

openings, allowing for intermittent respiration. Estivating *O. lactea* can take as little as 2–3 breaths per hour (Barnhart & McMahon, 1987). Thus, estivation in *O. lactea*, while aerobic, is characterized by long periods of apnea that are irregularly interrupted by short bouts of oxygen and carbon dioxide gas exchange (Barnhart, 1986). While physical adaptations such as the epiphragm and behavioral modifications such as the intermittent pneumostome opening contribute to water conservation, these adaptations also decrease ventilation of the lung. As a result, between breaths, snails experience a progressive hypoxia, extracellular and intracellular acidosis, and hypercapnia (Barnhart & McMahon, 1988). Between breaths, the partial pressure of carbon dioxide gradually rises and the partial pressure of oxygen gradually falls in the snail's tissues. It was determined that the interval length between breaths is determined by oxygen need (Barnhart, 1986). On the other hand, elevation of the partial pressure of carbon dioxide (or the resulting cellular acidosis that this creates) has been implicated as the molecular trigger for the suppression of metabolic rate (Guppy et al., 1994). Consistent with this notion, oxygen consumption (an index for metabolic rate) was reduced by 50% in active *O. lactea* that were exposed to carbon dioxide, but upon removal of carbon dioxide oxygen consumption quickly returned to normal levels in these snails (Barnhart & McMahon, 1988). Hence, elevated carbon dioxide can be considered a signal that triggers the substantial metabolic rate depression that occurs in estivating snails.

8.3 METABOLIC RATE DEPRESSION IS ESSENTIAL FOR SURVIVAL IN ESTIVATING ANIMALS

The transition into a hypometabolic state is advantageous to estivating animals because it extends the duration by which finite energy reserves can sustain life. This transition requires coordinated suppression of the rates of both energy consuming and energy producing pathways to create a new, lower balanced state of ATP turnover. Estivating snails typically show metabolic rates that are <30% of the corresponding resting rate in active snails (Bishop & Brand, 2000; Rees & Hand, 1990). The transition into a hypometabolic state requires coordinated suppression of the rates of both ATP-consuming and ATP-producing cell functions as well as a reorganization of the priorities for energy use to sustain essential cellular processes and suppress other energy-intensive functions such as growth, development, and reproduction (Storey & Storey, 2004). Carbohydrate oxidation was observed to be the primary energy reserve in two species of estivating mountain snails,

Oreohelix strigosa and *Oreohelix subrudis* (Rees & Hand, 1993). When carbohydrate stores were depleted after the first few months of estivation in these snails, only then was protein oxidation initiated; overall, there was little contribution at any stage from lipid breakdown (Rees & Hand, 1993). Carbohydrate reserves can therefore fuel life processes during estivation for months, and in extreme conditions, even years (Herreid, 1977), largely because these estivating snails can profoundly depress their metabolic rates.

Several levels of control can be involved in regulating entry into and/or arousal from dormancy including changes in gene expression, protein synthesis, posttranslational modification, and allosteric control of enzymes. Physiologic states of natural metabolic rate depression are not typically accompanied by wholesale changes in gene transcription (Storey & Storey, 2004). Modification of gene expression patterns is an elaborate, time-consuming, and energy-intensive process, and it can take many hours for a change in the gene transcriptional level to manifest in a measurable change in the corresponding protein level. For example, substantial physiologic increases in glucoregulatory hormone concentrations can bring about rapid and substantial alterations in glucoregulatory gene expression in vivo, such that mRNA levels of target genes can be increased many fold or decreased up to 90% within 30 min, but several hours are required for these changes to manifest in actual modification of corresponding protein levels (Ramnanan et al., 2010a, 2011). This type of regulation would not be ideal for animals that move into a hypometabolic state in response to adverse conditions, since these animals typically need to make this transition quickly, in response to environmental and physiological cues. Thus, control at the level of gene expression is typically limited to just a few selected genes that are either up-regulated or down-regulated in terms of mRNA expression. Furthermore, the manufacturing of proteins is energetically demanding, and ATP is at a premium for dormant animals. Hence, large scale protein synthesis (and also large scale protein degradation) is not compatible with the animal's need for energy conservation during dormancy (and our studies on the topic will be discussed later in this chapter). In addition, dormant animals can be aroused rapidly when the environment returns to more favorable conditions; for example, *O. lactea* arouse within minutes of sensing water (Hermes-Lima et al., 1998). Since metabolic capabilities need to be maintained in a state or readiness to facilitate a rapid return to normal life, major metabolic restructuring at the level of altering protein levels does not typically occur during dormancy. Instead, the changes in gene expression and protein content are relatively subtle, and regulation comes instead from reversible and energy-efficient controls on metabolism. One such mechanism that has been well

established to regulate a myriad of cellular functions across many hypometabolic states in several species is reversible protein phosphorylation.

8.4 REVERSIBLE PHOSPHORYLATION: KEY MECHANISM OF METABOLIC REGULATION

Reversible protein phosphorylation is an important and far-reaching theme in posttranslational regulation. The covalent binding of a phosphate group to an enzyme (mediated by protein kinases) or removal of a phosphate group (catalyzed by protein phosphatases) can have immediate, dramatic effects on enzyme activity and kinetic/regulatory properties, typically modifying enzymes from active (or more active) to inactive (or less active) conformations. Protein phosphorylation in animal cells typically targets serine or threonine residues (both having a free hydroxyl group) or tyrosine residues (featuring a phenolic group). The enzymes that mediate phosphorylation events are therefore typically classified as being either serine/threonine kinases or tyrosine kinases (and similar distinctions exist for the corresponding phosphatases). Moreover, serine/threonine kinases can be further subclassified based on their dependency on biochemical coactivators or second messengers, while serine/threonine phosphatases can be classified based on their substrate affinities, ion dependency, and sensitivity to natural or pharmacological inhibitors (Cohen, 1989).

The presence of saturating amounts of Mg^{2+}-ATP (a substrate and source of inorganic phosphate for kinase catalytic activity) and specific second messengers or molecular activators in experimental conditions can promote the activity of specific protein kinases. These kinases include: the cyclic AMP (cAMP)-dependent protein kinase (protein kinase A; PKA); the cyclic GMP (cGMP)-dependent protein kinase (protein kinase G; PKG): the calcium- and phorbol myristate acetate-activated protein kinase C (protein kinase C; PKC); the AMP-activated protein kinase (AMPK); and the calcium/calmodulin-dependent protein kinase (CaMK). Similarly, experimental conditions can be designed to be permissive for specific protein phosphatases, as protein phosphatases are known to be sensitive to different inhibitors and require certain ions for their function. Sodium fluoride (NaF) can inhibit all serine/threonine phosphatases, while freshly prepared sodium orthovanadate (Na_3VO_4) can inhibit global protein tyrosine phosphatase activity. Protein phosphatase of type-1 activity (PP1) and of type-2A (PP2A) are both ion-dependent and sensitive to the marine toxin okadaic acid, but can be differentiated due to PP2A being is 50–100-fold more sensitive to this inhibitor than PP1 (Cohen,

1989). Type-2B phosphatase activity (PP2B) is calcium- and calmodulin-dependent, whereas type-2C phosphatase activity (PP2C) is manganese- or magnesium-dependent. Moreover, these ion-dependent phosphatases can be inhibited by including chelating agents (EDTA or EGTA) in experimental conditions.

Covalent phosphorylation of a protein is a very stable mechanism for protein modification, and removal of phosphate(s) in vivo can only be achieved by the corresponding serine/threonine or tyrosine protein phosphatases. Modification of an enzyme via phosphorylation can have substantial effects on the flux of a metabolic pathway, at a much lower energetic cost than by other means of changing enzyme activities (i.e., protein synthesis or degradation). Another desirable feature of phosphorylation-mediated control is that the modification is readily reversible, and this allows the animal to respond quickly to environmental cues, and enter (or exit) the dormant state (and adjust metabolic rates) as rapidly (Storey, 2002). Metabolic pathways are commonly regulated by (1) controlling the rate of substrate entry into the pathway, and/or (2) controlling the rate of enzymatic reaction(s) that influence flux through the pathway. In every metabolic pathway, there is at least one nonequilibrium, highly exergonic (and essentially irreversible, under cellular conditions) reaction catalyzed by a low activity enzyme, the rate of which influences the rate of the entire pathway. These key pathway-controlling enzymes are likely targets for regulatory mechanisms such as feedback allosteric inhibition by end products and reversible posttranslational modification. As *O. lactea* relies primarily on aerobic catabolism of carbohydrates for energy to fuel life during dormancy, it could be expected that enzymatic activities influencing the rates of glycolysis and the Krebs cycle would be subject to modification to facilitate transition to the estivating condition.

8.5 ENZYMES OF CARBOHYDRATE METABOLISM ARE REGULATED BY PHOSPHORYLATION IN *O. LACTEA*

Several enzymes that can influence the rate of carbohydrate catabolism have been studied in *O. lactea*, and these proteins have all been implicated as targets of estivation-dependent covalent modification. These enzymes include (1) glycogen phosphorylase (GP) which regulates the catabolism of glycogen to provide the substrate for glycolysis; (2) PFK-1 which is recognized as the key control enzyme of glycolysis; (3) pyruvate kinase (PK) which catalyzes the essentially irreversible terminal reaction of glycolysis;

and (4) pyruvate dehydrogenase (PDH) which regulates the enzymatic reaction that is considered to be the entry point for carbohydrates into the Krebs cycle. Activities of GP, PFK-1, PK, and PDH were all reduced in estivating *O. lactea*, and in all cases these were linked to changes in the phosphorylation state of the enzymes (Brooks & Storey, 1992, 1997; Whitwam & Storey, 1990, 1991). As a consequence of change in phosphorylation state, these catabolic enzymes all displayed at least one of the following characteristics: reduced maximum activities (V_{max}), reduced affinity for substrates (increased K_m), or increased sensitivity to feedback inhibition by end products and inhibition by metabolites in general (decreased I_{50}). These parameters of enzyme kinetics will be explicitly defined later in the chapter. The decrease in activity of these regulatory enzymes of ATP-generating catabolism is in line with *O. lactea*'s priority to conserve carbohydrate stores during the estivating state.

8.6 THE ACTIVATION OF G6PDH DURING ESTIVATION FACILITATES PROTECTION AGAINST OXIDATIVE STRESS

Glucose-6-phosphate dehydrogenase (G6PDH), another enzyme of carbohydrate metabolism, is considered to be a key control or rate-limiting enzyme of the pentose phosphate pathway (or shunt). This enzyme displayed increased activity in the hepatopancreas of estivating *O. lactea* (Ramnanan & Storey, 2006a). The question arises as to why increased activity of this enzyme, or increased carbohydrate flux down this pathway, would be beneficial to an animal whose priority is to conserve carbohydrate stores during the dormant phase. The pentose phosphate pathway (alternatively termed the hexose monophosphate shunt) has several important functions, including (1) the production of pentose sugars for synthesis of nucleotides and nucleic acids, (2) serving as the entry point for dietary nucleotides and 5-carbon sugars into catabolic pathways, (3) rearranging the carbon skeletons of dietary carbohydrates into glycolytic/gluconeogenic intermediates, and (4) the generation of reducing equivalents in the form of NADPH (Ozer et al., 2002).

It is this latter function of the pentose phosphate pathway that is of particular relevance to the estivating snail. NADPH supplies the reducing power for the production of reduced glutathione and thioredoxin, two of the key antioxidant reducing agents in cells. Biosynthesis is not a priority for dormant animals, but protection against oxidative damage remains key for organisms that must remain viable over many weeks/months in a hypometabolic state

(Hermes-Lima et al., 1998; Storey & Storey, 2004). Indeed, although oxygen consumption is significantly reduced in estivation, and the generation of oxyradicals in tissues is generally proportional to oxygen consumption, the activities of a variety of antioxidant enzymes are elevated during estivation in *O. lactea*, including superoxide dismutase and catalase (Hermes-Lima & Storey, 1995; Hermes-Lima et al., 1998). The increase in antioxidant enzyme function during dormancy would prepare the estivating animal to deal with the large increase in oxyradical formation associated with arousal from dormancy, when oxygen is rapidly reintroduced in large amounts. Moreover, increased antioxidant capacity in estivating snails would be an adaptation that serves the animal well in dealing with intermittent sharp increases in tissue oxygenation (and oxyradical formation) brought about by discontinuous breathing patterns during estivation. For antioxidant defenses to be elevated during estivation, it follows that pools of reducing power must be available, and hence the regulation of the pentose phosphate pathway becomes important.

While preliminary analysis indicated no measurable changes in G6PDH enzyme kinetics in foot muscle or mantle tissues during estivation, G6PDH in hepatopancreas extracts from estivating *O. lactea* featured increased maximal enzyme velocities (V_{max}) in saturating substrate conditions, relative to G6PDH assayed from active snail extracts (Ramnanan & Storey, 2006a). This tissue-specific alteration of G6PDH in estivation is consistent with the notion that the hepatopancreas in snails, much like hepatic tissue in other animals, plays critical roles in biosynthesis, protection against xenobiotics, and protection against oxidative stress. Further analysis determined that the Michaelis constant (K_m), which represents the substrate concentration at which the reaction rate is one-half the V_{max}, for glucose-6-phosphate (G6P) was decreased during estivation in hepatopancreas. A reduced K_m can be interpreted to suggest that the enzyme's affinity for that substrate has increased. Given that G6P concentrations are known to be reduced by nearly 70% in the hepatopancreas of estivating *O. lactea* (Churchill and Storey, 1989), reduced K_m for G6P could be an adaptation that brings G6PDH enzyme kinetics in line with substrate availability. In any case, it appears that an estivation-dependent response in *O. lactea* hepatopancreas results in a more active form of G6PDH. Moreover, given that PFK (a key control enzyme of glycolysis) is regulated in an inverse manner to G6PDH [such that PFK displayed reduced V_{max} and increased K_m for its substrate fructose-6-phosphate (F6P)], it appears as these enzymes are regulated in a coordinated fashion to reduce G6P carbon flux through glycolysis and enhance

G6P carbon flux through the pentose phosphate pathway during the estivating state.

We then incubated hepatopancreas extracts in different conditions that stimulated specific endogenous protein kinases or specific protein phosphatases, prior to assay for G6PDH activity (Ramnanan & Storey, 2006a). Incubation of active snail hepatopancreas extracts in conditions that stimulated endogenous PKG activity increased G6PDH activity to levels comparable to those seen in estivating *O. lactea* hepatopancreas. Conversely, incubation of estivating snail hepatopancreas extracts in conditions that favored PP1 activity reduced G6PDH activity to levels seen in active snails. In addition, chromatographic isolation and profile of G6PDH activity from tissue extracts revealed two major peaks of enzyme activity, where the first peak featured enzyme kinetics consistent with the lower activity form of the enzyme and the second peak featured enzyme kinetics characteristic of the higher activity form of the enzyme (Ramnanan & Storey, 2006a). Notably, active snails featured a proportionally larger first peak of G6PDH activity and estivating snails featured a proportionally larger second peak of G6PDH activity. Finally, incubation of active snail hepatopancreas extracts in conditions that promoted endogenous kinases (particularly PKG) converted the subsequent chromatographic G6PDH profile into a peak pattern (i.e., larger second peak) resembling that of estivated snails. Similarly, incubation of hepatopancreas extracts from estivating snails in conditions that promoted PP1 activity was able to convert the subsequent chromatographic profile into a pattern that was similar to that of active snails (i.e., larger first peak). Taken together, these experiments support the notion that the pool of G6PDH enzymes in the hepatopancreas of estivating *O. lactea* features a larger proportion of relatively highly phosphorylated, higher activity enzyme with greater substrate affinity (Ramnanan & Storey, 2006a). While these studies in estivating *O. lactea* were the first to demonstrate hypometabolism-dependent changes in G6PDH activity were related to phosphorylation state, subsequent studies confirmed that G6PDH kinetics were similarly regulated in the hypometabolic states conferring anoxia tolerance in the marine mollusc *Littorina litorrea* (Lama et al., 2013), and the freshwater crayfish *Orconectes virilis* (Lant & Storey, 2011). Thus, the evidence suggests that carbohydrate flux through the pentose phosphate pathway, unlike flux through glycolysis, may benefit dormant animals by increasing the capacity for these animals to cope with oxidative stress, and this flux can be regulated by modifying the phosphorylation state of G6PDH.

8.7 ATP-DEPENDENT ION PUMPS ARE SUPPRESSED IN DORMANT SNAILS BY REVERSIBLE PHOSPHORYLATION

The Na^+K^+-ATPase has a critical function in the maintenance of plasma membrane potential difference in all animal cells, pumping sodium and potassium ions against their concentration gradients to maintain high sodium levels outside cells and high potassium levels inside cells. The Na^+K^+-ATPase consumes a considerable amount of cellular energy. In resting endotherms, this ion pump is responsible for 5–40% of total ATP consumption (Clausen, 1986). The activity of Na^+K^+-ATPase can be modified via reversible phosphorylation by several protein kinases (Lopina, 2001). It is essential that transmembrane sodium and potassium gradients are maintained, even during periods of dormancy, to permit cellular living conditions despite strongly suppressed rates of ATP turnover. This requires coordinated suppression of the rates of Na^+ and K^+ movements through ion channels (termed channel arrest) and oppositely directed ATP-driven ion pumps to match the rates of ATP availability from catabolic pathways (Hochachka, 1986). We hypothesized that Na^+K^+-ATPase activity would be decreased during the estivating state in tissues of *O. lactea*, thereby resulting in substantial ATP savings for the animal. Further, we proposed that the mechanism involved would be phosphorylation.

Our study of *O. lactea* Na^+K^+-ATPase determined that activity is strongly suppressed in multiple tissues during estivation, and this suppression was independent of any substantial alteration in protein content (Ramnanan & Storey, 2006b). The lower Na^+K^+-ATPase activity observed in estivating tissue extracts was coincident with reduced affinities for substrates (sodium ions, ATP) and co-substrates (magnesium ions). In addition, we assessed V_{max} over a range of assay temperatures to calculate Arrhenius activation energy. The Arrhenius activation energy associated with the estivating Na^+K^+-ATPase was 1.5-fold greater than that of the ion pump from active snails, consistent with a less active form of the enzyme. Finally, in vitro incubations promoting the activity of several protein kinases (PKA, PKC, and PKG) were shown to reduce Na^+K^+-ATPase activity in extracts of active snails to the levels seen in extracts isolated from estivating animals; conversely, stimulation of specific protein phosphatases (PP1 and PP2A) raised the activities measured in tissue extracts from estivated animals back to the levels seen in active snails. Phosphorylation can either inhibit or stimulate Na^+K^+-ATPase activity, depending on cellular context (Lopina, 2001). In *O. lactea*, it was clear that phosphorylation inhibits the enzyme, thereby contributing substantially to the overall decrease in ATP consumption in snail tissues that defines

estivation (Ramnanan & Storey, 2006b). The reduction of Na^+K^+-ATPase activity during a hypometabolic state was also associated with a change in phosphorylation state in the hibernating ground squirrel (MacDonald & Storey, 1999), indicating that this mechanism could be employed across animal species that utilize metabolic rate depression as a survival strategy.

Another energetically taxing ion pump in living cells is the sarcoendoplasmic reticulum calcium ATPase (SERCA). This enzyme serves to maintain and/or restore calcium gradients at a considerable ATP cost. Analysis of SERCA kinetics in *O. lactea* demonstrated that estivating tissues featured a lower activity form of the SERCA enzyme (Ramnanan & Storey, 2008). Interestingly, the low activity form that was present in estivation demonstrated increased kinetic (substrate affinity for Mg-ATP was maintained over varying temperatures) and conformational (increased resistance to denaturation in presence of increasing urea concentrations) stability. Again, we proposed that phosphorylation could have been responsible for the estivation-dependent alterations in SERCA (Ramnanan & Storey, 2008). In vitro incubations of tissue extracts from active *O. lactea* snails in conditions that promoted several endogenous kinases decreased SERCA activity to levels comparable to the low SERCA activity observed in estivating animals. Conversely, incubation of extracts from estivating animals in conditions that stimulated endogenous PP2A (in foot muscle) and endogenous PP2C (in hepatopancreas) resulted in elevated SERCA activity similar to levels seen in active animals. Taken together, our data suggested that SERCA could be downregulated in hypometabolic conditions, and this decrease was the result of changes in phosphorylation state. Estivation-dependent phosphorylation and alteration of SERCA activity has since been observed in both freeze-tolerant and freeze-avoiding insects (McMullen et al., 2010) and anoxia-tolerant turtle tissues (Ramnanan et al., 2010b), which suggests a high degree of conservation of this mechanism across species that naturally depress their metabolic rates in response to unfavorable environmental conditions.

8.8 PROTEIN TURNOVER IS REGULATED BY PHOSPHORYLATION IN ESTIVATING *O. LACTEA*

Much like ion pumping, protein synthesis is a very energy and resource intensive cellular process, hydrolyzing four ATP equivalents for every peptide bond synthesized. Not only are proteins continually being synthesized under normal metabolic conditions, but the protein population also is continually being turned over, as proteins are eventually degraded in a specifically

targeted, carefully regulated manner. It follows that a physiologic state of metabolic rate depression would include, as a key contributing component, a massive reduction in the rate of overall protein synthesis. Further, to maintain homeostasis, the overall rate of protein degradation would likely be reduced in concert in an estivating animal (Storey & Storey, 2004).

In vitro assay of protein synthesis revealed marked (~80%) reductions in *O. lactea* tissues early (2 days, the earliest time point measured) into the dormant period, and this suppressed level was maintained at later (14 days) stages of estivation (Ramnanan et al., 2009). Thus, the substantial suppression of protein synthesis could be considered as happening at an early stage of dormancy, thereby bringing about energetic savings at the onset of estivation. This was consistent with the reductions in protein synthesis previously observed in the estivating desert frog *Neobatrachus centralis* (Fuery et al., 1998) and the estivating snail *Helix aspersa* (Pakay et al., 2002). There are several levels by which protein synthesis rates could be regulated in estivating snails. We first looked at expression levels of key molecular regulators of ribosomal biogenesis. The transcriptional activator c-Myc and the transcriptional repressor Mitotic Arrest Deficient-1 (MAD1) are determining factors in the synthesis of the upstream binding factor (UBF), a key regulator of ribosomal DNA (rDNA) expression (Poortinga et al., 2004). We observed decreases in protein levels of c-Myc, increases in protein levels of MAD1, and reduction in UBF protein levels, consistent with reduced ribosome formation (Ramnanan et al., 2009). However, alterations in MAD1 and UBF were only apparent at 14 days, meaning that alterations in ribosomal biogenesis could not be the determining factor mediating the substantial decrease in protein synthesis evident after 2 days of estivation. These changes may, however, play a role in bringing about ribosomal machinery changes suited for longer term dormancy.

Protein synthesis can also be regulated by the level of covalent phosphorylation of key regulators of protein translation. In conditions of nutrient excess, the mTOR (mammalian target of rapomycin) protein kinase becomes hyperphosphorylated, which increases its protein synthesis-promoting activity (Gingras et al., 2001). The mTOR kinase then phosphorylates two key substrates that are, in turn, key regulators of protein translation. The first of these substrates is a regulatory kinase of approximately 70 kDa in size that phosphorylates and modifies several residues on the ribosomal S6 subunit protein. Phosphorylation of this 70 kDa S6 kinase (or p70S6K) by mTOR typically occurs during nutrient-rich conditions that favor growth and proliferation, and phosphorylated p70S6K results in enhanced rates of 5′ terminal oligopyrimidine tract (TOP) translation. The second of these mTOR targets is the binding protein of the eukaryotic initiation factor eIF4E (4E-BP).

In fasting or nutrient poor conditions, 4E-BP1 binds to eIF4E, preventing the association of eIF4E with eIF4G. In nutrient-rich conditions, 4E-BP1 becomes phosphorylated, releasing eIF4E from its inhibitory binding, which permits eIF4E and eIF4G interaction to promote cap-dependent translation. Although we characterized no alteration in mTOR kinase phosphorylation (Ramnanan et al., 2007), decreased levels of p70S6K and 4E-BP1 phosphorylation were observed in estivating snail tissues (Ramnanan et al., 2009). These decreases were consistent with and indicative of decreased protein translation in the dormant state.

That altered p70S6K and 4E-BP1 phosphorylation were observed, independent of measurable changes in mTOR phosphorylation (and therefore mTOR activity) in estivation, raised the possibility that other translation factors were differentially regulated in dormancy as well in this model system. We then performed a thorough analysis of the phosphorylation state of protein translation factors implicated in the control of protein synthesis. The eukaryotic initiation factor eIF2α plays an important role in driving translational initiation by facilitating the necessary GTP-GDP exchange activity of the eIF2B complex (Proud, 2006). This function of eIF2α is inhibited when this factor is phosphorylated. Similarly, the elongation factor eEF2 mediates ribosome translocation along the mRNA strand after addition of an amino acid, and phosphorylation of eEF2 by an upstream kinase inhibits binding of this factor to the ribosome, thereby suppressing translation. In estivating *O. lactea*, levels of total eIF2α and eEF2 both decreased relatively slowly (reduced protein levels were not observed after 2 days of estivation but were observed after 14 days of dormancy). On the other hand, levels of eIF2α and eEF2 phosphorylation were evident relatively early (after 2 days) in estivation (Ramnanan et al., 2009). Thus, there appears to be at least two mechanisms by which these loci are regulated to inhibit energetically taxing protein translation: phosphorylation events that happen early in dormancy and reduced protein levels that happen at a later point in the estivating state. It is probable that the phosphorylation events involving these two factors evident early in estivation played some role in the substantial decreases of protein translation measured in vitro. In any case, by 14 days of estivation the ratio of phosphorylated protein to total protein for both eIF2α and eEF2 had substantially increased in both foot muscle and hepatopancreas (Ramnanan et al., 2009), indicating that the capability for protein translation initiation and elongation were markedly reduced.

Possible estivation-dependent regulation of eIF4E or eIF4GI, two components of the eIF4F complex that is responsible for the rate-limiting process of cap dependent mRNA recruitment to the ribosome, was also

characterized (Ramnanan et al., 2009). Moreover, expression of eIF4E and eIF4GI proteins at the genetic level are determined by the interplay of the transcriptional activator c-Myc and the transcription repressor MAD1; c-Myc and MAD1 were observed to be upregulated and downregulated, respectively, in estivation by 14 days (Ramnanan et al., 2009). Generally, protein levels of eIF4E or eIF4GI were not altered in either foot muscle or hepatopancreas at either 2 days or 14 days of estivation, indicating that these loci were not regulated at the genetic level. However, the amount of phosphorylated eIF4E was decreased in foot muscle (but not hepatopancreas) and the level of phosphorylated eIF4GI was decreased in hepatopancreas (but not foot muscle). These decreases were only apparent after 14 days of estivation. Counter to the case with eIF2α and eEF2, phosphorylation of eIF4E and eIF4GI enhances the translational activity of the eIF4F complex (Proud, 2006). It appears that these two proteins are regulated in a tissue-specific manner in *O. lactea*, with the net result in either tissue being a reduction of translation (Ramnanan et al., 2009). While these phosphorylation events are temporally discordant with and do not contribute to the substantial reduction in protein synthesis rates observed in vitro from 2 days estivating snails, it may be that the reduced phosphorylation of eIF4E (in foot muscle) and of eIF4GI (in hepatopancreas) may be factors in sustaining the profound reductions in protein synthesis that will endure for the duration for dormancy.

Because we observed reductions in protein synthesis rates in vitro and characterized alterations in biochemical markers that would seem to suggest that protein translation and ribosomal biogenesis are downregulated in estivation, it would follow that protein degradation would be globally decreased in concert. This would permit an increase in the lifetime of intracellular proteins during the dormant phase. A coordinated decrease in protein degradation would prevent or at least delay animal tissues from entering a state of negative protein balance, which would facilitate a return to normal life function upon arousal from estivation. Protein carbonyl levels were not elevated in estivating *O. lactea* (Ramnanan et al., 2009), indicating that the level of oxidatively damaged proteins was not increased during estivation (an aerobic, oxidative condition). Moreover, expression of selected heat shock proteins was increased during estivation (particularly in hepatopancreas), suggesting that the estivating animal has a greater capacity to protect unfolded proteins from being degraded during dormancy (Ramnanan et al., 2009). The last question to address was whether protein degradation rates per se were downregulated in estivation.

The majority of intracellular protein degradation is mediated by the multicatalytic proteinase (MCP) complex (Orlowski & Wilk, 2003).

Proteolytic activity of the 20S proteasome (a significant component of the MCP complex) in vitro was markedly reduced in both hepatopancreas and foot muscle of estivating *O. lactea*. Decreased activity was generally associated with increased K_m values for substrates, suggesting lower substrate affinity (Ramnanan et al., 2009). These kinetic changes in 20S proteolytic activity were observed in the absence of any measurable decrease in 20S expression. As several subunits of the 20S proteasome are known to be phosphorylated, it was possible that the decrease in 20S proteasome function was mediated by an increase in phosphorylation state. Incubation of hepatopancreas extracts from active snails with 8-bromo-cGMP (a more stable, potent activator of PKG, as compared with endogenous, labile cGMP) decreased 20S proteasome activity to levels that approached the low proteasome activity seen in dormancy (Ramnanan et al., 2009). On the other hand, incubation of hepatoapancreas extracts from either active or estivating snails in conditions that permitted ion-independent phosphatase (PP1/PP2A) activity enhanced 20S proteasome activity. Further, this stimulation of the 20S proteasome was fully abolished when PP2A was completely inhibited with nanomolar amounts of okadaic acid. Thus, PKG and PP2A appear to mediate the phosphorylation and dephosphorylation, respectively, of the 20S proteosome in *O. lactea* in an estivation-dependent manner (Ramnanan et al., 2009). Thus, our data characterize a coordinated suppression of both global cellular protein synthesis and protein degradation rates that accompany transition to the estivating state, which serves the dormant *O. lactea* organism well in terms of conserving energy (Fig. 8.1).

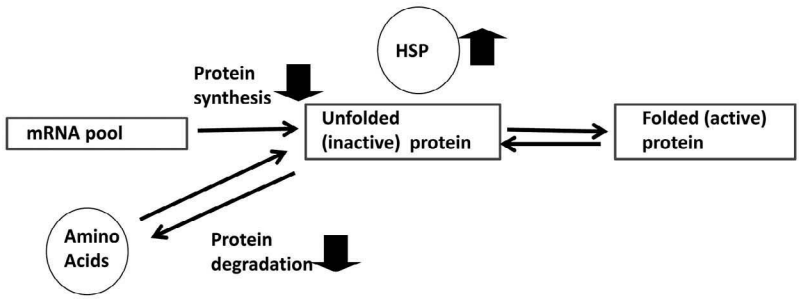

FIGURE 8.1 The global suppression of protein metabolism during estivation in *O. lactea*. Overall protein synthesis and protein degradation rates are both substantially suppressed, as indicated by various indices indicated in the text (differential phosphorylation states of protein translation initiation and elongation factors, reduced activities of protein synthesis, and multicatalytic protein as in vitro). In addition, prolonged lifetime of unfolded proteins may be facilitated by increased expression of chaperone heat shock proteins (HSPs), which serves the animal well upon arousal from the dormant state.

8.9 A ROLE FOR THE AKT SIGNALING PATHWAY IN ESTIVATION

The serine/threonine kinase Akt (also called protein kinase B; PKB) is well-known to be a key downstream regulator of growth, proliferation, and survival and has been perhaps best defined in terms of mediating the downstream response of the anabolic hormone insulin (Ramnanan et al., 2010a). Insulin binding to its receptor can trigger the phosphoinositide 3 kinase (PI3K)/3-phosphoinositide-dependent (PDK1) signaling cascade, which leads to the phosphorylation of Akt at several sites; assessment of Akt phosphorylation at the Ser473 residue is typically considered an index of Akt activation. Phosphorylated, active Akt mediates several different protein responses. In response to a physiologic rise in circulating insulin, for example, phosphorylated Akt can phosphorylate and inactivate glycogen synthase (GS) kinase 3β (GSK3β), which prevents GSK3β from phosphorylating and inhibiting GS, permitting anabolic carbohydrate metabolism and glycogen deposition. Indeed, the time course and magnitude of Akt phosphorylation and activation in vivo is closely related to the time course and magnitude of GSK3β phosphorylation (Ramnanan et al., 2010a, 2011–2013). As discussed previously, Akt can also bring about mTOR phosphorylation which enhances protein synthesis (Proud, 2006). In addition, Akt can phosphorylate transcriptional regulators such as members of the forkhead box, class O (FOXO) family of transcription factors. Dephosphorylated FOXO proteins reside in the nucleus where they are free to drive the expression of genes involved in gluconeogenesis, cell cycle arrest, and pro-apoptotic regulators (Ramnanan et al., 2010a). Upon phosphorylation, FOXO proteins exit the nucleus and are sequestered in the cytoplasm where they are transcriptionally inert (Rivera et al., 2010). Similarly, Akt can also mediate phosphorylation of the Bcl-2-associated death (BAD) promoter proteins. Dephosphorylated BAD proteins form a complex with Bcl-2, blocking Bcl-2 from inhibiting the pro-apoptotic activity of the Bax protein. On the other hand, phosphorylation of BAD by Akt releases Bcl-2 from BAD-mediated inhibition, which permits Bcl-2 to suppress the pro-apoptotic activity of Bax (Zhang et al., 2011).

We observed that Akt phosphorylation increased approximately 40% in both foot muscle and hepatopancreas, and these increases were correlated with twofold increases in Akt V_{max} and reduced affinity toward its synthetic peptide substrate (Ramnanan et al., 2007). These alterations in Akt enzyme kinetics were coincident with increased protein stability (as measured by increased resistance to denaturation in the presence of urea). Incubation of tissue extracts from active snails in conditions that stimulated endogenous protein kinases (likely PKA or PKG) led to increases in assayed Akt activity,

while incubation of tissue extracts from dormant snails in conditions that promoted endogenous protein phosphatases (likely PP2A or PP2C) reduced assayed Akt activity. The question arises as to why Akt, a kinase with well-characterized anabolic, ATP-consuming downstream effects, would be activated in estivating *O. lactea*, an animal model where ATP is at a premium and biosynthetic pathways are generally suppressed as part of a wholesale move to a depressed metabolic state. We confirmed that mTOR phosphorylation was no different in estivating versus active snails, and as discussed previously, multiple lines of evidence supported the notion that both protein synthesis and protein degradation rates were reduced in concert in dormant snails. With regards to carbohydrate metabolism, Akt activation was discordant with changes in GSK3β, where decreased phosphorylation and increased kinetic activity were both observed in estivation, changes consistent with suppressed glycogen synthesis (Ramnanan et al., 2007). On the other hand, phosphorylated Akt was correlated with increases in measured phosphorylation of both FOXO and BAD proteins. Thus, it appears as though Akt activation in this estivating model is uncoupled from energetically taxing anabolic processes involving both protein and carbohydrate biosynthesis, but is correlated with mechanisms that enhance cell survival and suppress apoptosis, which would facilitate cell survival throughout the estivating state (Fig. 8.2).

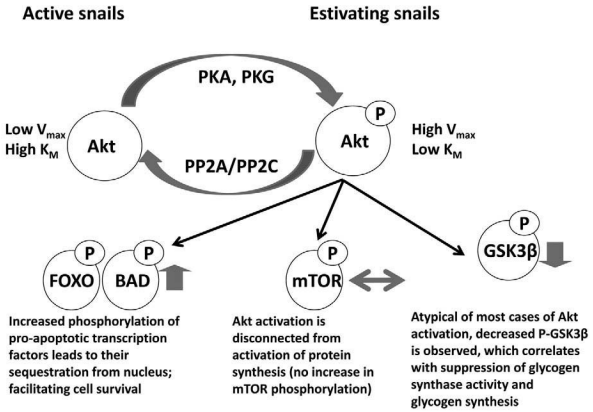

FIGURE 8.2 The stimulation of the master metabolic regulator AMPK during estivation in *O. lactea*. Increased activity and phosphorylation of AMPK in this model (likely mediated by either LKB1, a known upstream regulator of AMPK, or PKG, a kinase that has established regulatory capacity across the mollusk phylum) phosphorylates and regulates many metabolic enzymes, including acetyl-CoA carboxylase (ACC), a rate-determining enzyme of fatty acid synthesis. Increased ACC phosphorylation in estivation leads to decreased ACC activity and decreased formation of high activity ACC polymers, which is well-suited to the overriding priority of the animal to conserve energy stores during the hypometabolic period.

8.10 THE ROLE OF AMPK IN METABOLIC RATE DEPRESSION IN ESTIVATING *O. LACTEA*

Unlike Akt, a signaling system typically associated with conditions of nutrient excess or anabolic states, AMPK is characterized as a molecular regulator that is sensitive to elevated AMP levels (which demarcates catabolic or energy-depleted states) and responds to restore cellular energetic balance by suppressing ATP-consuming anabolic activities and promoting ATP-generating catabolic processes (Hardie & Carling, 1997). AMPK has been defined to suppress ATP-intensive processes including fat, cholesterol, protein, and glycogen biosynthesis in various animal and nonanimal cells and tissues. AMPK was therefore a likely candidate for estivation-dependent regulation in *O. lactea.*

Characterization of AMPK phosphorylation (an index of its activity) revealed substantial activation of the enzyme early (2 days) into the estivating condition, and AMPK phosphorylation was maintained at this elevated level at a later stage (14 days) in dormancy (Ramnanan et al., 2010c). Kinetic analysis of enzyme activity indicated that V_{max} was elevated as well in estivation. The observations that AMPK affinity for its peptide substrate increased and Arrhenius activation energy decreased in estivation supports the notion that AMPK functions at a higher level in dormancy in this model. While AMPK can be activated by either elevated AMP levels or phosphorylation, AMP levels do not measurably increase in estivating *O. lactea* tissues. As such, it is likely that phosphorylation of AMPK drives the increase in its activity seen in estivation. There are several protein kinases established as being upstream regulators of AMPK phosphorylation, and of these kinases, the phosphorylation of LKB1 was temporally associated with AMPK activation in this estivating model. Moreover, increased AMPK protein and activity were observed in LKB1 immunoprecipitation preparations from estivating extracts, indicative of an estivation-dependent increase in LKB1–AMPK interaction. In addition, our incubation experiments suggested that PKG and PP2A had the ability to phosphorylate and dephosphorylate, respectively, AMPK in a manner consistent with elevated AMPK activity in estivating snails and decreased AMPK activity in dormant snails (Ramnanan et al., 2010c).

After confirming that AMPK was activated in the estivating snail, likely by LKB1 in vivo, we next elucidated the consequences of elevated AMPK activity in estivating *O. lactea*. One of the best characterized substrates of AMPK activity is acetyl-CoA carboxylase (ACC), an enzyme that catalyzes the conversion of acetyl-CoA to malonyl-CoA. ACC exists in two forms:

ACCα is present in the cytoplasm and is the rate-limiting enzyme in fatty acid biosynthesis, while ACCβ co-localizes with mitochondria and serves to inhibit (via its product malonyl-CoA) the outer mitochondrial membrane enzyme carnitine palmitoyltransferase (CPT1), which regulates fatty acid transport into mitochondria for oxidation (Hardie & Pan, 2002). In the context of estivation, given that snails rely primarily on carbohydrates during the estivating period and repletion of fatty acid stores is not likely a cellular priority, it would be reasonable to predict that ACC would be deactivated in estivating *O. lactea*. Kinetic analysis of ACC activity revealed a less active form of the enzyme in estivation, one with decreased V_{max}, reduced substrate affinity for Mg^{2+}-ATP, and (in foot muscle only) reduced ability to be activated by citrate (Ramnanan et al., 2010c). Citrate is a powerful regulator of ACC activity in vivo and tends to accumulate in the fed (nutrient-rich) state, stimulating the aggregation of ACC monomers (where ACC has relatively low activity) into ACC polymers that promote increased ACC function. Tissue extracts (from active snail foot muscle) were incubated in different conditions before being subjected to gel-filtration chromatography (Ramnanan et al., 2010c). These experiments determined that ACC activity exists in two fractions in *O. lactea*, a large molecular weight polysome fraction and a small molecular weight monosome fraction. Incubation in conditions that stimulate AMPK shifts the ACC activity profile into one that featured the monosome fraction only, consistent with the principle that AMPK phosphorylates and deactivates ACC. On the other hand, incubation of extracts with a saturating concentration of citrate shifted the ACC activity profile into one that exclusively featured a polysome fraction (Ramnanan et al., 2010c). Finally, tissue extracts were incubated in conditions that included excess citrate and that were permissive to endogenous AMPK activity. In this setting, AMPK was able to prevent the ability of citrate to stimulate ACC activity and promote ACC polysome formation, providing insight into the mechanism by which AMPK modifies ACC activity in a physiologic state of metabolic rate depression (Fig. 8.3).

We then characterized the protein levels and phosphorylation state of AMPK targets in both foot muscle and hepatopancreas (Ramnanan et al., 2010c). In both of these tissues, phosphorylation of both ACCα and ACCβ were increased in estivation, suggesting that the enzymatic capability for fatty acid synthesis is suppressed and for fatty acid oxidation is increased during dormancy. However, given that net fatty acid oxidation does not occur in estivating snails until several months into dormancy (when carbohydrate reserves have been exhausted), it is unlikely that differential ACCβ regulation plays any early role in estivating *O. lactea* tissues. While gluconeogenic

and lipogenic gene expression were both suppressed in hepatopancreas (as would be expected in a state where AMPK is activated), gene expression related to mitochondrial biogenesis in foot muscle was not increased (which is discordant with typical states associated with AMPK activation). It may be that mitochondrial biogenesis may not be a driving priority in estivation as it would be in other states (such as exercise stress) that are associated with increased AMPK function. In any case, increased phosphorylation of GS was observed in foot muscle, consistent with the decreased levels of GSK3β phosphorylation (and increased levels of GSK3β activity) previously described in estivating *O. lactea* (Ramnanan et al., 2007). It must be noted that AMPK is suggested to drive opposite regulation of GS (inactivation) and GP (activation) to maintain whole body homeostasis in other physiological (hyperglucagonemia and/or hyperglycemia) conditions (Rivera et al., 2010). Our studies in *O. lactea* suggest that activated AMPK can play some part in the coordinated suppression of both GS and GP (decreasing rates of both glycogen synthesis and breakdown) in the context of a physiologic state of metabolic rate depression,, and that this suppression of glycogen metabolism is in line with the overall priority of the animal to suppress metabolism.

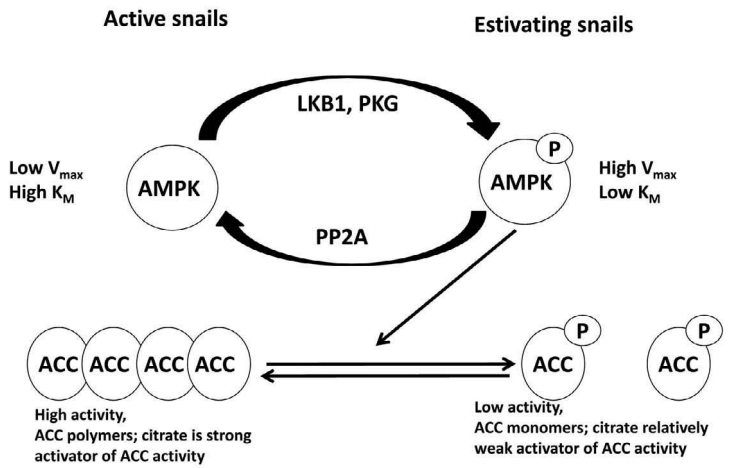

FIGURE 8.3 The phosphorylation and activation of Akt during estivation in *O. lactea*. In most physiologic systems, Akt activation leads to phosphorylation of targets that facilitate anabolism and growth. In the estivating snail, Akt is activated, and leads to phosphorylation and inactivation of pro-apoptotic transcription factors FOXO and BAD. However, activation of Akt does not result in increased levels of mTOR or GSK3β phosphorylation, and therefore does not facilitate protein synthesis or glycogen synthesis. This suggests that Akt can enhance pro-survival signaling in the absence of promoting energy-intensive anabolic cellular activities, in a state of hypometabolism.

8.11 DIFFERENTIAL REGULATION OF TYPE-1 PROTEIN PHOSPHATASE (PP1) ACTIVITY IN ESTIVATING SNAILS

To date, reversible phosphorylation has been clearly established as a dominant regulatory mechanism in estivating *O. lactea*. In this chapter, we have discussed in detail the role of phosphorylation in the estivation-dependent modification of proteins and enzymes involved in carbohydrate metabolism, ion pumping, protein synthesis,and degradation, transcriptional regulation, and signaling pathways (Akt, AMPK) with far-reaching downstream effects. If all of these processes are, at least in part, modified by differential phosphorylation state during the transition to the estivating condition, it stands to reason that protein dephosphorylation is differentially regulated as well. PP1 is a far-reaching phosphatase with known targets that bring about structural, metabolic, translational and transcriptional effects (Cohen, 1989). While PP1 did not have a major role in the estivating toad (Cowan et al., 2000), specific functions of this phosphatase have been elucidated in other forms of hypometabolism (MacDonald & Storey, 2002, 2007). Given its ubiquitous nature, we proposed that PP1 would be differentially regulated in estivating *O. lactea* (Ramnanan & Storey, 2009).

PP1 activity (V_{max}) was reduced in estivating tissues and several kinetic parameters were indicative of a less active enzyme in the estivating animal (Ramnanan & Storey, 2009). The PP1 catalytic subunit (PP1c) was subsequently purified and determined to be approximately 39 kDa in size, similar to sizes seen across other species, and with similar enzyme kinetics. However, the purified PP1c did not display altered enzyme kinetics between active and estivating snail tissue extracts, likely due to the fact that functional PP1c activity in vivo is largely determined by PP1c binding to regulatory subunits that target and localize PP1c activity (Cohen, 1989). This aspect of PP1c regulation is lost when assaying PP1 activity in crude extracts. As such, we prepared PP1 samples in a manner that would permit PP1c complexes with targeting proteins and subjected these preparations to gel-filtration chromatography (Ramnanan & Storey, 2009). This experiment yielded four major peaks of activity, including peaks associated with protein sizes of 257 ± 8 kDa (which featured high substrate affinity when assayed) and 76 ± 2 kDa (which featured low substrate affinity when assayed). Moreover, active snails had a greater proportion of PP1c activity associated with the 257 kDa/high substrate affinity complex and a relatively small proportion of PP1c associated with the 76 kDa/low substrate affinity complex. Conversely, the distribution of the PP1c population shifted in estivating samples, with less phosphatase activity (and PP1c content) associated with the 257 kDa peak,

and increased phosphatase activity (and PP1c content) associated with the 76 kDa peak. In addition, immunoblotting confirmed that the 76 kDa peak contained both PP1c and the nuclear inhibitor of PP-1, a known and relatively well-conserved regulatory targeting subunit of PP1c (Ramnanan & Storey, 2009). Finally, subcellular fractionation studies indicated that the PP1c activity in nuclear and glycogen-associated fractions were increased and decreased, respectively, in estivation (Ramnanan & Storey, 2009). The decrease in glycogen-associated PP1c fits with the overall suppression of glycogen metabolism described earlier. For example, in hepatic tissue, PKA and PP1 mediate opposing effects on the rate-determining enzymes of glycogenolysis and glycogen synthesis (Ramnanan et al., 2010a, 2011). PKA catalyzes the phosphorylation of GP and GS, favoring net glycogenolysis, and PP1 dephosphorylates GP and GS, resulting in net glycogen synthesis. That PP1c association with the glycogen fraction is decreased in estivation is consistent with the previous observation that PKA activity is suppressed during dormancy in this model, and is consistent with the observation that GS phosphorylation is relatively enhanced (and GS is relatively less active) in estivation (Ramnanan et al., 2010c). Decreased glycogen-associated PP1c in estivating *O. lactea* is consistent with a similar finding in hibernating ground squirrels (MacDonald & Storey, 2007).

8.12 SUMMARY AND FUTURE DIRECTIONS

While reversible protein phosphorylation has been established as a key intracellular theme biochemical adaptation in various hypometabolic states (Storey & Storey, 2004), the work done in the estivating land snail *O. lactea* over the previous decade has clearly established that this mechanism is critical in preparing for, and facilitating survival during, dormancy. In this chapter, we detailed the estivation-dependent changes in protein phosphorylation that have profound influences on carbohydrate metabolism, the production of reducing equivalents to support antioxidant capacity, the suppression of ATP-intensive processes such as ion pumping and protein translation, the coordinated reductions of protein synthesis and degradation rates, the differential targeting of protein phosphatase type-1, and selective activation of specific arms downstream of the Akt and AMPK signaling pathways. There are other candidate molecules that could have major regulatory capacity in dormant *O. lactea*. Given that cGMP levels are known to be elevated during the early hours of estivation (Brooks & Storey, 1996), and that cGMP and PKG has been implicated in the control of many of the

estivating-dependent alterations characterized earlier in the chapter, ongoing experiments are defining PKG enzyme kinetics in estivating *O. lactea*. PKG seems to be central to cellular regulation across the phylum Mollusca (del Pilar Gomez & Nasi, 2005; Sung et al., 2004), including other species that utilize hypometabolic survival strategies (Larade & Storey, 2004). To date, preliminary studies have indicated that PKG may have altered enzyme kinetics and differential localization in estivating *O. lactea* (Ramnanan and Storey, unpublished data). Future studies may prove that PKG is, indeed, a master regulator in the move to a depressed metabolism in this remarkable species of desert snail.

KEYWORDS

- **metabolic rate depression**
- **reversible protein phosphorylation**
- **metabolic pathways**
- **oxidative stress**

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CHAPTER 9

GASTROPOD ECOPHYSIOLOGICAL RESPONSE TO STRESS

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CONTENTS

ABSTRACT

Gastropods have colonized marine, freshwater, and terrestrial ecosystems, and the extraordinary diversification of their habitats makes them a group of high ecological importance. They may be exposed to various environmental stressors of natural or human origin, and thereby forced to acclimate or adapt to a highly changing and often stressful, if not toxic, environment. Using examples chosen from the most recent literature, this chapter reviews the various aspects of gastropod ecophysiological response to natural and anthropogenic stressors. Biological responses are presented in their diversity, from stress signaling to successive steps and components of the stress response including oxidative stress, detoxification systems, macromolecules alterations, and apoptosis. A specific section is devoted to the response of the immune system to stressors, including parasites. The chapter ends with some evolutionary perspectives associated with stress responses.

9.1 INTRODUCTION

Gastropods are by far the most speciose class of molluscs, widespread on nearly all possible environments on Earth (Hyman, 1967). As a unique feature in the phylum, gastropods have indeed colonized marine, freshwater, and terrestrial ecosystems, and the extraordinary diversification of their habitats makes them a group of high ecological importance. Gastropods are diverse in many other aspects, including reproduction (gonochorism, parthenogenesis, sequential or simultaneous hermaphroditism, self-fertilization ability), dispersal (from sedentary to pelagic life style), while retaining unique developmental and anatomical characteristics (e.g., torsion, radula). Last, gastropods are currently exposed to intense environmental pressure related to intensification of human activities, and thereby forced to acclimate or adapt to a highly changing and often stressful, if not toxic, environment.

Molluscs are particularly sensitive to chemical pollutants, which makes them well suited as bioindicators of environmental quality (Oehlman and Schulte-Oehlman, 2003). From the IUCN Red List (www.iucnredlist.org), 90% of molluscan species classified as near threatened, vulnerable, endangered, critically endangered, extinct in the wild, or extinct, are gastropods, with a total of 2541 species. Therefore, with regard to conservation, it is of utmost importance to improve the understanding of stress response in this group.

This chapter is devoted to gastropod ecophysiological response to environmental stress, including natural and anthropogenic stressors. Considering the broad scope of this topic, it was obviously impossible to provide a detailed analysis of all possible strategies developed by this group to deal with environmental variation and stress. As not treated elsewhere in this book, a specific section on immunological stress was also included. We have then opted for a general overview of the multiple facets of gastropod stress response, illustrated by examples chosen from the most recent literature. Following an introductory part devoted to stress definitions, biological responses are presented in their diversity, from stress signaling to successive steps and components of the stress response. The chapter ends with some evolutionary perspectives.

9.2 STRESS, STRESSORS, AND STRESS RESPONSE

9.2.1 MULTIPLE VIEWS ON STRESS IN BIOLOGY AND ECOPHYSIOLOGY

The term "stress" is probably among the most controversial biological terms. It originates in physics to describe pressure and deformation in a system. Following the work of Selye (1950) who recognized a similar suite of coordinated reactions to diverse noxious stimuli or "agents" in mammals as a "General Adaptation Syndrome", it has been widely applied into a biological context. Selye's model includes three stages. After an alarm initial stage, which includes a shock phase (changes in several organic systems) and a counter-shock phase (defensive response, including increased production of hormones), the organism attempts to resist or adapt to the stressor (resistance stage). The process eventually goes into an exhaustion stage, during which energy is depleted, presumably leading to death.

As stress can be applied to various levels of biological organization, the term is used in many different areas, especially in ecophysiology, ecology, toxicology, and ecotoxicology. In ancient works, "stress" has been used to refer both to the event or agent causing a response in an organism (e.g., change in temperature, attack by a predator) and to the response in itself. More recently, the triggering external stimuli have been named "stressors" while "stress" is the internal state brought about by a stressor (sometimes identified as a response syndrome), and "stress response" is a cascade of internal changes triggered by stress (Van Straalen, 2003).

Grime's definition of stress, "external constraints limiting the rates of resource acquisition, growth or reproduction of organisms" (Grime, 1989) has been widely used in ecology (see Borics et al., 2013), but it does not specify any component of temporal dynamics. "Biological stress" is used in the literature to describe any condition that forces living systems away from a physiological steady state (Kagias et al., 2012).

Kagias et al. (2012) defined "Physiological stress" as "the primary biological stress…defined as any external or internal condition that challenges the homeostasis of a cell or an organism." They also identified three dimensions in physiological stress: intrinsic developmental stress, environmental stress, and aging. "Intrinsic developmental stress" is associated with developmental events that may challenge the developing organism. For example, morphogenesis and changes in inner chemistry may yield stressful conditions that may trigger the activation of defense mechanisms. Indeed, transient upregulation of heat shock proteins (Hsps) followed by a subsequent downregulation has been described in tissues undergoing morphogenesis in several invertebrate species, including gastropods such as *Haliotis asinina* (Gunter and Degnan, 2007).

"Environmental stress" refers to the situation where environmental variations exceed certain levels and homeostasis is threatened. Aging is another source of stress that living organisms have to cope with. It may be viewed as the consequence of the stochastic accumulation of molecular damage over time (Hayflick, 2007; Holliday, 2006; Kirkwood and Melov, 2011; Kirkwood and Melov, 2011; Partridge, 2010; Rattan, 2006). The capacity of an individual to cope with aging and other stresses defines its longevity. Although gastropods are not frequently mentioned as model invertebrates for aging studies (Murthy and Ram, 2015), the pond snail *Lymnaea stagnalis* is a suitable model species for studying the process of neuronal aging and age-associated learning and memory impairment (Hermann et al., 2007; Watson et al., 2013).

Some authors make a difference between predictable and unpredictable stressors, especially in vertebrates (Wingfield, 1994; Wingfield et al., 1997). If environmental conditions change in a predictable way (e.g., seasonal reductions of temperature or food supply), individuals can prepare to such events that do not act in themselves as stressors. These individuals cannot be described as stressed but they may be more susceptible to the effects of real stress.

9.2.2 INTEGRATION OF STRESS AT VARIOUS LEVELS OF BIOLOGICAL ORGANIZATION

9.2.2.1 STRESS AND HOMEOSTASIS

Stress is frequently defined as the state of a biological system in which homeostasis (i.e., the mechanisms that maintain stability within the physiological systems and hold all the parameters of the organisms internal milieu within limits that allow an organism to survive) is threatened or not maintained (Moberg, 2000), and this definition applies to virtually all the biological systems. The shape of the relationship between external conditions and organisms internal conditions allows the distinction between "conformers," for which internal conditions are determined by environmental conditions, and "regulators" for which internal conditions are stable for a certain range of environmental conditions.

Stressors constantly challenge the homeostasis of biological systems (Charmandari et al., 2005). Various adaptations have evolved that provide organisms with the ability not only to survive, but also to reproduce under different, sometimes hostile conditions. Such adaptations are associated with specific molecular and cellular mechanisms, body structures and organism behaviors, tailored to a specific environment. Animals encounter ranges of environmental conditions in which performance is maximized as well as thresholds beyond which performance fails and tolerance becomes time limited (Hoffmann and Todgham, 2010). In an organism exposed to a stimulus (e.g., biotic or abiotic stressor), constitutive response processes are elicited to cope with the challenge. They operate within a dynamic range and are part of the perturbation response. If the response is efficient, homeostasis may be restored. Depending on the duration and strength of the perturbation, the dynamic capacity of the response may be exceeded, leading to the initiation of a stress response that involves both constitutive and inducible elements (Stefano et al., 2002).

9.2.2.2 ALLOSTASIS AS AN ALTERNATIVE TO HOMEOSTASIS

In order to take into account the existence of temporal rhythms (e.g., circadial or circannual) and of changes associated with the various life history stages of a species, the concept of "allostasis" was proposed for vertebrates and included in the Allostasis Model (Sterling and Eyer, 1988). Allostasis refers to the integrative adaptive processes maintaining "stability through

change," that is, a stability that is not within the normal homeostatic range. Allostasis is theoretically able to account for evidence that physiological variables are not constant. It is based on the concept that the goal of regulation is not constancy, but rather fitness under natural selection, that implies preventing errors and minimizing costs. Both needs are best accomplished by using prior information to predict demand and then adjusting all parameters to meet it (Sterling, 2003). Although the definition has been refined by various authors (see e.g., McEwen and Wingfield, 2003; Sterling, 2012), in all cases, allostasis emphasizes the dynamic behavioral and physiological mechanisms that are used to anticipate or cope with environmental change to maintain organismal function.

Associated with allostatis is the concept of "allostatic load," that is, the wear and tear associated with the chronic overactivity or dysregulation of allostatic mechanisms (McEwen and Stellar, 1993). At this point, the activation of allostatic mechanisms causes serious negative physiological consequences (McEwen and Wingfield, 2010). Two theoretical types of allostatic load have been defined. Type I occurs when the energy demand exceeds the available energy supply, resulting in reduced performance. Type II occurs when the prolonged activation of the allostatic mechanisms causes pathology, even when sufficient energy is available. It is therefore necessary to focus on the costs of mounting a response to an environmental change, and on the distinction between responses that are beneficial, those that may impose a significant cost and those that may actually cause harm (Schulte, 2014).

9.2.2.3 TOWARD AN INTEGRATIVE FRAMEWORK

There is no consensus on the usefulness of replacing homeostasis by allostasis (Dallman, 2003; Day, 2005), and instead, Romero et al. (2009) proposed to integrate homeostasis, allostasis, and stress in a common framework, the Reactive Scope Model (Fig. 9.1).

According to the Reactive Scope Model, four levels or ranges may be defined for the hormones or other physiological mediators of the stress response: homeostatic failure, predictive homeostasis, reactive homeostasis, and homeostatic overload.

Homeostatic failure occurs when concentration/level of a mediator is too low to maintain homeostasis, leading rapidly to death. Predictive homeostasis refers to the situation where the concentration/level of the mediator

necessary to cope with predictable environmental changes is met. Its range and temporal dynamics follow predictable life-history changes, circadian (area in dark gray in Fig. 9.1) and seasonal rhythms. The upper limit of the corresponding range usually lays a little bit above the peak values associated with the circadian cycle. Reactive Homeostasis occurs when unpredictable changes induce an emergency elevation of the mediator. This elevation corresponds to the classical "stress response." Together, the predictive and reactive homeostasis ranges form the normal reactive scope for that mediator. If the concentration/level of this mediator exceeds the upper limit of the reactive homeostasis range, the mediator itself may start to cause damage. Finally, homeostatic overload refers to the range above this threshold. "Chronic stress" refers to the periods here where the concentration/level of the mediator is in this range.

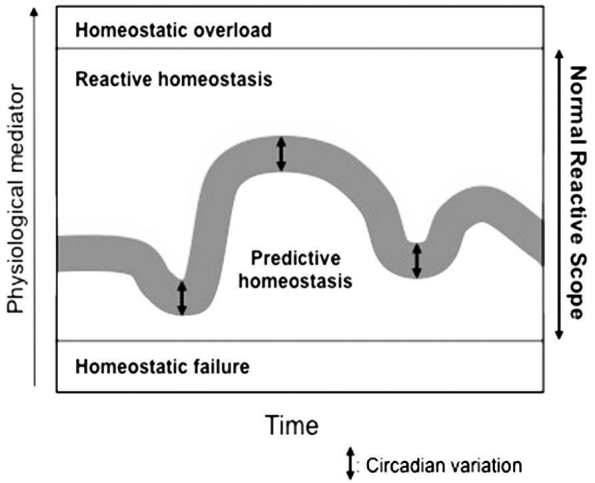

FIGURE 9.1 Schematic representation of the Reactive Scope Model (Adapted from "Using the Reactive Scope Model to Understand Why Stress Physiology Predicts Survival During Starvation in Galápagos Marine Iguanas" in General and Comparative Endocrinology, 2012 May 1;176(3):296-9, with permission from Elsevier.)

Reactive homeostasis is part of the normal physiology of the animal, but it consumes energy and resources that cannot be used in other systems, thereby increasing the allostatic load (i.e., the increase in workload required to maintain homeostasis). The result of this cost is an accumulation of wear and tear, or the depletion of energy or the directing of energy away from other tasks such as tissue maintenance.

9.2.2.4 STRESS RESPONSE AND BIOENERGETICS CONSEQUENCES

Various physiological reactions aim at minimizing the detrimental effects of the stressors. Usually, this involves reallocation of resources from nonessential functions to life-preserving processes (Buchanan, 2000). At the molecular level, studies of stress in animals have shown that various types of stressors induce a set of common responses, which include DNA and protein repair, apoptosis, lysis of molecules damaged by stress (e.g., proteolysis), and metabolic changes reflecting the transition from cellular growth to cellular repair (Sokolova and Lannig, 2008).

Energy reserves play a key role in the ability to elicit these responses. Indeed, stress can result in elevated basal metabolic demand due to the costs of upregulation of cellular protective mechanisms. Competition may therefore occur between these uses of reserves and other energy-demanding functions such as e.g., reproduction (Sokolova et al., 2012). The integration of all these dimensions has led to the concept of energy-limited tolerance to stress (Sokolova, 2013) that is presented below.

Stress responses have been recently reviewed by Kassahn et al. (2009), who also highlighted that oxygen imbalance (reduced partial pressure) and oxidative stress play a central role in the response to many stresses, through stress-inducible-signaling pathways (e.g., heat shock protein-Hsp expression modulated by the JNK pathway), redox-sensitive transcription factors (e.g., HFS1, which induces Hsp transcription, as a possible result of protein damaged by oxidative stress), and a group of genes known as "immediate early genes" (including transcription factors, cytokines, actin, fibronectin). The generic nature of these responses is interesting because it provides suitable conditions (larger datasets) to explore hypotheses about how stress responses relate across biological scales, in particular between molecular processes and higher phenotypic integration such as population divergence in life history traits.

Under aerobic conditions, substrates used for energy production include carbohydrates, lipids, and amino acids. The energetic costs involved in the defense strategies toward stressors may have consequences on the dynamics of these substrates in stressed organisms, suggesting that parameters linked with the status or use of these energy reserves may be used as biomarkers of stress (Huggett et al., 1992).

Polysaccharide level, especially glycogen, is one of the parameters that reflects the energetic and reserves status of an organism. Glycogen is used rapidly when organisms are under stress, and glycogen levels have been suggested as biomarker of general stress (Huggett et al., 1992; Vasseur

and Cossu-Leguille, 2003). In gastropods, glycogen is considered to be the principal energetic reserve (Livingstone and de Zwaan, 1983). Usually, this reserve is found in specific storage cells, glycogen cells, which are widely distributed in the whole body of the snail (Geraerts, 1992; Hemminga et al., 1985).

Convergent results showed that glycogen reserves of gastropods may be severely affected by parasitism by digenetic trematodes (Becker, 1983; Pinheiro and Amato, 1994; Schwartz and Carter, 1982; Tunholi-Alves et al., 2014). Parasite infection may lead to an acceleration of gluconeogenesis through increased consumption of glucose from the hemolymph by the larval trematodes and an acceleration of the catalytic activity of the glycogenolysis pathway (Tielens et al., 1992).

Decreased glycogen levels have also been reported following exposure to hypoxia (e.g., in *Nassarius conoidalis*; Liu et al., 2014). Hypoxia induces specific impacts on the metabolic pathways in stressed aquatic invertebrates. Information about anaerobic metabolism of gastropods is fragmentary (Liu et al., 2014; Santini et al., 2001). A number of anaerobic end products have been reported, including lactate, octopine, alanine, succinate, acetate, propionate, butyrate, strombine, and alanopine (Livingstone and de Zwaan, 1983) but with differences between taxonomic groups (Larade and Storey, 2002), and even within the same genus (Liu et al., 2014). Environmental conditions (Prabhakara Rao and Prasada Rao, 1982; Wieser, 1980), hypoxia duration (de Zwaan and van Marrewijk, 1973; Kluytmans et al., 1975), and type (Gäde, 1975; Gäde et al., 1984) may also have an influence on this metabolism. Hypoxia or anoxia usually induces the increase of glycogen consumption (the "Pasteur Effect"), due to an activation of anaerobic glycolysis in order to compensate for the lower production or synthesis of ATP (Silva-Castiglioni et al., 2010). Under hypoxia, four anaerobic pathways have been identified in invertebrates: (1) glucose–lactate pathway (end product: lactate); (2) glucose–opine pathway (end product: opines); (3) glucose–succinate pathway (end products: succinate); and (4) aspartate–succinate pathway (end products: succinate and alanine; Hochachka and Somero, 1984; Livingstone, 1983). Lactate is an important end product in terrestrial and freshwater gastropod but not in marine species (see Wieser, 1980 and references therein). In marine species, lactate is replaced by alanine, succinate, acetate, and propionate (Liu et al., 2014). The accumulation of these various end-products may be used to demonstrate the exposure of gastropods to hypoxia in the field.

A decrease in glycogen content may also reveal the exposure to toxic compounds such as inorganic toxicants (e.g., arsenic and lead in *Biomphalaria glabrata*; Ansaldo et al., 2006) or organic pesticides (trichlorfon

in *Lymnaea acuminata*; Mahendru and Agarwal, 1981; endosulfan, methyl parathion, quinalphos, and nuvanmay in *Bellamya dissimilis*; Padmaja and Rao, 1994; imidacloprid in *Helix aspersa* (*Cornu aspersum*); Radwan and Mohamed, 2013). Stimulation of the activity of polysaccharide-hydrolyzing enzymes has also been shown in *Lymnaea palustris* exposed to hexachlorobenzene (Baturo et al., 1995).

Since individuals only have a limited amount of carbohydrates, the next alternative source of energy to meet the increased energy demand associated with stress are proteins and, to a lesser extent, lipids. Radwan et al. (2008) showed a significant effect of two carbamate insecticides (methomyl and methiocarb) on the decrease of contents of total proteins and lipids in the tissues of *Eobania vermiculata*. Exposing *H. aspersa* (*C. aspersum*) snails to high concentrations of thiametoxam, Ait Hamlet et al. (2012) observed a significant negative effect of the two highest concentrations of the insecticide on the concentrations of total carbohydrates, total proteins, and total lipids. This effect was correlated with strong histological alteration of the hepatopancreas. In *Bellamya bengalensis*, Kumari (2013) showed that exposure to high detergent concentration caused a decrease in the protein content in the digestive gland, mantle, and foot of the exposed snails. In *L. stagnalis*, Bhide et al. (2006) reported that sublethal concentrations of baygon and nuvan induced depletion in the protein content. According to Padmaja and Rao (1994), the decrease in tissue proteins of snails exposed to pesticides could be due to various mechanisms such as the formation of lipoproteins which are utilized for the repair of damaged cells and tissue organelles or the direct utilization by cells to fulfill energy requirements.

However, decreasing lipid or protein content following acute exposure to a stressor is not always observed. For example, El-Gohary et al. (2011) and El-Shenawy et al. (2012) observed an increase in the lipid content of the digestive gland in *E. vermiculata* exposed to heavy metals or molluscicides, respectively. In *H. aspersa* (*C. aspersum*) exposed to imidacloprid, Radwan and Mohamed (2013) even observed an increase in the protein content of the digestive gland. This increase may be a consequence of an induction of the synthesis of proteic defense systems such as stress proteins.

Proposals have been made for modeling the bioenergetics of animals that may also integrate the costs associated with the responses to stressors and their consequences for fitness, such as the Scope for Growth (SfG) model (Winberg, 1960), the Dynamic Energy Budget (DEB) theory (Kooijman, 2010), the Ontogenetic Growth Model (OGM; Hou et al., 2008), the oxygen- and capacity-limited thermal tolerance (OCLTT) concept

(Pörtner, 2010, 2012), or the concept of energy-limited tolerance to stress (Sokolova, 2013). Some of these models have already been applied to assess the effects of individual or multiple stressors on bivalves (Widdows et al., 1987, 1995, 2002) and gastropods (Ducrot et al., 2007; Stickle et al., 1984; Wo et al., 1999).

The concept of energy-limited tolerance to stress has recently been proposed as a framework for assessing the effects of multiple environmental stressors that integrates some elements from DEB and OCLTT concepts (Sokolova, 2013). According to this framework, five main energy-demanding functions should be considered in an individual: basal maintenance, activity, reproduction/maturation, growth/development, and deposition of energy reserves. Relative allocation of energy to these different processes varies among species and life stages. These processes rely on ATP provided via aerobic or anaerobic metabolism.

Four levels or range may be defined regarding the response of an individual to stress. In the optimum range, the aerobic scope is maximal and aerobic ATP supply covers the basal maintenance and the investment in the other functions. The excess of energy is deposited in storage compounds (e.g., glycogen or lipids). During moderate stress (or pejus range), the maintenance costs increase in order to meet the needs for additional energy for protection from stress and for repairing damage. In some cases assimilation of food and/or capacity for aerobic metabolism may be impaired, therefore, leading to a decline in aerobic scope. Energy storage does no occur or it is reduced. During extreme stress (or pessimum range), the increase in ATP demand for maintenance and/or the impairment of aerobic metabolism overrides ATP supply via aerobic metabolism. Metabolism switches to partial anaerobiosis that fuels the essential maintenance costs and supports the time-limited survival of the organism. In some species, survival in the pessimum range can be enhanced by a metabolic rate depression that reduces rates of energy turnover at the expense of shutting down the ATP-demanding functions that are not essential for immediate survival. The energy balance is temporarily disrupted during transitions between the optimum, pejus, and pessimum ranges but is eventually reinstated, as the organism becomes acclimated to these conditions. In the lethal range, the balance of supply and demand of ATP is permanently disrupted, resulting in negative aerobic scope and death of the organism. This model may also serve as a theoretical basis to identify possible bioenergetics markers for the assessment of the impacts of stressors (Sokolova, 2013).

9.2.3 THE CASE OF MULTIPLE STRESSORS

A lot of studies have been performed to evaluate the effects of individual stressors on various gastropod species, usually in laboratory experiments where one stressor (e.g., temperature, salinity, toxic substance) is manipulated whereas all the other conditions are kept constant. However, under natural environmental conditions organisms may be simultaneously exposed to changes in various variables, including (a)biotic stressors that may therefore have an impact on the physiology of the exposed individuals (Todgham and Stillman, 2013).

These changes may have an additive, an antagonistic or a (non)linear synergistic effect on various parameters of interest (Crain et al., 2008; Darling and Côté, 2008; Holmstrup et al., 2010). Under additive hypothesis small shifts in multiple stressors may have a small effect on performance, whereas under synergistic hypothesis, small shifts in multiple stressors may have a great impact on physiological performance, thus generating unpredictable responses in terms of species' distributions and abundances. Inferences deduced from studies performed on single stressors are potentially misleading regarding the outcome of multiple stressors, including toxic stressors, under natural environmental conditions (see e.g., McBryan et al., 2013; Whitehead, 2013).

In addition, a first stressor may either cause the organisms to be more susceptible to a second stressor (cross-susceptibility; Sinclair et al., 2013) or increase tolerance to a second stressor (cross-tolerance; Horowitz, 2007; Sinclair et al., 2013). At the cellular level, "cross-talk" occurs when multiple pathways, each stimulated by a separate stressor, converge on one physiological function. "Cross-tolerance" is the situation where each stressor may modulate the same pathway, but producing distinct physiological outcomes (Todgham and Stillman, 2013). Both cross-talk and cross-tolerance may be adaptive, but with differing consequences for responding to future changes, due to their mechanisms of action.

The case of exposure to multiple stressors is highly relevant from a fundamental point of view and for environmental management. Several experimental studies have been performed on the combined effects of at least two stressors in gastropods: alkaline stress and copper (Paulson et al., 1983), temperature and salinity (Deschaseaux et al., 2010, 2011), temperature and rainfall (Dong et al., 2014; Williams et al., 2011), temperature and pH (Byrne et al., 2010, 2011), parasitism by a trematode and temperature, salinity, or hypoxia (Lee and Cheng, 1971; McDaniel, 1969; Sousa and Gleason, 1989; Tallmark and Norrgren, 1976; Vernberg and Vernberg, 1963),

acidified conditions, elevated temperature, and solar UV radiation (Davis et al., 2013), metals and predacious cues (Lefcort et al., 2013), combination of metals (Byzitter et al., 2012) or other toxicants (Nevo and Lavie, 1989), or starvation and temperature (Jeno and Brokordt, 2014).

Cross-susceptibility was frequently observed. For example, larval trematode infections have been shown experimentally to cause increased mortality at high water temperatures in various molluscan hosts, including the marine snails *Nassarius obsoletus* (Vernberg and Vernberg, 1963), *Nassarius reticulatus* (Tallmark and Norrgren, 1976), and *Littorina littorea* (McDaniel, 1969), and in the freshwater snail, *B. glabrata* (Lee and Cheng, 1971). Cross-susceptibility may also be two ways. In *L. palustris*, for example, a brief exposure to heavy metals (cadmium, lead, zinc) impaired the ability to avoid predacious cues and conversely after preexposure to crushed conspecifics, individuals not only failed to avoid metal treated water, but also they actually moved toward it (Lefcort et al., 2013).

When addressing the effects of multiple stressors, it is necessary to distinguish the cases where the induced stress remains moderate and compatible with the survival of organisms from those where stress is unsustainable and leads to death. As it was already mentioned, cellular defense and maintenance of homeostasis implies the diversion of energy from other function such as growth or reproduction. The rates of intake and assimilation of energy are limited for all organisms, as well as their metabolic capacity to convert ingested food to ATP (Guderley and Pörtner, 2010). Exposure to stressors such as toxic substances may induce a reduction in food consumption and/or assimilation (Edwards, 1980), therefore, leading to a decrease in the availability of energy for defense systems. When the energy reserves are not sufficient, the synthesis and activity of defense substances may be reduced. For example, starvation decreased the content of stored energy substrates of juveniles of *Concholepas concholepas*, an intertidal snail, as well as their ability to synthesize Hsp70 during emersion under thermal stress, especially at high temperatures (Jeno and Brokordt, 2014).

9.3 STRESS RESPONSE, A SEQUENTIAL PROCESS

Deleterious effects induced by environmental change and stress affect organisms at various levels of biological organization, including molecular and cellular processes, endocrine systems and physiology, immune functions, lifespan, and fitness. As noted earlier (see Section 9.1), homeostasis maintenance is critical for the ability of an organism to cope with stress. In this

respect, the ability to assure oxidative metabolism is of primary importance (Kassahn et al., 2009). Some processes are evolutionarily highly conserved, such as the cellular stress response and associated proteins (Kültz, 2003).

Stress response is a sequential process which involves stress-signaling pathways, followed with the activation of antioxidant systems and cellular/molecular mechanisms that are common to many stressors, and which include the repair of DNA and protein damage, cell cycle arrest or apoptosis, changes in cellular metabolism associated with cellular repair, as well as the release of stress hormones (Kassahn et al., 2009). Among gastropods, these processes have been studied in three main "stress" contexts, that is, natural environmental variation in intertidal snails, parasitism, and water chemical contaminants in freshwater snails. Indeed, once in the body, many xenobiotics may induce signal transduction events leading to various cellular, physiological, and pharmacological responses including homeostasis, proliferation, differentiation, apoptosis, or necrosis.

9.3.1 STRESS SIGNALING

Environmental information is processed by extra-and intracellular-signaling pathways and membrane transporters. The mitogen-activated protein kinases (MAPKs) are important-signaling molecules involved in relaying extracellular signals to intracellular targets (Storey and Storey, 2004). Members of the MAPK superfamily include the cJun-N-terminal kinases (JNKs) and p38 MAPK pathways, which are typically responsive to environmental stresses (Kyriakis and Avruch, 2001). Stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) are members of the MAPK family and are activated by a variety of environmental stresses, inflammatory cytokines, growth factors, and G-protein-coupled receptors (GPCR) agonists. Stress signals are delivered to this cascade by small GTPases of the Rho family (Rac, Rho, cdc42). As with the other MAPKs, the membrane proximal kinase is a MAPKKK, typically MEKK1–4, or a member of the mixed lineage kinases that phosphorylates and activates MKK4 (SEK) or MKK7, the SAPK/JNK kinases. Alternatively, MKK4/7 can be activated by a member of the germinal center kinase family in a GTPase-independent manner. SAPK/JNK translocates to the nucleus where it can regulate the activity of multiple transcription factors (see more on this topic at: http://www.cell-signal.com/common/content/content.jsp?id=pathways-mapk-sapk#sthash.BrLF8Hrm.dpuf).

Once activated during stress, these pathways mediate gene expression. For example, JNKs phosphorylate c-Jun protein, in combination with c-Fos, forms the AP-1 early response transcription factor (activator protein 1). This transcription factor regulates the expression of various genes, and controls several cellular processes and fate, including apoptosis. Other JNK-mediated transcription factors included ATF-2 (which regulates c-Jun transactivation, especially in response to genotoxic agents; Van Dam et al., 1995), Elk-1 (drug addiction, memory, breast cancer, depression), Myc, Smad3 (see TGF beta pathway), tumor suppressor p53, NFAT4, DPC4 and MADD, a cell death domain protein (Cowan and Storey, 2003). Cross-talks occur between heat shock response and JNK (Kassahn et al., 2009); for example, Hsp72 accumulation downregulates JNK and increases thermotolerance (through tolerance to caspase independent apoptosis) in humans (Gabai et al., 2000). Comparatively, p38 kinase pathway is a complex pathway known to be activated in mammalian response to various extracellular stimuli, including UV light, heat, osmotic shock, inflammatory cytokines, and growth factors (Zarubin and Han, 2005).

In gastropods, MAPKs have been characterized in a few representatives, the marine species *Aplysia californica* (cDNA, REF) and *Littorina littorea* (cDNA and protein characterization; p38, JNK, ERK; Iakovleva et al., 2006); and freshwater species such as *L. stagnalis* (protein characterization, activity assay; ERK; Plows et al., 2004) and *B. glabrata* (cDNA; Yoshino et al., 2001). Larade and Storey (2006) studied the response of JNK and p38-signaling pathways, as well as ERK pathway (extracellular signal-regulated protein kinases) to short-term anoxia in the common periwinkle *L. littorea*, a species adapted to high environmental variation, with respect to temperature, water, salinity, and oxygen. Anoxia was found to induce p38 phosphorylation in the digestive gland cells, by a twofold increase. Downstream effects of p38 activation were supported by the observed increase in phosphorylated Hsp27 (involved in cytoskeleton) and CREB (cAMP Response-Element Binding protein, which, among other, regulates c-fos expression), which suggests the involvement of MAPKAKP-2. By contrast, no changes in JNK and ERK pathways were detected. In this species, a specific ERK family member with high molecular weight was identified (p115 MAPK), that was transiently activated by freezing and anoxia (MacDonald and Storey, 2006). The role of ERK in regulating phagocytosis and immune response was assessed in *L. stagnalis* hemocytes (Plows et al., 2004). Recently, the role of post-transcriptional regulators of protein expression such as microRNAs has also been advocated (see Box 9.1). Altogether, these few examples, beyond the

merit of existing, also demonstrate the critical need to improve knowledge on stress signaling in gastropods.

BOX 9.1 miRNA and Gastropod Stress Response

As a particularly class of post-transcriptional regulators of protein expression, microRNAs are short noncoding RNAs (about 22 nucleotids) known to have pervasive roles in regulation of cellular processes, including biological development, cell differentiation, apoptosis, or cell cycle control. These regulators are transcribed as long RNAs which are first cleaved to pre-miRNAs by the nuclear processing enzyme *drosha*, and then processed into mature miRNAs by the cytoplasmic ribonuclease *Dicer*. Their involvement in mediating stress response was recently reviewed (Leung and Sharp, 2010 and references therein). For example, tumor suppressor p53, which can be induced upon DNA damage, induces the transcription of miRNAs of the 34 family, as well as their further processing through its association with a cofactor of *drosha*. These miRNAs in turn promote cell growth arrest and apoptosis. DNA damage also induces repression of another miRNA involved in the regulation of p53. More generally, upon stress, a miRNA can either be involved in homeostasis restoration or act as an enforcer of a new gene expression pathway.

The effects of anoxia and freezing on miRNAs have been recently explored in the common periwinkle *L. littorea* (Biggar et al., 2012). By focusing on highly conserved miRNAs with previously established roles in metabolic rate depression in other organisms, these authors were able to identify effects on miRNAs of particular relevance to stress response: miR-210 (hypoxia-inducible, likely to be regulated by transcription factor HIFalpha), miR-29b (involved in the PI3K/Akt pathway itself related to p53 regulation and apoptosis), miR-34a (transcription induced by p53), miR-125b (cold-inducible in fish, involved in p53 regulation and activating the antioxidant defense-related NF-κB pathway), etc. (see Biggar et al., 2012). To our knowledge, this is first attempt to decipher the role of miRNAs in gastropod response to environmental stress, and this pioneer study paves the way for future breakthroughs in this domain.

9.3.2 OXIDATIVE STRESS AND ANTIOXIDANT SYSTEMS

As a common consequence of many stressful conditions, oxidative stress is a major actor in eliciting responses to stressors. Signal transduction pathways (JNK, p38 MAPK) and transcription factors sensitive to redox imbalance include immediate early genes (c-fos, fosB, c-Jun, JunB, c-myc, egr-1, KC

and JE cytokines, actin, fibronectin), HIF1-alpha, NF-κB, and p53 (Kassahn et al., 2009).

In aerobic organisms, the production of reactive oxygen species (ROS, a term which encompasses both initial species produced by oxygen reduction as well as secondary reactive products; Winterbourn, 2008) is a normal process which results from the inherent dangerousness of oxygen ("oxygen paradox"). The superoxide anion radical, hydrogen peroxide, and the extremely reactive hydroxyl radical are common products of life in an aerobic environment, and these agents appear to be responsible for oxygen toxicity (Davies, 1995). The oxidative burden is contributed by various cellular processes. ROS are predominantly generated within mitochondria, as a consequence of oxidative phosphorylation, but also in the plasma membrane (e.g., NAPDH oxidases), in the peroxisomes (lipid metabolism), as a product of many cytosolic enzymes such as cyclooxygenases (Balaban et al., 2005). Despite their toxic effects, ROS also act as essential physiological regulators of various intracellular-signaling pathways (D'Autréaux and Toledano, 2007; Finkel, 2011; Poulsen et al., 2000). Notably, ROS play a critical role in innate immune defense, including in gastropods (see specific section below), and hydrogen peroxide was also shown to be involved in metamorphosis, such as in the nudibranch *Phestilla sibogae* (velar loss; Pires and Hadfield, 1991). Therefore, the management of oxidative stress represents a balance between meeting the functional requirements for ROS (e.g., as signaling molecules) and preventing or repairing oxidative damage (Dowling and Simmons, 2009; Monaghan et al., 2009).

The maintenance of intracellular redox homeostasis is dependent on a complex system of antioxidant molecules. These antioxidants include low molecular weight molecules such as glutathione, as well as an array of protein antioxidants that each has specific subcellular localizations and chemical reactivities (Finkel, 2011). Oxidative stress occurs when levels of ROS exceed the capacity of antioxidant defenses. Oxidative damage can affect most macromolecules, namely DNA, proteins, and lipids.

In gastropods, oxidative stress has been largely studied as part of the immune response (see specific section below), as well as in ecotoxicology (see Box 9.2). For example, in the latter field, the response of *L. stagnalis* to various herbicides was investigated by Russo and her collaborators. In this species, hemocyte ROS production (H_2O_2) was decreased by atrazine (Russo and Lagadic, 2004), whereas the peroxidizing herbicide fomesafen had the opposite effect (Russo et al., 2007). More detailed work on this topic is presented in Box 9.2.

BOX 9. 2 Toxicants as Exogenous Sources of Oxidative Stress

Environmental contaminants are often sources of stress to organisms and may impair population demography and fate. Furthermore, because these molecules are likely to interact with natural factors of stress, their effects need to be jointly estimated. Population ecotoxicology typically deals with this issue and a battery of tests have been developed to assess the toxicity of chemicals that are based on stress response at molecular, cellular, and organismic levels.

Oxidative stress is known to elicit the toxicity of many pollutants (e.g., aromatic hydrocarbons, pharmaceuticals, metals and pesticides; Isaksson, 2010; Regoli et al., 2002; Valavanidis et al., 2006). As active pro-oxidants, some pollutants (such as most heavy metals, as well as bipyridyl herbicides) directly increase the level of ROS. In other cases, pollutants can increase oxidative stress by inhibiting gene expression of antioxidants and, thereby, change the pro-oxidant/antioxidant balance (Limón-Pacheco and Gonsebatt, 2009). Therefore, antioxidant defense systems and biotransformation pathways are commonly used as biomarkers in ecotoxicology, including gastropods (e.g., Bouétard et al., 2013; Gust et al., 2013a,b). Antioxidant mechanisms are induced to counteract ROS production and overcome the stressful condition. When antioxidant defenses are overwhelmed by ROS generation, different forms of toxicity arise, including lipid peroxidation of cellular membranes, protein degradation, enzyme inactivation and damage to DNA (Regoli et al., 2000).

Aerobic organisms have developed defenses against oxidative damaging, which include enzymatic systems and antioxidant (nonenzymatic) molecules. In gastropods, most of these systems have been described and studied in link with immune, stress, and toxicological responses. A specific section of this chapter is devoted to immunity, so that only environmental stress and toxicological responses will be considered here. Some findings in gastropods are presented, in the framework of cellular defenses as well as detoxification processes.

Within cells, superoxide anions are reduced by superoxide dismutase (SOD) and produce hydrogen peroxide and singlet oxygen. Hydrogen peroxide is in turn converted to water by catalase (CAT) or glutathione peroxidase (GPX). GPX, using glutathione as cofactor, is a protagonist of the glutathione cycle which also involves glutathione reductase and the second phase detoxication enzymes glutathione *S*-transferases (GSTs). Singlet oxygen is quenched by other antioxidants, notably vitamin E. Peroxiredoxin enzymes also play an important ROS scavenging role in the

mitochondria (Balaban et al., 2005). Depending on tissues and species, the transcription factor retinoid X receptor (RXR) is recognized to play different roles in response to oxidative stress, such as being an activator for the phase II detoxication induction (Kang et al., 2005), an antiapoptotic factor, and an inhibitor of intracellular ROS generation by up-regulating CAT activity (Shan et al., 2008).

Many gastropods are naturally exposed and thus adapted to recurrent conditions generating oxidative stress. This is typically the case of intertidal snails, such as *L. littorea*, which is regularly exposed to high environmental variation (incl. heat and freezing, hypoxia, desiccation). Such organisms are thus expected to have elaborated an efficient antioxidant system. A nice example of this efficiency is given in the study of Pannunzio and Storey (1997). These authors showed that in *L. littorea*, anoxia significantly reduced enzymatic activities in the digestive gland (CAT, SOD, glutathione *S*-transferase, glutathione reductase, and glutathione peroxidase), in consistency with anoxia-induced deprivation of oxygen free radicals. The responsive ability of the digestive gland antioxidant defenses was evidenced by a rapid recovery of the four glutathione-linked activities when oxygen was reintroduced. On the other hand, the total content of glutathione (GHS, reduced, and GSSG, oxidized form) increased significantly, which may reflect the anticipated need for GSH during the recovery period in this facultative anaerobe species. Last, the ratio of the two forms (GHS/GSSG) remained stable during anaerobiosis, and increased only during the aerobic recovery period, which is consistent with a protective response to the oxidative damage associated with the reintroduction of oxygen, as well as with an involvement in detoxification of pro-oxidant products accumulated during anaerobiosis.

Terrestrial snails also have to cope with adverse environmental conditions, which led them to adapt through estivation. Arousal from estivation, by triggering abrupt changes from a hypometabolic state to an active one (reset of oxygen metabolism), sets up a condition of increased ROS formation, against which anticipatory mechanisms have evolved. For example, in the land snail *Otala lactea*, arousal triggered a strong induction of SOD, CAT, GST, and GPX activity, followed with a rapid return to normal activity for SOD and GPX (Hermes-Lima and Storey, 1995). Estivation induced the enzymatic preparation for oxidative stress in different snails species, with a protective key-role identified for the selenium dependent GPX (reviewed in Feirrera-Cravo et al., 2010). In the aquatic snail *Pomacea canaliculata*, urate, which has antioxidant properties, was found to increase during estivation

and its oxidation product, allantoin, to increase after arousal (Giraud-Billoud et al., 2013).

Using data from a broad taxonomic range of anoxia-tolerant species, Hermes-Lima and Zenteno-Savin (2002) identified that an increase in the baseline activity of key antioxidant enzymes, as well as "secondary" enzymatic defenses and/or glutathione levels in preparation for a putative oxidative stressful situation arising from tissue reoxygenation seem to be the preferred evolutionary adaptation.

However, the huge diversity of responses produced by hypoxia-tolerant animals demonstrates the lack of a general mechanism, as tolerance may result from different strategies, from high constitutive expression of antioxidant defenses, to high inducibility of antioxidant defenses during anoxia (preparation for oxidative stress upon reoxygenation). These processes were recently reviewed and the role of transcription factors and miRNAs as antioxidant regulators discussed into details (Welker et al., 2013).

The transcription and/or activity of antioxidant enzymes have been largely studied for their biomarker value in ecotoxicology, notably in *L. stagnalis*, in response to pesticides (Bouétard et al., 2013) and to waste water effluents and to pharmaceutical mixtures (Gust et al., 2013a,b), in *Lymnaea natalensis* exposed to various metals (Siwela et al., 2010), in the marine gastropod *Onchidium struma* exposed to copper (SOD, CAT; Li et al., 2009), in the terrestrial snails *Achatina fulica* faced to cadmium and zinc (Chandran et al., 2005) and *Chilina gibbosa* exposed to the organophosphate insecticide azinphos-methyl (Bianco et al., 2013). *B. glabrata* exhibited genetic variation in SOD and CAT responses to the same insecticide, between albino and pigmented strains (Kristoff et al., 2008). However, in all cases, mechanisms underlying observational and correlational data could not be clearly specified, despite the multimarker approach implemented. Such datasets provide nevertheless basic information that can be useful to guide further investigations on the early response of snails to oxidative stress.

9.3.3 DETOXIFICATION OF XENOBIOTICS

The exposure of organisms to toxic substances leads to various catabolic reactions that aim to degrade and eliminate them. Successive phases can be described, which correspond to different metabolic pathways. Drug metabolism includes the activation of so-called phase I (biotransformation), phase II (conjugation) enzymes as well as phase III transporters (Xu et al., 2005).

9.3.3.1 PHASE I ENZYMES (BIOTRANSFORMATION ACTIVITY)

Phase I enzymes mainly belong to the superfamily of cytochrome P450 (CYP). Types of P450-mediated reactions include hydroxylation, epoxidation, oxidative deamination, S-, N-, and O-dealkylations, and dehalogenation. The end results of P450 reactions are most often more hydrophilic and presumably more excretable products (Snyder, 2000). P450 enzymes may reside in the mitochondria and/or in the membrane of the endoplasmic reticulum (ER) (microsomal P450s). Detoxification is mainly performed by microsomal P450s, although some mitochondrial P450s show activity toward exogenous compounds (Rewitz et al., 2006).

In gastropods, these enzymes have been described and studied in the context of natural and xenobiotic stress. Due to their role in early response to chemical stress (phase I), CYPs have been proposed and used as exposure biomarkers (defined as "biochemical, physiological, or histological indicators of either exposure to or effects of xenobiotic chemicals"; Huggett et al., 1992), including in gastropods. For example, in the marine species *Avicularia gibbosula*, the content of CYP450 in the ER from the digestive gland cells was found significantly increased following an oilspill pollution (Yawetz et al., 1992).

The measure of CYP-related biotransformation activity is traditionally assessed using artificial substrates such as ethoxyresorufin (ethoxyresorufin-*O*-dealkylase activity, EROD) and related molecules, that is, pentoxyresorufin (pentoxyresorufin-*O*-dealkylase activity, PROD) and ethoxycoumarin (ethoxycoumarin-*O*-deethylase activity, ECOD), or benzo(*a*)pyrene (BaP-hydroxylase activity, BaPH). The value of CYP-related biotransformation activities as exposure biomarker was investigated in freshwater gastropods (*Valvata piscinalis*, *Potamopyrgus antipodarum*; Gagnaire et al., 2009). In *H. aspersa* (*C. aspersum*), naphthalene was found to modify some of these activities (EROD, ECOD) in an organ-dependent manner, that is, increase in the kidney and decrease in the digestive gland (Ismert et al., 2002). A CYP oxidase system was described in *L. stagnalis* (Wilbrink et al., 1991a) and *L. palustris* exhibited increased microsomal CYP450-related activity (BaPH) when exposed to the herbicide atrazine (Baturo, and Lagadic, 1996).

Altogether, results from these assays suggest that CYPs could be relevant exposure markers in gastropods, although P450-mediated detoxification is generally limited in molluscs (Gooding and LeBlanc, 2001). However, due to the current lack of knowledge on the diversity of the CYP superfamily in this taxonomic group, methodologies often rely on vertebrate models and assumptions (e.g., use of antibodies to vertebrate CYP1A) and may often

lead to departures from toxicological expectations, thus reducing the value of CYPs as biomarkers. Actually, the diversity of CYPs may be still higher in invertebrates than in vertebrates. In molluscs, a recent survey of available genetic resources has been performed in bivalves, providing a benchmark for future studies in ecotoxicology as well as for the understanding of CYP genes evolution (Zanette et al., 2010). Based on sequences from four species (*Mytilus calfornianus*, *Mytilus galloprovincialis*, *Crassostrea gigas*, and *Crassostrea virginica*) the authors identified 123 expressed sequence tags (ESTs) homologous to members of various vertebrate CYP families. The same approach could be developed in gastropods, as genetic resources are becoming substantial in a growing number of species, notably, *A. californica* (Moroz et al., 2006, see also http://www.genome.gov/Pages/Research/Sequencing/SeqProposals/AplysiaSeq.pdf), *L. stagnalis* (Bouétard et al., 2012; Feng et al., 2009; Sadamoto et al., 2012), *Lottia gigantea* (Veenstra, 2010), *B. glabrata* (Adema et al., 2010, available from https://www.vectorbase.org), *Radix balthica* (Feldmeyer et al., 2011), as well as *Crepidula fornicata*, *Crepidula plana*, *Physa acuta*, and *Physa gyrina* (see Romiguier et al., 2014). For example, NGS-based transcriptomic data obtained in *L. stagnalis* provided more than a hundred transcripts matching with various CYPs (M.A. Coutellec, unpublished).

It is also to be noted that besides activation related to biotransformation activity, some compounds may also affect the endogenous functions of CYPs. A remarkable example of such possible effect is found in marine gastropods, in which tributyltin (TBT, previously used as antifouling biocide agent) is known to cause imposex, that is, pseudohermaphroditism in females (at least 195 species; deFur et al., 1999; Oehlmann et al., 2007; Sternberg et al., 2010). Among the alternative hypotheses for TBT-induced imposex, the inhibition of CYP aromatase (CYP19) has received much attention. This steroidogenic enzyme converts testosterone to estrogen and has been shown to be inhibited by TBT, especially in vertebrates. However, in gastropods, conclusions are controversial and still subject to debate (Oehlmann et al., 2007; Rewitz et al., 2006; Sternberg et al., 2010; see Box 9.3).

BOX 9.3 Mechanism of Imposex Induction in Prosobranchs

Since the 1970, female masculinization has been documented in marine prosobranchs all over the world (Sternberg et al., 2010). This syndrome was named "imposex" because it involves the presence of one or more male reproductive organs surimposed onto the normal female apparatus (Smith,

1971). Tributyltin (TBT), a biocide compound used in antifouling paints, was rapidly identified as agent causing imposex and subsequent negative impact at the population level, sometimes down to population extirpation (Gibbs and Bryan, 1996; Minchin et al., 1996). Therefore, the total ban of TBT was voted in 1998, implying a worldwide prohibition on application (within 5 years) and presence (within 10 years) (see Sternberg et al., 2010).

Due to the general lack of knowledge on invertebrate endocrine systems, mechanisms underlying TBT-induced imposex are still not understood. Three main hypotheses have been proposed (all reviewed in details in Sternberg et al., 2010; see references therein):

1. The *steroid hypothesis*, under which TBT increases the level of testosterone by inhibiting steroid metabolizing enzymes, such as aromatase (CYP19, which converts testosterone to estrogen), sulfotransferase (SULTs) (which catalyses the sulfoconjugation and inactivation of steroid hormones), and acyl coenzyme A–steroid acyltransferase (ATAT, which catalyses the fatty acid esterification of testosterone).
2. The *neuroendocrine hypothesis*, under which TBT causes the aberrant secretion of neuropeptide APGWamide, involved in the regulation of male sexual differentiation, and putatively acting as penis morphogenic factor.
3. The *RXR agonist hypothesis*, which relies on the high affinity of TBT as ligand of RXR, and resulting disruption of retinoic acid-dependent-signaling pathways.

Sternberg et al. (2010) evaluated these hypotheses in the light of available results obtained from various prosobranchs. Within the *steroid hypothesis*, the ATAT inhibition would get strongest empirical support. However, the comparison of complete genomes representative of lophotrochozoans, arthropods, and vertebrates revealed that steroidogenic enzymes evolved independently in these three metazoan phyla, and that genes orthologous to the vertebrate steroidogenic enzymes are not present in lophotrochozoans (Markov et al., 2009). Consistently, recent reviews highlight the lack of undisputable evidence for a hormonal role of vertebrate sexual steroids in molluscs, which globally invalidates the steroid hypothesis (see Scott, 2013).

Recent work on prosobranchs provides strong support to the *RXR agonist hypothesis* (Lima et al., 2011; Pascoal et al., 2013; Stange et al., 2012). In particular, full transcriptomic data from *Nucella lapillus* individuals exposed to TBT suggest the RXR/PPAR (peroxisome profilerator-activated receptor) axis as the most plausible mechanism of TBT induction of imposex (Pascoal et al., 2013). Still, much has to be done to clearly demonstrate the molecular mode of action of TBT on prosobranchs.

9.3.3.2 PHASE II ENZYMES (CONJUGATION ACTIVITY)

In vertebrates, the phase II metabolizing or conjugating enzymes consist of many families of enzymes including SULTs and UDP-glucuronosyltransferases, DT-diaphorase or NAD(P)H: quinone oxidoreductase (NQO) or NAD(P)H: menadione reductase (NMO), epoxide hydrolases (EPH), GSTs and *N*-acetyltransferases (NAT) (Xu et al., 2005). In gastropods, several of these enzymes have been studied in an ecotoxicological context.

A focus will be given on GSTs, which are members of a superfamily of multifunctional proteins involved in cellular detoxification and protection against oxidative damage. GSTs catalyze the conjugation of a wide range of endogenous and exogenous electrophilic substrates to glutathione (Armstrong, 1997). The conjugates are too hydrophilic to diffuse freely from the cell, and must be pumped out actively by a transmembrane ATPase (phase III system). This results in the unidirectional excretion of the xenobiotic from the cell (Sheehan et al., 2001).

GSTs have been characterized in a few gastropod species (Wilbrink et al., 1991b), in link with parasitism (*Bulinus truncatus*; Abdalla et al., 2006), dietary toxins (*Cyphoma gibbosum*; Whalen et al., 2008), endocrine disruption (*Thais clavigera*; Rhee et al., 2008), or environmental contamination (*N. obsoletus* and *Cerithium floridanum*; Lee et al., 1988). Furthermore, a survey of Genbank nucleotide database (2015, March) indicates 291 gastropod GST sequences, obtained from seven species: *A. californica* (141 sequences), *L. gigantea* (122), the viviparid *Cypangopaludina cathayensis* (3), abalones *Haliotis discus* and *H. diversicolor* (18), *Th. clavigera* (4), and *C. gibbosum* (2). Although a very small fraction of the Gastropoda diversity is represented, this dataset actually constitutes most of the molluscan GST sequences available to date, since only 181 additional sequences are published, from cephalopods (57) and bivalves (124). It is to be noted that several GST cDNA sequences were also obtained in *L. stagnalis* (RNAseq; Bouétard et al., 2012).

GST activity has been studied in response to various environmental contaminants. It is not our purpose to give an exhaustive list of these studies here, yet a few findings can be highlighted. For example, in *Nucella lapillus*, copper inhibited GST activity with a lowest effect concentration of 0.044 mg/L, while cadmium had the opposite effect (although this was not significant). Several hypotheses were proposed to explain the inhibitory effect of copper, including ROS production and interaction with the enzyme, or depletion of glutathione. Comparatively, this enzymatic activity appeared

generally much lower and unaffected by these metals at the tested concentrations in *Monodonta lineata*, despite a similar acute sensitivity in the two species (Cunha et al., 2007). Specific patterns may relate to differences in bioaccumulation capacities, which seem particularly high in species of the *Monodonta* genus.

In the context of biological control and potential impact on non-target species, the biocide used against mosquito, *Bacillus thuringiensis* var. *israelensis*, was found to elicit GST activity in the freshwater snail *Physa marmorata* (Mansouri et al., 2013). In *L. palustris*, GST activity was inhibited by the herbicide atrazine, whereas the effect of the chlorinated insecticide HCB changed from slight inhibition to significant increase over 24-h exposure (Baturo and Lagadic, 1996).

Although biomarkers are generally not studied alone, but in a set of potentially responsive markers, based on previous knowledge of the toxicant mode of action or properties, only limited information can be provided by these observational approaches. A deeper understanding of the underlying mechanisms would evidently require a proper toxicological methodology, including gene knock-down technology.

9.3.3.3 PHASE III TRANSPORTERS (EXCRETION)

Phase III transporters, including P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and organic anion-transporting polypeptide 2 are expressed in many tissues such as the liver, intestine, kidney, and brain, where they provide a formidable barrier against drug penetration, and play crucial roles in drug absorption, distribution, and excretion. P-gp and MRP utilize the energy from the hydrolysis of ATP to substrate transport across the cell membrane and are called ATP–binding cassette (ABC) transporters (see references in Xu et al., 2005).

In this transporter family, several members of the ABCB (P-gp, MDR, or MXR) and ABCC (MRP) subfamilies function as highly promiscuous transporters, capable of trafficking a diverse array of moderately hydrophobic xenobiotics across cell membranes (Bodo et al., 2003).

Information on these transporters is very scarce in molluscs. The implication of multidrug resistance in the response to environmental contamination has been mainly investigated in bivalves (see Bard, 2000). A recent study provided new insight into the regulatory pathway of ABCB in *M. galloprovincialis*, confirming the role of phosphorylation activity of the

cAMP-dependent protein kinase PKA in mediating P-gp activity, as in mammalian models (Franzellitti and Fabbri, 2013). Among gastropods, P-gp has been previously characterized in *Monondota turbinata* (Kurelec et al., 1995). In this class of molluscs, ABC transporters have been recently studied in the particular context of dietary toxins and diet selection. The tropical ovulid *C. gibbosum* and nudibranch *Tritonia hamnerorum* are known to feed exclusively on allelochemically defended gorgonian corals. Using immunohistochemistry and functional approaches, Whalen et al. (2010) showed that in *T. hamnerorum* (the diet of which is more specialized than that of *C. gibbosum*), P-gp expressed specifically in the midgut epithelia and in the epidermis, two tissues in close contact with gorgonian toxins. This location is thus consistent with a role of P-gp in protection against ingested prey toxins. Comparatively, no immunoreactivity was observed for this transporter in *C. gibbosum*, despite its demonstrated occurrence as two ABCB isoforms. Besides, constitutive expression was found in the latter species for one MRP (ABCC) isoform. The differences may relate to the degree of specialism/generalism of the studied species in terms of prey diet, as MRP and P-gp are known to differ in substrate selectivity (see human resistance to anticancer agents; Kruh and Belinsky, 2003). In *L. stagnalis*, a transcriptomic analysis based on RNAseq highlighted several ABC transporter genes as upregulated in individuals exposed to the pro-oxidant herbicide diquat (Bouétard et al., 2012; M.-A. Coutellec, unpublished data).

9.3.3.4 THE CASE OF METALLOTHIONEINS

Metallothioneins (MTs) are a class of nonenzymatic cysteine-rich proteins of low molecular weight which lack aromatic amino acid residues. They are widespread in most living phyla and have unique metal-binding properties, which combine high thermodynamic stability and kinetic lability (Sigel et al., 2009). These proteins have various biological functions, with a pivotal role in the homeostatic control of essential metals (Cu, Zn) as well as the detoxification of nonessential metals (Cd, Hg, Pb, etc.) or excess of essential metals. Other roles have also been ascribed to MTs, including free-radical scavenging or oxidative stress protection, anti-inflammatory, antiapoptotic, proliferative, angiogenic, neuroprotectant (Blindauer and Leszczyszyn, 2010; Sigel et al., 2009). Consistent with their multiple activities, MTs are often present as multiple isoforms in a given species and are also highly diverse across phyla. In view of their high diversity, MTs have been

classified into three classes: class I (mammalian homolog, also present in molluscs and crustaceans), class II (nonhomolog to mammalian sequences, a highly heterogeneous group), and class III (phytochelatins, plant polypeptides derived from glutathione polymerization). A new classification has been proposed, in which classes I and II MTs are classified into 15 families, molluscan MTs belonging to family 2 (see Vergani, 2009). However, it is clear that MTs forms a superfamily of proteins within which phylogenetic relationships are still unresolved.

Due to their properties, MTs are now part of a core suite of biomarkers recognized at European level in monitoring programs (Amiard et al., 2006). Mollusks have high metal accumulation capacities, and therefore MTs have been widely used as biomarkers in this phylum, especially in marine bivalves, but also in intertidal gastropods (Bebianno et al., 1992, 2003). However, their biomarker value is still questioned, since MTs can also be induced by other contaminants, including organic and inorganic compounds.

In molluscs, MTs contains about 75 amino acids, except in gastropods, which MTs are shorter, due to an atypical N-terminal region (Vergani, 2009). MTs have been particularly studied in helicid snails by R. Dallinger's group. In these snails, two isoforms occur, a Cd-binding peptide involved in cadmium detoxification, and a Cu-binding peptide, involved in homeostatis metal regulation. In the Roman snail *Helix pomatia*, Cd-MT transcription is highly inducible by cadmium in the digestive gland and the gut tissue, whereas Cu-MT is constitutively expressed in rhogocytes (pore cells, present at high density in the mantle tissue of the lung), which are specialized in the synthesis of hemocyanin (a Cu-containing respiratory protein; Chabicovsky et al., 2003). Studies on metal accumulation associated to snail exposure from contaminated soils showed that copper accumulation is much more limited than accumulation of nonessential metals (see Dallinger et al., 2005, and references therein). In fact, copper is stored in rhogocytes in two ways, as bound to Cu-MT and as granular precipitations. The Cu-MT is a stable copper pool, whereas the granular form is highly inducible by Cu exposure, and thus most probably involved in metal excess detoxification. The noninducibility of Cu-MT by Cu exposure seems to be specific to the study species and contrasts with other findings, including in gastropods (*L. littorea*; Mason and Borja, 2002). In *H. pomatia*, Dallinger et al. (2005) showed that the two pools (Cu-MT and granules) do not coexist in the same cells, suggesting that rhogocytes have two distinct physiological states. Due to their phagocytic properties (see Section 9.5.1.), it is possible that these cells are responsible for the sequestration and excretion of copper excess in snails.

9.3.4 ALTERATION OF MACROMOLECULES: PROTEIN MISFOLDING, GENOTOXICITY, LIPID PEROXIDATION

Oxidative stress is known to alter most macromolecules, that is, nucleic acids, proteins, and lipids, with highly damaging consequences for the cell. Lipid peroxidation (LPO) leads to the formation of lipid peroxides and a disturbance of biomembranes (Gutteridge and Halliwell, 1990).

9.3.4.1 PROTEIN MISFOLDING

ROS also induce protein misfolding. Accumulation of misfolded proteins in stressed cells activates heat shock factors and results in the expression of Hsps (Kim et al., 2007). Hsps have the ability to restore the damaged proteins to their functional three-dimensional structure, to prevent aggregation of unfolded proteins, or to facilitate their degradation in case of irreversible damage (Grune et al., 2011; Qiu et al., 2006).

Hsp70, which may act with its co-chaperone Hsp40 (Kim et al., 2007) has been commonly assessed as a biomarker inducible by pollutants in aquatic snails (e.g., platinum in *Marisa cornuarietis*; Osterauer et al., 2010; pesticides in *L. stagnalis*; Bouétard et al., 2013; pharmaceuticals and wastewater effluents in the same species; Gust et al., 2013a,b), and terrestrial gastropods (heavy metals in *Deroceras reticulatum*; Köhler et al., 1996). *L. stagnalis* is used as model in neurophysiology. In this species, hypoxia suppresses sensory and motor behaviors, and Hsp70, induced as an early response to hypoxia, prevents degradation of synaptic proteins syntaxin and synaptotagmin I (Fei et al., 2007). In land snails, these enzymes have been extensively studied either as exposure biomarkers to dessication, overheat and solar radiations, as well as for their expected implication in physiological change related to estivation. *Xeropicta derbentina* is a land snail from Eastern Mediterranean recently introduced in Southern France, where it has been very successful, especially for the last decades. The dynamics of thermal Hsp70 induction was experimentally investigated over 8 h of heat-exposure (45°C) and compared to that of another Mediterranean land snail, *Theba pisana* (Scheil et al., 2011). In *X. derbentina*, besides a circadian evolution of Hsp expression noticed in control snails (peak at noon-time), the experiment demonstrated two temperature-induced expression peaks, one during the heat-exposure phase (after 2 h) and one during the post-heating phase (after 8 h of recovery at 24.7°C). The decrease in expression following the first peak was interpreted as an element of the noncompensation phase due to stress–protein

impairment, which was supported by histopathological data from the digestive gland. Beyond results, this experiment demonstrates that Hsp induction and activity is a rapid and dynamic process, and that studies based on single time point assays can be highly misleading. Furthermore, prior conditions encountered by organisms may also have consequences on results, and this may be particularly problematic when individuals are brought from the field to the lab. This may be illustrated by the results obtained for *Th. pisana*, the second species used in the study of Scheil et al. (2011, 2012). Despite being clearly more sensitive to heat stress, test individuals of this species subjected to similar conditions exhibited a very weak induction of Hsp70, which might reflect a preliminary depletion of this enzyme in the field, as snails were collected in summer. Altogether, these results suggest that extreme care is required to design experiments dealing with the issue of molecular responses to short-term stress.

A field experiment designed to identify proximal determinants of Hsp70 levels in *X. derbentina* showed that both morphological and behavioral factors (i.e., shell size and color morph, and diurnal location of snails above ground as well as shell aperture orientation relative to the sun) were effective and interacted. The fully white morph was predominant in larger snails, which also climbed higher above the ground and had lower Hsp70 levels, smaller snails tended to be darker (higher frequency of darker morphs), stayed closer to the ground level, and had higher Hsp70 levels (Di Lellis et al., 2012). Possible explanations for the observed discrepancy may relate to higher thermal thresholds for Hsp induction in more tolerant individuals, a size-dependence of the ability to tolerate desiccation (smaller snails being more sensitive), higher constitutive expression of Hsp associated to growth and maturity acquisition in smaller (also younger) snails as compared to adult ones (Di Lellis et al., 2012). Consistent results were obtained from a more narrow range of size, that is, avoiding very young and very old snails (Dieterich et al., 2015). On the other hand, no difference in thermal capacity was observed between dark and pale shell coloration morphs of the land snail *Theba pisana* (Scheil et al., 2012).

Similar differences in Hsp levels were also demonstrated between two land snails, the desert species, *Sphincterochila zonata*, and a Mediterranean, desiccation-sensitive species of the same genus, *S. cariosa*, in link with their ability to tolerate desiccation (Arad et al., 2010). Western-blot analysis of Hsps expression (Hps70, Hps90, and sHps) showed both species and organ-specific patterns of variation during estivating and active snails, as well as during arousal after estivation. In active snails, lower expression of Hsp70 occurred in the foot, kidney, and digestive gland of the desert-adapted

species *S. zonata*, compared to the level observed in the Mediterranean (desiccation sensitive) species (*S. cariosa*). Likewise, small Hsps (Hsp25 and Hsp30) expressed to a higher level in the kidney and digestive gland of active individuals of the latter species. By contrast, higher expression of Hsp90 was detected in the kidney of *S. zonata*. Transition from estivation to activity (arousal) is a stressful period, during which oxidative stress may be associated with the increase in metabolic rate and in oxygen consumption. Therefore, it is possible that Hsp induction might reflect a specific response to oxidative stress during this process. Interestingly, the authors of this study observed a stronger increase in Hsp72 expression in *S. cariosa* kidney and digestive gland during arousal, as compared to *S. zonata*. This might suggest a higher inducibility of Hsp70 in the sensitive species. However, a reversed relationship was detected in the foot. It is to be noted that, during estivation, endogenous expression of Hsp72 and Hsp90 in the kidney was higher in *S. zonata* than in *S. cariosa*. This result suggests a role of these enzymes in protecting organisms against the deleterious effects of high concentration of solutes such as urea, as a defense mechanism against water loss during dormancy. The higher osmolality of pallial fluid observed in *S. zonata* supports this hypothesis. As a chapter is fully devoted to regulatory pathways involved in estivation (Ramnanan, Bell, and Hughes, this volume), it is not the place here to further elaborate on this phenomenon.

9.3.4.2 LIPID PEROXIDATION

Oxidative stress may trigger LPO, which leads in turn to the formation of lipid peroxides and a disturbance of biomembranes. LPO is a complex phenomenon which encompasses various mechanisms, including free-radical-mediated oxidation. This mechanism proceeds by a chain mechanism, starting with one initiating free radical which can oxidize many molecules. The chain propagation is carried by lipid peroxyl radicals independent of the type of chain-initiating free radicals. LPO induces biomembrane disturbance, such as alteration of structure, integrity, fluidity, permeability, and functionality, and modifies low-density lipoproteins to proinflammatory forms, and generates potentially toxic, mutagenic, and carcinogenic products (Niki, 2009). Although many LPO products exert cytotoxicity, sublethal concentrations of LPO products induce cellular adaptive responses and enhance tolerance against subsequent oxidative stress through upregulation of antioxidant compounds and enzymes (Niki, 2009). Lipid hydroperoxides, the primary products of LPO, are substrates of GPX.

ROS-mediated damages to lipids can be quantified at different stage in the peroxidation process (Gutteridge and Halliwell, 1990). Conjugated dienes are an initial product of free radical attack on lipids, lipid hydroperoxides represent an intermediate product, and malondialdehyde is a terminal product of lipid breakdown that can be measured using the thiobarbituric acid reactive substances (TBARs) assay. These measures were applied by Pannunzio and Storey (1997) to study the effect of anoxia on *L. littorea*, along with a set of antioxidant enzymatic and nonenzymatic assays. Under anoxia, the only responsive marker in the digestive gland was the lipid hydroperoxide content, which was strongly reduced, and consistent with oxygen deprivation. By contrast, the stable and low level of conjugated dienes in this tissue could be explained by the likely activation of an efficient mechanism against their accumulation. TBARs were also stable in the digestive gland, but at a high level, which would indicate that aldehydes other than those produced from lipid degradation contribute to the TBARs content, or that the terminal products are not readily cleared from the tissue, particularly during anoxia. Comparatively, the foot muscle tissue exhibited a different pattern, with a significant increase of the two first stages of LPO, followed with a return to normal levels of hydrogen hydroperoxides only, during the recovery period. This was interpreted by the authors as reflecting the capacity of the foot tissue to reverse lipid hydroperoxides but not primary conjugates. These results were further interpreted in the light of the antioxidant response measured in parallel. Indeed, upon oxygen return, the three LPO indicators remained stable (did not increase) in the digestive gland, which reflects the efficiency of an antioxidant system naturally adapted to anaerobic to aerobic transitions (see Section 9.2.2). The abrupt and transient increase in TBARs, as evidence for arousal-induced oxidative stress and efficient response ability in estivating snails was also observed in the digestive gland of *O. lactea* (Hermes-Lima and Storey, 1995).

9.3.4.3 DNA DAMAGE

If DNA damage can be caused by many endogenous and exogenous agents, a significant portion of the damage is caused by ROS (Brazilai and Yamamoto, 2004). The occurrence of DNA lesions triggers that activation of an intricate web of signaling pathways known as the DNA damage response.

Some environmental contaminants are also genotoxic, for example, polycyclic aromatics hydrocarbons (PAHs), heavy metals, endocrine disrupters such as TBT (Hagger et al., 2006; Sarkar et al., 2014). DNA damage induced

by xenobiotics encompasses simple and double-strand break and the formation of micronuclei, DNA–adducts, DNA–protein cross-links, as well as chromosomal aberration. Therefore, assessment methods dedicated to such defects have been developed in the context of ecotoxicology (biomarkers of DNA integrity and DNA-strand breaks).

The Comet Assay (or Single Cell Gel Electrophoresis [SCGE]) is a simple method for measuring DNA-strand breaks (Box 9.4). Based on results obtained in cephalopods (Raimundo et al., 2010) and bivalves (*Ruditapes philippinarum*; Hartl et al., 2004), it seems that the digestive gland is not a proper organ for the use of Comet Assay. It was shown to yield levels of single-strand breakage likely too high (from autolytic processes) for a valid application of the assay without proper cell sorting and viability check (de Lapuente et al., 2015). Therefore, hemocytes are the most common target for genotoxicity assessment in vivo and in vitro in gastropods using the Comet Assay. In gastropods, this method has been successfully applied as biomarker of genotoxicity, mainly using marine species exposed to contaminants, for example, *Bullacta exarata* (An et al., 2012), *L. littorea* (Noventa et al., 2011), *Nerita chamaeleon* (Sarkar et al., 2015), *M. granulata* (Sarkar et al., 2014), *N. lapillus* (together with a micronucleus test; Hagger et al., 2006), *Patella vulgata* (Lewis et al., 2010), *Planaxis sulcatus* (Bhagat and Ingole, 2015), but also in terrestrial species such as *C. aspersum* and *Helix vermiculata* (Angeletti et al., 2013; Ianistcki et al., 2009).

As a different class of DNA alteration, epigenetics, which refers to modifications in gene expression that are influenced by DNA methylation and/or chromatin structure, RNA editing, and RNA interference without any changes in DNA sequences (Bird, 2002), should deserve particular attention with respect to stress. Molecular epigenetic studies in molluscs are rare, and Fneich et al. (2013) were the first to describe DNA methylation in a gastropod snail, *B. glabrata.* This domain is still only in its infancy, and we are aware of only one publication dealing with stress-mediated epigenetic modification, that is, the effect of immune challenge on *P. canaliculata* neurons (see Ottaviani et al., 2013).

BOX 9.4 The Comet Assay as a Standard Method for Determining Genotoxicity

The Comet Assay, or Single Cell Gel Electrophoresis (SCGE), has become a standard method for determining in vivo/in vitro genotoxicity (see review in Collins, 2014; de Lapuente et al., 2015). The assay is based on quantification

of the denatured DNA fragments migrating out of the cell nucleus during electrophoresis. The image obtained with this technique looks like a "comet" with a "head" consisting of intact DNA, and a "tail" which contains damaged or broken pieces of DNA. The amount of DNA liberated from the head of the comet during electrophoresis depends on the level of effect of the stressor under evaluation. Although the first demonstration of "comets" (though they did not use the word) was by Östling and Johanson (1984), the assay became popular following its improvement by Singh et al. (1988) and Olive et al. (1990).

Several versions of the assay are currently in use (de Lapuente et al., 2015). Basically, after a suspension of cells has been obtained, the basic steps include preparation of microscopic slides layered with cells embedded in an agarose gel, lysis of cells to liberate the DNA, DNA unwinding, electrophoresis, neutralization of the alkali, DNA staining and scoring. Various image analysis systems are available for assessing the resultant images. Basically, when dealing with relatively low damage levels the distance of DNA migration from the body of the nuclear core is used to measure the extent of DNA damage. This technique is not very useful in situations where DNA damage is relatively high, as with increasing extent of DNA damage the tail increases in fluorescent staining intensity but not in length. Collins (2004) proposed a scoring method that has been shown to give quantitative resolution which is sufficient for many purposes. Probably the most popular method for comet evaluation is referred to as "tail moment" calculated as measure of tail length × measure of DNA in the tail; Olive et al., 1990). It incorporates relative measurements of both the smallest detectable size of migrating DNA (reflected by the length of the comet tail) and the number of broken pieces of DNA (represented by the staining intensity of DNA in the tail).

The Comet Assay can detect DNA single-strand breaks as initial damage and those developed from alkali-labile sites under alkaline condition (pH > 12.6), and that formed during repair of base adducts or alkylated bases, which are not initial DNA damage (Collins, 2004). The sensitivity and specificity of the assay are greatly enhanced if the nucleoids are incubated with bacterial repair endonucleases that recognize specific kinds of damage in the DNA and convert lesions to DNA breaks, increasing the amount of DNA in the comet tail. DNA repair can be monitored by incubating cells after treatment with damaging agent and measuring the damage remaining at intervals. Alternatively, the repair activity in a cell extract can be measured by incubating it with nucleoids containing specific damage (Collins, 2014).

9.4 APOPTOSIS

Apoptosis is the primary cell death program by which cells are physiologically eliminated without inducing inflammation. In Metazoans, apoptosis is involved in important stress-unrelated functions, such as ontogenetic processes related to embryonic development and metamorphosis (organ ontogenesis, cellular maintenance, and repair associated to developmental plasticity and error).

Contrary to necrosis, apoptosis involves the nuclear condensation and organized fragmentation (200 bp fragments), cleavage of chromosomal DNA into internucleosomal fragments and packaging of the deceased cell into apoptotic bodies without plasma membrane breakdown (Edinger and Thompson, 2004). Apoptosis is an evolutionary highly conserved process (see Meier et al., 2000). A recent review of the available knowledge on these pathways in Mollusca was performed by Kiss (2010). Although the major part of this knowledge comes from bivalve models, the set of information gathered should be useful for further investigations in gastropods. Two different pathways can mediate apoptosis, the extrinsic (Fas and other tumor necrosis factor receptor [TNFR] superfamily members and ligands) and intrinsic (mitochondria-associated) pathways, and among which cross-talks are possible.

With an illustrative purpose, Figure 9.2 presents the apoptosis pathway as described by KEGG orthology for human (Kanehisa and Goto, 2000; http://www.kegg.jp/pathway/map04210), with *L. stagnalis* transcripts expressed in the digestive gland highlighted in red (M.A. Coutellec, unpublished). These data were obtained from RNAseq analysis, which led to a total of 202 contigs matching with KEGG terms involved in both extrinsic (e.g., TNF, TRAIL, FADD, caspase8) Bcl2 and intrinsic pathway (IAP). Differential expression induced by the pro-oxidant herbicide diquat was observed for several apoptosis-associated transcripts, namely, FASL, TRAILR, CASP7, transcripts annotated as IAP repeat-protein 2/3 and 7/8, E3 ubiquitin–protein ligase XIAP, and CASP9.

Apoptosis is a genetically controlled mechanism of cell death involved in the regulation of tissue homeostasis. The two major pathways of apoptosis are the extrinsic (Fas and other TNFR superfamily members and ligands) and the intrinsic (mitochondria-associated) pathways, both of which are found in the cytoplasm. The extrinsic pathway is triggered by death receptor engagement, which initiates a signaling cascade mediated by caspase-8 activation. Caspase-8 both feeds directly into caspase-3 activation and stimulates the release of cytochrome *c* by the mitochondria. Caspase-3 activation leads to the degradation of cellular proteins necessary to maintain cell survival and integrity.

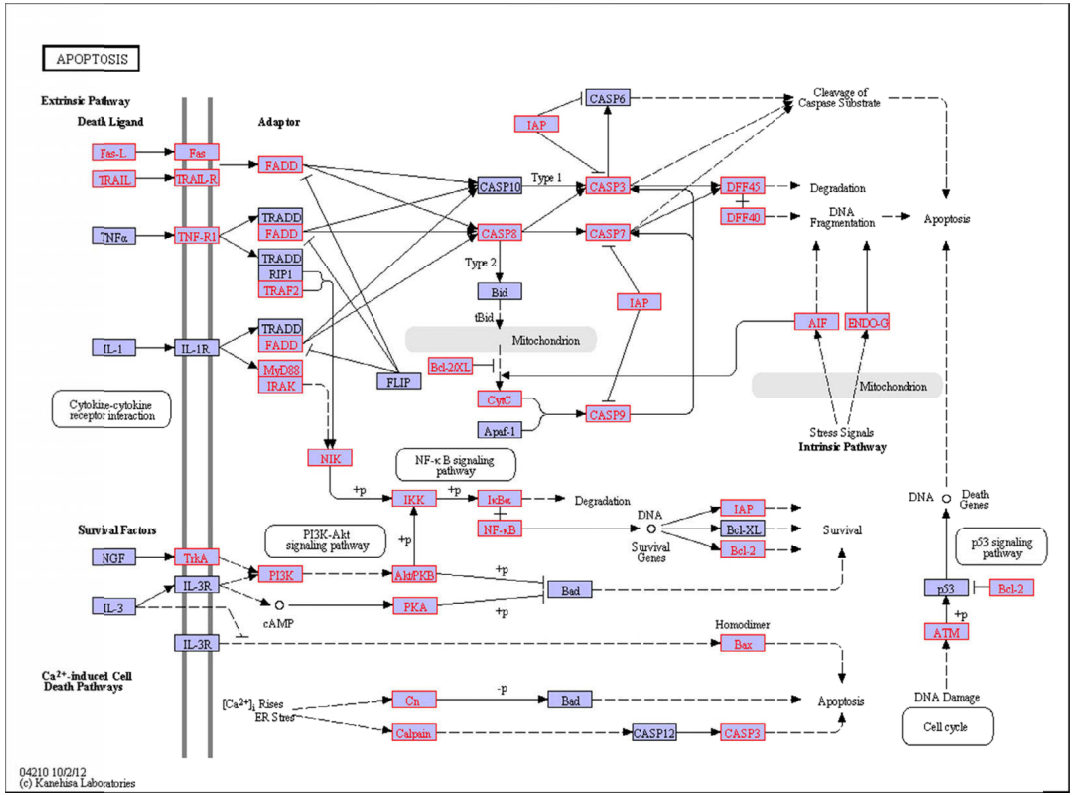

FIGURE 9.2 Apoptosis pathway. (From Romero LM, "Using the reactive scope model to understand why stress physiology predicts survival during starvation in Galápagos marine iguanas", Gen Comp Endocrinol. 2012 May 1;176(3):296-9. doi: 10.1016/j.ygcen.2011.11.004. Epub 2011 Nov 12. http://www.kegg.jp/kegg/kegg1.html; http://www.kegg.jp/pathway/map04210; Used with permission. (KEGG annotation resources, http://www.kegg.jp/pathway/map04210) with transcripts identified in *Lymnaea stagnalis* [in red]).

The intrinsic pathway occurs when various apoptotic stimuli trigger the release of cytochrome *c* from the mitochondria (independently of caspase-8 activation). Cytochrome *c* interacts with Apaf-1 and caspase-9 to promote the activation of caspase-3. Recent studies point to ER as a third subcellular compartment implicated in apoptotic execution. Alterations in Ca^{2+} homeostasis and accumulation of misfolded proteins in the ER cause ER stress. Prolonged ER stress can result in the activation of BAD and/or caspase-12, and execute apoptosis.

In gastropods, cellular responses have been particularly well studied in hemolymph. Phagocytosis parameters and lysosomal fragility as well as apoptotic processes were investigated in *L. stagnalis* hemocytes in response to pesticides (Russo and Madec, 2007; Russo et al., 2008). Interestingly, early apoptotic events could be related to mitochondrial membrane alterations and exposure of phosphatidylserine to the outer face of the plasma membrane, suggesting an implication of the intrinsic pathway in this pesticide-induced apoptosis. Again, such results provide a good basis for further investigations on gastropod cellular responses to stress, and vice versa. For example, *C. gigas* hemocytes were transfected with gastropod sequences of the Hsp70 promoter and a Ras-related gene (Rho) to study noradrenaline-mediated apoptosis in mollusk hemocytes (Lacoste et al., 2001).

Tissues nonspecifically implicated in immune or stress response are also subject to cell death processes. Membrane-associated hallmarks of both apoptosis and necrosis were detected in *L. stagnalis* neurons treated with the anesthesic lidocaine (Onizuka et al., 2012). Similarly, hydrogen peroxide induced both processes in *Aplysia kurodai* sensory neurons, as reflected by apoptotic (nuclear shrinkage and chromatin condensation) as well as necrotic (organelle swelling) features (Lim et al., 2002).

Interestingly, antipredatory or deterrent proteins have been identified in gastropods as being cytotoxic and inducing cell death. Among them, the glycoprotein Cyplasin was isolated from the California sea hare *A. californica* and studied for its potential biotechnological application to cancer treatment (Petzelt et al., 2002). Other molecules involved in the defensive system of this sea hare have been characterized, which may indirectly trigger apoptosis-related cascades of reactions. The L-amino acid oxidase Escapin, was isolated from the ink of *A. californica*. Ink results from the combined secretion of ink gland and opaline gland. Escapin reacts extremely quickly with L-lysin, which is present at high concentration in the secretion, to produce a diverse mixture of molecules, some of which are strongly bioactive. Intermediate and end reaction products result from the initial Escapin-induced deamination of L-lysin (leading to alpha-keto acids) and interaction

with H_2O_2 (EIP-K, EEP-K) with bactericidal effects (see details in Ko et al., 2008). Various L-amino acid oxidases (LAAOs) express specifically in different organs (purple ink, egg masses, and albumen gland). In another sea hare, *Dolabella auricularia*, smaller polypeptides with LAAO activity have been found in the skin, body wall, and coelomic fluid (Iijima et al., 2003). A phylogenetic analysis of orthologs and paralogs of *A. californica* Cyplasin showed a closer relationship of the latter with Aplysianin A (isolated from *A. kurodai*; Jimbo et al., 2003), whereas the sequence of Escapin was closer to *Aplysia punctata* ink toxin APIT1 (Butzke et al., 2004). In *A. californica*, Escapin occurs in the ink, where it functions as defensive molecule against attack, while the occurrence of its paralog Aplysianin (*A. californica* homolog) in egg masses is likely to have a protective function against egg consumption (Derby, 2007). A LAAO was also described in the mucus of the terrestrial snail *A. fulica*. Cell death caused by this enzyme was shown to be due to both H_2O_2-related cytotoxicity (necrosis or apoptosis) and to caspase-mediated apoptosis (Kanzawa et al., 2004).

Thus, both interspecific and intraspecific organ-dependent variation occurs in gastropod LAAOs, which discovery opens the way to further exciting studies on antipredatory functions of this defensive pathway.

9.5 STRESS AND THE IMMUNE SYSTEM

Available information on the response of gastropod immune system to stress mostly concern model species (e.g., *L. stagnalis*; van der Knaap et al., 1993) or species of commercial (e.g., abalones; Hooper et al., 2007) or medical interest (e.g., *B. glabrata*; Coustau et al., 2015). Since gastropods may be the intermediate hosts of various parasites, a lot of immunological studies also focus on the interaction and the larval stages of digeneans (digenetic trematodes) such as schistosomes. Besides, the immunobiology of the vast majority of gastropod species have never been studied (Loker, 2010) and gastropod immunobiology still remains largely unknown.

9.5.1 BRIEF OVERVIEW OF GASTROPOD IMMUNE SYSTEM

Gastropods lack the acquired immune system of vertebrates. Their immune systems rely on three basic mechanisms of immune defense: physical barriers, cellular defenses, and humoral mechanisms (Ellis et al., 2011). Cellular and humoral defenses, which are provided by the hemolymph, are

similar to the innate immune system of vertebrates (Rowley and Powell, 2007).

The mucus produced by the epithelium that covers gastropod body forms the first barrier against pathogens and foreign elements. Bioactive substances that contribute to the efficiency of this barrier have been isolated from mucus such as achacin in the giant African land snail *A. fulica* (Ehara et al., 2002) or dolabellanin B from the sea hare *D. auricularia* (Iijima et al., 2003).

Rhogocytes (also known as pore cells), which are involved in synthesis or processing of respiratory proteins and MTs (Dallinger et al., 2005; see Section 9.3.3.4), have also been implicated in ingestion of small foreign particles (Albrecht et al., 2001). Cells with phagocytic activity may be found in the connective tissue (*L. stagnalis*; Sminia et al., 1979) or in organs such as the digestive gland where they may form a fixed phagocyte system (*H. pomatia*; Reade, 1968) or be distributed throughout the entire gland (*Planorbarius corneus*; Ottaviani, 1990). Antigen-trapping cells have been described in blood sinus and kidney in *H. pomatia* (Renwrantz et al., 1981). Hemocyte islets are also found in the kidney of *P. canaliculata* and they show phagocytic activity and spheroid formation after bacterial or yeast injections (Cueto et al., 2013). In *B. glabrata*, fixed phagocytic cells are located in various tissues (Matricon-Gondran and Letocart, 1999). The relative contribution of these fixed cells to defense is not well understood and most data concern hemolymph-related defense mechanisms.

9.5.1 CELLULAR COMPONENT

Cellular defense in gastropods is coordinated by circulating hemolymph cells that functionally resemble mammalian macrophages (van der Knaap et al., 1993). These cells are usually designated as hemocytes (or haemocytes) but other names may be found in the literature, including amebocytes (Prowse and Tait, 1969; Sminia and Barendsen, 1980), leukocytes (Müller, 1956 in Sminia, 1981), granulocytes (Barracco et al., 1993), macrophages (Yamaguchi et al., 1999), lymphocytes (Kress, 1968), fibrocytes (Foley and Cheng, 1974), and hyaline cells (George and Ferguson, 1950).

No standard nomenclature of gastropod hemocytes has been defined so far. They are frequently classified into subpopulations based upon functional, morphological, and staining characteristics analyzed by light and electron microscopy. The use of flow cytometry or immunostaining has increased the opportunities of distinguishing between various additional cell types

(Dikkeboom et al., 1988a; Franceschi et al., 1991; Johnston and Yoshino, 2001).

Two types of immunocytes have generally been described in gastropods, granulocytes, and hyalinocytes (sometimes called agranulocytes), respectively, although the corresponding nomenclature and even the number of cell populations may vary according to species and between authors for a given species (Table 9.1). Granulocytes contain cytoplasmic granules, have a low nuclear-to-cytoplasmic ratio and are effective in phagocytising foreign materials; in contrast, hyalinocytes are smaller cells, have a high nuclear-to-cytoplasmic ratio, few cytoplasmic granules and a poor capacity to phagocytose foreign materials (Martin et al., 2007). Hemocyte subpopulations that differ both chemically and functionally are probably regulated in their activities or behaviors through specific receptors and the signals conveyed by their interaction with appropriate ligands (Humphries and Yoshino, 2003).

TABLE 9.1 Examples of Nomenclature of Gastropod Hemolymph Circulating Cells Recorded in the Literature.

Clade/Species	Hemocyte Cell Types (Synonyms or Subpopulations)	References
Vetigastropoda		
Haliotis discus discus	Blast-like cells and hyalinocytes	Donaghy et al. (2010)
Haliotis tuberculata	Large and small (blast-like cells) hyalinocytes	Travers et al. (2008a)
Megathura crenulata	Hemocytes	Martin et al. (2007)
Turbo cornutus	Blast-like cells, type I and II hyalinocytes, and granulocytes	Donaghy et al. (2010)
Architenioglossa		
Ampullaria cuprina	Type I, II, and III (spreading cells) hemocytes	Wojtaszek et al. (1998)
Bellamya bengalensis	Agranulocytes (blast-like cells, round hyalinocytes, and spindle hyalinocytes), semigranulocytes (semigranular asterocytes and round semigranulocytes), and granulocytes (round granulocytes, spindle granulocytes, and granular asterocytes)	Ray et al. (2013)
Pila globosa	Agranulocytes and granulocytes	Mahilini and Rajendran (2008)

TABLE 9.1 *(Continued)*

Clade/Species	Hemocyte Cell Types (Synonyms or Subpopulations)	References
Pila globosa	Agranulocytes (blast-like cells, round hyalinocytes and spindle hyalinocytes), semigranulocytes (semigranular asterocytes and round semigranulocytes), and granulocytes (round granulocytes, spindle granulocytes, and granular asterocytes)	Ray et al. (2013)
Pomacea canaliculata	Group I (small) and group II (large) hemocytes	Accorsi et al. (2013, 2014)
Pomacea canaliculata	Nongranular, granular with few granules, and granular will electron dense granules hemocytes	Cueto et al. (2007), Shozawa and Suto (1990)
Viviparus ater	Spreading hemocytes	Franchini and Ottaviani (1990), Ottaviani (1989)
Littorhinomorpha		
Littorina littorea	Hyalinocytes	Neves et al. (2015)
Littorina littorea	Hyalinocytes (Juvenile round cells, intermediate cells, and large mature hemocytes)	Gorbushin and Iakovleva (2006)
Oncomelania hupensis	Round cells with filiformfilopodia, acidophilic round cells, basophilic round cells without filiformfilopodia, and spindle cells	Zhang et al. (2007a)
Oncomelania nosophora	Type I (macrophage-like) and type II (lymphocytes-like) cells	Sasaki et al. (2003)
Neogastropoda		
Babylonia areolata	Granulocytes and hyalinocytes (Type I and II)	Di et al. (2011, 2013)
Heterobranchia		
Acteon tornatilis	Granulocytes and hyalinocytes	Yonow and Renwrantz (1986)
Doto coronata, D. pinnatifida, D. fragilis	Granulocytes and hyalinocytes	Kress (1968)
Hygrophila		
Biomphalaria glabrata	Granulocytes and hyalinocytes	Cheng (1975), Cheng and Auld (1977)
Biomphalaria glabrata	Amebocytes	Sminia and Barendsen (1980)

TABLE 9.1 *(Continued)*

Clade/Species	Hemocyte Cell Types (Synonyms or Subpopulations)	References
Biomphalaria glabrata	Small, medium and large hemocytes	Martins-Souza et al. (2009)
Biomphalaria glabrata	Granulocytes, hyalinocytes, and round cells	Noda and Loker (1989a)
Biomphalaria tenagophila	Granulocytes and hyalinocytes	Barracco et al. (1993)
Biomphalaria tenagophila	Small, medium, and large hemocytes	Martins-Souza et al. (2009)
Bulinus guernei	Granulocytes	Krupa et al. (1977)
Bulinus truncatus	Amebocytes	Sminia and Barendsen (1980)
Indoplanorbis exustus	Agranulocytes and granulocytes	Mahilini and Rajendran (2008)
Lymnaea stagnalis	Spreading and round amebocytes	Stang-Voss (1970)
Lymnaea stagnalis	Amebocytes	Sminia (1972), Sminia and Barendsen (1980)
Lymnaea stagnalis	Phagocytes (hemocytes)	Van der Knaap et al. (1993)
Lymnaea stagnalis	Round cells and granulocytes	Russo and Lagadic (2004)
Lymnaea truncatula	Round cells and spreading cells	Monteil and Matricon-Gondran (1993)
Planorbarius corneus	Spreading (SH) and round hemocytes (RH)	Ottaviani (1983), Ottaviani and Franchini (1988)
Sigmurethra		
Helix aspersa maxima	Type I (=granulocytes) and type II (=hyalinocytes) hemocytes	Adamowicz and Bolaczek (2003)
Helix pomatia	Granulocytes	Renwrantz (1979)
Helix pomatia	Type I, II, III (spreading cells), and IV hemocytes	Wojtaszek et al. (1998)
Incilaria bilineata	Type I (macrophage-like), II (lymphocyte-like), and III (fibroblast-like) hemolymph cells	Furuta et al. (1990)
Incilaria fruhstorferi	Type I (macrophage-like), II (lymphocyte-like), and III (fibroblast-like) hemolymph cells	Furuta et al. (1986)

TABLE 9.1 *(Continued)*

Clade/Species	Hemocyte Cell Types (Synonyms or Subpopulations)	References
Trachea vittata	Agranulocytes and granulocytes	Mahilini and Rajendran (2008)
Aplysiomorpha		
Aplysia californica	Hemocytes	Martin et al. (2007)

The hemopoiesis of molluscs has not yet been fully understood and two major theories have been proposed. Cheng (1981) and Auffret (1988) suggested that hyalinocytes and granulocytes might differentiate from two distinct cell precursors. However, juvenile cells containing granules (also named granuloblasts) have been extremely rarely observed, prompting Hine (1999) to suggest that one cell type might give rise to hyalinocytes that would further mature to become granulocytes. Hematopoietic organs (amebocytes producing organ or APO) have been described in pulmonate gastropods (Jeong et al., 1983; Lie et al., 1975; Rondelaud et al., 1982) and in *M. cornuarietis*, an ampullariid snail (Yousif et al., 1980). However, it has also been shown in littorinimorph and pulmonate gastropods that hemocyte proliferation occurs in peripheral vascular locations or in the circulation (Gorbushin and Iakovleva, 2006; Sminia, 1974; Sminia et al., 1983; Souza and Andrade, 2006).

Gastropod hemocytes can recognize and subsequently eliminate, or sequester, invading pathogens through various processes, including phagocytosis, encapsulation, and the production of lysosomal enzymes and bacteriostatic substances (see e.g., Nunez et al., 1994; van der Knaap et al., 1993; Yoshino and Vasta, 1996). They can also produce cytotoxic molecules such as reactive oxygen and nitrogen intermediates that play an important role in the destruction of microorganisms and parasites (Adema et al., 1994; Conte and Ottaviani, 1995; Dikkeboom et al., 1988b; Hahn et al., 2000, 2001; Zelck et al., 2005).

During these processes, increased amounts of oxygen are consumed, and the cells undergo an oxidative burst and produce a variety of cytotoxic ROS, including superoxide, hydrogen peroxide, hydroxyl radicals and possibly singlet molecular oxygen (Adema et al., 1994; Dikkeboom et al., 1988b). Several enzymes participate in the generation of ROS, including NADPH-oxidase complex generating superoxide (Adema et al., 1993), SOD

converting it to hydrogen peroxide and peroxidases that catalyze the transformation of hydrogen peroxide.

9.5.1.2 HUMORAL COMPONENT

Humoral factors play a fundamental role in the immune responses in molluscs. In addition to phagocytosis, hemocytes are able to secrete soluble antimicrobial peptides (AMPs) and other cytotoxic substances into the hemolymph. Together with other nonspecific humoral defense molecules, including lectins, bactericidins, nitric oxide (NO), lysozymes, and serine proteases, these form the humoral component of invertebrate immunity.

9.5.1.2.1 Bioactive Peptides

AMPs represent the most universal immune effectors and several compounds have been identified in molluscs since the mid-1990s, mostly in bivalves (see review in Li et al., 2011). However, as compared to many other invertebrates, the study of gastropod AMPs remains in its infancy (Loker, 2010). Among them, defensin has been identified in the marine gastropods *Haliotis discus hannai* (Hong et al., 2008) and *Haliotis discus discus* (De Zoysa et al., 2010). Abhisin, a 40 amino acids AMP was identified in *H. discus discus* (De Zoysa et al., 2009) and another peptide, littorein, has been observed in the plasma of the common periwinkle *L. littorea* (Defer et al., 2009).

Results from laboratory experiments on the antiviral activity of hemolymph and lipophilic extract of the digestive gland of the abalone *Haliotis laevigata* also suggest that abalone have at least two antiviral compounds with different modes of action (Dang et al., 2011). In addition, evidence for the existence of AMPs was found in *B. glabrata* (Mitta et al., 2005).

9.5.1.2.2 Lectins

Lectins are carbohydrate-binding proteins that bind to specific carbohydrate structures endogenous to the host or presented by microbial invaders. They play diverse roles in nonself-recognition and clearance of invaders as pattern recognition receptors (PRRs; Wang et al., 2011). Many lectins have been isolated from mollusc eggs, being involved in the immune protection of the eggs from bacterial invasions (Prokop and Köhler, 1967). For

example, *H. pomatia* agglutinin was first isolated from perivitelline fluid of eggs for which it provides efficient antibacterial protection (Sanchez et al., 2006). Several lectins, especially the C-type lectins, have been well documented (Wang et al., 2011) and they are found to mediate various innate immune responses such as pathogen recognition (Janeway and Medzhitov, 2002), agglutination (Song et al., 2011), opsonization (Yang et al., 2011), and phagocytosis (Canesi et al., 2002). A galectin present on the surface of circa 60% of *B. glabrata* hemocytes has been characterized, and in recombinant form binds to the tegument of *Schistosoma mansoni* sporocysts in a carbohydrate-inhibitable manner, suggesting it is a hemocyte-bound pattern recognition molecule (Yoshino et al., 2008). The presence of "counter receptors" on hemocytes, such as integrin-like molecules has also been suggested (Davids et al., 1998). These receptors could be bound by soluble forms of galectin, such that the galectin could also serve in cross-bridging hemocytes to a parasite surface (Yoshino et al., 2008).

Fibrinogen-related proteins (FREPs) are a family of lectins that contain one or two Ig domains and a fibrinogen domain, and they can bind soluble and surface antigens of parasites that infect the snail (Adema et al., 1997). They are considered to play a key role in the compatibility polymorphism that determines parasite/snail compatibility on an individual basis (see review in Coustau et al., 2015).

9.5.1.2.3 Lysozymes

Lysozyme is an enzyme existing in diverse organisms. It catalyzes the hydrolysis of β-1, 4-glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine of peptidoglycan, a major component of bacterial cell wall and causes bacterial cell lysis. Six distinct lysozyme types have been identified and three are found in animals, commonly designated as the c-type (chicken-type), the g-type (goose-type), and the i-type (invertebrate-type; Callewaert and Michiels, 2010). Although the data regarding gastropods are far less abundant than for bivalves, there is evidence that the three types may be found in these organisms. For example, c-type lysozyme has been isolated in the abalone *H. discus hannai* (Ding et al., 2011), g-type in the freshwater snails *O. hupensis* (Zhang et al., 2012) and *P. acuta* (Guo and He, 2014), and i-type in the marine conch *Lunella coronata* (Ito et al., 1999).

9.5.1.2.4 LBP/BPI Proteins

LBP lipopolysaccaride-binding protein and BPI (bactericidal/permeability-increasing protein) are components of the immune system that have been principally studied in mammals for their involvement in defense against Gram-negative bacterial pathogens (Krasity et al., 2011). Following specific binding to LPS, they increase the permeability of the bacterial membranes, and contributes to the elimination of bacteria (Elsbach et al., 1994). LBP/BPI genes have been detected in *B. glabrata* hemocytes and egg masses (Hathaway et al., 2010; Mitta et al., 2005). Baron et al. (2013) showed that a LBP/BPI (BgLBP/BPI1) of maternal origin is the major protein in *B. glabrata* eggs. It displays a strong biocidal activity against oomycetes in addition to its antibacterial activity, therefore, protecting *B. glabrata* offspring from lethal bacteria and fungi infections.

9.5.1.2.5 Phenoloxidase

Phenoloxidase (PO) catalyses the hydroxylation of L-tyrosine to L-DOPA (monophenoloxidase activity, MPO), as well as the oxidation of the diphenols to their respective quinones (diphenoloxidase activity, DPO). These quinones are capable of binding free amino groups in proteins to form protein crosslinkages, eventually leading to the formation of insoluble, chemically resistant protein polymers leading to the entrapment of foreign material in a capsule. This process has been referred to as sclerotization (sclerotin formation) or melanization (melanin formation; Waite, 1990).

PO is present in mollusc plasma in an inactive state (prophenoloxidase, proPO) that can be activated to PO by the endogenous proPO-activation system or some exogenous elicitors such as laminarin, SDS, LPS, etc. (Asokan et al., 1997). Numerous studies have demonstrated the importance of PO in the immunological defenses of bivalve molluscs (see e.g., Aladaileh et al., 2007; Butt and Raftos, 2008; Hellio et al., 2007). In gastropods, PO plays a role in sclerotization of shell (Nellaiappan and Kalyani, 1989). It can be released from circulating hemocytes into hemolymph when the animals are stimulated by physical injury or infestation (Asokan et al., 1997; Bahgat et al., 2002). It has also been found in the reproductive tract of *B. glabrata* and in its egg masses following transfer from the maternal organism (Bai et al., 1996, 1997; Hathaway et al., 2010). However, its role in immune defense of gastropods remains controversial (Scheil et al., 2013). Bahgat et al. (2002) did not show difference in PO activity in hemocytes of *B. glabrata* strains

susceptible or resistant to infection with miracidia of *S. mansoni*. PO-like activity was identified in the hemolymph of *L. stagnalis* (Leicht et al., 2013) but its response to immune elicitors was not consistent (Seppälä and Leicht, 2013). Furthermore, using histochemical methods, Vorontsova et al. (2015) recently showed that dopamine oxidation in this species involved peroxidase rather than PO activity.

9.5.1.2.6 Nitric Oxide

Nitric oxide (NO) plays an important role as a signal molecule throughout the animal kingdom, especially as an intercellular messenger in the central nervous system (Bruckdorfer, 2005). In gastropods, NO is involved in the control of feeding and locomotion in *Clione limacina* (Moroz et al., 2000), in the regulation of feeding in *A. californica* (Lovell et al., 2000), in food-attraction conditioning in *H. pomatia* (Teyke, 1996), in chemosensory activation of feeding in *L. stagnalis* (Elphick et al., 1995; Moroz et al., 1993), in the oscillation of olfactory neurons in the procerebral lobe in *Limax maximus* (Gelperin, 1994), and in the swimming rate in *Melibe leonine* (Newcomb and Watson, 2002).

Although it is not toxic itself, this short-lived radical, generated by nitric oxide synthase (NOS), also plays an important role in the elimination of pathogens as part of the innate immune response (Rodríguez-Ramos et al., 2010). Together with superoxide anions it forms peroxynitrile anion which is a highly toxic compound with antibacterial and antiviral activity (Beckman et al., 1996; Fang, 1997). The generation of NO (or its stable end products) in response to immunological challenge has been described in hemocytes of *V. ater* (Conte and Ottaviani, 1995; Ottaviani et al., 1993), *L. stagnalis* (Wright et al., 2006), and *B. glabrata* (Zahoor et al., 2009).

9.5.1.2.7 Other Factors

Other factors involved in immune response have been described in gastropods, such as aplysianin/achacin-like protein in the sea hare *A. kurodai* (Kisugi et al., 1989) and in *B. glabrata* egg masses (Hathaway et al., 2010), protease inhibitors, Gram-negative bacteria-binding protein (GNBP), and scavenger receptor cysteine-rich protein, C1q domain-containing protein, and protease inhibitor in the perivitelline fluid of the eggs of *P. canaliculata* (Sun et al., 2012).

Non-targeted transcriptomic studies have recently yielded a lot of original information. Mitta et al. (2005) have identified several hundred novel transcripts including 31 immune-relevant transcripts corresponding to various functional groups using random sequencing of a *B. glabrata* hemocyte cDNA library. For the first time transcripts displaying similarities with the mammalian cytokine MIF (macrophage migration inhibitory factor) and a PGRP (peptidoglycan recognition protein) were identified in a gastropod. Additional nontargeted studies helped identifying numerous candidates genes belonging to various functional groups including those coding for pattern recognition proteins, cell adhesion molecules, immune regulators, cellular defense effectors, proteases and protease inhibitors, or oxidative stress and stress-related proteins as well as candidates involved in regulatory networks and signaling pathways (review in Coustau et al., 2015; see also Adema et al., 2010; Deleury et al., 2012; Hanelt et al., 2008; Mitta et al., 2005).

9.5.1.3 HEMOCYTE-SIGNALING PATHWAYS

Deciphering the intracellular signal transduction pathways likely to be activated by exposure of hemocytes to exotic stimuli is a key to understanding hemocyte effector functions (Loker, 2010). Experimental studies with protein kinase C (PKC) activator showed that PKC is involved in H_2O_2 generation in *B. glabrata* (Bender et al., 2005), of superoxide anions in *L. littorea* (Gorbushin and Iakovleva, 2007), and of NO in *L. stagnalis* (Wright et al., 2006).

PKC activity is dependent on its phosphorylation status and studies have shown that phosphorylation of PKC's hydrophobic motif regulates the enzymes stability, phosphatase sensitivity, subcellular localization and catalytic function (Bornancin and Parker, 1997; Edwards and Newton, 1997). The fact that inhibitors of MAPKs also prevent hemocyte spreading or H_2O_2 production suggest that PKC activation is likely to result in activation via phosphorylation of MAPK-like ERK or p38 (Skála et al., 2014; Wright et al., 2006; Zelck et al., 2007). Natural stimuli such as laminarin also activate PKC and H_2O_2 production in *L. stagnalis* hemocytes (Lacchini et al., 2006). A role for phosphatidylinositol 3-kinase in controlling phagocytic activity has been shown in *L. stagnalis* hemocytes (Plows et al., 2006) and G-protein-coupled membrane receptors have also been reported from *L. littorea* (Gorbushin et al., 2009). Targeting by pathogens of components of

gastropod-signaling pathways such as p38 has been documented in abalones challenged by *Vibrio harveyi* (Travers et al., 2009).

Toll-like receptors (TLRs) are well-characterized PRRs of innate immunity, known to induce immune responses by interacting with evolutionarily conserved pathogen-associated molecular patterns (PAMPs). Homologs of TLRs are present in the *Lottia* genome, a Rel-like NF-κB transcription factor is known from abalones (Jiang and Wu, 2007), and AbTLR a TLR homolog from disk abalone (*H. discus discus*) was identified and characterized at molecular level (Elvitigala et al., 2013). Therefore, additional Toll-pathway homologs are likely to be present in gastropods, although their functional relevance remains to be assessed.

Acting upstream of Toll-signaling pathways is pattern recognition molecules like PGRPs and GNBP (or B-1-3 glucan recognition/binding protein or LGBP). Both short and long form PGRP-encoding genes are present in *B. glabrata* and at least three different GNBPs are also known from this species (Zhang et al., 2007b).

9.5.2 RESPONSE OF THE IMMUNE SYSTEM TO STRESS

9.5.2.1 THE CASE OF DIGENEAN TREMATODES

Along with the usual background of viral or bacterial challenge, gastropods face other pathogens that are unequivocally gastropod specialists, the best known being the digenetic trematodes, also known as digeneans or "flukes" (Loker, 2010). Studies on the interactions between these parasites and their gastropod host are probably the source of the major part of the available knowledge on gastropod immunity. This is undoubtedly the case for the investigations on the interactions between the freshwater snail *B. glabrata* and its trematode parasite *S. mansoni* (Coustau et al., 2015).

Detailed studies on the response of *B. glabrata* to different stressors, including infection by *S. mansoni* or other trematodes, have shown that exposed individuals may modulate their immunological response according to the stressor (Adema et al., 2010; Hanington et al., 2010a). In case of infection by a trematode, the snail-immune response may fail to clear the infection due to a combination of trematode-mediated avoidance and inhibition of snail defense mechanisms (Coustau and Yoshino, 1994; Douglas et al., 1993; Lie and Heyneman, 1977; Loker et al., 1986; Loker and Hertel, 1987; Noda and Loker, 1989a,b; Roger et al., 2008a). The identification of immune genes that could play a role in *B. glabrata* immune processes has been the

focus of many studies (Bouchut et al., 2006a,b, 2007; Deleury et al., 2012; Guillou et al., 2007a; Ittiprasert et al., 2010; Lockyer et al., 2007, 2008; Nowak et al., 2004; Raghavan et al., 2003; Vergote et al., 2005).

The dialog between the host snail and its parasite has been intensively studied. The comparative analysis of *B. glabrata* strains that are susceptible (M-line or NMRI strains) or resistant (13-16-R1 and BS-90 strains) to specific *S. mansoni* stocks have clearly demonstrated a strong genetic basis for the susceptibility of *B. glabrata* to *S. mansoni* (Lewis et al., 2001; Richards et al., 1992). Significant progress toward the identification of resistance genes have been made (Blouin et al., 2013; Bonner et al., 2012; Ittiprasert et al., 2013; Knight et al., 1999) but without complete achievement (Coustau et al., 2015).

Differences were shown in the short-term expression of genes in hemocytes from parasite-exposed and control groups of both schistosome-resistant and schistosome-susceptible strains. Genes involved in immune/stress response, signal transduction, and matrix/adhesion were differentially expressed between the two strains (Lockyer et al., 2013). Results suggest that resistant snails recognize parasites and mount an appropriate defense response. A lack of capacity to recognize and react to the parasite or an active suppression of hemocytes response by the parasite early in infection are the two main hypothesis that may explain the absence of defense in susceptible snails.

Studies on the mechanisms involved in the compatibility polymorphism characteristics in certain *B. glabrata*/*S. mansoni* populations allowed the identification of two repertoires of polymorphic and/or diversified molecules that were shown to interact: the parasite antigens SmPoMucs (*S. mansoni* polymorphic mucins) and *B. glabrata* FREP immune receptors (Roger et al., 2008a–c; Mitta et al., 2012; Moné et al., 2010). SmPoMucs are only expressed by larval schistosome stages that interact with the snail intermediate host. They are highly glycosylated and polymorphic. Each individual parasite possesses a specific and unique pattern of SmPoMucs. In *B. glabrata*, FREPs are also highly diversified (Adema et al., 1997; Hanington et al., 2010b; Loker et al., 2004), and they play a key role in the fate of the interaction between the snail and its trematode parasites. For example, FREP3 plays a central role in resistance to digenetic trematodes. It is up-regulated in *B. glabrata* infected with *S. mansoni* or *Echinostoma paraensei*, and functions as an opsonin favoring phagocytosis by hemocytes (Hanington et al., 2010b). Subsequent studies showed that FREP3 is important for successful defense against schistosome infections in *B. glabrata* and that its suppression by trematode parasites facilitate their establishment within the snail (Hanington et al., 2012). The cytokine-like molecule, BgMIF (*B. glabrata*

macrophage MIF) may also play a role in the anti-parasite response of *B. glabrata* (Baeza Garcia et al., 2010).

The availability of resistant and susceptible strains of *B. glabrata* has also stimulated innovative studies that provided support to several lines of evidence regarding the host–parasite interaction. Hemocytes from resistant snails possess a different allelic form of the Cu/Zn SOD and produce higher levels of H_2O_2, a substance lethal to *S. mansoni* sporocysts (Bayne, 2009; Bender et al., 2005, 2007; Goodall et al., 2004, 2006; Hahn et al., 2001). It remains unclear if there is a link between the two phenomena. Other putative immune effectors have been identified, including LBP, BPI, and AMPs (Guillou et al., 2007b; Mitta et al., 2005), but their functions remain to be determined. In addition, the first cytolytic β pore-forming toxin from a mollusc, biomphalysin, has recently been identified and characterized in *B. glabrata* (Galinier et al., 2013; Moné et al., 2010). Biomphalysin is only expressed in hemocytes and plays a sentinel role in preventing pathogen invasion. It has a hemolytic activity and binds parasite membrane, being highly cytotoxic.

Sporocysts of *S. mansoni* bear larval transformation proteins that may be involved in scavenging of ROS, therefore, offering a protection against hemocyte attack (Guillou et al., 2007b; Wu et al., 2009). A reciprocal coevolution has been demonstrated between ROS and ROS scavengers produced by sympatric populations of *B. glabrata* and *S. mansoni* (Moné et al., 2011). Other compounds such as glycoconjugates have been detected at the surface of sporocysts, which may serve as host-mimicking molecules (Peterson et al., 2009) and divert attack by humoral factors (Roger et al., 2008b,c).

As recently reviewed by Coustau et al. (2015), advances in the field of trematode/snail interaction clearly showed the multigenic and variable nature of the mechanisms underlying the success or failure of parasite development. Interactions include recognition mechanisms (e.g., lectin/glycan interactions) and effector and anti-effector systems (e.g., biomphalysin, LBP/BPI, and ROS/ROS scavengers). The relative importance of these different factors varies greatly among population of hosts and parasites. The consequences of additional phenomena such as interaction with environmental factors (e.g., temperature; Ittiprasert and Knight, 2012), immune priming associated with previous encounters with parasites (Portela et al., 2013), or epigenetic mechanisms (Perrin et al., 2013) remain to be studied in detail (Coustau et al., 2015). The current availability of the genome of *S. mansoni* (Berriman et al., 2009; Protasio et al., 2012) and the perspectives concerning the genome of *B. glabrata* (undergoing assembling and annotation; see https://www.vectorbase.org) offer great perspectives for forthcoming studies.

9.5.2.2 OTHER STRESSORS

Although there is more variation in neuroendocrine systems across invertebrate groups than there is within the vertebrates, similar molecules mediate some aspects of the acute stress response across phyla. In gastropods, the acute stress response originates in the endocrine system, with corticotropin-releasing hormone stimulating the release of adrenocorticotrophic hormone (ACTH), leading to the release of biogenic amines, noradrenaline, and dopamine into the hemolymph, which then mediate secondary effects (Malham et al., 2003). Although there is evidence that ACTH, a hormone that induces the release of stress hormones in vertebrates (Charmandari et al., 2005), may be present in molluscs (Ottaviani and Franceschi, 1996), its role in the response to acute stress in gastropods remains to be demonstrated (Adamo, 2012).

In the European abalone *H. tuberculata*, acute mechanical stress (mechanical shaking) increased the concentration of noradrenaline and dopamine (Malham et al., 2003). Concomitantly, a transient decrease was shown for the number of circulating hemocytes, their migratory activity, their phagocytic activity, and their respiratory burst response. This decrease was followed by an increase in some immune functions (e.g., phagocytic ability) a few hours later before returning to baseline, suggesting that acute stress can also have a delayed immunoenhancing effect in abalone. Evidence from studies in bivalves suggest that stress-related circulating biotic amines may inhibit hemocyte function following interaction with hemocyte β-adrenergic-like receptors that are coupled to a cAMP/protein kinase A pathway, similar to the receptors found in vertebrates (Lacoste et al., 2001). The existence of an identical pathway in gastropod has not yet been demonstrated.

One of the key questions when addressing the effects of stress on gastropods is the possible consequences on the immunocompetence of the stressed organisms. Immunocompetence, the general capacity of an individual to exhibit an immune response (Schmid-Hempel, 2003), is commonly assessed through the measurement of phagocytic activity (Hooper et al., 2007). Phagocytosis is probably the measure that has received the greatest amount of investigation when assessing the impact of changing environmental conditions on the mollusc immune response. It may be measured either through the proportion of hemocytes that are phagocytically active in a population or using the phagocytic index, that is, the number of bacteria or particles engulfed by each immune cell.

Phagocytic activity may exhibit natural seasonal variation caused by changes in organism physiology especially sexual maturation and spawning, sometimes leading to a natural increased susceptibility to infection at some

key moments of the life cycle. For example, a reduction in phagocytosis and phenoloxidase activity during gametogenesis has been reported in *H. tuberculata*, associated with an enhanced susceptibility to *V. hayeri* (Travers et al., 2008b). Therefore, measuring a change in phagocytic activity does not give information on the origin of this change neither on other possible cellular immune dysfunction. The measurement of additional parameters such as the abundance, morphology, or viability of hemocytes may provide information on the mechanisms behind phagocytic activity changes. In addition, although an increase in phagocytic activity might feasibly suggest an increase in the activity of the hemocytes themselves without any influence of hemocyte number as shown by Seppälä and Jokela (2010a) in *L. stagnalis* exposed to elevated temperature, this increase may also be an indirect effect of increased hemocyte numbers caused by an alteration in hemocyte proliferation or mobilization of cells from peripheral tissues (Hooper et al., 2011). Therefore, in measuring total hemocyte counts, it is possible to demonstrate a change in the number of hemocytes in context with changes in other cellular immunological measures (Perez and Fontanetti, 2011).

A number of authors have shown that gastropod immune system is sensitive to "natural" environmental changes. Significant reduction in hemocyte abundance, phagocytic activity, or respiratory burst have been recorded in gastropod species following exposure to changes in parameters such as temperature (Cheng et al., 2004a), salinity (Cheng et al., 2004b; Martello et al., 2000), air exposure (Cardinaud et al., 2014), and hypoxia (Cheng et al., 2004c), as well as changes in concentrations of ammonia (Cheng et al., 2004d) and nitrite (Cheng et al., 2004e).

Similar observations were made following exposure to anthropogenically induced stressors, including butyltins (Zhou et al., 2010), polyaromatic hydrocarbons (Gopalakrishnan et al., 2009), and pesticides (Russo and Lagadic, 2004; Russo et al., 2007). Experiments were also performed recently on primary cultured hemocytes with various chemicals, including metals and pharmaceuticals (Gaume et al., 2012; Ladhar-Chaabouni et al., 2014; Latire et al., 2012; Minguez et al., 2014; Mottin et al., 2010), but their results are not necessarily representative of the phenomena that may occur in whole animals.

In some cases, an increase in hemocyte number, ROS production and/or phagocytic activity following exposure to mechanic stress (Cardinaud et al., 2014; Hooper et al., 2011), municipal effluent (Gust et al., 2013b), metals (Itziou et al., 2011), or pesticides (Russo and Lagadic, 2000) was also observed.

Immunodulation by acute stress may lead to an increased in the susceptibility of gastropods to pathogens. For example, handling caused an alteration of all the immune parameter levels and a metabolic depression in *H. tuberculata*, associated with an enhanced susceptibility to *V. harveyi* infection (Cardinaud et al., 2014). A series of studies by Cheng et al. (2004a–e) reported an increase in susceptibility to *Vibrio parahaemolyticus* in the Taiwan abalone *H. diversicolor supertexta* exposed to elevated temperature, fluctuating salinity, high concentrations of organic compounds, and reduced oxygen levels.

The case of chronic exposure to toxic stress raises additional concerns. Chronic (several week-long) exposure to sublethal concentrations of toxic compounds was shown to modulate the immunocompetence in abalones (*H. diversicolor*, *H. diversicolor supertexta*) exposed to benzo[*a*]pyrene or TBT (Gopalakrishnan et al., 2011; Zhou et al., 2010). Short-term recovery following cessation of exposure was observed for benzo[*a*]pyrene but not for TBT (Gopalakrishnan et al., 2011).

Chronic stress is immunosuppressive in invertebrates with a decline in the expression of immune-related genes and in immunological function, and a loss of disease resistance (Ellis et al., 2011). However, the involved mechanisms are not well understood, especially regarding the links with stress hormones. In vertebrates, the common explanation is that elevated levels of stress hormones are initially adaptive but become pathological if they remain elevated for too long, due to the exhaustion of molecular resources, build-up of toxic compounds, and dysregulated pathways (Dhabhar, 2002; Hawlena et al., 2011; Romero et al., 2009). However, no evidence is available today regarding these processes in gastropods.

9.5.3 CURRENT PERSPECTIVES IN MOLLUSC IMMUNE SYSTEM

As stated earlier the immune system in molluscs is traditionally considered to lack specific immunity and to rely only on an innate immune system. However, increasing evidences have recently revealed the existence of specific or "primed" immunity in bivalves (Cong et al., 2008; Yue et al., 2013), and maternal transfer of immunity (i.e., immunity transferred via eggs from mother to offspring) is one of the highlighted instances to favor the presence of a kind of "specific" immunity in invertebrates (Yue et al., 2013; Wang et al., 2015). Many maternally derived immune factors have been identified in mollusc eggs or embryos (Wang et al., 2015). Antibacterial and lysozyme activities as well as agglutination against pathogens have

been demonstrated in terrestrial and marine gastropod eggs (Fiolka and Witkowski, 2004; Kamiya et al., 1986). Maternal transfer of lectins from mother to eggs/offspring has been reported in some gastropod species such as *H. pomatia* (agglutinin; Sanchez et al., 2006), *B. glabrata* (C-type lectin; Hathaway et al., 2010), *Pomacea scalaris* (scalarin; Ituarte et al., 2012), *Pila ovata* (anti-B-like agglutinin; Uhlenbruck et al., 1973), and *A. kurodai* (D-galactose-binding lectine; Kawsar et al., 2009). Primarily maternally derived lysozyme was found in snail *H. aspersa maxima* and *Achatina achatina* eggs (Fiolka and Witkowski, 2004).

Various factors have been shown to have an influence on the maternal transfer of immunity to offspring, being responsible for a "trans-generational immune priming" (TGIP). In molluscs, TGIP has been evidenced so far mostly in bivalves following exposure to pathogens (Gueguen et al., 2003; Oubella et al., 1994), changeable-environment conditions (Lacoste et al., 2002), and environmental pollutants (Pipe and Coles, 1995). Some biological factors such as age (Xu et al., 2011) and reproduction (Duchemin et al., 2007) may also have an influence on this phenomenon. Among gastropods, the effects of heat (in *L. stagnalis*; Leicht et al., 2013), nutrition (in *L. stagnalis*; Seppälä and Jokela, 2010b), and exposure to benzo[*a*]pyrene (in *H. diversicolor*; Gopalakrishnan et al., 2009) on TGIP have also been reported. Although the specific mechanisms underlying TGIP clearly deserve more investigation, it may be considered as a beneficial survival strategy of invertebrates.

In addition to the maternal transfer of immune factors from mother to eggs, it seems that maternal experience of pathogen or other immune stimulation also has a profound influence on the phenotype of offspring, probably through epigenetic inheritance or genomic imprinting (Yue et al., 2013). The first evidence of epigenetic modifications triggered by an immune challenge in a gastropod species was recently reported in *P. canaliculata* by Ottaviani et al. (2013).

9.6 STRESS AND LIFE-HISTORY TRADE-OFFS

Life history trade-offs are likely to have determinants of various origins: ecological (resource allocation), physiological (endocrine systems, oxidative stress), and genetic (genetic correlations due to linkage disequilibrium, pleiotropy, correlational selection).

9.6.1 DELAYED EFFECTS OF STRESS

"Latent" or "carry over" effects reflects the effects that originate in embryonic and larval experiences, but are expressed only in juveniles or adults. They are of particular interest in the ecological literature (see e.g., Altwegg and Reyer, 2003; Goater, 1994; Marshall et al., 2003; Ng and Keough, 2003; Pahkala et al., 2001; Phillips, 2002, 2004). Latent effects do not include the effects of adult conditioning on offspring quality, that is, "maternal effects" (Pechenik, 2006).

Latent effects have been observed for several groups of marine invertebrates (review in Pechenik, 2006), including gastropods. Most studies concern the consequences of starvation, pollution, or delayed metamorphosis (Table 9.2).

TABLE 9.2 Results of Experimental Studies on the Latent Effect of Stress on Gastropods.

Clade/Species	Treatment in larval or embryonic stage	Latent effect	Sublethal consequences	References
Vetigastropoda				
Haliotis asinina	Exposure to larval settlement cue (coralline algae)	Yes	Gene expression in metamorphosing postlarvae	Williams and Degnan (2009)
Coenogastropoda				
Crepidula fornicata	Starvation	Yes	Slower juvenile growth rate	Pechenik et al. (1996a,b, 2002)
Crepidula fornicata	Cadmium	No		Pechenik et al. (2001)
Crepidula onyx	Starvation	Yes	Reduced size, total organic content, and energy reserves of newly metamorphosed juveniles	Chiu et al. (2007, 2008)
			Reduced growth and filtration rate of juveniles	
Crepidula onyx	Hypoxia and low food availability	Yes	Reduced growth rate, dry weight, and filtration rate of juveniles	Li and Chiu (2013)

TABLE 9.2 *(Continued)*

Clade/Species	Treatment in larval or embryonic stage	Latent effect	Sublethal consequences	References
Crepidula onyx	Hypoxia and high food availability	No		Li and Chiu (2013)
Crepidula fornicata, Crepidula onyx, Crepipatella fecunda	Temporary reduction of salinity	No		Diederich et al. (2011)
Crepipatella dilatata	Reduction of salinity for brooding females	Yes	Reduced growth and reduced rates of oxygen consumption and feeding	Chaparro et al. (2014)
Nassarius festivus	Hypoxia	Yes	Reduced size of juveniles	Chan et al. (2008)
Heterobranchia				
Phestilla sibogae	Delayed metamorphosis with fed larvae	No		Miller and Hadfield (1990)
Phestilla sibogae	Delayed metamorphosis with starved larvae	Yes	Lower mean juvenile weight Decreased juvenile survival Decreased weight at reproductive maturity Longer mean time to reproductive maturity	Miller (1993)
Siphonaria australis	Elevated UVB, salinity and temperature	Yes	Reduced survival, larval growth, and length of velar cilia	Fischer and Phillips (2014)
Euopistobranchia				
Haminaea vesicular	Hypoxia	Yes	Reduced size of juveniles	Strathmann and Strathmann (1995)
Melanochlamys diomedea	Hypoxia	Yes	Reduced size of juveniles	Strathmann and Strathmann (1995)

The mechanisms for delayed or latent effects are not known in details. Depletion of energy stores in stressed larvae leading to a reduced availability of energy at metamorphosis is probably one of the main causes for

latent effect (Pechenik, 2006). However, some results indicate that this is not always the case. Several nonmutually exclusive hypothesis have been proposed: (1) larval stress would reduce the size of the juvenile feeding apparatus (e.g., abnormal gill size in juvenile *C. fornicata* issued from starved larvae; Pechenik et al., 2002), which in turns decreases the ability of the individual to feed after its metamorphosis thus causing a reduction in growth rate (Chiu et al., 2007, 2008; Marshall et al., 2003; Pechenik et al., 2002; Wendt, 1996); (2) interference with transcriptional or translational processes (Pechenik, 2006; Pechenik et al., 1998; Williams and Degnan, 2009); (3) direct damages to DNA and/or enzymes (Heintz et al., 2000); and (4) epigenetic effects (Jablonka and Raz, 2009). So far, the results obtained for gastropods only support the two first hypotheses.

Experimental studies with *C. onyx* showed that the latent effects of larval starvation varied depending on the timing of starvation (Chiu et al., 2008). The juveniles developed from larvae that had experienced starvation in the first two days of larval life had reduced growth and lower filtration rates than those developed from control larvae. Starvation experienced later in larval life caused a reduction in shell length, lipid content, and RNA:DNA ratio of larvae at metamorphosis. Furthermore, the corresponding juveniles performed poorly in terms of growth in shell length and total organic carbon content. Latent effects of some stressors may also vary according to the nutritional status of larvae. For example, Li and Chiu (2013) showed that hypoxia only exert a latent effect on juveniles under low larval food condition. When the food concentration during the larval stage was doubled there was no discernible effect on juveniles.

Williams and Degnan (2009) tested for latent effects of inductive cues (three different species of coralline algae) on gene expression in metamorphosing postlarvae of the tropical abalone, *H. asinina*. They showed that the expression profiles of 11 of 17 metamorphosis-related genes differed according to which species of algae the larvae settled upon. Several genes continue to be differentially expressed for at least 40 h after removal of the algae, clearly demonstrating a carryover effect of inductive cues on gene expression.

To conclude, the reasons why some stressors cause latent effects and others do not remain largely unknown. In addition, results are also often conflicting within a species since offsprings from different parents may exhibit different susceptibility, suggesting a genetic basis for this difference. Vulnerability depends on a variety of factors (e.g., intensity and duration of exposure, defense and repair mechanisms, physiological tolerance),

including the previous history of individuals, and even their parents. As stated by Fischer and Phillips (2014), though early life stages are particularly vulnerable to environmental stress and can be bottlenecks for populations, focusing too narrowly on immediate responses likely underestimate cumulative effects on populations and communities.

9.6.2 MEDIATORY ROLE OF OXIDATIVE STRESS

Among physiological mechanisms underlying life-history trade-offs, oxidative damage is thought to play a central role and to represent a universal constraint on life history evolution (Dowling and Simmons, 2009; Monaghan et al., 2009). The amount of cellular and molecular damage caused by oxidative stress increases with age, and this would in turn induce ageing and reduce lifespan (free radical theory of ageing, Harman, 1956; oxidative stress theory of ageing; Beckman and Ames; 1998). The theory is mostly supported by correlational data, which moreover come from vertebrates. For example, using a dataset from 10 mammalian and 2 avian species, Lambert et al. (2007) found a negative relationship between mitochondria ROS production and maximum lifespan. Similarly, lower ROS production and more efficient antioxidant defenses were observed in long-lived than in short-lived garter snakes (Robert and Bronikowski, 2010). Moreover, an age-related decreased activity of the proteasome (the enzymatic complex which is responsible for the degradation of abnormal and oxidized proteins) is observed in various mammalian tissues (Löw, 2011). In gastropods, though data are very scant, consistent results were obtained in land snails, at both intra- and inter-specific levels. *C. aspersum* subsp. *aspersa*, which is shorter-lived than its counterpart *C. aspersum maxima* (giant form) also exhibits higher ROS production upon bacterial challenge (Russo and Madec, 2011). Also, considering the positive relationship between lifespan and body size, the same authors found a similar trend between species (higher ROS production in smaller species, *O. lactea*, *Th. pisana*, *Cepaea nemoralis*, compared to the larger species *C. aspersum* and *H. pomatia*; Russo and Madec, 2013).

The causative role of oxidative stress in the ageing process, that is, that increased ROS production and/or decline in antioxidant defense are responsible for the accumulation of cellular damage, is far from being demonstrated. It is even strongly invalidated by recent findings, such as the increased levels of pro-oxidants triggered by increased antioxidant activity, or lifespan shortening by antioxidant dietary supply (see Gems and Doonan, 2009 and references therein). Also unsupportive of the oxidative stress theory of ageing

is the lack of relationship between ROS production or antioxidant defenses and exceptional longevity, such as in the naked mole rats; see Austad, 2010). With respect to reproduction, opposing conclusions are also drawn from observations, for example, increased oxidative damage in plasma proteins during lactation in the red squirrel (Fletcher et al., 2012) on the one hand, and reduced oxidative damage in reproducing female house mouse (Garratt et al., 2012) on the other hand. Therefore, to be solved, this issue clearly calls for new datasets, and we feel that gastropod models represent a promising and relevant study system in this respect.

9.7 CONCLUDING REMARKS AND EVOLUTIONARY PERSPECTIVES

Through this chapter, we have tried to give to the reader an overview of the diversity of stress responses in gastropods, by considering natural (biotic and abiotic) and anthropogenic stressors. Most examples cited revolved around oxidative stress, as oxygen imbalance is pivotal in eliciting and regulating stress responses at the organismal, cellular, and molecular level (Kassahn et al., 2009). Furthermore, oxidative stress plays a central role in life history, through its effects on life history trade-offs (Monaghan et al., 2009). However, in many instances, stress response pathways could not be fully described, due either to the merely correlational nature of available data, or more basically to the lack of mandatory genetic resources, and the need to resort on the knowledge based on phylogenetically remote models such as vertebrates. Thanks to recent technological advances in sequencing (NGS, next generation sequencing), the latter issue is hopefully becoming a less important obstacle to researchers, especially those working with nonmodel organisms, such as most gastropods. As mentioned earlier in this chapter, gastropod full transcriptomes are continuously appearing in the literature, which contain a wealth of information for those interested in stress molecular responses of their preferred species. However, for a full analysis of the pathways involved in these responses, an annotated genome would be evidently more appropriate. Currently, two species have their genome sequenced and annotated, *L. gigantea* (selected for its small genome size) and *A. californica*, but it is to be noted that a *B. glabrata* genome project is underway and already provides a large set of resources relevant to malacologists. Also, the genome of *L. stagnalis* is currently being sequenced, under the scientific coordination of a multidisciplinary consortium. As this species has been for long a model of choice in invertebrate zoology and

biology, and also serves as model to neurophysiologists, this new resource is keenly anticipated. Furthermore, at a deeper evolutionary scale, comparative studies based on full genomes provide a unique tool for hypothesis validation in gastropods (see e.g., evolution of steroid hormone-signaling pathway in Metazoans; Markov et al., 2009).

Throughout this chapter, variation in stress response has been raised, at both intra- and interspecific levels. While the latter level of variation can be attributed to phylogenetic determinants and/or to ecological niche differences, the former may result from shorter term (micro) evolutionary forces, such as natural selection or population isolation and associated random genetic drift, and of their interaction with environment on population genetic diversity and ability to adapt to new conditions.

Population response to stress has both a plastic and a genetic component. The latter forms the basis for adaptive evolution. Until recently, evolutionary processes related to adaptation have been mostly investigated through integrated polygenic characters (life-history traits, morphology). However, a growing number of studies focuses on adaptive evolution at the level of gene expression, and this is noticeably due to the huge breakthrough in genetical and population genomics that has been permitted by NGS (e.g., Jeukens et al., 2010; Stapley et al., 2010). High genetic variation in gene expression has been found in various taxa (Fay and Wittkopp, 2008; Gibson, 2008; Schadt et al., 2003; Whitehead and Crawford, 2006). Therefore, the role of regulatory changes in evolutionary adaptation evolution is a question that can now be investigated at the genome scale through population genomics. Gene expression is assumed to evolve mostly under stabilizing selection, although this hypothesis may be difficult to disentangle from neutrality (Gilad et al., 2006; Whitehead and Crawford, 2006).

Under the present context of global change and intensification of environmental pressures, the evolutionary component of population response to stress has also become an emerging issue in environmental sciences and in ecotoxicology (Bickham, 2011; Klerks et al., 2011; Lankau et al., 2011). Although evidences for evolutionary impact of pollutants are accumulating (see Coutellec and Barata, 2013 and references therein), very few studies have focused on gastropods so far, and basically on *L. stagnalis* and pesticides (Bouétard et al., 2014; Coutellec and Lagadic, 2006; Coutellec et al., 2011, 2013). Interestingly, this species exhibits high population genetic variation in copper tolerance (acute toxicity; Côte et al., 2015), as well as in transcriptomic expression, both constitutive and induced by the pro-oxidant herbicide diquat (Bouétard et al., 2013). It is expected that similar studies on

gastropods from marine, terrestrial, and freshwater habitats will help disentangling stress response pathways and their evolution in this ecologically important group.

KEYWORDS

- **gastropods**
- **environmental stress**
- **homeostasis**
- **stress signaling**
- **immune system**
- **life history**

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PHYSIOLOGY OF MOLLUSCS

Volume 2

A Collection of Selected Reviews

PHYSIOLOGY OF MOLLUSCS

Volume 2

A Collection of Selected Reviews

Edited by

Saber Saleuddin, PhD
Spencer Mukai, PhD

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Saber Saleuddin, PhD, is a University Professor Emeritus of the Department of Biology at York University in Toronto, Ontario, Canada. Dr. Saleuddin received his early education in Bangladesh. He received his doctorate in molluscan zoology from the University of Reading in the UK. After an NRC Research Fellowship at the University of Alberta, studying biomineralization in molluscs, he continued his research on biomineralisation in the laboratory of Karl Wilbur at Duke University. Though offered a position at Duke, he accepted a faculty appointment at York University in Canada, where he taught for 37 years. The university recognized his outstanding contributions to research, teaching, and administration by honoring him as a University Professor. He has published more than a hundred papers in international journals and has co-edited three books on molluscan physiology. He served as co-editor of the *Canadian Journal of Zoology* for 18 years and was president of the Canadian Society of Zoologists, from whom he was awarded the Distinguished Service Medal.

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Spencer Mukai, PhD, is currently an instructor and technician at York University's Glendon College campus (Toronto, Ontario, Canada), where he is facilitating the implementation of a new biology undergraduate teaching laboratory. Dr. Mukai's research interests are in the neuroendocrine regulation of reproduction, growth, and osmoregulation in molluscs. He has published in and served as reviewer for national and international journals. After receiving his BSc and PhD from the Department of Biology, York University, Dr. Mukai has spent time as a postdoctoral fellow and research associate as well as an instructor at York University's Keele campus. He has demonstrated labs in invertebrate physiology and zoology for many years and has taught a variety of courses, including invertebrate physiology and endocrinology, animal physiology, environmental physiology, histology, human physiology, parasitology, introductory biology, ecology, and conservation biology.

DEDICATION TO OUR MENTORS

Professor Alastair Graham, FRS
1906–2000

Professor Alastair Graham, born in Edinburgh, was one of the most distinguished molluscan biologists. Professor Graham started his teaching career at Sheffield University. After 20 years of teaching and administrative duties, including the Chair of Zoology and Dean of the Faculty at Birkbeck College in London, he took up the Chair of Zoology at the University of Reading in 1952 and later served as Deputy Vice Chancellor. During his career he published many papers in collaboration with Dr. Vera Fretter. One of their most important publications is the classic *British Prosobranch Molluscs.* He received many accolades for his scholarship, including a DSc and a Fellowship of the Royal Society. Professor Graham was also the editor of *The Journal of Molluscan Studies* for many years.

Duke Professor Karl M. Wilbur
1912-1994

Professor Wilbur was born in New York, and following his doctoral degree at the University of Pennsylvania, he joined the Zoology Department of Duke University in 1946. He became a James B. Duke Professor in 1961. His major interest in research was the physiology of mineralization, primarily in molluscs. Professor Wilbur was an eminent cell physiologist. In addition to many scientific papers, Professor Wilbur is best remembered for coediting the classical volume *The Physiology of Mollusca* and being Editor-in-Chief of the series *The Mollusca,* published by then Academic Press. One of Professor Wilbur's long-time collaborators was Professor Norimitsu Watabe of the University of South Carolina. Together they published many articles that made a significant advancement to the field of biomineralization.

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LIST OF ABBREVIATIONS

AKH	adipokinetic hormone
AREAC	The Aquatic Research and Environmental Assessment Center
CBIs	Cerebro-buccal Interneurons
CDCs	caudo-dorsal cells
CGCs	cerebral giant cells
ChL	chromatophore lobe
CNS	central nervous system
CPG	central pattern generator
CPG	cerebral plus pedal ganglion
CR	conditioned response
CSs	conditioned stimuli
CTA	conditioned taste aversion
DBD	DNA-binding domain
DNMT	DNA methyltransferase inhibitor
ELH	egg-laying hormone
ERR	estrogen-related receptor
GI	gonad index
GnRH	gonadotropin-releasing hormone
GVs	germinal vesicles
IP3	input 3 interneuron
IPSP	inhibitory postsynaptic potential
ITM	intermediate-term
LBD	ligand-binding domain
LTM	long-term memory
MIF	median inferior frontal lobe
MT	memory test
OA	octopamine
OMAF	oocyte maturation arresting factor
PKC	protein kinase C
PNS	peripheral nervous system
RIs	retention intervals
RPeD1	right pedal dorsal 1
SF	superior frontal

SO	slow oscillator
SV	subvertical lobe
TER	tentacle extension response
VD4	visceral dorsal 4
VL	vertical
VPF	vitellogenesis promoting factor

PREFACE

The first comprehensive treatment on the physiology of molluscs was published in two volumes, edited by K. M. Wilbur and C. M. Yonge in 1964 and 1966. Almost 20 years later, a landmark compendium in multiple edited volumes on the biology of molluscs was published between 1983 and 1988. This series dedicated two volumes (volumes 4 and 5) to review papers on molluscan physiology. K. M. Wilbur was the editor-in-chief of this important series. The volumes in 1964 and 1966 and those in the 1980s were all published by then Academic Press.

The only review series on selected aspects of molluscan physiology since the 1980s was a special volume of the *Canadian Journal of Zoology*, published in 2013, which was edited by Saber Saleuddin. As luck would have it, we were approached by Apple Academic Press in 2014 to edit another volume dedicated to molluscan physiology, which we enthusiastically agreed to undertake.

With the rapid development of cutting-edge proteomic, molecular biological, and cellular imaging techniques, our understanding of molluscan physiology, specifically in the areas of neurobiology, reproductive biology, and shell formation, has increased exponentially over the last several years. Therefore, we felt that compiling an edited volume of review papers was warranted, and we hope that this book will serve as an important resource for researchers, professors, and students.

Editing a review series is a daunting task. The major challenge of such an endeavor is not what areas we could cover but how to deal with topics where we were unable to find excellent contributors. Thus, the titles and areas of research included in this book are our personal choices based on availability of contributors and their willingness to write within the allotted time frame. Furthermore, in certain fields of physiology, such as osmoregulation and defense mechanisms, we felt that the fields have not advanced significantly enough to warrant reviews. To partially compensate for not covering certain fields, we have included two papers previously published in the *Canadian Journal of Zoology.* The only instructions we gave to contributing authors is that the coverage be comprehensive, with a brief introduction, present knowledge highlighting the significant recent findings, and finally, provide suggestions about future directions in the context of recent developments.

We are indebted to friends and colleagues around the globe who have kindly contributed to this volume. During the months of writing, rewriting, and editing, the authors have been unfailingly cooperative in all we have requested them to do. We gratefully thank the appraisers who provided an immense service by providing critical appraisal and evaluation of each paper. Each revised paper was so much better following the evaluation reports. The fact that this service is given freely attests to the generosity of our colleagues.

We had expected that a single volume should suffice, but as the project developed it became apparent we needed two volumes. In grouping papers for the two volumes, we tried to ensure that the majority of papers in each volume complemented each other and were aimed at specific readers. Thus, Volume 1 is on shell structure, mineralization, the dynamics of calcium transport, shell drilling, byssus proteins, locomotion, and reproduction. Volume 2 includes reviews on the neural mechanisms of learning, reproductive behavior, responses to environmental stress and hormones, and neurotransmitters. We believe that the reviews included in these two volumes make a significant contribution to our understanding not only of molluscan physiology but also the physiology of animals in general.

We are grateful to Sandra Jones Sickels, Ashish Kumar, and Rakesh Kumar of Apple Academic Press for their invaluable guidance and support not only at the planning stages, but also during the editing and printing processes. Finally, we are grateful to the Canadian Science Publishing of Ottawa for allowing us to reprint two papers from the *Canadian Journal of Zoology.*

CHAPTER 1

ASSOCIATIVE MEMORY MECHANISMS IN THE POND SNAIL *LYMNAEA STAGNALIS*

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CONTENTS

ABSTRACT

Lymnaea is an attractive model system for physiologists interested in understanding the fundamental mechanisms of associative learning and memory. Operant conditioning has been investigated in the respiratory system and classical conditioning in the feeding system. Many of the components of the neural networks that generate the respiratory and feeding behaviors have been identified, and it is possible to record the electrical activity of these neurons both during and after conditioning. In this chapter, we highlight the advances made in understanding the network and cellular and molecular mechanisms underlying the two types of associative conditioning.

1.1 INTRODUCTION

The freshwater pond snail *Lymnaea stagnalis* (Linnaeus 1758) has been used often in the field of Neuroscience. In particular, it has proven an exceptional model system for examining aspects of associative learning and memory at the behavioral, neuronal, and molecular level. Studies in *Lymnaea* have focused on two forms of associative learning; classical conditioning, which involves learning associations between two unrelated stimuli, and operant conditioning, which involves learning associations between a behavior and the consequences of that behavior. In general, for an animal to be appropriate for studying mechanisms of associative learning, it must produce behaviors that are both easily quantifiable as well as trainable. *Lymnaea* exhibits at least two such behaviors; the aerial respiratory behavior which can be operantly conditioned and the feeding behavior which can be classically conditioned.

It is known that implicit memories are stored within the neural networks that mediate the behavior. Since many of the neurons underlying both aerial respiration and feeding in *Lymnaea* have been identified, it has thus been possible to examine the changes correlated with associative memory formation in this species, both at the level of individual neurons as well as network properties. The *Lymnaea* central nervous system (CNS) consists of a central ring of 9 ganglia, with 2 additional buccal ganglia on the buccal mass, and contains an estimated 20,000 neurons. The CNS has been extensively "mapped," with many of the neurons on the dorsal and ventral surface of the ganglia individually identified or arranged into named, functional groups. The CNS has been studied at an electrophysiological level for many decades and the functions of many individual neurons are known. The synaptic

connections between many of these cells, as well as the transmitters released at some of these synapses, have also been described (Benjamin & Kemenes, 2009; Benjamin & Winlow, 1981; Benjamin, 2008; Magoski & Bulloch, 1997; Syed & Winlow, 1991; Syed et al., 1991; Winlow et al., 1981). In particular, the networks mediating aerial respiration and feeding have now been well characterized, including the individual components of the central pattern generators (CPGs) which generate the rhythmic outputs, as well as many of the sensory and motor components of the network.

In this chapter, we will describe the conditioning of respiratory and feeding behaviors in *Lymnaea*, and then highlight the advances made in our understanding of the cellular and molecular mechanisms underlying associative learning and memory of these behaviors.

1.2 OPERANT CONDITIONING OF THE AERIAL RESPIRATORY BEHAVIOR

1.2.1 THE AERIAL RESPIRATORY BEHAVIOR OF LYMNAEA STAGNALIS

Lymnaea is a bimodal breather and performs gas exchange either cutaneously (when submerged) or by means of aerial respiration at the water's surface. *Lymnaea* demonstrate a preference for aerial respiration when the dissolved oxygen content of the water declines (hypoxia), while cutaneous respiration is favored in well-aerated water (Jones, 1961; Syed et al., 1991). During hypoxic conditions, when aerial respiration is required, *Lymnaea* travel to the water's surface where they open their respiratory orifice, known as the pneumostome, to perform gas exchange (Fig. 1.1A). This aerial respiratory behavior is irregularly rhythmic, and *Lymnaea* might open and close their pneumostome a number of times before finally resubmerging (Jones, 1961; Syed et al., 1991).

The frequency with which *Lymnaea* perform aerial respiration changes over their lifespan; adult snails perform the behavior more frequently than juveniles, both in eumoxic (normal) or hypoxic conditions (McComb et al., 2005b). Interestingly, adult animals will perform this behavior even if they are prevented from doing so during their entire developmental period. *Lymnaea* can be raised from embryos in a manner that prevents them from surfacing or opening their pneumostome until they reach adulthood. As adults, they will however travel to the water's surface (when permitted to do so) and perform aerial respiration (Hermann & Bulloch, 1998; Khan &

Spencer, 2009), clearly indicating that this behavior is not learned, and is instead innate.

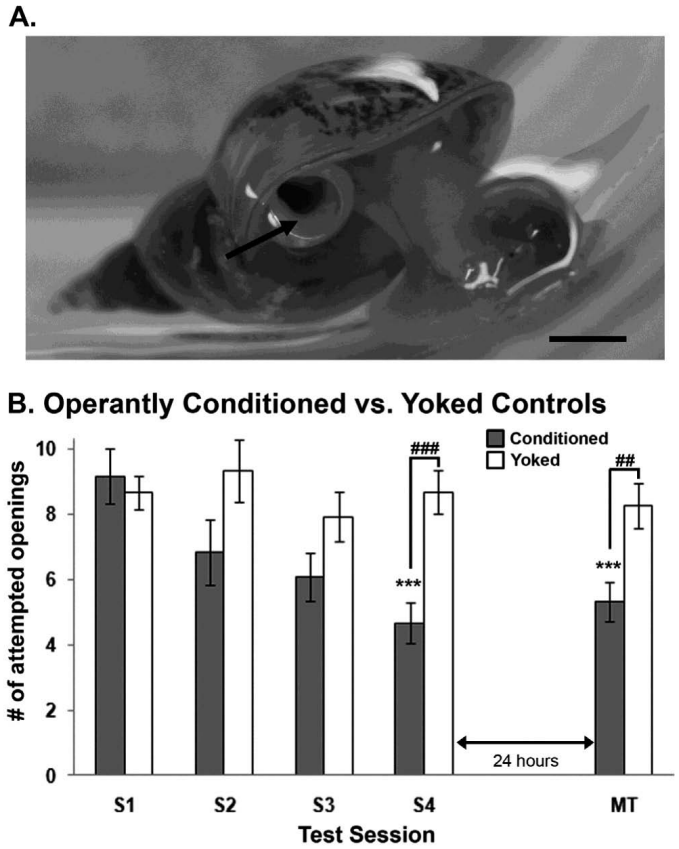

FIGURE 1.1 *The aerial respiratory behavior of Lymnaea stagnalis can be operantly conditioned.* (A) *Lymnaea stagnalis* opening its pneumostome (arrow) to perform gas exchange via aerial respiration at the surface of the water. Scale bar = 5 mm. (Photo courtesy of Dr. N. Vesprini). (B) Representative data from operantly conditioned and yoked control snails. Animals received four training sessions (S1–S4) and a memory test (MT) 24 h later. The operantly conditioned animals demonstrate a significant reduction in attempted pneumostome openings between S1 and S4 (indicative of learning) and this reduction in behavior is maintained for at least 24 h at the MT (***$p < 0.001$, relative to S1). However, yoked control animals demonstrate no significant change in aerial respiratory activity over time. Operantly conditioned snails also opened their pneumostome significantly less often than yoked controls during both S4 and the MT ($^{###}p < 0.001$; $^{##}p < 0.01$; $n = 12$ for both groups).

1.2.2 THE AERIAL RESPIRATORY BEHAVIOR OF LYMNAEA CAN BE OPERANTLY CONDITIONED

In order to use *Lymnaea* as a model organism to investigate neural correlates of learning and memory, it was first necessary to fully investigate the behavioral changes induced by specific training paradigms. Lukowiak et al. (1996) first developed the operant conditioning paradigm for aerial respiration in *Lymnaea*. Animals were exposed to hypoxic conditions (to increase the frequency of aerial respiration) and an aversive (punishing) tactile stimulus was presented to the snail's open pneumostome each and every time it opened at the water's surface. When this aversive stimulus was applied over a number of training sessions, the animals learned to associate the behavior with the punishment, which resulted in reduced respiratory behavior, including a reduction in the number and duration of pneumostome openings over time.

A typical learning curve following four training sessions is shown in Fig. 1.1B (gray bars). Depending on the number and length of training sessions administered, *Lymnaea* are capable of forming both intermediate-term (ITM) and long-term (LTM) memory (Lukowiak et al., 1998, 2000). ITM is generally defined as a memory that lasts for a few (1–3) hours, whereas LTM lasts for at least 18–24 h (as seen in the memory test (MT) in Fig. 1.1B). These stages of memory have differing dependencies on gene activation in *Lymnaea*; LTM requires both gene transcription and protein synthesis, whereas ITM requires only new protein synthesis (Sangha et al., 2003c).

A number of different control experiments have been carried out to verify the associative nature of this conditioning-induced reduction in respiratory behavior. For example, simply applying a random, punishing stimulus to the closed pneumostome of the snail is not sufficient to induce a change in respiratory behavior. Though such "yoked control" animals receive the same number of stimuli as trained animals, they fail to make any association between the stimulus and pneumostome opening, and thus show no subsequent changes in their aerial respiratory behavior (white bars in Fig. 1.1B; Lukowiak et al., 1996, 1998, 2000).

During the conditioning procedure, animals close their pneumostomes once stimulated and are thus prevented from breathing throughout the training session. In order to ensure that the reduced behavior is indeed a result of associative learning (and not simply a result of the animal becoming increasingly hypoxic), appropriate hypoxic controls are also carried out. *Lymnaea* are actually remarkably resistant to hypoxia; hypoxic

control animals prevented from surfacing and breathing for the entire duration of the training sessions, also show no subsequent reductions in their respiratory behavior (Lukowiak et al., 1996; Lowe & Spencer, 2006). In light of this experimental evidence, it can be concluded that the animals show effective learning and memory following operant conditioning of the aerial respiratory behavior, but this requires the consistent application of the stimulus to the open pneumostome.

This operantly conditioned response has since been extensively studied and shares many features with conditioned behaviors of vertebrates and mammals, one of which is context-dependency. Haney and Lukowiak (2001) showed that animals can be exposed to a different environmental context during training, such as a carrot odor. The presence of this odor does not itself affect either learning or memory, but its presence is later required for expression of the conditioned response. That is, animals only exhibit memory when tested in the *same context* as that in which they were originally trained. In addition to being context-dependent, the conditioned respiratory response can also be extinguished (due to the presence of interfering events; Sangha et al., 2003a) and reconsolidated (following a memory reactivation session; Sangha et al., 2003b). Interestingly, if memory is reactivated and animals undergo reconsolidation in a different context than that in which they were trained, memory for both contexts is observed (Lukowiak et al., 2007).

Another interesting observation regarding this operantly conditioned behavior is that snails from different locations have different memory-forming capabilities. Snails reared and trained in the Lukowiak lab (Calgary snails) require only two 30-min training sessions to produce LTM lasting 24 h (Braun & Lukowiak, 2011). However, in the Spencer lab (Brock snails), two training sessions produce only ITM lasting 2 h (but not 24 h) and LTM requires four training sessions (Lowe & Spencer, 2006; Rothwell & Spencer, 2014). We recently discovered that baby snails originating in the Spencer lab, but reared and trained in the Lukowiak lab, still do not form LTM with only two training sessions, despite being reared for at least 2 months in Calgary (Sunada, Dodd and Lukowiak, *unpublished observations*). This finding perhaps alludes to a genetic rather than environmental cause for the observed differences in memory formation. Braun et al. (2012) have also reported differences in memory-forming capabilities of wild strains of *Lymnaea* from close geographical locations in Alberta (discussed in more depth in the chapter by K. Lukowiak).

1.2.3 THE NEURONAL NETWORK UNDERLYING AERIAL RESPIRATION

The ability to perform electrophysiological recordings from individually identifiable neurons in the CNS of *Lymnaea* has allowed for the identification of many components of the neuronal network underlying aerial respiration. As with many other rhythmic behaviors, aerial respiration in *Lymnaea* is controlled by a CPG, the identity of which was described over two decades ago (Syed & Winlow, 1991). At least three neurons that comprise this CPG are now well characterized and have been mapped to specific ganglia within the CNS (Fig. 1.2A). Right Pedal Dorsal 1 (RPeD1) is a giant dopaminergic neuron located on the dorsal surface of the right pedal ganglion and is the neuron primarily involved in initiating the CPG network activity. RPeD1 forms connections with two other important CPG neurons, namely Input 3 Interneuron (IP3; located on the ventral surface of the right parietal ganglion) and Visceral Dorsal 4 (VD4; located on the dorsal surface of the visceral ganglion) (Syed & Winlow, 1991).

When RPeD1 becomes active, it excites the IP3 interneuron which controls pneumostome opening (expiration). Pneumostome closing (inspiration) is controlled by the activity of VD4. The rhythmical nature of the pneumostome opening and closing behavior is a consequence of the alternate bursting activity produced by IP3 and VD4, respectively, which is itself facilitated by the presence of reciprocal inhibitory synaptic connections between these two neurons (Fig. 1.2B). Such reciprocal inhibitory connections are common in many CPG networks and constitute a half-center model that allows for the alternate rhythmical firing activity and consequent motor output.

The role of these three CPG neurons in the generation of aerial respiration has been shown in semi-intact preparations (Syed & Winlow, 1991), in isolated CNS preparations (Spencer et al., 1999; Syed et al., 1990), and in vitro (cell culture; Syed et al., 1990). Indeed, the sufficiency and necessity of these three CPG neurons in generating rhythmic oscillations was demonstrated in cell culture by Syed et al. (1990). The three individual neurons were isolated and removed from the CNS and plated in cell culture, where they demonstrated robust neurite outgrowth and reformed their appropriate synaptic connections (Fig. 1.3A). Once synaptic connections were reestablished, Syed et al. (1990) were able to study the electrophysiological characteristics of these neurons and thus the connectivity of the CPG in detail. It was shown that the presence and activity of all three neurons was required in order to generate the rhythmic oscillations (Fig. 1.3B). In particular, this cell culture approach determined that neither VD4 nor IP3 were endogenous

bursters but were instead conditional bursters, requiring dopaminergic synaptic input from RPeD1 in order to produce the oscillatory behavior.

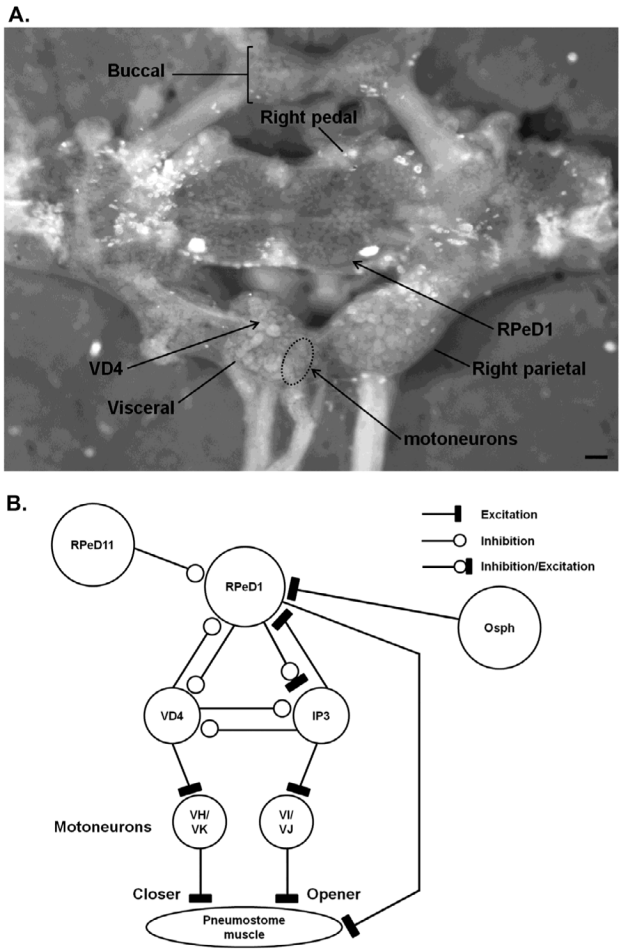

FIGURE 1.2 *The Lymnaea central nervous system and central pattern generator controlling aerial respiration.* (A) The central ganglia of *Lymnaea* (dorsal surface shown) consists of 11 ganglia (9 of which form a central ring). The arrows refer to the CPG neurons involved in aerial respiration (RPeD1: Right Pedal Dorsal 1; VD4: Visceral Dorsal 4). The motoneurons involved in aerial respiration are the opener motoneurons (VI/VJ) and closer motoneurons (VH/VK). Note that Input 3 Interneuron (IP3) is located on the ventral surface of the right parietal ganglion and is thus not shown. Scale bar = 100 μm. (B) Schematic illustration of the neural connections within the aerial respiratory CPG. Note that IP3 innervates the opener motoneurons, while VD4 innervates the closer motoneurons. Right Pedal Dorsal 11 (RPeD11) is responsible for triggering the whole body withdrawal response. "Osph" refers to the osphradial neurons which excite RPeD1 in hypoxic conditions.

The necessity for either RPeD1 or VD4 in generating network output was also demonstrated in cell-ablation studies, where the removal or destruction of either RPeD1 (Scheibenstock et al., 2002) or VD4 (Syed et al., 1992) in the intact CNS, abolished the respiratory behavior. The requirement of only three neurons in generating rhythmic network output for such an important homeostatic behavior may appear unusual. However, the reconstruction of the network in cell culture (Syed et al., 1990) demonstrated that these neurons were *sufficient* to generate the network behavior but did not rule out the involvement of other, as yet, unidentified neurons which may contribute to and/or modulate CPG network activity.

In addition to the identification of the respiratory CPG neurons, the sensory and motor components underlying the behavior have also been identified and studied. IP3, which controls pneumostome opening, excites a group of motoneurons known as the visceral I/J (VI/VJ) cells, whereas VD4, which controls pneumostome closure, excites visceral H/K (VH/VK) motoneurons (Syed et al., 1991). Both groups of motoneurons directly innervate pneumostome muscles. The excitatory input of IP3 onto the VI/VJ cells generates characteristic bursting and/or plateau potentials in these opener motoneurons, which is unlike any other input onto these cells. This has proven useful in the study of CPG activity in the intact CNS, as unlike the other two CPG neurons, IP3 is located on the ventral surface of the CNS and cannot be recorded from at the same time as RPeD1 or VD4, which are both located on the dorsal surface. The activity of IP3 can thus be monitored indirectly from recording its characteristic inputs onto the VI/VJ motoneurons (see Fig. 1.3Bi).

Chemo-sensory input to the respiratory CPG (particularly in response to hypoxia) likely originates in the periphery (Inoue et al., 2001). The neurite processes of the CPG neuron RPeD1 project to the periphery and contact the osphradium, an organ located near the pneumostome. Osphradial cells have been shown to excite RPeD1 under hypoxic conditions (Bell et al., 2007) and are proposed to be oxygen-sensing peripheral chemoreceptors. However, lesioning of the osphradial nerve has differing consequences on respiratory behavior (Bell et al., 2007; Karnik et al., 2012) and so the role of the osphradium and the identification of the chemoreceptors responsible for sensing peripheral oxygen still require further investigation. Interestingly, in addition to receiving chemo-sensory input, the neurite processes of RPeD1 also respond to mechano-sensory input; RPeD1 is also activated when the pneumostome breaks the surface of the water (Haque et al., 2006). Evidence also suggests that RPeD1 can bypass the entire motor program and can directly produce pneumostome opening, likely via direct synapses with

pneumostome muscles (Haque et al., 2006). Although the reason for this direct activation is not yet clear, it highlights the multifunctional nature of this important interneuron in the respiratory behavior.

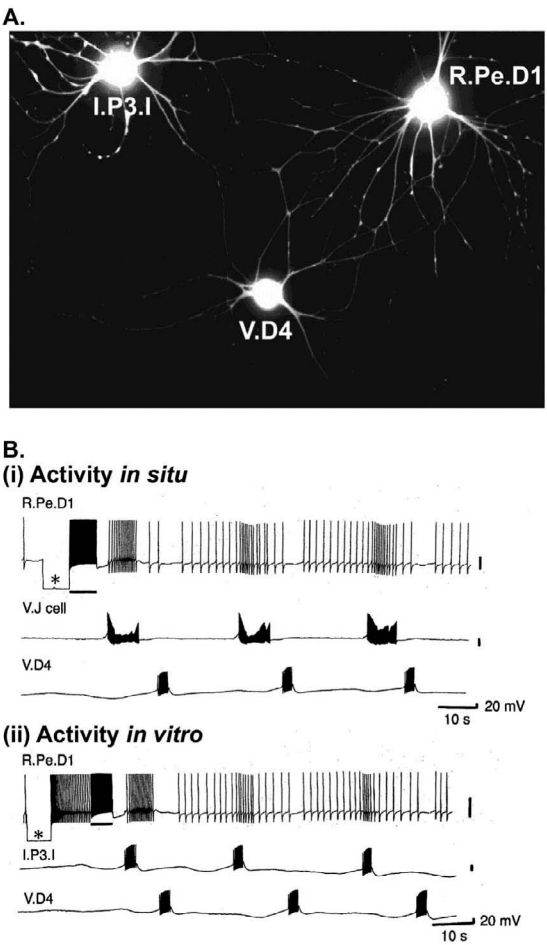

FIGURE 1.3 *The reconstruction of the Lymnaea respiratory central pattern generator in vitro.* (A) Photomicrograph of the three neuron respiratory central pattern generator in cell culture. (Photograph courtesy of N. I. Syed, University of Calgary and reproduced with permission of *Can. J. Zool.* **2013,** *91*(6), 384 (Part A); © NRC Research Press). (B) Electrophysiological recordings demonstrating activity within this central pattern generator both in situ (i) and in vitro (ii). Note that following stimulation of RPeD1 (indicated by the black bar on the trace), the respiratory rhythm is initiated. In situ, the VJ motoneuron was used to indirectly measure activity of the IP3 interneuron. (From Syed et al., reproduced with permission of *Science* **1990,** *250*, 284 (part B), © 1990 American Association for the Advancement of Science.)

1.2.4 CONDITIONING-INDUCED CHANGES IN THE NEURONAL NETWORK CONTROLLING AERIAL RESPIRATION

Intracellular electrophysiological recordings from individual neurons in the *Lymnaea* CNS have been the standard approach over the last few decades in the study of neural correlates of operant conditioning. Initial studies utilized the isolated CNS dissected from the conditioned (or yoked control) animals (Spencer et al., 1999). These recordings first highlighted important changes in the firing activity of RPeD1 following conditioning. While there were no significant differences in the resting membrane potential of RPeD1 in CNS from trained animals, a significantly higher percentage of RPeD1 cells were quiescent following conditioning, compared to controls. Such a reduction in activity of the neuron that initiates CPG activity is perhaps to be expected considering that the outcome of conditioning is a reduction in behavior. However, these studies also demonstrated a reduction in synaptic efficacy between RPeD1 and the IP3 neuron, suggesting that even if firing of RPeD1 occurred, it might be less likely to induce activity in IP3 and hence produce pneumostome opening.

One of the problems inherent to performing experiments on isolated CNS, is that peripheral projections to and from the pneumostome area are lost. Indeed, removal of the CNS, even from naive animals, tends to release inhibitory peripheral control of the CPG and rhythmic activity within the network consequently increases. The identification of changes in neuronal activity and synaptic connectivity thus required a preparation in which peripheral projections, especially to the pneumostome area, remained mostly intact. A number of research groups thus took the approach of using a semi-intact preparation, one that requires minimal dissection and permits electrophysiological recordings from the CNS, but maintains intact connections between the CNS and the pneumostome (Fig. 1.4A). The advantage of this preparation is that it continues to open and close its pneumostome, while corresponding neural activity in identified respiratory neurons can be simultaneously monitored.

It was of course essential to demonstrate that the dissection of the conditioned animal, despite being minimal, did not affect the animal's ability to retain the memory of the conditioned behavior. Indeed, a "reminder" punishing stimulus to an open pneumostome of the semi-intact preparation caused a subsequent reduction in the number of pneumostome openings (Khan & Spencer, 2009; Spencer et al., 2002), confirming the preparation's memory of the conditioned stimulus (yoked control preparations showed no such change). These semi-intact preparations were then used to validate

previous observations made in the isolated CNS; that RPeD1 was, indeed, more likely to become quiescent following operant conditioning (McComb et al., 2005a; Spencer et al., 2002).

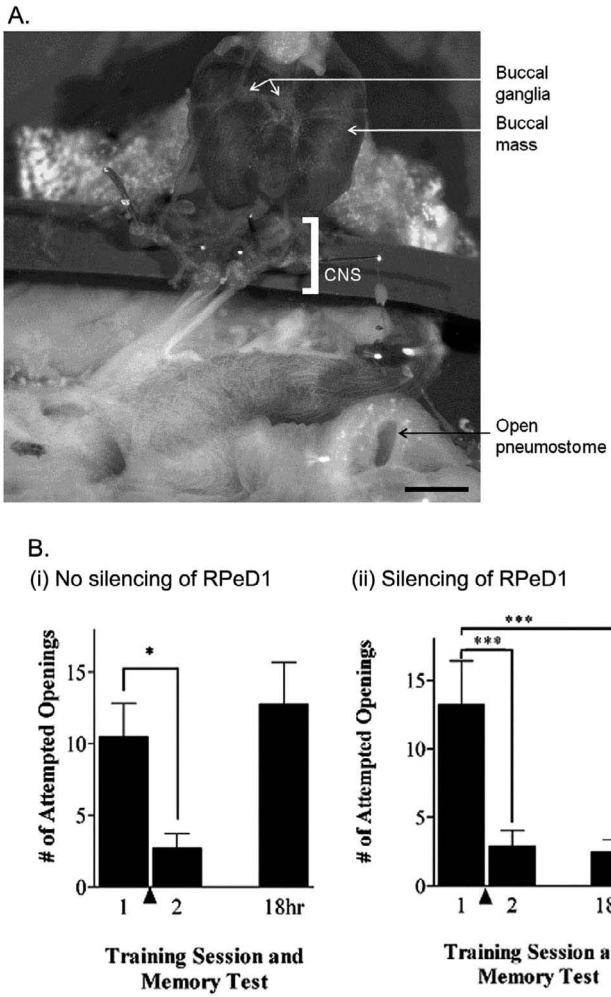

FIGURE 1.4 *Memory formation is enhanced following the hyperpolarization of RPeD1.* (A) A semi-intact *Lymnaea* preparation performing aerial respiration. Scale bar = 5 mm. (Bi) Two training sessions administered to sham control snails (in which RPeD1 was not silenced; $n = 10$) did not result in the formation of LTM lasting for 18 h. However, when RPeD1 was hyperpolarized immediately following the first training session (Bii; $n = 12$) LTM was present at 18 h. ***$p < 0.001$; *$p < 0.05$. (Adapted from Lowe and Spencer, reproduced with permission of *J. Exp. Biol.* **2006,** *209*(4), p 717–718 (part B), © 2006 The Company of Biologists Ltd.).

Researchers next developed a strategy to operantly condition naive semi-intact preparations "in the dish." McComb et al. (2005a) successfully operantly conditioned naïve semi-intact *Lymnaea* to produce ITM lasting 1 h. Following ITM, induced firing in RPeD1 was less likely to initiate pneumostome openings in conditioned preparations compared to yoked controls. Lowe and Spencer (2006) next developed a semi-intact preparation that could survive and behave for up to 24 h (Fig. 1.4A). This allowed researchers to operantly condition semi-intact naive *Lymnaea* preparations in the dish to produce memory lasting 18 h (LTM). The ability to record from RPeD1 during conditioning of the preparation ultimately shed light on the network changes occurring *during* changes in behavior as well as potential neural changes *leading* to changes in behavior. For example, during a punishing stimulus to the pneumostome, it was observed that RPeD1 ceased to fire. It was thus hypothesized that RPeD1 firing (or lack thereof), might encode information about the stimulus and that if RPeD1 was artificially silenced (in the absence of the stimulus), memory formation might be promoted. Normally, intact animals and semi-intact preparations require four training sessions to form LTM (Lowe & Spencer, 2006; Rothwell & Spencer, 2014). Two training sessions produce only ITM (lasting 2 h), but not LTM (lasting 18 h) (Fig 1.4Bi). However, when RPeD1 was artificially silenced (by injection of hyperpolarizing current) between the first and second training sessions, the preparations demonstrated LTM lasting 18 h after only two training sessions (Fig. 1.4Bii). Neither electrode impalement of RPeD1 (but no silencing; Fig. 1.4Bi), or depolarization of RPeD1 (increased firing), produced this memory-promoting effect. We speculated from these findings that the artificial silencing of RPeD1 mimicked the application of the aversive tactile stimulus and provided an additional "artificial" training session, resulting in enhanced memory formation.

These studies further emphasized the key role of this neuron in operant conditioning, but in this case, demonstrated a *causal* role for RPeD1 activity in associative memory. Gene transcription resulting from increased neuronal activity is an important mechanism underlying vertebrate memory formation, but in the case of this molluscan preparation, a reduction in firing activity seemed to be required. Interestingly, evidence from an entirely different experimental approach demonstrated the importance of transcriptional events in RPeD1 for LTM formation. Elegant studies by Scheibenstock et al. (2002) were carried out to surgically ablate the cell body of RPeD1 in intact animals. After allowing sufficient time to recover from the surgeries, the animals then underwent behavioral training. It was discovered that RPeD1-ablated animals (but not the sham-operated animals) showed

normal learning and ITM formation (processes that are independent of gene transcription; Sangha et al., 2003c), but failed to form LTM. This finding led the authors to conclude that gene transcription in RPeD1 must be required for LTM of the conditioned respiratory response.

Of importance to note here, is the feasibility of these soma-ablation studies in invertebrate CNS. Molluscan neurons are unipolar and following the loss of the soma, the primary neurite process emerging from the soma (containing both pre- and post-synaptic sites) can survive indefinitely and continue to function. Such features of a molluscan CNS differ from most vertebrates and mammals, where destruction of a neuron's cell body usually causes death of the entire cell (including its dendritic and axonal processes). Indeed, the ability of RPeD1's primary neurite to survive the surgery and to initiate CPG activity, as well as the ability of these animals to demonstrate normal baseline respiratory behavior following surgery was clearly shown (Scheibenstock et al., 2002).

If silencing of the CPG neuron RPeD1 is important for the formation of LTM following operant conditioning of respiration, the obvious question arises; how does this occur? The reduction in firing of RPeD1 might result from changes in synaptic influences. However, the only known excitatory drive to RPeD1 in the central ganglia is IP3 (which itself is excited by RPeD1), suggesting that the silencing of RPeD1 does not merely result from a suppression of central excitatory input. Lu and Feng (2011) recently provided evidence of a putative sodium leak current underlying the resting membrane potential and spontaneous firing of RPeD1. Partial knockdown of this sodium-leak channel resulted in silencing of RPeD1. This translated into behavioral changes that included a reduction in pneumostome openings and overall breathing time. It will be of future interest to determine whether changes in the channel expression or properties of this sodium-leak current occur following operant conditioning and LTM formation.

Another interesting finding is that a wild strain of *Lymnaea* with enhanced memory forming capabilities possess RPeD1 cells that exhibit lower input resistance (in the naïve state), thus conferring properties of reduced excitability in RPeD1 (Braun et al., 2012). Both changes in input resistance and/or the sodium leak conductance may thus reduce excitability and/or firing of RPeD1, emphasizing that non-synaptic changes in key neurons (and not merely synaptic changes), are likely required for effective memory formation. Interestingly, LTM of the conditioned respiratory response can survive nerve injury and subsequent nerve regeneration (Lukowiak et al., 2003). This further emphasizes that cellular and/or network changes occurring in response to conditioning might not be

confined to synaptic sites, which are more sensitive to disruption by nerve crush injury.

While it is clear that RPeD1 is important for the neural network plasticity underlying memory formation following operant conditioning, the network changes associated with memory formation are not restricted to this single CPG neuron. Activity of the IP3 interneuron (recorded as IP3-induced bursts in the VI/VJ motoneurons), normally produce pneumostome openings (Syed & Winlow, 1991; Syed et al., 1991). However, following conditioning, the incidence of IP3 activity (recorded as input onto either RPeD1 or the VI/VJ motoneurons) is significantly reduced (Khan & Spencer, 2009; McComb et al., 2005a). This is again perhaps an expected change in network properties; if pneumostome opening is reduced, so also will be the activity of the neuron responsible for this opening. However, perhaps less intuitive is that IP3-induced bursts in the VI/VJ opener motoneurons, when they do occur, are significantly less likely to produce corresponding pneumostome openings, or produce openings only after a significant delay (Khan & Spencer, 2009). These VI/VJ motoneurons are monosynaptically connected with the pneumostome opener muscles (Bell et al., 2008). How then does activity in the motoneurons not translate into pneumostome opening? Two possible explanations include either a change in axonal conduction of the motoneurons (as shown previously in Wolpaw, 1997), resulting in delayed pneumostome opening, or alternatively, a reduction in synaptic efficacy at the neuromuscular junction (resulting in failed pneumostome opening). Though the mechanism responsible has not yet been deduced, these findings clearly show that there are network-wide changes occurring as a result of conditioning, which include significant changes in the motor program controlling pneumostome opening (Khan & Spencer, 2009; McComb et al., 2005a).

Overall, it appears that a number of important sites of plastic change have been identified in the neuronal network underlying aerial respiration in *Lymnaea*, including the CPG neuron that initiates rhythmogenesis (RPeD1), the CPG neuron that controls pneumostome opening (IP3 interneuron) as well as the motoneurons (VI/VJ cells) that directly innervate and control the pneumostome muscles.

1.2.5 MOLECULAR MECHANISMS UNDERLYING THE OPERANTLY CONDITIONED RESPIRATORY RESPONSE

Our understanding of the underlying molecular changes occurring during operant conditioning are not yet as well established as the cellular and

network changes, or as advanced as our understanding of the molecular mechanisms underlying classical conditioning of feeding in *Lymnaea*. For example, though it is apparent that gene transcription in RPeD1 is important for the consolidation of the conditioned response into LTM (Scheibenstock et al., 2002), the transcriptional changes in RPeD1 have not yet been identified. The transcription factor, CREB, is known to be important for synaptic remodeling during long-term potentiation in mammals (Frank & Greenberg, 1994), memory formation in the mollusc *Aplysia* (Hawkins et al., 2006) and LTM formation in *Lymnaea* following classical conditioning (Ribeiro et al., 2003; Sadamoto et al., 2004). Guo et al. (2010) recently showed that transcription of CREB was increased during LTM following operant conditioning of respiration, and that partial knockdown of CREB prevented LTM. Furthermore, both phosphorylated CREB and total CREB were higher following operant conditioning of the aerial respiratory behavior in *Lymnaea*.

Protein phosphorylation is another apparently conserved mechanism important for both vertebrate (Barria et al., 2001; Genoux et al., 2002; Mulkey et al., 1993; Zhao et al., 1995) and invertebrate (Ezzedine & Glanzmann, 2003; Kuzirian et al., 2006) memory formation. Treatment of *Lymnaea* with either a protein kinase C (PKC) activator, or a protein phosphatase inhibitor, was shown to boost a 3-h memory (ITM) into a memory lasting 24 h (LTM; Rosenegger et al., 2008). Interestingly, soma-ablation of RPeD1 abolished these effects, again highlighting this cell as a potentially important locus of protein phosphorylation (though not ruling out its importance elsewhere in the network). Indeed, global phosphorylation levels in the CNS were increased 1 h (though not 24 h) after operant conditioning of *Lymnaea* (Silverman-Gavrila et al., 2011). These authors also demonstrate increased glycosylation levels as well as changes in expression of various CNS proteins 24 h after training, suggestive of an important role in LTM formation. Rosenegger et al. (2010) discovered 19 proteins uniquely detected in the CNS one h after a reinforcing training session following LTM formation. One of these proteins was PKC-*epsilon*, and Rosenegger and Lukowiak (2010) went on to demonstrate that PKC inhibitors, as well as inhibitors of NMDA receptors (known to be important in vertebrate hippocampal LTP), blocked the formation of memory in *Lymnaea*.

Though the identification of molecular mechanisms underlying operant conditioning of *Lymnaea* is still in its early stages, it is already apparent that many of the signaling molecules involved are those previously shown to be involved in vertebrate memory. Another example of a potentially conserved molecular mechanism that has recently come to light is that of the vitamin A metabolite, retinoic acid. Disruption of retinoid signaling in rodents impairs

spatial working memory (Chiang et al., 1998) and novel object recognition (Wietrzych et al., 2005) and also disrupts underlying hippocampal synaptic plascticity (long-term potentiation and depression; Chiang et al., 1998; Etchamendy et al., 2003; Misner et al., 2001). Inhibiting retinoid signaling in *Lymnaea* also results in significant changes in the operantly conditioned behavior; the animals are still capable of learning and forming ITM, but are incapable of forming LTM lasting 24 h (Rothwell & Spencer, 2014). These results are surprisingly reminiscent of the results following RPeD1 soma ablation. Furthermore, the soma of RPeD1 was only required *during* training; soma-ablation *after* training did not prevent LTM formation (Scheibenstock et al., 2002). Likewise, inhibition of retinoic acid signaling only prevents LTM when it occurs *before* or *during* training, but not when it occurs *after* training (Rothwell & Spencer, 2014). Considering that the soma of RPeD1 has already been identified as an important transcriptional locus in this conditioning model, it will be interesting to determine whether retinoid-mediated transcription is required in this cell during LTM formation. Interestingly, we have also recently demonstrated that application of retinoic acid to cultured RPeD1 neurons can cause them to become silent (Vesprini & Spencer, 2014).

In summary, the neuronal correlates of learning and memory following operant conditioning have so far revealed electrophysiological changes within the network generating the behavior, including interneurons and motoneurons, and have also shown that changes likely include both synaptic and non-synaptic mechanisms, dispersed throughout the network. Analysis of molecular mechanisms is now also underway and is revealing the involvement of many molecules that have previously been identified as important for memory formation in vertebrates.

1.3 CLASSICAL CONDITIONING OF THE FEEDING BEHAVIOR

1.3.1 FEEDING BEHAVIOR AND DIVERSITY OF SENSORY CUES

The feeding system of *Lymnaea* has been extensively used to investigate the physiological and molecular basis of classical conditioning. Feeding is a rhythmic motor behavior where repeated rasping (biting) movements of a toothed tongue, the radula, scrape at the surfaces of floating pond weed or other food substrates, leading to the ingestion of food. *Lymnaea* is a generalist feeder and so it is advantageous to learn about potential foods using several different sensory modalities. Thus, a wide variety of sensory cues,

visual, touch, and chemical, have been successfully used as conditioned stimuli (CSs) in the classical conditioning of feeding behavior. Visual stimuli are provided by floating plants and snails are able to move upward toward the water surface using this cue (Benjamin, 2008). Tactile cues potentially provide information about physical properties of food, such as texture. Touch is known to provide a normal component of the food stimulus to the lips and reinforces the stimulatory effects of chemical cues in initiating feeding in naïve animals (Staras et al., 1999b). Chemical cues provided by potential food substrates are important indicators of nutritional value and presumably this is why they are such effective CSs in classical conditioning experiments.

1.3.2 THE FEEDING CIRCUIT

During each rasp, the mouth opens and a toothed radula is scraped forward over the food substrate (protraction phase). Food is then lifted into the mouth (retraction phase), which closes while the food is being swallowed (swallow phase) and the sequence is repeated. Rhythmic movements of the feeding muscles located in the feeding apparatus, known as the buccal mass (Fig. 1.4A), are driven by a network of motoneurons (B1 to B10) that, in turn, are driven by synaptic inputs from a feeding CPG network of interneurons (Fig. 1.5). Each phase of the feeding rhythm is generated by one of three main types of CPG interneurons, N1 (protraction), N2 (retraction), N3 (swallow), providing sequences of excitatory and inhibitory synaptic inputs to motoneurons active in different phases of the feeding rhythm. Their intrinsic properties as well as their synaptic connectivity are important in generating the rhythmic feeding behavior (Benjamin, 2012). There are subtypes of these CPG interneurons and their synaptic connections are shown in Figure 1.5.

The N1M and N2 cells are of particular importance because they form the core oscillator of the CPG network (Vavoulis et al., 2007). CPG-driven rhythmic electrical activity can be recorded in the feeding network even in the absence of the feeding muscles of the buccal mass and this is called fictive feeding. Activity in the motoneurons and CPG neurons is modulated by identified higher order interneurons, such as the cerebral giant cells (CGCs), cerebro-buccal interneurons (CBIs) and the slow oscillator (SO). These higher order neurons have been the focus of the learning and memory studies. The CGCs act as gating neurons in the feeding circuit. Increased CGC spiking activity during feeding facilitates feeding responses to food. The CBIs are command-like neurons involved in the initiation of feeding.

One of the CBIs, the CV1a, has been of particular importance in learning and memory studies.

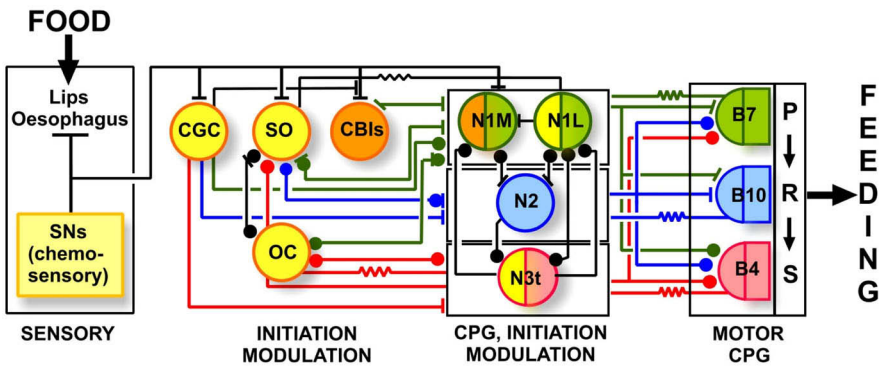

FIGURE 1.5 *Synaptic connectivity and functions of neurons in the feeding circuit of Lymnaea*. Modulatory function is indicated by yellow and initiating function by orange. CPG interneurons and motoneurons (B7, B10, B4) active during the three phases of the feeding rhythm are indicated by green (P, protraction), blue (R, rasp) and red (S, swallow). Neurons labeled with two colors have two functions. Dots indicate inhibitory chemical synapses, bars indicate excitatory chemical synapses, and resistor symbols indicate electrotonic (electrical) synapses. This figure emphasizes the point that many of the neurons have more than one function in the feeding network. Abbreviations: CGC: Cerebral Giant Cell; CBIs: cerebro-buccal interneurons; N1L: N1 lateral; N1M: N1 medial; N3t: N3 tonic; OC: octopamine neurons; SO: slow oscillator; SNs: sensory neurons.

Sucrose is an effective chemical stimulus for feeding and is therefore used as the unconditioned stimulus (US) in reward conditioning. At the cellular level, sucrose applied to the lips in semi-intact preparations induces fictive feeding in motoneurons and interneurons of the feeding network (Kemenes et al., 2001). As well as the CPG neurons, the modulatory CGC and CBI cells are activated in the sucrose-driven fictive feeding rhythm.

The main conditioned stimuli (CS) used for reward classical conditioning in *Lymnaea* are either a chemical (amyl acetate) or a tactile (a gentle brush stroke applied to the lips) stimulus. Recently, visual stimuli have also been used and the success of a black and white checkered pattern as a CS indicates that the eyes are involved (Andrew & Savage, 2000). Amyl acetate (CS) was previously thought to be a neutral stimulus and have no effect on feeding, either at the behavioral or electrophysiological levels, but has recently been shown to have stimulatory (low concentrations) or inhibitory (high concentrations) effects in naïve animals (Straub et al., 2006).

1.3.3 REWARD CLASSICAL CONDITIONING OF FEEDING TO A TACTILE CS

Lymnaea can be classically conditioned by repeatedly pairing touch to the lip with food (5–15 trials) (Kemenes & Benjamin, 1989). This type of learning shares important characteristics with associative conditioning in vertebrates, such as stimulus generalization and discriminative learning, classical–operant interactions and strong dependence on both external and internal background variables.

Two approaches have been used to investigate the electrophysiological basis of tactile appetitive classical conditioning in *Lymnaea* (Kemenes et al., 1997). One approach was based on the development of an in-vitro preparation where electrophysiological manipulation of a specific neuronal pathway was used to mimic the chemical US. A lip touch stimulus (the CS) was paired with intracellular activation of the modulatory SO neuron (the US), which can drive fictive feeding. After 6–10 pairings, presentation of the touch stimulus alone could activate a robust fictive feeding rhythm in feeding motoneurons. Interestingly, a correlate of tactile conditioning could also be recorded in the CPG network. A complex sequence of inhibitory synaptic inputs that occurs in the N1M CPG interneuron in response to lip touch in naïve animals changes to a strong sustained depolarizing synaptic input after conditioning and this drives a sustained plateauing pattern in the N1M cell (Benjamin & Kemenes, 2009). This is an example of synaptic plasticity involving a core member of the feeding oscillator.

A second approach is where behavioral conditioning is followed by electrophysiological recording of conditioned fictive feeding responses in a semi-intact preparation (Staras et al., 1999a). Fictive feeding patterns of neuronal activity in response to sensory stimuli are a correlate of the repetitive feeding behavior seen in the intact snail. A retraction phase feeding motoneuron, known as B3, is routinely used as a monitor of fictive feeding in the whole network (see Fig. 1.6B2). This cell is easy to identify, plays no role in pattern generation but receives synaptic inputs from all three types of CPG interneurons and so can be used as an indirect monitor of the conditioned activation of the CPG. It also receives an easy-to-identify early compound excitatory post-synaptic potential (EPSP), input that results from stimulation of the CS pathway (Staras et al., 1999a).

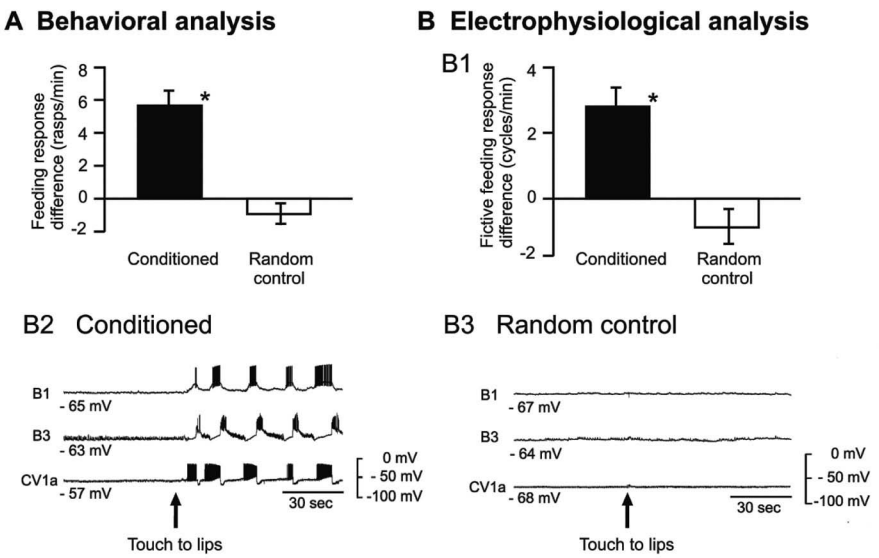

FIGURE 1.6 *Electrophysiological correlates of the LTM trace after multiple-trial tactile classical conditioning in Lymnaea.* (A) The behavioral response to the touch CS (conditioned stimulus) is significantly (*) stronger in the conditioned versus the control group. (B) Behavioral classical conditioning produces a CS-induced increase in the systems-level electrical activity (fictive feeding response). (B1) The electrophysiological response to the touch CS, which was calculated as the post- minus pre-stimulus difference in fictive feeding activity, is significantly (*) stronger in the conditioned versus the control group. (B2) The lip touch CS evokes rhythmic fictive feeding activity in a CBI neuron, CV1a (cerebro-ventral 1), and motoneurons B1 and B3 in a preparation from a conditioned animal. (B3) The lip touch CS evokes no rhythmic fictive feeding activity in a preparation from a control animal. Note that CV1a is more depolarized in the preparation from the conditioned animal than in the preparation from the control animal, a consistent observation in this type of experiment. Depolarization of CV1a contributes significantly to the enhanced feeding response following this type of conditioning (see text). (Adapted from Jones et al., reproduced with permission of *Curr. Biol.* **2003,** *13*, 1066; © Elsevier).

Using the lip touch behavioral-training protocol, snails are subjected to 15 training trials over 3 days and then dissected for electrophysiological analysis, starting on the day after the last training trial. Touching the lips of the intact snails from the experimental group after training induced a pattern of feeding movements not seen in controls (Fig. 1.6A). Similar significant differences were seen between experimental and control animals at the level of the electrophysiologically recorded fictive feeding pattern in motoneurons (Fig. 1.6B1). The CPG-driven activity in the motoneurons depends ultimately on activity of neurons at all levels of the feeding network, so

the conditioned fictive feeding recorded in the motoneurons is a systems level "readout" of the memory trace in the whole feeding system. However, more detailed changes can also be recorded in different parts of the network (Staras et al., 1999a). One of these is the early EPSP that occurs in the B3 motoneuron before the onset of the fictive feeding pattern. The amplitude, but not the latency and duration of the EPSP, was significantly enhanced after conditioning. In sated snails, the conditioned fictive feeding response to touch was lost but the increase in the EPSP amplitude persisted. This suggests that there is unlikely to be a causal link between increases in amplitude in B3 and generation of the fictive feeding pattern.

Electrical correlates of conditioning were recorded also at other levels within the feeding circuit and these could all be potential sites of plasticity. Sites quite early in the CS pathway which could be involved in conditioning were revealed by extracellularly recording mechanosensory fibers in the connective between the cerebral and buccal ganglia (Staras et al., 1999a). Tactile responses could be recorded in these fibers and, following conditioning, the number of spikes occurring early in this response increased compared with controls.

One candidate for initiating CPG activity following conditioning is the modulatory interneuron cell type known as CV1a. This neuron is capable of driving a fictive feeding rhythm via its monosynaptic excitatory connections with the N1M cells of the CPG network (Kemenes et al., 2001). After conditioning, the CV1a cells are significantly more active following touch in conditioned snails (Fig. 1.6B2) compared with controls (Fig. 1.6B3). More detailed experiments on the role of CV1 cells in tactile conditioning revealed that non-synaptic electrical changes play a role in tactile memory. A long-lasting membrane depolarization of 11 mV was recorded in CV1s from conditioned compared with control snails (Fig. 1.6B2 and B3), that persisted for as long as the electrophysiological and behavioral memory trace (Jones et al., 2003). The depolarization makes the cells more responsive to the CS and can account for the activation of the feeding response after conditioning, due to the CV1 cells' strong excitatory synaptic connection with the CPG. The importance of this result is emphasized by experiments where the membrane potential of the CV1 cells is manipulated to either reverse the effect of behavioral conditioning or to mimic the effects of conditioning in naïve snails. These experiments showed that the persistent depolarization of the CV1 cells were both sufficient and necessary for the conditioned response in the feeding network.

A more complex form of learning, differential appetitive conditioning, was also demonstrated using the tactile CS (Jones et al., 2001). Here, the

lips and tentacles were used as sites for application of the CS. In one group of animals, touch to the lips was reinforced using sucrose (the CS+ site); in the other group, the tentacles were used as the touch site without sucrose (the CS− site). Electrophysiological responses in the semi-intact preparation were recorded from B3 motoneurons to record the fictive feeding activity underlying behavioral conditioned responses. Following behavioral training, touch-evoked fictive feeding responses were present in the CS+ group, but not in the CS− group, indicating the success of the differential conditioning experiment.

1.3.4 REWARD CLASSICAL CONDITIONING OF FEEDING TO A CHEMICAL CS

In the original successful formulation of appetitive conditioning in *Lymnaea*, snails were subjected to a chemical conditioning protocol using amyl acetate as the CS and sucrose as the US (Audesirk et al., 1982). Following training, the explicitly paired (CS + US) experimental group showed significantly greater feeding responses to amyl acetate over their own naïve responses and all the standard control groups (Random, Explicitly Unpaired, CS alone, US alone). As might be predicted for appetitive conditioning, both age and motivational state (hunger versus satiety) influenced learning. Both hungry and sated young snails could acquire the conditioned response, but in the latter group its expression was only apparent when the animals were starved before testing. On the other hand, old snails could only acquire the conditioned response, if they were maintained in a hungry state during training. The significance of motivational state became even more apparent when it was realized that if snails were starved long enough (for 5 days) before and throughout the experiment, even a single pairing of amyl acetate and sucrose resulted in LTM which lasted for at least 19 days (Alexander et al., 1984).

As a first step toward understanding the electrophysiological mechanisms underlying chemical conditioning, an electrophysiological correlate of the conditioned behavior following single-trial chemical conditioning was recorded as changes in the fictive feeding responses in motoneurons. Then, electrical activity following conditioning was recorded in other parts of the feeding system to localize sites of plasticity (Straub et al., 2004). The cell bodies of chemosensory neurons are located in lip epithelial tissue and project to the cerebral ganglia via the lip nerves, where they synapse with cerebral ganglion neurons like the CV1a cells. Extracellularly recorded spike

responses to both the CS and US can be recorded in the lip nerves from naïve animals, and these peripheral responses do not change after conditioning.

In contrast, neuronal output from the cerebral ganglia is significantly enhanced in response to the CS after conditioning. This indicates that chemical conditioning affects central but not peripheral processing of chemosensory information, with the cerebral ganglia an important site of plasticity. The fibers that were recorded extracellularly to indicate cerebral plasticity were axons of the CBI interneurons, command neurons for feeding, so their activation is particularly significant. Confirmation that the CBIs do increase their activity after conditioning was obtained by showing increases in feeding patterns to the CS recorded intracellularly in CV1a neurons (Kemenes et al., 2006b). From this work it is suggested that chemical synapses between the primary chemosensory neurons and the CBIs are a major site of plasticity (Fig. 1.7A). Two other CS pathways, including an inhibitory one, are present in naïve animals but these are not affected by conditioning (Straub et al., 2006).

The current network model for chemical conditioning also includes non-synaptic neuronal plasticity. The CGCs are persistently depolarized by about 10 mV between 16 and 24 h to 14 days after conditioning. This increases the strength of postsynaptic responses to CGC stimulation by a process that involves an increase in intracellular calcium concentration in the proximal dendritic processes of the CGCs (Kemenes et al., 2006b). The local targets for CGC depolarization are the CV1a cells and artificial depolarization of the CGCs in naïve snails increases the response of the CV1a cells to the CS, mimicking the effects of behavioral conditioning. It appears that the CGCs are increasing the strength of the CS to CBI synapse by pre-synaptic facilitation.

Recently, it has been shown that the conditioning-induced depolarization of the CGCs is due to an increase in the amplitude of a cyclic AMP-sensitive persistent sodium current (Nikitin et al., 2006, 2008). The measured increase in the size of the current is sufficient to depolarize the CGC by the required amount. There was also an increase in the density of CGC sodium channels caused by conditioning, revealed by immunocytochemical methods (Nikitin et al., 2008).

Surprisingly, the depolarization of the CGCs does not cause a change in the firing rate of the CGCs or their spike shape. In order to understand the ionic mechanisms of this novel combination of plasticity and stability, a Hodgkin–Huxley-type computer model of the CGCs was constructed (Vavoulis et al., 2010). The model was used to elucidate how learning-induced changes in a measured somal persistent sodium current and a delayed rectifier potassium

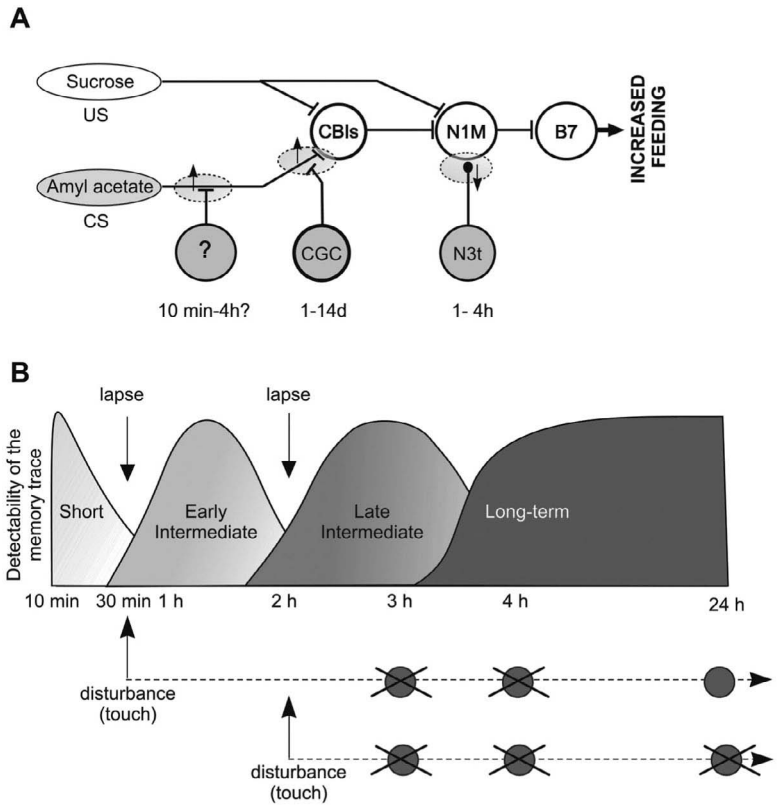

FIGURE 1.7 *Temporal patterns of electrophysiological and behavioral changes underlying the consolidation of the memory trace following one-trial reward classical conditioning of feeding in Lymnaea*. (A) Three types of electrophysiological changes are suggested to underlie consolidation. The first conditioned response is recorded at 10 min. This is due to the enhancement of the CS-CBI neuronal pathway. The CBI interneurons drive feeding responses by exciting the CPG interneurons (only the N1M is shown) which then drive rhythmic feeding by their synaptic effects on motoneurons (e.g., the B7). This early conditioned response occurs during STM (short-term memory). A reduction in the N3t-mediated tonic inhibition of the N1M, also contributes to the conditioned response, but the onset is at 1 h, at the time point when ITM is first observed and continues through to LTM at 4 h. Pre-synaptic facilitation of the CS to CBI synapse is first recorded at 1 day when LTM is well established and continues for at least 14 days after conditioning. (B) Schematic representation of the time course of four phases of memory following associative conditioning. Time since conditioning is plotted against the detectability of memory expression. Each curve represents a different form of memory (short-term, early intermediate, late intermediate and long-term). The absence of a lapse in memory between 3 and 4 h is because the expression level of the memory trace is above the threshold for memory recall. Upward arrows indicate the time points when the mechanical disturbance was applied and the behavioral effect on subsequent phases of memory indicated by the gray spots. Crossed gray spots indicate failure of recall. Disturbance was applied at other time points but had no effect (omitted for clarity).

current could lead to a persistent depolarization of the CGCs while maintaining firing rate. The maintenance of firing rate is required for the normal modulatory function of the CGCs in the feeding network and has no role in learning. Included in the model was an increase in the conductance of a high-voltage activated calcium current that allowed the spike amplitude and spike duration to be maintained. A balanced increase in these three types of conductances was sufficient to explain the electrophysiological changes observed after conditioning.

Cellular changes that occur in the CGCs are sufficient to explain the enhancement of the CS effects on feeding following conditioning, but they cannot be the whole story because the onset of CGC depolarization is at 16–24 h after training, whereas a behavioral memory trace is present as early as 10 min after training, so an alternative mechanism must also be present to explain the early memory trace. Significantly, there is another type of electrical change in the feeding circuit that occurs as early as 1 h after one-trial in-vitro conditioning. This is a conditioning-induced reduction in tonic (continuous) inhibitory synaptic input to the feeding CPG. By reducing the frequency of this "background" inhibitory input, the threshold for the CS to activate the feeding network is reduced, making it more likely that the feeding CPG will respond to the CS (Marra et al., 2010). This inhibitory input originates from one of the CPG interneurons known as the N3t (N3tonic) that has multiple roles in the feeding network (Benjamin, 2012). This threshold-setting mechanism has been simulated in a computer model of the feeding network by assuming that the conditioning-reduced monosynaptic inhibitory input from the N3t to another CPG interneuron, the N1M, is responsible for controlling the enhanced fictive feeding response to the CS (Marra et al., 2010).

In summary, there are at least three electrophysiological mechanisms responsible for memory consolidation after single-trial chemical conditioning (summarized in Fig. 1.7A). One mechanism increases the strength of the response in the CS chemosensory pathway at 10 min and may persist for up to 4 h after conditioning. The mechanism(s) underlying this response is unknown but its effect is to enhance the CS response in CBI interneurons such as the CV1a. A second mechanism, due to a reduction in tonic inhibition, starts 1 h after conditioning and persists for at least 4 h. A third, mediated by presynaptic facilitation, is first recorded at 16–24 h and persists until 14 days after conditioning. Comparing the time course of these electrophysiological mechanisms with the phases of memory consolidation revealed by molecular analysis (Fig. 1.7B) shows that the earliest mechanism with a 10-min onset coincides with STM. The reduction of tonic inhibition is

present during ITM and continues through to LTM at 4 h. The time course of presynaptic facilitation suggests that it is a mechanism for the maintenance of LTM (Kemenes et al., 2006b).

1.3.5 REWARD CLASSICAL CONDITIONING OF FEEDING TO A VISUAL CS

Recent work shows that feeding can be conditioned by pairing visual stimuli (CS) with sucrose (US). *Lymnaea* has a lens that is capable of forming an image on the retina under water (Benjamin, 2008) and visual approach and reaching behavior can be evoked by a black and white checkered pattern (Andrew & Savage, 2000). One-trial conditioning is possible when snails approaching a black panel along a gutter (the CS), are reinforced with sucrose (the US). After conditioning, approaching the black pattern elicits more rasping movements compared with un-reinforced trials. Successful reward conditioning was also obtained with a checkered pattern that was discriminated from a gray pattern of equal luminance, but in this experiment multiple trials were necessary (4 trials per day for 4 days). A memory trace for this visual stimulus was recorded 4 days after conditioning and is presumed to be LTM.

1.3.6 AVERSIVE CLASSICAL CONDITIONING OF FEEDING TO A CHEMICAL CS

In pioneering experiments, an aversive classical conditioning paradigm (conditioned taste aversion [CTA]) was developed based on pairing sucrose (the CS) with an aversive stimulus, KCl (the US), which inhibits feeding and evokes a withdrawal response when applied to naïve animals (Kojima et al., 1996). After eight trials, trained animals show a significantly weaker feeding response to sucrose than did controls and this memory lasted for over 1 month. A neural analysis of CTA was carried out on isolated brains dissected from conditioned and control animals (Kojima et al., 1997). In particular, the synaptic connection between the modulatory CGCs and the CPG interneuron, N1M, was examined. In conditioned animals compared to controls, a significant increase in the size of inhibitory post-synaptic potentials (IPSPs) recorded in the N1M was observed following an artificial depolarization of the CGCs. Since the CGCs are known to play a critical gating role in feeding behavior and the N1M is a pivotal member of the

feeding CPG, this enhanced IPSP may be an important cellular correlate of the conditioned taste-aversion learning.

This aversive paradigm requires multiple training trials to be successful, but more recently a single trial paradigm has been developed (Kemenes et al., 2011). This pairs low concentrations of amyl acetate as the CS and the aversive chemical, quinine, as the US. At these low concentrations (10% of normal), amyl acetate stimulates feeding but after a single pairing these responses are significantly reduced compared with controls. The role of inhibitory circuits located outside of the feeding ganglia, which were previously shown to mediate aversive responses in naïve snails, was then examined. These ganglia ("the rest of the brain") were removed after conditioning and this resulted in a loss of the CS inhibitory responses on fictive feeding responses, compared with controls. This shows that the inhibitory circuits located in the rest of the brain play a key role in aversive classical conditioning. As expected, a similar removal of the ganglia after reward chemical conditioning has no effect on this type of memory trace because its locus is in the retained cerebral ganglia.

1.3.7 PHASES OF MEMORY CONSOLIDATION

All the types of associative conditioning paradigms described in this chapter are capable of inducing LTM lasting from hours to many days. LTM requires new RNA and new protein synthesis and the blocking of memory by the injection of protein synthesis inhibitors (e.g., anisomycin, ANI) and RNA synthesis inhibitors (e.g., Actinomycin D) confirmed the formation of LTM. Injecting ANI into intact snails at various time points after one-trial reward-induced chemical conditioning and testing for the presence of the 24-h memory, showed that there is a single critical period of sensitivity to protein-synthesis blockers in the first hour after conditioning (Fulton et al., 2005). Subsequent work using both protein synthesis and RNA synthesis blockers showed that there was an LTM trace as early as 4 h after single-trial conditioning. Other work showed that consolidation of 24-h LTM can be blocked by cooling snails immediately after training and before the time period 10–60 min after training, when protein synthesis blockers are known to be effective (Fulton et al., 2008). It is suggested that this very early blocking of the 24-h memory trace by cooling is interfering with enzymic cascades, including kinases that are known to be activated by chemical conditioning (see below).

A more detailed analysis of the phases of memory based on recent in-vitro electrophysiological training experiments (Marra et al., 2013) has identified

STM at 10 min after conditioning, ITM from 1 to 3 h after conditioning and LTM at 4 h, persisting for 24 h after conditioning (Fig. 1.7B). STM was defined by its lack of sensitivity to both protein synthesis and RNA synthesis inhibitors injected immediately after conditioning and ITM by its sensitivity to protein synthesis but not RNA synthesis inhibitors injected at the same time point. Further experiments divided ITM into an early and late phase, based on their sensitivity to protein kinase inhibitors (Marra et al., 2013). Injecting the protein kinase C (PKC) blocker bisindolylmaleimide-1 (Bis) into intact snails, immediately after training and testing for memory recall up to 24 h after training, blocked behavioral memory during ITM at only 1 and 2 h after conditioning, identifying an early phase of ITM. Late ITM at 3 h after conditioning differed from the early ITM in its independence of PKC but its dependence on a constitutive variant of PKC, protein kinase M (PKM). The known PKM blocker, Chelerythrine, was used to block this atypical member of the atypical PKC family when it was injected immediately after training. Protein kinase A (PKA) blockers (H-89) blocked both early and late ITM and so did not distinguish between early and late phases.

1.3.8 LAPSES IN MEMORY EXPRESSION

An intriguing aspect of this work on memory phases has revealed specific time points in memory consolidation when lapses in memory expression occur (Marra et al., 2013). These coincide with the transition between different phases of memory (Fig. 1.7B). The memory lapse at 30 min after conditioning occurs at the transition between STM and ITM and the second at 2 h after conditioning at the transition between early ITM and late ITM. There was no lapse in memory at the transition between ITM and LTM. These lapses were initially identified in the in-vitro electrophysiological experiments but later were also found to occur in behavioral experiments at the same time points. It must be emphasized that the lapses in memory recall are only temporary. The memory trace only becomes inaccessible for brief periods before continuing into LTM.

Reports of memory lapses during early memory consolidation are widespread. They have been observed in many types of organisms including human subjects raising general questions about the function. By application of novel sensory stimuli, such as touch to the skin, during the lapses (but not at other time points) we have found that memory consolidation becomes vulnerable and leads to the blocking of the subsequent progress of consolidation. Application of touch or other disturbing stimuli at 30 min blocks

memory at 3 h and 4 h, and application of touch at 2 h blocks the memory at 3 h, 4 h, and 24 h after conditioning (Fig. 1.7B). We have speculated (Marra et al., 2013) that the lapses represent choice points that allow the memory trace to be expressed adaptively according to the variety of novel external stimuli that the animal is exposed to in the environment.

1.3.9 GENE-REGULATING CASCADES

Regulation of gene expression following one-trial reward chemical conditioning of feeding is known to involve transcription factors like the cyclic AMP-responsive element binding protein (CREB). The highly conserved CREB gene and CREB-like proteins have been identified in *Lymnaea* (Ribeiro et al., 2003; Sadamoto et al., 2004). Consistent with a role for CREB in LTM is the observation that levels of phosphorylated CREB1 are increased in the CGCs following reward conditioning (Ribeiro et al., 2003). In more recent work, it was shown that CTA learning increased LymCREB1 gene expression (Sadamoto et al., 2010), so it seems that classical conditioning can increase both the level of the expression of the gene of this transcriptional activator and the level of its activation by phosphorylation.

Other highly conserved molecular pathways that have been implicated in LTM are the PKA (Michel et al., 2008) and mitogen-activated protein kinase (MAPK) signaling pathways (Ribeiro et al., 2005). Inhibition of PKA catalytic subunit activity or MAPK phosphorylation without blocking sensory or motor pathways blocks LTM and levels of both PKA activity and MAPK phosphorylation were increased, with PKA activation first detected at an even earlier time window (5 min). Memory consolidation after retrieval at 6 h post-training is both PKA and protein-synthesis dependent, whereas reconsolidation after retrieval at 24 h depends on protein synthesis but not PKA activity (Kemenes et al., 2006a) (Fig. 1.8). This interesting finding indicates that depending on the "age" of the consolidated memory, different molecular pathways are activated by memory retrieval and contribute differentially to memory reconsolidation.

Like reward conditioning, aversive chemical conditioning (CTA) of feeding involves conserved molecular pathways linked to the PKA and the CREB transcriptional regulatory system, such as the CAAT Element Binding Protein (Hatakeyama et al., 2006). The injection of cAMP or PKA into the soma of the CGC leads to a long-term enhancement of the strength of the synaptic connection between the CGC and the B1 feeding motoneuron and presumably other neurons of the feeding network (Nakamura et al.,

1999; Nikitin et al., 2006). An exciting new finding has been that the Type II Calcium Calmodulin dependent kinase (CaMKII) is intrinsically activated (by phosphorylation at T286) in a later post-training time window, around 24 h after food-reward training. This activation occurs independently of NMDA receptor activation and is a necessary molecular component of late memory consolidation (Wan et al., 2010).

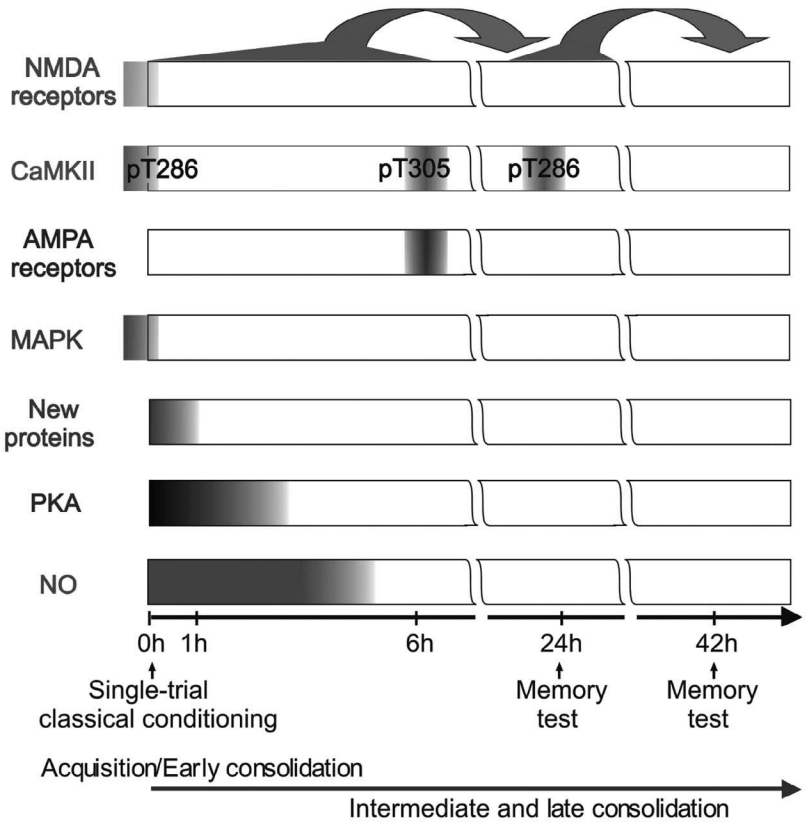

FIGURE 1.8 *Time windows of known molecular requirements for LTM after one-trial food-reward classical conditioning in Lymnaea.* The large curved arrows indicate the requirement for the activation or synthesis of molecules in the acquisition/early consolidation phase for the expression of memory at 24 h and in the late consolidation phase for the expression of memory at 42 h, respectively. In the late memory consolidation phase, around 24 h post-training, of the molecular mechanisms investigated so far, only activation of CaMKII by autophosphorylation at T286 is required for memory expression at 42 h. In the intermediate-term consolidation phase, around 6 h post-training, there is a requirement for the activation of AMPA receptors for memory expression at 24 h, which is dependent upon autophosphorylation of CaMKII at T305 in the same time window.

More recently, another novel CaMKII-dependent mechanism has been identified by which a learning-induced increase in AMPA receptor (AMPAR) levels is stabilized for on-going consolidation of 24 h LTM after single-trial food-reward classical conditioning (Naskar et al., 2014). Six hours after training, the levels of both autophosphorylated pT305-CaMKII and GluA1 type AMPAR subunits were significantly elevated in the buccal and cerebral ganglia that contain the *Lymnaea* learning circuits. Treatment with a CaMKII inhibitor (CaMKIINtide) significantly reduced the learning-induced elevation of both pT305-CaMKII and GluA1 levels and impaired the consolidation of associative LTM. Inhibition of proteasomal activity offset the deleterious effects of CaMKIINtide on both GluA1 levels and LTM. These findings suggest that increased levels of pT305-CaMKII play a role in AMPAR dependent memory consolidation by reducing proteasomal degradation of GluA1 receptor subunits.

1.3.10 TRANSMITTERS AND MODULATORS

An increasing number of neurochemicals, known to be important in chemical communication between neurons, are involved in various phases of memory in *Lymnaea*. For instance, consolidation of the 24-h memory trace following one-trial chemical conditioning is dependent on the nitric oxide (NO)-GMP signaling pathway. There is a critical period of sensitivity up to 5 h after conditioning when blocking this pathway by drug injection prevents behavioral LTM formation (Kemenes et al., 2002). Further evidence for a role in LTM comes from experiments on the CGCs. These neurons express mRNA transcripts from the two related nNOS (neuronal nitric oxide synthase) genes (*Lym-nNOS1* and *Lym-nNOS2*) (Korneev et al., 2005). Six hours after chemical conditioning, *Lym-nNOS1* is up-regulated compared with controls. This up-regulation of the NOS coding transcript may be due to an earlier down-regulation of the *Lymnaea antiNOS* that is known to be inhibitory on NOS transcript production. NO is known to modulate the strength of serotonergic transmission between the CGCs and motoneurons in the feeding system (Straub et al., 2007) and this could be involved in conditioning.

As well as NO, the monoamine dopamine plays a key role in reward conditioning in *Lymnaea*. Injection of the specific D1-like receptor antagonist SCH23390, but not the D2 antagonist sulpiride, 10 min after one-trial chemical conditioning blocks the 24-h memory trace. Because the drug was injected after conditioning, it suggests that dopamine is involved with consolidation of LTM (Kemenes et al., 2011). Figure 1.8 provides a summary

of published data on the known molecular requirements for the early and late phase of the consolidation of LTM expressed >24 h after single-trial food-reward classical conditioning in *Lymnaea.* It is interesting to note that the requirement for PKA and NO outlast the requirement for protein synthesis, indicating that these molecules are required for some as yet unidentified transcription and translation-independent processes underlying memory consolidation in the 1–6-h-post-training time window.

1.4 SUMMARY AND DISCUSSION

We have reviewed here what is known as a "top-down approach" to studying the mechanisms of associative conditioning in *Lymnaea*. This approach starts from the investigation of behavioral aspects of learning and then aims to establish causal links between the learned behavior and the underlying electrical and molecular changes in the nervous system. The advantage of the *Lymnaea* system is that behavioral studies can be directly related to network and cellular levels of analysis. This results from the ability to identify individual neurons, with their known electrical properties and synaptic connectivity, within both respiratory and feeding networks. Semi-intact preparations made from trained animals were instrumental in allowing a direct correlation of electrical changes with behavioral changes. In initial experiments, the firing patterns of motoneurons or CPG interneurons were recorded to give a systems level correlate of the behavioral memory trace. Later, more detailed mechanistic studies of individual neurons and their synaptic interactions were carried out.

Not all the neuronal mechanisms underlying conditioning-induced changes are fully understood, because they are surprisingly complex. However, we do know that these changes occur at multiple sites within both the feeding and respiratory circuits. The previously favored model for explaining associative conditioning in molluscs involved restriction of synaptic changes to single sites (such as the sensory-motor synaptic locus; Kandel, 2001). However, evidence presented in this chapter and elsewhere (e.g., Crow & Jin, 2013) show that more numerous sites of plasticity are likely to be involved in memory formation within a circuit, especially where there are intermediate layers of (interneuron) organization occurring between sensory and motoneurons. In this chapter, we present evidence for several types of modulatory and CPG interneurons being significant sites of plasticity in the feeding and respiratory networks.

It has long been recognized that changes in synaptic strength play a major role in molluscan learning and memory, but an increasing number of examples of non-synaptic plasticity have now been demonstrated (Benjamin et al., 2008). These non-synaptic mechanisms include changes in membrane resistance, membrane potential, and threshold for plateau initiation. Changes in these non-synaptic properties usually alter the excitability of the neurons and result from learning-induced changes in intrinsic ion currents. Non-synaptic changes (e.g., learning-induced depolarization of modulatory interneurons (Fig. 1.6) and silencing of a key CPG interneuron (Fig. 1.4), play a major role in associative learning in *Lymnaea* and these are "pioneering" examples of this type of plasticity.

The general significance of the work described in this chapter is brought out by comparisons with work carried out in vertebrates where similar types of phenomena occur. Although initial studies in *Lymnaea* focused on simple forms of operant and classical conditioning, *Lymnaea* are also capable of more complex forms of learning, whose features are similar to those found in vertebrates. For instance, stimulus generalization (responding to related stimuli), goal tracking (moving toward the US), and context dependency (increased learning in a novel environment) were found in tactile classical conditioning.

The importance of environmental context in LTM formation was emphasized by more detailed work carried out using the operant conditioning paradigm. For example, snails trained in the presence of a food odor did not demonstrate LTM unless they were later tested in the presence of the odor. Extinction of the memory trace (loss of LTM when pneumostome opening was not followed by the punishing stimulus) is also influenced by context; extinction of the memory trace did not occur if extinction "training" was carried out in a context that was different from the one used during the initial training. It is also of interest that the molecular mechanisms of consolidation and reconsolidation in *Lymnaea* are similar to their vertebrate counterparts.

A temporal sequence of phases defined by their sensitivity to protein synthesis and RNA synthesis has defined short-term, intermediate-term, and long-term phases of memory in both types of *Lymnaea* associative memory and in a variety of vertebrates where this has been examined. A requirement for PKC defines ITM in both operant and classical conditioning in *Lymnaea*; protein kinases like PKA and MAPK are ubiquitous in memory formation and play important roles in the first few hours after conditioning. Regulation of gene expression is known to involve transcription factors like the highly conserved CREB protein, which plays a critical role in the switch from short to long-term memory in *Lymnaea*, as well as a variety of other species.

Although molecules and pathways involved in many learning and memory paradigms are well known to be conserved between invertebrates and vertebrates, it is now also apparent that *Lymnaea* may utilize these same pathways to alter its behavioral outcome over many hours and days following conditioning paradigms.

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- **freshwater pond snail**
- **memory-forming capabilities**
- **electrophysiological recordings**
- **rhythmic behaviors**
- **operant conditioning**
- **classical conditioning**

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CHAPTER 2

FROM LIKES TO DISLIKES: CONDITIONED TASTE AVERSION IN THE GREAT POND SNAIL (*LYMNAEA STAGNALIS*)[1]

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CONTENTS

[1]This chapter is one of a series dealing with trends in the biology of the phylum Mollusca. "From likes to dislikes: conditioned taste aversion in the great pond snail (Lymnaea stagnalis)" by E. Ito, S. Kojima, K. Lukowiak, and M. Sakakibara was originally published in the Canadian Journal of Zoology, 2013, 91(6): 405-412, 10.1139/cjz-2012-0292.

ABSTRACT

The neural circuitry comprising the central pattern generator (CPG) that drives feeding behavior in the great pond snail (*Lymnaea stagnalis* [L., 1758]) has been worked out. Because the feeding behavior undergoes associative learning and long-term memory (LTM) formation, it provides an excellent opportunity to study the causal neuronal mechanisms of these two processes. In this chapter, we explore some of the possible causal neuronal mechanisms of associative learning of conditioned taste aversion (CTA) and its subsequent consolidation processes into LTM in *L. stagnalis*. In the CTA training procedure, a sucrose solution, which evokes a feeding response, is used as the conditioned stimulus (CS) and a potassium chloride solution, which causes a withdrawal response, is used as the unconditioned stimulus (US). The pairing of the CS–US alters both the feeding response of the snail and the function of a pair of higher order interneurons in the cerebral ganglia. Following the acquisition of CTA, the polysynaptic inhibitory synaptic input from the higher order interneurons onto the feeding CPG neurons is enhanced, resulting in suppression of the feeding response. These changes in synaptic efficacy are thought to constitute a "memory trace" for CTA in *L. stagnalis*.

2.1 INTRODUCTION

In many respects, the birth of modern neuroscience occurred in the 1950s. In our view, two seminal events happened. The first was the brain surgery performed on a patient known as HM that leads to Milner's observations of human memory which ultimately showed that hippocampal neural circuits were necessary for the formation of declarative memory (Milner et al., 1998). Those observations ultimately lead to the proliferation of studies concerned with the neuronal changes that occurred within the hippocampal circuits which are necessary for memory formation. However, the techniques and knowledge needed to undertake those studies depended on a second happening, the realization that molluscs possess large, identifiable neurons which controlled interesting, tractable behaviors.

For example, early studies occurring about the same time in France and Monaco by Tauc (1954) and Arvanitaki and Chalazonitis (1955) using the central nervous system (CNS) of the sea hare (genus *Aplysia* L., 1758) laid the foundation for the use of these model systems to study the causal neuronal mechanisms of learning and memory. We can get an appreciation

of this situation by reading the report of Strumwasser (1971). He wrote that "I had come to Woods Hole to receive instruction from Angelique Arvanitaki and her husband Nick Chalazonitis in the methodology work on the *Aplysia* CNS. In 1955, Arvanitaki and Chalazonitis and quite independently, Tauc, had performed the first cellular recordings from the large neurons of *Aplysia*." We feel that Kandel and Tauc's (1965) discovery of heterosynaptic facilitation in a molluscan preparation laid the groundwork for hypotheses developed later to explain the neuronal basis of learning and the subsequent formation of memory. These studies utilizing for the most part molluscan preparations (California seahare, *Aplysia californica* J.G. Cooper, 1863) culminated in Kandel being awarded the *Nobel Prize for Medicine and Physiology* in 2000 "for the discoveries concerning signal transduction in the nervous system" (Kandel, 2001).

In addition to the genus *Aplysia*, many other gastropod molluscs were used in these early days of neuroscience to perfect the intracellular techniques still used today in attempting to elucidate the causal mechanisms of memory formation. Great strides have been made by a number of groups using such model systems. For example, Alkon and his colleagues have used the marine snail hermissenda (*Hermissenda crassicornis* [Eschscholtz, 1831]). They concentrated their efforts on associative learning by developing a classical conditioning procedure utilizing light as the conditioned stimulus (CS) and vibration as the unconditioned stimulus (US) (Alkon, 1975). Their data showed that a specific type of photoreceptor, the B-type, was a key site for long-term memory (LTM) formation (Ito et al., 1994; Kawai et al., 2004a). Crow continues to use Pavlovian conditioning of *H. crassicornis* (Crow & Tian, 2006). Gelperin and his group demonstrated the remarkable learning and memory capabilities of the giant garden slug (*Limax maximus* L., 1758) (Gelperin, 1975). Matsuo and his colleagues are enthusiastically advancing the cellular and molecular neurobiology of the three-band garden slug (*Limax valentianus* [Férussac, 1823]) (Matsuo et al., 2011).

The great pond snail (*Lymnaea stagnalis* [L., 1758]) (Fig. 2.1) is another useful gastropod mollusc and has become an important model system for studying the causal neuronal mechanisms of associative learning and the subsequent formation of LTM. The initial studies utilizing *L. stagnalis* to study the associative learning involved in feeding behaviors were begun in the 1980s (Alexander et al., 1982; Audesirk et al., 1982; Kemenes & Benjamin, 1989a,b), and some of the studies in the subsequent decades utilized both classical and operant conditioning of a number of different behaviors, including various aspects of feeding, withdrawal, and aerial respiratory behaviors (Hermann & Bulloch, 1998; Kemenes & Benjamin,

1994; Kemenes et al., 1997, 2011; Kawai et al., 2004b; Kita et al., 2011; Lukowiak et al., 1996; Staras et al., 1998a,b; Spencer et al., 1999; Straub et al., 2004; Sakakibara, 2006; Suzuki et al., 2008; Whelan & McCrohan, 1996). In addition, CNS preparations have also been utilized to study neural analogues of associative learning in vitro (Kemenes et al., 1997; Sunada et al., 2012; Veprintsev & Rozanov, 1967).

FIGURE 2.1 The great pond snail (*Lymnaea stagnalis*). All the snails used were originally the gift of Vrije Universiteit Amsterdam and have been maintained in our laboratories. We generally used snails with a 20 mm shell length.

In our opinion, the most important reason for adapting the *L. stagnalis* model system to study learning and memory is the fact that the underlying neuronal circuitry has been worked out better than in any other model system to study associative learning. We base this opinion on the following facts. (1) The underlying neuronal circuitry of the central pattern generator (CPG) that drives feeding behavior has been worked out better than in other molluscan preparations (Benjamin & Rose, 1979; Benjamin et al., 2000, 2008; Benjamin, 2012; Elliott & Benjamin, 1985a, b; McCrohan & Benjamin, 1980; Rose & Benjamin, 1979). (2) The CPG that drives aerial respiration is the only neuronal circuit that we know of where both the sufficiency and

the necessity of the 3-neuron circuit has been experimentally demonstrated; in addition, one of the three CPG neurons, RPeD1, has been shown to be a necessary site for LTM formation (Lukowiak, 1991; Lukowiak et al., 2010; Syed et al., 1990, 1992; Scheibenstock et al., 2002; Sangha et al., 2003a,b; Taylor & Lukowiak, 2000; Winlow & Syed, 1992). (3) *Lymnaea stagnalis* feeding behaviors undergo both appetitive and aversive classical conditioning (Ito et al., 1999; Kawai et al., 2004b; Staras et al., 1999a,b; Straub et al., 2006; Whelan & McCrohan, 1996). (4) Aerial respiratory behavior can be operantly conditioned, and the memory formed following learning can be modified by environmentally relevant stimuli (Lukowiak et al., 1996, 1998, 2000, 2003a–c, 2008, 2010). (5) Both feeding and aerial respiration are tractable behaviors that exhibit associative learning (classical conditioning and operant conditioning) and the subsequent consolidation of the learning into LTM (Azami et al., 2006; Fulton et al., 2008; Teskey et al., 2012). (6) Finally, both behaviors undergo one-trial learning that leads to LTM formation, which allows investigators to more accurately investigate the time course of the molecular and neural events leading to LTM formation (Alexander et al., 1984; Fulton et al., 2005; Martens et al., 2007; Sugai et al., 2007).

Thus, because the neuronal circuitry has been so well worked out and the behaviors mediated by those circuits exhibit associative learning and LTM formation, the advantages offered by the *L. stagnalis* model system are second to none. This point has been brought out previously by Chase (2002) in his excellent book comparing various molluscan preparations.

In the present chapter, we present an outline of cellular mechanisms underlying aversive conditioning in the feeding behavior of *L. stagnalis*.

2.2 CONDITIONED TASTE AVERSION IN *L. STAGNALIS*

The Ito group, with help from M. Sakakibara and K. Lukowiak, has so far noted one remarkable learning ability in *L. stagnalis*. This is the capacity to establish taste aversion and consolidate it into LTM. This phenomenon is referred to as conditioned taste aversion (CTA) (Kojima et al., 1996). To produce CTA in *L. stagnalis*, an appetitive stimulus (e.g., sucrose) is used as the CS. Application of the CS to the lips increases the feeding response (i.e., the number of bites) in snails. An aversive stimulus (e.g., KCl) is used as the US. Application of the US to the snails inhibits feeding behavior. In the taste aversion training procedure, the CS is paired with the US. After repeated temporal contingent presentations of the CS and US, the CS no longer elicits

a feeding response (Fig. 2.2), and this taste aversion persists for more than a month (Kojima et al., 1996).

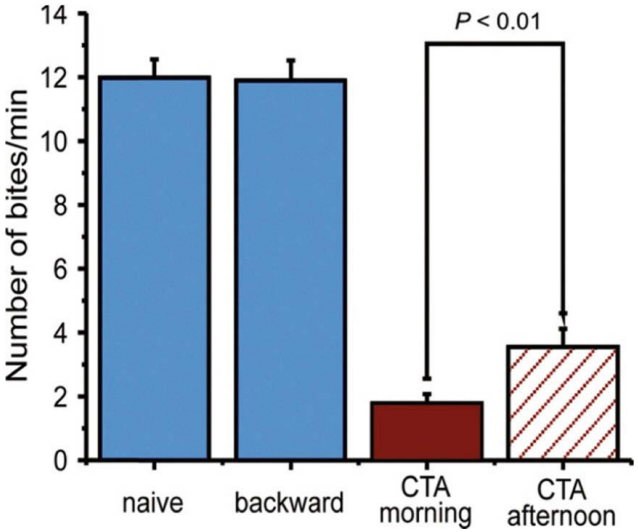

FIGURE 2.2 Learning scores after taste aversion training in the great pond snail (*Lymnaea stagnalis*). Taste aversion training was brought about by pairing 10 mmol/L sucrose (conditioned stimulus: CS) and 10 mmol/L KCl (unconditioned stimulus: US). The duration of both the CS and the US was 15 s, with an interstimulus interval between the onsets of CS and US of 15 s. A 10-min intertrial interval was interposed between each pairing of the CS–US. Snails received 10 paired CS–US trials. We also used a backward conditioned (US–CS) control group and a naive control group to validate associative learning. For the naive control group, only distilled water was applied to the lips instead of the CS and US. The *y* axis shows the number of bites/min after training that was evoked by application of sucrose (i.e., CS). The learning score by taste aversion training (CTA) is better when snails are trained in the morning than in the afternoon (see Wagatsuma et al., 2004). Data are expressed as the mean + SE.

2.3 ENHANCEMENT OF THE INHIBITION ON FEEDING CENTRAL PATTERN GENERATOR NEURONS

Based on the above behavioral experiments, we proposed a working hypothesis for CTA in *L. stagnalis* (Figs. 2.3A, 3B). We hypothesized that when the CS (sucrose) is followed by the US (KCl) in the training session, the association of the CS and US causes a potentiation of an inhibitory neuronal pathway, resulting in suppression of the feeding response to the CS (Fig. 2.3A; Kojima et al., 1996). Taking into account the underlying neural circuits worked out

by the Benjamin group (Benjamin & Elliott, 1989; Elliott & Kemenes, 1992; Ferguson & Benjamin, 1991a,b; Inoue et al., 1996a,b; Kemenes et al., 2001; McCrohan & Kyriakides, 1992; Syed & Winlow, 1991; Staras et al., 1998b; Straub & Benjamin, 2001; Straub et al., 2002; Yeoman et al., 1994a,b, 1996), our model further proposes that sensory neuron(s) activated by the appetitive sucrose (CS) excite the feeding CPG neurons which drive motor neurons in the CS pathway to induce a feeding response. Similarly, sensory neuron(s) activated by the aversive KCl stimulus (US) excite withdrawal interneurons that activate motor neurons in the US pathway, resulting in a withdrawal response. The withdrawal response takes precedence over the feeding response. With the pairing of CS–US, the CS is no longer capable of eliciting feeding. It is the association of the CS and US in the key interneurons that result in the CS no longer being able (i.e., while LTM persists) to elicit the feeding response (Fig. 2.3B).

Previous studies have shown that the cerebral giant cells (CGCs) exert both a weak excitatory monosynaptic influence and a strong inhibitory polysynaptic influence on the neuron 1 medial (N1M cells) of the feeding CPG, and that the repetitive firing of the CGCs results in inhibitory influences on the N1M cells (Yeoman et al., 1996). The CGCs act as a pair of interneurons, one located in the right and the other in the left cerebral ganglia. We showed that the CS and US are associated in the CGCs and alter the activity of the CGCs (Nakamura et al., 1999a,b). Because we applied CS and US only to the lips, but not the neurons in the CNS, these solutions were used as tastes for the lips, such as sweet and bitter, respectively. The concentrations of the sucrose solution (CS) and the KCl solution (US) that we used in our conditioning paradigm are each 10 mmol/L. We have in control experiments shown that if these solutions are directly applied to the CNS, no responses are recorded at the CGCs. Thus, the CGCs were a logical site for further explanation to elucidate the neuronal mechanisms of CTA.

With further experiments, we found that a polysynaptic inhibitory postsynaptic potential (IPSP) recorded in the N1M cells by activation of the CGCs was larger and lasted longer in the taste aversion trained snails than that in the control snails (Fig. 2.4; Kojima et al., 1997). These data suggested to us that an enhanced IPSP in the N1M cells underlies the suppression of feeding response in the CTA of *L. stagnalis*. Interestingly, when the amplitude of the IPSP recorded in the N1M cells in the CNS taken from good memory performers was compared with the IPSP amplitude recorded in the poor memory performers, we found that there was a much greater variance in the amplitude of the IPSP from the poor performers. This suggested to us

that this greater variance in IPSP amplitude in the poor performers corresponds to the instability of the input elicited by the US in those key target neurons. Thus, the polysynaptic IPSP from the CGCs to the N1M cells in memory-poor performers is not able to suppress the feeding response.

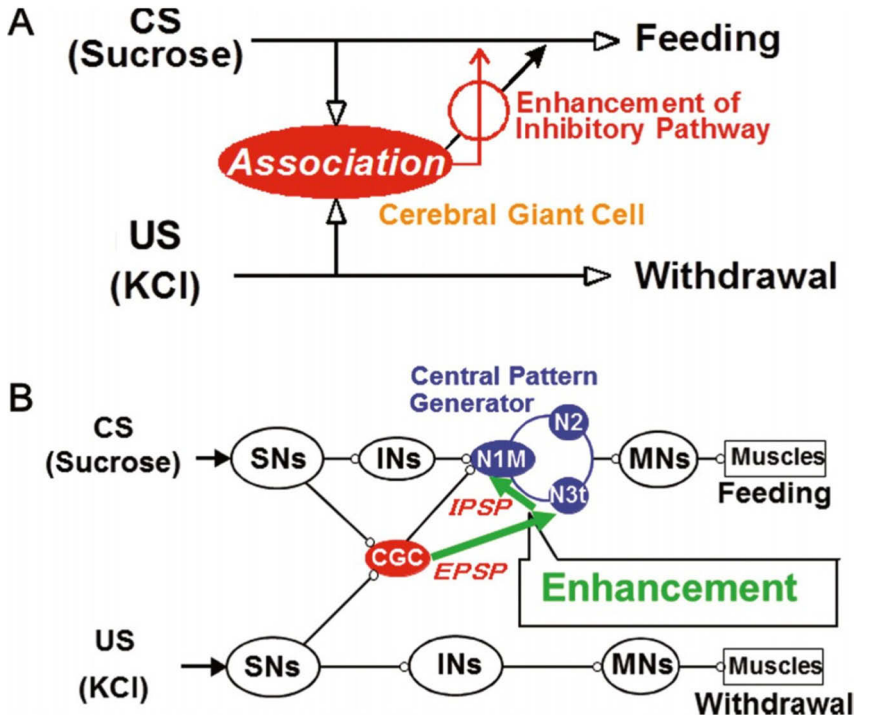

FIGURE 2.3 Our working hypothesis for conditioned taste aversion in the great pond snail (*Lymnaea stagnalis*). (A) Neuromodulatory model. When sucrose (CS) is followed by KCl (US) in the training session, the association of these stimuli occurs at one or more loci in the central nervous system. Then this association enhances an inhibitory pathway, resulting in suppression of the feeding response to sucrose (CS). (B) Neural circuitry model. The sensory neurons (SNs) sensitive to sucrose excite the interneurons (INs), including the feeding central pattern generator neurons, and the motor neurons (MNs) to induce a feeding response, whereas the sensory neurons to KCl excite the interneurons and the motor neurons in the withdrawal pathway, resulting in a withdrawal response. Based on previous observations by many researchers, we hypothesized that a pair of cerebral giant cells (CGCs) receive the information of the above two stimuli and that the CGC exerts a strong polysynaptic inhibitory influence on one of the feeding central pattern generator neurons (neuron 1 medial (N1M) cell) via the neuron 3 tonic (N3t) cell. Modified from Kojima et al. (1997).

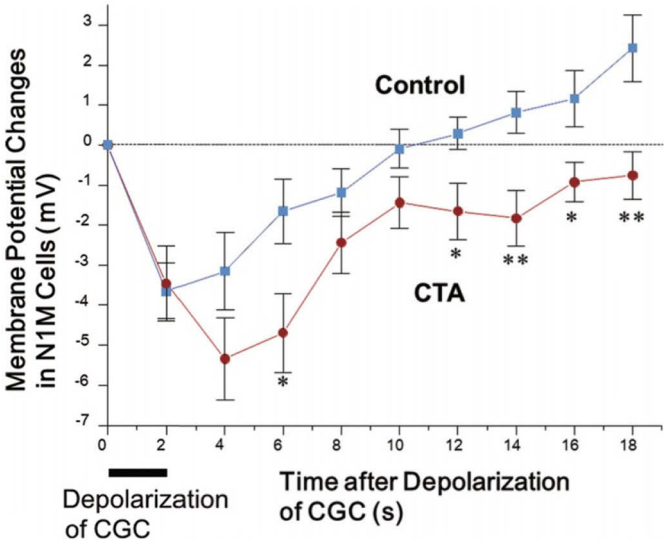

FIGURE 2.4 Enhancement of polysynaptic inhibitory postsynaptic potential (IPSP) in the neuron 1 medial (N1M) cells by activation of the cerebral giant cells (CGCs) after taste aversion training in the great pond snail (*Lymnaea stagnalis*). The CGCs were depolarized for 2 s. The IPSP was larger and lasted longer (2-way repeated-measures ANOVA, $P < 0.01$) in the taste aversion trained snails (CTA) than in the naive or backward snails (control). $*P < 0.05$; $**P < 0.01$. Data are expressed as the mean ± SE. Modified from Kojima et al. (1997)

2.4 MULTIPLE SITE OPTICAL ANALYSIS OF CONDITIONED TASTE AVERSION

When we published our electrophysiological data and our interpretation of those data, we received a substantial amount of criticism. The criticism primarily centered on whether the changes we observed (i.e., the long-lasting synaptic change between the CGCs and the N1M cells) were the only changes that occurred in the CNS of the taste aversion trained snails. In other words, we had no information about any other changes occurring in synaptic strength in other CNS neurons. To attempt to answer this criticism, we used an optical recording technique to measure changes that could occur in other CNS neurons in response to taste aversion training (Kojima et al., 2001). To perform these experiments, we used isolated CNS preparations obtained from taste aversion trained snails, stained them with a voltage-sensitive dye RH155, and simulated the presentation of sucrose (i.e., the CS) with electrical stimulation of the median lip nerve. The median lip nerve transmits chemosensory signals of appetitive taste to the CNS.

We optically detected a large number of spikes in several areas of the buccal ganglion after electrical stimulation of the median lip nerve. The effects of behavioral taste aversion training on the spike responses were examined in two areas of the buccal ganglion where the most active neural responses were seen. In one area that accounted for the N1M cells, the number of spikes after median lip nerve stimulation (i.e., the simulated CS) was significantly reduced in taste aversion trained snails compared with control snails. In another area positioned between the buccal motor neurons (i.e., the B3 motor neuron and the B4 cluster cells), the evoked spike responses elicited by median nerve stimulation were unaffected in the taste aversion trained preparations. These data showed that the appetitive signal transmitted via the median lip nerve to the N1M cells is suppressed following CTA. This results in a decrease of the fictive feeding response. However, even with our optical recording technique, we still cannot rule out the possibility that changes in neuronal activity in other areas of the CNS occur with taste aversion training.

2.5 MEMORY TRACE IN THE FEEDING CENTRAL PATTERN GENERATOR IN CONDITIONED TASTE AVERSION

As described above, the polysynaptic IPSP recorded in the N1M cells by activation of the CGCs in taste aversion trained snails was larger and lasted longer than the IPSP in control snails (Fig. 2.4). However, the neural circuit between the CGC and the N1M cell consists of two types of synaptic connections: (1) the excitatory monosynaptic connection from the CGC to the neuron 3 tonic (N3t) cell and (2) the inhibitory monosynaptic connection from the N3t cell to the N1M cell (Fig. 2.5A). As a next step, we had to determine which synaptic connection is more changed following the acquisition of CTA.

The recent studies on appetitive conditioning of feeding behavior in *L. stagnalis* by the Benjamin group made three points (Marra et al., 2010). (1) Tonic inhibition in the feeding network is provided by the N3t cell. This interneuron makes a monosynaptic inhibitory connection with the N1M cell. (2) There is a reduction in N3t spiking after appetitive conditioning, and this reduction in N3t firing inversely correlates with an increase in the conditioned fictive feeding response. (3) Computer simulation of N3t–N1M interactions suggests that changes in N3t firing are sufficient to explain the increase in the fictive feeding activity produced by appetitive conditioning. These data showed that appetitive conditioning of feeding behavior in

L. stagnalis occurs because of the combined effects of reduced tonic inhibition and enhanced excitatory synaptic connections between the CS pathway and the feeding command neurons (Fig. 2.5A).

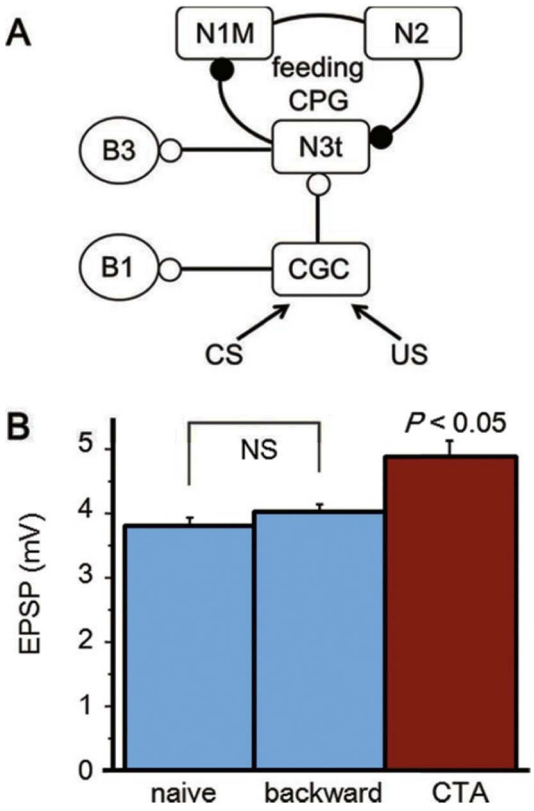

FIGURE 2.5 Enhancement of spontaneous excitatory postsynaptic potential (EPSP) in the B3 motor neurons after taste aversion training in the great pond snail (*Lymnaea stagnalis*). (A) Schematic presentation of the neural circuitry underlying taste aversion training. The signals of sucrose (conditioned stimulus: CS) and KCl (unconditioned stimulus: US) are associated in the cerebral giant cells (CGCs). Rectangles and circles indicate interneurons and motor neurons, respectively. At synapses, open circles and solid circles indicate excitatory monosynaptic inputs and inhibitory monosynaptic inputs, respectively. The neuron 1 medial (N1M), neuron 2 (N2), and neuron 3 tonic (N3t) cells form part of the feeding central pattern generator (CPG). (B) Spontaneous EPSP in the B3 motor neurons. The EPSP recorded in the B3 motor neurons can be used for monitoring the changes in the N3t–N1M synaptic connection. The B3 EPSPs recorded from the taste aversion trained snails were significantly larger (1-way ANOVA, $P < 0.05$) than those observed for the backward conditioned and naive control snails. Data are expressed as the mean + SE. Modified from Ito et al. (2012).

Next, we hypothesized that "taste aversion learning" would occur via a mechanism which was the inverse of the mechanism proposed for "appetitive conditioning". That is, there would be an increase in N3t spiking after conditioning, and this increase in N3t firing would inversely correlate with a reduction in the conditioned fictive feeding response. We thus hypothesized that taste aversion learning in *L. stagnalis* is also due to the combined effects of reduced tonic inhibition and enhanced excitatory synaptic connections between the CS pathway and the feeding command neurons.

However, because the N3t cells are too small to access consistently by standard sharp electrode recording techniques, in the present study the synaptic inputs from the CGCs to the N3t cells and those from the N3t cells to the N1M cells were inferred by monitoring the monosynaptic excitatory postsynaptic potential (EPSP) recorded in the large B1 and B3 motor neurons, respectively. The evoked monosynaptic EPSPs of the B1 motor neurons in the brains isolated from the taste aversion trained snails were identical to those in the control snails, whereas the spontaneous monosynaptic EPSPs recorded in the B3 motor neurons were significantly enlarged (Fig. 2.5B; Ito et al., 2012).

These data suggested that, after taste aversion training, the monosynaptic inputs from the N3t cells to the follower neurons, including the N1M cells, are facilitated. That is, one of the neural correlates of CTA–LTM is an increase in neurotransmitter release from the N3t cells. We thus conclude that the N3t cells suppress the N1M cells in the feeding CPG, in response to the CS in *L. stagnalis* CTA.

2.6 DEVELOPMENT OF THE LIFE CYCLE AND DEVELOPMENT OF THE LEARNING ABILITY

By using the taste aversion training procedures in *L. stagnalis*, we can assess the common changes that occur, both development the life cycle and development of the learning ability of this organism (Karasawa et al., 2008; Ono et al., 2002; Sunada et al., 2010a). We examined developmental changes in the acquisition and retention of CTA in *L. stagnalis* (Yamanaka et al., 1999). Our data showed that snails developed their ability to form CTA–LTM through the three critical stages: (1) stage 25 embryos (veliconcha) start to respond to appetitive sucrose, (2) stage 29 embryos just before hatching acquire CTA, but not LTM, and (3) immature snails with a 10-mm shell are able to learn and remember which foods can be safely eaten. That is, the development of learning ability in snails is coincident with the major changes in their life cycle.

We then examined the relationship between the learning ability for CTA and the development of the CGC for CTA. Using Lucifer-yellow staining of the CGCs and Azan staining for the ganglion sections, we found that the CGCs mature at the early developmental stages and that the number of buccal and cerebral neurons in immature snails is similar to that seen in adult snails (Sadamoto et al., 2000). The immunoreactivity of serotonin, which is one of the main neurotransmitters employed in the feeding circuitry (Hatakeyama & Ito, 1999; Kemenes et al., 1989, 1997; Kemenes, 1997; Kawai et al., 2011; Nakamura et al., 1999c), was first observed in the CGCs at stage 29 (Yamanaka et al., 2000). After hatching, the neuropile of CGCs developed faster than other cells in the buccal and cerebral ganglia, resulting in their early innervation at the immature stage. Thus, the developmental changes in the CGCs correlate well with the ability to form CTA.

2.7 IDENTIFICATION OF INTERNEURONS INVOLVED IN THE WITHDRAWAL RESPONSE THAT AFFECT THE CEREBRAL GIANT CELLS

Although we have some evidence that there are input pathways onto the CGCs from higher order interneurons which mediate the withdrawal response elicited by the KCl stimulus (Nakamura et al., 1999a,b), these interneurons have not been positively identified (Ferguson & Benjamin, 1991a,b). However, two identified neurons are good candidates. One is the pleural–buccal neuron (PlB) and the other is the right pedal dorsal 11 neuron (RPeD11). The PlB is FMRFamidergic and was reported to inhibit all the neurons in the feeding circuit, including protraction and retraction motor neurons, feeding CPG interneurons, buccal modulatory interneurons, and the CGCs (Alania et al., 2004, 2008).

On the other hand, the Sakakibara group with the help of K. Lukowiak demonstrated that the RPeD11 sends an inhibitory input onto the CGCs (Sunada et al., 2012). The RPeD11, a well-known interneuron receiving sensory input from the right parietal dorsal 3 (RPD3) and sending output to the motor cluster neurons right pedal G (RPeG) and the right cerebral A (RCeA), exerts withdrawal behavior in response to multimodal noxious stimuli such as mechanical prodding, KCl application, and shadow presentation (Sunada et al., 2010b). An aversive stimulus to *L. stagnalis* employed in CTA as a US basically resulted in withdrawal behavior. The Sakakibara group's studies on neuronal mechanisms in the CTA used sucrose application as the CS and weak mechanical prodding to the animal's head as the US

(Kawai et al., 2004b). After acquisition of learning, the conditioned animals responded to decreases in the feeding response against the CS application. The presentation of the US, irrespective of whether KCl application or mechanical prodding was used, increased excitability in the RPeD11. Even with the application of weak mechanical prodding, the RPeD11 was excited, thereby decreasing the feeding response. The strong excitation induced by positive current injection into the RPeD11 resulted in inhibition of the CGCs, as evidenced by such effects as a decrease in spontaneous firing activity. This inhibitory effect was transmitted to the CGCs via mono-chemical synapses. The isolated preparations with mouth, buccal, and esophageal ganglia may provide a common platform for the CTA in vitro conditioning model using sucrose application as the CS and current injection into the RPeD11 as the US (Sunada et al., 2012).

2.8 FUTURE QUESTIONS

In our studies designed to determine how long CTA–LTM persists, it became apparent that snails continued to eat their normal diet of lettuce or similar leafy plants in their home aquaria while still exhibiting CTA–LTM. Thus, it was unclear what the relationship was between a CTA for a specific CS and other appetitive food stimuli. If snails can successfully differentiate between appetitive food stimuli, where in the CNS does this occur? Our previous experiments showed that snails can be differentially conditioned to avoid one appetitive CS following taste aversion training while continuing to be responsive to a different appetitive food CS that has not been paired in a forward manner with an aversive US (Sugai et al., 2006). That is, *L. stagnalis* can distinguish between tastes during CTA. The neurons responsible for taste discrimination may be located in the CNS and most probably exist upstream of the CGCs, but we have to carefully address this question and attempt to find neurons involved in taste discrimination in the *L. stagnalis* CNS in the future.

2.9 CONCLUSION

Researchers investigating CTA in rats and other mammals are often surprised to see that relatively simple invertebrate model systems, such as *L. stagnalis*, are also capable of acquiring CTA. In fact, Bernstein (1999) concluded from our data that the neural circuitry required for this learning is fairly primitive.

However, we consider that this perceived weakness of the *L. stagnalis* model is actually an advantage (e.g., Murakami et al., 2013), because the use of simple invertebrate systems can provide answers to important basic questions before we move on to more complex mammalian systems.

The molecular mechanisms that regulate serotonin release from the CGCs have also been clarified in *L. stagnalis*. The key players in the molecular cascades are cAMP, protein kinase A, cAMP response element binding protein, CCAAT/enhancer binding protein, and serotonin transporter (Hatakeyama et al., 2004a,b, 2006; Nakamura et al., 1999c; Sadamoto et al., 2004a,b, 2008, 2010, 2011; Wagatsuma et al., 2005, 2006). We think that regulation of the amount of serotonin released from the CGCs plays an important role in CTA. These cascades will be reviewed elsewhere.

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KEYWORDS

- **conditioned taste aversion**
- **feeding**
- **long-term memory**
- ***Lymnaea stagnalis***
- **withdrawal**

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CHAPTER 3

STRESS, MEMORY, FORGETTING AND WHAT, *LYMNAEA* CAN TELL US ABOUT A STRESSFUL WORLD

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CONTENTS

ABSTRACT

The ability of animals to learn and remember during their lifetime allows them to successfully adapt to various environmental stressors. Stress modulates (either enhancing or diminishing) the ability to learn and the ability to form memory and the ability to recall memory. We have attempted to use environmentally relevant stressors (e.g., crowding, low levels of calcium, predator detection and thermal shock) to determine how the various stressors change the ability to learn, form memory and to recall that memory. It is difficult, if not impossible, to predict ahead of actually doing the experiment to say with any certainty how a specific stressor will alter memory formation and its recall. It is even more difficult to predict how a combination of stressors alters these cognitive events. Identical stressor stimuli affect different strains of *Lymnaea* (e.g., smart vs. average) differently. It may be that one of the "costs" of being "smart" is a poor ability to handle stress.

The birth of modern neuroscience occurred in the 1950s. A number of seminal events associated with scientists and clinicians from the Montreal area played important but sometimes forgotten roles in establishing what we now call Neuroscience. One event was the brain surgery performed on a patient known as HM that lead to Brenda Milner's team observations on human memory, which ultimately showed that specific neural circuits were necessary for different forms of declarative and non-declarative memory (Milner et al., 1998). Those observations formed the basis of many experiments that ultimately led to our present understanding of the molecular events occurring in specific neurons, which are necessary for memory formation. However, the techniques and knowledge needed to undertake those studies at the neuronal and circuit level depended in large measure on the realization that molluscs possess large, identifiable neurons, which controlled interesting, tractable behaviors. A second and a third event were the ideas put forward by Donald Hebb in the 1950s (the Hebb synapse (Hebb, 1949) and the inverted U shape function regarding stress and memory formation; Fig. 3.1). Interestingly, this latter idea is most often attributed to a paper by Yerkes and Dodson in 1908; but in reality Hebb (Hebb, 1954) conceived the notion of what is now commandingly referred to as the Yerkes–Dodson law (Lukowiak et al., 2015).

Foundational studies that were also necessary for Neuroscience to become a "science" were occurring in France and Monaco led by Tauc (1954) and Arvanitaki and Chalazonitis (1955), respectively, using the central nervous system (CNS) of the sea hare (genus *Aplysia*). These studies

laid the groundwork for the use of molluscan model systems to investigate the causal neuronal mechanisms of learning and memory. Building on these earlier studies, Kandel and Tauc's (1965) discovery of heterosynaptic facilitation in a molluscan preparation laid the experimental groundwork for hypotheses developed subsequently to explain the "Hebb synapse" and the neuronal basis of learning and the subsequent formation of long-lasting memory. Ultimately, the research performed by a multitude of investigators using a wide variety of molluscan preparations culminated in Eric Kandel being awarded the *Nobel Prize for Medicine and Physiology* in 2000 "for the discoveries concerning signal transduction in the nervous system" (Kandel, 2001).

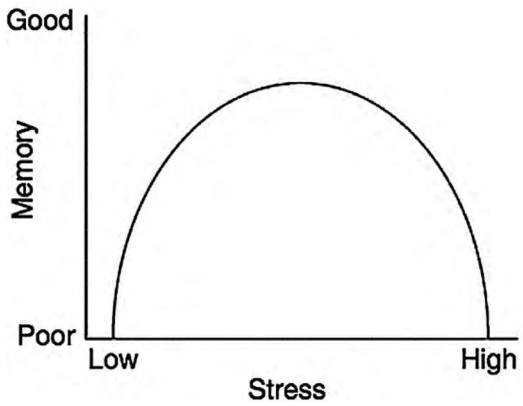

FIGURE 3.1 *The Hebb Curve (aka Yerkes–Dodson law)*. On the *x*-axis, the subject's perceived stress level, which runs from "low" to "high" is plotted. On the *y*-axis, LTM spanning the range from "poor" to "good" is plotted. At low levels of stress, there is "inattention" and this results in a "poorer" memory, whilst at high levels of stress poor LTM is also observed. At the higher stress levels, the subject finds it difficult to cope (i.e., lack of resilience) and maintain homeostasis. In between the high and low stress levels is the region of "good" stress. "Good" stress level is that which coping occurs, but which is sufficient to keep our attention. Thus, at some levels of stress, memory will be better than at others. This curve can change depending on the age of the individual, their previous history and the difficulty of the task.

In this chapter, I will tell two main stories. One a compelling (I hope) series of stories of how learning, memory, and forgetting are all altered by environmentally relevant stressors; and, two, why it is important to understand the neuroecology of the model system one works with.

3.1 *LYMNAEA STAGNALIS* AS A MODEL SYSTEM

Lymnaea stagnalis (often called the great pond snail, Fig. 3.2) is an important model system for studying the causal neuronal mechanisms of associative learning and the subsequent formation of long-term memory (LTM). The initial studies utilizing *Lymnaea* to study associative learning involved in feeding behaviors were begun in the 1980s (Alexander et al., 1982, 1984; Audesirk et al., 1982; Benjamin, 2012; Kojima et al., 1996), and these studies are reviewed in the chapter in this book by Gaynor Spencer and Paul Benjamin. As well, a recent review by Ito et al. (2013) paid particular attention to aversive food conditioning in *Lymnaea.* In that chapter, a descriptions of both feeding behavior and aerial respiratory behavior are given, thus I will not spend time here describing aerial respiration here.

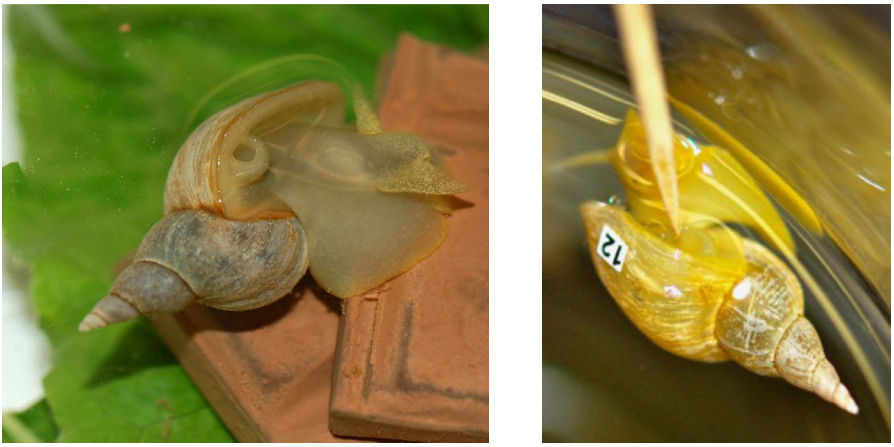

FIGURE 3.2 *The great pond snail, Lymnaea stagnalis.* The left hand picture shows a freshly collected *Lymnaea stagnalis* from a pond along the Trans Canada Highway, just west of Calgary. The shell length of this individual is approximately 30 mm. Also in this picture are two pieces of dark chocolate (high in the flavonol) epicatechin, which causes an enhancement of LTM formation. On the left is a picture of a lab-reared snail of approximately 25-mm shell length. This snail (no. 12) is being trained. It is receiving a tactile stimulus delivered to its pneumostome area as it attempts to open it. The pneumostome of the freshly collected snail is open and shows that this behavior is easy to detect. This behavior, aerial respiration, is driven by a 3-neuron central patter generator (CPG) shown below.

In my opinion, the most important reason for adapting the *Lymnaea*-model system to study learning and the subsequent formation of LTM is the fact that the underlying neuronal circuitry of the behavior I study, aerial respiratory behavior has been worked out better than in any other model

system to study associative learning. The central pattern generator (CPG) that drives aerial respiration (Fig. 3.3) is the only neuronal circuit that I know of where both the *sufficiency and the necessity* of the 3-neuron circuit have been experimentally demonstrated (Syed et al., 1990, 1992; Lukowiak, 1991; Taylor & Lukowiak, 2000). Importantly, one of the three CPG neurons, RPeD1, is a necessary site for LTM formation, reconsolidation, extinction, and forgetting (Lukowiak et al., 2010; Sangha et al., 2003a,b; Scheibenstock et al., 2002). Additionally, differences in cognitive ability between different strains of snails (i.e., ease of forming LTM and its longer duration following operant conditioning training) are reflected in RPeD1's activity in the naive state (Braun et al., 2012). Finally, the behavior and observed long-lasting changes in RPeD1 activity are seen following one-trial learning (Martens et al., 2007). Such ability allows us to more accurately investigate the time course of the molecular and neural events leading to LTM formation.

3.2 THE HEBB INVERTED *U* (AKA YERKES–DODSON) LAW AND MEMORY FORMATION

Stress is hard to define. Hans Selye, often called the Father of Stress, said the following "Everyone knows what stress is and nobody knows what it is" (Selye, 1973). Here, I define stress as any significant state that requires a physiological, psychological, or behavioral readjustment or modification necessary to maintain the wellbeing of the organism. No matter how it is defined, it is a certainty that stress modulates the ability to learn and to form memory, and it also affects the ability to recall the previously formed memory. The "fact" that stress alters memory has been a part of the scientific literature since the 17th century (Bacon, 1620). Typically when stress is mentioned in regards to learning and memory one immediately thinks of the so-called Yerkes–Dodson (Y–K) law. It is important here that I discuss exactly what the Yerkes and Dodson (1908) paper reports and then discuss where the typical inverted *U* curve attributed to that paper actually arises. In their 1908 paper, Yerkes and Dodson report on the relationship between stimulus strength and rapidity of learning in rodents. Their basic finding was "an easily acquired habit may be readily formed under strong stimulation, whereas a difficult habit may be acquired only under relatively weak stimulation." However, as most of us who took Psychology 101 or read a popular psychology textbook know the Y–K "law" is an inverted *U* function. The "law" (Fig. 3.1) is interpreted to mean that there is an optimal level of stress at which memory forms best. This "law" however, is actually derived

from a presentation made by Donald Hebb. In his presidential address (his Figure 2) to the American Psychological Association (Hebb, 1955), Hebb was championing the notion that the psychologists of that time must be cognizant of the "best brand of neurology we can find" in order to understand how arousal and motivation affect learning. Thus, Hebb hypothesized that there is an optimal level of learning and that with too little or too much "arousal" (i.e., stress) learning is not optimal (i.e., the inverted-*U* function). A more detailed description of the so-called Yerkes–Dodson law is given in Diamond et al. (2007) and Lukowiak et al. (2015).

In any case, the "law" posits that stress is an important element in determining both whether information becomes stored as LTM and for how long. Too much or too little stress impedes LTM formation, whereas an optimal level of stress enhances LTM formation. Since there is a "cost" to LTM formation (i.e., altered gene activity and new protein synthesis in neurons) organisms may only decide it is worth the "cost" to form LTM to "relevant" events. An important relevancy factor that helps determine whether a specific "event" will be encoded into memory is the level of stress perceived by the organism at the time of the occurrence and how the stress relates to the event. But as Hebb pointed out too little or too much stress blocks the formation of memory.

3.3 STRESS AND WHY USE *LYMNAEA*

There are large disagreements in the literature concerning the effect of stressors on learning and memory formation (Shors, 2004). This is not too surprising, given the complexities of the vertebrate brain and the different, often complex behaviors tested. Since I use a much "simpler" system and a relatively simple behavior to analyze, I don't typically encounter many of the problems other researchers have to deal with. However, it is important to remember that the "state" of the organism in large measure determines how it perceives a stimulus as a stressor. The perception of the stimulus, rather than the stimulus itself, acts as the stressor.

The stressors I have typically employed are ecologically and behaviorally relevant (Dalesman and Lukowiak 2012a). That is, these are the type of stressors snails in a pond would encounter. In addition, I have in many cases not only used laboratory-reared snails, but have also used freshly collected snails that have differing memory-forming capabilities. These are two important points that must always be considered in assessing how stressors

alter learning and memory. I have to say that quite often the stressors used in experiments, especially on very inbreed rodent preparations, do not make ecological sense. Thus, the conclusions drawn from these experiments may only really be applicable to those specific laboratory animals and not to animals, including us, in general. As Rudy Boonstra has pointed out the "bio-medical" view of how stress alters animal behavior is not the same as it occurs in wild, behaving animals (Boonstra, 2013). Based on laboratory model systems, the overall conclusion often drawn is that chronic stressors result in maladaptations (i.e., pathology). However, in "wild animals" long-term naturally occurring stress is part of their normal experience, and while there may be some "costs" the responses made by the animals are adaptive (i.e., not pathological). I believe we have to view how a stressor(s) affects LTM formation in this light rather than as a "pathology." Blocking LTM formation may in many instances actually be adaptive.

With regard to how stress alters memory formation I believe that I make good use of the so-called Krogh (1929) principle. This principle can be summarized as follows: there is an animal best suited to be used to study that particular problem. In my opinion, *Lymnaea* may be Krogh's "animal of choice." *Lymnaea* has frequently been used to study learning and memory due to their relatively simple neuronal network that mediates interesting, tractable behaviors. I make use of aerial respiratory behavior in the *Lymnaea* model system because (1) the neural circuit that drives this tractable behavior is well understood (Fig. 3.3); (2) this behavior can be operantly conditioned, and long-lasting memory can be demonstrated (Lukowiak et al., 1996); and (3) it is possible to demonstrate that a single neuron in the snail is a necessary site of LTM formation (Scheibenstock et al., 2002).

In the natural environment, suboptimal conditions may act as a stressor on the snails and alter their cognitive abilities (i.e., learning and LTM formation). My overall basic finding is that *Lymnaea's* ability to form LTM following associative learning is pliable because memory formation, its persistence, and its recall are all significantly altered by ecologically relevant environmental stressors (Lukowiak and Dalesman, 2013). Whether memory formation and its maintenance are enhanced or blocked depends on (1) the nature of the stressor; (2) the timing of the application of the stressor relative to both the learning procedure and the consolidation process; (3) how the stressor is perceived by the snail (i.e., the state of the snail); (4) when the stressor is encountered; and (5) whether the individual stressors are applied separately or in combination.

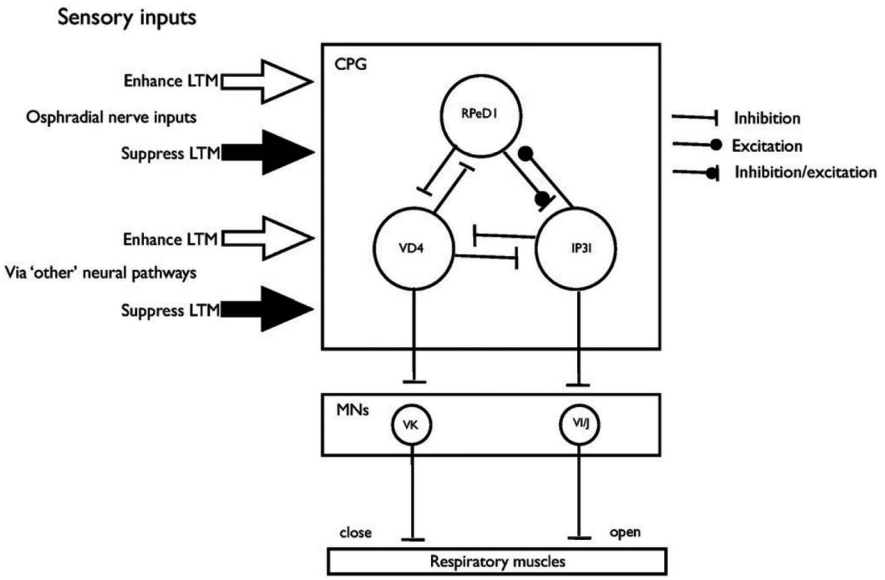

FIGURE 3.3 *The central pattern generator (CPG) that drives aerial respiratory behavior, associated higher order interneurons, sensory inputs and motor neuron outputs.* The three CPG neurons (VD4, IP3, and RPeD1) have been experimentally shown to be both necessary and sufficient to drive aerial respiratory behavior. RPeD1 has been demonstrated to be a necessary site for LTM formation, reconsolidation, extinction, and forgetting. The "state" of RPeD1 activity predicts the ability of the snail to make LTM. Rhythmogenesis is an emergent property of this network. Input from the periphery via the osphradial nerve, for example, plays a major role in determining the level of spontaneous activity of neurons such as RPeD1. Also shown are a representative closer (VK) motor neuron and a (V/J) opener pneumostome motor neuron. White arrows signify enhancement of LTM; while black arrows signify suppression of LTM formation.

A final observation before describing some experimental results is that in many instances the different stressors employed only alter memory formation but not learning (Fig. 3.4). The data in this figure show that learning is statistically similar in the three cohorts of snails exposed to the two different stressors and the control cohort but that the ensuing memory as a result of being exposed to the stressor is dramatically different (i.e., enhanced in some cases blocked in others). I believe that studying why this phenomenon occurs will give great insight into how stressors alter memory formation and may give valuable clues as to why certain memories may be either more or less difficult to forget.

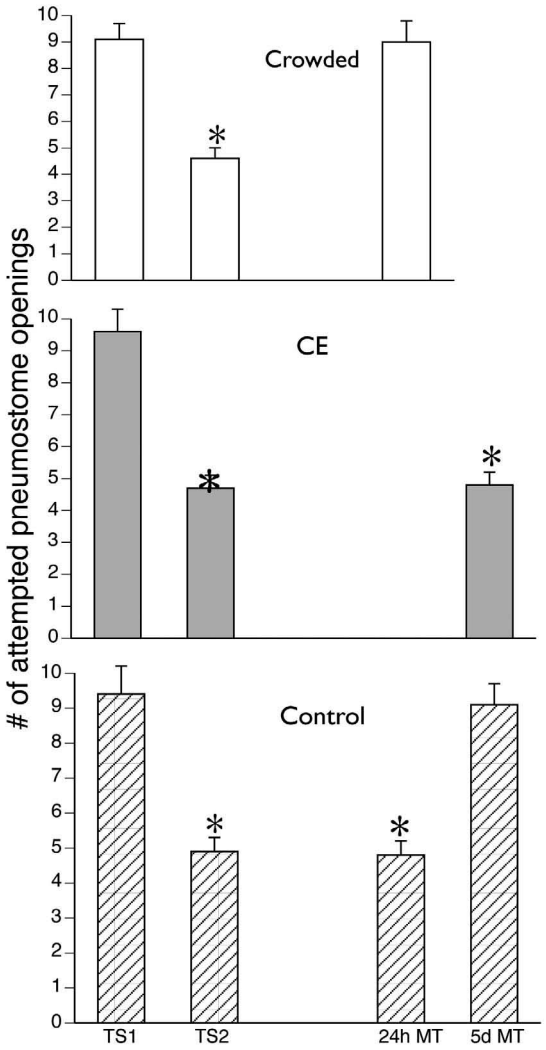

FIGURE 3.4 *Similar learning but very different memory*. Training of *Lymnaea stagnalis* consisted of two 0.5-h training sessions separated by 1 h (i.e., TS1 and TS2). Three different cohorts of snails ($N = 15$ per cohort) all showed learning (i.e., TS2 is significantly less than TS1, asterisk). (A) Crowded for 1 h before training in pond water; (B) trained in pond water following crayfish effluent (CE) exposure; (C) trained in control pond water. There was no LTM following exposure to crowding (A); there was a 5-day memory following exposure to the CE stressor (B); and there was a 24-h memory with training in pond water but not a 5-day memory (C). However, the difference between TS1 and TS2 (i.e., learning) for all three cohorts was the same. There was also no difference in TS1 between the three cohorts. Likewise, there was no difference in TS2 between the three cohorts.

A working hypothesis is that during the learning phase both "memory-making" and "forgetting-making" processes are activated (Lukowiak et al., 2014a). Certain stressors favor one process over the other. Whether memory forms and how long it persists is thus dependent on the interaction between the "memory" and "forgetting" processes. Such a "dual process" theory has previously been invoked to explain behavioral habituation (Groves & Thompson, 1970; Lukowiak & Jacklet, 1972).

3.4 ECOLOGICAL STRESSORS AND THEIR EFFECT ON LTM FORMATION

There are a number of factors that are important in the life of a snail, changes that could lead to stressful times. *Lymnaea*, to live long and prosper, requires adequate food, a good source of calcium, which it absorbs directly from the water to grow its shell (see below), and the ability to detect predators (Dalesman & Lukowiak, 2010; Lukowiak et al., 2014b). Restrictions of either food or calcium lead to stunting and reduced reproduction. Thus, food or decreased calcium availability act as environmental stressors. Crowded conditions may increase competition for resources. Thus, crowding would appear to be a stressor that *Lymnaea* could encounter. Conversely, as a preferentially out-crossing hermaphrodite, isolation, and therefore not being able to find a mate is also not ideal. Consequently, either crowding or isolation could act as social stressors.

Typically, *Lymnaea* are found living in slow flowing or stagnant shallow water bodies. In temperate regions, these habitats may be subject to rapid temperature fluctuations. During a summer day in Alberta with the sun shining, water temperature may rapidly rise in a shallow pond, and then with frost overnight (yes, even in summer around Calgary) could drop equally rapidly to close to freezing. These rapid temperature fluctuations are not uncommon, and may act as a stressor.

In addition to natural environmental factors that may act as stressors, my lab has also studied the effects of human generated pollutants. Anthropogenic pollutants are a wide-spread problem in freshwater environments; whilst pollutants may not be classified as a "natural" stressor, they are a factor that will affect natural populations. My lab has assessed the acute effects of heavy metals (cadmium and zinc) and hydrogen sulphide on LTM formation. Rather than assessing the concentration of a heavy metal on mortality of the snails, we have studied the effects of heavy metals on LTM formation. Thus far, my laboratory has studied the acute effects of H_2S

and toxic "heavy" metals, specifically Zn and Cd (Rosenegger et al., 2008; Byzitter et al., 2012).

3.5 H_2S AND HEAVY METALS

We initially choose to look at H_2S because of the abundance of "sour-gas" in Southern Alberta. It has been shown that the concentration of H_2S can get quite high in ponds close to well sites. In addition, in a number of communities surrounding Calgary, there is concern of low levels of the gas being present and altering the behavior of bees. Thus, we decided to investigate whether relatively low levels, such as could be found near drilling sites, would alter behavior in *Lymnaea* (Rosenegger et al., 2004). The effects of H_2S on LTM formation following operant conditioning in *Lymnaea* were assessed using a range of doses from 50 to 100 µmol/l that the snails were exposed to during operant conditioning (Fig. 3.5). At lower concentrations (50 & 75 µmol/l) while LTM was present, it was not as good as in controls. That is, those snails while still being capable of forming LTM did so more poorly. Each snail was given a grade in order to access how good the memory was compared to their initial response at the start of training. These H_2S exposed snails exhibited "poorer" grade distribution compared to controls (i.e., fewer A's more F's, etc. Fig. 3.5A). However, when *Lymnaea* were exposed to 100 µmol/l H_2S both learning and memory were blocked (Fig. 3.5B). Thus, this environmental pollutant at lower concentrations negatively impacted the snails' ability to form good memory; while when the higher concentration was used both learning and memory were negatively affected.

LTM formation was also found to be altered by sublethal levels of heavy metals but only when they were exposed to combinations of heavy metals (Byzitter et al., 2012). Levels such as these are often found in areas with mining and oil extraction. This is something that needs to be discussed. Too often organisms are only exposed to a single heavy metal over a range of concentrations to determine the "lethal" dose of that metal. However, I believe that one should investigate combinations of heavy metals at lower concentrations, as that scenario most often is what the snail would be exposed to in a pond. My lab found that two heavy metals, Cd and Zn in combination at levels below those allowable in municipal drinking water (Zn: 1100 µg/l; Cd: 3 µg/l) affect learning and memory in *Lymnaea* following an acute exposure (~48 h) prior to and during training/testing (Fig. 3.6). When *Lymnaea* was exposed to either metal in isolation, they were able to learn and form memory equally well as the control animals. However, when exposed to

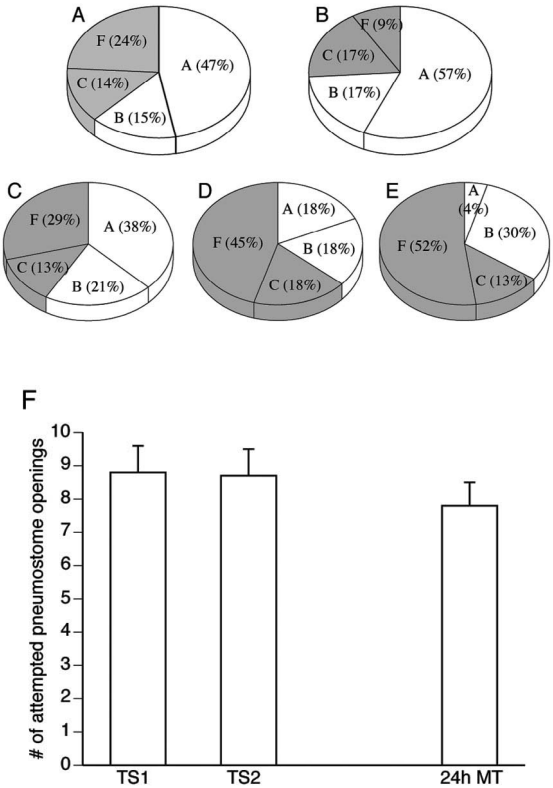

FIGURE 3.5 *H_2S and LTM formation*. Snail LTM "grade distributions." Snails were given grades based on their individual performance. Grades were calculated as follows: a 50% reduction or greater from the first training session to the memory test session is an A, a B is a reduction of 35–49.99%, a C is a 20–34.99% reduction, and an F is a reduction of less than 20%. (A) Grade distributions observed to occur under standard hypoxic conditions ($N = 2301$). (B) Distribution of grades for snails presented with the "more intense hypoxic challenge' ($N = 23$). These snails showed a statistically greater number of A grades and fewer F grades than controls ($P = 0.0007$). (C) Grade distributions seen for snails trained in the hypoxia + 50·μmol·/l H2S condition ($N = 24$). (D) Distributions for snails trained in the hypoxia + 75·μmol/l H2S ($N = 11$). (E) Distributions for training in hypoxia 100·μmol/l H2S condition ($N = 23$). As can be seen as the H2S increases the % of F's increase. (F) The cohort ($N = 23$) of snails whose marks are depicted in E underwent the training and testing protocol in hypoxia + H2S (100·μmol/l). These snails showed neither learning nor memory. That is, the data in session 2 were not significantly different than that in session 1 (i.e., no learning) and the memory test session (MT) was not significantly different from session 1 (i.e., criteria for memory not met; ANOVA $F(22,2) = 2.3095$, $P = 0.1112$; no significant difference between sessions).

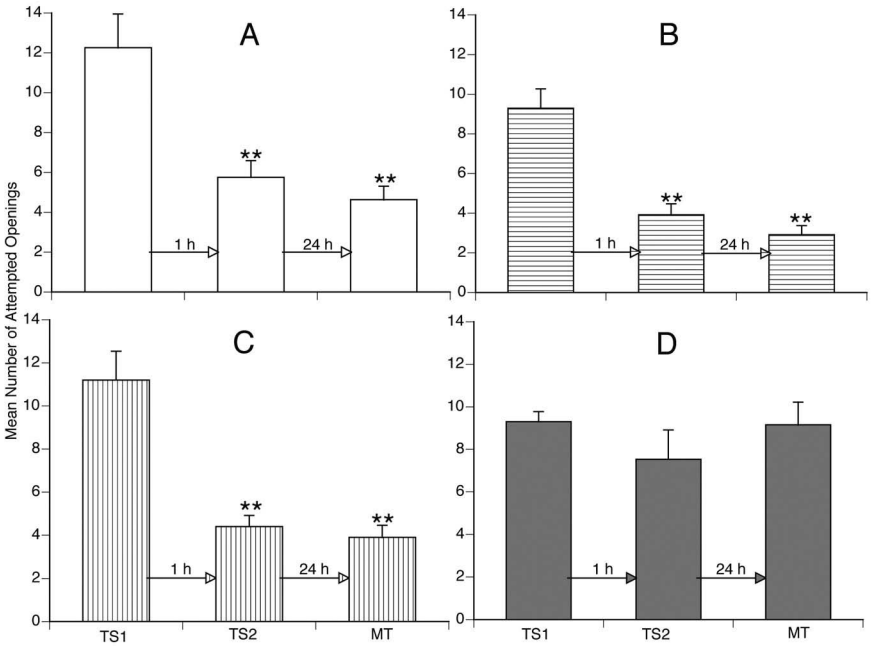

FIGURE 3.6 *The combination of heavy metals (zinc and cadmium) block LTM*. Mean number of attempted pneumostome openings (±SEM) in 30 min in the first (TS1) and second (TS2) training sessions and the memory test (MT) 24 h later following exposure to (a) control pond water, white columns (N = 16); (b) zinc (1100 µlg/l), horizontal stripe (N = 12); (c) cadmium (3 µlg/l), vertical stripe (N = 10), and (d) combined zinc and cadmium, dark gray (N = 13) 24 h prior to training and between training and testing. **Significantly different from TS1 (paired t test, $P < 0.01$).

these metals in combination both learning and LTM were blocked. It also turns out that the sensory pathway used by the snails to detect the heavy metals in the pond water was found. It was the osphradial nerve pathway that runs from the osphradium, a sensory organ near the pneumostome, to the CNS. If the osphradial nerve was severed placing snails in the Cd and Zn combined pond water had no effect on either learning of memory. Thus, it was the detection of the heavy metals by sensory neurons in the osphradium that altered the ability of the snail to form LTM and not a direct effect of the heavy metals crossing the snails' integument (Byzitter et al., 2012). Thus, the stress effects on neuronal functioning may be far more sensitive to very low levels of heavy metal pollution than other behavioral traits. In addition, these data show that toxic compounds such as heavy metals may have synergistic effects at far lower concentrations than those required to show detrimental effects in single toxin exposures.

3.6 PREDATOR DETECTION

I will not go into the details as to why my laboratory started to use freshly collected snails in addition to our laboratory-reared snails; that story has been told (Lukowiak et al., 2010). The laboratory-reared snails were derived from snails originally collected from polders in the Netherlands near Utrecht. They have been maintained in various labs since the 1950s. We found, however, that a batch of freshly collected *L. stagnalis* from the Belly River area of Southern Alberta (referred to as Belly snails) exhibited enhanced LTM-forming capabilities compared to our lab-reared snails, which I will refer to as Dutch snails (Fig. 3.7). I will discuss the different strains of snails in a section below. One of the first hypotheses to explain the difference between the Belly and Dutch snails was that the Dutch snails were not as smart because they were reared in a laboratory environment. That is, rearing them in the lab "dumbed" them down. Although we later disproved this hypothesis, we initially attempted to "enrich" the Dutch snails environment by exposing them to a predator threat. It was found that Dutch snails exposed to pond water the crayfish was in (called crayfish effluent, CE), had enhanced memory-forming capabilities (Orr et al., 2007). These data (Fig. 3.8) lead us to investigate how environmentally relevant stressors alter cognitive abilities in snails.

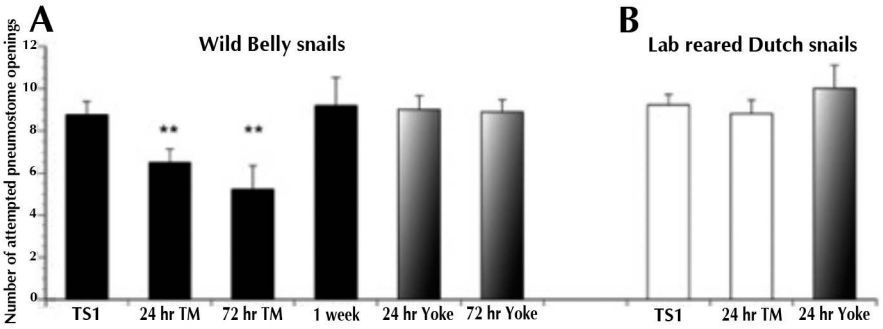

FIGURE 3.7 *LTM formation in freshly collected Belly snails and lab-reared Dutch snails*. A single 0.5-h training session (TS1). (A) Operant conditioning of Belly snails results in an LTM that persists for 24 and 72 h. Yoked control snails do not demonstrate memory at these same time periods. (B) Dutch lab-reared snails do not demonstrate LTM after a single 0.5-h training session. Results are shown as means + s.e.m. ** $P < 0.001$.

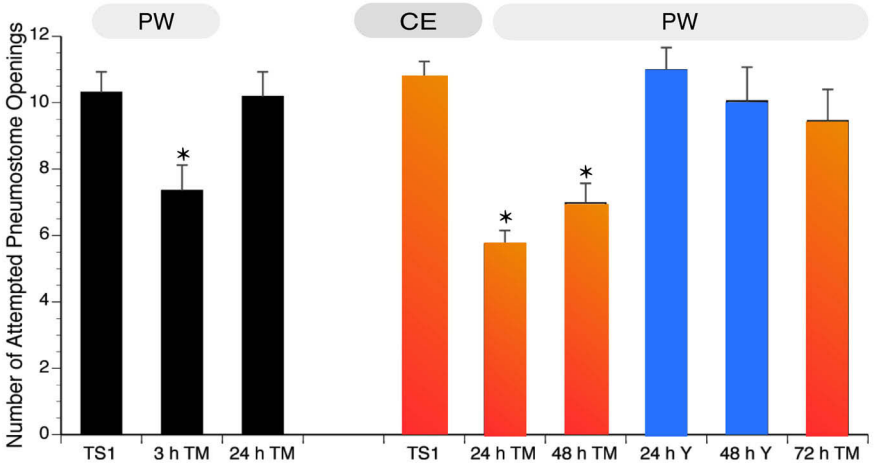

FIGURE 3.8 *Crayfish effluent (CE) enhances LTM formation in lab-reared Dutch snails*. Lab-reared Lymnaea after a single 0.5-h training session in either PW or CE. The single training session (TS1) in PW (black bars) results in a 3-h memory but does not result in LTM. However, the single training session (TS1) in CE results in LTM at 24 and 48 h, but not in 24 and 48 h yoked control groups or 72 h after training. ** $P < 0.001$.

These experiments demonstrated that an environmentally relevant stressor could enhance LTM formation. That is, in the presence of CE, operant conditioning training resulted in quicker, longer lasting and better LTM (i.e., more A's and less lower marks as presented in Fig. 3.5). Crayfish are natural predators of *Lymnaea* in the Netherlands and even though the Dutch snails have been reared in the laboratory since the 1950s (without coming into contact with this predator), they still maintained their ability to detect and respond appropriately to the predator smell. As can be seen in Figure 3.8 when Dutch snails detect the presence of a predator (i.e., trained in CE), LTM formation is enhanced. This is also evident at the electrophysiological level in recordings made from RPeD1. How do we know that detection of a predator is a stressful situation for the snail? We found that CE elicits a change (a heightened response) in the snails' so-called "vigilance" or "risk assessment" behaviors (Orr et al., 2007). For example, snails in CE respond to a shadow stimulus with a much exaggerated withdrawal response than in pond water. Hyper-arousal is considered a behavioral phenotype associated with stress. An alternative explanation was that a smell from any predator would elicit similar responses. This was not the case as tiger salamander effluent (SE) did not elicit vigilance behaviors in our lab-reared snails nor did it enhance LTM formation (Orr, 2009a,b). Tiger salamanders are not a sympatric predator of

Dutch snails. It is also interesting that the crayfish used in the Lukowiak lab to produce the CE were not a European species of crayfish but were species originating in both the USA and Australia. Whether the European species of crayfish would produce an even larger effect has not been experimentally tested; however, effects produced by the USA and Australian species do not seem to differ. Both result in enhanced LTM formation.

Experiments performed in my laboratory showed that sensory neurons in the osphradium were involved in predator detection (Il-Han et al., 2010), just as they were for heavy metal detection (Byzitter et al., 2012) and the low Ca environment (Karnik et al., 2012). Following severing of the osphradial nerve, Dutch *Lymnaea* at both the behavioral and neurophysiological levels no longer respond to predator scent. Changes in both the neural and the behavioral response elicited by predator scent are also abolished by injection into the animal or superfusing the semi-intact preparation with a broad spectrum serotonin blocker, mianserin. Thus, it was concluded that predator detection is mediated by osphradial sensory elements that ultimately involve serotonin as the neurotransmitter to enhance memory formation (Il-Han et al., 2010). More recently, working in collaboration with Barb Sorg's lab, it has been shown that CE's enhancing effect on LTM formation involves "epigenetic" changes (i.e., DNA methylation or histone acetylation; Lukowiak et al., 2014). Epigenetics here is not used in the sense first described by Waddington.

The data presented here are all consistent with the hypothesis that predator detection significantly alters adaptive behavior in that LTM formation was significantly enhanced and various defensive behaviors were also altered. Finally, neural correlates of these stress-modulated behaviors were detected in RPeD1 (Orr & Lukowiak, 2008).

Once snails detect the predator "smell" (i.e., CE), they make a decision to alter their behavioral activities in a manner that would prove beneficial to survivorship (i.e., "keeping a low profile" or getting out of harm's way quicker). In other words, on detecting the presence of a predator, they make a risk assessment and take the appropriate actions to reduce that risk. This is not surprising given that predator detection via kairomones not only gives information regarding predator presence, but also potentially gives information regarding the proximity, physiological state, and even diet of potential predators (Dalesman et al., 2006; Kats & Dill, 1998; Wisenden, 2000). At the neuronal level, it has not yet been determined exactly how this is accomplished. While changes in RPeD1 activity do occur, I feel that this is not the "choice" making neuron. I take this position because RPeD1, while it is a necessary site for LTM formation of aerial respiratory behavior, does not

play a role in the mediation of other behaviors altered by predator detection. For example, in CE snails respond to a shadow stimulus in a significantly more robust manner. A shadow stimulus will only evoke the whole-animal withdrawal response when the snail detects the predator. Thus, I believe that higher order interneurons such as RPeD11 (Sunada et al., 2010), which do alter RPeD1 activity, are more likely to be part of the decision making process. However, we do have preliminary data that the whole-animal withdrawal neural circuit, of which RPeD11 is an integral member, is itself also under control by even other as yet unidentified "higher order" neurons. One of the laboratory's long-term goals is to identify the neuronal circuits and the mechanisms by which CE alters adaptive behaviors in the snail.

CE is detected by sensory neurons in the pneumostome and/or osphradial ganglion and this activity in the peripheral nervous system (PNS) modulates aerial respiratory behaviors. The interaction between the CNS and PNS of molluscs, especially as regards mediation of adaptive behaviors involving respiratory organs, is complicated, interesting, and controversial (Lukowiak & Colebrook, 1988; Peretz et al., 1976; Lukowiak & Jacklet, 1972).

With respect to the "Y–D law," any predator–prey encounter where the prey is aware of a predator presence, yet escapes the interaction with its life, should fall within a range close to the "optimal stress intensity" for memory formation and, therefore, should augment memory formation. Unfortunately, attempts to confirm this theory in lab-reared rodents experimentally have yielded mixed results (see Kim & Diamond, 2002; Shors, 2004).

Finally, we have looked at a number of different predators that are able to and in fact do prey on *Lymnaea* in the laboratory. What we have found, however, is that only the detection of sympatric predators causes enhancement of LTM formation. *Lymnaea* do not appear to posses the ability to either detect or "learn" to respond to cues released by a non-sympatric predator.

3.7 SOCIAL STRESSORS

In ponds that we collect snails from in Alberta, Saskatchewan, and the Somerset Levels in the United Kingdom (see below), I have noticed that, *Lymnaea* population density fluctuates widely within and between years. I have found broad fluctuations in density both between sampling periods (weeks apart) and within a site on a single day. Possible factors causing this would seem to be food availability or temperature gradients. In Alberta ponds, temperatures can fluctuate over 20°C during the course of a single day. Consequently, a snail may experience rapid changes in both its "social"

and physical context. Anecdotally, it was observed in the lab that having too many snails in an individual aquarium altered negatively the production of growth factors obtained when "cultured media" was produced for cell-culture experiments (Syed et al., 1990; 1992). This and other data suggested to us that *Lymnaea* might well be experiencing stress at high population density. Thus, it was not a leap in logic to ask whether crowding *Lymnaea* before or after operant conditioning training would in any way alter their ability to learn and form LTM.

Crowding for as little as 1 h is sufficient to block LTM but not ITM formation (de Caigny & Lukowiak, 2008). Interestingly, crowding for up to 23 h did not prevent snails from recalling an already formed LTM. As LTM requires both altered gene activity and new protein synthesis, whereas ITM requires only new protein synthesis, it is thought that crowding interferes with the necessary genomic activity to produce LTM in neurons, such as RPeD1, that are necessary for LTM formation. For crowding to block LTM formation it must occur either immediately before or after the training procedure and it can be as short as 1 h. If crowding occurs and then a period as short as 10 min is inserted before training the previous, crowding has no effect. These data are interpreted to mean that snails are able to quickly determine that a stress is no longer present and then modify their nervous system back to the original state such that LTM could be formed. At present, the sensory pathway by which snails sense they are crowded is unknown, but the osphradium does not mediate crowding (Dalesman & Lukowiak, 2011a). These data are consistent with the notion that memory modification is time dependent, that is, it does not occur instantaneously. It is also clear that the "factor" mediating the crowding effect on LTM formation is not water borne, as water from crowded snails does not block LTM formation. Presently, the working hypothesis is that it is the mucus of snails that is doing this and that physical interaction between snails (e.g., crawling over is other) is necessary for the crowding effect.

Whether chronic crowding (i.e., days to weeks) would have any different effect(s) on aerial respiration and/or memory formation remains to be determined. There is an interesting addendum to this story as very preliminary data suggest that strain differences play a role in how crowding alters LTM formation (Dalesman & Lukowiak, 2011b). It appears that if a different strain of *Lymnaea* is used to crowd our Dutch strain, LTM formation is unaffected. If these data are confirmed, it would mean that part of snail sociability is the ability to determine (although it's not clear how) how closely related is another snail.

3.8 LOW LEVELS OF [CA++] IN THE ENVIRONMENT

There has been a wide-spread decline of dissolved calcium in ponds and lakes across North America as a result of the leaching of calcium cations from the soil by acid rain (Dalesman & Lukowiak, 2010). This has had serious consequences on freshwater aquatic organisms, including snails. Snails rely on calcium for growth of their shell and are highly dependent on calcium for survival. In some respects snails such as *Lymnaea*, which are classified as being calciphile, meaning they must obtain the required calcium from pond water and not from food sources, are most affected by this situation (Dalesman & Lukowiak, 2010). Thus, reduced dissolved calcium in pond water is considered a major stressor for *Lymnaea.* In low-calcium pond water (<20 mg/l), we have found that snails have thinner shells, lay fewer eggs and have increased mortality compared to those maintained in our "high" calcium pond water (>80 mg/l). For example, the speed of locomotion was significantly reduced in snails exposed to low environmental calcium (20 mg/l) compared to snails exposed to the high (80 mg/l) calcium environment. The slower speed observed in the low calcium is due to increased metabolic demands on *Lymnaea*, where a greater proportion of energy is required for calcium acquisition, reducing energy available for motility. We further found that in low calcium, there was a significant increase in cutaneous respiration (i.e., oxygen consumption was higher) consistent with our hypothesis that the increased metabolic demands of calcium acquisition at low dissolved calcium levels reduce the energy available for locomotion. Thus, exposure to a low-calcium environment alters basic physiological and behavioral traits, respiration, and locomotion.

So, the question was asked what would maintaining snails in a low-calcium environment do to learning and LTM formation? Following even short periods of exposure to a low-calcium environment, *Lymnaea* were no longer able to form LTM, although they still demonstrated intermediate-term memory (ITM; Fig. 3.9). There was no alteration in the electrophysiological activity of RPeD1 in naive animals relative to calcium availability, but significant differences between calcium environments were apparent in RPeD1 24 h following training. That is, in our normal-calcium environment RPeD1 activity was significantly depressed relative to the naive state, in low calcium only a partial change in activity occurred, insufficient to result in a significant alteration in breathing behavior Thus this environmental stressor, similar to crowding, blocked LTM formation but not ITM (Dalesman et al., 2011a).

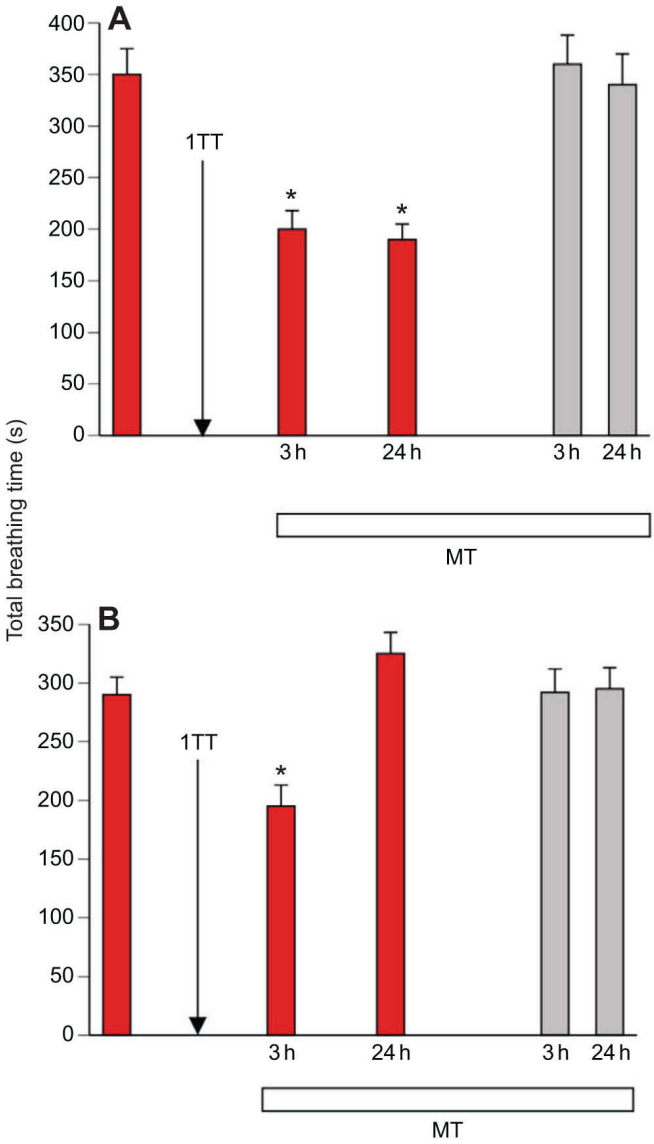

FIGURE 3.9 *Memory following a 1-week exposure to normal or low-calcium pond water environments*. Mean total breathing time in standard (A; 80 mg l/l) and low (B; 20 mg l/l) environmental calcium prior to and following one-trial conditioning (1TT, arrow). *Memory present ($P < 0.05$). The yoked control data (gray) show that only contingent pairing of the pneumostome opening attempt results in memory formation. As can be seen in both A and B, memory was present at 3 h, while LTM was only present in normal-calcium pond water (A).

Having seen that sensory neurons in the osphradium detected the toxic heavy metals in pond water and CE in pond water, it was hypothesized that neurons in the osphradium would also detect changes in the dissolved calcium concentration. Thus, the osphradial nerve was severed and now in low-calcium conditions, the osphradially cut group formed LTM, equivalent to animals maintained in our standard calcium PW. However, LTM was blocked in the sham-operated animals. Therefore, osphradial input is required to mediate the memory-blocking effect of low environmental calcium (Dalesman et al., 2011b; Karnik et al., 2012).

3.9 THERMAL STRESS

As mentioned above, *Lymnaea* are found in shallow, often stagnant environments (hence the name *stagnalis*) in northern temperate zones. Thus, *Lymnaea* encounter broad temperature fluctuations over the course of seasons and days. For example, on a summer day (~17 h sunlight) in Alberta shallow water bodies will frequently warm to around 30°C in mid-afternoon and cool down to the single digits on other days. We, therefore, hypothesized that temperature may act as an environmentally relevant stressor that will affect learning and memory formation in *Lymnaea* (Teskey et al., 2012).

Cooling snails (to 4°C) for an hour after training blocks the formation of both ITM and LTM (Sangha et al., 2003c,d). This blockage may in part be due to inhibiting new protein synthesis that is a necessary component for both ITM and LTM. However, cooling *Lymnaea* to 4°C for just 10 min then rapidly warming them up back to 23°C, either immediately prior to or following training, enhanced their ability to form LTM, most probably due to the stress elicited from the rapid temperature fluctuation (Martens et al., 2007).

As regards, higher temperatures, lab-reared Dutch snails that are exposed to a brief period of heat (1 h at 30°C), either before, during, or immediately after operant conditioning exhibited enhanced LTM formation (Fig. 3.10). These data are all consistent with the hypothesis that the memory enhancement seen is a result of the heat acting as a stressor on snails, which alters their ability to form LTM, following associative learning. Interestingly, maintaining Dutch snails for periods longer than a few hours most often resulted in their death. However, "wild" snails in ponds we collect from often have water temperatures above 30°C for many hours, yet those snails remain viable. Preliminary data on snails from such a pond indicate that a 1-h 30°C temperature step (from room temperature ~20°C) does not result in enhanced LTM.

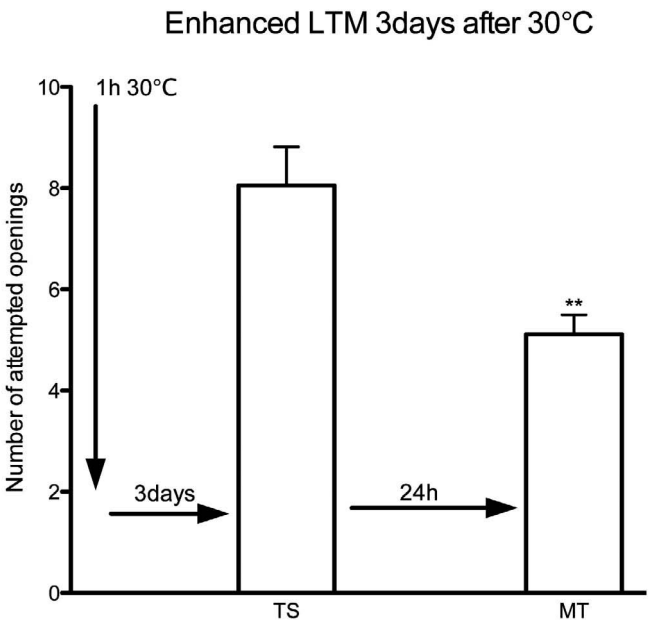

FIGURE 3.10 *The thermal stressor presented 3 days prior to training enhances LTM formation*. A naive cohort of snails ($N = 20$) were exposed to the thermal stressor for 1 h 3 days prior to receiving the single 0.5-h TS. Memory was tested 24 h later and, as can be seen, LTM is present. Thus, this is an example of a stressor enhancing LTM formation as it typically takes at least two training sessions to cause LTM formation. Data are means ± s.e.m. (**$P < 0.01$).

We hypothesize that in the lab-bred Dutch snails the 30°C thermal stressor session acts directly on neurons responsible for LTM formation and not via a peripheral sensory pathway. Severing of the osphradial nerve did not block the enhancing effect of the thermal stimulus (Teskey et al., 2012). In a series of new experiments done in collaboration with Ted Henry and Nicola Foster looking at heat shock proteins (HSPs; Foster et al., 2015), it was found that and both *HSP40* and *HSP70* were rapidly induced in the *Lymnaea* CNS within 30 min of the end of the application of the thermal stress. The induction of *HSP70* was greater than that of *HSP40*, with over four times the relative fold change These results demonstrate that exposure to an acute thermal stress of 30°C was sufficient to increase the synthesis of HSPs above constitutive levels. The observed increase in HSP synthesis suggests that these proteins may play a role in LTM formation in relation to a thermal stimulus. Thus, further investigation of the role of HSPs in memory formation is warranted.

3.10 INTERACTION BETWEEN STRESSORS

I have often stated that if I had to only deal with one stressor at a time, it would be heaven on earth. Sadly, the truth is that I and everyone else I know must deal with multiple stressors, sometimes simultaneously other times in a very tight temporal sequence. However, few if any studies combine stressors. This is much like the situation mentioned above where typically only a single toxic heavy metal is studied at one time; whereas in actuality the organism encounters a combined cocktail of heavy metals. Yet the results are very different. Thus I wished to begin studies on the combined effects of stressors experienced simultaneously and determine their combined effect on learning and memory formation. For example, can we predict how different stressors will interact to alter learning and memory based on their individual effects? To address this question, *Lymnaea* were exposed to combinations of different stressors, where we already knew what their individual effects on learning and memory were. The basic answer coming from these experiments is "anything can happen." That is, the interaction of stressors on memory formation exhibits emergent (i.e., unpredictable) properties.

One of our first experiments assessed the combined effects of CE and crowding (Dalesman & Lukowiak, 2011b). These stressors as outlined above have opposing effects on LTM formation (i.e., CE enhances LTM; whereas crowding blocks LTM). When *Lymnaea* were crowded immediately prior to training in CE and tested for LTM 24 h later, snails did not show memory. Thus, crowding effectively "trumped" the effects of CE. Recent (unpublished) work indicates that the interaction between CE and crowding may not be this simple and may be dependent on other environmental factors.

A second memory-blocking stressor is low environmental calcium (20 mg/l). Similar to crowding, low-calcium availability blocks the ability of the Dutch laboratory strain to form LTM, but they are still able to learn and form ITM. When we combined the low-calcium stressor with CE and then trained the snails they were able to form LTM lasting 24 h, showing that training in CE prevents the LTM-blocking effects due to low calcium. However, in normal-calcium pond water, the Dutch strain normally forms LTM lasting 24–48 h following two 0.5-h training sessions. However, with such a training procedure CE enhances that memory such that LTM persists for up to 8 days. But with the combined low calcium and CE stressors only a 24-h LTM was seen. Thus it appears in this case, the two stressors "cancel each other out." I have spent quite some time attempting to model the combination of these or any other stressors to alter the Y–D curve for the snail. It is unclear from the literature (very few papers are published using combinations of stressors and

studying the outcome on adaptive behavior). It is not clear to me that trying to combine the Y–D curve for each individual stressor yields the observed behavioral outcome.

A third environmental factor we decided to consider in combination with other environmental stressors is that of social isolation (Dalesman & Lukowiak, 2011b). This was particularly interesting to us because we were somewhat surprised that this "stressor" did not alter learning and memory formation by itself in Dutch snails. Socially isolated snails trained in the presence of CE did not demonstrate any difference relative to grouped animals; that is, CE still enhanced LTM in those snails. However, when we trained isolated *Lymnaea* in low-calcium pond water, which normally blocks LTM, these snails now formed LTM lasting 24 h. This was very unexpected.

We hypothesized this may be due to their change in their reproductive behavior as a result of social isolation. Dutch adult *Lymnaea* normally lay 100–300 eggs per week, and in each egg case, there is a deposit of calcium. Thus, egg laying has a substantial calcium cost. Isolated Dutch snails typically do not lay eggs, and it appears that they assume more "male" behavioral characteristics (i.e., they prefer to be a sperm donor). Thus, it is likely that in isolation, the low calcium does not create as great a stress as it does when snails are not in isolation.

While the combination of social isolation and low calcium produced an unexpected effect (i.e., LTM was formed) we encountered an even greater unexpected result when we combined three stressors (isolation, low calcium, and CE). Dutch snails exposed to these three stressors and then operantly conditioned demonstrated learning but not LTM (Dalesman & Lukowiak, 2011b). I cannot quite figure out why that happens as normally CE causes enhanced LTM, it cancels out the negative effects of low calcium (i.e., LTM still occurs although not enhanced LTM) and with socially isolated snails in low–calcium, LTM was still seen. It may be that within the context of being socially isolated CE has deleterious effects. If we attempt to interpret these data in respect to the Y–D law, we are perplexed as to why we are in the "poor" part of the curve. It may be that isolated snails no longer have a "herd-effect" to protect them. Thus, the stress resulting from predator detection is "different" than what we typically see when we train a group of snails in CE. We are attempting to explore whether the concept of herd protection occurs in *Lymnaea.*

Finally, we have the situation when we combine crowding with the low-calcium environment (remember each of these stressors individually blocks LTM formation but learning and ITM still occur), this combination of stressors blocked associative learning and all memory processes

(i.e., learning, short-term, ITM, and LTM; Dalesman et al., 2013). Thus, it appears that the effects of the stressors were additive.

Together, these data show that we cannot successfully predict the effects that combination of stressors have on learning and memory formation based on the effect that each stressor has when experienced alone (Lukowiak et al., 2014a). That is, combining stressors have emergent properties. This work provides an intriguing avenue for future exploration, and we already have a wealth of environmental stress combinations to test. Importantly, this raises our awareness and, it is hoped, that of others reading this work in detailing other environmental factors experienced by the animal when testing for stress effects. It is also interesting, although not discussed here, how such stressors may produce different effects on learning and memory if experienced if snails are in a different developmental stage (e.g., juvenile vs. young adult vs. older snails).

3.11 BIO-ACTIVE SUBSTANCES

In addition to examining the effects of different stressors on learning and memory, we have also investigated the effects of various bioactive substances on memory formation. I will mention one of these, epicatechin. I mention this one because we have used that substance in combination with a stressor and the results are interesting.

It has also been shown that a flavonoid, epicatechin, found in dark chocolate, green tea, and red wine enhances LTM formation in *Lymnaea* (Fruson et al., 2012). However, the enhancement brought about by epicatechin occurs via a different mechanism than that brought about by predator detection. That is, epicatechin's enhancing effect is not mediated via the osphradial nerve nor does it appear that serotonin plays a role in the enhancement process.

Exposing snails to epicatechin significantly enhanced the ability of snails to form LTM (Fruson et al., 2012). The epicatechin-enhanced LTM formed faster, persisted longer, and was more resistant to extinction. Moreover, epicatechin did not alter other behavioral tests (locomotion, baseline breathing rates, etc.) in *Lymnaea*, thus exemplifying specificity for the drug to interact with the memory-forming neuronal pathways (Fruson et al., 2012). Exposure of *Lymnaea* to epicatechin alters the ability of neurons to cause new protein synthesis and altered gene activity essential for LTM formation to occur. Exactly how epicatechin does this at the molecular level is not yet known.

As both the environment and life-style choices (e.g., diet) alter LTM formation, we questioned whether a substance such as epicatechin could overcome

the negative effects of a stressor that blocks LTM formation in *Lymnaea*. Because snails exposed to low-calcium pond water (20 mg/l) are not able to make new LTM (Dalesman et al., 2011a; Knezevic et al., 2011), would the addition of epicatechin to the low-calcium pond water reverse or mitigate the effects that low calcium has on memory? This is an important question to answer because it is unclear whether in the continued presence of a stressor, the effects of the stressor can be overcome by life-style choices such as diet.

The data obtained (Fig. 3.11) show that epicatechin has the ability to overcome the suppressive effects of a low environmental calcium stressor on LTM formation (Knezevic and Lukowiak, 2014). This is a significant finding because it shows that a food product has the ability to counteract the effects brought about by an ecologically relevant stressor on LTM formation. Epicatechin, which is present in dark chocolate, has been thought to improve various aspects of cognition in rodents and humans, with some reports suggesting that it has positive effects on mood (i.e., anxiolytic), which may be why chocolate is often consumed under emotional distress (Yamada et al., 2009; Nurk et al., 2009; Messaoudi et al., 2008; Parker et al., 2006; Nehlig, 2013). To our knowledge, however, the ability of epicatechin to overcome the negative cognitive effects of stress has not previously been demonstrated in a model system used to elucidate the causal mechanisms of learning and memory formation.

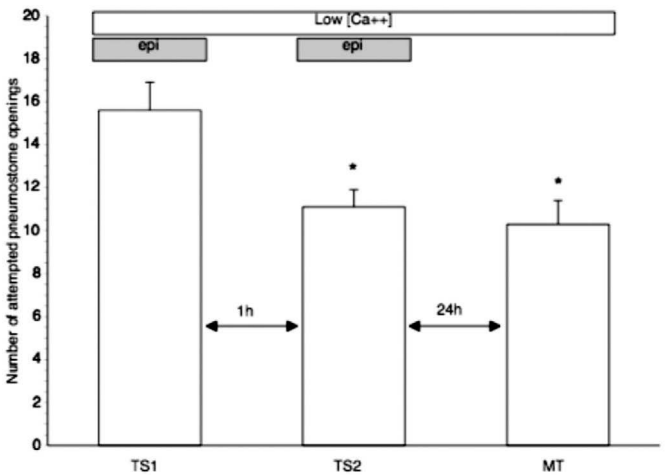

FIGURE 3.11 *Epicatechin overcomes the suppressive effect of low calcium on LTM formation*. Mean ± s.e.m. pneumostome openings for snails (N = 19) during two 0.5-h training sessions (TS1 and TS2) separated by 1 h, and a 0.5-h memory test session 24 h later. Snails were kept in low calcium (20 mg l/l) water throughout the experiment, and 15 mg l/l epicatechin was added during TS1 and TS2. *$P < 0.001$ compared with TS1.

3.12 STRAIN DIFFERENCES IN THE ABILITY OF *LYMNAEA* TO FORM LTM

In the summer of 2006, we trained freshly collected *Lymnaea* (Belly snails) and were surprised to find that they possessed superior LTM-forming capabilities compared to our Dutch inbreed snail-line. That is, they had the capability of forming LTM following a single 0.5-h training session. This led us to construct a number of different hypotheses that can be summarized as follows: (1) Lab-bred snails had become dumber due to being reared under laboratory conditions; and (2) Alberta snails were smarter than European snails. However, both those hypotheses were shown to be incorrect. For example, we found that freshly collected snails from other Alberta ponds did not display superior memory-forming capabilities; freshly collected snails from the Netherlands did not exhibit superior memory-forming capabilities, and finally Belly snails grown in the laboratory conditions for a number of generations continued to exhibit superior memory-forming ability (Orr et al., 2008, 2009a,b). Moreover, we also found that *Lymnaea* from some select ponds in the United Kingdom (Dalesman et al., 2011b) as well as other ponds in Alberta exhibited enhanced memory-forming capabilities. We thus concluded that there are strain differences (i.e., snails from different ponds) in memory-forming capabilities. Simply put, snails from one pond are "smarter" than those from another pond, and the ponds can be as close to each other as 500 m; defining "smart" as having the ability to form LTM following a single 0.5-h training session.

A question that we posed was "What is different?" between a "smart" snail and an "average" snail. One thing we found was that a neuron, RPeD1 that is a necessary site for LTM formation is in a different "state" between the two strains (Braun et al., 2012). In the smart strain, the excitability of RPeD1 in naive preparations (i.e., not trained) is significantly lower than in the preparations from naive average snails. Interestingly, the level of excitability of RPeD1 in the smart naive preparations is very similar to that seen in an average preparation that has received a single 0.5-h training session. In other words, it appears that RPeD1 in smart snails is "primed" for memory formation. At this time, we do not know how this is achieved or maintained.

In keeping with the focus of this chapter, our present working hypothesis is that "smart" snails are more easily stressed than our "typical, average" snails. The preliminary data that I have so far gathered suggests that the "smart" snails respond to certain environmentally relevant stressors differently than do average snails, especially when combinations of stressors are used. For example, smart snails from the United Kingdom respond

to predator scent in a manner similar to the way that "average" snails do. That is, predator detection further enhances their already superior memory-forming capabilities. However, social isolation which does not alter the memory-forming capabilities of average snails, blocks LTM formation in these "smart" snails. However, much more experimentation must be undertaken before we can confidently assert that smart snails have a greater difficulty in handling stress.

3.13 FORGETTING IN SNAILS

Basically, there are two dominant theories of forgetting: (1) decay in some part of the "memory trace;" and (2) retrograde interference (Wixted, 2004; Sangha et al., 2005). Thorndike (1913; the law of disuse) hypothesized that memory decays, unless regularly used, similar to the way leg muscle atrophy occurs when a person is in a cast. Historically, decay (Woodworth, 1929; Gates, 1930) was assumed to be a passive process; but is now viewed as an active process (Hardt et al., 2013), active in the sense as loss of the "memory-substrate (e.g., atypical protein kinase C isoform M-zeta [PKMz]). For example, transiently inhibiting PKMz activity often abolishes fully established memories (Serrano et al., 2008; Shema et al., 2009).

Retrograde interference (RI) was also hypothesized to cause forgetting. That is, learning and forming memory of something new (an active process) conflicts with the established memory (Jenkins & Dallenbach, 1924). Both human (nonsense syllables; sleep vs. awake and the amount of forgetting) and invertebrate data (forcing cockroaches to move after conditioning decreased retention of the learned response; Minami & Dallenbach, 1946) lend support for this hypothesis.

In *Lymnaea*, we have performed a number of different experiments to determine how forgetting occurs. It is important to keep in mind that forgetting allows an animal to react more appropriately to current conditions (a positive consequence), rather than continuing to exhibit a previously learned, possibly maladaptive behavior based on previous experience. Our initial *Lymnaea* data readily supported the RI hypothesis, but more recent data (Lukowiak et al., 2014b) are more supportive of the active-decay hypothesis.

A typical forgetting curve is shown in Figure 3.12. As can be seen, following conditioning forgetting occurred approximately five days after training. We thus concluded that this training procedure was sufficient to result in a memory that could persist for about five days.

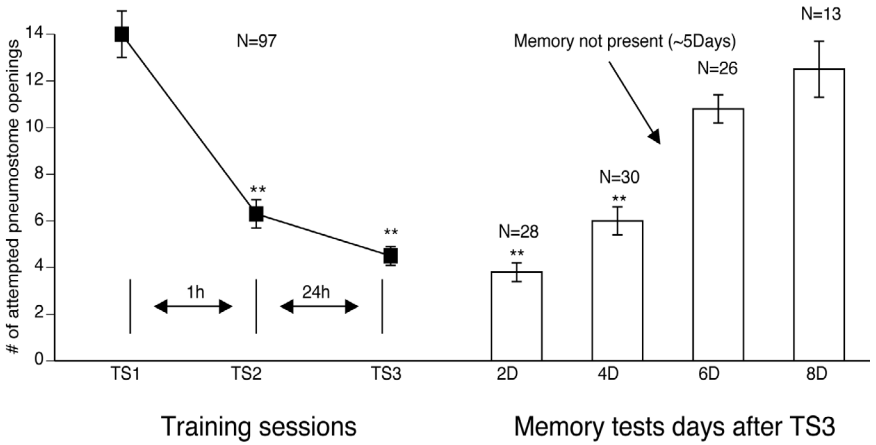

FIGURE 3.12 *Typical forgetting curve in Lymnaea.* A naive cohort (N = 97) of snails received two 0.5-h training sessions (TS1 and TS2) separated by a 1-h interval on day 1. They then received a third 0.5-h training session (TS3) 24 h later. Snails were randomly sampled to test for memory 2 days (2D, N = 28), 4 days (N = 30), 6 days (N = 26), and 8 days (N = 13) after TS3. Snails tested 2 and 4 days after TS3 met the criteria for LTM. Snails tested on days 6 and 8 after TS3 did not meet the criteria for LTM. Thus memory was forgotten by day 5.

An experimental result that appeared to support the RI hypothesis was a submersion experiment in which trained snails that could not come to surface to perform aerial respiration did not forget as fast as snails maintained in the same aquarium but which could perform aerial respiration (Fig. 3.13). That is, snails prevented from performing aerial respiration had their memory extended. This experiment complemented the experiment performed in Miami and Dallenbach in 1946, on cockroaches, which historically supported the RI hypothesis. It was shown in that study that after intervals of inactivity in which the cockroaches were immobilized in small boxes filled with tissue paper, memory retention and relearning were far superior to those insects that received corresponding intervals of normal rest. In addition, this same study illustrated that forced activity following learning led to savings scores that were much poorer than after corresponding intervals of normal rest. That study and the previous study in humans (Jenkins & Dallenbach, 1924) demonstrated that it is not the passage of time that results in memory decay; rather it is a result of interference from new events (Minami & Dallenbach, 1946).

So, we went back and performed the submersion experiments again, but this time we injected into the snails 5-AZA, a DNA methyltransferase inhibitor (DNMT), which blocks DNA methylation. It is thought that DNA methylation plays a prominent role in LTM formation (Zovkic et al.,

2013). We trained snails in a similar manner to those in Figure 3.13, but now injected half of the snails with saline and the other half with 5-AZA. All snails were placed under the barrier. We found that snails injected with saline had extended LTM, in agreement with our previous findings (Sangha et al., 2003e, 2005). However, snails injected with 5-AZA did not exhibit LTM (see Fig. 3.4, Lukowiak et al., 2014). We concluded in that study that the persistent memory (i.e., delaying forgetting) induced by submersion in *Lymnaea* is blocked by the DNMT inhibitor, 5-AZA, given prior to training. Thus, it appears that DNMTs play an important role in enhanced memory persistence. It is possible that the DNMTs play a role in maintaining the substrate necessary for LTM support and thus interference with this substrate by 5-AZA injection causes forgetting. Thus, these data appear to favor the decay hypothesis.

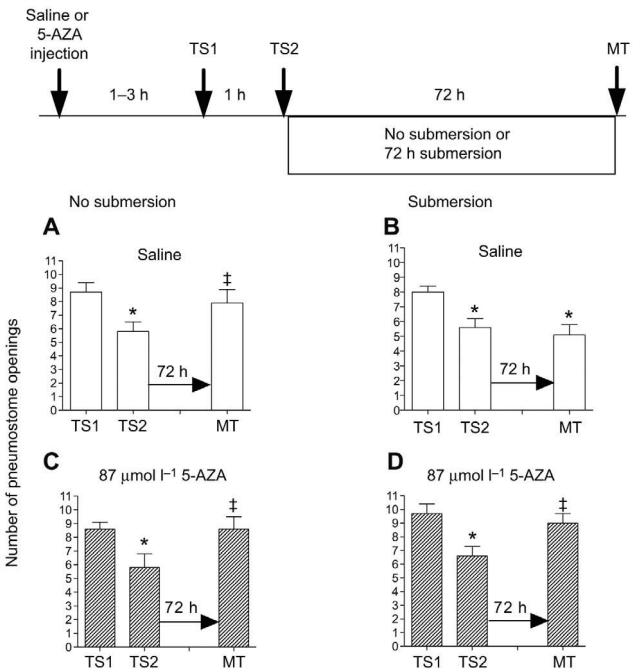

FIGURE 3.13 *Submersion of L. stagnalis extends memory that is prevented by 5-AZA treatment*. (A–D) Mean ± s.e.m. number of pneumostome openings for the following treatments: (A) saline injection 1–3 h prior to TS1, no submersion (N = 18); (B) saline injection 1–3 h prior to TS1, 72 h submersion (N = 30); (C) 87 µmol/l 5-AZA injection 1–3 h prior to TS1, no submersion (N = 17); (D) 87 µmol/l 5-AZA injection 1–3 h prior to TS1, 72 h submersion (N = 23). Injection of 5-AZA prevented the memory-enhancing effects of submersion on memory measured 72 h later. Timeline for treatment is shown at the top: *P < 0.05, comparing with TS1; ‡P < 0.05, comparing MT with TS2.

3.14 OVERALL SUMMARY

Memory formation is a dynamic process that can be modulated, for example, by stressful events occurring during, before or after the learning and memory consolidation processes (Lukowiak et al., 2014a). The effect that stressors have on memory formation varies greatly depending on the type of stress experienced, the level of stress applied and the timing of the stressful event relative to the learning process, and can either enhance or block memory formation. What has not often been acknowledged or rigorously tested is the stressors may also be altering the forgetting process. It is possible that the stressors that "block" memory formation are actually enhancing the "forgetting" processes. It will be interesting to attempt to design experiments that address this question in the future. Stress is often seen as being detrimental to the fitness of the organism. However, I do not take that view here as stress can improve learning and memory formation. It may be that such a "positive" view of stress needs to be embraced by researchers studying stressors that are ecologically relevant to organisms that are either in their "natural" environment or at least in an "enriched environment" rather than the manner in which most research organisms are maintained.

KEYWORDS

- **modern neuroscience**
- ***Lymnaea***
- **stressors**
- **maladaptations**
- **snails**

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CHAPTER 4

LEARNING AND MEMORY IN THE LIVING FOSSIL, CHAMBERED *NAUTILUS*

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CONTENTS

ABSTRACT

We study learning in the ancient cephalopod *Nautilus pompilius* as a way of understanding how behavioral and neural complexity may have evolved in its more derived relatives, the coleoid cephalopods (octopuses, cuttlefish, and squid), whose brains are heavily invested in learning and memory. Nautilus retains a relatively primitive brain whereas coleoid brains are among the most complex of all the invertebrates, containing several discrete lobes dedicated to learning and memory storage—the vertical and frontal lobe complexes. The nautilus CNS lacks known homologues or analogues of these regions, and instead the brain more likely approximates those of the ancestors of the two extant subclasses. Nautilus is therefore uniquely placed in this lineage for studies of the evolution of complex behavior, including learning and memory. We identify and characterize learning, memory formation, composition, and retrieval in Nautilus through controlled classical conditioning studies and also behavioral assays with free-moving animals. In studies of classical conditioning where a light predicted the reward of food, nautiluses learned rapidly, expressing temporally separated short-term and long-term memory (STM and LTM). STM expression in Nautilus was similar to results from coleoids, but LTM was of shorter duration, perhaps due the lack of dedicated learning and memory centers found in more derived cephalopods. Spatial memory studies in complex mazes have revealed far more complex information processing than the "primitive brain" of nautiluses might predict. By providing increasingly complex cue arrays to be learned for spatial navigation to a goal, we investigated how nautiluses perform in complex cognitive tasks similar to those tested in coleoids. Nautiluses were trained to escape from a shallow-water, brightly lit arena (aversive) into deep, dark water (reward) via a hole in the arena—with the aid of either a beacon proximate to the goal or multiple visual cues in the arena located proximate and distant from the escape point. We found evidence for rapid learning, memory of long duration, and dynamic switching among learned navigational tactics when the cue arrays were altered. Current studies focusing on what information is retained, processed, and used to navigate reveal that nautiluses can simultaneously learn and remember net displacement (dead reckoning of turns and distance traveled) and beacon information (both associative and spatial) proximate to the escape point as they navigate toward the goal location.

4.1 GENERAL INTRODUCTION

Cephalopods (Subclasses Coleoidea and Nautiloidea) are an exceptional taxon for the comparative study of learning and memory. They are well known for their complex behaviors and brains—brains that share convergent features with vertebrate neural organization. There are substantial differences in the natural histories of cephalopods that are thought to contribute to the variety in their behaviors, allowing researchers to investigate the role ecology plays in shaping cognition. Importantly, this lineage contains a living representative of the ancestral condition, the Chambered *Nautilus*, providing an opportunity to examine historical evolutionary contributions to complex behavior, including learning, memory, and cognition, in this class as a whole. An understanding of the behavior of nautiluses and supporting neural structures allows researchers to identify adaptive pressures that lead to fast and flexible processing in brains in this lineage as a whole.

Cephalopods have representatives in almost every ecological niche in the sea—benthic, pelagic to abyssal, deep-ocean regions. Familiar cephalopods include squid, cuttlefish, octopus, and nautiluses. At present, at least 700 species of living cephalopods are recognized (Jereb & Roper, 2005; Nesis, 1987; Norman, 2000). All living species belong to either the subclass Coleoidea (*reduced or absent shell*) including squids, octopuses, cuttlefishes (700–800 species) (Jereb & Roper, 2005; Norman, 2000) or the subclass Nautiloidea (*external, chambered shell*) an ancient group limited to 2 genera and perhaps 5 species (Bonnaud et al., 2004; Saunders & Landman, 1987; Ward & Saunders, 1997). The chambered nautilus, *Nautilus pompilius* L. is one of only a few extant representatives of a shelled cephalopod lineage that flourished between 450 and 60 million years ago (Ward, 1987; Ward & Saunders, 1997; Wray et al., 1995). Nautiloids lack many of the derived features that characterize the more modern coleoids, which have numerous adaptations to support their fast, predatory lifestyle (Aronson, 1991; Packard, 1972) including lensed eyes and complex brains that contain dedicated lobes for learning and memory. Contemporary nautiloids are comparably pleisiomorphic. Nautilid eyes have no lenses, they lack chromatophores, and they retain many primitive anatomical features (Sasaki et al., 2010; Shigeno et al., 2008, 2010; Tanabe et al., 2010). Recent embryological evidence (Shigeno et al., 2008) suggests that nautilus retains features of the neural arrangement of its ancestors, a lineage that goes back millions of years. Thus, the study of modern nautiloid behavior and its neural support is an important component in identifying shared and divergent adaptive constraints on cognition in the class as a whole.

4.2 EVOLUTION

The divergent modern lifestyles of nautilids and coleoids are thought to have arisen during a period of intense competition with bony fishes in the Mesozoic, canalizing these two lineages into entirely different niches: "*live fast, die young*" (coleoids) versus *"live slow, die old"* (nautilids) (Aronson, 1991; Chamberlain, 1990, 1993; Landman & Cochran, 1987; Packard, 1972). The cephalopod fossil record supports the view that the ancestors of coleoids separated about 380 MYA, radiating into three orders and hundreds of species (Anderson, 2000; Bonnaud et al., 2006; Carlini et al., 2001; Clarke, 1998a,b; Packard, 1972; Strugnell et al., 2005, 2006; Teichert & Matsumoto, 1987; Teichert, 1988; Young & Vecchione, 1996; Young et al., 1998). The coleoid expansion in direct competition with fishes is thought to be responsible for the evolution of their vertebrate-like eye, their speed, and their complex behavior (Aronson, 1991; Grasso & Basil, 2009; Hanlon & Messenger, 1996; Hochner et al., 2006; Packard, 1972). Coleoids are thought to have developed their fast-and-flexible cognitive abilities as a means to hide and escape from their fast, visual predators. Nautilids, on the other hand, moved to deep-water niches, avoiding direct competition with fishes and adopted a slow-moving, scavenging lifestyle (Chamberlain, 1993; Packard, 1972). Today, they are represented by only two genera, *Nautilus* and *Allonautilus* (Bonnaud et al., 2004; Saunders & Landman, 1987; Ward & Saunders, 1997). It has long been assumed that their slower lifestyle would not support much in the way of complex behavior including cognition. However, they are long lived, and while they occupy a different niche from coleoids, they too must find food and avoid predation without the complex predatory (speed, poison) and defensive (crypsis) tools of the coleoids. Learning and returning to reliable foraging and hiding places along the coral reef is likely to be adaptive to nautiluses. The modern behaviors of nautiluses, including learning, memory, and spatial cognition, supports the notion that the brain of cephalopods prior to the fish expansion may already have been capable of supporting complex behaviors (Basil et al., 2012; Basil & Crook, 2013; Grasso & Basil, 2009).

4.3 ECOLOGY

To place the behavior of modern nautiluses into an adaptive framework, it is necessary to have an understanding of their sensory ecology. *Nautilus* lives among the coral reefs of the Indo-Pacific, surviving in dimly lit waters for most of its life at depths up to 300 m. They undergo daily migrations moving

along the steep contours of their coral-reef habitat, traveling to shallower, warmer waters (75 m) to forage amidst deeper corals during night-time hours, with bouts of vertical excursions throughout the day (Carlson et al., 1984; Dunstan et al., 2010, 2011; Hanlon & Messenger, 1996; Saunders, 1985; Saunders & Landman, 1987; Saunders & Ward, 1987; Ward et al., 1984; Ward, 1987; Wells et al., 1992). At these depths, nautiluses are likely to rely heavily upon chemical and tactile information to solve important problems, especially since they are primarily nocturnal or crepuscular (Basil et al., pers. Obs). The structure of the large but primitive pinhole eye of *Nautilus* (Fig. 4.1) suggests vision may not be the most essential sensory system for foraging (Messenger, 1991) as it is for other modern cephalopods (Hanlon & Messenger, 1996; Messenger, 1971; Muntz & Raj, 1984; Muntz, 1986, 1987a,b; O'Dor et al., 1993) which have well-developed eyes, complete with a lens (Budelmann, 1994). However, the eye of *Nautilus* is extremely large to capture as much light as possible in its dim environment and is tuned to wavelengths (470 nm) commonly produced by bioluminescent organisms.

FIGURE 4.1 *Nautilus pompilius, external anatomy*. External shell is chambered, with the animal residing in the front chamber only. Pin-hole camera eye is evident below the hood and posterior to the tentacles. Up to 90 distinct tentacles can be retracted into sheaths when at rest. Olfactory rhinophores lie, one below each eye, exposed to the exterior by a narrow pore (Photo J. Basil).

They are likely to use vision to help them track the substrate as they move along the coral reefs, so they do not stray into open waters. In the laboratory, nautiluses are indeed attracted to light (Muntz, 1986, 1987a,b) and nautiluses can use visual stimuli to make orientation and navigation decisions (Basil et al., 2012; Crook, 2008; Crook & Basil, 2008b, 2013; Crook et al., 2009). Vision may therefore play a greater role than previously thought in their natural habitat.

4.4 SENSORY BIOLOGY AND BEHAVIOR

Nautiluses seem to be specialized for a "smelling and groping" lifestyle along the coral reefs where they live (Hanlon & Messenger, 1996; Saunders, 1985). They bear a pair of olfactory organs (rhinophores), one located below each eye and open to the exterior by a narrow pore (Barber & Wright, 1969; Ruth et al., 2002). The rhinophores of *Nautilus* are similar to the olfactory organs in *Octopus* and other cephalopods but are significantly larger, as are the olfactory lobes in the brain of *Nautilus* to which they project (Young, 1965a). The epithelium of their rhinophores possesses cells that are similar in ultrastructure to chemoreceptors located in the sucker of *Octopus*, the olfactory structure of squids, and the lip of *Sepia* (Emery, 1975,; Graziadei, 1964; Gilly & Lucero, 1992; Lucero et al., 1992; Ruth et al., 2002). Odor is likely an important source of information for nautiluses as they ascend and descend the reefs, staying along the slopes to avoid open waters.. Nautiluses are adept at detecting and tracking odors—at distances of at least 10 m in the laboratory (Bidder, 1962; Basil et al., 2000). Following turbulent patterns of odor in plumes emanating from a distant odor source, nautiluses rely upon their paired rhinophores to interpret directional information within the plume to find the source (Basil et al., 2000, 2005). Initial detection of odor, on the other hand, is probably accomplished by both the rhinophores and the tentacles (Basil et al., 2005).

Nautilus tentacles are arranged in a large array of up to 90 that retract into sheaths when the animals are at rest and extend when the animals are aroused (Bidder, 1962; Basil et al., 2000, 2005; Ruth et al., 2002). When nautiluses come into contact with odor, they extend their tentacles in a stereotyped posture ("cone of search") and swim toward the source of the odor (Bidder, 1962; Basil et al., 2000). Tentacles fall into both anatomical and functional groups: digital tentacles (medial and lateral) and pre- and postocular tentacles (Bidder, 1962; Fukuda, 1980; Kier, 1987; Willey, 1902). Cells found in pre and postocular tentacles, the lateral-digital tentacles, and in the olfactory

pits of the rhinophores are well suited for far-field chemoreception, including long tufted ciliated cells, characteristic of those modified for long-distance chemoreception (Ruth et al., 2002). Other cell types (near the base of the rhinophores) are primarily mechanosensory, while the medial-digital tentacles carry receptors that are used for contact chemoreception (Ruth et al., 2002). Stimulation of the digital tentacles initiates near-field food-searching behavior: nautiluses extend their longer digital tentacles, swim toward the substrate, and then probe the substrate with their medial-lateral tentacles (digging, etc.; Basil et al., 2005).

In naturalistic studies, nautiluses effectively detect, track using the characteristic "cone of search" posture, locate, and then dig for food items buried completely in sand, using their tentacles and siphon to excavate the item from the substrate (Barord et al., in preparation; Barord, 2015 PhD thesis). The sequence of foraging behaviors, cone of search, tracking, skimming the substrate with the long digital tentacles, excavation, and removal of food using the medial digital tentacles, has been documented both in the laboratory under controlled conditions and also in the field using remote underwater camera observations of nautiluses attracted to bait (Barord et al., in preparation; Barord, 2015). This stereotyped sequence of events, with nautiluses adopting the cone of search up to 10 m away in the laboratory and 5 m away in the field (Barord et al., in preparation; Barord, 2015) is likely adaptive to an opportunistic scavenger that must track any item of food it can find. Once found in the substrate via far-field rhinophore detection and near field digital tentacle search, it may be that the nautilus then decides whether to accept the food item or not using the medial digital tentacles.

Nautiluses also use odor to discriminate between males and females (Basil et al., 2002; Westermann, 2001; Westermann & Beuerlein, 2005). In a choice-maze experiment, males were attracted to conspecific odor of any kind (male or female) while females actively avoided female odor, preferring male odor or even a blank stimulus instead. Westermann and Beuerlein (2005) have demonstrated that a substance found in the rectal gland of male and female nautiluses is the key to their ability to discriminate between the sexes. Clearly odor and tactile stimuli are of great importance to these animals in their deep-sea, coral-reef habitat where little light penetrates.

Nautiluses may also benefit from the ability to detect mechanical and acoustical stimuli in their coral-reef hugging habitat. Nautiluses do indeed respond to underwater acoustical stimuli, slowing their ventilations in the presence of vibrations (Soucier & Basil, 2008). Large vibrations at positions close to the animal had the strongest effect on the animals' rate of breathing. As nautiluses live in dark conditions where visual information is often

limited, they may benefit from including vibrations in the suite of stimuli to which they can respond. Predators may create pressure and acoustic disturbances as they approach a nautilus—slowing their ventilation may make it less likely they will be detected by the oncoming predator. Potential prey items (e.g., snapping shrimp) may also emit acoustic signals detected by a foraging nautilus.

The statocysts of nautiluses provide information about angular acceleration that the animal may use to make decisions about movements in space (Budelmann, 1976; Budelmann and Tu, 1997; Neumeister and Budelmann, 1997; Soucier & Basil, 2008; Young, 1988). One alternative is that nautiluses to utilize "path integration," "dead reckoning," or "route memory" (Gallistel, 1993, in vertebrates) to navigate directly back to a goal by continuously keeping track of their movements in space as they orient on the outbound journey. Karson et al. (2003) and Alves et al. (2007b) demonstrated that cuttlefishes can use either route memory or place behavior to find a goal location (a dark spot with a sandy bottom). Nautiluses are therefore capable of detecting and using detailed information on many scales from a variety of environmental stimuli. Learning to associate these stimuli may be advantageous for an animal with few defenses living in a complex deep-sea environment. Nautiloid longevity, unique coral-reef-hugging niche, large foraging range and susceptibility to predation (see Hanlon & Messenger, 1996) are likely to promote learning, memory, and spatial cognition, including supporting neural structures, despite their differences from coleoids (Basil et al., 2012).

4.5 NEUROETHOLOGY

Learning, memory, and spatial cognition require neural support. The nervous system of cephalopod molluscs is one of the largest and most complex among invertebrates. Far more is known about the anatomy and function of the coleoid brain than that of nautiloids. Coleoid cephalopods have developed complex brains that include *dedicated learning and memory centers* (Fig. 4.2, *Vertical Lobe and Superior frontal lobe*; Agin et al., 2003; Boycott & Young, 1955; Dickel et al., 2001; Graindorge et al., 2006; Hochner et al., 2006; Sanders, 1970, 1975; Shomrat et al., 2008; Young, 1960a, 1965b, 1961, 1965b,c, 1971, 1988, 1991). Coleoid brains have been proposed to converge with the vertebrate brain in organization (Hochner et al., 2006, *review*; Boyle, 1986; Kandel, 1976; Nixon & Young, 2003; Wells, 1978; Young, 1991; Young, 1965c, 1971), with shorter connectives and synaptic

morphology that supports fast and flexible neural processing (Hochner et al., 2006). When the vertical lobe is lesioned in octopuses and cuttlefishes, learning and memory of certain stimuli are abolished (Boycott & Young, 1955; Boycott, 1961; Graindorge et al., 2006; Maldonado, 1965; Wells, 1978; Young, 1991). Even observational learning (Fiorito & Scotto, 1992) requires the presence of the vertical lobe (Fiorito & Chichery, 1995). Ecological differences are reflected as variation in both memory abilities and supporting neural systems among coleoids. For instance, many octopods have a well developed chemotactile memory system (*Subfrontal lobe*) suited for their benthic habitat. This area of the brain is absent or poorly developed in pelagic octopods which do not display complex chemotactile learning and memory (Young, 1964). Teuthids too have a poorly developed subfrontal lobe, while cuttlefish lie somewhere in between octopods and squids. Both groups have chemotactile habits that correspond to these differences in neural lobe development (Hanlon & Messenger, 1996).

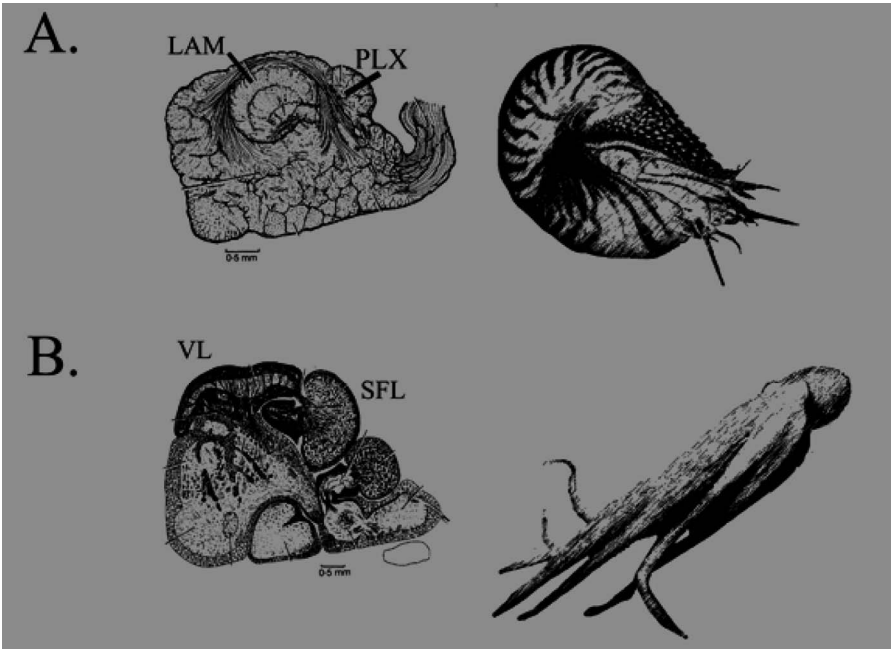

FIGURE 4.2 *Comparison of Nautilus and Octopus brains.* VL: Vertical lobe complex; SFL: superior frontal lobe complex, LAM: laminated area of olfactory lobe, PLX: plexiform layer. (Modified from Maddock & Young, 1987; this figure reproduced with permission from S. Karger AG, Medical and Scientific Publishers, Basel. *Grasso, F. W.; Basil, J. A. Brain Behav. Evol.* **2009,** *74*, 231–245.) (A) Nautiloids and (B) octopods. The LAM of the olfactory lobe in nautiloids lies in a similar position to the VL in coleoids.

Despite their demonstrated learning, memory and flexible spatial abilities, the brain of nautiluses is considerably different from those of coleoids. They have a ring-shaped brain with little internal differentiation (Young, 1965a; Fig. 4.2), possessing only 13 lobes in contrast to the 38 found in squid and 40 in *Octopus* (Hochner et al., 2006; Young, 1965c, 1971), although it is significantly larger and more developed than the brains of other molluscs (*Aplysia*: Kandel and Schwartz, 1982; Carew and Sahley, 1986; Baxter and Byrne, 2006; *Hermissenda*: Alkon et al., 1990; Blackwell, 2006; *Snails*: Gelperin and Cullgan, 1984; *Lymnaea:* Sahley and Crow, 1998; Lukowiak et al., 2000; Syed et al., 1990; Kemenes and Benjamin, 2009). Embryological evidence (Shigeno et al., 2007) suggests that nautilus retains features of the neural arrangement of its ancestors. Specifically, they lack any defined structures analogous to the vertical or superior frontal lobe complexes of coleoids (e.g., Boycott & Young, 1955; Young, 1960a,b, 1971). It is not surprising that their brain has a greatly expanded olfactory lobe, with their reliance on olfactory stimuli. One possible analog to coleoid learning-and-memory centers is the laminated area of the olfactory lobe and surrounding cerebral nerve cord, located in a similar region in nautilus as the vertical lobe in coleoids (Young, 1965a). This laminated area receives input from the plexiform region, which integrates information from the visual and tactile systems of the animal (Young, 1965a). Based on its location, structure and multisensory inputs, the plexiform region may be compared to the frontal and vertical lobes of coleoids. It may be that the basic neural arrangement of nautiloids is conserved although greatly expanded in coleoids or that nautiluses have co-opted another area of the brain entirely to support their learning.

4.6 LEARNING

4.6.1 CLASSICAL CONDITIONING

Given their natural history and ecological pressures, nautiluses are likely to learn to associate two stimuli that commonly occur together to find food, mates, and avoid predators (Basil et al., 2012; for example, light and odor emanating from a decaying prey item covered in bioluminescent bacteria). Our laboratory has taken advantage of a robust behavior in nautiluses (tentacle extension when they are aroused in some way, as their tentacles can be retracted into sheaths) to reliably classically condition them to two associated naturalistic stimuli, with tentacle extension as a measure of the learned response (Barord & Basil, 2014; Crook & Basil, 2008a,b).

In Pavlovian conditioning, an initially neutral CS (light) becomes associated with the US and subsequently elicits a response on its own (the CR) previously only initiated by the US (Bitterman et al., 1983; Pavlov, 1927). Models of Pavlovian conditioning (e.g., Pavlov, 1927; Rescorla & Wagner, 1972) posit that (1) learning can be thought of in terms of a change in association strength between the CS and an aspect of the US and that (2) variation in stimulus salience and CS/US timing can contribute to changes in associative strength. A hallmark of classical conditioning is that the CS must precede the US. The period between them should not be too great or the associative strength will lessen. This is true across a variety of animal phyla, including nautiluses (e.g., *bees*, Bitterman et al., 1983; Hammer and Menzel, 1995; *Vertebrates*: Rescorla & Wagner, 1972).

Nautiluses can be classically conditioned easily: forming an association with a light pulse (CS) and a food-odor reward (US) and responding (CR) by extending their tentacles (tentacle extension response [TER], analogous to the proboscis-extension response (PER) in bees, Bitterman et al., 1983; Smith, 1997; Smith & Cobey, 1994). In this paradigm, they produce a biphasic memory curve (Crook & Basil, 2008a,b) similar to that of cuttlefishes (Agin et al., 1998, 2006a; Messenger, 1973; Wells & Young, 1970). Excitatory conditioning is thought to be pairing dependent, with the contingency that the CS must precede the US for conditioning to occur. To determine that excitatory conditioning is occurring in nautiluses, we also assessed learning in a group receiving CS–US pairings with an explicitly unpaired group where the US was presented at random intervals relative to the CS. It differed from the experimental group only in the temporal relationship between CS and US. We then tested for retention of this association in both groups at various retention intervals (RIs) after training.

In these associative-conditioning experiments, we used a within-subject design with each animal serving as its own control to control for individual variation among wild-caught animals. Naïve adult animals were randomly assigned to receive the *CS+* training or *Explicitly Unpaired* for each memory RI. RIs included 3-min, 30-min, 1-h, 6-h, 12-h, and 24-h post-training and were tested on separate occasions in a counterbalanced design to control for order effects (Fig. 4.3). Experiments took place during the natural activity period for these animals (nocturnal, Barord & Basil, 2014; O'Dor et al., 1993; but see Dunstan et al., 2010) in an experimental tank with a harness to hold the shell of each animal while the hood and tentacles remained free to move.

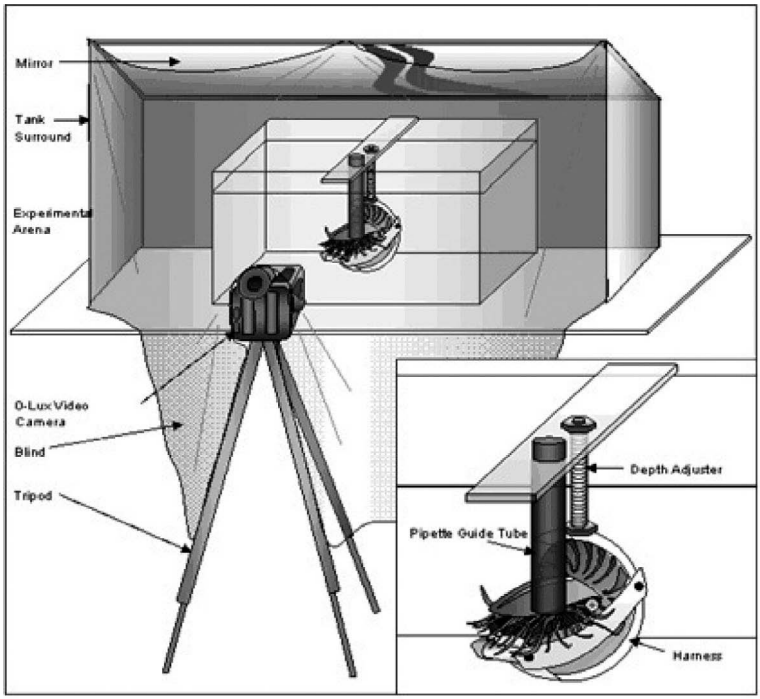

FIGURE 4.3 *Pavlovian conditioning apparatus* (This figure is reproduced with permission from Company of Biologists, Ltd. Crook, R.; Basil, J. *J. Exp. Biol.* **2008a,** *211*, *1992–1998*.) A small light is flashed onto the back of the tank (CS) to create a dispersed 470-nm illumination of the tank for 1 s. The harness holding the shell (inset) aids in controlled delivery of the odor reward and are free to respond to either of the stimuli.

In excitatory conditioning, or CS+ trials, a food stimulus (US) was paired with a blue light (CS, 470 nm to match the natural spectral sensitivity of the nautiluses) flashed for 500 ms against the back of the tank. Within 1 s of the flash, the odor was released directly onto the rhinophore of the nautilus producing a strong conditioned response (CR), or tentacle extension (Crook and Basil, 2008a,b). Retention-interval testing began after the last training trial to map the time course of short-term (STM) and long-term (LTM) memory. If the animal had learned the association, we expected to see TER to the CS (light) alone after training, as it would have salient predictive power of the ensuing US (odor). For each RI, animals were tested with a single unrewarded presentation of the CS (light) and the proportion of TER was measured.

Like other cephalopods, nautiluses exhibited a biphasic learning curve with temporally separate STM and LTM stores (Fig. 4.4) as found

in cuttlefishes (Agin et al., 2006a; Messenger, 1973). There was a peak in response at about 1 h, a decline between hours 1 and 6 (likely when STM is consolidated into LTM, see below), and then a rise to about 12 h, with LTM declining until 24 h. Retention in this simple task did not last much longer than 12 h. In explicitly unpaired controls, CS (light) unpaired with the US (odor) did not lead to asymptotic increase in the response to the CS (CR) (Fig. 4.4). This difference between paired and unpaired controls is due to the different temporal arrangement in the two conditions. More CRs in response to the CS in the paired group indicates that excitatory learning occurs only when there is a predictable temporal relationship between the CS and the US. We have further identified that the intermediate-term or LTM consolidation occurs between 1.0 and 3.0 hours post-training (Basil et al., in preparation).

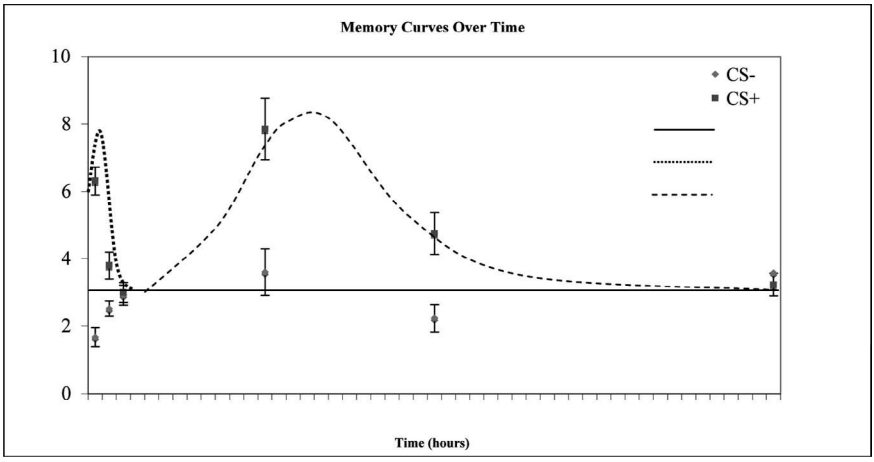

FIGURE 4.4 *Biphasic memory curve. Y*-axis: proportion of trials with a TER response; *X*-axis: time after initial training. In excitatory forward-paired, CS+, trials (black), there is a peak of memory at 1 h after training that drops and then rises again at about 6 h, declining until 24 h. No such learning curve is detected in explicitly unpaired, CS−, trials (gray). Bars indicate SEM. *profile (This figure is reproduced with permission from Cephalopod Cognition (2014). Cambridge U. Press.)*

These associative learning experiments, using stimuli commonly encountered in the habitat of nautiluses, demonstrate that they can learn to associate naturally occurring stimuli and that their memory processes include short-term and long-term components. While STM, in this task at least, is comparable to cuttlefishes—it is of shorter duration, perhaps due to the lack of a vertical lobe in nautiluses. In nautiluses STM may be processed in a different area of the brain compared to coleoids. Future experiments will

determine if the olfactory lobe is responsible (Hochner, Crook, and Basil, in progress)—it is much larger in nautilids than coleoids and is found in a similar neuroanatomical location to the vertical lobe of coleoids (VL, Young, 1965a). Since the plexiform layer and subesophogeal nerve cords of nautilus have been hypothesized to be the antecedents of the superior and inferior subfrontal lobes in *Octopus* (Young, 1965a), also important learning centers, perhaps the VL developed as an expansion of these areas during the cretaceous period when coleoids radiated in direct competition with the bony fishes (Packard, 1972; Aronson, 1991; Grasso & Basil, 2009).

4.6.2 SPATIAL MEMORY

Because of their unique daily vertical migration in the wild as they move up and down coral-reef slopes to forage (Carlson et al., 1984; Dunstan et al., 2010; Ward et al., 1984), the capacity for spatial learning and memory in *Nautilus* is also of interest. It is possible that nautiluses have the capacity to collect and use spatial information on a large scale during these vertical movements, relying upon smaller scale spatial cues (visual, tactile, olfactory) as they hug the coral-reef face during foraging. Especially important is to maintain visual contact with the contours of the coral reef to avoid entering open (and more dangerous) waters. To orient and navigate successfully, animals must be able to detect and perhaps learn the relevant topography of their environment (Tolman, 1948; O'Keefe and Nadel, 1978). Nautiluses can remember a series of turns to return to a goal (as in other animals Gallistel, 1993, *response strategies, route reversal*; in nautiluses, Crook & Basil, 2013, Barod et al., in progress, Barord, 2015,) or they can use single visual cues (as in other animals Gallistel, 1993, *beacon homing;* in nautiluses, Crook et al., 2009; Crook & Basil, 2013; Barord et al. in progress, Barord, 2015,) coding the location of a goal in space with a landmark nearby (e.g., *Octopus,* Mather, 1991). A more complex alternative is arranging more than one beacon in a "chain" to be followed to a goal (coleoids, Alves et al., 2007a,b; arthropods, Collett et al., 1992; Gallistel, 1993). Animals may also use numerous landmarks to cue the location of a goal (*Piloting or place learning*, Gallistel, 2003, e.g., Kamil & Jones, 1997, 2000), using multiple bearings from these various landmarks to increase their accuracy (Kamil & Cheng, 2001). Karson et al. (2003) and Alves et al. (2007b) demonstrated that cuttlefishes can use either algorithmic/route or place behavior to find a goal location (a dark spot with a sandy bottom). Depending upon the kinds of cues available and/or reliable (extramaze and intramaze), cuttlefishes can

switch their response tactic to find their goal location. Cuttlefishes can also switch flexibly between tactics to find a familiar spatial goal, using cues within the maze or outside the maze to varying degrees. Given their complex natural environment and the importance of quickly locating home, food, and safety, it is not surprising octopuses and cuttlefish would have complex spatial abilities, probably based upon constellations of visual cues in their environment. Another homing strategy found extensively among invertebrates and vertebrates is *dead reckoning*. Animals calculate their net displacement from "home" by keeping track of the number of turns and distance traveled to determine an absolute vector to return to the goal (Gallistel, 1993). This may occur in octopuses (Forsythe & Hanlon, 1997; Mather, 1991). An alternative is that octopuses are using patterns and changes in polarization patterns to solve these homing problems (Shashar, 2014). For nautiluses living along current-swept coral-reef slopes, dead reckoning or route memory in combination with odor tracking may be a vitally important source of information for nautiluses searching for food and mates (Basil et al., 2000, 2002) and perhaps avoiding predators.

Learning: Beacon Homing: In the laboratory, nautiluses are adept at solving spatial problems and are cognitively flexible in the solutions they use: relying upon a variety of landmark cues in various subsets to locate a learned escape hole in a shallow-water maze (Crook et al., 2009; Crook & Basil, 2013). In beacon-homing experiments, when an escape hole in a horizontal, 1-m diameter, shallow-water maze is cued only with a simple visual beacon surrounding the hole (Fig. 4.5; a white-striped ring Boal (1991, 1996), Boal et al. (2000)) nautiluses learned the goal quickly and memory persisted for up to 3 weeks, rivaling the performance of octopuses (Figs. 4.6 and 4.7, Crook et al., 2009). When the beacon was shifted from the goal, nautiluses first directed their search over the beacon, only finding the goal after extensive trial-and-error search of the maze. At least in this case, route-memory tactics did not compensate to allow the animals to find the goal quickly using route memory, with the learning and presence of the beacon taking priority. In a later control series and with more experience, however, route memory was invoked when the beacon was removed entirely (Crook, 2008; Crook & Basil, 2013). Later studies demonstrate that nautiluses can dynamically switch between beacon homing and route memory to find a goal in response to changes in the environment (Barord, Derman, Ju, Travis, Vargas, and Basil, in preparation; Barord, 2015, PhD Thesis). However, prior training experience with the beacon impacted the speed with which nautiluses switched to route memory, demonstrating overshadowing of that source of egocentric information by the beacon cue. Learning to navigate to

a goal without a beacon, using route memory alone, was successful, but took longer (Barord et al., in preparation; Barord, 2015, PhD Thesis).

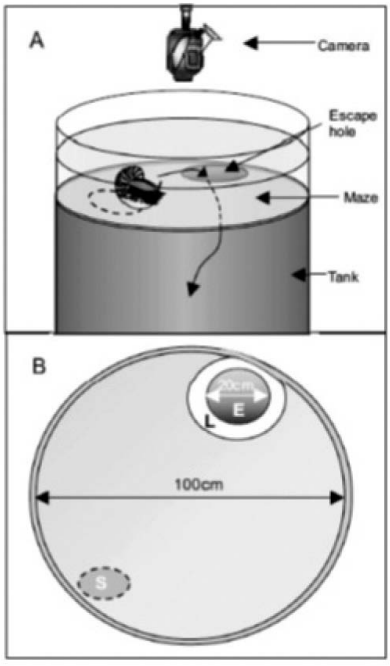

FIGURE 4.5 *Inverse Morris Water Maze with Beacon*. Total depth of tank is 1.5 m and width is 1 m. Maze depth is the shell diameter of the animal subject and width matches that of the tank. Goal located 80 cm from animal's start position and is 18 cm in diameter. Beacon is a 5-cm-wide concentric ring of white-striped bubble wrap surrounding the escape hole. (This figure is reproduced with permission from American Psychological Association. *Crook, R.; Hanlon, R.; Basil, J. J. Comp. Psychol.* **2009,** *123*, 264–274.)

Learning: Navigation: Nautiluses use visual cues as beacons or as a complex array of cues to locate the goal and are flexible in how they use cues, depending upon availability and reliability (Crook et al., 2009; Crook & Basil, 2013). Crook and Basil (2013) trained and tested nautiluses to find a goal with a constellation of objects positioned in the maze in various subsets. Nautiluses could use each subset flexibly, both those within the maze (both proximate to the goal and distant from the goal) and those outside the maze, to navigate to the escape point (Figs. 4.8 and 4.9). Interestingly, nautiluses were proficient at using visual cues to navigate, despite the limited acuity of their eyes (Muntz, 1984, 1986, 1987b). Nautiluses also expressed a hierarchy for cues that shifted with cue reliability (Crook & Basil, 2013). The

salience of a particular cue and the choice to use it to make spatial decisions was dependent on the array of other cues that were present during learning and also their reliability in testing. Similarly, Alves et al. (2007b) showed that cuttlefish (*Sepia officinalis*) make spontaneous choices between response- and place-based strategies based on the type of cues available and the perception of their reliability.

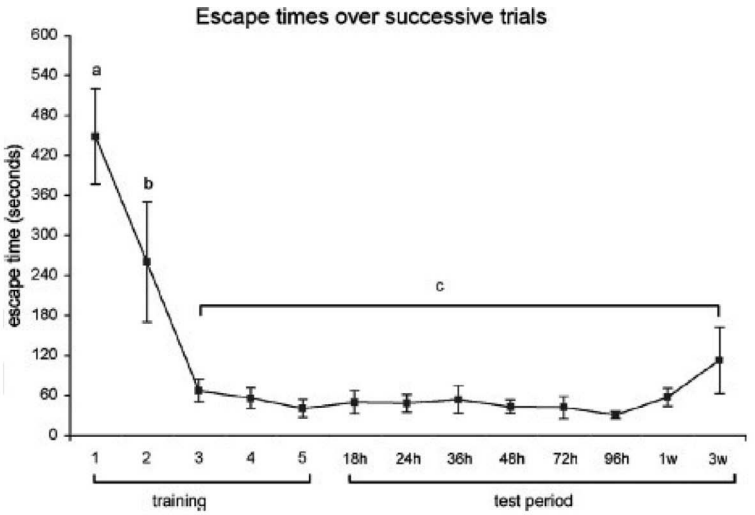

FIGURE 4.6 *Long-term retention of spatial information.* Time to find exit hole marked by a beacon in the spatial maze on successive 10-min trials improves rapidly across five training trials and is retained for at least 3 weeks. (This figure is reproduced with permission from American Psychological Association. *Crook, R.; Hanlon, R.; Basil, J. J. Comp. Psychol.* **2009,** *123*, 264–274).

In these studies (Crook & Basil, 2013), the relative importance of egocentric cues versus geocentric cues (and global (distant) and local (proximate) cues) were examined separately by removing one set of cues or creating conflicting information among the cue types. Numerous hypotheses were tested: (1) Nautilus uses beacon-based homing to locate an exit hole in a platform in shallow water. A beacon was defined as a single, proximate cue located close to or directly over the goal. (2) Nautilus uses path integration/route memory to locate the exit. Path integration/route memory involves animals using internal information such as body position and movement direction to locate a goal. (3) Nautilus uses geometric relationships among cues (local and/or global) to locate the exit. (4) Nautilus can use a combination of these features, depending upon which ones are reliable and/or available.

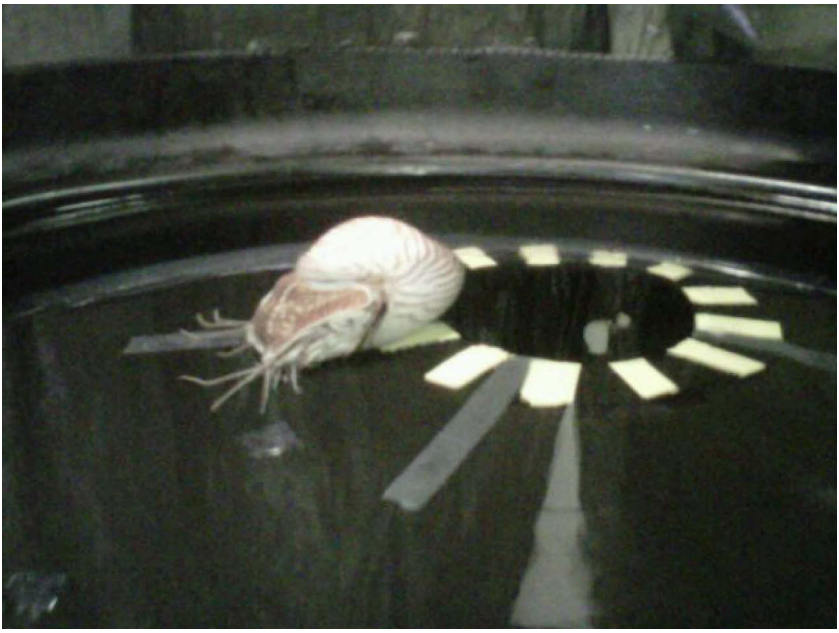

FIGURE 4.7 *Nautilus* in "*Inverse Morris Water Maze*". Animal is attached to maze at escape exit to deeper water, surrounded by striped visual beacon (*Photo, J. Basil*).

Six spatial configurations were tested (Fig. 4.8). (1) *Geocentric, single proximal cue*: The hypothesis that animals were using beacon-based homing (S–P) was tested by training with a maze configuration identical to that of Crook et al. (2009). During training, the beacon surrounded the escape hole. The start position and escape hole were kept constant in training and probe trials, but beacon position differed. During the probe trial, the beacon was moved counter clockwise, while the escape hole itself was unmarked and remained in the training position. (2) *Egocentric, path integration or route memory*: To test whether animals were using route memory (MR) to locate the exit hole, animals were trained as above with the beacon around the hole. During the probe trial, the start and exit points were kept constant, but the beacon was removed, and all external landmarks were obscured by a blind around the tank. (3) *Egocentric, route memory, novel start*: In a second test of route memory (MR–NS), training was identical to that described above. However, in the probe-test trials the start position was moved 90° clockwise to the quadrant adjacent to the training start position. (4) *Geocentric, multiple distal cues*: There were three landmarks marking the exit hole in a geometric arrangement relative to the hole, but not at the exit (a white airstone, a white PVC x-junction, and a white piece of plastic egg crate).

During probe trials, the landmarks remained in a constant position relative to the exit and to the experiment room. The start position, however, was shifted 90°. (5) *Geocentric: multiple distal, single proximal*: To test whether animals were relying on proximal (close to the exit) or distant (around the edge of the arena) intra-maze navigational cues, the training trials were repeated with both the beacon (proximal cue) and the landmarks (distant cues) present. In probe trials, the beacon was moved to the adjacent quadrant to the exit, but the landmarks and the start position remained constant. (6) *Geocentric: global versus local cues*: Animals were trained in the maze with the three distant landmarks (local, intra-maze cues) present, positioned as described above. In probe trials, the maze and the intra-maze cues array were rotated 180° relative to their training position and to the experiment room. Animals started their probe trials in the "correct" start position relative to the intra-maze cues, not global extra-maze cues.

Nautilus paths during testing in each of the above configurations (Fig. 4.8) are displayed in Figure 4.9. In the test of Beacon/Single Proximal Cue homing, when the beacon was moved away from the exit, all of the subjects oriented initially toward the beacon. This suggests that the proximate visual cue was highly salient for navigation, and is consistent with results obtained by Mather (1991) in a similar experiment using octopuses. When animals were given the proximate cue (beacon) together with the distant intra-maze landmarks (air-stone, egg-crate, and pipe fitting), they ignored the repositioning of the beacon and navigated successfully using the multiple, distant cues. In this case, prior learning of a beacon during training did not affect the ability of nautiluses to subsequently use local intra-maze cues alone in tests as long as they did not shift from training (e.g., *rats*, Thein et al., 2008). Animals were able to switch strategies, from navigating using the beacon to navigating using distant landmarks, easily. Similarly, Alves et al. (2007b) showed that cuttlefish (*S. officinalis*) were capable of making spontaneous choices between response-and-place-based strategies based on the type of cues available and the perception of their reliability.

A similar strategy was apparent in the test of local intra-maze cues versus global extra-maze cues (Fig. 4.9). Animals were able to navigate using the three intra-maze; however, when the maze (and all local intra-maze cues) was shifted 180° with respect to the experiment room, all of the animals searched repeatedly over the previous location of the hole *relative to the experiment room* (*global cue*). The directed search over the previous exit position relative to the room suggests that animals were using navigational cues that were located outside the maze over local cues within the maze.

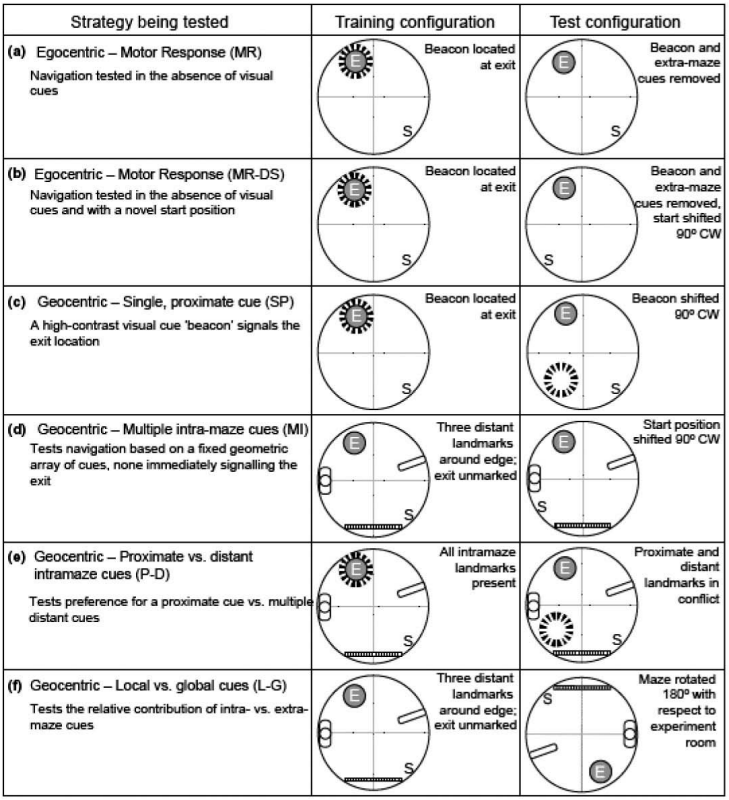

FIGURE 4.8 *Maze configurations to test navigational tactics*. Training configuration on the left, testing configuration on the left. Visual cues were beacon (ring with white stripes), white PVC x-fitting on one end (white rectangle with central circle), a white airstone laid on its side (white rectangle), and white flourescent light grating on one edge (rectangle with hash marks). E and S are exit and start position, respectively. (From Crook, R.; Basil, J. Flexible Spatial Orientation and Navigational Strategies in Chambered Nautilus. *Ethology* **2013,** *119*, 77–85, (Used with permission from John Wiley & Sons, Inc.).

When animals were tested in a novel start position using local intra-maze cues, the animals searched across the maze until the point when an animal crossed close to the original start location (Fig. 4.9). At this point subjects oriented toward the exit and adopted a straight trajectory toward it. Thus, this is not a true "cognitive map" as animals could not compute a new, direct route from a novel start position (Bennet, 1996; Healy, 1998; Shettleworth and Hampton, 1998). However, nautiluses had encoded the spatial relationships of the landmarks relative to the goal, and recognized when they were in a position where their internal representation matched prior visual input,

similar to the retinotopic "snapshot" described in other invertebrates (ants: Antonsen & Wehner, 1995; *Drosophila*: Dill et al., 1993).

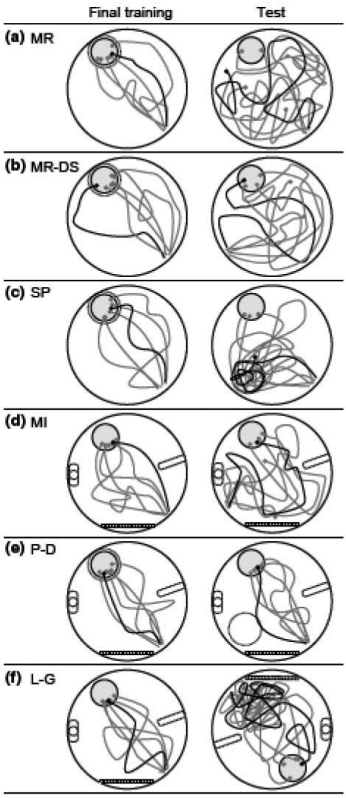

FIGURE 4.9 *Animal paths for all six navigational-tactic conditions*. Training paths on the left, testing paths on the right. One animal is compared, in black, in both training and test trials. All other animals shown in gray. (Labels as above. From Crook, R.; Basil, J. Flexible Spatial Orientation and Navigational Strategies in Chambered Nautilus. *Ethology* **2013,** *119*, 77–85. Used with permission from John Wiley & Sons, Inc.).

In general, nautiluses seem to prefer the more distant landmarks of each array (Fig. 4.9). When a single proximate beacon was the only cue available, animals oriented toward it even when it conflicted with any egocentric route-memory, a result also observed in octopuses (Mather, 1991). When given a choice between beacon and distant intra-maze cues, animals navigated successfully using the distant landmarks, and when intra-maze and extra-maze cues were in conflict, animals chose to navigate using the global cues. Taken together, these results offer some interesting insights into navigation

in the wild. The reliance on the most distant cue type available in several experiments suggests that perhaps animals in the wild might orient to their surroundings using a global cue such as the onshore–offshore light gradient, a cue that remains constant and is likely to be visible for at least some part of the daily migratory cycle. The reliance upon local visual cues for small-scale orientation may be useful in the more brightly lit surface waters, but is unlikely to be of great use at the depths where nautiluses spend most of their time. Given that animals probably scavenge for food, remembering the location of already depleted foraging areas may be of some use. The ability to find and relocate appropriate safe locations for egg deposition is likely adaptive as well. Ecologically, nautiluses' accommodation of the spatial changes they encounter on their nightly vertical migrations along coral reefs would benefit from such a flexible system.

4.6.3 LEARNING

In our prior studies examining beacon homing which has both an associative (beacon = hole reward) and a spatial (beacon = landmark near goal) component, animals did not seem to rely heavily on route memory when the beacon was removed for a few control probe trials (Crook et al., 2009). Most animals eventually found the escape hole, but they appeared to search the maze randomly to do so. However, in the later study with training using more complex landmark arrays, when the beacon was removed entirely animals *were* able to find the exit hole in the absence of any other landmarks (Crook & Basil, 2013). From this, we inferred that perhaps route memory is possible, but is learned at a slower rate than beacon homing (requiring more training trials). However, when trained without a beacon in the first place, route memory was indeed a viable solution to the problem (Barord et al., in preparation; Barord, 2015, PhD Thesis). These different rates of learning raise the idea that perhaps (1) learning the landmark array and/or beacon competes with learning the kinesthetic information as they navigate, and only when no other information is available do they access and use the route memory tactic. Or, that (2) there are different substrates supporting storage of the two tactics, with information learned and stored at different rates, as opposed to shared memory space. Were beacon homing and route memory sharing memory space we would predict (1) that prior learning of the beacon would overshadow subsequent learning of route (Gibson & Shettleworth, 2005, Shettleworth & Sutton, 2005, Cheng et al., 2007), and they would perform less well with only route memory as an option than with

a beacon present. Animals trained only with route memory as a solution with no competition of the learned beacon, would therefore perform better in tests requiring route memory than those with prior beacon training, and we test that possibility currently (Barord et al., in preparation). Having both a beacon and a route-memory module processing information simultaneously, and equally accessible given a test trial with no interference (2), would be indicative of complex spatial processing. This, in turn, may require more complex neural organization than learning a simple association of contiguous events.

We examined the nature of memory space by testing nautiluses in a spatial overshadowing paradigm, one group (1) using a compound stimulus of distinct modalities (beacon and route memory information) during training (Barord et al., in preparation; Barord, 2015 PhD thesis; Cheng et al., 2007; Gibson & Shettleworth, 2005; Shettleworth & Sutton, 2005) and comparing them with a group (2) trained only with route-memory information. Both groups are then tested with no beacon or with the beacon positioned away from the exit hole (thus, unreliable). If there is overshadowing by the beacon (CS1) in group 1, then egocentric route memory information alone (CS2) should not be as efficient when the animal is trying to find the exit hole during testing. These two sources of information would likely then share memory space. If prior testing with a compound stimulus (Beacon and route memory, CS1 and CS2) does *not* affect the ability of animals to find the goal with kinesthetic egocentric information alone (CS2), with them performing as well as those trained with kinesthetic information and no beacon, then this argues against overshadowing and, importantly, shared memory space. Preliminary results from experiment 1 indicate that animals were more likely to use route-memory information when the beacon was absent, or in an unreliable location, than when it was still located at the exit hole during testing. Animals trained with no beacon performed better than animals with prior beacon training: the beacon training overshadowed learning of the route, thus supporting the idea of a shared memory space (Barord et al., in preparation). Field testing has also recently begun with beacons marking nautilus lures near the coral reefs of the Indopacific (Barord et al., in preparation).

4.6.4 VERTICAL SPATIAL MEMORY

Given their vertical reef-hugging habitat, it may not be surprising that nautiluses also solve vertical three-dimensional spatial problems. In an artificial reef constructed from black and white cinder blocks, nautiluses detected

when the three-dimensional configuration of the artificial coral reef was altered (Crook et al., 2009), but not when the black and white components of the reef simply swapped colors, with the three-dimensional configuration remaining the same (Fig. 4.10). This kind of local familiarization with vertical configurations, or environmental silhouettes, should be adaptive for locating hiding places or reliable foraging spots in the wild.

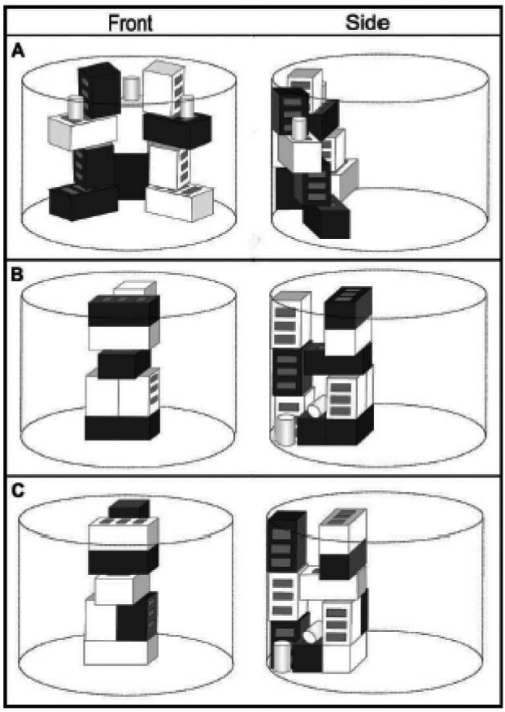

FIGURE 4.10 *Three 3D Reef Configurations (A–C).* (A) Reef 1 is a step like stack of blocks and pipes. Reef 1 and Reef 2 (B) differ in spatial configuration. (B) Reef 2 and (C) 3 are identical in configuration, but differ in visual pattern. (This figure is reproduced with permission from American Psychological Association. Crook, R.; Hanlon, R.; Basil, J. *J. Comp. Psychol.* **2009,** *123*, 264–274, permission granted.)

Summary: By applying an ecological approach to the study of this ancient animal, deriving hypotheses based upon its natural history and then using a multiprocedural approach to test them, we have learned (1) that nautiluses learn and remember, expressing both short-term and long-term components (Crook and Basil, 2008a,b), (2) that nautiluses are adept at both horizontal

and even vertical spatial problems (Crook et al., 2009), and that (3) nautiluses can dynamically use a variety of cues in their environment, both visual (Crook et al., 2009; Crook & Basil, 2013) and route-based (Barord et al., in preparation; Barord, 2015, PhD Thesis), to locate goals in space. We continue to use these techniques to probe fundamental questions regarding the nature of their memory storage, organization and retrieval, and to guide electrophysiological and anatomical study of their brain.

The study of the learning, memory, and complex spatial abilities in nautilus is valuable because of their close phylogenetic relationship to the most neurologically complex invertebrate, their unique ecological niche, and their own neurological organization, which, despite lacking the known learning centers of coleoids, is more complex than those of other molluscs (for which much is known of their mechanisms of learning and memory). Despite these differences from the coleoids (i.e., the lack of the vertical lobe), nautiluses still learn, remember, and perform complex spatial tasks, dynamically switching among solutions depending upon the information available. This supports the argument that historically the behavioral complexity of cephalopods predates the expansion of bony fishes (Packard, 1972; Grasso & Basil, 2009). Nautilus is thus positioned at a critical point in the cephalopod lineage to reveal how selective pressures resulted in heavy investment in fast and flexible behavioral and neural processing over evolutionary history. Their simpler neural architecture, relative to coleoids at least, belies their true capabilities.

4.7 CONCLUSIONS

Our long-term goal is to understand the contributions of phylogeny and ecology to learning and memory using the comparative, synthetic approach (Kamil, 1988). Because of their well-known complex behaviors, cephalopod molluscs are an exceptional lineage for this kind of comparative study. While a variety of behaviors and supporting neuronal structures have been well described in coleoid cephalopods, until recently very little was known of their pleisiomorphic relative, Chambered *Nautilus*, for comparison. Our research has revealed shared capabilities, despite marked differences in their brains, underscoring the role that convergence may have played in the development of neuronal complexity in this lineage. Beyond understanding, how these animals learn, remember, and navigate in a complex environment, our research also addresses:

4.7.1 ECOLOGY AND CONSERVATION

Field research on nautiloid populations is extremely difficult. They are solitary animals that live in extremely deep waters for most of their long lives. Population levels are estimates at best, and there is growing concern over anthropogenic disruption of their coral-reef habitat and also direct impact of over fishing (for their "decorative" shells—they are not a major food source, Dunstan et al., 2010). An understanding of their behavior and the role that environmental information plays in their ability to navigate, forage, and find mates is therefore extremely important to the management and care of natural populations. It is a goal of our laboratory to use our study of their behavior to develop more accurate methods to measure natural populations, track their movements, and make predictions about factors that are important to their survival.

4.7.2 BEHAVIORAL ECOLOGY

Our proposed research will complement studies of the costs and benefits of learning and memory. Coleoid and nautiloid cephalopods occupy vastly different niches (fast/visual versus slow/smelling and groping, respectively) and life-history strategies (semelparous coleoids vs. iteroparous nautiloids). By understanding the abilities of animals with widely divergent lifestyles, we aim to identify ecological constraints and contributions to learning and memory.

4.7.3 NEUROSCIENCE

Comparative studies have identified fundamental organizational principles that underlie speedy and flexible information processing in the brains of vastly different animals (e.g., short connectives and "en passant" synapses in both vertebrates and coleoid molluscs, Hochner et al., 2006). Much is known of the neuroanatomy and functional anatomy of the molluscan (particularly coleoid cephalopod) brain. Recent successes with isolated slice preparations in coleoids (Hochner et al., 2006) have allowed researchers to identify cellular properties in these complex brains. Our ongoing studies, by determining the characteristics of learning and memory in a close relative with simpler, pleisiomorphic neural architecture will provide context for future study of (1) general neural organizational principles underlying complex

behavior and (2) specific homologous and analogous systems in the cephalopod brain.

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KEYWORDS

- **cephalopods**
- **nautiloids**
- **environmental stimuli**
- **conditioned response**
- **landmark**

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CHAPTER 5

THE CEPHALOPOD BRAIN: MOTION CONTROL, LEARNING, AND COGNITION

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CONTENTS

ABSTRACT

Outgrowing their molluscan ancestors coleoid cephalopods have developed a remarkable complexity of behaviors and the underlying nervous system that controls them. Like in other phyla, such as teleost fish, cephalopods follow a trend for cephalization the concentration and evolution of a head holding acute sense organs an a highly concentrated central brain. The brains of cephalopods developed many de novo structures termed lobules by previous researchers. On the other hand the peripheral nervous system, having to control the complex motor output in its muscular hydrostat body has retained a remarkable size and level of independence. The flexible muscular hydrostat movement system theoretically has unlimited degrees of freedom, and octopuses are models for "soft bodied" robots. The decentralized nervous system, particularly in the arms of octopuses, results in decision making at many levels. Cephalopods developed a camouflage/signaling system in their skin that is unmatched in complexity and speed. This gives cephalopods to on one hand communicate with conspecifics and on the other hand to use a system of active camouflage to disappear from the sight of predators and prey alike. Modern cephalopods represent an alternative model to the vertebrates for the evolution of complex brains and high intelligence, which has as yet been only partly explored.

5.1 INTRODUCTION

Cephalopods have by far the most complex and the most centralized of all invertebrate brains (Nixon & Young, 2003; Young, 1971, 1976, 1979).

The variations between the brains of different cephalopod species mirror the wide variety of ways of life and morphological body adaptions. While especially the coleoid—or modern cephalopods—show a dramatic centralization and the formation of distinct lobulated brain areas, the gross morphological properties remain the same. One of the most basic concepts in the building of an invertebrate brain is the distinction between outer layers containing nuclei while the more central areas contain the fibers of the neurons. This means that, as is the case in other invertebrates, cephalopod neurons are unipolar, and groups of neurons are organized with outer cell body layers and an inner neuropil (Fig. 5.1A and B).

However, recent studies show that neurotransmitters, neuromodulators, and synaptic plasticity mechanisms are a combination of adaptations of the molluscan mechanisms and convergent evolution of "vertebrate-like"

mechanisms such as the neural network organization underlying learning (Brown & Piscopo, 2013; Hochner et al., 2006 Hochner & Shomrat, 2013; Shomrat et al., 2015). While many aspects of our chapter deal with all cephalopods—there are two classes of cephalopods, Coleioidea or modern cephalopods, which comprise all living taxa of cephalopods apart from the Nautilioidea. While the Nautiloids are being covered in a chapter by Basil (same volume), in this, we aim to describe some of the behavioral and physiological plasticity and the complex motor programs, coordinated and controlled by coleoid cephalopod brains.

5.2 THE ORGANIZATION OF THE BRAIN

The neuroanatomy of cephalopods has been extensively and faithfully compiled into several in depth works (Nixon & Young, 2003; Young, 1971). Using a variety of histological methods, Young (1971) and colleagues described in detail the cells and tracts of the nervous system revealing the structure and connections between brain lobes and between the peripheral and central nervous system (Fig. 5.1). The brain can be morphologically divided into subesophageal, periesophageal, and supraesophageal masses, and these can be further subdivided into many lobes.

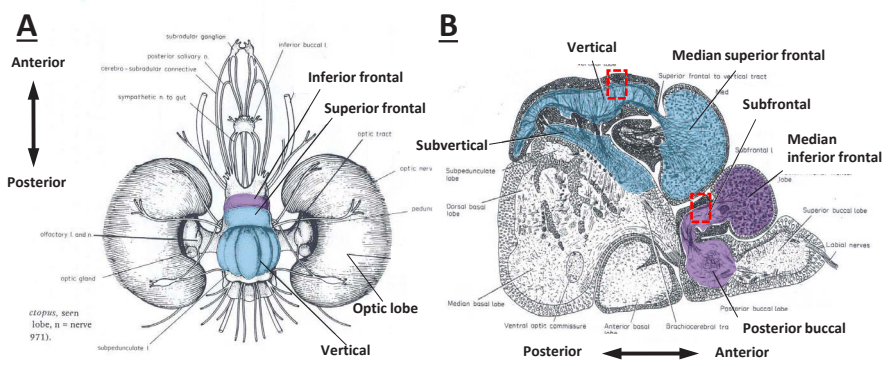

FIGURE 5.1 The octopus CNS and its two separated learning and memory systems (visual – blue, chemo-tactile – purple): (A) The centralized brain as seen from above. The visual learning system located at the most dorsal part of the brain composed of the median superior frontal lobes (MSF) and the vertical lobes (VL). Part of the touch learning system can be seen beneath the superior frontal lobe. **(B)** A sagittal slice from the medial part of the supraesophageal brain mass. Note the resemblance in the gross morphology between the MSF-VL region and subfrontal (SubFr)- median inferior frontal (MIF) regions (figure modified from Young, 1971).

It is generally assumed that the subesophageal lobes are mostly lower motor centers. The lower motor centers are those which contain the final motorneurons innervating different area of the body. Studies on the behavioral responses of *Octopus vulgaris* to direct electrical stimulation by wire electrodes, as well as several brain lesion studies, helped ascribe different, although somewhat overlapping, motor functions to major lower motor lobes.

The intermediate motor centers send fibers that synapse at least once before reaching the target muscle.

Finally, the higher motor centers are located in the supraesophageal mass. Electrical stimulation of these areas evoked a variety of coordinated or complex movements. One clear difference from higher motor areas in vertebrates is that so far no somatotopy, that is, no exact topographic representation of the body in the brain, has been found. Therefore, Zullo et al. (2009) suggest that the organization might be arranged into overlapping circuits. However, somatotopy cannot be ruled out, especially in lower motor areas.

As is the case for the motor centers, the sensory centers are likely organized in a hierarchical way. Apart from the sensory input originating from the arms and body (covered later in this chapter), several of the sensory organs are in close the central brain. Vision is likely an important sense for all coleoids. They all have large lens type eyes, a remarkable case of convergent evolution. Behind each eye is an optic lobe, and the retina projects directly to it. However, not much is known about the processing of visual information. The brain also receives vestibular input from the statocysts, which are located behind the brain.

Although not encased in the brain capsule as other lobes, the large paired optic lobes make up 2/3 of the CNS (Nixon & Young, 2003). The optic lobes, connected directly to the retina, are presumed to take part not only in visual processing, but also in the learning and memory network. However, lesions of the optic lobes do not disturb tactile learning or tactile memory. Additionally, electrical stimulation of the center of the optic lobes elicits motor responses similar to those elicited by stimulation of the higher motor centers (Boycott, 1961). Another area, thought to have critical importance in motor control, sensory integration, and learning and memory, is the peduncle lobe. There are two peduncle lobes, each situated at the connection between the optic lobes and the central brain. The peduncle lobes are found in all coleoids, and as is the case for many other lobes, their exact location and relative size differ between species. Several morphological features have lead researchers to compare the peduncle lobes to the cerebellum, collecting information from different sensory systems and playing a role in regulating

motor programs (Camm et al., 1985; Hobbs & Young, 1973; Saidel, 1981; Wells, 1978).

5.3 LEARNING AND MEMORY

One of the most intriguing aspects of cephalopods is their learning capabilities and "vertebrate-like" behavior. Cephalopods are phylogenetically remote from vertebrates (Wray et al., 1996) and their advanced behavior is achieved with a brain that is relatively simple compared to the brains of vertebrates with similar cognitive abilities. This makes cephalopods as an ideal subject for comparative analysis of brain mechanisms, evolutionarily selected, for the mediation of complex, flexible behaviors. Identifying the similarities and differences with other biological learning systems, as the mammalian hippocampus and the insect mushroom-bodies, has the potential of revealing essential and universal principles of learning and memory networks (Brown & Piscopo, 2013; Hochner & Shomrat, 2013; Shomrat et al., 2015).

The visual learning system in coleoids comprises of the vertical (VL) and superior frontal (SF) lobes (along with the subvertical lobe (SV), Figs. 5.1 and 5.2). These lobes, lying in the supraesophageal mass, are involved in long-term memory formation. However, lesions of SF–VL have only a mild effect on the retrieval of well-established memories, learned pre-lesion (Shomrat et al., 2008; Young, 1961). Similarly, short-term learning does not depend on the SF–VL, which only modulate the acquisition rate (Boycott & Young, 1955; Shomrat et al., 2008). Hence, simple short-term memory can be formed outside the SF–VL system. (It is hypothesized that it takes place directly at the circuitry controlling the behavior [Shomrat et al., 2008].) The octopus SF–VL system contains only three types of morphologically identifiable, typical invertebrate, unipolar neurons. The MSF lobe contains only one type of neuron (~1.8 M), which convey integrated signals to the VL where they innervate ~25 millions small amacrine interneurons (AM) en passant. These ~25 million AMs then converge onto only ~65 thousand large efferent neurons (LN). The synaptic inputs to the AM are glutamatergic, while the synaptic connections from AM to the LN are cholinergic (Hochner et al., 2003; Shomrat et al., 2011). Thus, the neural network is organized in a three-layered feed-forward "fan-out fan-in" connectivity (Fig. 5.2b). Interestingly, this network arrangement resembles that of a "perceptron," an artificial neural-network found to be efficient as classification networks (Shomrat et al., 2011; Vapnik, 1998).

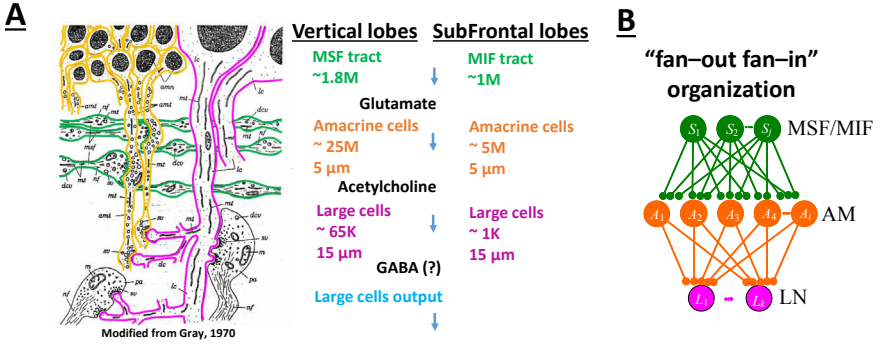

FIGURE 5.2 **(A)** Areas of the VL and SubFr within the red rectangles in Figure 1B. The VL and the SubFr regions are similar in their network organization and types of neuron cells. The neurotransmission of the SubFr still needs to be examined. **(B)** The connectivity in the VL/SubFr is organized in a fan-out fan-in network (in resemble to a multilayer perceptron—typical for biological and 'machine learning' classification networks).

Although phylogenetically disparate, there are striking functional and morphological similarities between the cephalopod SF–VL network and the mammalian hippocampus. Functionally, like their mammalian analogue, the SF–VL lobes are essential for the consolidation of new memories but not for short-term memory or for the retrieval of established memories (Sanders, 1975; Shomrat et al., 2008). Morphologically, like the hippocampus CA1 region, the SF–VL network is organized as a series of matrices of crossing fibers, and the input from the SF innervates the VL neurons in en passant manner (Young, 1995). At the cellular and molecular level a non-NMDA, activity-dependent, LTP has been shown at the SF to AM synapses, resembling the LTP mechanism found in the mammalian mossy fiber synapses (Hochner et al., 2003; Nicoll & Schmitz, 2005). In octopus, as in other molluscs, serotonin (5-HT) acts as a facilitator for synaptic transmission in the VL, yet unlike its function in other molluscs, it produces no long-term modulatory effects. However, 5-HT indirectly enhances the activity-dependent LTP induction. Octopamine (OA) has a similar short-term facilitatory effect, but unlike 5-HT, it attenuates LTP induction and de-potentiates consolidated LTP (for a review, see Hochner & Shomrat, 2013; Shomrat et al., 2015). Thus, 5HT and OA might act, respectively, as positive and negative reinforcement signals during learning. The origin and the level of 5-HT and OA during learning is unclear. However, additional inputs to the VL enter from the SV (Young, 1971), these might carry modulatory input like serotonin (5-HT) (Shomrat et al., 2010) and (OA). A similar function was suggested for dopamine and noradrenaline in the mammalian hippocampus

(Lemon & Manahan-Vaughan, 2006; O'Dell et al., 2010; Yagishita et al., 2014), and octopamine (OA, *p*-hydroxyphenylethanolamine) and dopamine in insects' mushroom body (Giurfa, 2013; Perry & Barron, 2013).

Recently, using immunostaining for serotonin, octopus gonadotropin-releasing hormone (oGNRH), and octopressin–neurophysin (OP–NP), Shigeno and Ragsdale (2015) showed that the five lobules forming the VL gyri as well as the SF in *Octopus bimaculoides* (Fig. 5.3) have distinct neurochemical signatures. This intricate internal anatomy of the octopus SF–VL system might reflect the presence of functional subsystems within cephalopod learning circuitry.

Uniquely, the octopus brain possesses two separated learning and memory systems (Fig. 5.1). The visual learning system described above and the chemo-tactile (Hanlon & Messenger, 1996) learning system are composed of the subfrontal lobe (SubFr) and median inferior frontal lobe (MIF) (along with the lateral inferior frontal and the posterior buccal lobes (PB), Fig. 5.1). The general morphological structure of the SF–VL complex (described above) is shared by the MIF–SF system (Fig. 5.1), which seems to play a similar role, but in chemo-tactile learning (Young, 1983, 1991). The MIF–SubFr system is much smaller than the SF–VL containing ~1 million MIF neurons that convey integrated signals to the SubFr, innervating the ~5 million AM interneurons en-passant. The ~5 million AMs then converge onto only ~1000 large efferent neurons (larger than those of the VL (Young, 1971). Thus, as is the case in the SF–VL network, it is organized in a three-layered feed-forward "fan-out fan-in" connectivity (Fig. 5.2b). From an evolutionary point of view, in contrast to the SF–VL system, which exists in all the coleoids, yet is absent the nautilus (Crook & Basil, 2008). The MIF–SubFr system is poorly developed or absent in decapods (Hanlon & Messenger, 1996). The MIF–SubFr system morphologically resembles the SF–VL system, however, it is significantly smaller. In the MIF, Shigeno and Ragsdale (2015) found neurochemical heterogeneity within single fiber bundles. Although the SF–VL system has some role in tactile learning, the MIF–SubFr system does not influence visual learning (Sanders, 1975). Taking all these into account, the MIF–SubFr system might have evolved in context with the SF–VL and in correspondence with the development of the octopus arms.

There are many fascinating unknowns remaining. Recent developments in imaging techniques provide the chance to get large-scale morphological data on the development of brain areas in cephalopods. As demonstrated in a study by Kerbl et al. (2013), state-of-the-art X-ray microtomography (micro-CT) can be used for 3D imaging of soft-bodied organisms. Using

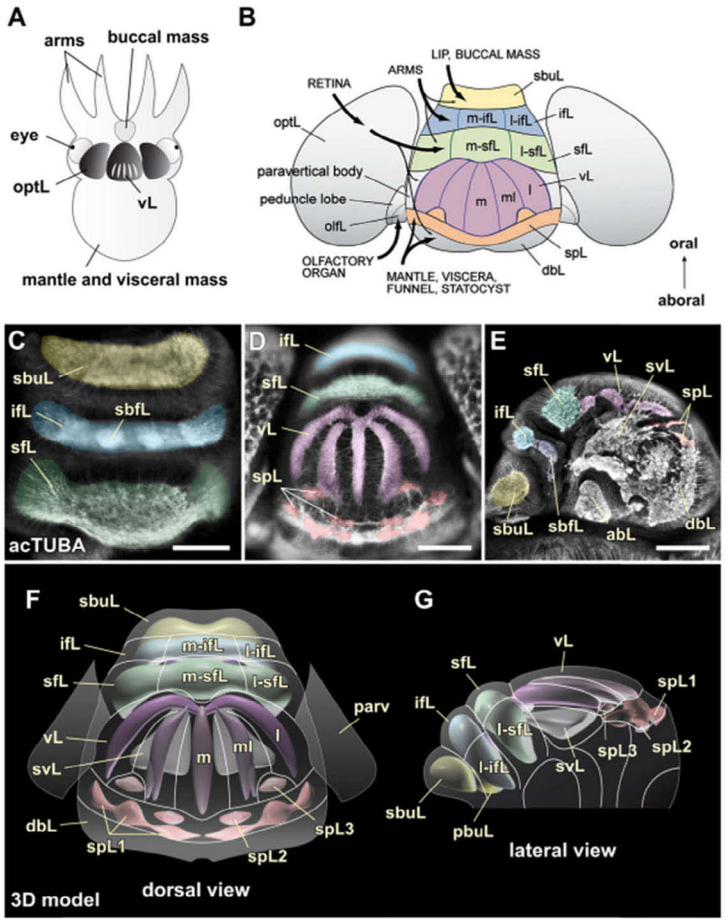

FIGURE 5.3 Distinct labeling of neurotransmitters in the gyri of the vertical lobe of *O. bimaculoides* (Shingeno & Ragsdale, 2015). (A) Cartoon of a hatchling octopus showing the traditional orientation of central brain and optic lobes, which are shaded in dark gray, along the oral (buccal mass)–aboral (mantle) axis. This dorsal view and oral–aboral orientation is employed for all whole mount images presented in this report. (B) Schematic drawing of the supraesophageal mass and optic lobes. Major sensory inputs to the supraesophageal brain are illustrated with arrows. The color code shown is followed in the false coloring of (C)–(E) and in the three-dimensional reconstructions of (F) and (G). (C)–(E) Hatchling brain whole mounts processed for acetylated α-tubulin (acTUBA) immunohistochemistry, which labels the supraesophageal neuropils. Illustrated are surface (C and D) and midline cut (E) views. Due to the pitch of the brain's surface (E), separate images are provided to capture the superior buccal lobe orally (C) and the vertical and subpedunculate lobes dorsally (D). The position of the subfrontal lobe, which lies deep to the inferior frontal lobe, is indicated in (C) and (E). (F) and (G) Three-dimensional space-filling model of the supraesophageal neuropils discussed in this report are presented in dorsal (F) and lateral (G) views. Scale bar = 100 μm in (C); 200 μm in (D) and (E).

contrast-enhancing substances, such as iodine or phosphotungstic acid, can provide detailed 3D information on the anatomy of cephalopod embryonic structures including the nervous system (Fig. 5.4). Thus, combining micro-CT with immunochemistry and gene expression studies opens up a variety of new avenues to investigate the functional organization of cephalopod brains.

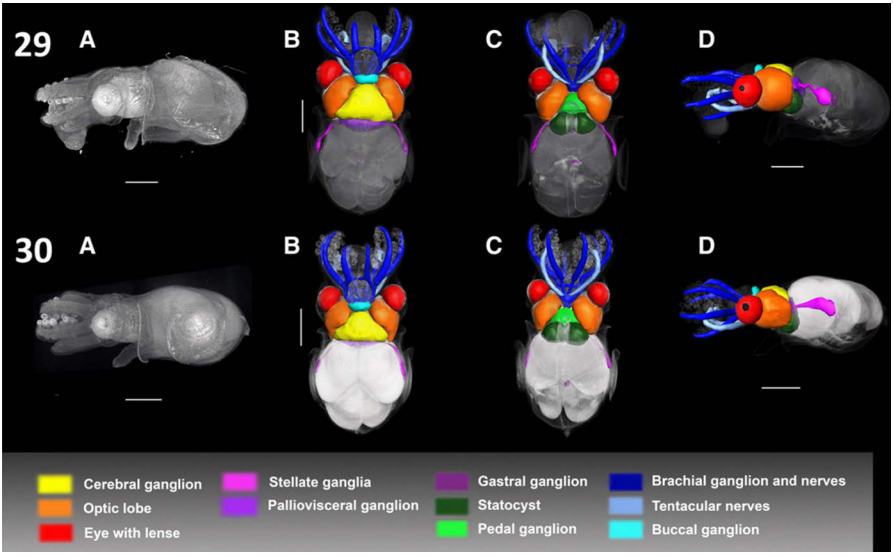

FIGURE 5.4 Kerbel et al. (2013): Volume renderings (A) and combined images of volume and surface renderings ((B) dorsal, (C) ventral, (D) lateral) focusing on the nervous system of *Euprymna scolopes* of stages 29 and 30 (hatchling). The different ganglia, prominent sensory organs, and nerve strands are color-coded. At hatching, most of the ganglionic mass is strictly concentrated in the head. From stage 29 onward, the gastral ganglion close to the stomach can be observed. Scale bars are 500 μm.

5.4 THE NEURAL CONTROL OF MOTION

Behavior relies on movement, so any review of cephalopod behavior necessarily includes a description of cephalopod movement. In many animals, the control and execution of movement is closely related to the skeletal support system. In coleoid cephalopods, this skeletal support system, the muscular hydrostat, is singular, as it does not involve any hard structures (Kier & Thompson, 2003; Yekutieli et al., 2009). This system has no endo- or exoskeleton, instead the muscles themselves arranged in a three-dimensional array, and assisted by connective tissue, co-contract and act as skeletal support as well as vehicles for movement. One of the most fundamental

aspects of a muscular hydrostat system is that it has a constant volume; for example, if we represent an octopus arm as a cylinder, then, when the cylinder elongates, the volume remains constant, therefore the cross section is reduced (Yekutieli et al., 2007). These movements are produced by muscle fibers, arranged in bundles in three mutually perpendicular directions. In the octopus arm, the bundles are transverse, longitudinal and oblique to the main axis of the arm, with an additional thin circular muscle layer (Smith & Kier, 1989; Kier & Stella, 2007). With no rigid skeleton muscular hydrostats are present in many structures, such as the arms and tentacles of squid (Kier & Schachat, 2008), the skin and mantle musculature of cuttlefish (Hanlon & Messenger, 1996), the adhesive octopus suckers (Kier & Smith, 2002; Tramacere et al., 2013, 2015) and the buccal musculature of the octopus beak (Uyeno & Kier, 2007), each requiring appropriate neural control.

Stiffness, bendability, the ability to exert force and the accuracy of localizing movement (Yekutieli et al., 2009) are some of the basic characteristics of any motor system. Each octopus arm theoretically has almost unlimited degrees of freedom, the ability to move in any direction at any place along the arm, and octopuses are famous for the amount of force an arm can exert (Dilly et al., 1964). Kier and Stella (2007) point out that the octopus arm movements must be some combination of elongation, shortening, bending, stiffening, and torsion. Examples of this variety of movements can also be seen in the arm postures of Caribbean reef squid (Mather, 2010), especially those used by young squid for camouflage. Arms and paired tentacles bend at one or many positions along their lengths, shorten, extend, splay away from the midline, and rotate around their own axis. One specific posture, called "Bad Hair," combines all of these movements. In octopuses, the "Flamboyant" posture (Packard & Sanders, 1971) involves positioning the arms similarly.

Squid and cuttlefish also have a pair of extensible tentacles which can elongate by over 80% at peak extension, have peak velocities of over 2 m/s and attach to a prey with suckers only at the terminal club (Kier & van Leeuwen, 1997).

Unlike the extendable tentacles, each arm subsystem contains hundreds of suckers along its length, the fine morphological details of which vary between species (Nixon & Dilly, 1977).

5.5 THE SUCKERS

The suckers, apart from being effective adhesion structures, also carry a huge number of primary sensory cells devoted to both chemical and mechanical

senses. While the arms of decapod cephalopods clearly differ from tentacles, octopus arms are unique, as they are the main method for interaction with the environment, from locomotion to exploration, holding on to items and catching food. Because octopus arms, and their control are the most thoroughly investigated, most of the research reported here is about that system. In octopus, a single sucker of 3-mm diameter contains several thousands of sensory cells (Graziadei, 1971; Wells, 1978), and the whole skin of the octopus is estimated to contain up to 2.4×10^8 sensory cells. Additionally, deep receptor-like stellate neurons are found at many sites within the arms and suckers (Graziadei, 1965). Wells (1978) suggested these branched neurons that may serve as proprioceptors, monitoring muscle deformation. Consequently, the amount of potential information that can be derived from even one single region on one arm is enormous. The possibility to utilize this information from the arms is further complicated by the flexibility, which results in changing distances between sensory areas as arms may elongate or shorten.

The control of sucker, stalk and arm all at once is therefore not a trivial task (Grasso & Basil, 2009), and it is still unclear how much of this control is restricted to the peripheral nervous system and how much is centrally controlled. Clearly, despite the complexity, octopus arms can perform many tasks, from bending back and grooming the body surface (Mather, 1998), to snaking through an opening to explore the substrate for hidden prey (Yarnall, 1969), and accurately bringing caught prey to the mouth (Sumbre et al., 2001).

5.6 MOTOR CONTROL AT THE LEVEL OF A SINGLE ARM OR SEVERAL ARMS

Although octopuses have large central brains, more than 3/5 of the nervous system is dispersed throughout the body and arms (Hochner, 2010, 2012). Available knowledge of the octopus nervous system identified several levels of sensory integration. In the arm axonal tract, the relatively small number of axons suggest that a great deal of processing of motor commands and sensory information takes place at the level of the arm nervous system itself (Altman, 1971; Graziadei, 1971; Sumbre et al., 2001). Although the axons of some of the primary sensory receptors travel directly to the arm nerve cord ganglia, the first level of integration takes place at the level of the individual suckers. Many of the primary receptors on the sucker rim synapse onto the cell bodies and processes of a relatively small number (hundreds

in comparison to tens of thousand) of large encapsulated neurons, mainly touch receptors, which ended (their dendrite) in a capsule organ instead of the other type, which is called "free nerve ending." These encapsulated cells travel to the arm nerve cord ganglia. The extraordinary mobility of amputated octopus arms (Altman, 1971; Sumbre et al., 2001; Wells, 1962, 1978; Wells & Wells, 1956) indicates the extensive role of the axial nerve cord circuitry in controlling arm movements. The axial nerve cord contains networks that can generate a phase lag between the activity in adjacent ventral and dorsal nerve roots without requiring feedback (Gutfreund et al., 2006). Several of the coordinated movements performed by the arm are reflexive (ten Cate, 1928; Uexkül, 1892) and can be reproduced in amputated arms, without central nervous system input (Altman, 1971). One of the reflexes of the octopus arm is the grip reflex (Altman, 1971; Wells, 1978). Sufficiently strong mechanical or chemical stimulation of a sucker causes the arm to bend and adjacent suckers (both distal and proximal) to serially protract toward the stimulus. Since ventral roots of the sucker ganglion project directly to the sucker apparatus, stimulating a ventral root may mimic the sensory signal (mechanical and chemical) from a single sucker and the burst of activity in ventral roots from neighboring ganglia may reflect the motor output to neighboring suckers. Such reflexes are present not only within a single arm, but also as coordinated movements of several arms (Altman, 1971; Rowell, 1963; ten Cate, 1928; Uexkül, 1893). The axial nerve cords of each individual arm, apart from sending fibers to and from the CNS, link the arms by two groups of fibers that together comprise the interbrachial commissure. These fibers serve as a direct connection between neighboring arms, and a circular connection of all arm tracts (Graziadei, 1971). Thus, provided the interbrachial commissure remains intact, neighboring amputated arms will "join" a mechanically stimulated arm (Graziadei, 1965). Coordinated arm movements are the basis of many of the octopus's behaviors; web-over food catching behavior, walking, arm swimming, and food manipulation are several examples.

However, it seems that coordinated arm movements cannot be reproduced in a subject with a removed supraesophageal mass, and, therefore, must involve CNS control or interaction (Shomrat & Kuba, unpublished observation). Anatomically, the large peripheral nervous system of the arms (~350 M neurons, in octopus) is connected by a relatively small number of fibers to the central brain (in octopus ~45 M), suggesting that they send highly processed information to the brain, and that the arms receive mostly high-order motor commands from the brain.

5.7 CENTRAL NERVOUS CONTROL OF MOVEMENTS

One adaptive solution for centrally controlling the highly flexible arms is movement stereotypy. Gutfreund et al. (1998) showed, in octopus, the reaching movement, extending an arm toward a target, is stereotypical. Movement direction is determined by the angle at the arm base, and then, with an initial driving force, a single propagating wave of muscle activation, from arm base to tip, produces a directed, stereotypical, reaching movement. This stereotypical movement can also be produced by stimulation of amputated arms (Sumbre et al., 2001). However, the activity recorded in the isolated nerve cord is not sufficient to account for the complete, whole arm behavior, of arm extension (Gutfreund et al., 2006). Therefore, the suggested mechanism for arm reaching control is that local motor programs, in the arms peripheral nerve system, are sufficient to continue, yet not initiate, the motor program. Gutfreund et al. (2006) demonstrated that mechanosensory information is transmitted from the intrinsic muscles in the axial nerve cord. Thus, control of the complex flexible arm for reaching a target, is simplified by execution of stereotypical movements. In a reaching task, octopuses strongly prefer to use the frontal arms (Byrne et al., 2006), but each one has a "favorite" of these four. In recruitment of a second arm to accomplish a reaching task, a principle of "neighborliness" holds, perhaps because of nervous impulses spread around the interbrachial commissure. Similarly, the octopus fetching movement, bringing an object caught by the arm directly to the mouth, is another example of stereotypical movement. By bending the arm into three "pseudo joints" separated by stiffened arm segments, octopuses use a skeleton-like strategy to execute a point-to-point fetching of an object to their mouth. The location of these joints is determined by the location of the object on the arm, and it is therefore suggested that their formation is a result of a collision of proximal (from CNS toward arm) and distal (from grasping site toward CNS) activation signals (Sumbre et al., 2005). Such a mechanism would again, simplify the movement control, while ensuring success. Richter et al. (2015) were the first to introduce a physical constraint, entering the arm through a small hole, and investigate the adaptability of stereotypical bend propagation in reaching movements and the pseudo-limb articulation during fetching (Fig. 5.5). They showed that octopuses immediately adjusted their movements to the constraint. In addition to reaching movements, the authors described an additional movement, which they termed a waving like movement. Octopuses obtain the food reward using one of the two movements; the retrieval of this food reward, however, was not done by a standard fetch movement but by pulling in the

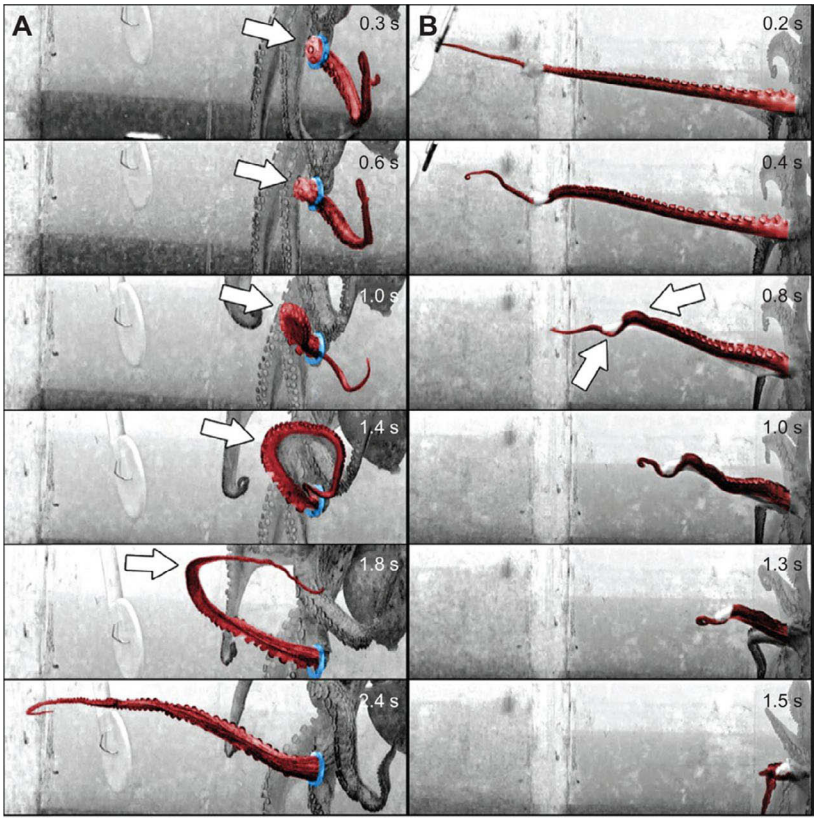

FIGURE 5.5 Octopus movements under constrained conditions (Richter et al., 2015). Octopuses are behind a Perspex wall and reach or fetch through a hole with a single arm (indicated in red). (A) Straight reaching toward a target is done with a typical bend propagation, which is set up by building up an arm loop at the hole. White arrow indicates arm bend. Seconds 0.3–0.6 show building up of the arm loop; seconds 1.4–1.8 show bend propagation toward the target; 2.4 s frame shows arm hitting the target. Blue ring highlights the hole. (B) Straight fetching of a food item (white object). Frame at 0.2 s shows the arm attached to target; seconds 0.4–1.5 show linear point-to-point pull-in movement. Arrows at 0.8 s mark two bends of the S-shaped grip of the food item (white). Colors, brightness and contrast were altered to highlight arm movements.

arm until the food reward had passed through the hole. While these octopus arm movements are the most studied, others are likely to exist. For example, squid and cuttlefish tentacle extension when striking food might be a good candidate action (Messenger, 1973) for future detailed kinematic and physiological analysis. While a variety of movement strategies were documented in octopuses (Huffard, 2006), how the coordination of arms during crawling

is achieved is an unsolved question. While soft-bodied robots used a simple octopus inspired method of crawling along the sea floor (Calisti et al., 2011) the exact documentation of this behavior in octopus was missing. Levy et al. (2015) showed that, to create a thrust in a given direction, octopuses use a simple pushing by elongation mechanism. Using a simple model, Levy et al. (2015) showed how octopuses could crawl in any direction independent of their body orientation.

Another adaptive solution to movement control is to use "modal action patterns," in the case of these MAPs, some of aspects of the behavior stay invariant while others are modified in response to a stimulus or situation (see Barlow, 1968; Pellis et al., 2009). These behavior sequences, unless interrupted, will continue to the end even without sensory input. Such pattern behaviors have been studied in many species (fish, reptiles, birds, mammals, insects), however, only few have been scientifically investigated in cephalopods. The predatory sequence of cuttlefish hunting composed of three stages: orientation, approach and strike (Messenger, 1968), is one such example. More thoroughly studied are the sand-digging behaviors of sepiid and sepiolid cuttlefish (Anderson et al., 2002, 2004; Mather, 1986; von Boletzky & von Boletzky, 1970). Sepiolids blow the sand out from under them with alternating forward and backward bending of the flexible funnel, then sweep a particular pair of arms across the sand surface anterior-laterally to cover their dorsal surface with a thin layer of sand. This sequence is not completely fixed and can be modified by variations in the environment, like substrate granularity and depth (Mather, 1986). *Rossia*, when placed on gravel, attempts arm sweeps even though no gravel was picked up (Anderson et al., 2004). There are consistent differences in these "modal action patterns" amongst different sepiolid species (von Boletzky & von Boletzky, 1970), similar species differences can be found in other animals, for example, courtship postures in ducks (see Bradbury & Vehrencamp, 2011).

The CNS control of movements in cephalopods follows a hierarchical structure (Young, 1971). However, there has been only little progress in our knowledge on the identity and amount of CNS involvement in motion control, planning, and execution (for a review, see Nixon & Young, 2003; Zullo & Hochner, 2011). In more recent work, Zullo et al. (2009) stimulated different areas of the higher motor regions, in the supraesophegeal lobes, and they found no evidence of somatotopic motor representation. Movements were not represented in specific spatial regions of higher motor areas, as they are in vertebrates. Gutnick et al. (2011) highlighted our lack knowledge about how the movements of the arms are controlled by CNS and visual system. They showed, contrary to previous assumptions, that octopuses

could complete an operant task by controlling single arms in complex movements using visual guidance. Although, this has provided the first evidence that octopuses have knowledge and control over single arm location and movement, however, the extent of this control remains unknown.

5.8 CEPHALOPOD CAMOUFLAGE SENSORY-MOTOR SYSTEM

While we tend to think of motor control in terms of actions, cephalopods have a second unique motor system, the chromatophore skin pattern system. The active camouflage system of coleoid cephalopods is a sophisticated system that has a unique physical structure. Cephalopods used to avoid predators, stalk prey and for inter- and intraspecies communication (Borelli et al., 2005; Messenger, 2001; Hanlon, 2007; Hanlon & Messenger, 1996; Wells, 1978).

Chromatophores, small pigment sacs surrounded by radial muscles, are each under direct motor control (Fig. 5.6). Chromatophore muscle activation results in an expansion of the pigment sac, revealing the pigment. Muscle relaxation, by contrast results in the masking of the pigment (Budelmann et al., 1997). There are several chromatophore colors—yellow, red, brown, and black, arranged three-dimensionally within the skin (Messenger, 2001). Chromatophore density ranges from 8 to 230/mm^2, depending on species, and also varies across the lifespan (Packard, 1985), with pelagic paralarval stages of the animal often having few and widely spaced chromatophores. Thus, the relative area covered by the pigments can control the intensity of a small patch of skin. By changing the relative size of the pigment "color channels" the color of a given skin patch can be varied. Additionally, when the chromatophores are contracted, the underlying leucophores and iridophores are revealed (Denton & Land, 1971). Iridophores are multilayer stacks of thin electron-dense platelets, made of chitin or protein (Cooper et al., 1990), usually arranged parallel to the skin, providing the blue and green components of skin color. While they had been assumed to be passive reflectors, Cooper et al. (1990) showed that ACh hormones could change the state of the material in the platelets and result in a change in iridescence, which may be involved in signaling. Leucophores are broad-band reflectors (Packard & Sanders, 1971), while they appear as "white spots" (Cloney & Brocco, 1983), it was shown that they reflect the predominant wavelengths in the environment, and are likely important for the matching the color background general color resemblance to the background that cephalopods can use as part of camouflage (Hanlon & Messenger, 1996). Due to the fast time scale

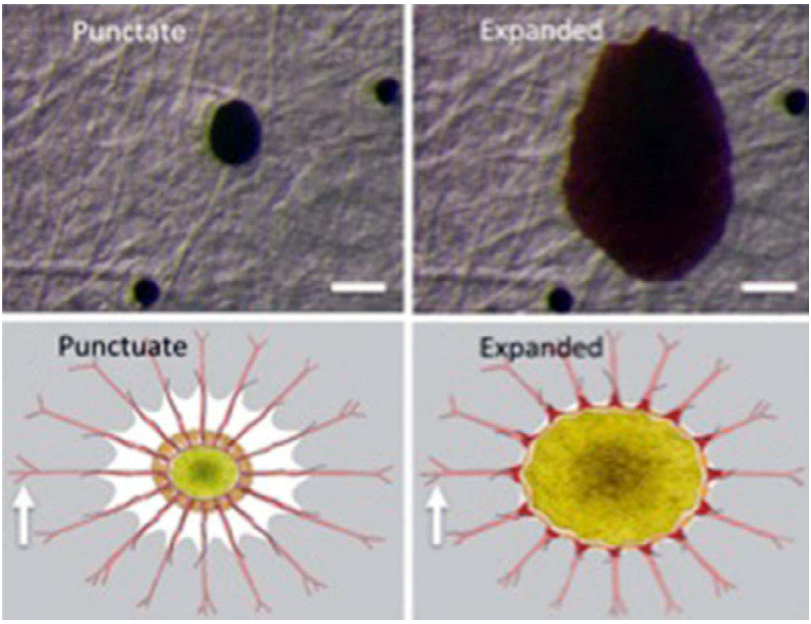

FIGURE 5.6 (A) A single retracted chromatophore. Scale bar=150 μm. (B) A single expanded chromatophore. Scale bar=150 μm. (C) Schematic diagram of a retracted chromatophore. (D) Schematic diagram of an expanded chromatophore. In (D) and (E), the pigment cell (yellow) and portions of the radial muscle fibers (salmon) are in the chromatophore sinus (white). Pigment cells, spherical when retracted (D), have a highly folded cell membrane (orange) that unfurls when the pigment cell expands into a disk. Muscle cells extend radially from the pigment cell, through the sinus, then arborize deep within the connective tissue (white arrows). The muscle fiber active zone is in the chromatophore sinus; compare relaxed and contracted (red, in (E)) muscle fibers. Diagrams are not drawn to scale.

of muscle contraction, switching between patterns can occur in hundreds of milliseconds. Finally, benthic octopuses and cuttlefish possess the capability, unique in the animal kingdom, to dramatically and quickly change their skin from smooth and flat to rugous and three-dimensional (Packard, 1995). The organs responsible for this physical change are the skin papillae. In a study by Allen et al. (2013), small dorsal papillae from cuttlefish (*Sepia officinalis*) were preserved while in their retracted or extended state, and examined with a variety of histological techniques including brightfield, confocal, and scanning electron microscopy. Analyses revealed that papillae are composed of an extensive network of dermal erector muscles, some of which are arranged in concentric rings while others extend across each papilla's diameter. Like cephalopod arms, tentacles, and suckers, skin papillae appear to function as muscular hydrostats (Allen et al., 2013, Fig. 5.7).

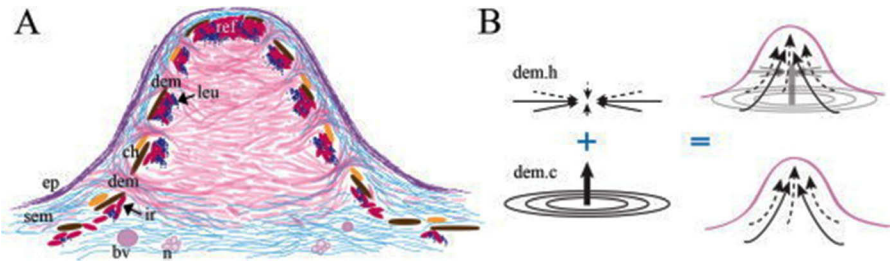

FIGURE 5.7 From Allen et al. (2013): Schematic drawing illustrating small dorsal papilla structure and functional morphology. (A) Drawing of an extended SDP in cross section with various skin elements labeled. (B) Model of SDP extension with force directions. Circular dermal erector muscles (dem.c) near the base of the papilla contract and lift superjacent skin layers away from the mantle (left, bottom). Simultaneously, horizontal dermal erector muscles (dem.h) contract to pull the outer skin layers toward the center of the muscular core (left, top). Together, these muscle contractions result in papilla extension (right). Blood vessel (bv), dark purple; chromatophore pigment cell (ch), yellow or brown; dermal erector muscle (dem), circular (dem.c) or horizontal (dem.h), pink; epidermis (ep), purple; iridophore (ir), dark pink; leucophore (leu), dark blue; nerve (n), light purple, structural reflectors (ref).

The central control of dynamic camouflage depends on retinal input. In *O. vulgaris* when visual input is disrupted by retinal lesion, color changes stop and octopuses assume a pale body coloration (Wells, 1978). However, camouflage is not affected by concealing the body from the animal's own eyesight, indicating that visual feedback plays no role in pattern formation (Hanlon & Messenger, 1996). Although cuttlefish are capable of creating colorful patterns, they are color blind, and easily "tricked" by presenting stimuli of varying color but equal brightness (Marshall & Messenger, 1996; Mäthger et al., 2009). Recently, Kingston et al. (2015) discovered that cephalopod dermal tissues, and specifically chromatophores, may possess the requisite combination of molecules required to respond to light. Subsequently, Ramirez & Oakley (2015) demonstrated that in *O. bimaculoides*, there is eye-independent, light-activated chromatophore expansion (Fig. 5.8).

Scientific investigation of cephalopod camouflage has concentrated on several levels of patterning behavior. The first, which covers the widest variety of cephalopods, is a descriptive analysis of body patterns and their environmental and behavioral contexts (summary in Hanlon & Messenger, 1996, visual catalogue in Borelli et al., 2005). More recent work includes descriptions of *Octopus insularis* (Leite & Haimovici, 2009; Leite & Mather, 2008), the reproduction patterns of *Sepioteuthis australis* (Jantzen & Havenhand, 2003), and the graphic model of Caribbean reef squid, *S. sepioidea* (Byrne et al., 2003).

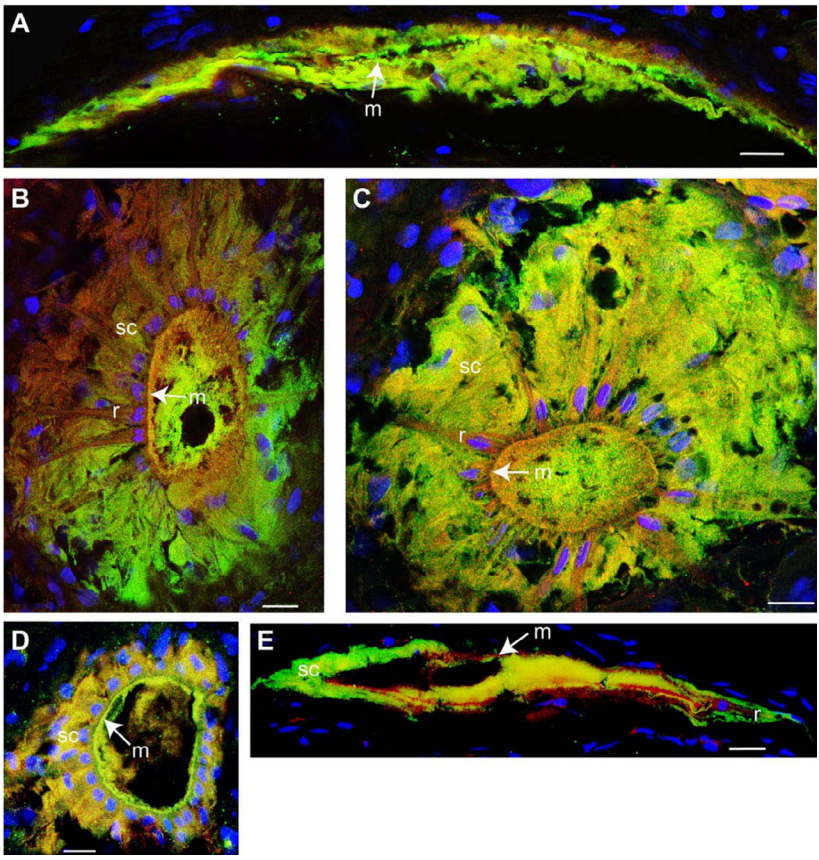

FIGURE 5.8 Immunohistochemical labeling of rhodopsin and retinochrome in various tissues of *D. pealeii* (Kingston et al., 2015). (A) Ventral mantle, (B) dorsal mantle, (C) fin, (D) arm 1, (E) tentacle. Rhodopsin (green) and retinochrome (red) are present in chromatophore (pigment cell) membranes, radial muscle fibers and sheath cells. Yellow indicates overlap of rhodopsin and retinochrome label, suggesting that some of these cells express both proteins. Blue represents DAPI labeling of nuclei. m, pigment cell membrane; r, radial muscle fiber; sc, sheath cell. Scale bars: 25 μm.

The second level of analysis has focused on the connection between visual perception and pattern. Dynamic camouflage is the result of analysis of the visual field, and the extraction and processing of relevant information. The resultant information is then used for the selection of a body pattern. However, which features of the visual background are important is only partially understood, using patterns as the measured response for discerning the perception and processing of visual information. These experiments, which have focused mainly on cuttlefish, have outlined three camouflage

patterns: dimantic, mottle, and uniform. These three categories are likely an over simplification of a much more complex pattern system, however, using visual stimuli specifically designed to elicit these three patterns (like checkerboards driving cuttlefish deimantic patterns) many important aspects of visual perception have been explored. Mäthger et al. (2009) shown that checkerboard patterns would normally result in deimantic patterns, when composed of yellow and blue of matched intensity resulted in a uniform pattern, thus demonstrating that cephalopods are color blind. Similarly, the effects of contrast (Barbosa et al., 2008), spatial frequency and edges (Chiao et al., 2013), intensity (Chaio et al., 2007), and polarization (Shashar et al., 1996) have been tested, as well as differences in camouflage between cuttlefish species (Shohet et al., 2007).

The last aspect of camouflage discussed in this chapter is the issue of central and local motor control of chromatophores. The cell bodies of the motorneurons directly innervating the chromatophore muscles are situated in the subesophageal lobe, the chromatophore lobe (ChL). The nerves reaching largest chromatophore field, in the mantle, leave the ChL in the palial nerve bundle, pass through the stellate ganglion and then spread to different areas of the mantle (and in squid and sepia the fins). Each motor-neuron innervates a small number of chromatophores, and each chromatophore is innervated by several motorneurons. Additionally, in some cases there is electrical coupling between muscles of adjacent chromatophores, and there are receptors for several neurotransmitters (Andrews et al., 1983; Gaston & Tublitz, 2002, 2006; Loi & Tublitz, 2000; Loi et al., 1996; Messenger, 2001). In order to create coherent patterns, the activation of chromatophores, from the level of single chromatophores to activity of entire fields, must be coordinated. Packard outlined some control principles that might underlie this system (Packard, 2006; Kelman et al., 2008). Generally, there is evidence for multiple levels of coordinated activity: from motor units, chromatophores innervated by the same motor neuron, to elements or components, distinct pattern features of coordinated chromatophore activity (eye spots), and behavioral components, coappearing elements creating a full pattern. Several recent studies have attempted to isolate these pattern building blocks from images of cuttlefish patterns using methods such a principle component analysis and independent component analysis (Anderson et al., 2003). Understanding the hierarchies and connections of pattern elements can then suggest the organization of the neural control.

One example of making such a connection is the recent work by Laan et al. (2014). While many cephalopods show traveling wave patterns on their skin (Mather & Mather, 2004; Laan et al., 2014), few attempts have been

made to look at the clear function of this pattern. Traveling waves on cephalopod skin can generally be seen in at least two different circumstances. One is seen in uncoordinated waves of pattern activation that follows no specific direction or pattern. This can either be induced by application of neurotransmitter (Tublitz et al., 2006) or in parts of the skin no longer connected to the central nervous system. Laan et al (2014) were the first to see the potential use of these traveling waves—or passing clouds—as a general model to study wave activities in organisms. They showed that there are four on the body that have different directionalities of wave patterns (Fig. 5.9). While these regions are not always active simultaneously, they are synchronized

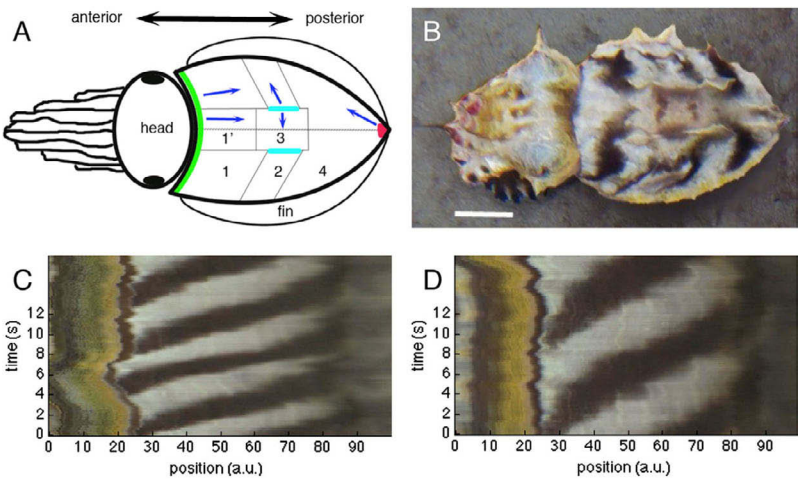

FIGURE 5.9 Laan et al. (2014) traveling waves on the cuttlefish *Metasepia tullbergi*. (A) Schematic of the animal with depiction of the four regions of wave travel on the dorsal mantle. The regions and wave displays are almost always bilaterally symmetric. Regions of wave travel (with boundaries as thin lines) are indicated on left side of the animal; direction of travel within each region is denoted by arrow on right side. Each region supports only one direction of travel, and waves do not cross-region boundaries. The green line marks the mantle rim wave initiation zone; the cyan line indicates the middle wave initiation zone; the crimson spot indicates the third wave initiation zone at the mantle tip. Region names are as used in text: 1 indicates anterior, 10 indicates anterior dorsal, 2 indicates middle, 3 indicates central, and 4 indicates posterior. (B) Still video image of one of the experimental animals during wave display. Intensity of back-ground is reduced, and contrast of the animal is enhanced to improve visibility. Scale bar represents 1 cm. (C) and (D) Time position intensity plots for two wave displays with two different wave propagation velocities (fast in (C), slow in (D)). Each column depicts the pigmentation of a one-pixel-wide parasagittal strip in region 1 of the animal's body over time (sampling rate is 5 Hz); rows show the same strip along the body of the animal at successive times. Motion thus appears as diagonal lines, whereas static patterns form vertical columns. Jaggedness is due to superimposed motion of the animal or contractions of the mantle.

in activity and maintain a constant wavelength and a period-independent duty cycle. While traveling these waves can even disappear transiently and reappear in a different position ("blink"), revealing ongoing but invisible propagation. Their findings rule out wave propagation mechanisms based on delayed excitation from a pacemaker, but are consistent with two other alternatives, such as coupled arrays of central pattern generators and dynamic attractors on a network with circular topology.

It is clear, that the motor system of cephalopods is both unique and fascinating. There are many promising avenues of research, from the behavioral variables of the camouflage system and its variations given a wide variety of cephalopod habitats to its use in conspecific signaling and mimicry.

5.9 LEARNING AND COGNITION

The modern study of cephalopod neurobiology dates to the 1940s, when J. Z. Young, at the Stazione Zoologica in Naples, began a comparative study of the behavior, learning and neuroanatomy in cephalopods (Borelli et al., 2008; Nixon & Young, 2003). *O. vulgaris*, and to a lesser degree *S. officinalis*, emerged as excellent laboratory animals, and have remained the central cephalopod models for behavioral and electrophysiological research. Over the years, there have been many studies on learning in cephalopods (in no way comparable to the wealth of studies on vertebrates), but we will only cover several important examples highlighting the unique place of cephalopods in the animal kingdom (for a detailed review, see Hanlon & Messenger, 1996).

Unlike the last remaining noncoleoid cephalopods the Nautiloidea, which are limited to a narrow ecological niche, coleoid cephalopods are predators, evolutionarily adapted to a variety of ecosystems. As is the case for many animals, their life is centered on three major missions: eat, avoid being eaten, and reproduce. Perhaps, as a result of coevolution, and therefore competition, with bony fish, coleoid cephalopods are highly mobile, in some cases generalist predators, capable of fast learning, and possessing many complex, "vertebrate like," behaviors (Packard, 1972).

5.10 EARLY LIFE

Every animal must either rely on an embedded, genetically acquired, set of behaviors, or it must learn new behaviors during its lifetime. The study of

post-hatching behavior, and even the effects of pre-hatching stimulus exposure, has been essential for understanding both the innate behaviors and the plasticity of these early stages (Darmaillacq et al., 2014). However, many cephalopods have a paralarval stage, lasting weeks to months, in which hatchlings are small and free swimming and often have subtle morphological differences from the adults (Villaneueva et al., 1995). Thus, much of the research has focused on the easily reared, and reasonably abundant, *S. officinalis*, which hatches as a miniature, yet relatively similar, version of the adult (Guerra, 2006; von Boletzky, 1983) and hunts using the same visual cues.

Early research (Wells, 1962) on the innate preferences of naïve, newly hatched cuttlefish demonstrated that hatchlings had a strong preference for linear figures extending vertically. They hypothesized that this preference is a result of a resemblance between the shape and movement of the stimuli, and the crustacean *Mysis*, a common prey item. The innate preference of hatchlings to vertical shapes was confirmed when it was found that they had a strong preference for shrimp over crabs and fish (Darmaillacq et al., 2004). However, this innate preference is not unchangeable, exposure of newly hatched cuttlefish, and even eggs in the last stages of development, to various stimuli shapes their subsequent preferences (Darmaillacq et al., 2014). Hatchlings fed only crabs, showed a preference for crabs at 7 days; the length and intensity of exposure during this sensitive period predicted the strength of the preference (Darmaillacq et al., 2004). These preferences can be fine-tuned, while normal preference is directed toward black crabs, exposure to only white crabs will shift the preference toward them (Guibé et al., 2012). This fast, age sensitive but persistent learning, eludes toward the process of imprinting. Surprisingly, this early learning is possible even before hatching, as the crab-prey preference learning can be induced in the week before the cuttlefish hatch (Darmaillacq et al., 2008). *S. officinalis* eggs are opaque, and the egg capsule is deposited together with ink, however, as the eggs grow and the capsule stretches they become "clearer." At this stage, presentation of different shapes in the egg vicinity shifts preference post-hatching (Darmaillacq et al., 2008).

In contrast to these fast and long-lasting changes, other associations are not learned as easily. Hatchlings do not learn to stop attacking an unavailable prey item (presented in a clear tube). In the first month of life, cuttlefish exhibited short-term memory, but were did not have long-term memory (Messenger, 1971; also see Dickel et al., 1997; Agin et al., 2006). This increase in learning capacity as the animal develops is concomitant with the continued development of the vertical lobe post-hatching (Dickel et al., 1997), a brain area

important in the acquisition and encoding of information storage. Similarly, in some vertebrates it was shown that the neural substrate underlying the processes of early learning and predisposition is different and even localized in different brain regions (Cole et al., 2005; Bolhuis & Honey, 1998).

5.11 NON-ASSOCIATIVE LEARNING

Two of the simplest forms of learning are habituation and sensitization. Habituation is the relatively persistent waning of a response as a result of repeated stimulation without reinforcement. In an experiment with a blind octopus (Wells & Wells, 1956), a plastic cylinder placed on the arm of the animal is passed under the web to the mouth, examined, and rejected. If the same object is repeatedly presented, after a few trials, the octopus stops passing it to the mouth. After more trials the octopus spends only a few seconds examining it. In intact octopus, habituation has been shown in visual tasks (Mather & Anderson, 1999; Kuba et al., 2006a,b). Recently, Samson et al. (2014) showed that cuttlefish (*S. officinalis*) habituate to repeated sound stimuli. Cuttlefish reduced the number of inking and jetting responses over 30 exposures to a 200-Hz tone stimulus. Habituation of a visual response has been demonstrated in bay squids *Lolliguncula* (Long et al., 1989), showing the decline of escape jets and ring patterns on the mantle with repeated presentation of a fish predator model. The squids also showed dishabituation to the predator model after a threat stimulus.

Sensitization, or the increased likelihood of an animal responding to a stimulus, has been demonstrated clearly in *O. vulgaris*. Either reward or punishment that takes place before the presentation of a test shape will, respectively, decrease or increase the likelihood of an attack on the stimuli. This has been shown for tactile discriminations (Wells & Wells, 1956; Wells, 1978) as well as olfactory stimuli (Chase & Wells, 1986). The importance of sensitization usually dwindles during discrimination training, as longer term changes become entrenched.

5.12 ASSOCIATIVE LEARNING

Associative learning involves long-term changes in behavior as a consequence of an association between particular sets of events. Although learning experiments have investigated the use of several other senses, especially in the case of the octopus, most research has focused on operant conditioning

using visual stimuli (Hanlon & Messenger, 1996). Classical conditioning has been tested in a color vision study with *Eledone* and *Octopus*, and in an appetitive conditioning study in *Octopus cyenea* (Sanders, 1975; Papini & Bitterman, 1991). Early reports of avoidance learning (see Sanders, 1975) were later demonstrated in experiments in which hermit crabs carrying anemones, or other food species next to an anemone, were offered to various octopuses. After being stung by the anemone, octopuses avoided approaching the food items (Boycott, 1954; Sanders, 1975). MacLean (1983) compared the ability of *Octopus joubini* and *Calappa* sp. crabs in attacking hermit crabs carrying large anemones. While the octopuses learned to take indirect routs and managed to get to the hermit crab without being stung, the crabs never managed to overcome the direct approach trying to break down the shell getting stung and quitting. The study of operant conditioning in cephalopods has been more popular (for a review, see Hanlon & Messenger, 1996; Sanders, 1975). Octopuses learn to attack one of a pair of stimuli when reinforced either with a food reward or with a small electric shock. Using the innate inclination of octopuses to attack, using both positive and negative reinforcement, learning is quick with easy discriminations. However, octopuses never reach the levels of performance achieved by mammals in discrimination tasks. In visual discrimination training, the simultaneous presentation of the stimuli achieves higher scores than the successive presentation, which is also true for vertebrates (Sutherland & Muntz, 1959). An important output of the series of discrimination experiments was that octopuses show some stages of visual information processing similar to mammals. Octopuses show stimulus generalization, which can be demonstrated in a "transfer test." For example, octopuses that were trained to attack a small solid square attacked a small outline square much more frequently than a large outline square, even without a reward (Sutherland, 1960). Other cases of stimulus generalization were shown for size invariance and brightness invariance (Messenger, 1981) and for degrees of roughness (Wells & Young, 1970). Octopuses also show receptor generalization. After being trained to make a visual discrimination using one part of the retina only, the performance was significantly better than chance when the task was presented to a different part of the retina (Muntz, 1962). The same applies to interocular transfer (Muntz, 1961) and tactile discriminations (Wells, 1959). An octopus trained to accept and pass an object to the mouth, or reject an object when presented to one arm, rejected the same object when tested on other arms (see Wells, 1978). However, the transfer between arms is not immediate and took 1–3 h to reach the same level of performance in other arms. Cue-additivity has also been demonstrated in octopuses. With two or more relevant cues, the

animals learned significantly faster than with only one cue (Sanders, 1975). This has been tested with combinations of size and shape (Sutherland et al., 1965) and with brightness and orientation (Messenger & Sanders, 1972). Interestingly, the octopuses do not attend equally to both features of the stimulus. Some learn the discrimination in terms of one cue, some in terms of the other. Reversal learning has also been studied in *Octopus* (Mackintosh, 1965), octopuses made fewer errors with progressive reversals, but the performance seems to depend on earlier training regimes, similar to rats. There is some evidence that in *Octopus* the visual and tactile memories may interact (Messenger, 1983). Octopuses were trained first with a tactile and then a visual discrimination (plastic balls in black, white, or clear; rough or smooth). It was found that a negatively associated visual memory was sometimes able to interfere with a previously learned positively associated tactile memory (Messenger, 1983).

While many experiments were conducted with *O. vulgaris*, visual discrimination experiments have been carried out with various species of Octopus (for reviews, see Sanders, 1975; Hanlon & Messenger, 1996; Wells, 1978). In decapods, *Sepia* (Messenger, 1977), *Lolliguncula* (Allen et al., 1985), and also *Todarodes* (Flores, 1983) have been tested on visual discriminations.

5.13 SPATIAL LEARNING

For many mobile animals, goal-directed movement in space is vital and has been studied in species from several phyla. Field observations of various cephalopod species have shown that cephalopods are mobile, often over long distances (Hanlon & Messenger 1996). Several octopus species have been observed traveling between semipermanent dens and foraging areas, some as far as 40 m away (for a review, see Alves-Jozet et al., 2014). In order to achieve such goal oriented travel requires the ability to remember locations, to orient in space, and to create new maps when new areas are entered. In laboratory conditions, octopuses and cuttlefish have been shown to explore novel environments (Boal et al., 2000; Karson et. al., 2003).

The representation of spatial information, and therefore the ability to orient within it, has been divided into several hierarchical levels of complexity (Wiener et. al., 2011). For various octopus and cuttlefish species, several aspects of navigation, spatial orientation, and spatial memory have been tested, within the lower levels of spatial representation complexity. Laboratory experiments have tested “Sensorimotor Processes”: the simplest

level, which includes behaviors, such as taxes and beacon homing; and "Spatial Primitives": the second level, which includes behaviors such as view matching, combining beacon with movement pattern, and navigation using multiple route cues. Recently, Crook and Basil (2013) showed that the chambered Nautilus is capable of navigation and spatial orientation.

Testing *Octopus maya*, in a dry T-maze, Walker et al. (1970) showed that octopuses could be trained to find a goal location with water. Octopuses were placed in starting box in the center of the T, and learned to consistently crawl into one side of the T, opposite any initial side preference. However, since the visual information from room was not occluded, it could not be determined whether octopuses used information about self-motion and turning information, extra-maze visual cues or any combination of cues. Demonstrating spatial memory in *O. bimaculoides*, Boal et. al (2000) placed octopuses for 23 h in a novel environment with one open and one closed escape burrow. After a 24-h delay, when octopuses were reintroduced to the environment, and the water level was lowered, the octopuses went first to the open burrow. Spatial memory in *S. officinalis* and *S. pharaonis* was shown using an arena with two exits, and a central cue indicating which of the two exits was open (Hvorecny et al., 2007). Cuttlefish learned to select the correct exit, even when two different central cues were intermixed. Alves et al. (2007) showed that most of the tested cuttlefish (*S. officinalis*) used motor cues (turn made at T) to solve a T-maze task, rather than distal visual cues. However, when presented with proximal, rather than distal, visual cues, half the cuttlefish used the visual cues and the other half used motor cues. In a similar experiment, Cartron et al. (2012) showed that when trained with a combination visual cue (polarized light and visual pattern), after learning, cuttlefish could solve the task using either of the visual cues. Therefore, during learning, the animals must have processed both visual cues.

Although it was shown that cuttlefish could use both motor and visual cues, Alves et al. (2008) found that preferred orientation strategies varied with differences in sex and age. While mature males were more likely to use visual cues, mature females and immature cuttlefish preferentially used motor sequence learning strategy.

Graindorge et al. (2006) investigated the effects of vertical lobe lesions on the performance of cuttlefish in a navigation task. Lesions in different parts of the vertical lobe resulted in different behavioral alterations. If the lesions were applied to the dorsal part of the vertical lobe system, the only significant difference from control animals was an elevated general activity in an open field test. They hypothesized, that as the vertical lobe is thought to be involved in inhibition of behavior (Sanders, 1975), a lesion might

result in the loss of a central "off" switch regulating the amount of locomotor activity. Interestingly, these behavioral alterations were caused by removal of very small (only 3–5%) amounts of vertical lobe tissue. A second group of animals, that had lesions applied to the ventral part of the vertical lobe, showed significantly reduced long-term retention of a learned navigation task. However, as the authors point out, these lesions might also have damaged the tracts that run between the frontal lobe and the vertical lobe system.

Although phylogenetically disparate, there are striking functional and morphological similarities between the cephalopod VL and the mammalian hippocampus (Young, 1991, 1995). Like their mammalian analog, the vertical lobes are essential for consolidation of new memories but not for short-term memory or retrieval of old memories. Morphologically, the vertical lobe network is organized as a series of matrices of crossing fibers, and the input to the lobe from the superior frontal lobe innervates the vertical lobe neurons in en passant manner, resembling, for example, the hippocampal CA1 regions. In the hippocampus, place cells are pyramidal neurons that fire selectively as a function of the animal's spatial location in the environment (the neuron's "place field") and are considered essential for spatial memory (Hafting et al., 2005). In addition, it has been suggested that place fields are shaped largely by some form of "reverberatory" network dynamics (also suggested to be important for working memory). Based on anatomical studies which revealed reciprocal connections, Young (1971) suggested that working memory is implemented by a reverberating loop in the vertical lobe system), and as mentioned above, Graindorge et al. (2006) showed that lesions in the VL impair spatial learning in cuttlefish. Taken together, this evidence makes it tempting to speculate about the existence of hippocampal-like place cells in the vertical lobe system. Such a system would indicate the strength of the biological principle of neuronal units for mapping and remapping space. Moreover, it would provide the opportunity to explore those mechanisms on the simpler neural circuitry found in cephalopods. Such a research, for example, may shed light on the hypothesis of cognitive mapping and episodic memory processes.

5.14 "HIGHER ORDER" BEHAVIOR—A CASE FOR THE OCTOPUS

In July of 2012, a group of scientists from a wide variety of areas concerned with consciousness, signed the "Cambridge Declaration on Consciousness," which declared that nonhuman animals, including mammals, birds and even

octopuses, had the neural substrate to generate consciousness. Octopuses represent an important and interesting and unique model for comparative cognition (Fig. 5.10). They are highly specialized animals with a unique morphology, intricate behavior and the most complex central nervous system among all invertebrates. Animal cognition has advanced greatly in recent years, and new findings in both vertebrates and invertebrates have raised the general interest in the evolution of cognition in the animal world. While most research focuses on primates, birds and to some extent, insects, and although cephalopods show great potential, experimental data on higher order cognition in cephalopods is scarce.

FIGURE 5.10 Different brain areas involved in conscious processes in mammals, birds and octopuses The nature and origin of brain areas responsible for higher cognition in cephalopods remains understudied and speculative. (Reprinted from David B. Edelman, Bernard J. Baars, Anil K. Seth, "Identifying hallmarks of consciousness in non-mammalian species)," in Consciousness and Cognition, 2005 Mar;14(1):169-87. Used with permission from Elsevier.

Gallup et al.'s (2002) mirror test has been used as the benchmark for self-awareness in animals. When an animal looks into a mirror, does it link exploratory behavior seen in the image to itself, does it recognize the image in the mirror as "me" by other means? Octopuses failed even the easier aspect of the mirror test. Octopuses are clearly capable of social learning even if the exact mechanisms that octopuses can employ to extract information from a conspecifics' actions are not yet fully understood (Fiorito & Scotto, 1992; Fiorito & Chichery, 1995). When presented with a mirror (Mather & Anderson, pers. com.), octopuses oriented toward their image in the mirror, but there was no difference in their behavior in this condition, compared with a viewing a conspecific. Interestingly, in fish (Elwood et al., 2014), only an inverted mirror image resulted in an appropriate response. Therefore, octopuses might have similar issues of behavioral lateralization—only the appropriate side of an opponent's body might trigger a response. Additionally, octopuses had equal difficulty carrying out a detour task when the prey was visible through a window (Wells, 1964). Tricarico et al. (2011) used a

familiarity paradigm to suggest that octopuses reacted differently to known individuals. This interesting topic clearly warrants further in depth research, and it is an important step toward the understanding of self and other.

Self-awareness and consciousness are standard properties of highly developed nervous systems (Baars, 1997; Bekoff & Sherman, 2004). Often they co-occur with development of social systems, or living in environments with highly unpredictable resources, and in animals that undergo long-developmental stages (Fig. 5.2). In vertebrates, they have been correlated to the thalomocortical system and the mesencephalic reticular formation (Srinivasan et al., 1999; Griffin, 1976; Griffin & Speck, 2004; Edelman et al., 2005).

Octopuses are reported to be opportunistic feeders, forage over large territories and migrate vertically and horizontally (Kuba & Mather, 2013). Although the juvenile stage of octopuses is not long, it is planktonic paralarvae differ in morphology, physiology, ecology, and behavior from the adult benthic stage. Especially, brain areas responsible for the processing of tactile information and also part of the VL system are not fully developed until a certain age is reached (Nixon & Young, 2003). The octopus brain with about 140 million neurons (Hochner, 2010; Wells, 1978; Young, 1971) is large and complex compared to other invertebrate brains. Their brain-weight-to-body-weight ratio is comparable to that of vertebrates and the vertical lobe, which is involved in long-term memory shares some functional features with the vertebrate hippocampus and the insect mushroom bodies (Hochner, 2012; Zullo & Hochner, 2011; Young, 1995). In many behavioral studies, octopuses show complex learning and memory abilities, solve discrimination tasks (Kuba & Mather, 2013; Nixon & Young, 2003), and even exhibit exploration and play like behaviors (Kuba et al., 2014).

Neisser (1967), talking about humans, described cognition as "all the processes by which the sensory input is transformed, reduced, elaborated, stored, recovered and used." Shettelworth (2010) produced a similar definition for animals as "the mechanisms by which animal acquire, process, store and act on information from the environment." She also mentions that we should not be concerned with first-order processes (operating on direct perceptual input, as cuttlefish camouflage might do) when we investigate cognition.

Why did cephalopods evolve into fast learners with high behavioral flexibility? Packard (1972) pointed out that the coleoids had evolved in the Jurassic period, at the same time that bony fishes were undergoing an explosive radiation. These fishes, which still dominate the world's oceans and have complex behavior themselves (Brown et al., 2011), act as cephalopods' prey,

predators, and even scavengers. Richardson (2010) suggests that animals developed cognitive ability when other animals became significant. He sees the animal as needing the ability to integrate complex and fleeting information into an internal image of the object, and then using this to predict the behavior of the object and to produce useful responses to it. Cephalopods abandoned the protective shell, speeded up the molluscan metabolic efficiency, adopted fast locomotion and condensed a centralized brain to control these calculations. Godfrey-Smith (2002) suggests that a demanding environment will produce pressure for cognitive development and Mather et al. (2014) points out that cephalopods evolved in the most complex and demanding near-shore oceanic environment.

Scientists have also been inspired by Griffins' (1976) contention that animals have awareness. He contended that animals have conscious intentions, beliefs and self-awareness, and they think about alternatives and make plans. Although the mental lives of animals (and often humans) is difficult to perceive and often nearly impossible to prove, the suggestion has stimulated much research. Mather (2008) has suggested that they may analyze and store information using a process similar to Baars' (1997) "global workspace," with an attentional spotlight. Certainly, advancement in understanding both their complex brain and acute cognitive ability will help us understand brain behavior relationships in a cephalopod model. With their formidable learning ability, complex brain, domain generality, and acute sensory perception, octopuses are compelling candidates for joining the group of "thinking" animals.

KEYWORDS

- **cephalopods**
- **brain-behavior linkage**
- **movement control skin display system**
- **learning and intelligence**

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CHAPTER 6

ENDOCRINE CONTROL OF GAMETOGENESIS AND SPAWNING IN BIVALVES

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CONTENTS

ABSTRACT

Reproductive process represented by gametogenesis and spawning accompanied by oocyte maturation and sperm motility activation in bivalves undergoes under the endocrine and neuroendocrine control as well as other animals. General morphology of germ cell and somatic cell supporting development of germ cell in bivalves are described in this chapter. Endocrine and neuroendocrine factors and their signal transduction which involve in reproductive process are additionally reviewed based on the literatures described morphological, physiological, pharmacological, biochemical, and molecular biological aspect in reproduction.

6.1 INTRODUCTION

The class name of Bivalvia is a synonym of Pelecypoda in taxonomy, which can be easily recognized because of two bisymmetric valves to cover their soft bodies. Bivalve species are commercially important organisms harvested as capture and aquaculture products in fishery industry. The study on the reproduction of bivalve species is an essential subject for the biological evaluation of brood stock and environmental evaluation of aquaculture area. Reproduction and broad-ranging biological phenomena controlled by the nervous system in molluscs have been well reviewed by Joosse and Geraerts (1983), although the knowledge was limited to a gastropod. Very little is known of endocrine control of gametogenesis and spawning of bivalve mollusks, while the artificial seed production based on reproductive control is thirsted to improve productivity in aquaculture.

Development of eggs and sperms during gametogenesis process is a common phenomenon among oviparous organisms. In oogenesis, the primary germ cells (stem cell) undergo mitotic division and give rise to oogonia (Sastry, 1979), and then develop into oocytes accumulating yolk materials. In the testis, the stem cells undergo a series of mitotic division with decreasing cytoplasmic volume and successively give rise to spermatogonia (Sastry, 1979) and then undergo meiosis to form spermatozoa through spermatocytes and spermatids followed by spermiogenesis. The gametogenesis normally undergoes an annual cycle and the changes in the gonadal development have been qualitatively classified into several stages; in the scallop undifferentiating, early differentiating, growing, mature, spawning, post-spawning, and degenerating stages (Osanai, 1975), based on the characterization of overall morphology of the gonads, but not on the quantitative analysis of the germ cells (Fig. 6.1).

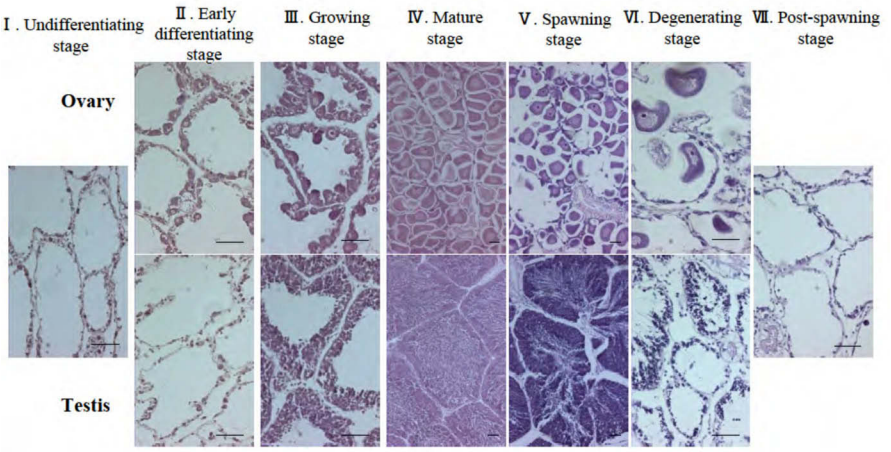


FIGURE 6.1 Morphological changes in gametogenesis of the scallop *Patinopecten yessoensis*. Bars, 50 µm. (From Osada, M.; et al. Fish. Sci. 2007, 73, 1318, with permission.)

In this chapter, the histology of germ cells and cells related to gametogenesis, the mitotic process in the early development of germ cells, vitellogenesis resulting in oocyte growth and spawning based on oocyte maturation and sperm motility in bivalves are described. Most of the descriptions will be focused on bivalve reproduction from the point of view of comparative endocrinology.

6.2 HISTOLOGY OF GERM CELLS AND CELLS RELATED TO GAMETOGENESIS

Bivalves possess single gonads. The gonad most frequently takes the form of a mass, surrounding the digestive gland in the visceral region (oysters, clams). In mussels, it also invades paired mantle lobes, which run along the internal lining of the shell and ramify into the digestive gland. In scallops, the gonad is a discrete organ, and therefore the gonad index (GI) which represents the weight of the gonad as a proportion of total body weight, is used to assess gametogenic cycles.

Histological examinations are necessary to describe reproductive events concerning gamete development. Bivalve reproductive maturation is categorized into several stages; differentiation, growing or developing, ripening of gametes, spawning, and resting stages. In *Crassostrea gigas*, the gonad is a diffuse organ, which consists of numerous tubules invaginated in a

connective tissue. Expansion of the gonadal tubules in the visceral mass varies broadly with the progression of gametogenesis. At undifferentiated stage, the gonadal area is largely filled with the vesicular connective tissue cells. Genital tubules are reduced in size and number and contain only undifferentiated cells. Sex is not distinguishable. At developing stage, oogonia or spermatogonia develop from undifferentiated cells and divide actively. Gonoduct distributed near the surface of the gonad are lined with oogonia and oocytes or spermatogonia on the inner side and a ciliated epithelium on the outer side (Eble & Scro, 1996). Genital tubules gradually enlarge, ramify, and invade the surrounding vesicular connective tissue. In females, oocytes are distributed along the inner wall of ovarian genital tubules. Vitellogenic oocytes with prominent germinal vesicles (GVs) predominate. In males, sperm can be found in the lumina of genital tubules. The vesicular connective tissue can be still observed partly. At ripe stage, genital tubules are filled with full-grown oocytes or sperm and occupy most of the region between the mantle epithelium and the digestive diverticulae. The vesicular connective tissue can hardly be observed at this stage. At spawning stage, genital tubules are partly empty because some of eggs or sperm are discharged during spawning. After completion of spawning, undischarged eggs or sperm and spermatocytes are re-absorbed. The vesicular connective tissue cells proliferate again.

The gonadal development of the scallop *Patinopecten yessoensis* is histologically classified into seven stages (Fig. 6.1). The scallops at the undifferentiated stage show the lowest value of GI. A few oogonia and oocytes, or spermatogonia and occasionally spermatocytes are distributed along the epithelia of germinal acini in the ovary and testis, respectively, at the early differentiating stage. The scallops at the growing stage have growing oocytes or an increased number of spermatogonia and spermatocytes and the gonads developed to the mature stage. Disappearance of oogonia and the increased number of growing oocytes in the ovary, and the increased number of spermatogonia, spermatocytes, spermatids, and spermatozoa in the testis are observed at the mature stage. In the scallops at the spawning stage, the germinal acini are filled with fully grown oocytes in the ovary and mostly spermatozoa with spermatogonia, spermatocytes, and spermatids in the testis. A spawned trace is partially seen. After spawning, the gonad enters the post-spawning and degenerating stages. The GI continuously increases from the early differentiating stage to the spawning stage for both females and males and decreases after spawning to a similar level as that at the undifferentiating stage. Similarly to the GI profile, the oocyte diameter increases from the early differentiating stage to the spawning stage.

6.2.1 ACCESSORY CELLS IN FEMALES

In bivalves, the ovarian acinus is a simple structure containing mainly oocytes and associated accessory cells within a thin germinal epithelium. The relationship between intra-acinal accessory cells termed "follicle cells" and the successive stages of the developing oocyte were shown in *Mytilus edulis* (Pipe, 1987a). During the early stages of development, oogonia are located along the internal wall of the acini (Fig. 6.2A) and develop into oocytes, which are surrounded by a limited number of small follicle cells (Figs. 6.2B and 3). With development of the oocyte, the follicle cells are

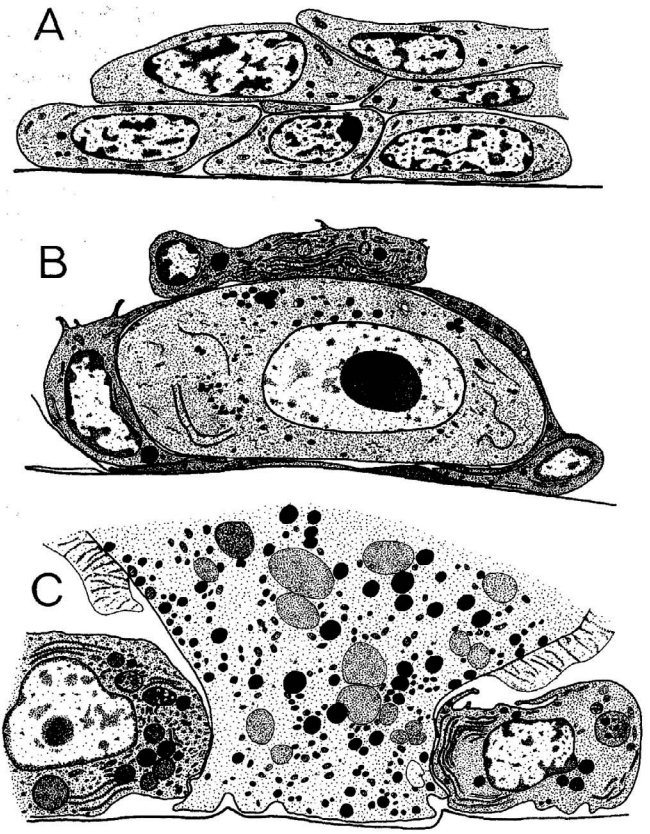


FIGURE 6.2 Relationship between follicle cells and the developing oocyte. (A) Primary oogonia. (B) Developing oocyte surrounded by small follicle cells. (C) Basal region of post-vitellogenic oocyte showing follicle cells in contact with stalk region of oocyte. (From Pipe, R. K. Oogenesis in the Marine Mussel Mytilus edulis: An Ultrastructural Study). Mar. Biol. 1987a, 95, 405. With kind permission from Springer Science and Business Media.

restricted to the stalk region, which attaches the oocyte to the acinus wall (Fig. 6.2C). A few follicle cells are also observed in association with oocytes at all stages of *Crassostrea virginica* (Eckelbarger & Davis, 1996a). These cells of *Pecten maximus* are called "auxiliary cells" (Dorange & Le Pennec, 1989b). Auxiliary cells as well as follicle cells are closely associated with developing oocytes (Fig. 6.4). The contact between the follicle cells and the developing oocyte is maintained by means of desmosome-like gap junctions. The presence of desmosome-like gap junctions indicates that exchange of small molecules and ions may take place between follicle cells and developing oocytes. Ovarian follicle cells or auxiliary cells are the site of vitellogenin (Vtg) synthesis in *C. gigas* and *P. yessoensis* (Matsumoto et al., 2003; Osada et al., 2004a).

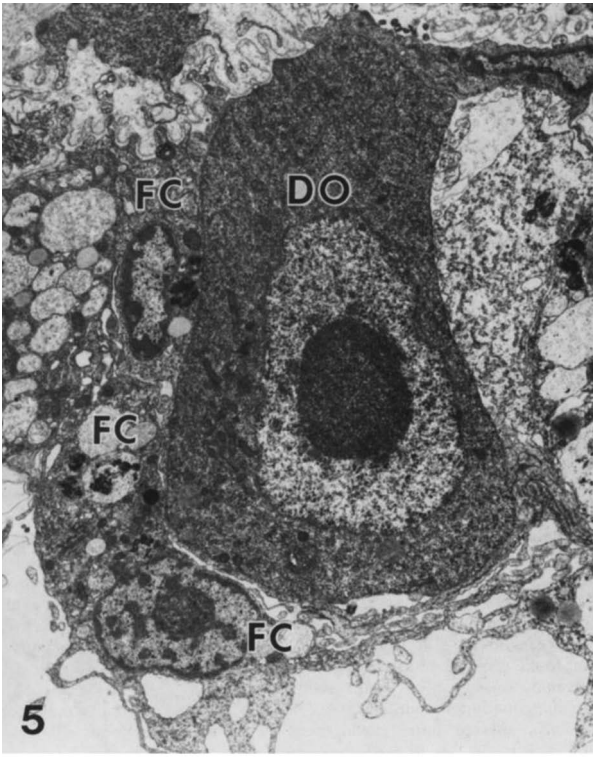


FIGURE 6.3 Developing oocyte (DO) surrounded by small follicle cells (FC) (×6500). (From Pipe, R. K. Oogenesis in the Marine Mussel Mytilus edulis: An Ultrastructural Study). Mar. Biol. 1987a, 95, 405. With kind permission from Springer Science and Business Media.

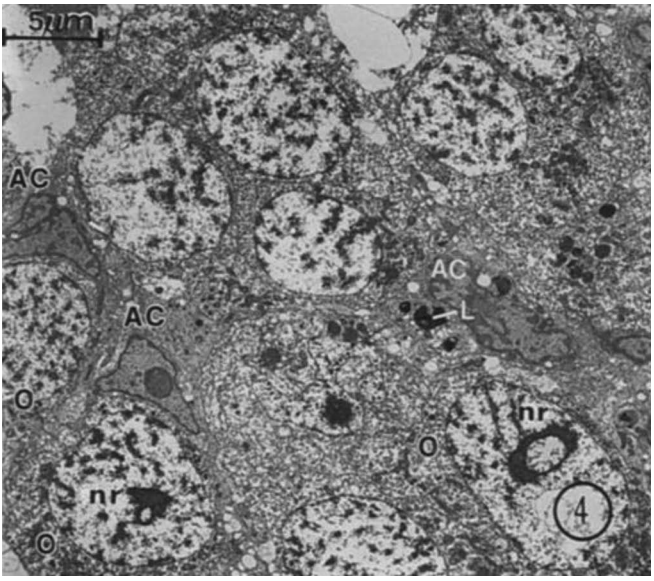


FIGURE 6.4 Auxiliary cells (AC) in close contact with the young oocytes (O); nr: nucleolar ring in oocyte. An auxiliary cell displays dense lysosomal-like organelles (L). (From G. Dorange, M. Le Pennec, "Utrastructural study of oogenesis and oocytic degeneration in Pecten maximus from the Bay of St. Brieuc", Marine Biology (1989) 103: 339). doi:10.1007/BF00397268. With kind permission from Springer Science and Business Media.

6.2.2 ACCESSORY CELLS IN MALES

Bivalve testes contain accessory (somatic) cells which presumably play important roles in the processes of spermatogenesis. In the ultrastructural study of spermatogenesis in *C. gigas*, somatic intragonadal cells are observed among the male germ cells (Franco et al., 2008). With light microscopy, such cells are difficult to identify because their cytoplasm do not stain differentially from that of germ cells. Intermediate junctions or zonula adherens and phagolysosomes containing degenerating sperm cells are noticed in some somatic cells. Intragonadal somatic cells are observed in numerous species of bivalves, but the terminology used to describe these cells differs among the studies (Eckelbarger & Davis, 1996b). In *C. virginica*, accessory cells are closely associated with all sperm stages except the mature spermatozoon (Fig. 6.5). Accessory cells appear to be assigned the term "Sertoli cells" in *M. edulis* (Pipe, 1987b), and "Sertoli-like cells" in *P. maximus* (Dorange & Le Pennec, 1989a).

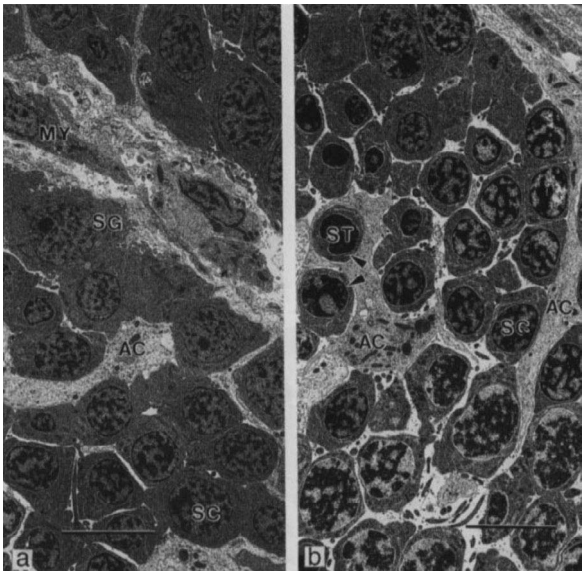


FIGURE 6.5 Accessory cells closely associated with sperm cells. (a) The accessory cell (AC) is distributed in close association with spermatogonia (SG) and spermatocytes (SC); MY: myoepithelial cell. (b) Spermatocytes (SC), spermatids (ST) and accessory cells (AC). One accessory cell surrounds two adjacent spermatids (arrowheads). Scale bar = 4.0 μm. (From K. J. Eckelbarger, C. V. Davis, "Ultrastructure of the gonad and gametogenesis in the eastern oyster, Crassostrea virginica. II. Testis and spermatogenesis", Marine Biology (1996) 127: 89). doi:10.1007/BF00993648. With kind permission from Springer Science and Business Media.

6.2.3 ESTROGENIC CELLS IN GONADS

In *P. yessoensis*, estrogenic cells were identified immunohistochemically in the gonad. Immunoreactivities against P450 aromatase and estradiol-17β (E_2) were detected in the cells along the inside of the acinus wall of the testis (Osada et al., 2004b), whereas the cells are distributed along the outside of the acinus wall in the ovary (Matsumoto et al., 1997). The localization of estrogen synthesizing cells in the testis is similar to that of a Sertoli cell in the testis of vertebrates. In contrast, the distribution of the same type of cells in the ovary is similar to that of Leydig cells in the testis of vertebrates.

6.2.4 GENERAL ANATOMY OF THE CENTRAL NERVOUS SYSTEM

The central nervous system (CNS) that controls the reproduction of the bivalves has been studied in detail in *P. yessoensis* (Matsutani & Nomura,

1984). The CNS of the scallops is composed of the cerebral, pedal, and visceral ganglia. The cerebral ganglia, consisting of an anterior lobe and a posterior lobe, respectively, are located on either side of the pedal ganglion, which lies at the base of the foot. The cerebrovisceral connective exits the posteroventral corner of each cerebral ganglion and extends under the gonad to the visceral ganglion. The visceral ganglia are the largest and have the most intricate structure of the central ganglia. They are situated on the ventral side of the adductor muscle. The accessory ganglia are positioned at the point of the lateral lobes of the visceral ganglia. The width of the cerebropedal commissure and the visceral ganglia in adult is approximately 5.5 mm and 3.4 mm, respectively.

In contrast to other bivalves, CNS of oysters is simple. It includes a pair of very tiny cerebral ganglia located at the bases of the labial palps. The coalesced visceral ganglia are at the ventral end of the visceral mass on the anteroventral border of the adductor muscle. The cerebrovisceral connectives run from the cerebral ganglia to the visceral ganglia. There is no foot and, therefore, there is no pedal ganglion.

6.2.5 NEUROSECRETORY PRODUCTS

There are both aminergic and peptidergic secretions from CNS. The existence of monoamines including dopamine and serotonin (5-hydroxytryptamine, 5-HT) has been demonstrated in the scallop by histofluorescence methods (Matsutani & Nomura, 1984). Green fluorescent cells which presumably contained dopamine were detected in the anterior lobe of cerebral ganglion and the lateral lobe of visceral ganglion, whereas all the cells of accessory ganglion showed yellow fluorescence which suggested the presence of serotonin. In the gonadal area, the wall of the gonoduct contained both green and yellow fluorescent fibers. Green fluorescent varicose fibers were distributed in the epithlium around the gonad and along the intestinal epithelium. Moreover, the localization of 5-HT neurons in CNS and the gonad of the scallop was examined immunohistochemically (Matsutani & Nomura, 1986a). In CNS, 5-HT neurons were distributed in a part of the anterior lobe and the posterior lobe of the cerebral ganglion, the pedal ganglion and the accessory ganglion, whereas no nerve cells containing 5-HT were found in the visceral ganglion. All central ganglia, including the visceral ganglion, had numerous 5-HT varicose fibers in their neuropil. In the gonadal region, a number of bundles of 5-HT nerve fibers, most of which appeared to be ramifications of the cerebrovisceral connectives, were found to run into the gonad through

the pallial epithelium of the gonad near the adductor muscle. Bundles of 5-HT nerve fibers were also observed in the subepithelial layer of the gonoduct, and the varicose fibers were distributed along the germinal epithelium. The development of 5-HT and catecholamine neuron during larval period and their distribution has been also examined in detail using immunocytochemical and histofluorescent techniques in mussel species from the point of view of different development among trochozoan groups (Voronezhskaya et al., 2008).

Several kinds of neuropeptides have been identified in bivalve mollusks. The cardioexcitatory neuropeptide FMRFamide was first isolated in the clam *Macrocallista nimbosa* and its cardioexcitatory effect on the cardiac muscle were confirmed in other bivalves (see Zatylny-Gaudin & Favrel, 2014). Presence of FMRFamide-like peptides was immunologically evidenced in the scallop *Placopecten magellanicus* through the CNS and peripheral organs of juveniles and adults (Too & Croll, 1995). Immunoreactivity of the neuropeptide, APGWamide, which functions as a neurotransmitter within the CNS of gastropods, was detected in the CNS and the axonal terminals within peripheral tissues including gonads of bivalves (Smith et al., 1997). Gonadotropin (GTH)-releasing hormone (GnRH) is a neuropeptide that plays key roles in the regulation of reproduction in vertebrates. The demonstration of GnRH neurons has been made in the nervous system of bivalves using immunohistochemical techniques. In the scallop, the GnRH-like neurons detected with anti-mammalian GnRH antibody were distributed sparsely in the pedal ganglia and predominantly in the anterior lobe of the cerebral ganglia of both sexes at the growing stage, whereas no GnRH nerve fibers were found in the gonad (Nakamura et al., 2007). A study carried out in the nervous system of *C. gigas* and *M. edulis* detected neurons, which react positively to antibodies raised against octopus GnRH (Bigot et al., 2012). GnRH neurons and fibers were scattered in the visceral ganglia of *C. gigas*. In *M. edulis*, both cerebral and pedal ganglia contained GnRH neurons and fibers. In addition, anti-GnRH immunoreactivity was not found in the male and female gonad of *M. edulis* in the previous study (Pazos & Mathieu, 1999).

6.3 MULTIPLICATION OF GONIAL CELLS

Quantification of development of gonial cells is necessary to understand endocrine control of early development of germ cells in bivalves. The mitotic division of gonial cells has been quantified in bivalve mollusks. In blue mussel, *M. edulis*, [^{3}H] thymidine incorporation into young germinal

cells, particularly spermatogonia, in the mantle tissue was demonstrated by autoradiography (Mathieu, 1987). The [^{3}H] thymidine incorporation into dissociated cell suspensions from the mantle tissue associated with estimation of aspartate transcarbamylase activity was applied to quantitatively evaluate the mitogenic influence of an endogenous factor on spermatogonial multiplication (Mathieu, 1985, 1987). In scallop, *P. yessoensis*, BrdU incorporation into gonial cells during early development showed that the proliferation of gonial cells in the scallop could be divided into phase I and II (Osada et al., 2007) (Fig. 6.6). In phase I, oogonia and spermatogonia show a slow proliferation. In phase II, oogonia terminate proliferation followed by development into the vitellogenic oocytes while spermatogonia show a rapid proliferation. In vertebrates, developing spermatogonia have been classified into two types: Non-proliferated type A spermatogonium as a stem cell and type B spermatogonium as a differentiated spermatogonium (Miura & Miura, 2001). Osada et al. (2007) proposed that spermatogonia in bivalves could be classified into a spermatogonial stem cell and a series of development of differentiated spermatogonium during early spermatogenesis on the basis of proliferation potency of spermatogonia.

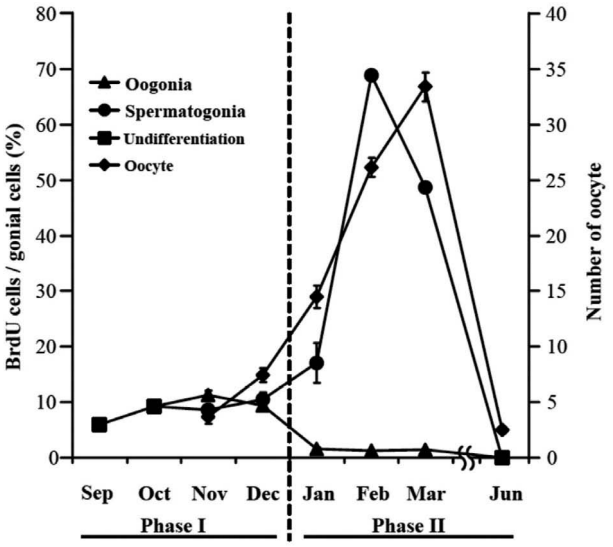


FIGURE 6.6 Changes in percentage of BrdU immunopositive gonial cells per gonial cells in germinal acini of the ovary and testis and the number of oocyte in germinal acini of the ovary of *Patinopecten yessoensis* during sexual maturation. Each value represents the mean ± SE (n = 7–10). ((From Nakamura, S.; Osada, M.; Kijima, A, "Involvement of GnRH Neuron in the Spermatogonial Proliferation of the Scallop, Patinopecten yessoensiss. " Molecular Reproduction and Development . 2007, 74(1), 108. Copyright © 2006 Wiley–Liss, Inc. Used with permission.)

Mollusks are useful to scientists involved in the study of neurophysiological responses due to their extremely large nerve cells. The effect of neural factors on gonial mitosis in mollusks was first reported in *M. edulis* of bivalve. The cerebral ganglia stimulated gonial mitosis, the reinitiation of meiosis in males and previtellogensis and vitellogenesis in females in organ culture experiment of the mussel in vitro, although the ablation of the ganglia did not affect such a reproductive function in vivo (Lubet & Mathieu, 1982; Mathieu & Lubet, 1980). Involvement of neural factors in multiplication of germ cells has been reported also in the slipper limpet, a gastropod (Le Gall et al., 1987). Mathieu et al. (1988) found occurrence of a neural factor named gonial mitosis-stimulating factor from the cerebral ganglia of *M. edulis* with molecular mass of less than 5 kDa, which stimulated [^{3}H] thymidine incorporation into dissociated cell suspensions of the mussel mantle tissue and existed in the hemolymph and circulatory cells. This neural factor was supposed to be primarily concerned with the GnRH of bivalve mollusks.

GnRH superfamily, which includes GnRH, adipokinetic hormone (AKH), corazonin (Crz), and AKH/Crz-related peptides, is almost ubiquitous throughout the bilateral animals (Roch et al., 2011). In vertebrates, GnRH is synthesized in the hypothalamic region, and is then transported to, and acts on the pituitary to promote the release of GTH consisting of follicle-stimulating hormone, and luteinizing hormone (LH). The connection between the brain and the gonads via the pituitary is called the hypothalamus–pituitary–gonadal axis (HPG axis), which forms the basis for both the neural and endocrine regulation of reproduction in all vertebrates. Interestingly, α-mating factor, a tridecapeptide mating pheromone of yeast has been identified as a homologue of GnRH with LH-release activity from gonadotroph at high doses, suggesting a conservation of the structural and functional properties of GnRH-related peptides during evolution (Loumaye et al., 1982).

Since then, the existence and function of GnRH-like peptides in mollusks have been demonstrated using heterologous GnRH antibodies. In cephalopod, *Octopus vulgaris*, immunopositive GnRH-like peptides were shown to be present in the optic gland, which is a major endocrine organ (Di Cosmo & Di Cristo, 1998). GnRH was found throughout the CNS and in both the male and female reproductive ducts of octopus (Di Cristo et al., 2002), suggesting that GnRH in octopus could have some reproductive function. In gastropod, *Hellisoma trivolvis* nervous system showed characteristics consistent with the existence of GnRH-like peptide functionally similar to

mammalian GnRH (m-GnRH) (Goldberg et al., 1993). It was shown that GnRH-like neurons are present in the nervous systems of the freshwater snails *H. trivolvis* and *Lymnaea stagnalis* and a possible role for reproduction was hypothesized (Young et al., 1999). The possible presence of multiple forms of GnRH-like peptides was reported in the marine sea hare, *Aplysia californica* (Zhang et al., 2000) and the expression were refined to specific sites inside the CNS of the *Aplysia* using heterologous GnRH antibodies (Tsai et al., 2003).

As mentioned above, the first research that involved the indirect use of mollusk GnRH deeply was reported by Mathieu et al. (1988), who showed that extracts from the cerebral ganglion and hemolymph of the mussel *M. edulis* were capable of promoting the incorporation of [^{3}H] thymidine, thereby promoting mitosis of gonial cells. Subsequently, the same group showed that vertebrate GnRHs could also affect mitosis of gonial cells in the mussel and the Pacific oyster and the GnRH-like neurons were identified in the CNS of mussel (Pazos & Mathieu, 1999) (Fig. 6.7). GnRH signal may be transduced into cells through the membrane receptor as a GnRH receptor ortholog was cloned from the gonad of oysters (Rodet et al., 2005, 2008). And then two GnRH-related peptides (pQNYHFNSNGWQP-NH_2Cg-GnRH-a; amidated undecapeptide and pQNYHFNSNGWQPG Cg-GnRH-G; non-amidated doecapeptide) were confirmed by mass spectrometry from the CNS of the Pacific oyster (Bigot et al., 2012). However, a specific affinity of the GnRH receptor orthologs to endogenous oyster GnRH-like peptides identified (Bigot et al., 2012) have not yet been confirmed. In the Japanese scallop, Nakamura et al. (2007) reported an occurrence of GnRH-like peptide in the scallop CNS-regulating development of the scallop gonad. The GnRH-like neurons detected with anti-m-GnRH antibody were distributed in the CNS of the scallop, whereas no immunopositive GnRH nerve fibers were found in the gonad and both the neural factor extracted from the CNS and m-GnRH strongly stimulated mitosis of spermatogonia in vitro. The reactions of the neural factor and m-GnRH were abolished by absorption with anti-m-GnRH antibody and competition with m-GnRH-specific antagonists, which interfere GnRH function on a GnRH receptor, suggested occurrence of endogenous GnRH-like peptide and GnRH receptor-like receptor. And the same mitotic activity as the neural factor and m-GnRH was found in the hemocyte lysate, but not in the serum. These results suggested that the neural factor has a similar antigenicity to m-GnRH and the function of the factor may be mediated through m-GnRH receptor-like receptor in the testis under a neuroendocrine pathway (Fig. 6.8).

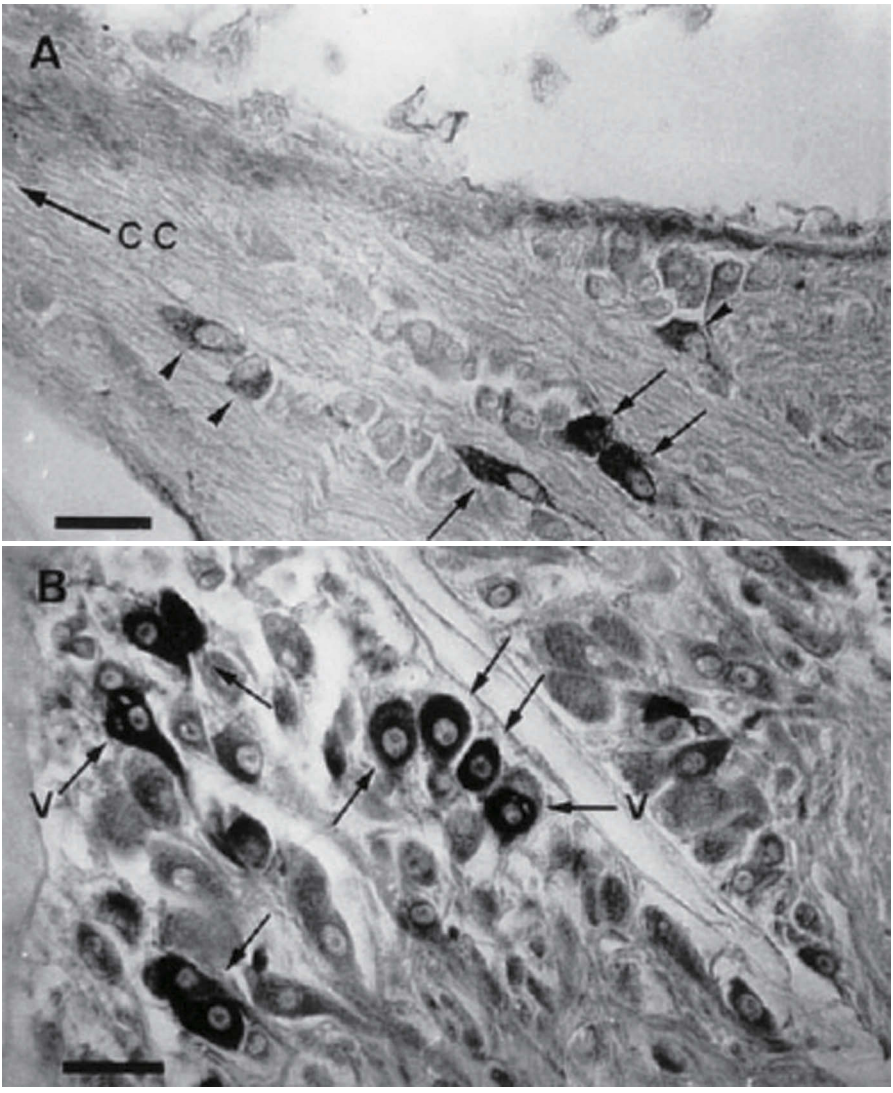


FIGURE 6.7 GnRH-like immunoreactivity in the central nervous system of *M. edulis*. (A) Intensely (arrows) and weakly (arrowheads) stained cells in the cerebral ganglia (CC, direction toward the cerebral commissure). (B) Immunoreactive cells (arrows) in the pedal ganglia. Two stained cells present vacuoles (V). Bar, 20 µm. (Reprinted from A.J. Pazos, M. Mathieu "Effects of Five Natural Gonadotropin-Releasing Hormones on Cell Suspensions of Marine Bivalve Gonad: Stimulation of Gonial DNA Synthesis", in General and Comparative Endocrinology, Volume 113, Issue 1, January 1999, Pages 112–120. With permission from Elsevier.)

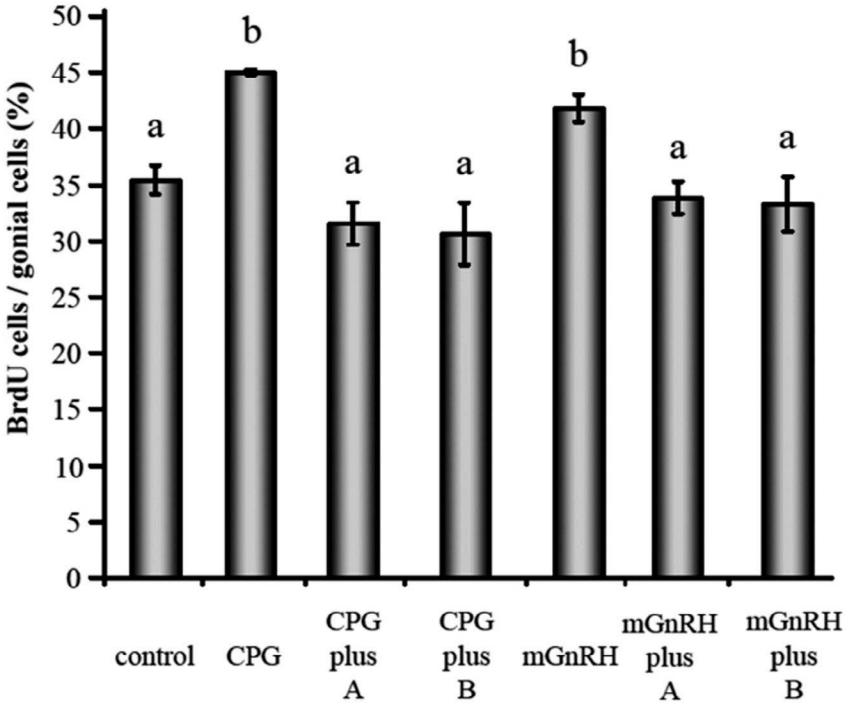
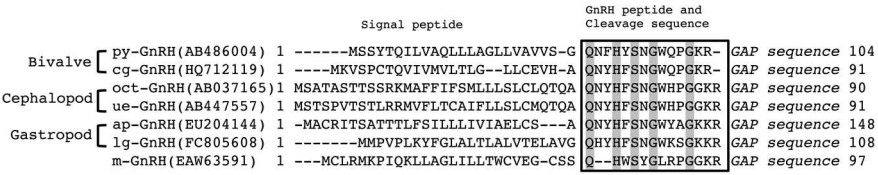


FIGURE 6.8 Effect of two types of antagonists (1×10^{-6} M each), antide (A) and [D-pGlu[1], D-Phe[2], D-Trp[3,6]] GnRH (B), of m-GnRH on 1×10^{-6} M m-GnRH and CPG extract-induced spermatogonial proliferation of *P. yessoensis*. Each value represents the mean ± SE (n = 3–4). Values with different letters are significantly different ($p < 0.05$). (From Nakamura, S.; Osada, M.; Kijima, A, "Involvement of GnRH Neuron in the Spermatogonial Proliferation of the Scallop, Patinopecten yessoensiss. " Molecular Reproduction and Development . 2007, 74(1), 108. Copyright © 2006 Wiley-Liss, Inc. Used with permission.)

The full cDNA sequences of GnRHs were cloned from the Japanese scallop and Pacific oyster as py-GnRH and cg-GnRH, respectively. The GnRH-like peptide sequences of both bivalve species have a high similarity to oct- and ap-GnRHs (Treen et al., 2012). The extra dipeptide insertion after N-terminal pyro-glutamate residue was recognized in both bivalve species in common with other molluscan species (Fig. 6.9) (Osada & Treen, 2013). The logically predicted pQNFHYSNGWQP-NH_2 (py-GnRH-P-NH_2), an amidated undecapeptide peptide, was synthesized and was shown to stimulate proliferation of spermatogonia in testicular tissue culture of the scallop as well as the previous culture with m-GnRH (Treen et al., 2012). However, the peptide failed to induce LH release from quail pituitary, suggesting that molluscan GnRHs might conserve a fundamental molecular structure similar

to that found in other animals, but may not bind to vertebrate receptor (Treen et al., 2012).



	Postulated function	Ref
py-GnRH	spermatogonial proliferation, steroidogenesis	Nakamura et al., 2007; Osada and Treen, 2013
oct-GnRH	steroidogenesis, brain function	Iwakoshi–Ukena et al., 2004; Kanda et al., 2006
ap-GnRH	behavior	Tsai et al., 2010

FIGURE 6.9 Amino acid alignment of prepro-GnRH sequences from molluscan species deduced from their coding DNAs and their postulated functions. Alignment was created by CLUSTALW2, conserved amino acids is shaded. The sequences containing the GnRH peptide with the cleavage site are boxed. Signal peptide and GAP regions are also indicated. EMBL/GenBank accession numbers: *Patinopecten yessoensis*: py-GnRH (AB486004), *Crassostrea gigas*: cg-GnRH (HQ712119), *Octopus vulgaris*: oct-GnRH (AB037165), *Aplysia californica*: ap-GnRH (ABW82703), *Lottia gigantea*: lg-GnRH (FC805608), *Uroteuthis edulis*: ue-GnRH (AB447557), *Homo sapiens*: m-GnRH (EAW63591). (Reprinted from Makoto Osada, Nicholas Treen, "Molluscan GnRH associated with reproduction," in General and Comparative Endocrinology, Volume 181, 15 January 2013, 254. Copyright © 2012 Elsevier Inc. With permission from Elsevier.)

In mollusks, several physiological functions for mollusk GnRH have been suggested other than spermatogonial proliferation. The immunopositive nerve fibers of oct-GnRH were identified in both CNS and peripheral organs, suggesting that the GnRH may act as a modulatory factor in controlling higher brain functions as well as a reproductive factor (Iwakoshi et al., 2004; Kanda et al., 2006). The oct-GnRH did modulate the contraction of the heart and oviducts (Iwakoshi et al., 2000, 2004). In addition, the oct-GnRH stimulated production of progesterone, testosterone, and E_2 in both testis and ovary of the octopus, suggesting the reproductive role of the oct-GnRH (Kanda et al., 2006) because ovarian development in octopus was found to be associated with fluctuation of the sex steroid hormone (Di Cosmo et al., 2001; Di Cristo, 2013). The ap-GnRH, distributed in the central tissue, functioned as a modulator of behavioral attributes controlling parapodia, foot and head movement, not as an acute reproductive trigger for development of ovotestis and secretion of egg-laying hormone (ELH) (Sun & Tsai, 2011; Sun et al., 2012; Tsai et al., 2010). The presence of multiple forms of GnRH

in *A. californica* was suggested (Zhang et al., 2000) and then ap-AKH shared common ancestry with AKH/RPHC was newly identified in addition to ap-GnRH, which inhibited feeding resulting in reduction of body and gonadal mass (Johnson et al., 2014). Bigot et al. (2012) identified two kinds of GnRH by mass spectrometry in the CNS of the Pacific oyster, while each function has not been confirmed in the oyster. In vivo administration of the scallop py-GnRH into scallop gonad accelerated spermatogenesis associated with a significant increase in testis mass and conversely inhibited oocyte development associated with an apoptosis, implying early phenotypic alteration to masculinization (Nagasawa et al., 2015a).

In view of the roles of GnRH in reproduction of molluscs without a pituitary, it is suggested that GnRH could be involved in spermatogonial proliferation by mediating gonadal steroidogenesis (Osada & Treen, 2013). Estrogen synthesizing cells have been immunologically found along the outside and inside acinar wall in the ovary and testis of the scallop, respectively, and their localization is similar to Leydig cells and Sertoli cells in the testis, respectively (Matsumoto et al., 1997; Osada et al., 2004b). Aromatase activity and E_2 content increased in synchronization with reproductive progress (Osada et al., 2004b). E_2 is involved in spermatogenesis in vertebrates (Hess et al., 1997; Pierantoni et al., 2009). Interestingly, oct-GnRH was capable of inducing steroidogenesis of testosterone, progesterone and estrogen (Kanda et al., 2006). It is possible that py-GnRH stimulates the estrogen synthesis that in turn promotes spermatogonial mitosis because the py-GnRH and E_2-induced spermatogonial proliferation was blocked by an estrogen antagonist (Osada & Treen, 2013). It was suggested that spermatogonial proliferation stimulated by py-GnRH might be mediated through estrogen whose synthesis was induced by py-GnRH (Fig. 6.10) (Osada & Treen, 2013).

Steroid production and function on mollusk reproduction has been well examined using vertebrate steroids in the past; quantification of steroids by immunological method and HPLC, etc., identification of putative sites of steroid production, and induction of sex reversal and oogenesis have been demonstrated (see Lafont & Mathieu, 2007). However, steroid function in mollusks is recently in contention because convincing evidence for biosynthesis of vertebrate steroids by mollusks and the biological effects of steroids on mollusks is required (Scott, 2012, 2013). Series of enzymes composing steroid synthetic pathway, structure determination of endogenous steroids and corresponding receptors should be uncovered hereafter.

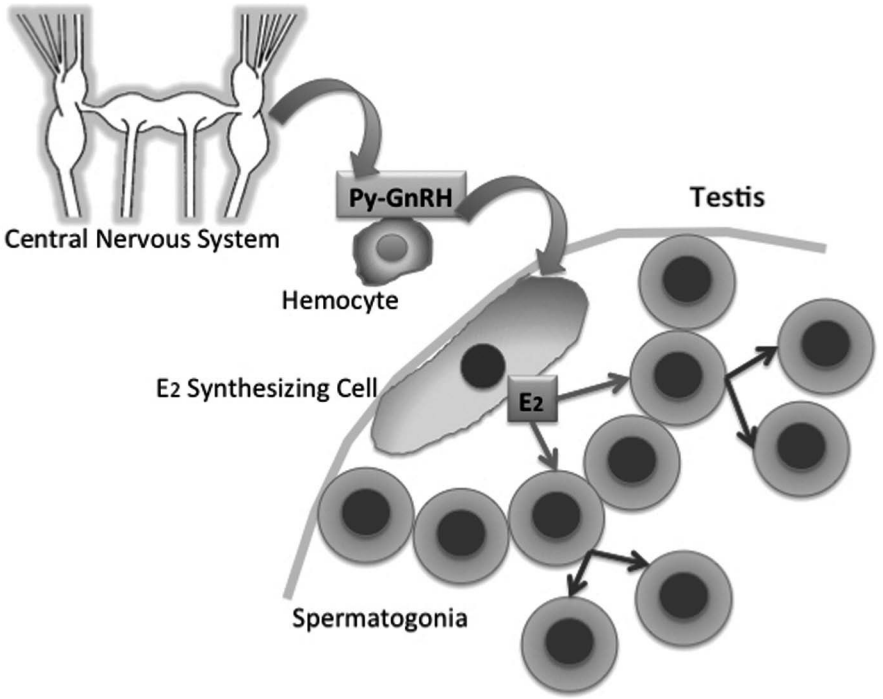


FIGURE 6.10 Transduction mechanism of py-GnRH for inducing spermatogonial proliferation in bivalve molluscs. The py-GnRH secreted from the anterior lobe of the cerebral ganglion may be transported by the hemocytes in the circulation system and received by estrogen synthesizing cells to stimulate the secretion of estradiol-17β, resulting in the induction of spermatogonial proliferation. (Reprinted from Makoto Osada, Nicholas Treen, "Molluscan GnRH associated with reproduction," in General and Comparative Endocrinology, Volume 181, 15 January 2013, 254. Copyright © 2012 Elsevier Inc. With permission from Elsevier.)

6.4 OOCYTE GROWTH

In the developing or growing stage, the oocyte rapidly increases in size. In these periods, a large amount of yolk protein is accumulated in oocytes. Vitellin (Vn) is a major yolk protein stored in yolk granules of oocytes and used as a nutrient during embryogenesis. In vertebrates such as fish, amphibians, and birds, yolk protein is synthesized from a precursor, Vtg, produced by the liver and is transported to the oocytes via the blood circulation system. In bivalves, a few biochemical studies of yolk proteins were carried out (Osada et al., 1992b; Suzuki et al., 1992). Yolk protein was purified and using a specific antiserum against purified protein, the distribution

of the protein was examined in *C. gigas* and *P. yessoensis*. The presence in the oocytes indicated the protein is a Vn. The ovarian Vn content of *C. gigas* increased as the oocyte developed and became low after spawning. This variation agrees with the profile of the oocyte diameter and the results of histological observation of the ovaries (Li et al., 1998). In scallops, Vn content showed low level till the growing stage and thereafter showed the highest value at the mature stage. At the degenerating stage after spawning, the level of Vn content decreased markedly. GI value at the early differentiating stage showed the lowest level and gradually increased, reaching the highest value just before spawning stage. Vn content in the scallop ovary increased in parallel with the ovarian development (Osada et al., 2003). These results indicate that oocyte growth depends on the accumulation of Vn within the oocytes during vitellogenesis. Since no organ other than the ovary reacted with anti-Vn serum, it was predicted that Vn was synthesized inside the ovary, not in the other tissues such as digestive diverticula. In addition, the immunoreactivity against anti-scallop Vn antibody was also observed in the auxiliary cells, suggesting the possibility of Vtg synthesis in these cells (Osada et al., 2003). Vn is slightly detectable in hemolymph of mature females, in particular, during the spawning season. This appears to originate from degenerated oocytes. Oocyte degeneration and resorption is not unusual in bivalves and may be brought about by a variety of environmental conditions (Pipe, 1987b).

Molecular investigations were carried out to elucidate the mechanism involved in vitellogenesis (Matsumoto et al., 2003; Osada et al., 2004a). The expression of Vtg mRNA indicated maximum level at the developing or growing stage and the level was retained during the mature stage. In RT-PCR analysis, Vtg mRNA was detected in the ovary but not in the digestive diverticulae, which was consistent with the results of immunohistochemistry. In situ hybridization indicated Vtg mRNA signals were detected in the follicle cells in the ovary. Similarly, scallop Vtg mRNA expression is demonstrated in the auxiliary cells. In marine bivalves, autosynthetic yolk formation in the oocytes had been thought to be the main type of vitellogenesis, based on the morphological evidence (Dorange & Le Pennec, 1989b; Eckelbarger & Davis, 1996a; Pipe, 1987a; Suzuki et al., 1992). In oyster and other bivalve species, the ovarian acini contain only developing oocytes and associated follicle cells within a thin germinal epithelium. The functions of follicle cells in the bivalve ovary were not well understood, though they were suspected of playing some roles in oocyte nutrition. Results of RT-PCR and in situ hybridization, together with the immunocytological results support Vtg is

synthesized in the auxiliary cells surrounding the vitellogenic oocyte through a heterosynthetic pathway and directly passed to oocyte. In common with bivalve Vtgs, the abalone Vtg gene is expressed in the follicle cells in the ovary (Matsumoto et al., 2008). In abalone species like in bivalves, follicle cells are present on the stalk of developing oocytes in the maturing ovary. A recent study in abalone showed the immunoreactivity in the follicle cells with anti-Vn antibody and indicated that transcription and translation of the Vtg gene occur in the ovarian follicle cells (Awaji et al., 2011). In addition, positive reactions with the antibody appeared first in the stalk part of the oocytes at the early phase of yolk accumulation, and the follicle cells adjacent to the stalk of these oocytes were also stained positively. These observations imply that Vn or Vtg is transported from the follicle cells to the oocyte through an extracellular space around the oocyte stalk. In the scallop, Vn immunoreactivity was observed in the oocytes and the auxiliary cells (Osada et al., 2003). Similar systems of yolk protein transport can be expected in the oyster and the scallop.

It is clearly established that the Vtg gene expression is regulated by E_2 through estrogen receptor (ER) in oviparous vertebrates (Polzonetti-Magni et al., 2004). In marine bivalves, the presence of steroids was reported; the structural identity of endogenous steroids as vertebrate-type sex steroids (progesterone, androstenedione, testosterone, E_2, and estrone) has been demonstrated in the mussel by gas chromatography and mass spectrometry (Reis-Henriques et al., 1990). In oysters and scallops, E_2 is detected in the ovary, and its contents showed a synchronous profile with gametogenesis on the basis of HPLC (Matsumoto et al., 1997). In the ovary of scallops, estrogenic cells were demonstrated from observation of immunoreactivities against P450 aromatase and E_2, which distributed along the outside of acinus wall. In addition, aromatase activity and E_2 showed maximum values at the mature stage before spawning (Osada et al., 2004b). The Vtg synthesis terminates at the mature stage (Osada et al., 2003, 2004a). These results suggest that E_2 synthesized in the estrogenic cells through P450 aromatase may be involved in the induction of Vtg synthesis. Physiological function of estrogen on vitellogenesis has also been reported in various mollusks, though it is still under scientific debate (Scott, 2013). Estrogen-induced Vn synthesis has been demonstrated in the scallop (Osada et al., 2003) and oyster (Li et al., 1998). In vitro E_2 treatment resulted in an increase in Vn content in the ovarian tissue. Further in scallop, in vitro ovarian tissue culture with the cerebral plus pedal ganglion (CPG) extract resulted in a greater increase in Vn content. This indicates the CPG contains a vitellogenesis promoting factor (VPF) which regulates Vtg synthesis. However, Vtg

mRNA expression in the ovarian tissue cultured in vitro showed no change with VPF, while it was promoted by E_2 (Osada et al., 2004a). VPF appears to promote vitellogenesis at the level of translation (Fig. 6.11). ER-like immunoreactivity was found in the growing oocyte and the auxiliary cells, in which Vn immunoreactivity and Vtg mRNA were also found (Osada et al., 2003). These findings suggest that E_2 may be involved in the control of vitellogenesis mediated by ER.

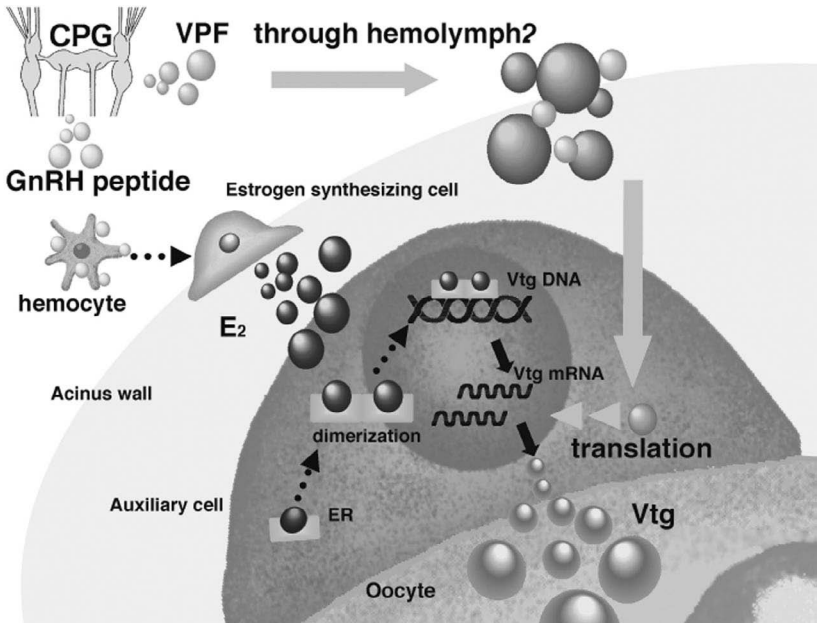


FIGURE 6.11 Central nervous system-gonadal control of vitellogenesis in scallops. Vitellogenin (Vtg) is synthesized in the auxiliary cell closely associated with growing oocyte. The transcription of Vtg mRNA is promoted by estradiol-17β (E_2) and the translation is enhanced by vitellogenesis promoting factor (VPF) from the cerebral plus pedal ganglion (CPG). The GnRH peptide secreted from CPG may be transported by the hemocyte in the circulation system and received by estrogen synthesizing cell along the outside of the acinus wall to stimulate the secretion of E_2. It remains unknown whether vitellogenesis is mediated by estrogen receptor (ER), though ER immunoreactivity is found in the auxiliary cells.

ERs are members of the nuclear receptor (NR) superfamily, which have a number of common features and their proteins can be divided into six domains. The DNA-binding domain (DBD) and the ligand-binding domain (LBD) are most highly conserved among species. The DBD recognizes and binds to specific responsive elements in DNA. The LBD regulates hormone-dependent transcription of target genes, such as Vtg.

To understand the estrogen signaling in the vitellogenesis of bivalves, the oyster ER homolog was isolated, which is highly similar to ERs of other species (Matsumoto et al., 2007). In invertebrates, ER has been cloned from mollusks, *A. californica* (Thornton et al., 2003), *Thais clavigera* (Kajiwara et al., 2006), and *O. vulgaris* (Keay et al., 2006). The amino acid sequence of oyster ER protein revealed a high identity to other mollusk ERs. The phylogenetic analysis indicated that oyster ER is most closely related to the other mollusk ERs. This indicates that the oyster ER isolated is an ortholog of Aplysia ER, snail ER, and octopus ER. The oyster ER also did not activate luciferase expression in the presence of E_2 and constitutively activated the reporter transcription. The addition of E_2 did not induce the further enhancement of the upregulation. This is consistent with the results on other mollusk ERs.

Mollusk ER orthlog is called ER because of high sequence similarity to vertebrate ERs; however, it activates transcription in the absence of ligand and does not bind steroid hormones. Annelid ER exceptionally conserved architecture of ligand pocket and responded to estrogen (Keay and Thornton, 2009). The X-ray crystal structure of oyster ER was determined and found that its ligand pocket is filled with bulky residues that prevent ligand occupancy (Bridgham et al., 2014). The oyster genome possesses 43 putative NR sequences (Vogeler et al., 2014). It contains two members of NR3, the sex steroid hormone receptor analogs, an ER homolog identified as oyster ER and an estrogen related receptor homolog, which is constitutively activated and unlikely to bind estrogen. Additional NR3 members, which could interact with vertebrate sex steroids, were not identified. Given prior studies of vitellogenesis induced by estrogens, these imply the effects must be mediated by mechanisms other than ER activation in the presence of estrogens.

Although the function of ERs from mollusks remains unsolved, snail ER mRNA was expressed in the ovary and cerebral ganglia (Kajiwara et al., 2006) and octopus ER expression was observed in both sexes, with the highest levels in ovary (Keay et al., 2006). The oyster ER mRNA was detected in all tissues tested, with the higher expression in ovary. The immunohistochemical localization of oyster ER using the antiserum to synthetic oyster ER peptide was found in the nuclei of follicle cells, the site of Vtg synthesis, and oocytes (Matsumoto et al., 2007). In *M. edulis*, two novel forms of ER-like genes have been isolated as an ER and an estrogen-related receptor (ERR), respectively, which were localized in the oocytes and follicle cells in contact with developing oocytes in the ovary and Sertoli cells in the testis, and in the ciliated cells of the gill (Nagasawa et al., 2015b). And a significant increase in ER not ERR as well as Vtg mRNA expression was

observed when mussels were exposed to estrogens during the early stage of gametogenesis (Ciocan et al., 2010). We cannot rule out the possibility that E_2 might be mediated through alternative NRs and activate Vtg gene expression in the ovary. Nevertheless, the question of what roles mollusk ERs have remains unanswered.

6.5 SPAWNING

Spawning in marine invertebrates including bivalve mollusk is suggested to correlate with changes in temperature, lunar age, illumination, salinity, abundance of phytoplankton, food availability, physical shock, tidal surge, drying, and radical oxygen, and such an environmental fluctuation has been thought to be a natural cue to induce spawning mediated through endogenous regulation mechanism (Giese & Kanatani, 1987). Blake and Sastry (1979) have reported that a neurosecretion associated with stage V of the neurosecretory cycle in the CNS has been associated with spawning in the bay scallop, *Argopecten irradians*. The involvement of the cerebral ganglion in spawning has been suggested on the basis of the histological change in the ganglion associated with spawning and induction of spawning by ablation of the ganglion (see Barber & Blake, 2006). The implication of the CNS in bivalve spawning has been predicted for many years, although endogenous and specific factor controlling spawning in bivalves has not been found by this time.

The relationship between specific neurosecretory substances and spawning has been first investigated in Japanese scallop, *P. yessoensis*. Exogenous serotonin (5-HT) has been found to strongly induce spawning in the scallop in vivo and suggested to play an important role in mechanism of the spawning in bivalves (Matsutani & Nomura, 1982). The reproducibility of 5-HT-induced spawning has been ascertained in a considerable number of other marine bivalves (Braley, 1985; Gibbons & Castagna, 1984; Tanaka & Murakoshi, 1985). The 5-HT neuron has been immunologically identified in the pedal ganglion, cerebral ganglion, and accessory ganglion adjacent to visceral ganglion, and 5-HT nerve fibers have been found to distribute around the gonoduct and along the outside of germinal acini of the scallop (Matsutani & Nomura, 1986a). The localization of the 5-HT neuron and nerve fiber strongly supported a regulation of spawning process in the gonad by endogenous 5-HT. The UV ray-irradiated seawater is well known to induce spawning of the scallop as well as abalone, *Haliotis discus hannai* (Kikuchi & Uki, 1974; Uki & Kikuchi, 1974). The mechanism of induction

of spawning with UV ray-irradiated seawater of the scallop has been pharmacologically demonstrated. The UV ray-irradiated seawater stimulates serotonergic mechanisms via dopaminergic mechanisms and induces spawning, which is modulated with prostaglandins (Matsutani & Nomura, 1986b).

A quantitative analysis of monoamine and prostaglandin during spawning of bivalves has been attempted to understand the roles of each endogenous substance in the spawning. In scallop, *P. yessoensis*, Osada et al. (1987) showed that significantly decreasing level of dopamine in the CNS and gonad of both genders after spawning induced by UV ray-irradiated seawater, suggesting that the release of dopamine might stimulate serotonergic mechanisms for induction of spawning. Thermal stimulation-induced spawning in other kind of scallop, *Argopecten purpuratus*, has in turn resulted in changes of levels of dopamine, noradrenaline and serotonin in the CNS, muscle and gonad (Martínez et al., 1996). Seasonal variations in the levels of prostaglandin F2α (PGF2α) and prostaglandin E_2 (PGE_2) in the gonad were closely related to the reproductive cycle, suggesting that PGF2α and PGE_2 might be involved in the sexual maturation and spawning of the scallop (Osada & Nomura, 1990). PGF2α and PGE_2 in the gonad have been reported to significantly decrease during spawning of females and conversely increase during spawning of males. It was suggested that these PGs in females and males are suppressive neuromodulators of 5-HT-induced egg release and acceleratory neuromodulators of 5-HT-induced sperm release from the gonad tissue of scallop, respectively (Osada et al., 1989). In in vitro experiment, PGF2α significantly inhibited 5-HT-induced egg release from ovarian tissue and PGE2 enhanced the 5-HT function, suggesting that PGF2α and PGE2 play a role as a suppressive and acceleratory modulators of 5-HT-induced egg release, respectively (Matsutani & Nomura, 1987). Taken together, PGF2α particularly may be a suppressive modulator in the spawning of the females and an acceleratory modulator in the spawning of the males (Osada et al., 1989).

5-HT function to induce spawning was also modulated by steroid as well as by PGs. The response to artificial stimulation with UV-irradiated seawater tended to become higher during sexual maturation (Uki & Kikuchi, 1974), suggesting that the sensitivity to 5-HT on spawning could rise, depending on maturity. Estrogen has been thought to be a stimulator on gametogenesis in bivalve because of its seasonal variation associated with gametogenesis and vitellogenesis accelerated by estrogen (Matsumoto et al., 1997; Li et al., 1998; Osada et al., 2004a,b). The promoting effect of estrogen on 5-HT-induced egg release in the scallop was observed when the ovarian

pieces of the scallop were pre-treated with estrogen before induction of egg release with 5-HT (Osada et al., 1992a). In bivalves, there have been several pharmacological characterizations of 5-HT receptors on the surface of germ cell to transduce 5-HT signal into the cell. The 5-HT receptor was pharmacologically characterized as a mixed profile of $5\text{-HT}_1/5\text{-HT}_2$ subtypes in the oocyte membrane of scallop and 5-HT_1 subtype in that of oyster (Osada et al., 1998). A unique type that showed mixed pharmacological properties (Kyozuka et al., 1997) in the oyster, a mixed $5\text{-HT}_1/5\text{-HT}_3$ type (Bandivdekar et al., 1991, 1992) or a novel type that was distinct from any mammalian 5-HT receptors (Krantic et al., 1991, 1993a,b) in the surf clam, and an original type in zebra mussels (Fong et al., 1993) were also reported in the oocyte and sperm. Osada et al. (1998) reported that the expression of the 5-HT receptor in the oocyte membrane of the scallop was induced by E_2 via a genomic action in their pharmacological experiment, suggesting that 5-HT receptors induced by estrogen distribute in the oocyte membrane and increase during oocyte growth, leading to a rising sensitivity to 5-HT involved in spawning (Fig. 6.12).

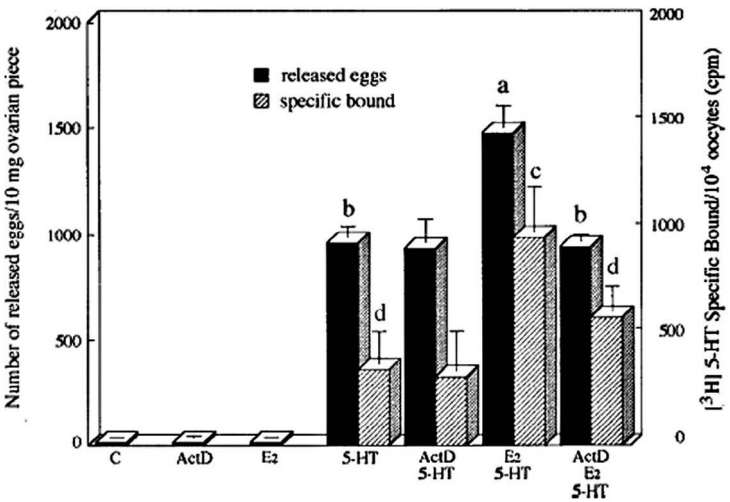


FIGURE 6.12 Effects of estradiol-17β and actinomycin D on 5-HT-induced egg release from ovarian pieces and specific bound of [^{3}H] 5-HT to scallop oocyte membranes teased from ovarian pieces before 5-HT induction. 2×10^4 cells of prepared oocytes were incubated with 0.3 μM [^{3}H] 5-HT. Each value represents the mean ± SE ($n = 3$). [a]Significantly different from b ($p < 0.005$). [c]Significantly different from d ($p < 0.01$). C; control, ActD; actinomycin D (From Makoto Osada, Ayumi Nakata,Toshie Matsumoto, Katsuyoshi Mori, "Pharmacological characterization of serotonin receptor in the oocyte membrane of bivalve molluscs and its formation during oogenesis," in Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 1 June 1998, 281, 124. Reprinted by permission of John Wiley & Sons, Inc.)

The 5-HT acts as a neurohormone to directly mediate meiosis reinitiation of prophase-arrested oocytes (Gobet et al., 1994; Guerrier et al., 1993; Hirai et al., 1988; Krantic et al., 1991; Osanai & Kuraishi, 1988; Osanai, 1985; Varaksin et al., 1992) as evidences by germinal vesicle breakdown (GVBD) (Matsutani & Nomura, 1987) using isolated oocytes in *Spisula solidissima*, *Spisula sachalinensis*, *C. gigas*, and *Ruditapes philippinarum*. In the scallop in which it is impossible to isolate oocyte from the ovary due to cytolysis after detachment from the germinal epithelium, GVBD oocytes were observed in paraffin section of ovarian tissue treated by 5-HT with dose dependent manner, which was identical to a variation of egg release induced by 5-HT (Tanabe et al., 2006) (Fig. 6.13). These results suggested that the induction of oocyte maturation is a primary role of 5-HT for their spawning. These facts suggest a distribution of 5-HT receptor on the surface of membrane of germ cell as mentioned before.

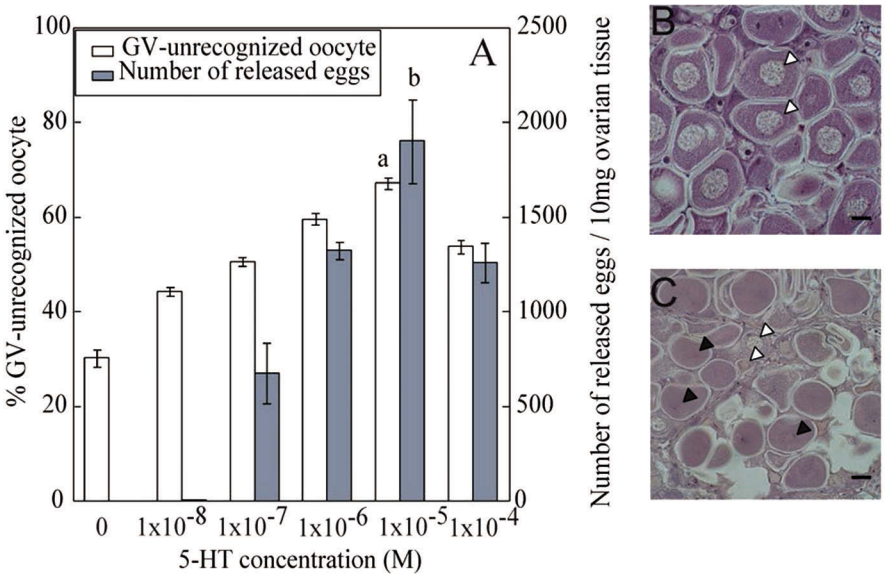


FIGURE 6.13 (A) Effect of various concentration of 5-HT on the induction of oocyte maturation and egg release of *Patinopecten yessoensis*. (B) A germinal vesicle (white arrowhead) can be seen in the section of ovarian tissue incubated without 5-HT. (C) Spindle fibers (black arrowhead) and a few germinal vesicles (white arrowhead) can be seen in the section of ovarian tissue incubated with 5-HT. [a,b]Significantly different from the others ($p < 0.05$). Each value indicates the mean ± SE ($n = 4$). Scale bar indicates 20 μm. (From Tanabe, T. et al. in "A novel oocyte maturation arresting factor in the central nervous system of scallops inhibits serotonin-induced oocyte maturation and spawning of bivalve mollusks," in General and Comparative Endocrinology, 2006, 147, 352. Reprinted with permission from Elsevier.)

In mollusk, primary structures for seven 5-HT receptors have been determined by molecular cloning. Six 5-HT receptor cDNAs were cloned from CNS and reproductive system of gastropods, pond snail *L. stagnalis* and sea hare *A. californica* (Angers et al., 1998; Barbas et al., 2002; Gerhardt et al., 1996; Li et al., 1995; Sugamori et al., 1993). The full-length cDNA encoding a putative 5-HT receptor has been isolated from the ovary of the scallop as a 5-HT_{py} (Tanabe et al., 2010). The 5-HT_{py} was classified into a vertebrate serotonin receptor 5-HT_1 subtype based on molecular architecture, homology searches, and phylogenetic analysis. The features of the absence of introns in the coding region of the gene, a relatively long third cytoplasmic loop, and a short fourth inner terminal domain (C-terminal tail) characterized the 5-HT_{py} as an ancestral 5-HT receptor and a member of the 5-HT_1 receptor family coupled with G protein with high probability (Paul & Mario, 2001; Tierney, 2001). A positive 5-HT_{py} signal was ubiquitously observed in any peripheral tissue as well as the nervous system, interestingly in the spermatid, oocyte and ciliated epithelium of gonoducts in both male and female gonads (Tanabe et al., 2010) (Fig. 6.14). These results suggested that effects of 5-HT on a series of events of spawning consisting of induction of oocyte maturation, sperm motility and transportation of mature oocyte and sperm through the ciliated epithelium of the gonoducts could be mediated by5-HT_{py}. The gene expression of 5-HT_{py} in the ovarian tissues was significantly up-regulated by E_2 (Tanabe et al., 2010), which supported the pharmacological results that the expression of the 5-HT receptor in the oocyte membrane has been induced by E_2 via a genomic action leading to a rising sensitivity to 5-HT in relation to spawning, and explained a higher sensitivity to external stimuli for spawning depending on maturity (Osada et al., 1998; Uki & Kikuchi, 1974).

After the binding of 5-HT to 5-HT receptors on the membrane of oocyte and sperm, the 5-HT signal is transmitted into the cells. Sperms are in a quiescence state just before ejaculation from the testis. The 5-HT signal transduction into the sperm mediated through 5-HT receptor was suggested to initiate 5-HT-dependent and osmolality-independent sperm motility in marine bivalve mollusks, which is associated with a K^+ efflux and Ca^{2+} influx via voltage-dependent ion channels under alkaline conditions (Alavi et al., 2014). And Na^+ influx was thought to be also important for the initiation of sperm motility probably via regulation of Ca^{2+} exchange (Fig. 6.15) (Alavi et al., 2014). Oocytes of oviparous animal are generally arrested in late prophase of meiosis I just before ovulation. At this stage, the oocytes posses a developmental stage-specific nucleus, the GV corresponding to the dictyate stage of oocyte. Meiosis reinitiation is a process of oocyte maturation

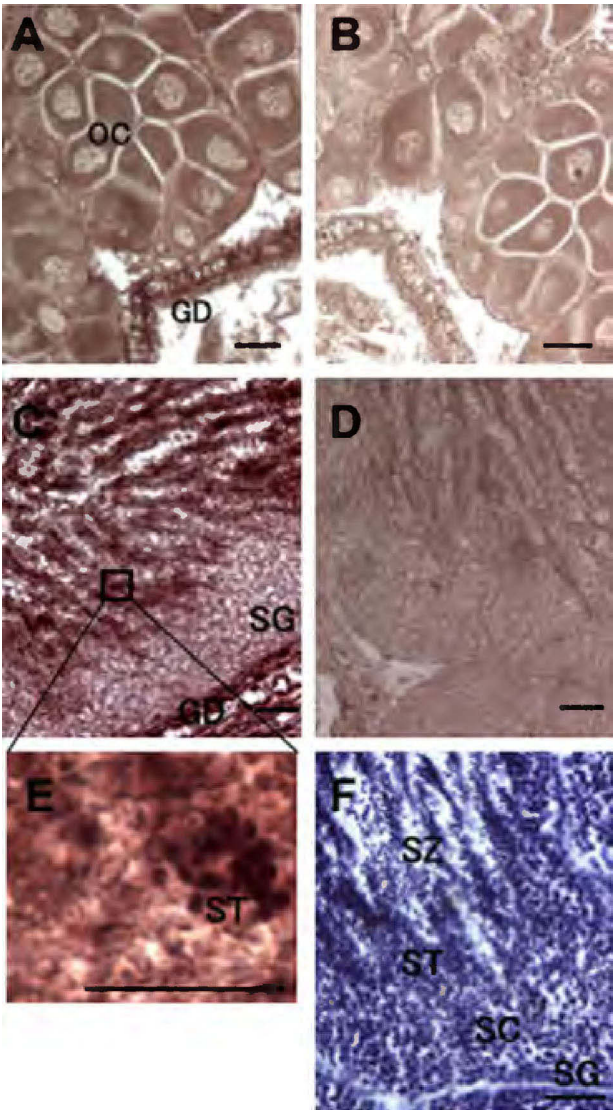


FIGURE 6.14 Localization of 5-HT$_{py}$ mRNA in the scallop ovary and testis. The ovary tissue hybridized with the antisense (A) or sense (B) DIG-labeled 5-HT$_{py}$ cRNA probe. The testis tissue hybridized with the antisense (C and E) or sense (D) DIG-labeled 5-HT$_{py}$ cRNA probe or was stained by hematoxylin–eosin (F). (E) is the large scale of the boxed part in C. Abbreviations: OC, oocyte; GD, gonoduct; SG, spermatogonia; SC, spermatocyte; ST, spermatid; SZ, spermatozoa. The scale bar represents 10 μm in E and 20 μm in A, B, C, D, and F. (From Tanabe,T. et al. in "The role in spawning of a putative serotonin receptor isolated from the germ and ciliary cells of the gonoduct in the gonad of the Japanese scallop, Patinopecten yessoensis," in General and Comparative Endocrinology, Volume 147, Issue 3, July 2006, 166, 620. Reprinted with permission from Elsevier.)

as evidenced by GVBD. It has been demonstrated that oocyte maturation and spawning of invertebrates are initiated by 1-methyladenine, cubifrin, and serotonin in starfish, sea cucumber, and surf clam, respectively (Hirai et al., 1988; Kanatani et al., 1969; Kato et al., 2009; Osanai & Kuraishi, 1988). 5-HT-induced oocyte maturation in bivalves was suggested to be resulted from a combination of the transduction mechanisms of 5-HT signal and their cross-talk involved in 5-HT-induced oocyte maturation (Krantic & Rivailler, 1996). The 5-HT signal may reduce cyclic AMP in the oocyte cytoplasm inhibiting adenylate cyclase coupled to G_i protein. The 5-HT signal simultaneously induces conversion of phosphatidylinositol-4, 5 bisphosphate (PIP2) into inositoltriphosphate (IP3) and diacylglycerol (DAG) activating phospholipase C coupled to G_0 protein, and increase in Ca^{2+} uptake activating membrane voltage-dependent Ca^{2+} channels coupled to G_x protein. These pathways activate protein kinases (PKA and PKC) and mitogen-activated protein kinase (MAPK), which activate maturation promoting factor (MPF) consisting of cdc2 and cyclin B and result in oocyte maturation.

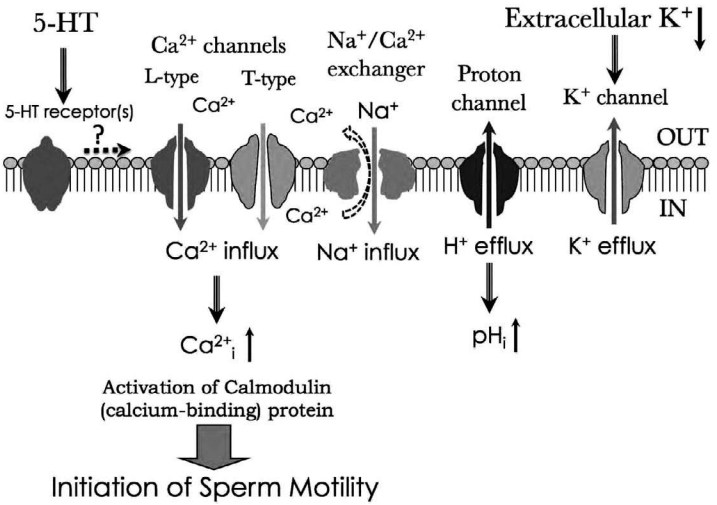


FIGURE 6.15 Ionic fluxes required for the initiation of sperm motility in marine bivalve mollusks. The stimulatory effect of serotonin (5-HT) on the initiation of sperm motility were associated with potassium (K^+) efflux, proton (H^+) efflux and calcium (Ca^{2+}) influx through a voltage-dependent K^+ channel, a proton channel and both L-type and T-type voltage-dependent Ca^{2+}, respectively. A sodium (Na^+)/Ca^{2+} exchanger regulates Na^+ influx to control intracellular Ca^{2+} during motility period. These steps stimulate Ca^{2+}-dependent or calcium-calmodulin (CaM) protein phosphatase(s) in the flagellum for the initiation of sperm motility. (From Alavi et al. in "Roles of extracellular ions and pH in 5-HT-induced sperm motility in marine bivalve", Reproduction 2014, 147, 331. Reprinted with permission from Elsevier.)

Given the 5-HT functions in oocyte maturation and initiation of sperm motility, an administration of exogenous 5-HT into bivalves must be able to induce spawning in vivo. Interestingly, it has been reported that the full-grown oocytes of bivalves at the mature stage are arrested at the dictyate stage corresponding to late prophase of meiosis I, which is commonly observed in marine invertebrates (Krantic & Rivailler, 1996). The arrest state is held for 2 months after reaching the highest 5-HT-specific binding to oocyte membrane until the spawning stage (Osada et al., 1998). Furthermore, the application of exogenous 5-HT in the induction of spawning does not always succeed and the number of released eggs widely varied among individuals (Matsutani & Nomura, 1987). These observations indicate the occurrence of modulatory mechanisms acting on 5-HT-induced oocyte/sperm maturation and spawning by maturation-competent extracellular signals. Oocyte maturation inhibitor and granulosa cell factor found in mammals have been reported to be heat-stable polypeptides with masses of less than 2 and 6 kDa, respectively (Hillensjo et al., 1985; Franchimont et al., 1988; Sato & Koide, 1984; Tsafriri & Pomerantz, 1986). In the bivalve *S. solidissima*, substances originating from oocytes that inhibit oocyte maturation induced by 5-HT have been identified as a *Spisula* factor with a mass of less than 1 kDa (Kadam & Koide, 1990; Sato et al., 1985) and an oocyte membrane component with a mass of more than 18 kDa (Sato et al., 1992). However, the gonad of bivalves has no such follicle structure like vertebrates and the roles of the *Spisula* factor in the endocrine mechanisms have not been well demonstrated. PGF2α has been suggested as a suppressive neuromodulator of 5-HT function in spawning of bivalves as mentioned before. In fact PGF2α certainly blocked 5-HT-induced egg release through the gonoduct from ovarian tissue, but did not inhibit 5-HT-induced oocyte maturation, suggesting that the cilioexcitatory activity of 5-HT in the gonoducts to transport mature eggs may be inhibited by PGF2α (Tanabe et al., 2006). In addition to PGF2α, a novel inhibitor of 5-HT-induced egg release from ovarian tissue was found in the CNS of the scallop *P. yessoensis* of both genders. A main action of the neural factor was to arrest 5-HT-induced oocyte maturation, named "oocyte maturation arresting factor," OMAF (Tanabe et al., 2006) (Fig. 6.16). The OMAF was a universal substance for bivalve species in both genders and thought to be transported from the CNS to the ovary through blood flow based on occurrence of OMAF function and its identification in the hemolymph. The OMAF may prohibit 5-HT-induced oocyte maturation and sperm motility due to the interference of extracellular Ca^{2+} influx into oocytes, eventually resulting in the inhibition of spawning (Tanabe et al., 2006; Yuan et al., 2012) (Fig. 6.17). Internal amino acid

sequences of the OMAF with a molecular mass of 52 kDa were determined and an antibody against the partial peptide of OMAF strongly amplified the 5-HT-induced release of egg and sperm due to the release from suppressive activity of OMAF by neutralizing endogenous OMAF with antibody. These results confirmed that the OMAF acts as an inhibitor of 5-HT-induced oocyte maturation and sperm motility (Yuan et al., 2012). Taken all together, 5-HT is an essential neurohormone for crucial events of oocyte maturation and sperm motility and following spawning of bivalves. The mode of actions of 5-HT on activation of germ cell and gonoduct regulated by OMAF and PGF2α is supposed to explain an arrest of oocyte maturation and sperm motility until spawning and simultaneous spawning in nature. Moreover, it is necessary for us to uncover a mystery as to receptor mechanisms of them and how bivalves can be released from the negative control by OMAF and PGF2α and secret 5-HT to induce simultaneous spawning.

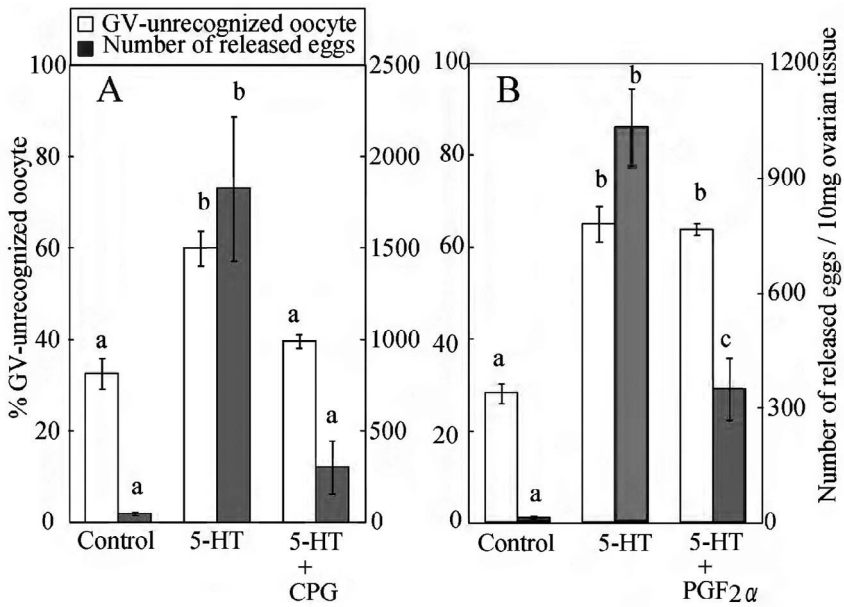


FIGURE 6.16 Effects of cerebral and pedal ganglia (CPG) extract and prostaglandin PGF2α on 5-HT-induced oocyte maturation and egg release of *Patinopecten yessoensis*. 1×10^{-5} M 5-HT was applied to the ovarian tissues with CPG extract (A) or 1×10^{-6} M PGF2α (B). Values with different letters within each section A and B, and among parameters are significantly different ($p < 0.05$). Each value indicates the mean ± SE ($n = 4$). (From Tanabe, T. et al. in "A novel oocyte maturation arresting factor in the central nervous system of scallops inhibits serotonin-induced oocyte maturation and spawning of bivalve mollusks," in General and Comparative Endocrinology, 2006, 147, 352. Reprinted with permission from Elsevier.)

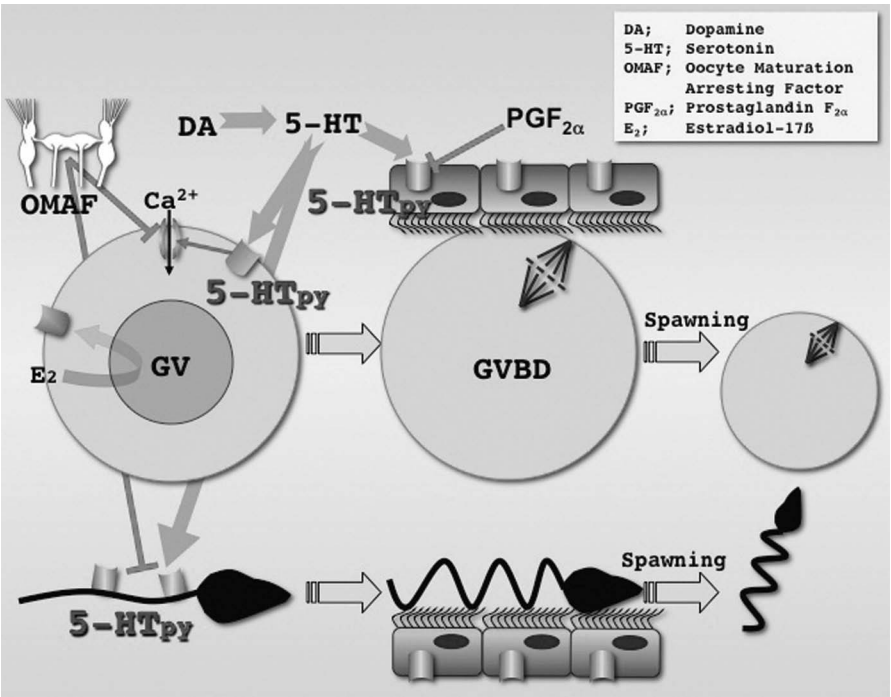


FIGURE 6.17 Illustration of transduction of extracellular signals for regulation of oocyte maturation, sperm motility and spawning. The stimulatory effect of 5-HT on oocyte maturation and sperm motility via 5-HT receptor was negatively regulated by OMAF mediated through Ca^{2+} ion. Transportation of activated oocyte and sperm through gonoduct is regulated with 5-HT and PGF2α.

6.6 PROSPECTIVE

A genetic research focused on the animal group of mollusk was not many compared with that of other animals including model invertebrates such as fruit fly, nematodes, etc. Recently, genetic analysis data are accumulated in mollusks and molecular works are well contributing to realize the molecular mechanisms involved in physiology of bivalve mollusks. Development of Next Generation Sequencer accelerated to read sequences of genome and transcripts on a massive scale in any kind of organisms. Pearl oyster, *Pinctada fucata* (Takeuchi et al., 2012), and Pacific oyster, *C. gigas* (Zhang et al., 2012), genomes have been sequenced and draft genome database has been constructed. The genome database was utilized as a platform for identification of specific genes for calcification, which is an essential phenomenon

to produce an ornamental pearl, stress adaptation, shell formation, and larval development. The released draft genome of the pearl oyster has been analyzed to screen reproductive-related genes (Matsumoto et al., 2013). A de novo transcriptome sequencing and analysis has led us to understanding of sex determination and differentiation, oocyte maturation, growth, and stress response (Dheilly et al., 2012; Ghiselli et al., 2012; Hou et al., 2011; Pauletto et al., 2014; Teaniniuraitemoana et al., 2014). For the future, the application of genome and transcriptome database is supposed to be a strong tool for research on comprehensive understanding of the mechanism of reproduction in bivalve mollusk because it is generally difficult to isolate a specific gene on the basis of the conserved region obtained from an alignment of a few mollusk sequences or vertebrate sequences which are taxonomically far from mollusks. The biological function of genes screened from genomic and transcriptomic resource should be proven in each animal to understand their primary physiological function in bivalve mollusks.

KEYWORDS

- **bivalvia**
- **gonad**
- **gametogenesis**
- **spawning**
- **intragonadal somatic cells**
- **endocrine**
- **neuroendocrine**

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CHAPTER 7

THE PHYSIOLOGY OF REPRODUCTION IN CEPHALOPODS

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CONTENTS

ABSTRACT

Physiological and behavioral aspects of reproduction in cephalopods have been widely addressed. In this chapter, new as well as old data on cephalopods reproduction are reported.

In these complex mollusks, sexes are separated with sexual maturity peaking at different stages of lifespan cycle, depending on the sex. Males mature earlier than females, the latter showing generally a semelparous behavior.

Although some differences among decapods and octopods during courtship and mating strategies, these orders show great anatomical similarities in reproductive organs. Fertilization is external (decapods) or internal (octopods), but in both cases sperm-attracting factors seem to be involved.

The bulk of knowledge on the physiological control of reproduction comes from studies on *Octopus vulgaris*. From the pivotal hypothesis of Wells and Wells (1959), more studies have been accumulated and a more complex frame of nervous and endocrine control has been hypothesized (Di Cristo, 2013).

New data from genomics and transcriptomics analyses could enrich and clarify some key steps of reproduction in these animals.

7.1 INTRODUCTION

Reproduction is one of the most studied biological aspects of cephalopods. In this class of short-lived mollusks (generally 1–2 years only), the sexes are separate and there are no hermaphrodites or sex reversals. In most species males and females are rather alike in form, but there are exceptions: many loliginids and the secondarily pelagic octopod *Argonauta* show extreme sexual dimorphism, with minute males. Sexual maturity in cephalopods differs between the sexes. Males are reproductively capable for the greater part of their life cycle. Females, which can mature later in life, can receive and store sperm for the majority of their lives, so that fertilization and egg laying are temporally independent of mating.

Most cephalopods reproduce only once, death occurring soon afterward (Boyle, 1983, 1987). The immediate causes of this apparently universal mortality are not clearly understood, but the sequence of physical changes brought about by the optic gland hormone seems to be irreversible. Some direct evidence for this hypothesis is available from experiments in which

the optic glands from mature octopuses were excised; their gonads regressed, while feeding and growth was resumed (Tait, 1986; Wodinsky, 1977).

However, such semelparous behavior is not universal in cephalopods: *Octopus chierchiae* is iteroparous (Rodaniche, 1984) and some oceanic squids such as *Sthenoteuthis oualanimsis* (Harman et at., 1989; Nigmatullin & Laptikhovsky, 1994) and the deep sea cirrate octopods *Opisthoteuthis* spp. (Villaneuva, 1992) produce series of mature eggs throughout most of their life cycle. Estimates of individual fecundity vary widely between species, and there is a trade-off between egg size and fecundity. Compared with other marine invertebrates, the eggs of cephalopods are large, well-protected, and produced in relatively low numbers.

The anatomy of the cephalopod reproductive system is rather similar across orders. The essential physiological processes of the onset and progress of reproductive maturity in females are the meiotic maturation of the oocyte, the production and sequestration of yolk in the oocyte (vitellogenesis), the development of organs for the formation of protective individual egg coats (oviducal glands), and the encapsulation of the spawned egg mass (nidamental glands). In most shelf species of cuttlefish, squid and octopus, the enlargement of oviducal and nidamental glands marks the beginning of breeding competence and reproduction. Such processes seem to be controlled by neurohormones and hormones (see Di Cristo, 2013 and references). In males, sexual maturity is reached when mature spermatozoa are packaged into complex spermatophores stored in the spermatophoric (Needham's) sac. Even these aspects seem to rely on hormones (D'Aniello et al., 1996; Wells & Wells, 1959).

Fertilization is achieved after individual mating in which spermatophores are transferred from the male to the female using an arm specially modified for the mating process (the *hectocotylus*). There may be competition for mates, but multiple mating is common. Egg masses are attached to the rocks (most octopuses, loliginid squid, and cuttlefish) or released into the water column in fragile gelatinous masses (most squid families).

This chapter presents new, as well old, data on cephalopod reproduction. The physiology and endocrinology of the reproductive system of these animals are however not well understood, even in the common octopus, which, for these studies, still remains the best-studied model in cephalopod. In fact, the bulk of studies on the endocrinology of cephalopod reproduction have been made on the *Octopus vulgaris*. New insights from the study of the nervous (and non-nervous) control of *Octopus* reproduction (Di Cristo, 2013) have added new aspects to the classical studies of Wells and Wells

(1959), but a clear and detailed picture of the control of cephalopod reproduction is still missing.

It is first necessary to describe the anatomy of reproductive systems of cephalopods to better understand their control. Separate anatomical and physiological descriptions from the Octopoda and the Decapoda will be used, in view of the differences between the two groups. Squids are the subject of extensive commercial fisheries, and a good deal is known about their growth rate and breeding seasons. Their eggs are laid in large masses at predictable times of year; embryological material has always been readily available, and much of the classical work on cephalopod development has been done with this. In contrast, little is known about their reproductive physiology; the animals are, in general, difficult to keep in laboratories and they do not respond well to surgery. For the octopods, a different spectrum of information is available. Several species can live, grow, and breed in aquariums, and *O. vulgaris* in particular has been the subject of very extensive behavioral experiments. Because this species is resistant to surgical interventiont it has proved possible to manipulate their glandular condition and so discover a good deal about the mechanisms regulating the condition of the gonads. Against this, and again in contrast with decapods, there is comparatively little information about seasonal migration, synchronization of spawning, and growth rates (Boyle & Rodhouse, 2005).

For these reasons, this chapter is concerned largely with the physiology of reproduction of *O. vulgaris*, since this is the animal we know most about. No comparable body of information exists for the open-water octopods or about the reproductive behavior of decapods, with the exception of *Sepia*, which is atypical in being a bottom-living form.

7.2 CEPHALOPOD REPRODUCTIVE SYSTEMS

7.2.1 FEMALE REPRODUCTIVE SYSTEM

The female reproductive system in decapods consists of five main components: a median single ovary, a single oviduct with thin-walled and glandular portions, paired nidamental glands, paired accessory nidamental glands, and a seminal receptacle (Döring, 1908; Williams, 1909) (Fig. 7.1).

Eggs, once shed, pass in into the ciliated funnel of the oviduct and are stored free in the proximal thin-walled portion of the oviduct until copulation. Here, they are coated with a layer of egg jelly in a glandular region of the oviduct on the left side of the mantle.

The funnel-shaped opening of the oviduct is closely interposed with the openings of the nidamental and accessory nidamental glands in spawning females. The paired nidamental glands (Fig. 7.1) appear as white, elongate structures on the midline between the branchial hearts and the arms. These glands produce a large amount of mucus that coats the forming egg capsule and has ciliostatic effects on other microorganism and marine embryos (Atkinson, 1973; Atkinson & Granholm, 1968). The accessory nidamental glands appear in sexually mature female as an orange to red mass and are continuous with the anterior portion of nidamental glands. The accessory nidamental glands form the jelly that coats the egg capsule when it is passed from the oviduct funnel into the siphon prior to laying.

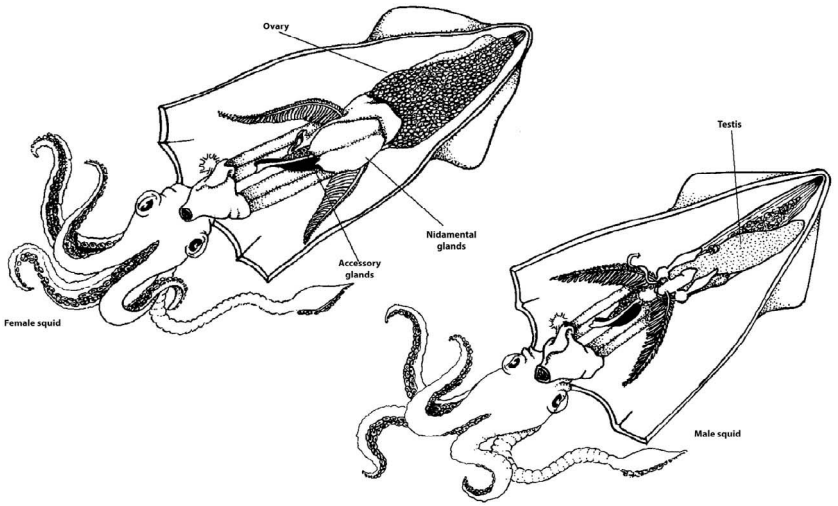

FIGURE 7.1 Drawing of the anatomy of female and male squids. The ventral side of the mantle has been medially sectioned to expose internal organs. Gonads and reproductive ducts are indicated. Note the ovary full of eggs.

A set of regulatory peptides produced by the ovary are involved in the egg laying process and control the contraction of both glands and oviduct in *Sepia officinalis* (Bernay et al., 2004–2006).

In octopods, there is a single ovary, an oviducal gland and two oviducts (Fig. 7.2A), one opening on either side of the midline about halfway along the mantle cavity (Wells, 1978).

In the ovary, as the eggs develop, each oocyte becomes enveloped in a double layer of follicle cells (Fig. 7.3), the inner layer cuboid and secretory, the outer a thin skin of flattened epithelium (Buckley, 1977).

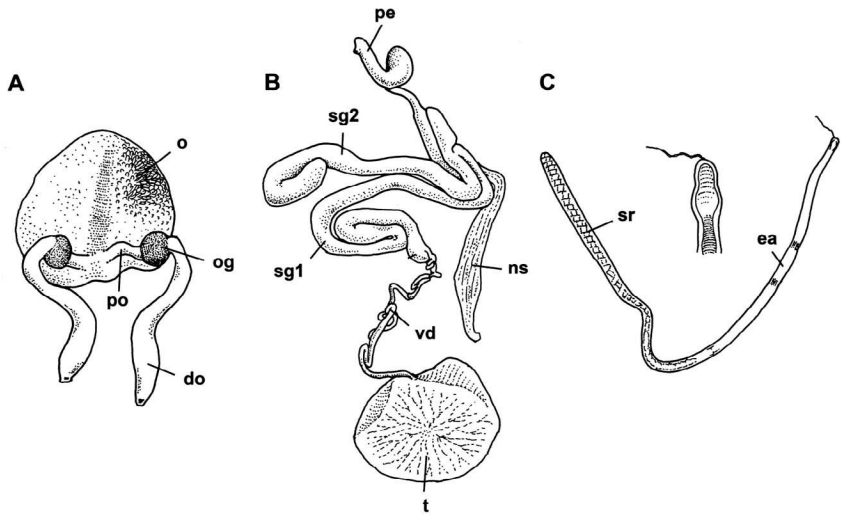

FIGURE 7.2 Anatomy of the reproductive system of female and male octopus. (A) Reproductive system of female: ovary, oviducal glands and oviducts. (B) Reproductive system of male after removal of the connective capsule. All the ducts are stretched to show their fine structure. (C) Spermatophore with an enlargement of the "female-oriented" region. Abbreviations: Female reproductive tract: do, distal oviducts; o, ovary; og, oviducal gland; po, proximal oviduct. Male reproductive tract: ns, Needham's sac; sg1, spermatophoric gland (seminal vesicle); sg2, accessory spermatophoric gland (prostate); t, testis; pe, penis; vd, vas deferens. Spermatophore: ea, ejaculatory apparatus; sr, sperm reservoir. (Modified from Norman MD, Hochberg FG, Boucher-Rodoni R, in "A Revision of the Deep-Water Octopus Genus Scaeurgus (Cephalopoda: Octopodidae) with Description of Three New Species from the Southwest Pacific Ocean," in Journal of Molluscan Studies, Volume 71, Issue 4, Pp. 319-337. With permission from Oxford University Press.)

The former secrete most of the yolk in the egg, and probably also the chorion (Fig. 7.3), which hardens to form a tough eggshell before the eggs are laid. Contractions of muscle fibers in the egg stalk move the eggs in the ovisac. The ovary, the oviducal gland, and the oviduct develop parallel always (Di Cosmo et al., 2001).

The oviducts swell and stiffen with muscular and connective tissue when the animal matures. As sexual maturity approaches the oviducal glands (Figs. 7.2A and 7.4) enlarge and become grooved, resembling the segments of an orange grouped around the oviduct. The region closest to the more distal portion is a brilliant white; the region closest to the ovary is brown. It is possible to divide the interior of the gland into two regions, each of which secretes mucus in a series of ducts, which converge in the lumen of oviduct (Fig. 7.4). This secretion seems to be controlled by neuropeptides and sex steroids (Di Cosmo et al., 1998, 2001, 2002; Di Cristo et al., 2002, 2005; Di Cristo & Di Cosmo, 2007).

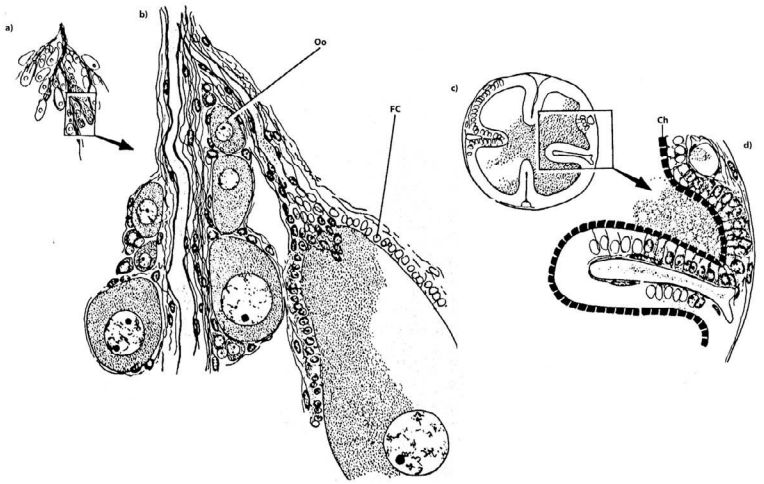

FIGURE 7.3 Structure of the *Octopus vulgaris* ovary. (a) A strand of eggs radiating from the midline of the ovary. (b) Magnification of (a) to show the internal organization and maturation of eggs. Lines of follicular cells are starting to wrap a large oocyte. (c) As the ovary matures the follicle cells divide and enlarge until they form a series of folds invading the oocyte cytoplasm. (d) Detail of a fold of follicle cells forming the chorion around the oocyte. An extensive capillary blood system envelops each egg. The chorion and the main bulk of the proteinaceous yolk are secreted simultaneously. Abbreviations: Ch, chorion; FC, follicle cells; Oo, oocytes. (Modified from M. J. Wells, "Octopus—Physiology and Behaviour of an Advanced Invertebrate," in Behavioural Processes 3(4):356–357 · December 1978. With permission from John Wiley & Sons, Ltd.)

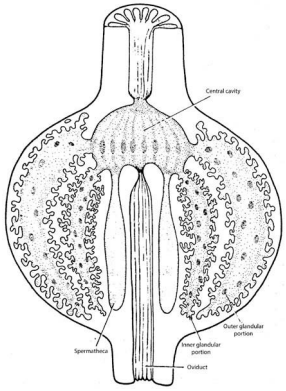

FIGURE 7.4 Drawing of the oviducal gland of *Octopus vulgaris* opened by a longitudinal cut. Note the three sets of compartments which open into the central cavity: the spermathecae, the inner, and the outer portion of the gland. (Modified from M. J. Wells, "Octopus—Physiology and Behaviour of an Advanced Invertebrate," in Behavioural Processes 3(4):356–357 · December 1978. With permission from John Wiley & Sons, Ltd.)

In addition to the two glandular portions, the oviducal glands contain a third series of convergent lumina, opening into a ring of thin-walled spermathecae (Fig. 7.4), the destination of sperm released from spermatophores placed in the oviducts. Animals as small as 180 g have been observed with spermathecae packed with spermatozoa. The sperm penetrate deeply into the walls of the sacs with their spiral acrosomes and then become immobile. They remain viable for long periods (Di Cosmo et al., 2001; Hanlon & Messenger, 1996; Mangold & Boletzky, 1973), unless reactivated by sex steroids (Tosti et al., 2001) and factors released by the eggs (De Lisa et al., 2013).

Not all octopods store sperm in spermathecae. In *Eledone*, for example, the bladder of sperm discharged by the spermatophore is carried up directly into the ovary.

7.2.2 MALE REPRODUCTIVE SYSTEM

The male reproductive system of decapods is composed by five components: the median testis, the vas deferens, the complex spermatophoric organ, the vas efferens, and the spermatophoric sac (or Needham's sac), which continues into the muscular penis (Fig. 7.1, but see also Fig. 7.2B). The mature testis is pure white and lies medially in the apex region of the mantle. Mature sperms, from the central lumen of the testis, are placed in the surrounding space and transported by the ciliated funnel of the vas deferens. In *Doryteuthis pealeii*, there is a progressive increase in the diameter of the vas deferens. In *Doryteuthis opalescens*, it first increases in diameter, then decreases approaching the spermatophoric organ (Arnold & Williams-Arnold, 1997).

The spermatophoric organ is a system of glandular tissue and lumina, which empty into a central canal or "sperm tract." While the sperm passes through this organ, it is compressed into a spiral mass, termed the spermatophore, and covered with different membranes and tunics. The mature spermatophores are moved one at a time, by the vas efferens, to the spermatophoric sac to be stored prior to mating.

The spermatophores are aligned in parallel arrays with their aboral (nonopening) end pointing distally.

The penis is on the left of the anus and appears as a muscular extension of the spermatophoric sac and through which pass several spermatophores during mating. There is great variation in the shape and size of the penis and spermatophores in different species. Many species have curved spermatophores, and a few species with exceptionally large spermatophores hold

them in a bent or even coiled condition, presumably because of the restrictive size of the spermatophore sac (Arnold & Williams-Arnold, 1997).

The male genital system in octopods is characterized by an unpaired testis and duct, opening on the left side of the mantle cavity (Fig. 7.2B). The testis appears as a compact mass of cells that surrounds a series of channels that converge on a ventral outlet from the coelomic surrounding the gonad. The germinal epithelium forms the wall of the radiating ducts within the testis and through its entire length. The germ cells proliferate and the spermatocytes differentiate as they move inward. Subsequently, free sperms migrate in the testis sac and then in the male duct, where they are packed into the spermatophore (Fig. 7.2C). In animals over 150 g, white spermatophores can usually be seen in the anterior more transparent parts of the duct.

The rest of the system is composed of three blind-ending diverticula (vas deferens, seminal vesicle or first spermatophoric gland, and prostate or second spermatophoric gland) and a storage compartment, the Needham's sac, in which the finished spermatophores accumulate prior to mating (Fig. 7.2B) (Belonoschkin, 1929a; Brock, 1878; Mann et al., 1970; Marchand, 1907; Peterson, 1959).

The sperm that leave the testis sac and arrive at the proximal vas deferens, emerge from this region compacted as a "sperm rope," with the sperm heads aligned closely. This rope will be coiled in a spiral when it leaves the vas deferens, and covered by mucilaginous secretion in the wider part of the duct (the first section of the first spermatophoric gland, which is subdivided into three sections).

The sperm rope with its mucin coating is moved through the first section of the spermatophoric gland. As it passes, a membrane (the sperm membrane or middle tunic) is added, sealing off the rope.

Some coatings are added in the following two sections of spermatophoric gland, together with the gel column, which is the heart of the ejaculatory apparatus, which at this stage lies posterior to the rest of the spermatophore (Mann et al., 1970).

The spermatophore continues into the second spermatophoric gland, a blind ending sac, where the ejaculatory apparatus is probably completed.

Spermatophores that emerge are stored in the Needham's sac. According to Peterson (1959) a "general finishing and hardening or dehydration" occurs here. Before it is placed in the groove of the hectocotylus, the packet will be shunted into yet another blind sac, the diverticulum of the penis. Usually, in a mature male, there is only one spermatophore in this region, correctly oriented toward the hectocotylus and ultimately to the oviduct of the female.

An *O. vulgaris* of 500–700 g that has not recently mated will normally carry about 50 spermatophores. No estimates of the number of sperm this represents have been made.

Reproductive male and female organs are innervated by the fusiform ganglion (Young, 1967, 1971). Neurons from this ganglion send neuropeptidergic terminals to the musculature of the wall of ducts and glands, possibly controlling their contraction (Di Cristo et al., 2002; Di Cristo & Di Cosmo, 2007).

7.3 GAMETES

Depending on the species considered, average female fecundities vary from a few hundred eggs (e.g. sepiolid squids), to several thousands (e.g., sepiid cuttlefish), to hundreds of thousands (e.g., merobenthic octopods like *O. vulgaris* or *O. cyanea*), or around 1 million, as in the pelagic octopod *Ocythoe tuberculata* (Salman & Akalin, 2012). Males generally produce large quantities of sperms stored in elaborated spermatophores (Mann et al., 1970). Cephalopods never show state of sexual rest (regression of reproductive organs).

7.3.1 EGGS MATURATION

The main processes of the onset and progress of reproductive maturity in the female are egg maturation (oogenesis and vitellogenesis), and the development of organs for the formation of protective individual egg coats (oviducal glands) and the encapsulation of the spawned egg mass (nidamental glands). Oogenesis, the production of eggs, is described for representative types such as *Sepia* (Dhainaut & Richard, 1976; Richard, 1971), *Loligo* (Knipe & Beeman, 1978; Selman & Arnold, 1977; Selman & Wallace, 1978), several ommastrephid squid species (Lipinski, 1979; Schuldt, 1979; Takahashi, 1978), and *Octopus* (Bolognari et al., 1976; Di Cosmo et al., 2001).

There are very few data about the meiotic maturation of oocytes in cephalopods. In *O. vulgaris*, L-type Ca^{2+} currents play a role in oocyte growth and cytoplasmic maturation, and possibly in preparing the plasma membrane for the interaction with the spermatozoon (Cuomo et al., 2005). A large germinal vesicle is characteristic of the immature oocytes (100–300-µm diameter), while in subsequent stages of growth (up to 1000-µm diameter) the nucleus is no more visible and the metaphase spindle appears. This suggests that in

O. vulgaris, oocytes are arrested in the first meiotic prophase up to the early-vitellogenic stage and resume meiosis at this stage up to a second block, presumably in metaphase I. Progesterone probably functions as hormonal stimulus for the first prophase–metaphase meiotic transition (Cuomo et al., 2005).

Small oocytes first arise from a germinal epithelium and are present from a very early stage (Fig. 7.3). Three phases of oocyte development are recognized along oocyte maturation (see Bottke, 1974 for the loliginid *Alloteuthis*; Boyle & Chevis, 1992 for *Eledone cirrhosa*, Di Cosmo et al., 2001 for *O. vulgaris*).

In the first stage (immature-previtellogenic phase), oocytes are less than 1 mm in length, and each is attached to the connective tissue of the ovary by a short stalk (Fig. 7.3). In the second stage (early vitellogenic phase), there is a migration of flattened follicles from the stalk in a single layer over surface of the oocyte. At this point the oocyte increases in volume, while the proliferation of the follicular cell layer increases at a greater rate, forming a deeply infolded double layer well supplied with blood vessels, giving the egg a reticulated appearance (Fig. 7.3).

In the third stage (full to late vitellogenic phase), cells of the follicular cell layer acquires columnar characteristics and secretes proteinaceous yolk into the lumen of oocyte. The eggs, consisting of an oocyte and follicular cells complex, undergo a rapid increase in volume. When the accumulation of yolk is full, the follicle cell layer degenerates and the chorion is formed. The formation of the oocyte/follicular cell complex seems to be the key to the rapid growth of cephalopod ovary during sexual maturation, since it seems that this complex accelerates the process of vitellogenesis. The extraordinary rate of development of the follicular cell layer is achieved by high rates of follicular cell division, with many nuclei becoming polyploid at this time (Boyle & Chevis, 1991). This follicular cell division and the subsequent huge release of vitellogenin into the ooplasm seem to be controlled by vertebrate-like steroids (Abate et al., 2000; Di Cristo et al., 2008). Progesterone induces vitellogenin synthesis in previtellogenic ovaries of *O. vulgaris* and is responsible for follicle cell proliferation (Di Cristo et al., 2008). The morphological changes in the oviduct and oviducal gland observed throughout the reproductive cycle in *O. vulgaris*, mirror progesterone and estradiol fluctuations, suggesting that both steroids might work in synergy to sustain the growth and differentiation of the female reproductive system in this species (Di Cosmo et al., 2001). Moreover, the localization of both progesterone and 17β-estradiol receptors in the nuclei of the follicle cells in the ovary of *O. vulgaris* sustains the theory that there is a

role for these steroids in the regulation of yolk synthesis (Di Cosmo et al., 1998; 2002). Finally, the presence of steroidogenic enzymes in the follicle cells, namely the 3β-hydroxysteroid dehydrogenase (3β-HSD) supports the concept of gonadial synthesis of steroids in *Octopus*.

At full maturity, the chorion is fully formed and encloses the egg, which detaches from its formative epithelial stalk. The size of eggs growing from the same string of germinal epithelium is not constant, but varies along the length of the string, and these differences are a further index of the stage of maturation of the animal (Boyle & Chevis, 1992; Di Cosmo et al., 2001).

Mature eggs are temporarily accumulated in the proximal oviduct (Boyle & Rodhouse, 2005). In *Octopus*, during spawning, eggs pass through a large section of the oviduct, the oviducal gland, in the distal portion. The glandular region of the oviducal gland provides secretion for the protection of the eggs and also preserves the accumulated sperm after mating of the female (Froesch & Marthy, 1975). Nidamental glands in the female squid also secrete the mass of jelly material or fragile gelatinous globes.

Once eggs are mature, they fall through the proximal oviduct in the fertilization chamber of the oviducal gland. Here, spermatozoa are immobilized in the mucosa of the spermathecae (Hanlon & Messenger, 1996; Di Cosmo et al., 2001). In *O. vulgaris*, it has been showed that stimulation of spermatozoa collected from the spermatophores in the male reproductive tracts with progesterone or the Ca^{2+}-ionophore induces an acrosome-like reaction (Tosti et al., 2001). Supporting the hypothesis, spermatozoa stored in the spermathecae of the female oviducal gland do not show any acrosomal region (Tosti et al., 2001).

Moreover, when the mature eggs fall into the fertilization chamber, they release a chemo-attractant factor, named sperm-attracting peptide (Octo-SAP) that induces spermatozoa mobilization toward a concentration gradient, which is followed by Ca^{2+} mobilization and membrane protein phosphorylation (De Lisa et al., 2013). These events facilitate egg fertilization, although the molecular mechanisms underlying this event are far from being fully characterized.

In *S. officinalis*, various sperm attractant factors have been purified (Zatylny et al., 2002). These chemotactic molecules were characterized as being small peptides synthesized in the embedded oocytes during vitellogenesis, released in the external media during egg laying, and able to facilitate fertilization by increasing the chance of gamete collision.

7.3.2 SPERM MATURATION, THE SPERMATOPHORE AND THE SPERMATOPHORIC REACTION

The median single testicle is the site of sperm production. Mature sperm are released into the vas deferens (and following spermatophoric glands) where they are packaged in spermatophores (Fig. 7.2C) and stored in the spermatophoric (Needham's) sac.

During mating, spermatophores are pumped through the distal portion of the vas deferens to the single excurrent duct. This region of the vas deferens is referred to as the penis, but it does not act as intromittent organ. Spermatophores are transferred to the female by the hectocotylized arm. The structure and position of the hectocotylus vary greatly between species. Many oceanic squids have no hectocotylus, and it is absent from *Vampyrotheuhis* and the finned octopus *Cirrotheuthis* (Arnold, 1984; Murata et al., 1982). Some sepioids and teuthoids have two arms hectocotylized. In cuttlefishes and some squids, the hectocotylus is evident only by the reduction or absence of suckers on the arm. In octopuses, the whole arm is usually modified by a groove along its ventral surface, terminating near the arm tip, which is modified and often spoon-like. During copulation, the hectocotylized arm acquires the spermatophore (either by reaching in with the arm tip, as in squids and cuttlefishes; or by extension of the penis to the base of the hectocotylized arm as in octopods) and transfers the spermatophore to the female in a variety of ways (Hanlon & Messenger, 1996).

The mature sperm often has a species-characteristic morphology, and detailed descriptions are available for several squid, cuttlefish, and octopus species (Fields & Thompson, 1976; Franzen, 1955, 1956, 1967; Healy, 1989, 1990, 1993; Maxwell, 1974, 1975; Richard, 1971).

The mature spermatophore is a coiled mass of sperm and an ejaculatory apparatus wrapped in a tunic (Fig. 7.2C).

In decapods spermatophores are stored in seminal receptacles, which are quite distant from oviducts; generally they are situated just below the mouth or in a ring around the mouth (Drew, 1911; Ikeda et al., 1993). In some squids, males attach spermatophore onto a pad located on the inner wall of the mantle (Hanlon & Messenger, 1996).

In *O. vulgaris* typically spermatophores are 2–3 cm long and about 0.5 mm across at their blunt, sperm rope end. They are tubular and turgid. There is a posterior or "male-oriented" part that is opaque white, filled with compacted sperm. The other, anterior, region is more translucent; it ends in a thin thread, which is carried back along the body of the tube.

This "female-oriented" region is an invaginated folded tube, the "ejaculatory apparatus," the anterior end of which forms a cap (Wells, 1978). These features vary considerably from one species to the next (Marchand, 1913).

The spermatophore has a proteic and elastic outer tunic. Inside, a transparent viscous fluid, the spermatophoric plasma, surrounds the sperm rope. In front of the sperm rope lies the gelatinous rod of the ejaculatory apparatus, separated from the sperm rope by a region filled with an amber-colored syrupy cement (Mann et al., 1970).

During mating in *Octopus*, the spermatophore, held in the muscular penis, is transferred to the guide and carried down to the groove in the hectocotylized arm. The arm tip is then inserted into the mantle cavity of the female to reach the opening of distal oviduct. After transfer to the female or release into seawater, the spermatophore swells and bursts, ejaculating the contained sperm by the spermatophoric reaction (Mann, 1970).

The uptake of seawater at the hind end of the spermatophore provokes this "spermatophoric reaction." The sperm rope moves away from the blunt end of the capsule, pushing the ejaculatory apparatus, until the breaking of tunic and ejaculatory apparatus extrusion. This latter evaginates slowly, pushed forward by the advancing sperm rope and sperm plasma, but suddenly there is an explosive increase in the rate of evagination and the whole apparatus and the front part of the outer tunic of the spermatophore swell to form a bladder, into which the rest of the sperm rope passes. The driving force for extrusion of the sperm bladder appears to be dilution of the spermatophoric plasma by seawater coupled with the considerable elasticity of the outer tunic of the spermatophore (Mann et al., 1970).

The fate of the sperm bladder apparently differs from one genus to the next. In *O. vulgaris*, it bursts, liberating sperm into the oviduct, which carries them toward the oviducal glands by peristalsis. Here, they accumulate in the spermathecal sections of the oviducal glands, where they are presumably stored until the eggs are ready (Belonoschkin, 1929b; Wells, 1960).

7.3.3 FERTILIZATION, EGG DEPOSITION, AND EGG CARE

In cuttlefishes, external fertilization occurs within the arm bundle. Sperm are released from the seminal receptacle on the buccal region or in the mantle cavity as eggs are extruded from the oviducts. Sepia eggs are often exceptionally large (up to 2-cm diameter; Arnold, 1984; Corner & Moore, 1980). These eggs, usually blackened with ink to obscure the developing embryo and perhaps reduce predation, are attached to plants or stones. Males in

some species accompany females during the several hours needed for egg laying (e.g., Boletzky, 1983; Corner & Moore, 1980); this form of temporary mate guarding is thought to be important because of sperm competition. Females usually die shortly after spawning, although spawning can extend over several weeks or even months in the laboratory (Boletzky, 1983, 1987, 1988). No form of egg care has ever been reported.

In squids, fertilization is also external, but there are two mating styles: eggs can be fertilized either as the capsules pass out of the oviduct (when spermatophores have been placed in the mantle cavity) or amidst the arms (when spermatozoa are released from the seminal receptacle near the mouth). Then eggs are deposited either as large communal masses on the substrate, or small groups laid on rocks, seagrasses or corals. The behavioral cues initiating egg laying are not understood. Female loliginid squids such as *D. pealei* or *D. opalescens* are spent after producing large numbers of eggs. Parental care of eggs or young is unknown among squids.

In octopods, fertilization is internal, occurring in the oviducal gland (where sperm are stored) as eggs pass down the oviduct. Females lay their eggs (many thousands of eggs; 1 × 3 mm) in strings, attaching them to the rocks. The operation seems to be preceded by an attempt to clean off the area by continually running over it and plucking with the suckers (Arakawa, 1962). Suckers manipulate laid eggs, pressing them onto the rock and one another, sticking them together by the stalks, so that they hang freely in bunches from a common stem. Eggs are deposited by two methods: either cemented individually to a hard substrate in the den; or individual eggs are intertwined with other eggs in clusters of many tens, and then cemented to a substrate. Females guard and care for the eggs, and there is a vital behavioral and physiological change associated with this phase of the life cycle. Basic metabolic changes occur in *O. vulgaris* females that approach full maturity, with somatic growth stopping (O'Dor & Wells, 1978), and the ovary becoming full of ripe eggs; in some octopods the other internal organs become squeezed in the mantle cavity. Feeding behavior becomes erratic. After egg laying, the female never leaves the den, does not feed, and continuously cleans and aerates the eggs while she broods them, which can be for as long as 1–3 months, perhaps a quarter of her life span. Declining physiological conditions in spawning females are often recognizable by the production of more or less disorganized egg masses (Boletzky, 1987). Females invariably become emaciated and die soon after hatching occurs. Males also die at about the same age, due to the same physical degradation as females: hormonally induced muscle catabolism, high amino acid levels in the blood, and consequent high metabolic rate (O'Dor & Wells, 1978).

This semelparous behavior, as well as the ensuing death, also seems to be controlled by the optic gland hormone (Wodinsky, 1977).

7.4 REPRODUCTIVE STRATEGIES, MATING, AND SPERM COMPETITION

Like other animals, cephalopods use several strategies to reach the maximal reproductive fitness. Although promiscuity is common in squids, cuttlefishes and octopuses, their very different life styles and body forms could explain why the mating systems among these animals is quite different (Hanlon & Messenger, 1996).

In fact, at least two different mating systems exist: (1) in some cuttlefishes and squids there is elaborate agonistic behavior, extensive courtship and brief copulation, but no protection of the eggs; (2) in octopuses there is little, if any, agonistic or courtship behavior, yet copulation is relatively lengthy, and females care for the eggs (Hanlon & Messenger, 1996). Some of these behavioral aspects of reproduction are controlled by hormones/paracrine substances (Cummins et al., 2011; Enault et al., 2012; Woodinsky, 1977).

Whether or not the choice of female (as well as the intrasexual selection of males) in decapods could be aided by some attractiveness by males, the question remains open in octopods. Display behavior of decapods, apparently absent in octopods, could be interpreted as a warning signal for other males and "ornament" to attract females. The rationale underlining such a large difference between decapod and octopod reproductive behavior is still a key question in cephalopods.

Fighting among males is a common feature in decapods, and competitive aggression is a means by which males gain access to preferred females for mating. Males of cuttlefishes and squids generally approach females in small groups (cuttlefishes, two to three males for female) or large groups (courting parties in squids). This approach has been observed at the moment of mating in *Sepia*, and may be present [?] in *Sepioteuthis* or *Doryteuthis* (Hanlon & Messenger, 1996). Generally decapods use body patterns (deriving from the chromatophores and other structural "elements" in the skin: Hanlon & Messenger, 1996) to communicate intra- and intersexually. Such chromatic displays, coupled with specific arm movements, can also be indicative of the sex of the animal; that is, during fighting, courting and mating, decapods display their sex.

In *S. officinalis*, agonistic contests are characterized by an Intense Zebra Display from the males (see Hanlon & Messenger, 1988, 1996 for references) with the arms either stretched forward or arched toward other males. The visual display can lead to intense fighting, culminating in the winning male approaching the female and copulating with her. Generally in cuttlefishes, the male–female pair mates in a "head-to-head" position. Interestingly, courtship and mating in *S. officinalis* can be highly tactile; a male will hover over and alongside the female, continually drawing his arms softly over her mantle, head, and arms (Hanlon & Messenger, 1996). Unfortunately, there are as yet no clues as to how this tactile aspect of male courtship affects the receptivity of the female. During mating, the male will pass spermatophores that are stored mainly in the seminal receptacle below the mouth.

In squids too, visual communication plays a very important role in the intrasexual as well as intersexual relationship during mating. Displays are made in the shoal, where courting parties (one female to four to five males) are formed. There is often a large male that approaches the female trying to drive away other males. Such intrasexual and intersexual messages are transmitted by specific body patterns and, surprisingly, females can use these to attract males other than the one close to her. Males engage in agonistic contests among themselves until a winner emerges who keeps away all the others from his partner. As a pair becomes isolated, mating begins. In squids, head-to-head as well as male-parallel positions are used: spermatophores are passed to the female and stuck on the mantle, around the head or in the seminal receptacle, according to the species (Hanlon & Messenger, 1996).

Females synthesize a protein named *Loligo β*-microseminoprotein (*Loligo β*-MSP) in their reproductive exocrine glands and embed the protein in the outer tunic of egg capsules (Cummins et al., 2011). This protein is able to immediately and dramatically changes the behavior of male squid from calm swimming and schooling to extreme fighting, even in the absence of females. Males are attracted to the eggs visually, but upon touching them and contacting *Loligo β*-MSP, they immediately escalate into intense physical fighting with any nearby males. Such evidence highlights the role of chemical communication during reproduction in cephalopods.

In contrast, octopuses are solitary animals that show little agonistic or courtship behavior before mating. In *O. vulgaris*, it does not seem to exist any specific display that would distinguish between the octopus sexes (Wells, 1978), so cohabitation is not present (Hanlon & Messenger, 1996). *O. cyanea* (Wells & Wells, 1972) seems to show some striking males-discouraging

displays. Packard (1961) suggested that *O. vulgaris* males show the larger suckers at the base of their second and third pair of arms as a signal of "maleness." However, there is no definitive evidence for this, and it can only be supposed either that there are subtle visual clues that we have not so far detected (Wells & Wells, 1972) or that sex recognition is chemotactile in this species.

Males octopuses fight, and when the smaller cannot escape, he is killed. A female typically submits to the demands of the male that starts to insert the hectocotylus into her mantle cavity.

Males, which carry about 50 spermatophores, each 2–3-cm long, then place a spermatophore at the base of the hectocotylized arm with an "arched" posture followed by an explosive "pumping" action that somehow sends the spermatophore down the hectocotylized arm and into the oviduct.

Competition among males for female is common in *Octopus*. Several observations report of two to three, or even six octopi mating with one female (Voight, 1991; Wood, 1963; but see Hanlon & Messenger, 1996).

In fact, there is some evidence to suggest that male cephalopods may mate completely randomly and that females are either promiscuous or practice "simultaneous polyandry." A stable pair almost never occurs in octopods, nor in decapods, where a temporary pair may be formed (sometimes "joined" by a sneaker male, Hanlon & Messenger, 1996).

Sperm competition was defined originally by Parker (1970) as the competition within a single female between the sperm from two or more males for the fertilization of the ova. Sperm competition occurs whenever a female mates with more than one male in one breeding cycle, and it has been found to occur in many phyla (Smith, 1984).

The ability to produce large quantities of sperm, sperm packaging in spermatophores, sperm storage by females, the physical nature of the oviduct and spermathecae, the existence of "polygamous" mating systems and nonsynchronous sexual maturation are all strong hints that sperm competition exists in cephalopods, although no clear evidence has been reported (Hanlon et al., 1997).

In decapods, there is some evidence that the female can choose the spermatophores that are stuck around the head or in the mantle. Moreover, their seminal receptacle seems to be too small to contain millions of sperms. It is possible that the sperm from the most recent mating displace that from earlier matings.

Similarly, in octopods, sperm competition is not unlikely. Sperm may be stored in the spermathecae of the oviducal gland, even months before

the fertilization. It has been suggested that the tip of hectocotylus, which is spatula-like, could be inserted into the oviduct and reach spermathecae, removing old sperm (or sperm from another octopus), before depositing new.

7.5 THE CONTROL OF REPRODUCTION IN CEPHALOPODS

In many invertebrates, growth and functional maturation of the gonads depend on the action of gonadotropic hormones (Engelmann, 1994). These are generally synthesized and released by endocrine glands, although in invertebrates, they are often released from neurons. In the classical concept of input–output relations, regions in the nervous system receive and integrate coded external stimuli (inputs such as temperature changes, photoperiod, food intake, partner availability) to generate internal responses that affect endocrine glands and, subsequently, gonad growth and maturation, egg laying, and sexual behavior (outputs).

The activity of endocrine glands involved in sexual maturation of several invertebrates is negatively controlled by the nervous system (Engelmann, 1994). Generally neurons produce factors that block gonadotropin release by endocrine cells unless coordinated (from different lobes) and synergistic inputs (time-linked) would reverse such inhibition by either shutting down a negative control or turning on a positive one. The onset of sexual maturity in cephalopods seems to be controlled in this way: the subpedunculate lobe in the central nervous system negatively controls the activity of the optic gland, which is assumed to release an as yet unidentified gonadotropic hormone (Wells & Wells, 1959). It is also possible that the glands have other, catabolic functions (Froesch & Mangold, 1976; Mangold & Froesch, 1977).

In female cephalopods, the essential processes of the onset and progress of reproductive maturity are the production and sequestration of yolk in the oocyte (vitellogenesis), egg laying, the development of organs for the formation of protective individual egg coats (oviducal glands) and the encapsulation of the spawned egg mass (nidamental glands). In most cuttlefish, squid and octopus, the enlargement of oviducal and nidamental glands marks the beginning of breeding competence and reproduction. Male sexual maturity, which is reached earlier than females and that is characterized by the production of spermatophores and ripening of reproductive tracts, has also been shown to depend on hormones (Wells & Wells, 1972).

Most of the evidence for the hormonal control of reproduction has been obtained from a series of studies on *O. vulgaris*. In the course of studies on the central nervous system, it was noticed that individuals in which an

experimental lesion either directly or indirectly affected the innervation of the optic glands, became precociously sexually mature within a few weeks (Boycott & Young, 1956; Wells & Wells, 1959). Subsequent experiments showed that the secretion(s) from the optic glands of female *Octopus* increased the rate of protein synthesis for yolk production and was essential for vitellogenesis (Wells et al., 1975). The suggestion that in some squid the process of copulation itself induces female maturation has not been confirmed (Ikeda & Shimazaki, 1994).

Optic gland material transplanted from one octopus to another apparently stimulates the recipient regardless of the sex of the donor. Implants from other octopodids (*Octopus macropus*, *E. cirrhosa*) are also effective (Wells & Wells, 1975). Removal of the optic glands from maturing *Octopus* has been shown to cause the gonads to regress, although mating behavior is apparently unaffected (Wells & Wells, 1972). Since reproduction is the terminal event for these species, it seems that the sequence of physiological processes brought about by the optic gland hormone cannot naturally be reversed. This idea received some direct support from the experiments of Wodinsky (1977) and Tait (1986), in which the excision of optic glands from maturing octopuses caused gonad regression and a subsequent resumption of feeding and growth.

In captivity, some cephalopods reduce their feeding rate or stop feeding altogether as sexual maturity progresses. This is consistent with the view that, at least in octopodids, the physiology of the prespawning female switches over from somatic growth to vitellogenesis in the ovary. The rapid secretion of large amounts of protein-rich yolk while food intake is reduced or stopped is apparently achieved by the remobilization of somatic protein (O'Dor & Wells, 1978). This results in the flaccid and weakened muscular tissues of "spent" animals. In field populations of squid in which this effect has been examined, the results have been inconsistent. In *Alloteuthis subulata* (Rodhouse et al., 1988), *Nototodarus gouldi* (McGrath & Jackson, 2002), and *Illex argentinus* (Rodhouse & Hatfield, 1992), evidence for loss of somatic protein was lacking, but for *Photololigo* spp. (Moltschaniwskyj, 1995) and *Moroteuthis ingens* (Jackson & Mladenov, 1994), wasting of somatic musculature was associated with breeding. It is unlikely that the loss of musculature alone can result in the high level post-spawning mortality so characteristic of cephalopods, but it seems that the process of remobilization of nutrients, mainly proteins to be sequestered in yolk, cannot be reversed (Boyle & Rodhouse, 2005).

7.5.1 THE STRUCTURE AND INNERVATION OF THE OPTIC GLANDS

The optic glands are small rounded bodies that lie on the optic tracts (Fig. 7.5). In young *O. vulgaris*, they are pale yellow in color, becoming swollen and orange as the animal matures. Owen (1832) figures them in a drawing of the brain of *Sepia*, and they are illustrated and labeled as "glandula ottica" in *O. aldrovandi* by Delle Chiaie (1828). Subsequent descriptions in the literature nearly all reported them as nervous tissue, but Boycott and Young (1956) had no doubt that these bodies were endocrine glands. Their fine structure has been described by Bjorkman (1963), Nishioka et al. (1970) and by Froesch (1974).

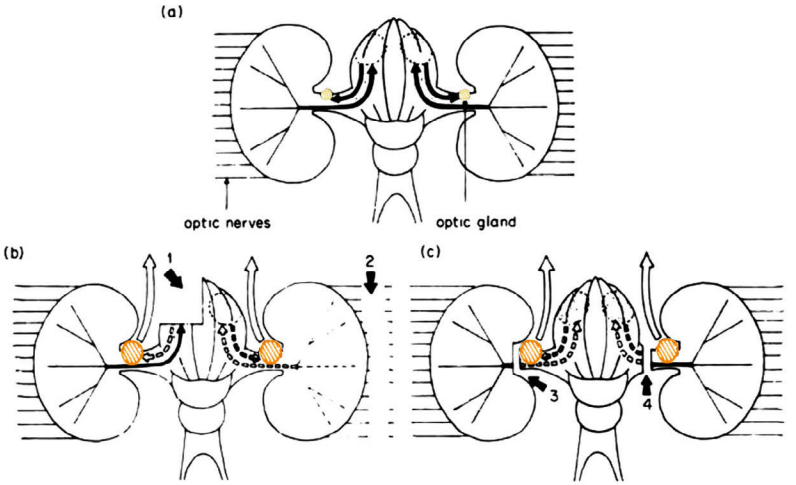

FIGURE 7.5 The nervous control of gonad maturation in *Octopus vulgaris*. (a) Immature and not activated *Octopus*; secretory activity optic glands (pale yellow) is inhibited by the nerve supply. (b) Lesion of the subpedunculate lobe (1) or section of the optic nerves (2) causes the optic glands (orange) to secrete a presumed gonodotropin. (c) Optic lobe removal and optic tract section have the same effect upon the gonads. (Modified from M. J. Wells, "Octopus—Physiology and Behaviour of an Advanced Invertebrate," in Behavioural Processes 3(4):356–357 · December 1978. With permission from John Wiley & Sons, Ltd.) (1978).

The glands are heavily vascularized (Young, 1971). Apart from those associated with blood vessels, there are two types of cell in the gland: large stellate cells, with massive (10–15-µm diameter) rounded nuclei, called chief cells. These are responsible for the production of the optic gland hormone since they change in size and appearance as the gland matures. A smaller group of supporting cells, probably fibrocytes responsible for the production

of a connective tissue framework to the gland, is dispersed among chief cells (Boycott & Young, 1956, Wells & Wells, 1959). The cytoplasm of the large stellate cells contains many mitochondria, extensive Golgi apparatus and large numbers of ribosomes. The endoplasmic reticulum (in the resting gland) consists of a sparse system of fine tubules. The fine structure of the supporting cells is similar with fewer mitochondria and ribosomes. The cyto-architecture of the gland suggests that it is of nervous origin. The supporting cells resemble glial cells (Bjorkman, 1963).

No trace of the optic glands is visible in the embryonic stages and they cannot be found in the planktonic larvae. They appear in young animals and seem to develop from nerve cells close to the olfactory lobe (Bonichon, 1967).

These glands are innervated from the subpedunculate lobe at the back of the supraoesophageal brain through the optic gland nerve. Removal of this lobe is followed by degeneration of at least some of the nerves, which enter the gland from a bundle running along the optic stalk (Wells & Wells, 1959; Froesch, 1974).

Ultrastructural analyses of the optic glands (Bjorkman, 1963) revealed that, in the immature optic gland, there are two types of synapses, axoaxonal (among fibres of optic gland nerve) and axoglandular (contacts on glandular chief cells), whereas there is only one type of synapse, the axoglandular, in the mature gland (Froesch, 1974). It was proposed that the axoaxonal synapses might inhibit the axoglandular synapses, according to the widespread pattern of presynaptic inhibition (Froesch, 1974). This idea was supported by the absence of axoaxonal synapses in the gland of adult animals, which is supposed to be active only in the absence of inhibition.

In a study of the neuropeptidergic innervation of the optic gland of *Sepia*, Le Gall et al. (1988) found that the neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) is the substance present in the optic gland nerve that apparently inhibits optic gland activity. Later it was demonstrated that in *Octopus*, too, the optic glands are innervated by FMRFamide immunoreactive fibers originating from neurons in both the subpedunculate and the olfactory lobes (Di Cosmo & Di Cristo, 1998).

7.5.2 THE FUNCTION OF THE OPTIC GLANDS

In 1956, Boycott and Young noted in *O. vulgaris* that the optic glands were enlarged in a proportion of the animals that they were using for experiments on brain function in learning. Animals in which the optic tracts had been

sectioned had large glands and grossly enlarged gonads. Similar effects were observed after destruction of the subvertical lobes.

The optic glands are now recognized as the endocrine organs that control the maturation of the reproductive system. Wells and Wells (1959) hypothesized that optic glands secrete a gonadotropic hormone, and clarified the relationship between the gland and the central nervous system (CNS).

They demonstrated that either cutting the optic gland nerve or making a surgical lesion in the subpedunculate lobe resulted in an enlargement of the gland and a subsequent hypertrophy of the gonads (Fig. 7.6).

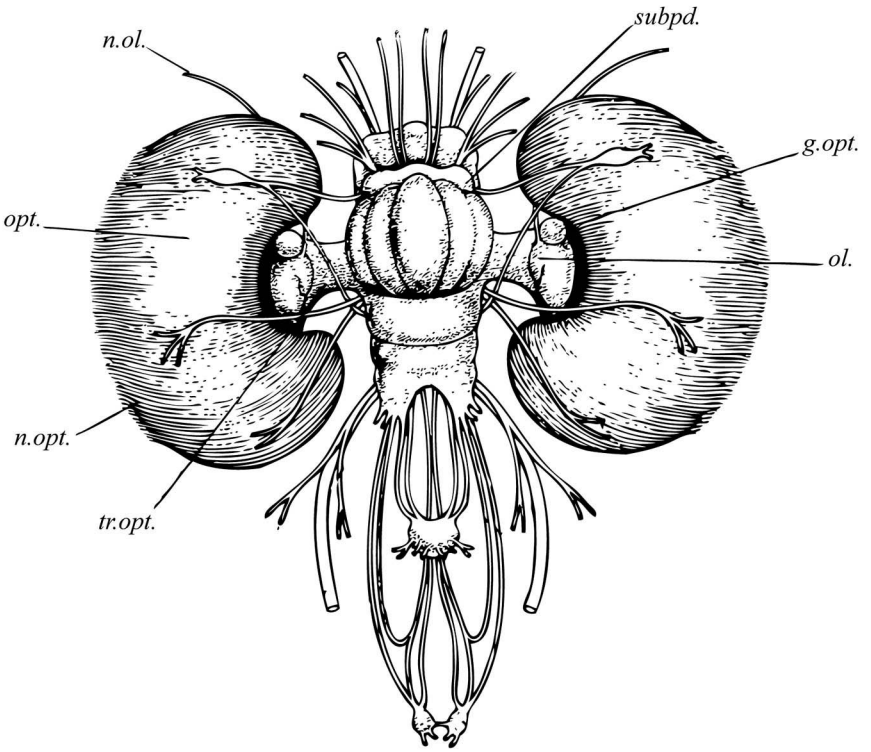

FIGURE 7.6 Diagrammatic drawing of *Octopus* CNS as seen from above (modified from Di Cosmo et al., 2004). Abbreviations: g.opt., optic gland; n.ol., olfactory nerve; n.opt., optic nerves; ol., olfactory lobe; opt., optic lobe; subpd., subpedunculate lobe; tr.opt., optic tract.

A similar (but weaker) hypertrophy of both optic gland and gonads is produced by cutting the optic nerves or removing the optic lobes (Fig. 7.6). If immature animals are blinded by optic nerves section, the optic glands

enlarge and the octopuses mature precociously. This observation led Defretin and Richard (1967) to test the effect of reducing the daylength on the state of the glands in *Sepia*. They kept cuttlefish in tanks in the dark for 22 out of every 24 h. A control group remained in continuous light. The stellate cells in the glands of animals maintained in short daylength enlarged greatly, with a massive increase in endoplasmic reticulum and Golgi apparatus. Budding from the Golgi apparatus were large numbers of dense vesicles 100 nm in diameter, absent in the resting glands and presumably containing the glandular secretion, which is presumably shed into the bloodstream.

Wells and Wells (1959) then proposed that the CNS exerts an inhibitory control on the optic gland via the optic gland nerve and that photoperiod also plays a crucial role in controlling gonad maturation: darkness activates it.

The gonad remains small, whatever the operation, if the optic glands are removed. Since the effects of blinding and more central brain lesions were not additive, it was postulated that control of the condition of the glands always ran through the subpedunculate lobe, so that the full chain of control is

Light → subpedunculate lobe → optic glands → gonad.

In this chain, excess of light triggers subpedunculate lobe inhibition of optic gland activity. The last part of this chain is excitatory and hormonal.

Subsequent work has in general confirmed these hypotheses. Removal of the subpedunculate lobe is always followed by enlargement of the optic glands. If the operation is unilateral, only the gland on that side enlarges. Activation of one or of both of the optic glands always produces enlargement of the gonad. The effect is most spectacular in females, where the ovary may increase in size from about 1/500th to as much as 1/5th of the body weight within a month of the operation. Even in males, these activating lesions induce weight increase of the testis, particularly of small animals.

Removal of the optic glands from males that are already producing sperm is followed by a decrease in the weight of the testis and the eventual cessation of sperm and spermatophore production; the effect is most marked in the largest males. The optic glands in males weighing less than 1000 g are generally small and not obviously secreting. It has been hypothesized that even glands that are not visibly enlarged must be producing a trickle of secretion which is sufficient to stimulate sperm production in males but not enough to stimulate yolk production in the ovary (Wells & Wells, 1972).

If the relation between subpedunculate lobe, optic gland, and gonad seems almost obvious, the link between daylength and secretion of the gonadotropin is less certain (Wells, 1978). When *Sepia* is kept under short

daylength conditions, it matures precociously (Defretin & Richard, 1967; Richard, 1967) and some wavelengths seem to have an inhibitory effect on gonad development.

Attempts to replicate the cuttlefish experiments using *Octopus* have yielded only equivocal results: only a few of the animals appear to respond (Buckley, 1977). The effect of blinding the animals, moreover, could just possibly be unconnected with daylength, since other operations that damage structures in the orbit, or the implantation of foreign bodies into the orbit can also result in optic gland enlargement, albeit much more slowly than optic nerves section (Wells, 1978).

A possible explanation of such a discrepancy could come from physiological studies indicating that neuropeptides and dopamine can modulate retina adaptation in female *Octopus* either to light or to dark, according to the sexual maturity of the animal (Di Cristo et al., 2003). This would imply that the brain control of optic gland secretion involves assessment of photoperiod, chemical stimulus, and modulation of photoreceptor activity.

Starvation has also been claimed as a cause of precocious maturation in *Eledone* (Mangold & Boucher-Rodoni, 1973), although again the results have not been confirmed with *Octopus* (Wells & Wells, 1975). It would appear that a variety of stresses could trigger optic gland and gonad enlargement in the laboratory.

Activated optic glands have been implanted into the system of blood sinuses behind the eyes of recipient animals. Once these glands attract an arterial blood supply, they begin to secrete. A month later the gonads of recipients that would otherwise be immature are noticeably enlarged. Neither the sex of the donor animal, nor the condition of the glands (active or inactive) when implanted makes any difference to the final result (Wells & Wells, 1975). It has been also demonstrated that the implant could be species and genera independent, the optic gland of different species (or genus) being active and inductive in the recipients. Implanting activated optic glands from decapods into octopods has no effect, although there is reason to believe that the hormone produced by the optic glands of decapods is very similar to that found in *Octopus* (Wells & Wells, 1975).

In vitro experiments have been conducted in fragments of the ovary or testis of *Sepia*. In culture, fragments of young cuttlefish ovary will grow normally in the presence of optic gland cells; the germinal epithelium continues to divide to produce oogonia, these in turn produce oocytes, which acquire a coating of follicle cells, which start to divide inducing oocytes swelling. The system breaks down at this stage, presumably because the

nutritive demand of the swelling eggs is too great for the nutrient medium (Wells, 1978).

Fragments of ovary cultured without optic glands, will survive for as long but the cells of the germinal epithelium and the follicle cells fail to divide. The oogonia already present when the culture is set up all transform into primary oocytes during the first 3 weeks, and these develop normally to the point where their further enlargement depends upon the follicle cells (Durchon & Richard, 1967; Richard, 1970).

A more recent approach (Di Cristo et al., 2010) has revealed that there are two different patterns of yolk protein in the eggs of *Octopus*. These mirror the two main periods characterizing the reproductive cycle of the female of *O. vulgaris*: the non-vitellogenic period and the vitellogenic period. It seems that progesterone plays a role in *Octopus* vitellogenesis (Di Cristo et al., 2010) by inducing proliferation of follicle cell (the site of synthesis of yolk protein, see below). This suggests that this steroid, together with the optic gland hormone, may be involved in vitellogenesis in *Octopus* (Abete et al., 2000; Di Cristo et al., 2010).

Similar experiments have also been performed on cultures of testis. With optic glands present, spermatogonia are produced from the germinal epithelium and divide to give spermatocytes and eventually sperm. Without optic glands, the spermatocytes develop normally, but the system eventually slides to a halt because there are no further divisions of the spermatogonia (Richard, 1970).

When octopus oocytes from the vitellogenic phase were used in in vitro experiments, O'Dor and Wells (1973) showed that the ovary is the site of synthesis of yolk proteins. Injected-labeled amino acid, rapidly disappears from the blood and begins to accumulate in the ovary. Uptake and synthesis stop if the optic glands are removed.

These experiments however did not show which part of the ovary is responsible. Experiments on oocytes and follicle cells in cuttlefish (Lankester, 1875; Yung Ko Ching, 1930) indicated the follicle cells as the likely site of yolk secretion. These light microscope studies have been followed by investigations at electron microscope level in *Octopus* (Buckley, 1977; Wells et al., 1975) that confirmed such hypothesis.

The follicle cells are packed with rough endoplasmic reticulum and active Golgi apparatus. Electron-dense material, originating from the Golgi apparatus, is exported to the oocyte down finger-like processes. Follicle cells from an animal deprived of its optic glands 2 or 3 days previously have ceased to produce dense granules (Wellset al., 1975).

In contrast to this, the oocyte itself has few organelles and shows no effect of hormone deprivation. Its main phase of synthetic activity comes earlier in development, in the period of carbohydrate and lipid accumulation, which precedes the full development of the follicle cells wrapping. This stage is independent of the optic gland hormone (Buckley, 1977). It seems that the hormone peaks twice during oocyte development: in early oocyte development (which corresponds roughly to the juvenile stage), it is required for oogonia division. There follows a period during which the ovary will grow, apparently at a normal rate, in the absence of the optic glands. The glands become essential again only in the last phases of ovarian maturation, when the animal begins to accumulate the massive quantities of vitellogenin (Wells, 1978). Interestingly, sex steroids, namely progesterone, seem to be required in both the circumstances (Cuomo et al., 2005; Di Cristo et al., 2010). This opens a new window in the endocrine control of cephalopods gonad (at least in female) development; the combined action of the unknown optic gland hormone, which seems to act as a trophic hormone, and the sex steroids that are locally produced (Di Cosmo et al., 1998; 2001; 2002; Di Cristo, 2013; Di Cristo et al., 2010). Nothing is known about the presumptive feedback action of steroids on the brain, however.

The optic gland hormone exerts also an enlarging effect on the oviducts and oviducal glands, as well as the apparatus responsible for packaging the sperm into spermatophores. The effect is greatest in the smallest animals (Froesch & Marthy, 1975; Wells, 1960).

Castration does not appear to alter the condition of the ducts in males or females nor hectocotylus regeneration (Callan, 1940; Wells & Wells 1972). Such experiments suggest there is no hormone produced by both male and female gonads.

Although no physiological evidence has been reported, an endocrine role for the cephalopods gonads has been proposed (D'Aniello et al., 1996; Di Cosmo et al., 1998, 2001, 2002; Tosti et al., 2001). The emergence of data on the presence of "vertebrate" sex-steroids, of steroidogenesis and of steroids-binding moieties in cephalopod gonads supports this hypothesis.

Although few studies have been done of the effects of hormones on reproductive behavior in cephalopods, Wells and Wells (1972) demonstrated that either castration or surgery affecting the condition of optic glands in mature octopus males did not interfere upon the reaction of males to females placed in their tanks. Octopuses with their testis and male ducts removed approached females, inserted the hectocotylus and passed imaginary spermatophores down the groove in the hectocotylus into the mantle of the female; even the entire set of stereotyped movements during the mating were not impaired by

that surgery (Wells & Wells, 1972). The only operation that prevents correct behavior is removal of the tip from the hectocotylus; evidently some signal from the tip indicates that it has located an oviduct in the mantle and initiates spermatophore transfer. Males will copulate with females having the distal ends of the oviducts removed, but do not pass spermatophores.

7.5.3 THE OLFACTORY LOBE AND THE OLFACTORY ORGAN

In cephalopods, the olfactory lobe lies on the optic tract, close to both the peduncle lobe and the optic gland and is subdivided into three lobules (anterior, middle, and posterior in *O. vulgaris*) (Young, 1971; Messenger, 1967; Fig. 7.6). From the anatomy, one could infer that olfactory lobe receives and processes distant chemosensory information (olfaction sense) (Hanlon & Messenger, 1996; Young, 1971). In fact, the name of this lobe comes from its connection, through the olfactory nerve, to the olfactory organ, a chemoreceptor organ (Woodhams & Messenger, 1974). However, the function of olfactory organ, at least in *Octopus*, is still far from clear, although a role of olfaction in this mollusk reproduction has been hypothesized (Polese et al., 2015).

Knowledge on the olfactory organ in cephalopods comes from studies on *Nautilus* (Basil et al., 2000; Ruth et al., 2002) and decapods (Lucero et al., 1992, 1995, 2000; Mobley et al., 2007; Mobley et al., 2008a,b; Piper & Lucero, 1999; Villanueva & Norman, 2008). In squid, the olfactory organ is a sensory epithelium made of ciliated supporting cells and sensorial bipolar neurons. Each receptor neuron is connected to the olfactory lobe and other areas of the brain (Messenger, 1967; Messenger, 1979). Recently, Walderon et al. (2011) investigated the role of the olfaction in the distance chemoreception of conspecifics in *Octopus bimaculoides*, but almost nothing is known about the mechanisms, functions, and modulation of the olfactory organ in octopods.

It should be emphasized that in *Octopus*, which uses its arms for detecting food, there are millions of chemoreceptors on the suckers (Graziadei, 1964). The role of olfactory organs and olfactory lobes is therefore presumably related to distance chemical reception (olfaction), rather than chemotactile perception, via the suckers (*taste by touch*, Wells, 1962; see also Hanlon & Messenger, 1996). The presence in this lobe, particularly in the posterior lobule of peptidergic neurons that innervate optic gland cells, has led to a critical revision of the role played by the olfactory lobe in reproduction (Di Cosmo & Di Cristo, 1998). In these neurons of the posterior olfactory

lobule, the neuropeptide gonadotropin-releasing hormone (GnRH, see Section 7.5.4), a neuropeptide belonging to the family of GnRH of vertebrates (Millar, 2005), has been found. GnRH-containing neurons send their axons to the chief cells of optic gland (Di Cosmo & Di Cristo, 1998; Iwakoshi et al., 2002; Iwakoshi-Ukena et al., 2004), possibly mediating the control exerted by olfactory system on optic gland activity.

Whether GnRH affects reproduction in cephalopods, however, is still an open question. Some cues favor such a hypothesis, but others, coming from comparative studies, seem to exclude a direct role of GnRHs in reproduction in mollusks.

Apparently, some of the GnRH (and other neuropeptidergic) neurons in the olfactory lobe send their axons also to the mucosa of the olfactory organ (Polese et al., 2015). According to these data, signals from the chemical world outside the animal are relayed to the optic gland, modulating its activity, through the two-way connection between olfactory organ and lobe.

If this is a concrete anatomical aspect, we cannot however formulate any definitive theory about the role that both chemical stimuli and GnRH could have on optic gland and its hormone. There is not yet evidence of any molecule perceived by the olfactory organ that could trigger sexual activity, nor a clear "receptive" role in reproduction has been described for olfactory organ. According to Wells and Wells (1972), chemotactile stimuli could have some importance, suggesting a role of suckers in reproductive behavior (at least in *Octopus*).

Incidentally, another neuropeptide has been found in the posterior olfactory lobule: the tetrapeptide Ala-Pro-Gly-Trp-NH_2 (APGWamide; Di Cristo et al., 2005; Di Cristo, 2013); its mRNA is present in the CNS transcriptome of *Octopus* CNS (Zhang et al., 2012). APGWamide is known to plays a key role in male sexual behavior in gastropods (de Lange & Joosse, 1998; de Lange & van Minnen, 1998). In *Octopus* CNS, APGWamide-immunoreactive cell bodies are confined, other than olfactory lobe, to the inferior frontal system. This system of lobes is involved in the reception and analysis of chemosensorial information coming from suckers in *Octopus* (Young, 1971).

The presence of this neuropeptide in these lobes of *Octopus* nervous system reinforces the hypothesis that in *Octopus*, and probably in other cephalopods, olfaction, as far as more likely chemotactile experience, could mediate aspects of sexual behavior (Di Cristo et al., 2005). Interestingly, a waterborne pheromonal attractant has been purified from both *S. officinalis* egg mass (Zatylny et al., 2000; Boal et al., 2010) and female reproductive ducts (Enault et al., 2012), and waterborne pheromonal peptides are present in the gastropod *Aplysia* (Cummins et al., 2008). Chemotactile inputs

evoke the intra-male competition for mates (in *Loligo*: Buresch et al., 2004; Cummins et al., 2011), and relatively quiet octopus males become highly actives as females touch them, or vice versa (Wells, 1978). This observation of mating behavior in octopuses suggests that physical, and probably chemical, contact might play a part in sex recognition (Hanlon & Messenger, 1996).

7.5.4 CEPHALOPOD GnRH

The cephalopod GnRH dodecapeptide (*ceph*GnRH) is a member of the wide family of GnRH neuropeptides, which in vertebrates are essentially involved in the activation of gonadotropin release from the pituitary gland (Morgan & Millar, 2004). The ancient origin of this peptide was suggested by the recent description of orthologous GnRH in many invertebrates, including nonvertebrate chordates (Roch et al., 2011; Tsai & Zhang, 2008). In mollusks, GnRH has been sequenced in three cephalopods (Di Cristo et al., 2009; Iwakoshi et al., 2002; Onitsuka et al., 2009), two bivalves (Bigot et al., 2012; Treen et al., 2012) and three gastropods (De Lisa et al., 2013; Tsai & Zhang, 2008; Zhang et al., 2008). The presence in the nematode *Caenorhabditis elegans* of a peptide with the structural features of both GnRH and insect adipokinetic hormone (termed GnRH-AKH) (Lindemans et al., 2009) suggests the presence of a GnRH peptide superfamily (Roch et al., 2011).

In contrast to the sequence elucidation of GnRHs, very few functional data are available in mollusks. Many studies have been performed primarily using vertebrate GnRH peptide isoforms (Goldberg et al., 1993; Gorbman et al., 2003; Nakamura et al., 2007; Pazos & Mathieu, 1999; Young et al., 1999; Zhang et al., 2000), complicating data interpretation. To date, physiological studies based on homologous GnRH administration have been conducted on few species. In *O. vulgaris* octGnRH stimulates oviduct contraction and gonadal steroidogenesis, suggesting a role in the reproductive process (Iwakoshi-Ukena et al., 2004; Kanda et al., 2006). In *Aplysia*, however, homologous GnRH has little effect on the activation of reproduction, although it modulates activity of diverse central neurons and inhibits after discharge in bag cells (Sun & Tsai, 2011; Tsai et al., 2010). Finally, putative scallop GnRH-like peptide stimulated spermatogonial cell division in cultured scallop testis (Treen et al., 2012).

Interestingly, in *C. elegans*, although homologous peptide administration was not performed, RNA interference that disrupted the production of

GnRH-AKH prohormone resulted in delayed egg-laying (Lindemans et al., 2009).

This mismatch between structure and function in GnRH in invertebrates has no simple explanation, and we should probably ask why reproduction seems to be affected by GnRH only in some species. One possible line of enquiry that could help in addressing this problem would be to study the presence, expression, pharmacology, and physiology of GnRH receptors in invertebrates. In *Octopus*, a GnRH receptor has been identified and characterized (Kanda et al., 2006). This receptor is distributed throughout nervous and peripheral tissues, and interestingly in the olfactory lobe, optic gland, as well as in the gonads. It responds specifically to its native peptide (octGnRH) to activate the classical GnRH-induced signal transduction pathway; this results in steroidogenesis in reproductive tissues during bioassays (Kanda et al., 2006). However, without data on the *Octopus* gonadotropin, it is impossible to define the role of GnRH in such a function.

7.5.5 OTHER NEUROPEPTIDES

As well as GnRH, FMRFamide, and APGWamide, the olfactory lobe also contains several peptides. Immunoreactive-like signals for galanin (Suzuki et al., 2000), neuropeptide Y (NPY; Suzuki et al., 2002), corticotropin-releasing factor (CRF; Suzuki et al., 2003) have been reported in *O. vulgaris*. Some of these results are exclusively based upon immunological data and need to be supported by strong molecular data, but a nonapeptide, designated peptide tyrosine phenylalanine (PYF) has been isolated from brain of the squid *Loligo vulgaris* (Smart et al., 1992). This peptide shows high homology with the C-terminal end of the other molluscan NPYs (seven out of nine residues are identical) and could be a processed form of a genuine *Loligo* NPY neuropeptide.

Galanin (and galanin-like peptides), NPY and CRF, whose presence in the olfactory lobe has been hypothesized, play important roles in the balance between metabolism and reproduction not only in vertebrates (Crown et al., 2007), but also in invertebrates (see de Jong-Brink et al., 2001 for the role of NPY in invertebrates). In vertebrates: (1) neuropeptides, including galanin-like peptide (GALP) and NPY, all reside in the hypothalamic area involved in the regulation of metabolism and reproduction; (2) neurons producing these peptides are targets of metabolic hormones, such as leptin and insulin, and (3) these neuropeptide either directly or indirectly affect feeding and metabolism, as well as the secretion of GnRH and gonadotropins.

7.5.6 STEROIDS

Sex steroids are key molecules in the endocrine mechanisms of vertebrates (Bentley, 2001). In many mollusks, "vertebrate-like sex steroids" were essentially believed to act as "endocrine disruptors" (Lafont & Mathieu, 2007). However, the discovery that these animals synthesize steroids and show tissue expression of steroid receptors has resulted in a reconsideration of the role these molecules may play in invertebrate reproduction (Kohler et al., 2007).

The presence of steroids in *Octopus* (but also in *Sepia*) has been widely reported (Cuomo et al., 2005; D'Aniello et al., 1996; De Lisa et al., 2012; Di Cosmo et al., 1998, 2001; Di Cosmo et al., 2002; Di Cristo et al., 2008, 2010; Tosti et al., 2001). These papers cover the biochemistry, physiology, and pharmacology of vertebrate-like sex steroids in this species and establish three key points: (1) *Octopus* and *Sepia* are capable of synthesizing steroids in both gonads and brain; (2) these steroids interact with specific receptors localized in the gonads, reproductive tracts and in specific brain regions; and (3) these steroids can affect the physiology of the gametes of *Octopus*.

One of the key aspect concerning steroids in cephalopods is the presence of putative steroidogenic enzymes in tissues of these animals. Some canonical enzymatic reactions on "cholesterol-derived backbones" are likely to occur in brain and gonads of cephalopods. However, there is not yet any structural evidence on the presence of the (presumptive) cephalopod steroids.

The absence of studies on the structural characterization of steroids in cephalopods could also explain some different results on steroid receptors in *Octopus*.

An orthologous gene of the estrogen receptor has been cloned in *O. vulgaris* (*oct*ER, Keay et al., 2006) and localized in the olfactory lobe (De Lisa et al., 2012). While this receptor seems to be able to bind estradiol pending conformational changes (De Lisa et al., 2012), previous findings reported that the same receptor was constitutively active as transcription factor and unable to bind estradiol (Keay et al., 2006). Interestingly, stimulation of *Octopus* with estradiol increases the *oct*GnRH transcript, as well as the *oct*ER transcript, in the olfactory lobes (De Lisa et al., 2012). This result links the expression of GnRH in the olfactory lobe to the presence of estrogens, whose level fluctuates throughout the entire life cycle of, at least, the female of *Octopus* (Di Cosmo et al., 2001), peaking just before egg laying. It is noteworthy that the level of *oct*ER gene in the olfactory lobe also fluctuates (Di Cristo, personal observations).

Finally, it cannot be excluded that these receptors bind exogenous molecules or catabolites of metabolism (see below).

7.5.7 THE NERVOUS CONTROL OF REPRODUCTION AND THE OPTIC GLAND HORMONE IN OCTOPUS

The basis of our understanding of the neuroendocrine control of reproduction in *Octopus* is still based on the Wells and Wells (1959) findings (Fig 7.7A). All their studies on the function and action of optic gland and its hormone are milestones in the study of reproductive endocrinology in cephalopods (Wells, 1978).

Subsequent work contributed by adding biochemical information (O'Dor & Wells, 1973, 1975; Wells et al., 1975; Wells & Wells, 1975), but did not result in the purification the gonadotropic hormone from the optic gland, which led him to abandon this area of research.

More recent studies suggest that the olfactory lobe, as well as the subpedunculate lobe, is involved in the control of the activity of the optic gland (Di Cosmo & Di Cristo, 1998). These data led to the neuroanatomical evidence that the olfactory lobe might influence the optic gland by releasing a positive factor (GnRH) to counterbalance the inhibitory effect of FMRFamide from the subpedunculate lobe on the glandular cells. This model, consisting of two centers controlling the activity of the optic gland, constitutes a simple feedback loop to switch optic gland activity on and off (Fig. 7.7B).

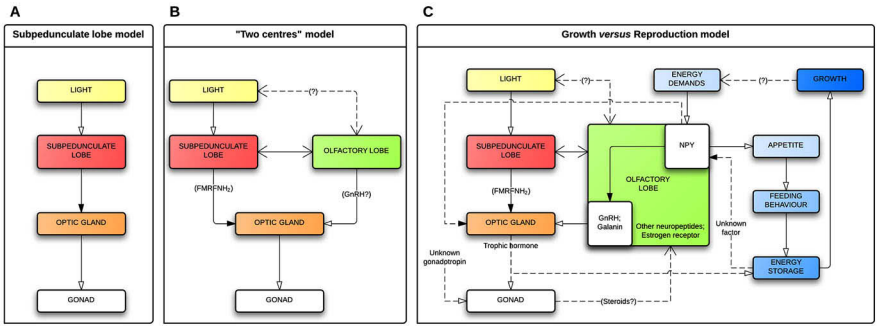

FIGURE 7.7 Different models of the nervous control of *Octopus* reproduction. (A) Model proposed by Wells and Wells (1959). (B) Model proposed by Di Cosmo and Di Cristo (1998). (C) Model proposed by Di Cristo (2013). Black arrowheads represent inhibitory control; white arrowheads represent excitatory control; arrows represent unknown relationships. Dashed lines indicate supposed pathways. (From Di Cristo, C. "Nervous Control of Reproduction in Octopus vulgaris: A New Model," in Invert Neurosci. 2013, 13(1), 27–34. Reprinted with kind permission from Springer Science+Business Media.)

Anatomical data support the hypothesis these two lobes are coupled in the control of optic gland activity. They both derive from the dorsal part of supraesophageal mass of *Octopus* brain and is confirmed in *Sepia* and *Loligo*, where the neuropils of the olfactory and dorsolateral lobe are continuous (Boycott, 1961; Messenger, 1979).

Even if the "two centers" model is validated, however, many more details are required to complete it. We need to know what activates the switch; and above all, we need to know what the optic gland hormone actually does, and if it is the only gonadotropic hormone.

Incidentally, the neuropeptides that are present in the olfactory lobe are involved in the neurocoding of inputs about the state of stored energy by the animal, possibly from food intake. In the gastropod *Lymnaea stagnalis*, for example, NPY is involved in regulating energy flow (de Jong-Brink et al., 2001). Although it does not affect food intake, it may stop the main energy consuming processes, like reproduction. When infected by endoparasites, the NPY gene in *Lymnaea* is up-regulated, causing egg laying to stop immediately (de Jong-Brink et al., 1999).

In all animals, reproduction is a high energy demanding physiological process that involves generally a shift of metabolic energy from somatic growth to reproductive maturation. The nervous system (and in turn gonadotropic glands) generally tempers the fertility of individuals to match nutritional availability. *Octopus* is no exception and its feeding behavior is obviously linked to reproduction.

In one of the last studies made by Wells on the optic gland (O'Dor & Wells, 1978), it was demonstrated in *Octopus* that optic gland hormone (and, hence, the nervous control of this gland) suppresses protein synthesis in the muscles. This is associated with an increase in the concentration of free amino acids circulating in the blood. In females, these events are associated with a rapid growth of the ovary and its ducts, and a loss of weight elsewhere. It was further established that such effect could be also dependent on an unknown hormone from the gonads.

According with such evidence, the production of yolk-rich eggs (vitellogenesis) by females of cephalopods, that requires the conversion of energy from food into the large amount of requested yolk, is dependent on the optic gland hormone (O'Dor & Wells, 1973).

Reproduction is also the terminal event in the life cycle in cephalopods; the animals breed once and then die. In females, egg laying is preceded by a period during which the ovary enlarges considerably, followed by a period during which the female broods the eggs. By the time the eggs hatch, the female appears notably emaciated and soon afterward dies. The death of

female octopuses is perhaps not very surprising; the animals starve and at the same time produce great quantities of yolk.

Male octopuses also die, and at about the same time as their mates; they too cease to feed during the last few weeks of their lives, and, like females, they show degenerative changes that range from a failure to control skin color and texture to the development of skin lesions that fail to heal (van Heukelem, 1973).

The situation in males suggests that the terminal condition of females is not simply due to starvation and the demands of a developing ovary. In fact, this semelparous behavior, as well as the ensuing death, also seems to be controlled by the optic gland hormone. Wodinsky (1977) demonstrated in *Octopus hummelincki* that removal of the optic glands from females that had laid and were brooding eggs induced a complete change of behavior. These animals abandoned their eggs, resumed feeding and growth and lived for considerably longer than mature animals retaining the glands. A resumption of growth is in agreement with the role of optic gland hormone in shifting energy balance in cephalopods (O'Dor & Wells, 1978). All the evidence points to activation of the optic glands being an irreversible process, so that the postreproductive animals continue to waste away until they die.

According to all these data, some hints on the functional role of optic gland hormone can be deduced. It is undoubtedly a gonado*trophic* hormone (from the Greek word *τροφή*, "food"). The early steps of gametogenesis, follicular cell division and vitellogenesis strictly depend on it. All these stages point to gonad growth. It is still unknown whether such an effect is direct or indirect. Progesterone has the same effect (Abate et al., 2000; Cuomo et al., 2005; Di Cosmo et al., 2001; Di Cristo et al., 2010). Moreover, while there is some evidence that progesterone is involved in the meiotic maturation of oocytes nothing is known about the meiotic maturation of spermatocytes. No similar role has been described for optic gland hormone.

In a new scheme of nervous control of reproduction in *Octopus* (Di Cristo, 2013; Fig. 7.7C), it is proposed that during the early stages of life (Di Cosmo et al., 2001), NPY neurons "perceive" the energy demands. They could then affect feeding behavior and, at the same time, shut down optic gland activity almost completely, in coordination with the subpedunculate lobe, either directly or by negatively modulating the activity of GnRH neurons. This would shift energy to body growth rather than reproduction.

When internal signals of satiation indicate that a discrete level of stored energy has been reached and can be converted into yolk, these inputs could inhibit NPY neurons. This would release from inhibition those neurons activating optic gland (GnRH; galanin) and stop the activity of the subpedunculate

lobe. Under the effect of the raising optic gland hormone titer, energy flow will be shifted to reproduction and vitellogenesis and gonadal maturation could then be initiated.

Such a process could be affected also by sex steroids, as well as their receptors, whose levels fluctuate during the life cycle (Di Cosmo et al., 2001). The role of an estrogen receptor in *Octopus* in the olfactory lobe is still unclear (De Lisa et al., 2012; Keay et al., 2006, see above). Certainly some of the classical "vertebrate" steroidogenic enzymes, are not present in mollusks (Markov et al., 2009), but enzymatic reactions on cholesterol-derived steroidal backbones are likely to happen, at least in *Octopus* and *Sepia*. This might indicate that either the real ligand of this receptor is still unknown or, as suggested elsewhere, it could work as a sensor, binding endogenous (or exogenous) hydrophobic molecules present in the diet (Markov & Laudet, 2011). If the last hypothesis were confirmed, the pathway mediated by this receptor would be influenced by the "metabolic milieu."

The whole hypothetical scenario implies that there is an unknown factor that signals the "*state of* satiety". Its release would positively depend on the optic gland hormone titer.

The most intriguing aspect of this topic concerns the optic gland hormone: Is it just a trophic hormone, which induces gonad ripening and vitellogenesis, as gastropods dorsal bodies that function this way (Roubos et al., 1980)? Or is it involved also in meiotic maturation and in egg laying? Wells and Wells (1959) reported that only three out of sixty-nine females with lesions activating the optic gland laid eggs.

This suggestion of an optic gland "trophic" hormone obviously poses another question: What is the hormone responsible for gametogenesis and egg laying in *Octopus*? Is there an octopus gonadotropin hormone like the egg laying hormone in *Aplysia* or the caudodorsal cells hormone in *Lymnaea* (Di Cosmo & Di Cristo, 2006)? And where is it released from?

In a certain sense, such a suggestion would give possible answers about the control of sexual maturation in male cephalopods. In fact, this unknown hormone could explain why *Octopus* males, as well as other cephalopod males, produce mature spermatophores long before optic gland enlargement (O'Dor & Wells, 1978). It can be inferred that the continued production of spermatozoa in young males probably does not rely on a high-energy requirement. This would imply that the trophic optic gland hormone is not needed, confirming the existence of another hormone regulating gametogenesis and gamete release.

7.5.8 THE NEUROSECRETORY SYSTEM OF THE VENA CAVA

The neurosecretory system of the vena cava system (NSV) is localized in the ventral part of posterior subesophageal mass of cephalopods, particularly in the ventral median vasomotor lobe. It is undoubtedly a neurosecretory system. The endings of its many nerve cells penetrate the walls of the vena cava and are filled with a variety of large vesicles (Martin, 1968). From the vena cava, secreted products can reach visceral organs located in the mantle cavity. The peripheral NSV system was described by Alexandrowicz (1964, 1965), and the neurosecretory cells reported from the visceral lobe by Bonichon (1967) and Martin (1968) are almost certainly parts of the same system. The neurosecretory cells are present and active in animals of all ages and at all times of year (Laubier-Bonichon, 1973).

In an alternative approach, Bianchi (1969), Bianchi and De Prisco (1971), and Berry and Cottrell (1970) made extracts of the peripheral part of the NSV system and were able to show that these had marked effects upon the heartbeat of *Octopus* or *Eledone*. This effect is probably brought by FMRFamide, which is a potent cardioaccelerator (Di Cosmo & Di Cristo, 1998).

Apart from its role in cardioregulation, the importance of the NSV system in the reproduction of cephalopods comes from the discovery that it contains two members of the vasopressin/oxytocin superfamily: cephalotocin and octopressin (Reich, 1992; Takuwa-Kuroda et al., 2003), two neuropeptides that in *Octopus* have been shown to be released into the vena cava to reach target organs. These peptide have also been found in *S. officinalis* (Henry et al., 2013).

Cephalopods seem to be the only invertebrates with two members of the oxytocin/vasopressin superfamily of peptides. Receptors for these peptides have been also identified and cloned (Kawada et al., 2004, 2005).

Interestingly octopressin evoked contractions of the smooth muscles of the oviduct, while cephalotocin has no effect on this tissue. Octopressin mRNA is also expressed in the buccal lobes, which control feeding behavior (Young, 1971). Interestingly, oxytocin may act as a “satiety hormone” in rats (Arletti et al., 1990).

We should recall here that in *Sepia*, other peptides released from eggs and the female reproductive ducts are able to control the contraction of oviducts (Bernay et al., 2004–2006; Zatylny et al., 2000a,b).

7.6 CONCLUSIONS

Apart from some small differences in the anatomy of the reproductive systems and in specific reproductive behaviors that may relate to different life styles all coleoid cephalopods show very similar reproductive features. These can be summarized as follows:

- Sexes are separate in these animals. Males are reproductively active for the greater part of their life cycle. Females mature later in life and are able to store sperm. Promiscuity seems to be common and sperm competition is likely to be widespread.
- Gametes undergo complex phases of maturation; fertilization (whether internal or external) and egg laying is temporally independent of mating.
- They are mainly semelparous, death coming suddenly after breeding.
- Sexual maturity in cephalopods (at least in females) strongly depends on activation of the optic gland, an endocrine gland whose activity is controlled by the central nervous system.
- The array of optic gland hormone actions goes beyond its reported gonadotropic effect.
- Experimental evidence supports the idea that the optic gland hormone has mainly a trophic action; it might play a major role in shifting energy from somatic growth to reproduction.
- Physiological and molecular studies support the hypothesis that the gonads of cephalopods might synthetize and release hormones. Steroids are candidate molecules.
- A set of neuropeptidergic systems controls and regulates activity of the optic gland.
- Olfaction and chemotactile reception might have a profound effect on both sexual maturity and reproductive behavior.

Many questions still remain without answers in the study of the reproductive physiology of cephalopods. In these animals, some systems have provided spectacular models for solving general problems: the giant squid axon is the perhaps the most striking example (Young, 1939). However, serious technical difficulties continue to hinder advance in our understanding of the undoubtedly complex control of reproduction in these "advanced invertebrates" (Wells, 1978). The housing and maintenance of cephalopods in captivity present many problems, as does molecular manipulation (see Fiorito et al., 2014). New genomic and transcriptomic approaches (Albertin

et al., 2012, 2015; Moroz et al., 2011) may open new windows to understanding some key steps of reproduction in these animals. Among these are identification of the optic gland hormone, the possible existence of an egg laying hormone, the hormonal role of the gonads, the role of olfaction and chemotactile stimuli and sex recognition are just some of the issues that urgently require investigation.

KEYWORDS

- **cephalopods**
- **octopus**
- **cuttlefish**
- **squid**
- **gametes**
- **mating**
- **optic gland**
- **neuropeptides**
- **steroids**
- **growth**

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CHAPTER 8

THE PHYSIOLOGY OF PRE- AND POSTCOPULATORY SEXUAL SELECTION IN SIMULTANEOUSLY HERMAPHRODITIC FRESHWATER SNAILS

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CONTENTS

ABSTRACT

Organisms evolve by natural selection toward traits that increase survival, while sexual selection creates an often opposing selective force toward traits that enhance reproductive success. Sexual selection can act on behavioral and physiological processes that take place before and after copulation. Precopulatory processes include competition for mates and mate choice; postcopulatory processes include sperm competition and cryptic female choice. While much-investigated in separate-sexed species, simultaneous hermaphrodites have remained underexposed in this field of research. This is surprising, because hermaphroditism is a common reproductive strategy for many organisms and allows for addressing outstanding questions that cannot be tackled in separate-sexed animals. Therefore, using hermaphroditic freshwater snails, this chapter aims to provide an overview of the importance of pre- and postcopulatory sexual selection and link this to the underlying physiological mechanisms. Several freshwater snail species have served as model organisms for decades, since they are amenable to a variety of different approaches, including cellular, biochemical, neurobiological, endocrinological, behavioral, and ecological. At the precopulatory level, the main focus of this chapter will lie on the neurobiological and physiological detection mechanisms that could underlie mate choice and strategic sperm allocation, with a special focus on the involvement of pheromonal cues. At the postcopulatory level, the physiological mechanism involved in effects caused by seminal fluid proteins, such as on behavior, paternity, and sex allocation, will be reviewed. Attempting to connect the ultimate and proximate levels of sexual selection leads me to the identification of a number of gaps in our knowledge and important research opportunities that should be exploited in future. Such integration will ultimately lead to a synthesis that is necessary for a full understanding of the evolution of hermaphroditism as a reproductive strategy in freshwater snails and will provide new insights into the process and validity of sexual selection in general.

8.1 INTRODUCTION

Organisms evolve under the influence of two, often opposing, selective forces: natural and sexual selection. Natural selection favors traits that increase survival to reproductive age and beyond, whereas sexual selection favors traits that enhance reproductive success. Darwin (1871) phrased

sexual selection mainly in a precopulatory context to explain extravagant male secondary sexual characteristics. Parker (1970) substantially extended this view by highlighting that sexual selection could also act postcopulation. Currently, precopulatory sexual selection includes competition for mates and mate choice, while postcopulatory sexual selection encompasses sperm competition and cryptic female choice (reviewed in Koene, 2012).

By extending pre- and postcopulatory sexual selection theory, research has firmly established that sexual encounters are usually accompanied by conflicts of interest between partners (e.g., Arnqvist & Rowe, 2005; Koene, 2012). Such sexual conflicts arise because traits that are advantageous for one sex can be harmful to the other. As a result, these conflicts can trigger coevolutionary arms races leading to extreme, costly, and sometimes bizarre mating behaviors (Arnqvist & Rowe, 2002; Morrow & Arnqvist, 2003; Rice, 1996). For species with separate sexes, many recent studies have focused on sperm competition, sexual conflict, and their consequences (reviewed in Arnqvist & Rowe, 2005; Birkhead et al., 2008). These investigations have shown that conflicts between the sexes can have severe implications on the evolution of behavior and (secondary) sexual characteristics that play important roles at the pre- and/or postcopulatory level (e.g., Parker & Birkhead, 2013; Schärer et al., 2012).

Although much research has been done on precopulatory sexual selection in general, especially with respect to pheromones (e.g., Wyatt, 2014), postcopulatory sexual selection has only received serious attention relatively recently (reviewed in, e.g., Nakadera & Koene, 2013). Moreover, when it comes to attempts to thoroughly understand the mechanisms underlying sexual selection, and its ensuing conflicts, detailed studies are still very rare. One notable exception is the work on postcopulatory effects of male accessory gland proteins of the fruit fly *Drosophila melanogaster* (e.g., Arnqvist & Rowe, 2005; Avila et al., 2011; Liu & Kubli, 2003; Kubli, 2003; Perry et al., 2013). Importantly, nearly all research has concentrated on species with separate sexes, while hermaphroditism is present in 24 of the 34 animal phyla (Anthes, 2010; Michiels, 1998; Schärer, 2009), representing approximately 5–6% of all animal species (30% when insects are excluded; Jarne & Auld, 2006), and most plant species (Eppley & Jesson, 2008), making this a far-from-trivial mode of reproduction (Schärer et al., 2014). I will here focus on simultaneously hermaphroditic freshwater pulmonates, the Basommatophora (or Hygrophila).

8.1.1 SEXUAL SELECTION IN HERMAPHRODITES

Darwin thought that sexual selection could not act in hermaphrodites, mainly because they are male and female at the same time (1871). Yet in recent years, experimental evidence demonstrating the opposite has accumulated (e.g., Anthes et al., 2010; Bedhomme et al., 2009; Hoffer et al., 2010; Koene & Chase, 1998; Koene & Chiba, 2006; Koene & Ter Maat, 2005, 2007; Schärer & Pen, 2013). In addition, these findings highlight that sexual selection processes can be fundamentally different from the separate sexed situation, because a simultaneous hermaphrodite (hereafter, hermaphrodite) can gain fitness through its male and female function, within each reciprocal mating or reproductive period, and often has the option to fertilize itself (e.g., Anthes et al., 2010; Charnov, 1979; Michiels & Koene, 2006). This difference might explain the bizarre mating systems, sophisticated sperm allocation and complex reproductive morphologies found in hermaphrodites. Examples include hypodermic insemination in tropical flatworms (Michiels & Newman, 1998), partner sedation in sea slugs (Anthes & Michiels, 2007), hypodermic injection of glandular products in earthworms (Koene et al., 2005), highly complex sperm design in flatworms (Schärer et al., 2011), and love-dart shooting in land snails (reviewed by Arnqvist & Rowe, 2005; Koene, 2012; Schilthuizen, 2005).

From such studies, it becomes clear that sexual selection is currently considered an important evolutionary driving force for hermaphrodites (at least equally important as in separate sexes). And, even though postcopulatory processes are thought to be the predominant driving force behind sexual selection in hermaphrodites, because of the apparent lack of clear precopulatory sexually selected traits (Nakadera & Koene, 2013; Schärer & Pen, 2013; Schärer et al., 2014), precopulatory processes cannot be neglected and are worthy of detailed investigation. Hence, it is now both timely and pertinent to investigate pre- and postcopulatory sexual selection in hermaphrodites, and to do this not only at the ultimate level but also consider the proximate levels involved. The latter requires that connections are made between findings at the ultimate level and the mechanism that could underlie effects that have been observed in terms of precopulatory mating decisions and postcopulatory effects of insemination.

8.1.2 SEXUAL SELECTION IN HERMAPHRODITIC FRESHWATER SNAILS

I do intend to keep this chapter general for all Basommatophora/Hygrophila, which is an order that shows a wide diversity in species that mostly occur in freshwater and are all simultaneously hermaphroditic (Jarne et al., 2010). Despite this species diversity, some of the following will necessarily focus on the most investigated species, the great pond snail *Lymnaea stagnalis*. This species has a long history as a model organism for neuroendocrine, behavioral, toxicological, molecular, and evolutionary research (e.g., Koene, 2010). As a result, a large body of background knowledge on many biological processes is already available for this species, along with a select few (reviewed in Chase, 2002; Koene, 2010). Despite this focus, I will include information about other species wherever possible throughout this chapter.

When thinking about sexual selection, it is important to have a grasp of the reproductive behavior and morphology of the species at hand. Therefore, although this has been reviewed recently and extensively (e.g., Jarne et al., 2010; Koene, 2010), a brief summary seems appropriate and will hopefully facilitate the reference to specific aspects of reproductive behaviors and organs later on in this chapter.

Copulation generally starts with mounting on the shell by one of the two mating partners, the sperm donor. This is followed by this individual crawling, in a circular fashion following the shell's winding direction, over the shell of the prospective sperm recipient. In high-spired species, the donor does this to locate the shell's tip in order to subsequently crawl to the shell's margin. Once the shell's margin is reached, the donor positions itself for intromission. Before intromission is achieved, the now fully everted preputium is used to locate the female gonopore (probing). Intromission is achieved when the penis, which everts at the tip of the preputium, is inserted into the female gonopore. During such an insemination, sperm (from the seminal vesicles) and seminal fluids (from the prostate gland) are transferred. Since these are simultaneous hermaphrodites, sexual roles can be swapped immediately afterward (see below).

Egg laying starts with a so-called resting phase, during which ripe eggs are ovulated, locomotion stops, the shell is held still, and slightly pulled forward over the tentacles and no rasping (i.e., feeding movement) occurs. This is followed by a turning phase, where locomotion starts again, the shell is turned back and forth and the surface is rasped. The actual oviposition, where the egg mass is attached to the substrate, is relatively short after which the animals can inspect the mass before leaving it behind. In many

freshwater snail species, egg masses are laid relatively frequently, often several per week, and can contain many eggs.

During the performance of these reproductive behaviors, the reproductive organs do their work. The reproductive system can be divided in a hermaphroditic, female, and male part. The hermaphroditic part consists of, from anterior to posterior, the ovotestis, seminal vesicles, hermaphroditic duct, and carrefour (or fertilization pouch). Both eggs and sperm are produced, stored, and transported here. Beyond the latter, the male tract splits of and transports sperm via the sperm duct, prostate gland, vas deferens, penis (with a preputium sheath), and preputium. The female tract contains the albumen gland, pars contorta, muciparous gland, and oothecal gland for egg packaging, and the allosperm duct and bursa copulatrix for transport and digestion of received sperm, respectively.

In the following, I will put forward that research has shown that both pre- and postcopulatory sexual selection pose important driving forces in these hermaphroditic snails. In separate sections, I will deal with this evidence, exploring to what extent we can link what is known about mating and investment decisions at the ultimate (evolutionary and functional) level, to proximate (physiological and developmental) mechanisms that may mediate these processes. Knowledge about both levels, and especially the integration thereof, allows one to address pertinent questions emerging from physiological mechanisms at an evolutionary level, and vice versa (e.g., Koene, 2010). That this can lead to insights that can only be uncovered through integration is illustrated by the work on the "decision" to mate in the male role in *L. stagnalis* (e.g., De Boer et al., 1997; Koene & Ter Maat, 2005), which is treated in more detail later on in this chapter. Finally, linking proximate and ultimate mechanisms will also identify gaps in our knowledge that need to be addressed to better understand the reproductive strategies that these species employ to optimize their reproductive output.

8.2 PRECOPULATORY SEXUAL SELECTION

Many freshwater snail species seem able to self fertilize, at least to some extent, and they normally do this internally by mixing their own eggs and sperm and not via auto-/self-copulation (e.g., Jarne et al., 2010). However, while selfing is an assurance for producing offspring when no mates are around, most species seem to prefer to outcross their eggs, meaning that they fertilize their own eggs with sperm received from a mating partner (allosperm), to avoid the negative effects of inbreeding (Escobar et al., 2011;

Tsitrone et al., 2003). This means that a suitable mate needs to be found that is willing to receive and/or donate sperm. The latter already implies that there are a number of conditions that need to be met before successful insemination can take place.

First, potential mates need to be located in the animal's environment. Even though population densities are not necessarily low in freshwater snails, a considerable amount of time can be invested in locating adult conspecifics. One clear advantage that simultaneous hermaphrodites have over separate-sexed species is that every encountered conspecific is a potential mating partner (Ghiselin, 1969; Puurtinen & Kaitala, 2002).

Second, once a potential mating partner has been located, the partners need to decide which sexual role to play. Since most studied hermaphroditic freshwater snails seem to mate via unilateral insemination—that is, within one mating interaction one individual is the sperm donor and one the sperm recipient—this means that the sexual roles need to be divided. After an initial interaction, the roles can be swapped. Although some species tend to exchange sexual roles with the same partner, this is not necessary for successful sperm transfer in only one direction (Koene & Ter Maat, 2005). There are species, however, where simultaneously reciprocal matings have been reported (*Biomphalaria glabrata*: Trigwell et al., 1997; *Helisoma trivolvis*: Abdel-Malek, 1952), although observations on whether both partners transfer sperm are lacking (see also Lamy et al., 2012; Soldatenko & Petrov, 2012).

Third, besides the mating role, each individual also needs to choose whether the potential mate is suitable to give sperm to and/or to receive sperm from. Especially this latter decision has been shown to be influenced by a number of different factors and is typically referred to as the actual mate choice. Reasons for being choosy, as male or female, are often found in various costs and risks of copulating. Examples include energy expenditure, time investment, resource investment, predation, survival, infection risk, and injury or manipulation inflicted by mates (Nakadera & Koene, 2013). Evidently, the ultimate goal of each mating partner will be to optimize its mate choice in such a way that the total reproductive success—meaning its male plus female reproductive success—of the individual is maximized (e.g., Charnov, 1979; Nakadera & Koene, 2013). Given that each individual is trying to achieve this, but that these objectives need not coincide between the mating partners, this often leads to a conflict of interest (Charnov, 1979; Nakadera et al., 2014a; Schärer et al., 2014). In the following, a number of the most important and best investigated factors involved in mate choice will be highlighted, but it should be noted that this field of research is still wide

open for much more in-depth investigations of the specific traits and cues involved as well as for determining whether particular choices are male or female decisions.

8.2.1 MATING HISTORY

One of the prominent factors that affect mate choice is mating history of both the donor and recipient. For example, while from the male perspective it will be beneficial if the individual is able to fertilize many sperm recipients (Anthes et al., 2010; Bateman, 1948), there is generally some limit to the number of inseminations that an animal is willing to perform. Part of this is due to the (substantial) costs involved in the production and transfer of ejaculates (Dewsbury, 2005; Hoffer et al., 2010). These male reproductive costs have been shown to be roughly equal to the female costs in *L. stagnalis* by experimental elimination of the male function (De Visser et al., 1994; Hoffer et al., 2010, the physiological mechanism underlying this is explained in the next section). As a consequence, from the male point of view hermaphroditic snails should choose to inseminate mates with a high reproductive potential. This can, for instance, be achieved by preferentially mating with virgins and/or by avoiding partners that have recently mated, since both choices would lead to avoiding sperm competition (i.e., inseminating individuals that already have allosperm from other partners stored in their storage organ; Jarne et al., 2010; Koene, 2010).

With a virgin partner, a sperm donor can potentially monopolize the reproductive resources of its mate. In the freshwater snail *L. stagnalis*, male acting individuals do not seem to discriminate between virgins and mated individuals, the likelihood of mating with either is the same (Koene et al., 2008; but they do transfer more sperm once mating takes place, Loose & Koene, 2008, see Section 8.4). Because inseminations are energetically costly, when an individual has already inseminated a partner, it would be predicted to prefer to inseminate a novel partner. This effect has become known as the Coolidge effect and can be tested by offering individuals the choice between familiar and novel partners (Koene and Ter Maat, 2007; Wilson et al., 1963). When this is done in *L. stagnalis*, it can be seen that this species prefers a novel over a familiar partner (Koene & Ter Maat, 2007). Especially when ejaculates are expensive to produce and transfer, it is beneficial for the male role to choose a novel partner, because the donor can expect to fertilize more eggs in that novel partner than in the partner it has already given sperm to (and invested an ejaculate in). Such a decision

to allocate sperm optimally over many mating partners, rather than to give everything to only one, is predicted to lead to a higher male reproductive success in the long run, to help avoid potential fertilization incompatibilities and effects of inbreeding (if some of the partners happen to be related to the sperm donor) as well as local sperm competition (among sperm from the same donor; e.g., Schärer; 2009).

The Coolidge effect found in *L. stagnalis* seems to depend on chemical cues, given that this preference was no longer observed when the mating trials were performed in a clean aquarium. Whether this is then due to the mucus trail, or to specific chemicals released by the recipients remains to be investigated (Koene & Ter Maat, 2007). Interestingly, the Coolidge effect was also tested in the freshwater snail *B. glabrata*, but not found (Häderer et al., 2009). One explanation for this different result may be that *B. glabrata* does not obtain sufficient benefits from discriminating between different mating partners. While this is partially supported by the higher mating rates of this species, which can range from 4 to 13 times within 12 h (Vernon & Taylor, 1996), we will see below that they do distinguish between their mates based on other traits. It would now be interesting to see whether partner novelty (and also virginity) affects mate choice in other basommatophoran species than the two that have been tested so far (next to the opisthobranch *Aplysia fasciata*: Ziv et al., 1989), since this could give an indication of the importance of such cues for mate choice (as well as sperm competition). For instance, the Coolidge effect may lie at the basis of why snails tend to perform more inseminations when they are in larger groups, a situation in which they encounter many novel partners (e.g., Koene & Ter Maat, 2007).

8.2.2 BODY SIZE AND AGE

Based on the size-advantage model (Ghiselin, 1969; Munday et al., 2006), it is generally assumed that body size is an indicator of mate quality. Indeed, the trend seems to be that larger individuals invest relatively more in egg production (e.g., Anthes & Michiels, 2007; Jordaens et al., 2007). This might make it beneficial for a sperm donor to choose to mate preferentially with larger partners, which would then lead to size assortative mating. Studies on various simultaneously hermaphroditic species have reported this preference (e.g., sea slugs, Anthes et al., 2006; earthworms: Domínguez & Velando, 2013; Monroy et al., 2005; Michiels et al., 2001). However, there seems to be no consensus for this within freshwater snails, even though there are numerous studies that have investigated this (e.g., *Physa gyrina* and *Physa*

heterostropha: DeWitt, 1996; *Physa acuta*: Ohbayashi-Hodoki et al., 2004; *Radix lagotis*: Yu & Wang, 2013; *H. trivolvis*: Norton et al., 2008; *L. stagnalis:* Hermann et al., 2009; see the review by Nakadera & Koene, 2013 for more examples). There are several important reasons for why reports may vary so much, as also outlined in Nakadera and Koene (2013).

First, body size is not restricted to one sexual function in a simultaneously hermaphroditic individual. As a consequence, such a shared cost trait will affect the female as well as the male reproductive success, thus leading to a trade-off in mate choice. On the one hand, mating with a large partner may be beneficial for the sperm donor, because its large partner will produce more offspring. On the other hand, and crucially at the same time, if it is likely that the mating partner will also become a sperm donor with the same individual, a larger sperm donor can transfer a larger ejaculate that contains more seminal fluid components that may negatively affect the recipients' female and male reproductive success (e.g., Nakadera et al., 2015; see also Section 8.5).

Second, under several circumstances, body size may not be a precise enough indicator of the current reproductive potential of a hermaphrodite. For example, for *Physa* it has been shown that it displays plasticity in growth under influence of temperature, and that as a consequence, fecundity cannot always be predicted based on size (Arendt, 2015). Another example, where the relationship between size and fecundity is lost, is when freshwater snails are infected with trematode parasites, which are common in the field (e.g., Nakadera et al., 2015). Such infection eventually leads to disproportionate growth (gigantism) as well as cessation of reproduction. Obviously, even though these snails grow very large, they should not be preferred as mating partners; this might even lead to selection against choice based on large body size (Ballabeni, 1995; Nakadera & Koene, 2013; Sorensen & Minchella, 2001).

Third, even when a choice based on body size is found, one should realize that body size is often correlated with age. This is especially true for freshwater snails given that they show indeterminate growth. Hence, if age is unknown in a mate choice experiment, one cannot conclude that body size is the trait that this choice is based on (e.g., Norton et al., 2008; Nakadera & Koene, 2013). To get around this confounding factor, experiments are required where both age and size are included as factors in a mate choice experiment. Recent work on *L. stagnalis* evaluated the effects of age and body size on sex role decisions separately (Nakadera et al., 2015). The results revealed that both age and size are important determinants of mating

decisions, where both young and small snails tend to perform the male role more often. It now remains to be shown whether this is a male- or female-driven choice.

8.2.3 GENETIC BACKGROUND: RELATEDNESS AND INFECTION RESISTANCE

Behavioral work on several freshwater snails has shown that these hermaphrodites can display behaviors that discourage the mating partner from inseminating or even dislodge the sperm donor from the shell. For example, in *P. acuta* this is done by vigorous shaking of the shell and biting in response to a conspecific mounting the individuals shell (DeWitt, 1996). In *Bulinus globosus*, the mounted individual twists its body (and hence its shell) in such a way that the prospective sperm donor cannot reach the female gonopore for insemination (Rudolph & Bailey, 1985). Similar insemination avoidance behaviors may also be present in *L. stagnalis* (Koene et al., unpubl.; as indicated by Van Duivenboden & Ter Maat, 1988), but this remains to be fully confirmed.

There are several convincing studies that show that such behaviors are indeed used in mate choice. One example comes from *P. acuta*, where the use of insemination avoidance behaviors has been shown to depend on the genetic background of the mating partners (Facon et al., 2006). In that experiment, the female acting (mounted) snails showed a higher frequency of shell swinging and biting of the partner's preputium (phallus) when these partners were related (siblings, Facon et al., 2006). Given the high inbreeding depression that this species shows, such insemination avoidance behavior may help in preventing inbreeding, via earlier termination of unwanted mating attempts. In contrast, a closely related species from the same genus, *P. gyrina*, has been reported to show more intense avoidance behavior when mated by partners from other populations (McCarthy, 2004). These findings, taken together, may indicate that these species prefer to mate with sympatric individuals as long as they are not highly related (i.e., siblings).

To complicate things, two species from the genus *Biomphalaria* seem to prefer to mate with sympatric individuals. The authors of that study interpreted this finding as a local adaptation against parasitic infection (Rupp & Woolhouse, 1999). Other work confirms that this can be an important selection pressure. As already mentioned, parasite infection can have a large impact on pulmonate reproduction and growth (reviewed in Jordaens et

al., 2007). Clearly, it would be of great benefit if snails could base their mate choice on traits related to infection (level) or even resistance. So far, such choice has only been experimentally tested and shown to occur in *B. glabrata* (Webster et al., 2003; Webster & Gower, 2006).

At the furthest extreme of population differences, there are differences in whole body morphology that could also play a role. One example of this is the so-called handedness of snails, where two different, mirror-image morphs of a species co-occur (Schilthuizen et al., 2007; Koene & Cosijn, 2012; Davison et al., 2016). In *L. stagnalis*, as in many snail species, this is a maternally inherited trait that results in a complete mirror-image development of the animals (Asami et al., 2008). While this species, unlike many land snail species, is still able to copulate successfully (albeit with some difficulty), at least the normal right-winding (dextral) morph seems to prefer to inseminate its own morph and not the left winding (sinistral) morph (Koene & Cosijn, 2012)

In sum, the foregoing illustrates that there are numerous factors that are relevant for the assessment of the quality of potential mates. These factors include body size and shape, morphology, parasite infection, disease resistance, genetic relatedness, mating history, mate identity, and population origin (summarized in Fig. 8.1). The following will explore which proximate mechanism possibly lie at the basis of such mating decisions and which cues (such as water-borne chemicals, substances in mucus trails, or tactile information) may be involved.

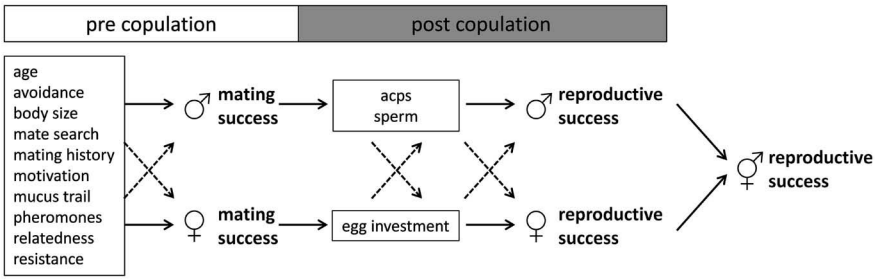

FIGURE 8.1 Schematic overview of the factors involved in pre- and postcopulatory sexual selection in hermaphrodites. The arrows indicate the influence that these factors can have on the male and female mating and reproductive success. The dashed arrows indicate that traits can also have positive and/or negative cross-sex effects, meaning that they can affect either one or both sexual functions of the hermaphrodite.

8.2.4 DETECTION OF MATE CHOICE CUES VIA MUCUS TRAILS AND PHEROMONES

So, a crucial question that emerges from the above is: How are traits and reproductive states of potential mating partners detected by sperm donors? Obviously, given that these animals have rather limited visual and acoustic senses, much of this is detected via chemical information (e.g., Chase, 2002; Cummins & Wyeth, 2014). However, even though several studies have demonstrated that pheromones and/or mucus trails are important for such mate choice decisions (e.g., Ng et al., 2013), the mechanistic link to how the animals detect these cues has remained very underexposed. In the following, I will explore what is known and what remains to be investigated in order to obtain a clearer picture of the involved physiology.

Nearly all gastropods release mucus, a sticky slime-like substance. They do this ventrally, via their foot, for which they are often equipped with mucous glands that are embedded in the foot (sometimes called the pedal gland). This mucus is used for gliding over surfaces and for adhering to these same surfaces. Via the dorsal surface of their skin they also produce mucus that prevents desiccation and dilutes or washes off irritants (Chase, 2002). Moreover, chemical or tactile irritants that discourage predators can be present and are produced in epidermal gland cells. For example, in *L. stagnalis*, eleven different epidermal gland types can be distinguished in the skin. Three types of gland cells are found in numerous places in the snail's skin, while other types are specific to the foot, lip or mantle regions (Zijlstra, 1972).

The mucus is composed of water in combination with proteins, carbohydrates, and lipids (Davies & Hawkins, 1998; Zijlstra, 1972). The major structural components of molluscan mucus are glycosaminoglycans and proteoglycans (proteins that are highly glycosylated). These proteoglycans are thought to provide stability to other proteins present in the mucus and are therefore less easily degraded. In the mucus trail of *L. stagnalis* at least three types of high molecular weight glycoconjugates, which are mucin-like, have been identified and have the ability to form gels (Ballance et al., 2004).

In addition to the general constituents that make the mucus sticky, it also contains specific pheromones and other types of chemicals that can provide information about the animal. In aquatic environments, such substances can often dissolve and/or diffuse into the water, and can thus travel over long distances. In these cases, such pheromonal information can lead to aggregations, not only for mate searching purposes, but also by way of food sharing and group defense (Kuanpradit et al., 2012). Several of such pheromones, the best known of these is a protein pheromone called attractin, have been

shown to have such functions in the Opisthobranch sea hare *Aplysia californica* and its congeners (and is released from the egg cordon; e.g., Cummins et al., 2004; Painter et al., 2003, 2004).

A similar pheromone to one of the components of the *Aplysia* blend, called temptin, seems also to be produced by the hermaphroditic freshwater snail *B. glabrata* (Hathaway et al., 2010), as well as the broadcast spawning separate-sexed abalone *Haliotis discus* (Kuanpradit et al., 2012), for which it has been shown that females are attracted to pheromones released by the male prior to spawning (Nhan et al., 2010). This suggests that some of these pheromones are highly conserved, even across sexual systems and habitat types. This is also supported by the fact that *L. stagnalis* is also attracted to the *Aplysia* pheromone attractin (Painter et al., 2004; Koene et al. unpublished). However, whether these pheromones play a similar role as the one proposed in *Aplysia*—aggregation for egg laying and mating—remains to be convincingly shown in any species.

Pheromones that are released into the water column may serve the more general purpose of locating potential mating partners (or suitable habitat or egg laying sites). But mucus trails can also contain pheromonal information that works at the closer range for finer scale location of conspecifics that have secreted the trail. This can often result in so-called trail following, where individuals follow the mucus trail (laid by themselves or others). Several reviews indicate that such trail following can serve many adaptive purposes, which include homing, prey location, and mate finding (e.g., Ng et al., 2013). Obviously, the latter is of relevance here. Such research has, for example, shown that sexually motivated *B. glabrata*, are more likely to display trail following that non-motivated individuals (Townsend, 1974). And both *B. glabrata* and *P. acuta* seem to even be able to detect the direction in which the trail was laid (respectively, Townsend, 1974; Wells & Buckley, 1972), possibly due to a chemical gradient in the trail (Bousfield et al., 1980).

8.3 PHYSIOLOGY OF PRECOPULATORY PROCESSES

The overview given above highlights two main topics for which we can now explore the underlying mechanisms and physiology. On the one hand, variation is observed in the motivation to mate. On the other hand, when a snail is motivated to mate, there are still factors that influence with which partner it will mate in the male and/or female role. What is known about the physiology of both of these processes will be reviewed below, which

will also include an exploration of how these snails may gather information about their mates.

8.3.1 MOTIVATION TO MATE

There are, of course, many environmental factors that influence whether mating will take place or not. One can think of the influence of density, temperature, food availability, parasites, and the like (e.g., Nakadera & Koene, 2013). But even when all these factors are optimal, one can still observe that a period of sexual isolation will influence the likelihood of mating. Such increased eagerness to mate after sexual isolation seems to be a common phenomenon in a range of simultaneous hermaphrodites (*A. fasciata*: Ziv et al., 1989; *Helix aspersa*: Adamo & Chase, 1990; *Dugesia polychroa*: Peters et al., 1996). There are two main reasons for this that I will explore here, either it is profitable to mate (in one of the sexual roles) or the time since the last mating as male and/or female (i.e., mating history) has temporarily (de)motivated the individual.

Aspects of profitability to mate have been best investigated and indicate that one of the main factors causing variation in motivation to mate is sexual isolation. This is often used experimentally to increase the likelihood of mating (Koene & Ter Maat, 2005). For example, for the description of the copulation strategy of *Stagnicola elodes* and *B. glabrata*, sexually isolated individuals were used (respectively, Rudolph, 1979a; Vernon & Taylor, 1996). Given that these animals are hermaphroditic, but generally mate unilaterally, it is important to consider which role drives this decision to mate.

This has been investigated in detail in *L. stagnalis*, which has also been shown to become more motivated after sexual isolation. More importantly, the motivation to mate in the male role seems decisive. For example, previous work has shown that individuals that have been sexually isolated for a longer time than their partner will act as sperm donors (Van Duivenboden & Ter Maat, 1985). When both individuals are motivated to donate sperm, role alternation will take place so that both individuals of the mating pair get to donate (and receive) sperm (Koene & Ter Maat, 2005). The one that inseminates first is generally referred to as the primary donor, and the other as the secondary donor (e.g., Nakadera et al., 2014b, 2015). To ensure the chance to also inseminate, the secondary donor may even display a typical mating posture in which it already holds onto the shell of the partner, ready to mount, well before this one has finished inseminating (Koene & Ter Maat, 2005). However, if more than one partner is around, that is, if the pair

is not confined to a small mating arena, but rather in a group, the secondary donor need not inseminate its primary donor, but is just as likely to choose a different partner to donate sperm to (e.g., Koene & Ter Maat, 2007). In other words, this species mating behavior is based on unconditional unilateral mating and reciprocity is not conditional for initiating a mating (Koene & Ter Maat, 2005).

So how does the individual "decide" about when to be male or, in other words, what is the underlying physiological mechanism. One of the main factors determining this decision seems to be the availability of seminal fluid, which is produced in the prostate gland. After sexual isolation, the animal has had time to fully replenish the seminal fluids that have been transferred in previous matings, which can be measured in terms of gland weight (De Boer et al., 1997). Electrophysiological experiments have demonstrated that increases in gland size are detected by the central nervous system via a small branch of the penial nerve (np1, De Boer et al., 1997; Fig. 8.2). This nerve branch feeds into the different regions within the central nervous system that are known to be involved in male mating behavior (reviewed in Jarne et al., 2010; Koene, 2010). That this information is crucial for the execution of the male role, has been demonstrated by lesioning this nerve, which results in complete elimination of the male function, that is, feminization (De Visser et al., 1994; Hoffer et al., 2010; Koene et al., 2009a; Van Duivenboden et al., 1985).

A study on *P. heterostropha pomilia* also shows that isolated individuals tend to mate more often as males than mated individuals (Wethington & Dillon, 1996). Moreover, role alternations were predominantly seen between isolated individuals (Wethington & Dillon, 1996; Koene & Ter Maat, 2005). That this may be a general pattern is further supported by reports that role alternation is reportedly very rare in "spontaneous" copulations, i.e., copulations between non-isolated snails (*B. globosus*: Rudolph, 1979b; *L. stagnalis*: Van Duivenboden & Ter Maat, 1988; *P. heterostropha pomilia*: Wethington & Dillon, 1996). What now remains to be demonstrated for these species is whether the underlying physiological mechanism is the same as in *L. stagnalis*.

The importance of the prostate gland for the regulation of the male function, suggests that the presence of sufficient seminal fluid is crucial for successful fertilization. The latter will be further expanded upon in the section about postcopulatory sexual selection. The following will first focus on the means by which these snails detect biochemical information relevant for mate choice. The two organs of interest are the osphradium and the cephalic sensory organs (Fig. 8.2).

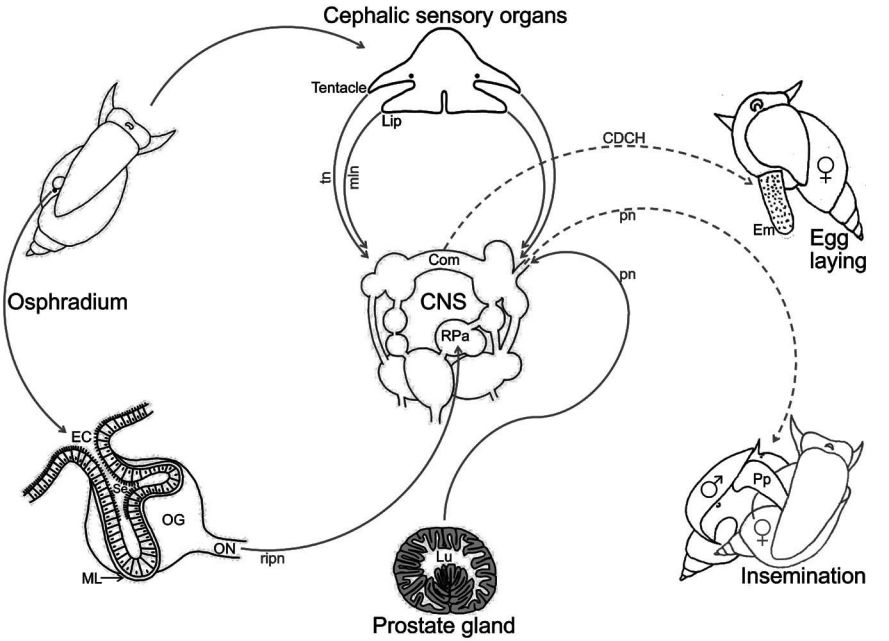

FIGURE 8.2 Schematic representation of the main processes and organs involved in reproductive decisions that are dealt with in the text. The input of information relevant for mating decisions is indicated with solid lines, the output behaviors with dashed lines. The nerves via which information travels are indicated in small letters along the arrows. The location of the osphradial pore is indicated in the snail on the right side of the figure. The snails on the left side illustrate the different reproductive behaviors, where the female and male symbols indicate sexual roles. Egg laying, for which the stage is shown where the egg mass (Em) is oviposited, is initiated by the release of caudo-dorsal cell hormone (egg laying hormone, CDCH). For insemination, the drawing illustrates how the top snail, performing the male role (sperm donor), has its preputium (penis-carrying organ, Pp) everted and inserted under the shell of the sperm recipient, where the female opening (gonopore) is located. Abbreviations: CNS, central nervous system; Com, Commissure; EC, Epithelial canal; Lu, lumen; ML, muscle layer; mln, median lip nerve; OG, osphradial ganglion containing subepithelial receptor cells (latter not shown); ON, osphradial nerve; pn, penial nerve; Pp, preputium; ripn, right internal parietal nerve; RPa, right parietal ganglion; Se, sensory epithelium; tn, tentacle nerve.

8.3.2 OSPHRADIUM

In the Gastropoda, the osphradium is a peripheral sensory organ that has mechano-, osmo-, and chemosensory properties (Chase, 2002; Cummins & Wyeth, 2014). This organ is widespread within the Mollusca and is generally

located in the mantle cavity (e.g., Haszprunar, 1987). The osphradium of aquatic pulmonate snails is found just anterior to the pneumostome, the animal's lung opening (Chase, 2002; Cummins & Wyeth, 2014). The general morphological layout of the osphradium is similar within all aquatic pulmonates that have been investigated. Among these are the blood fluke planorb *B. glabrata* (e.g., Michelson, 1960), the great rams horn *Planorbarius corneus* (e.g., Benjamin & Peat, 1971), and the great pond snail *L. stagnalis* (e.g., Wedemeyer & Schild, 1995).

In *L. stagnalis*, again the species in which this organ has been studied most extensively, the organ is described as having a Y- or λ-shaped epithelial canal that has a connection to the outside via a small pore. Although the exact shape of this epithelial canal may differ between species, they all conform to the following morphological description. From the osphradial pore, the epithelial canal leads inwards and its epithelium can be divided into three regions (e.g., Nezlin et al., 1994; Townsend, 1973; Wedemeyer & Schild, 1995; Fig. 8.2). In the region closest to the opening of the canal, the epithelium is ciliated, and subepithelial receptor neurons are present. The inner, sometimes bifurcated, part of the canal is covered with secretory cells. The middle, where the canal bifurcates in *L. stagnalis*, consists of both ciliated cells and numerous sensory cell dendrites that terminate at the surface (Kamardin et al., 1998; Nezlin, 1995; Fig. 8.2). The latter is referred to as the sensory epithelium (Wedemeyer & Schild, 1995).

The osphradial canal is surrounded by a layer of muscle cells and an osphradial ganglion that contains the subepithelial receptor neurons, the sensory neurons and other ganglion cells. The few studies that have been performed on these osphradial neurons indicate that several regulatory substances are present (γ-amino-butyric acid/GABA, FMRFamide, serotonin/5HT, methionine-, and leucine-enkephalin; Nezlin & Voronezhskaya, 1997; Nezlin et al., 1994). Besides sensory neurons, serotonin-containing motor neurons are present (Nezlin et al., 1994; Benjamin & Peat, 1971) that have been proposed to control the contractions of the osphradial muscle cells to force the water surrounding the animal in and out of the epithelial canal (Nezlin et al., 1994).

The osphradial nerve leads into the right internal parietal nerve that connects this ganglion to the parietal ganglion of the central nervous system (Crisp, 1973; Nezlin, 1997; Wedemeyer & Schild, 1995; Kamardin et al., 1998; Fig. 8.2). The osphradial nerve runs very superficially under the skin, just behind the female gonopore, and is therefore relatively accessible in an anesthetized snail. Hence, experimental lesioning of this nerve has been instrumental in answering several questions about the function of the

osphradium. For example, osphradectomy (i.e., the denervation or removal of the osphradium) has revealed that the organ is involved in detecting changes in respiratory gas concentrations as well as in chemoreception of organic compounds (Wedemeyer & Schild, 1995). This is also corroborated by neurophysiological experiments demonstrating that the osphradial receptor neurons detect changes in O_2, CO_2, NaCl, KCl, amino acids as well as more complex odor molecules (Wedemeyer & Schild, 1995; Kamardin, 1995). However, whether food cues are also detected via the osphradium remains unclear, especially because studies using an osphradectomy approach in *B. glabrata* have come up with mixed results (e.g., Michelson, 1960; Townsend, 1973). Of course, freshwater pulmonates do locate food successfully but may use different chemosenses (Bovbjerg, 1968). The fact that amino acids are detected by the osphradium does suggest that food detection may play a role as well (at least in *L. stagnalis*: Wedemeyer & Schild, 1995). More relevant for the topic at hand, the osphradium has been shown to pick up chemosensory information from injured conspecifics (Dalesman et al., 2006), low calcium availability (Dalesman et al., 2011) and predators (in the form of kairomones; Dalesman & Lukowiak, 2011). Moreover, such cues have been shown to affect respiration and behavioral responses to food sources and predator presence (Dalesman et al., 2011) and can even interfere with long-term memory formation that is thought to be related to stress (e.g., Karnik et al., 2012).

Besides detecting environmental cues important for survival, detected olfactory information seems to extend to nonaquatic compounds that are known to stimulate olfactory neurons in nonaquatic animals (Wedemeyer & Schild, 1995). All this does indicate that the osphradium is capable of mediating information needed for homeostatic responses as well as real olfactory responses involved in distance chemoreception (Cummins & Wyeth, 2014; Kamardin et al., 1998; Wedemeyer & Schmid, 1995). Whether this extends to detection of information that is relevant for reproductive decisions is less clear (Wedemeyer & Schild, 1995). There are two suggestions indicating that this may be the case. For marine pulmonate gastropods, of the genus *Siphonaria*, it has been suggested that the osphradium could play a role in homing (Kamardin, 1983, 1988). In addition, Townsend (1973) speculated about the osphradium's potential role in pheromone detection in *B. glabrata*.

The ospradium does seem to be involved in the decision to lay eggs, which is partly regulated by the presence of clean water (Ter Maat et al., 1988). It has been shown that spiking activity in the osphradial nerve increases when clean water comes through the epithelial canal, while this activity decreases with dirty water (containing urea; Kamardin et al., 2001).

Since neurons in the osphradial ganglion project all the way into the cerebral ganglia, and given that there are even two bilateral neurons with projections into the osphradial nerve (Nezlin, 1995), it is possible that information about water quality is relayed to the caudo-dorsal cells (CDCs). This latter bilateral cluster of neurosecretory cells, which is responsible for releasing the egg laying hormone and associated peptides that sets ovulation and egg laying in motion, is known to become active after transfer from dirty to clean water (see below, reviewed in Koene, 2010; Ter Maat et al., 1988). Interestingly, osphradectomy increased, rather than reduced egg laying, suggesting that this organ generally has a tonic inhibitory effect on egg laying under the circumstances this was tested (Nezlin, 1997). Nevertheless, the above does reveal that information coming from the osphradium about water quality, which correlates with (un)favorable egg laying conditions, is used for this reproductive decision (Nezlin, 1997; Kamardin et al., 2001).

Albeit admittedly rather speculative, there may also be a connection to the regulation of male mating behavior. The central neuronal network that innervates the penial complex includes many peptidergic neurons that are found clustered in the anterior and ventral lobe of the right cerebral ganglion, the I-cluster of the right pedal ganglion cluster, and dispersed cells in the right pleural and parietal ganglia (De Boer et al., 1997; El Filali et al., 2003, 2015; Smit et al., 1992). Interestingly, the two right parietal neurons that are backfilled via the osphradial nerve seem to be part of the B cells, very close to where the abovementioned parietal dispersed cells are also found (Kamardin, 1995, El Filali et al., 2003, 2015). Some of these latter neurons do have projections in both the penis nerve and the right internal parietal nerve. Whether these connect to the osphradium, or whether the neurons in this B-cluster integrate chemical information from the osphradium into the network regulating male behavior, is unknown, but worth investigating.

8.3.3 CEPHALIC SENSORY ORGANS: LIPS AND TENTACLES

Besides the osphradium, much of the perception of the external world in relation to feeding, homing, aggregation, mating, escape, and avoidance happens via the cephalic sensory organs (e.g., Chase, 2002; Wyeth & Croll, 2011). When referring to the cephalic sensory organs, one simply means the lips and tentacles (Fig. 8.2). These organs are involved in most of the chemosensory and mechanosensory reception of gastropods, meaning that they are also used in both contact and distance chemoreception (Chase, 2002).

Basommatophora have only one set of tentacles with an eye spot at the base of each tentacle. This is in contrast to Stylommatophora, which have two sets of tentacles, the inferior and superior (or cephalic) ones; the latter bearing the eye spots and the chemosensory receptors at their tips. In the Basommatophora, species generally have chemosensory receptors all over the tentacles, but there are exceptions where a specific patch of chemosensory receptors is present at the base of each tentacle (e.g., *Biomphalaria* and *H. trivolvis*: Emery, 1992).

The pair of triangular tentacles of *L. stagnalis* is covered with sensory endings of various types and also contains free nerve endings (Nezlin, 1995; Zaitseva, 1999). Their chemoreceptors respond to food items, their extracts, and amino acids (Bovbjerg, 1968; Zaitseva, 1994), while they do not react to sucrose (Nakamura et al., 1999a,b). Furthermore, experimental work has shown that it is possible that the chemoreceptors in the tentacles are peripheral neurons that either have central projections themselves or have peripheral synapses onto other peripheral neurons that project centrally (Cummins & Wyeth, 2014; Wyeth & Croll, 2011). Investigations into the central projection, which occurs via the left and right tentacle nerves, reveal that many neurons in nearly all ganglia of the central nervous system are innervated (*P. corneus*: Zaitseva, 1999; *L. stagnalis*: Nakamura et al., 1999a,b; Zaitseva & Bocharova, 1981). This clearly indicates that such information is used extensively by many biological functions of the animal (Wyeth & Croll, 2011).

The lips of *L. stagnalis* are innervated by the median and superior lip nerves that originate from both cerebral ganglia (Nakamura et al., 1999a,b). The superior lip nerve mainly innervates the mouth and the dorsal side of the head, while the median lip nerve mainly innervates the lips (Nakamura et al., 1999a,b). Afferent fibers from the lip and mouth area have been shown to not only terminate in the cerebral ganglia, but also in the pleural, parietal, and pedal ganglia (reviewed for *L. stagnalis* in Cummins & Wyeth, 2014; *P. corneus*: Zaitseva, 1999).

The chemosensory neurons in the lips detect sugar, and much of the work on the control of feeding behavior has used this stimulus to unravel the mechanisms underlying feeding (e.g., Ito et al., 2013; Kemenes et al., 1986; Nakamura et al., 1999a,b). Such sugar stimulation seems to be mainly registered via the superior but not the median lip nerves (Nakamura et al., 1999a,b). The median lip nerves may therefore be more involved in mediating signals from mechanoreceptors that are used in the search for food and may be important in detecting contact pheromones.

Four types of peripheral sensory cells could be identified in the lips and tentacles. These include bipolar catecholaminergic, histaminergic, and nitrergic cells with dendrites that penetrate the sensory epithelia. The fourth cell type are unipolar nitrergic cells (Cummins & Wyeth, 2014; Wyeth & Croll, 2011). Information from these sensory cells reaches the central nervous system via the bilateral tentacle and lip nerves. And this information reaches many ganglia of the central nervous system, as evidenced by the extensively branched networks that can be revealed by backfilling these nerves. The fact that the further complexity of the peripheral sensory neuroanatomy has only recently been revealed indicates that there is still lots of scope for discoveries in this area of research, making it currently very difficult to integrate what is known into a comprehensive picture (e.g., Wyeth & Croll, 2011). Finally, it should be noted that much of the above evidence is based on work looking at the control of feeding, using relevant stimuli for this, leaving the field of pheromonal detection via the cephalic sensory organs wide open, as well as how such information might reach the central neurons involved in reproductive processes.

8.4 POSTCOPULATORY SEXUAL SELECTION

Once sperm has been transferred from the donor to the recipient, we enter the realm of postcopulatory sexual selection (Fig. 8.1). At this level of sexual selection it becomes important where the sperm end up and whether they are used for fertilizing the recipient's eggs. As in many species, the receipt of sperm and the fertilization of eggs are decoupled in time (Birkhead et al., 2008; Jarne et al, 2010). This has a number of consequences for what happens to the sperm within the female reproductive tract. For example, sperm need to be stored and kept viable until the moment where they are used for egg fertilization. And, as we have already seen above, these animals receive sperm more than once so the site of sperm storage will contain sperm from several different donors. This already indicates that sperm competition and cryptic female choice are relevant in these animals.

8.4.1 SPERM

Previous paternity analysis work has indeed shown that there is scope for sperm competition in *L. stagnalis* as eggs are fathered by multiple partners

(Koene et al., 2009b; Nakadera et al., submitted). Moreover, sperm from a single sperm donation can be stored and used for 2 months (Nakadera et al., 2014b). Because individuals will usually have mated more than once, one can expect that when sperm from several mating events is stored, such recipients can produce outcrossed eggs for an extended period of time. The latter may be suggested by the work of Cain (1956), who showed that sperm can be used for over 3 months when the number of inseminations is not experimentally controlled for. Similar lengths of sperm storage were reported for two species of the genus *Bulinus*: 70 days for *Bulinus cernicus*, a study in which enzyme variation was used as a genetic marker (Rollinson & Wright, 1984); 123 days for *Bulinus africanus*, a study in which a polymorphism in pigmentation was used as a genetic marker (Rudolph & Bailey, 1985). Several studies, using albinism as a genetic marker (backcrossed or not), have shown that also *B. glabrata* and *P. acuta* store sperm for prolonged periods of time (respectively, Dillon et al., 2005; Vianey-Liaud et al., 1996).

When thinking about sperm storage, it is useful to consider what happens to the sperm right after transfer. The, often large, ejaculate, is received in the vaginal duct right behind the female gonopore (Jarne et al., 2010). Within 3 h, the bulk of the ejaculate has already been transported into the bursa copulatrix, where most of the sperm gets digested within 6 h (Koene et al., 2009b). Only a small proportion makes its way toward the distally placed female organs, where received sperm can be followed up to the seminal vesicles. Beyond that point, the recipient's own sperm is also present, so one needs a specific marker to distinguish auto- from allosperm. The lack of a suitable marker has been the obstacle in determining where allosperm is stored in freshwater snails. Some older work does suggest the ovotestis as the likely place, but the methodology of those studies is not watertight (Lobato Paraense, 1976; Monteiro & Kawano, 2000; see also Koene et al., 2009b). Although no one found a clearly defined sperm storage organ, as is present in most stylommatophorans (see the chapter by Baur & Baur in this volume), the sperm must indeed be stored somewhere, either in or near the seminal vesicles or the ovotestis of the recipient.

Clearly, sperm storage and digestion are important factors that can affect the paternity success that a sperm donor achieves in its partner(s). After all, only the sperm that gets stored has a chance to fertilize eggs. Fertilization takes place in the window of opportunity from the moment that ripe eggs are ovulated until the packaging of the eggs start in the female tract (beyond the carrefour area; Jarne et al., 2010; Koene, 2010).

8.4.2 ACCESSORY GLAND PROTEINS

In addition to sperm, seminal fluid is added to the ejaculate when it passes through the lumen of the prostate gland (Jarne et al., 2010). Such seminal fluid components, which are often referred to as accessory gland proteins, have been shown to play a major role in postcopulatory sexual selection processes in many animals (e.g., Arnqvist & Rowe, 2005; Perry et al., 2013). The presence and use of accessory gland proteins has only been investigated in one hermaphroditic freshwater snail, so far. The prostate gland of *L. stagnalis* has been shown to produce a number of proteins and peptides that are transferred to the partner (Koene et al., 2010). The receipt of one of these, referred to as Ovipostatin or LyAcp10, induces a delay in egg laying of recipients (Koene et al., 2010). This delay seems to result in a higher investment per egg and has been proposed to enhance storage duration and/or fertilization chances of the donated sperm (Hoffer et al., 2012). The latter might occur because egg laying is relatively frequent in this species, and a delay might enable the donated sperm to reach the sperm storage site before a new set of eggs is fertilized and an egg mass is laid.

Very recent work has revealed that two of the identified proteins affect the male function of the recipient, something that is unique to hermaphrodites. These novel proteins cause a snail to transfer half the amount of sperm to its next partner. This sperm-number-reduction effect has been demonstrated both after natural insemination and artificial injection (via the female gonopore) with the isolated protein. Moreover, it has been shown that this reduction in sperm numbers is relevant for paternity success, because such snails achieved less paternity with their recipient (Nakadera et al., 2014a; Schärer 2014). The finding demonstrates that these hermaphrodites directly influence their partner's male reproductive success (called a cross-sex effect: Anthes et al., 2010, see below), while one normally expects males to affect female physiology. This novel insight profoundly affects the way in which one thinks about postcopulatory sexual selection in general (see also Fig. 8.1). Clearly, this now also needs to be further investigated and verified in other hermaphroditic snails as well as other species where males can influence other males.

8.4.3 BEHAVIORAL AVOIDANCE

The foregoing indicates that some of the effects triggered by accessory gland proteins may cause conflict since the recipient's male and female

reproductive output is changed. This could imply that snails may want to avoid being inseminated too often. As mentioned earlier, freshwater snails have several behaviors at their disposal that they can use to avoid being inseminated. The mounted individual can twists its body to make the female gonopore inaccessible (*B. globosus*: Rudolph & Bailey, 1985), and it can vigorously shake its shell and bite its partner (*P. acuta*: DeWitt, 1996). Such behaviors can also be seen as a postcopulatory effect of mating (I discussed this earlier in the precopulatory context of mate choice). This could occur because individuals may try to avoid being inseminated again to avoid the costs of reduced egg laying and paternity, induced by the received accessory gland proteins. Alternatively, it could be the accessory gland proteins that induce such behavior, to make sure that the mating partner does not initiate a mating with another sperm donor (thus decreasing the initial sperm donor's chances on fertilization), or with a sperm recipient (thus saving energy for egg production, by not investing in an ejaculate and male behavior).

These ideas have not received much attention yet, but one recent study indicates that male motivation is not affected by the receipt of accessory gland proteins (Nakadera et al., 2015). This is in agreement with the fact that sperm donors, even after being inseminate themselves, tend to be eager to inseminate a novel partner (Koene & Ter Maat, 2007). However, female motivation may well be lower after recent insemination. Given the already-known effects of accessory gland proteins on the recipient (e.g., Koene et al., 2010; Nakadera et al., 2014a), one expectation could be that behavioral avoidance of surplus matings by the female-acting individual will especially be observed when a certain level of (specific) accessory gland proteins has been reached.

8.5 PHYSIOLOGY OF POSTCOPULATORY PROCESSES

As becomes evident from the preceding section, there are numerous processes that are being coordinated during and after the receipt of an ejaculate. This is a field of research that has only emerged relatively recently, especially when considering research on hermaphrodites, so some of the following will be rather speculative. Nevertheless, because a lot is known about the physiological regulation of many of the male and female reproductive processes that are involved, some tentative links can be made.

The identified accessory gland proteins, which have been shown to immediately affect postcopulatory processes, are the best place to start (see above; Koene et al., 2010; Nakadera et al., 2014a). For example, for the delay in

egg laying that ovipostatin (LyAcp10) causes, one prediction could be that this directly, or indirectly, affects the excitability of the main neuroendocrine center that controls egg laying: the bilateral CDC cluster in the cerebral ganglia. While the CDC system has been described in detail in *L. stagnalis* (e.g., Ter Maat et al., 1983, see below), morphologically similar cells have been described in a number of other freshwater species (*Stagnicola palustris*, *Radix ovata*, *B. truncatus*, *Planorbis planorbis*, *Planorbis vortex*, *P. corneus*, and *B. glabrata*: Boer et al., 1977; Roubos & Van der Ven, 1987).

That these cells are in control of egg laying was initially shown by cauterizing them, which resulted in an elimination of egg laying (Geraerts & Bohlken, 1976). Because they are electrically coupled, and send axons through the cerebral commissure to the contralateral cell cluster, these cells show a synchronous, long-lasting discharge (respectively, De Vlieger et al., 1980; Ter Maat et al., 1986). These cells also project into the neurohemal area of the cerebral commissure (De Vlieger et al., 1980; Joosse, 1964; Roubos, 1976; Wendelaar Bonga, 1971), where they release their neurosecretory products (e.g., Dogterom & Van Loenhout, 1983; Geraerts & Bohlken, 1976). These neuropeptides are all encoded on the CDCH-gene (Ferguson et al., 1993), named after the key egg laying hormone CDCH, because the injection of this hormone alone triggers egg laying (Ter Maat et al., 1987; Fig. 8.2).

This egg laying process seems to be influenced by accessory gland proteins, like ovipostatin. There are different ways in which this could work. First, this could be achieved by the protein being taken up into the blood of the recipient and directly targeting the activity of the CDCs. Second, peripheral receptors within the female reproductive tract may relay information relevant to egg laying to the CDCs. Ovipostatin could then either be a protein that serves as a cue telling the female system to delay egg laying because the received ejaculate needs to be processed, or it could act as a manipulative agent "hijacking" receptors that are normally used for the regulation of egg laying. Clearly, to find out it will be necessary to identify the receptor on which ovipostatin acts as well as its site of action.

As with the female system, the accessory gland proteins (LyAcp5 and 8b) that have been shown to influence the male function of the recipient could also act via different routes. For example, given the decrease in sperm numbers, the release of ripe sperm from the seminal vesicles may be targeted. Alternatively, the transport of the ejaculate could be slowed down. This transport is controlled by the peristalsis of the vas deferens, which pumps the ejaculate across during copulation. The transport process has been proposed to be caused by the antagonizing effects of the neuropeptides APGWamide

and conopressin (Van Golen et al., 1995). But there is a whole legion of other neuropeptides that is involved in the regulation of the male function, so it remains to be investigated how accessory gland proteins bring about their immediate sperm-number-reducing effect (Koene, 2010; Nakadera et al., 2014b).

Finally, although this remains to be investigated, accessory gland proteins may also target other processes in the recipients, such as allosperm storage and digestion, the recipient's male or female behavior during mating interactions, as well as sex allocation. The latter, which is defined as the allocation toward the male and female reproductive function (Charnov, 1982; Schärer, 2009; Koene, 2016), could potentially also explain some of the effect that accessory gland proteins have in *L. stagnalis*. While one generally measures effects on reproductive output in a specific sex function, it should not be forgotten that these are simultaneous hermaphrodites that perform both sexual functions. Therefore, assuming a fixed reproductive budget, a decrease in one sex function may actually result in an increase in the other (Charnov, 1979; Schärer, 2009). Hence, rather than targeting specific male or female processes, these accessory gland proteins could also be targeting the sex allocation decision of the recipient. In other words, by reducing the amount of sperm transferred, less new sperm will need to be produced and these left-over resources can be put into egg laying (either by increasing the total number of eggs produced or by increasing the quality and/or size of eggs; see also Schärer, 2014). The latter has been suggested to be the case in *L. stagnalis*, where repeated receipt of ejaculates increases the investment per egg (Hoffer et al., 2012). This is also in agreement with previous work showing that this species is very flexible in its resource investment (Koene et al., 2009a; Van Duivenboden et al., 1985). But while it has been shown that the elimination of the male function, by lesioning the aforementioned np1-nerve, leads to double the amount of eggs laid (De Visser et al., 1994; Koene et al., 2009; Hoffer et al., 2010), it remains unknown how such allocation decisions are executed by the central nervous system (Koene, 2010).

8.6 CONCLUSIONS AND FUTURE DIRECTIONS

As becomes clear from the presented overview, there is lots of scope for investigating ultimate and proximate aspects of pre- and postcopulatory sexual selection processes. For a better understanding of precopulatory sexual selection it will be highly relevant to understand how potential mating partners are detected and evaluated. For postcopulatory selection, it

is especially pertinent to elucidate sperm storage and the physiology underlying effects of accessory gland proteins. To conclude this chapter, I will briefly discuss a couple of directions of research that I think will be particularly fruitful and should therefore be encouraged.

The recent literature has highlighted the relative importance of postcopulatory sexual selection for hermaphrodites (Schärer & Pen, 2013; Nakadera & Koene, 2013). However, it should be pointed out that this is partly influenced by the absence of visible sexual dimorphism, in either size or specific secondary sexual traits (ornaments, armaments) in simultaneous hermaphrodites (Pélissié et al., 2012). This is the case by definition, because many visible hermaphroditic traits are shared by the male and female function (shared-cost traits), such as body size or tentacle length. Therefore, there seems to be little scope for sex-biased traits (Schärer & Pen, 2013; Nakadera & Koene, 2013). Nevertheless, a large part of this chapter has dealt with issues relating to precopulatory sexual selection. Hence, mate choice based on chemical cues deserves more attention, and may reveal that this is where we should be looking for sex-biased traits.

While I did point out that there is scope for mate choice based on chemical cues, the link with the organs detecting these still needs to be made satisfyingly. The main players are the osphradium, lips and tentacles. What is now required is that the relevant pheromones and their chemoreceptors are identified and localized (Cummins & Wyeth, 2014). For this, the ongoing genome projects on *L. stagnalis* and *B. glabrata* will be very valuable, as well as the already published transcriptome data (e.g., Bouétard et al., 2012; Davison & Blaxter, 2004; Feng et al., 2009; Hathaway et al., 2010; Sadamoto et al., 2012). Eventually, the identification of pheromones and chemoreceptors will help to elucidate how such information is processed in the central nervous system and how this influences mate choice. As also pointed out by Cummins and Wyeth (2014), understanding the chemosenses of gastropods might even lead to insights that can be used to our benefit in aquaculture and pest control.

The investigation of mate choice in simultaneous hermaphrodites can also be greatly facilitated by making use of some of the features of hermaphroditic freshwater snails. Besides their amenability to many different experimental approaches, one other advantage of these snails over many other hermaphroditic taxa, is that sperm donation usually occurs (or can occur) unilaterally. Hence, within one mating interaction there is one individual that donates sperm to the other (sexual roles can often be swapped afterward: Koene & Ter Maat, 2005). This allows for clear (experimental) separation of the male and female function, which is often impossible in hermaphrodites that exchange sperm simultaneously, which can be instrumental when investigating pre- and/or postcopulatory processes.

Previous work has already shown that knowledge about the underlying mechanism that determines male motivation, can be used to eliminate the male function in *L. stagnalis*. When this is done, enough reproductive resources become available in the operated animal to produce twice as many eggs (De Visser et al., 1994; Hoffer et al., 2010). On the one hand, this indicates that either the male function spends a lot of resources on searching mates and performing the mating behavior, or on the other hand, the production of the ejaculate itself is just as costly as egg production. Both aspects are worth investigating in more detail by measuring, respectively, respiration during mating trials and caloric values of ejaculates. The latter will also be informative for understanding the evolution of sperm digestion, since it is generally assumed that some of the received energy can be reused by the recipient (Greef & Michiels, 1999; Yamaguchi et al., 2012).

Besides sperm digestion, the issue of sperm storage also needs resolving. As indicated, the most likely places for sperm storage to take place seem to be the seminal vesicles or ovotestis (Koene et al., 2009b; check Jarne et al., 2010). To unequivocally uncover the site of sperm storage, it will be necessary to track donated (allo)sperm in recipients. Specific labeling will be necessary for this, given that the recipient's own sperm is also produced and stored there (Kupfernagel et al., 2013; Schärer et al., 2004, 2007). Once the site of sperm storage is identified, it will be possible to establish how sperm of different partners is being stored and whether more sperm are stored under influence of accessory gland proteins.

As outlined, these accessory gland proteins, together with sperm storage and digestion processes, are of prime importance for understanding postcopulatory sexual selection. The earlier mentioned genome and transcriptome work, in combination with functional studies (e.g., knock-outs/-downs, RNAi, transgenics and CRISPR genome editing), will also be instrumental for obtaining a more complete picture about the workings of accessory gland proteins. The established artificial insemination method, where individual proteins can be tested for activity, will also be instrumental here (Van Iersel et al., 2014). Clear expectations are that they have direct effects on processes that are known to be involved in the neurobiological regulation of male and female reproductive biology (see above and Koene, 2010). However, this may also uncover effects on more general signaling pathways involved in immune or stress responses (e.g., stress-activated protein kinases) as well as evolutionary differences between species.

Another clear expectation would be that such an approach will uncover molecular evidence for cross-sex effects. As pointed out by Anthes et al. (2010), aside from mating success in the male role affecting an individual's

male reproductive success, it can also affect its female reproductive success, and vice versa (Fig. 8.1). These interactions between the sexual functions of the individual are referred to as cross-sex effects (Anthes et al., 2010; Pélissié et al., 2012, 2014). A point in case is the observation that receipt of accessory gland proteins does not only affect regulation of the female function, but also the recipient's male function. If this can be backed up by molecular data, this would provide a strong mechanistic underpinning of the predicted cross-sex effects between mating success and reproductive success in hermaphrodites.

In sum, I have pointed out that by combining proximate approaches looking at (neuro)physiology and molecular biology with ultimate approaches looking at behavioral mating decisions and reproductive investment, much can be learned about sexual selection processes in freshwater snails. Moreover, I fully expect that such integration across different levels of biology will lead to novel insights that pertain to the evolution of hermaphroditism in general. Additionally, by focusing on hermaphrodites, the validity of the theory of sexual selection is put to the test by investigating its general assumptions and predictions.

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KEYWORDS

- **accessory gland product**
- **allohormone**
- **egg production**
- **freshwater snails**
- **mating**

- **mate choice**
- **natural selection**
- **pheromone**
- **reproduction**
- **sexual conflict**
- **sexual selection**
- **sperm competition**
- **selfing**

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CHAPTER 9

REPRODUCTIVE STRATEGIES IN STYLOMMATOPHORAN GASTROPODS

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CONTENTS

ABSTRACT

In most sexually reproducing species, males and females follow different strategies to maximize their reproductive success. Sex-specific strategies evolved because males can increase their reproductive success by mating frequently and promiscuously, whereas females benefit from being selective. Simultaneously hermaphroditic animals produce both eggs and sperm at the same time but the reproductive investment in the two sexual functions might be asymmetric. This may lead to a conflict of interests in hermaphrodites because the male and female functions retain their separate interests even though they are united in the same individual. Consequently, in simultaneous hermaphrodites selection on male reproductive traits also influences female traits and vice-versa. This chapter reviews reproductive strategies in stylommatophoran gastropods, a highly diverse group of molluscs exceeding 30,000 species, all of them being hermaphrodites. Stylommatophoran species show diverse and very complex reproductive organs, bizarre mating behaviors and variable breeding systems including cross-fertilization, self-fertilization, and a mixture of both. Stylommatophoran gastropods also developed a variety of morphological structures and behaviors such as egg cannibalism, egg retention and ovoviviparity to increase offspring survival in quite different environments. In stylommatophoran gastropods, almost all reproductive characters appear to be shaped by both natural and sexual selection, and there is increasing evidence that intersexual counter-adaptations may drive correlated reproductive character evolution in this group. Behaviors and traits to enhance survival of both eggs and hatchlings—the other important component of reproductive strategies—are shaped by natural selection.

9.1 REPRODUCTIVE STRATEGIES

Reproductive strategies are structural, functional, and behavioral adaptations that improve the chances of fertilization and/or increase the number and survival of offspring under given local conditions. In most sexually reproducing species, males and females follow different strategies to maximize their reproductive success, which is defined as the number of offspring produced that survive to adulthood (i.e., next generation). Reproductive success can be subdivided into different components: mating success, fecundity, reproductive life span, and offspring survival to breeding age

(Clutton-Brook, 1988). Sex-specific strategies evolved because males can increase their reproductive success by mating frequently and promiscuously, whereas females prefer to be selective (Birkhead & Møller, 1998). In general, the production of sperm is less costly than the production of eggs, resulting in different strategies (Bateman, 1948; Trivers, 1972). Different strategies may also be used after insemination because each male is interested in having all his sperm survive so that a maximum number can fertilize eggs. The female, however, may benefit from mating with other males resulting in genetically more diverse offspring and attracting more resources from different males.

Sexual conflicts exist in hermaphrodites because the male and female functions retain their separate interests even though they are united in the same individual (Michiels, 1998). The male function can increase its fitness by mating frequently and promiscuously, whereas the female function might be selective with respect to mating partners and their sperm (Michiels, 1998). Furthermore, it is important to note that in simultaneous hermaphrodites selection on male reproductive traits also influences female traits and vice versa.

Stylommatophoran gastropods are a large and highly diverse group, probably exceeding 30,000 species (Solem, 1984), all of them being hermaphrodites (Heller, 1993). They occur in a wide variety of terrestrial habitats such as river embankments, grasslands, soil, leaf litter in forest, exposed cliff walls, stone deserts, decaying wood and trees (Heller, 2001). Stylommatophoran species show diverse and very complex reproductive organs (e.g., allosperm-storage organ or spermatheca, sperm-digesting organ, dart-sac, penial appendages) and bizarre mating behaviors (e.g., dart-shooting, aerial mating with sperm exchange at the tips of extremely extended penises, apophallation), and variable breeding systems (i.e., cross-fertilization, self-fertilization, or a mixture of both) (Baur, 1998, 2010; Duncan, 1975; Jordaens et al., 2007; Leonard, 2006; Tompa, 1984). Furthermore, in stylommatophoran gastropods, a variety of morphological structures and behaviors have been developed to increase offspring survival in quite different environments (e.g., egg cannibalism, egg retention, ovoviviparity) (Baur, 1994a; Tompa, 1984).

There is abundant information on the morphology of reproductive organs in the literature, primarily as a result of systematic studies (for reviews see Barker, 2001; Duncan, 1975; Gomez, 2001; Luchtel et al., 1997; Nordsieck, 1985; Runham, 1988; Tompa, 1984). However, detailed functional information is restricted to a few model species and thus not representative of the

phylogenetic diversity in this animal group. Various aspects of reproduction and other life-history traits in stylommatophoran gastropods have been reviewed by Tompa (1984), South (1992), Baur (1998, 2010), and Jordaens et al. (2007). Nakadera and Koene (2013) summarized conceptual and empirical approaches of reproductive strategies in pulmonate gastropods, focusing on sexually selected traits.

We review characters and behaviors that determine reproductive success, and reflect on, for which of these characters and selective events stylommatophoran gastropods ought to serve as particularly suitable research models for simultaneous hermaphrodites. We apply evidence to current theory, and in doing so, we uncovered huge gaps in empirical studies. In this group of hermaphrodites several reproductive traits appear to be shaped by both natural and sexual selection. Phylogenetic analyses provide evidence for several cases of co-evolution between male and female reproductive traits. Many of the aspects are intimately interrelated which makes it difficult to consider them separately. Hence, the subdivision given below is partly artificial and other subdivisions may prove equally justified. By compiling the existing information, this review should stimulate future studies on reproductive strategies in terrestrial gastropods.

9.2 GENITAL MORPHOLOGY AND FUNCTION

Stylommatophoran gastropods exhibit a great diversity in their reproductive system, reflecting their phylogeny (Barker, 2001). The different functions of the reproductive system include: (1) production of ova and sperm; (2) storage and transport of mature gametes in a suitable medium; (3) structural and physiological roles in courtship and copulation; (4) transfer of endogenous sperm (autosperm) to the mating partner's reproductive duct; (5) reception and (long-term) storage of exogenous sperm (allosperm); (6) supplying a site and proper medium for fertilization of ova; (7) covering the zygote with nutritive and protective layers; (8) oviposition; and (9) resorption of remnant and excess sperm and other reproductive products (Baur, 2010; Gomez, 2001).

The terminology of the morphology of the gastropod reproductive tract is often confusing, partly due to the use of the same term for different structures. For simplicity, we use descriptive terms throughout this chapter (Fig. 9.1).

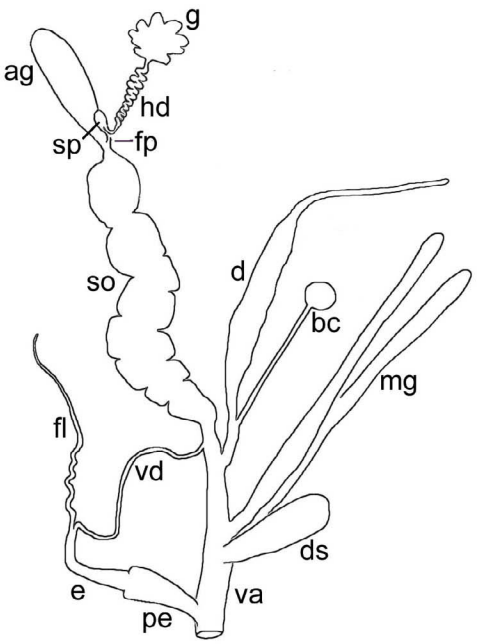

FIGURE 9.1 Schematic drawing of the reproductive morphology of *Arianta arbustorum*, a stylommatophoran gastropod with one dart and a diverticulum. ag, Albumen gland; bc, bursa copulatrix; d, diverticulum; ds, dart sac; e, epiphallus; fl, penial flagellum; fp, fertilization pouch; g, gonad; hd, hermaphroditic duct; mg, mucous glands; pe, penis; so, spermoviduct; sp, spermatheca; va, vagina; vd, vas deferens. The carrefour consists of the spermatheca and the fertilization pouch. Not to scale. (From Chapter 10: Stylommatophoran Gastropods, by Bruno Baur in Evolution of Primary Sexual Characters in Animals, edited by Edited by Janet Leonard and Alex Cordoba-Aguilar, 2010. Reprinted by permission of Oxford University Press, USA.)

9.2.1 GONAD AND GONODUCT

Stylommatophorans have only a single gonad, the ovotestis, which produces both oocytes and spermatozoa (Fig. 9.1). The gonad, located among the lobes of the digestive gland toward the posterior part of the body, consists of numerous acini containing both male and female germ cells (South, 1992). In most species, the male germ cells differentiate and mature earlier in the life cycle than the female germ cells (Duncan, 1975; Luchtel et al., 1997; see Section 9.3.2). The ovotestis opens to a gonoduct (= hermaphrodite duct). When released, both male and female gametes pass along the hermaphroditic duct. Thereafter, they follow separate paths (Fig. 9.1). The hermaphrodite duct varies in complexity between higher taxa (Duncan, 1975; Luchtel et al., 1997).

In many species, autosperm are stored in the seminal vesicle of the hermaphrodite duct throughout the year (Lind, 1973). Phagocytosis of autosperm by the hermaphrodite duct epithelium has been reported in *Helix pomatia* and *Oxychilus cellarius* (Rigby, 1963). Sperm can be expelled from the hermaphrodite duct at times other than copulation to be eventually digested (as are allosperm) in the bursa copulatrix (see Section 9.2.7).

9.2.2 CARREFOUR

In stylommatophoran gastropods, the carrefour includes structures for allosperm storage (the spermatheca or female sperm-storage organ), and for oocyte fertilization and the coating of zygotes with the albumen layer (Gomez, 2001). The fertilization of oocytes occurs in a specialized region of the carrefour, which has the form of a pouch in most species. Secretory cells occur in the walls of the fertilization chamber; their secretions are thought to provide a medium for gamete fusion (Gomez et al., 1991).

Sperm received (allosperm) travel through the spermoviduct to the spermatheca (Fig. 9.1). They reach the carrefour within 4 h of copulation in the slug *Deroceras reticulatum* (Runham & Hogg, 1992). In *H. pomatia* and *Cornu aspersum* (formerly *Helix aspersa*), only 0.02–0.1% of the allosperm transferred reach the storage organ; the majority of them within 12 h of copulation (Lind, 1973; Rogers & Chase, 2001). In the spermatheca, allosperm can be stored for long periods. Viable allosperm have been found up to 4 years after the last copulation in the sperm-storage organ of stylommatophoran gastropods (see Section 9.6.1).

There is an enormous variability in the structure and morphology of the carrefour in stylommatophorans. For example, the carrefour is not divided into separate spermatheca and fertilization chamber in the South African dorcasid *Trigonephrus gypsinus* (Brinders & Sirgel, 1992). *Oxychilus draparnaudi* and *Bradybaena fruticum* have a single spermathecal tubule beside the fertilization chamber (Bojat et al., 2001a; Flasar, 1967). In *Succinea putris*, 2 spermathecal tubules occur (Rigby, 1965), and 34 tubules have been recorded in the spermatheca of the South American bulimulid *Drymaeus papyraceus* (van Mol, 1971). There is also a considerable within-species variation in the number of spermathecal tubules (e.g., 3–5 in *H. pomatia*, Lind, 1973; 2–9 in *Arianta arbustorum*, Baminger & Haase, 1999; Haase & Baur, 1995; 4–19 in *C. aspersum*, Chase & Darbyson, 2008; Koemtzopoulos & Staikou, 2007).

The blind-ending tubules unite to a common duct, which opens into the fertilization chamber. In *A. arbustorum*, the musculature surrounding the spermathecal tubules is arranged in a complex three-dimensional network (Bojat et al., 2001bc). If there were a selective activation of the muscles of each tubule (which has not yet been examined), this would allow the animal to expel sperm stored in single tubules and thus promotes a selective fertilization of eggs. The ciliation of the common duct is probably responsible for the distribution of incoming sperm among the tubules. The spermatheca is expandable and can accommodate more sperm than would be expected from the initial volume (Beese & Baur, 2006).

As a consequence of the large intraspecific variation in the number of spermathecal tubules, different individuals might have different possibilities to store allosperm from more than one mating partner. Mixing of sperm from different mates would be more likely in a less structured spermatheca, whereas a large number of tubules would allow better separation of spermatozoa from different mates. Baminger and Haase (1999) examined whether the variation in number of spermathecal tubules and the amount of allosperm stored are influenced by the risk of sperm competition, as indicated by the local density of adult *A. arbustorum* in six natural populations in the Eastern Alps, Austria. The number of spermathecal tubules ranged from two to nine. However, snails from the six populations did not differ in either the mean number of spermathecal tubules or the cumulative length of the tubules. Individuals from different populations did not differ in the amount of sperm stored, neither was the amount of sperm stored correlated with population density. Similarly, no correlation was found between the number of spermathecal tubules or the cumulative length of all tubules and the local density of five *C. aspersum* populations in Greece (Koemtzopoulos & Staikou, 2007). This suggests that the risk of sperm competition does not affect the number of spermathecal tubules.

A variety of adaptive explanations have been proposed to explain the diversity of female sperm-storage organs (Beese et al., 2009). One hypothesis claims that the differentiation of sperm-storage organs is dictated by demands of sperm storage capacity arising from differences in animal longevity and/or egg productivity, or by selection for functional design to match sperm morphology in order to efficiently store and utilize sperm (Pitnick et al., 1999). Females, which live long or produce multiple clutches in consecutive years, may require more specialized organs to provide nourishment or protection (e.g., through anchoring the sperm inside the storage organ) to maintain the viability of sperm (Smith & Yanagimachi, 1990). Consequently, the evolution of sperm-storage organs should be coupled

with life history. Comparing six populations of *A. arbustorum*, Beese et al. (2006a) found a correlation between mean spermatheca volume and the average number of sperm transferred. This suggests that post-copulatory mechanisms drive a correlated evolution between sperm characteristics and female reproductive traits in hermaphroditic gastropods.

The evolution of female morphology may also simply track sperm length that evolves due to selection independent of female sperm stores (Pitnick et al., 1999), which might result in evolutionary correlations between the length of sperm-storage organs and sperm length documented in several gonochoristic animals with internal fertilization (Presgraves et al., 1999). A similar pattern of correlated morphological evolution between sperm length and carrefour length was found in a comparative analysis of 17 stylommatophoran gastropods (Beese et al., 2009).

Another widely supported hypothesis is that postcopulatory sexual selection has played an important role in the evolution of this trait, due to the potential influence of female sperm stores on the extent of nonrandom paternity (Eberhard, 1996). A prerequisite for sexual selection via sperm competition is that the sperm of two or more males coexist within the reproductive tract of the female at the time of fertilization (Parker, 1970). In the past few years, increasing attention has been paid to the possibility that females of many gonochoristic species (insects, birds, and mammals) are active not only in precopulatory choice, but also in controlling the processes of sperm storage and use (Birkhead & Møller, 1998; Eberhard, 1996; see Section 9.6.1). The presence of storage organs may allow females to maintain viable sperm from multiple mates and thus selectively bias the fertilization success of sperm in relation to male behavior (Siva-Jothi & Hooper, 1995) or male genotype (Ward, 1998). The presence of female sperm-storage organs should therefore be linked with the presence of complex or peculiar reproductive traits. Moreover, diverse mating systems that impose different levels of selection pressure on postcopulatory processes are expected to covary with the presence of sperm-storage organs and their complexity.

Beese et al. (2009) examined morphologically the presence and complexity of the spermathecae in the carrefour in 47 species of stylommatophoran gastropods and used partial 28 rDNA sequences to reconstruct a molecular phylogeny for these species. The phylogenetic reconstruction supported several gains and losses of the spermathecae in stylommatophorans indicating rapid evolutionary changes, which could have gone jointly with explosive radiations of families during Mesozoic and the Late Cretaceous/Early Tertiary. Moreover, a complex spermatheca was associated with the occurrence of love darts or any kind of auxiliary copulatory

organ (see Section 9.2.8), the presence of a long flagellum at the penis and cross-fertilization as the predominant mating system. However, the results of Beese et al. (2009) also suggest associations of carrefour complexity with body size, reproductive strategy (semelparity vs. iteroparity), reproductive mode (oviparity vs. ovoviviparity), and with habitat type.

9.2.3 ALBUMEN GLAND

The albumen gland is a compound tubular gland that produces albumen or perivitelline fluid for the egg. Secretory cells in this gland contain large amounts of galactogen (Duncan, 1975). In stylommatophorans, the gland increases in size with sexual maturation of the animal (Gomez, 2001). The number of eggs that can be produced at any one time appears to depend on the size of the albumen gland (Tompa, 1984). Protein concentration of eggs deposited varies among species and can change in successive egg batches produced by an individual snail (see Section 9.10.1).

9.2.4 SPERMOVIDUCT, FREE OVIDUCT, AND VAGINA

In stylommatophoran gastropods, the male and female gametes follow separate pathways from the carrefour along a common duct (=spermoviduct), and separate male and female ducts then diverge at the distal end of the spermoviduct. In many species, the lumen of the spermoviduct is incompletely divided into two grooves (Luchtel et al., 1997). These grooves are lined by ciliated and secretory cells and are unequal in size, with a larger female groove and a narrower male groove (South, 1992). The female groove produces supporting layers of the eggs, while the male groove produces seminal fluid (Gomez, 2001). As the egg descends along the oviductal channel, the perivitelline membrane, the jelly or organic matrix of the inner egg cover, and the outer egg cover are sequentially deposited (Bayne, 1968). In species with heavily calcified egg shells, the calcium content of the outer egg cover increases gradually as the egg passes along the oviductal channel (Tompa, 1984). In ovoviviparous species (see Section 9.10.3), the distal portion of the oviductal gland, together with the adjoining free oviduct, functions as a uterus for brooding of young after their hatching from eggs retained in the female duct (Szybiak & Gabala, 2013; Tompa, 1979a). Both the free oviduct and vagina have a thick muscular wall, which may be attributed to their role in copulation and oviposition (Gomez, 2001).

9.2.5 VAS DEFERENS, EPIPHALLUS, AND SPERMATOPHORE

The vas deferens is a ciliated, narrow, and partly folded duct, which functions to transport autosperm (Fig. 9.1). Peristalsis in the wall, together with ciliary action, contributes to the movement of seminal fluids along the duct, including their expulsion during mating (Runham, 1988). When present, the epiphallus is usually a highly muscular organ, with the lumen larger and more folded than the vas deferens lumen. In a variety of stylommatophoran species, the spermatophore is formed by the epiphallus (head filament and sperm container) and flagellum (=epiphallic caecum; tail) during copulation. The spermatophore is largely composed of secretory material containing glycosaminoglycans and mucoproteins (Mann, 1984). In *Arianta arbustorum*, spermatophore formation is initiated more or less synchronously in mating partners a few minutes after penis intromission (Baminger & Haase, 2001). The part of the spermatophore that contains the sperm increases in size until shortly before the spermatophore is transferred (approximately 90 min after penis intromission). Growth and final size of the spermatophore, however, are not adjusted between the mating partners (Baminger & Haase, 2001).

There is evidence that morphological characters can influence fertilization success. Garefalaki et al. (2010) found that the length of the epiphallus relative to the length of the spermatophore-receiving organ (see Section 9.2.7) of the mating partner was positively correlated with fertilization success in *C. aspersum*. Similarly, longer spermatophores of *Euhadra peliomphala* required more time to be transported to the gametolytic organ (see Section 9.2.7) than shorter spermatophores (Kimura & Chiba, 2013). Consequently, in long spermatophores an increased number of sperm may escape digestion and reach the sperm-storage organ (spermatheca) of the recipient.

Spermatophores have a species-specific shape and surface structure, which is of taxonomical significance (Baur, 1998; Mann, 1984). Spermatophores may be smooth, elaborately spined, calcified or uncalcified (Tompa, 1984). In *H. pomatia*, the spermatophore is 6–8-cm long and consists of a distinctive tip, a body (sperm container), and a long tail (Meisenheimer, 1907). In species with spermatophores, snails form a single spermatophore at each mating and exchange it reciprocally in most cases.

The adaptive significance of the spermatophore in stylommatophorans with well-developed copulation organs and internal fertilization is unclear. In *H. pomatia*, sperm leave the spermatophore body through the spermatophore tail in the stalk of the bursa copulatrix and migrate into the spermatheca (Lind, 1973). The spermatophore and any remaining sperm are digested later

in the bursa copulatrix. Lind (1973) suggested that the function of the spermatophore is to ensure that a number of sperm can migrate into the oviduct and reach the spermatheca without coming into contact with the digesting bursa copulatrix (see below). The significance of this way of sperm transfer may be to allow only the most active sperm to pass to the spermatheca and thus can be considered as a means to mitigate sperm selection of the recipient (which still might occur in the spermatheca).

9.2.6 PENIS

Penial morphology of the Stylommatophora is highly variable and species-specific (Barker, 2001; Reise, 2007). It has been suggested that the penis is the prime species recognition character during mating and in determining copulation success (Gomez, 2001).

The penis of stylommatophorans is a muscular organ that is everted at copulation and is typically inserted into the genital atrium and vagina of the mate. The basic structure of the penis is a tube consisting of a non-ciliated, nonglandular epithelium, surrounded by a thick muscular wall with inner circular and outer longitudinal layers (Gomez, 2001). The contraction of the penis wall affects the hydrostatic pressure necessary to evert the penis, while a muscle affects retraction. The epithelium lining the lumen of the penis is often folded transversely, and several types of raised ridges or folds, known as pilasters and spines, may function as stimulator or holdfast surfaces during copulation (Baur, 1998). Special glandular regions can be present in the penis wall of some species. For example, the appending penial gland of slugs of the genus *Deroceras* consists of one or more finger-like appendages located at the end of the penis. In *Deroceras gorgonium*, the penial gland is particularly large, consisting of a huge bundle of branched processes (Reise et al., 2007). In most *Deroceras* species, the appending glands are everted during copulation and spread on the partner's upper body wall transferring a secretion (Reise et al., 2007). The gland of *D. gorgonium*, however, was also spread underneath the partner's body. Reise et al. (2007) suggested that this secretion has a similar manipulative function as that involved in dart shooting (see Section 9.2.8). Benke et al. (2010) observed that individuals of *Deroceras panormitanum* tried to lick the received secretion off their own body and suggested that the external application of this gandular substance could function as either a pheromone or allohormone.

In those stylommatophoran species that have a penis, the animals insert it simultaneously or sequentially into the partners' genital pore during mating.

Some stylommatophorans, however, have external sperm exchange, by which sperm are deposited on the mate's everted penis without intromission (e.g., in some species of Succineidae, Polygyridae, Helicodiscidae, Limacidae, and Agriolimacidae; see Emberton, 1994; Jordaens et al., 2009; Reise, 2007; South, 1992).

Several slug species exhibit spectacular aerial matings (Chace, 1952; Falkner, 1992; Gerhardt, 1933). Copulating pairs are hanging on thick mucus ropes suspended from trees or vertical walls with everted penises. The penises entwine and exchange sperm at their tips, completely outside of the body. In these species, the penis is often remarkably long in relation to body length. For example, the uncoiled penis reaches a length of 60 cm in the 12–15 cm long slug *Limax corsicus* and 85 cm in the 13–15 cm long *Limax redii* (Falkner, 1990). In other species with external sperm exchange, courtship and mating are usually performed on horizontal surfaces such as old leaves. The mating partners are side by side and exchange sperm masses from penis to penis, for example, in *Deroceras rodnae* (Reise, 1995).

Other stylommatophoran species, especially certain slugs (e.g., *Ariunculus isselii*, several *Arion* species; Hutchinson & Reise, 2015), normally lack penises altogether; mating in these species may be accomplished by pressing the genital pores together directly, or by using nonhomologous penis-like structures (derived from other parts of the terminal genitalia) for sperm transfer (Tompa, 1984).

9.2.7 DIVERTICULUM, BURSA COPULATRIX, AND PENIAL FLAGELLUM

The bursa tract diverticulum, when present, is a blind-ended tube, which is especially long in several species (e.g., in *Eobania vermiculata*; Tompa, 1984). As a lateral continuation of the lower part of the bursa duct, the diverticulum is widespread in stylommatophoran species and apparently plesiomorphic (Barker, 2001; Koene & Schulenburg, 2005). The diverticulum is specifically positioned relative to the bursa duct opening (Fig. 9.1). During mating, it functions as the site of spermatophore uptake (Barker, 2001). Within the lumen of the diverticulum of *Arianta arbustorum*, the spermatophore wall is dissolved or at least partly broken down. The digested material is taken up by epithelial cells and accumulated in cells of the connective tissue (Beese et al., 2006b). In *H. pomatia*, the length of the diverticulum is highly variable; in some individuals, it may even be reduced or entirely lacking (Hochpoechler & Kothbauer, 1979). In a sample of 79 individuals of

H. pomatia, 27 (34%) had no diverticulum, while in the remaining snails its length varied from 1 mm to 9 mm (van Osselaer & Tursch, 2000).

The bursa copulatrix (=gametolytic gland; Tompa, 1984) is a sacculate reservoir, commonly connected to the female reproductive system via a thin duct. Sperm received (allosperm) travel up the spermoviduct to reach the spermatheca, where they are stored until fertilization (Lind, 1973). The vast majority of allosperm (99.98% in *C. aspersum*; Rogers & Chase, 2001), however, is transferred into the bursa copulatrix. The function of the bursa copulatrix is the extracellular digestion and subsequent resorption of excess gametes (primary allosperm) and other reproductive products, such as secretions from the albumen gland, oviductal glands, seminal channel, and remnants of the spermatophore (Beese et al., 2006b; Gomez et al., 1991). The stalk of the bursa copulatrix can exhibit strong peristaltic waves toward to bursa (Lind, 1973).

In snail species without a diverticulum, the spermatophore is directly deposited in the digesting bursa copulatrix (Barker, 2001). Until the spermatophore wall is dissolved, spermatozoa have the chance to excape through the spermatophore tail and to move into the spermatheca. In stylommatophoran species with a sperm digesting organ connected to the female part of the reproductive tract, there is—at least theoretically—an opportunity for sperm selection by the female function of the hermaphrodite (cryptic female choice; cf. Eberhard, 1996). In these species, the spermatophore is placed in the diverticulum and spermatozoa are either transported into the bursa copulatrix by the strong unidirectional peristalsis of the bursa stalk or they may reach the spermatheca. In *A. arbustorum*, the length of the diverticulum shows a positive allometry and a high phenotypic variation compared to snail size (Beese et al., 2006b). It has been suggested that the diverticulum in *A. arbustorum* and other stylommatophoran species has evolved in response to selection pressures imposed by divergent evolutionary interests between the male and female function (Beese et al., 2006b). Indeed, a comparative study across stylommatophoran species indicates counter-adaptations between presence, relative length and placement of the diverticulum and the penial flagellum length (Koene & Schulenburg, 2005).

The penial flagellum produces the tail of the spermatophore (Gomez, 2001). The length of a penial flagellum might be of importance when it increases the owner's reproductive success. It has been hypothesized that sperm are most successful at reaching the storage organ when the spermatophore's tail is protruding into the vagina duct of the mating partner (Lind, 1973). In a comparative analysis considering 47 species of stylommatophoran gastropods, the presence of a long penial flagellum was positively

associated with the presence of a carrefour with fertilization pouch and a subdivided spermatheca (Beese et al., 2009).

9.2.8 AUXILIARY COPULATORY ORGANS

The reproductive system of the Stylommatophora is plesiomorphically equipped with an auxiliary copulatory organ that plays an active role during mating (Gomez, 2001). This auxiliary organ has been thought to facilitate reciprocal copulation (Nordsieck, 1985) and mutual exchange of male gametes (Tompa, 1984). More recently, its potential role in sperm competition has begun to be explored. Reflecting the great diversity in morphology, numerous terminologies have been applied to the auxiliary organ and its components (for reviews see Barker, 2001; Tompa, 1984).

For many stylommatophorans, the auxiliary copulatory organ comprises a tubular gland opening through a prominent papilla into the penis (Gomez, 2001). During copulation, the papilla is protruded from the genital pore and pressed against the partner's body or genitalia, and may even be introduced into the mate's genitalia. This activity can be accompanied by expulsion of secretory material from the gland of the auxiliary copulatory organ, as found in *D. gorgonium* (Benke et al., 2010; Reise et al., 2007; see Section 9.2.6).

In many stylommatophorans, the papilla of this auxiliary organ is equipped with a sharp, calcified or chitinous dart within a so-called dart sac (Davison et al., 2005). One or several glands, in a compact glandulous mass or elongate tubules, open to the sac. The dart is used to pierce the body of the mating partner during courtship. Even though darts may wound a partner, the elaborate structure of the dart apparatus suggests that it serves some adaptive function (see Section 9.5.4).

9.3 VARIABILITY IN BREEDING SYSTEMS

9.3.1 BREEDING SYSTEMS

In theory, simultaneous hermaphrodites may reproduce through a variety of breeding systems. Individuals may (1) cross-fertilize as male (sperm donor), as female (sperm recipient) or both; (2) reproduce uniparentally by self-fertilization or parthenogenesis; or (3) produce offspring uniparentally and by cross-fertilization (i.e., mixed breeding system) (Jordaens et al., 2007). Currently, no stylommatophoran species is known to reproduce

parthenogenetically and uniparental reproduction in this group seems to be restricted to self-fertilization (Heller, 2001; Jordaens et al., 2007; McCracken & Selander, 1980).

Simultaneous hermaphroditism is advantageous when mates are hard to find. In such situations each sexually mature conspecific encountered is a potential mating partner. Numerous stylommatophoran species are predominant cross-fertilizers. Other species have a mixed breeding system, but self-fertilization is widespread (e.g., *Vallonia pulchella* [Whitney, 1938]; *Punctum pygmaeum* [Baur, 1989]) and in slugs (Arionidae, Philomycidae, Limacidae; South, 1992). Reviews of breeding systems in stylommatophoran species are given in Duncan (1975), Peake (1978), Selander and Ochman (1983), Heller (1993), and Jordaens et al. (2007).

Self-fertilization provides advantages including assurance of reproduction in the absence of mating partners, preservation of highly fit genotypes, and reduced energy allocation to both sperm production and mating behavior (Thornhill, 1993). On the other hand, self-fertilization results in inbreeding depression and low heterozygosity. Therefore, one of the key reproductive decisions is to decide when an individual should start to reproduce by self-fertilization in the absence of mating partners. In this situation, the optimal delay of reproduction balances the potential benefits of cross-fertilization and the costs of delaying the onset of reproduction, potential inbreeding depression, resource allocation, and survival (Tsitrone et al., 2003).

In the Stylommatophora, the frequency of self-fertilization varies greatly among species and even among populations (Heller, 2001). In some species, it is rare, in others it occurs occasionally, and still in others self-fertilization occurs regularly. Heller (1993) listed 18 genera (of 12 families) as capable of self-fertilization. Geographical variation in the degree of self-fertilization has been recorded in various taxa, including *Carinarion* (Jordaens et al., 2000), *Arion intermedius* (Reise et al., 2001), *Rumina decollata* (Selander & Hudson, 1976), and in at least five species of *Partula* (Johnson et al., 1977, 1986; Kobayashi & Hadfield, 1996; Murray & Clarke, 1966). In a breeding experiment with *Partula taeniata*, Murray and Clarke (1966, 1976) found an age-dependent variation in the rate of self-fertilization. Individuals produced approximately 20% of their progeny by self-fertilization in the early part of reproductive life, whereas almost all progeny produced later were cross-fertilized resulting in an average of 2% self-fertilized offspring throughout an individual's life.

Self-fertilization has evolved in several phylogenetically independent lines (Heller, 1993). In general, self-fertilization can be concluded to occur

either by observing reproduction in animals isolated from birth, or by applying genetic markers such as microsatellites (Heller, 2001). Molecular techniques also allow an assessment of the frequency of self-fertilization in snails living in their natural environment. It is therefore not surprising that the occurrence of self-fertilization can be demonstrated in even more species. Interestingly, a low frequency of self-fertilization may also occur in species so far considered as obligate cross-fertilizers. For example, helicids were long believed to be self-incompatable. Chen (1993, 1994) kept unmated individuals of *Arianta arbustorum* isolated over 3 years and compared their reproductive success with snails kept in pairs. Thirty-nine percent of the unmated snails produced fertile eggs, mostly in the second and third years. The number of hatchlings produced was 1–2% of that from mated animals, although survival was similar for offspring from unmated and mated snails. This indicates that *A. arbustorum* can self-fertilize, but with a great fitness reduction (Chen, 1993, 1994). These laboratory results were confirmed by two independent field studies using microsatellite markers. In a natural population of *A. arbustorum*, low frequencies of self-fertilization were found in two out of 41 mother–progeny arrays: Two mother snails produced 2.0% and 18.2% of their offspring by self-fertilization, while the remaining 39 mother snails reproduced exclusively by cross-fertilization (Kupfernagel & Baur, 2011). In another study comparing levels of multiple paternity in four subalpine populations of *A. arbustorum*, Kupfernagel et al. (2010) found a low rate of self-fertilization in one population but not in the other three populations. The overall level of inbreeding was relatively low. However, moderate levels of inbreeding were found in the population with a low rate of self-fertilization as well as in another population (Kupfernagel et al., 2010). Similarly, a low frequency of self-fertilization has been reported in laboratory breedings of *B. fruticum*, with self-fertilized eggs showing a reduced hatching success (Kuznik-Kowalska et al., 2013).

Strong inbreeding depression was also observed in *Triodopsis albolabris* (McCracken & Brussard, 1980), a species that predominantly reproduces by cross-fertilization. Isolated individuals are able to reproduce by self-fertilization but the number of eggs was much lower and their hatching success only 3% of that of paired individuals (McCracken & Brussard, 1980). In contrast, inbreeding depression appears to be absent in *Deroceras agreste* (South, 1992). Self-fertilizing individuals were two to four times more fecund, had a longer life span and the growth rate of their offspring was higher than that of cross-fertilizing individuals. Similarly, inbreeding depression was very low in self-fertilizing *Balea perversa* (Baur & Baur, 2000; Wirth et al., 1997).

9.3.2 PROTANDRIC PHASE

In stylommatophoran species, sperm production precedes egg production by a few weeks in the otherwise simultaneously hermaphroditic animals, resulting in a short protandric phase (Luchtel et al., 1997). In natural populations and in laboratory breedings, subadult individuals, which have not yet finished shell growth, have been observed to be engaged in copulations, a phenomenon called "precocious breeding," for example, in *Monacha cantiana* (Chatfield, 1968), *Theba pisana* (Cowie, 1980), *Arianta arbustorum* (Baur, 1984), *C. aspersum* (Chung, 1987; Herzberg & Herzberg, 1962; Pos, 1994), *H. pomatia* (Tischler, 1973), and in vitrinids (Uminski, 1975) and zonitids (Mordan, 1978). In iteroparous species, snails in the protandric phase may copulate toward the end of the reproductive season, but delay egg production until the succeeding season. Thus, a higher proportion of "males" is present in natural populations at any time of the reproductive season, resulting in intensified sperm competition (see Section 9.5).

Species of the family Clausiliidae also have a protandric phase. However, maturation of reproductive organs is delayed in relation to cessation of shell growth in this family, as shown in *Vestia gulo* and *Vestia turgida* (Maltz & Sulikowska-Drozd, 2011). Spermatogenesis starts during formation of the closing apparatus. Three months after growth completion, all reproductive organs are well developed and the snails begin to mate. Four to 6 months after shell growth completion, the snails are capable of retaining fertilized, developing eggs in their oviducts (Maltz & Sulikowska-Drozd, 2011).

9.3.3 PHALLY POLYMORPHISM

Phally polymorphism refers to a male genital polymorphism, in which two or three sexual morphs co-occur. The penis can be reduced or absent, in which situations the animals are referred to as hemiphallic and aphallic, respectively. The female reproductive organs are, however, always fully developed. Euphallic individuals, in contrast, have fully developed male and female reproductive organs. Hemiphallic and aphallic individuals cannot transfer sperm to mating partners; they can theoretically only reproduce uniparentally (i.e., by self-fertilization) or by cross-fertilization as females, whereas euphallic individuals can reproduce by cross-fertilization as male and female, as well as by self-fertilization. Aphallic and hemiphallic individuals have been reported in numerous basommatophoran and stylommatophoran species (Boycott, 1917; Jordaens et al., 1998; Leonard et al., 2007;

Pokryszko, 1987; Watson, 1923). The proportion of aphallic individuals varies widely among populations. In the rock-dwelling land snail *Chondrina clienta*, the frequency of aphally varied from 52% to 99% in 23 natural populations in Sweden (Baur et al., 1993) and from 1% to 89% in 21 populations of *Chondrina avenacea* in Switzerland (Baur & Chen, 1993). Jordaens et al. (1998) recorded frequencies of hemiphallic individuals of 81–100% in 17 European populations of *Zonitoides nitidus*, with differences between geographic regions (Belgium, Germany, and Sweden).

Phally polymorphism evolved at least 13 times independently in basommatophoran and stylommatophoran species, each time with euphally as the ancestral condition (Schrag & Read, 1996). The determination of aphally, however, is still unclear. In the basommatophoran snail *Bulinus*, breeding experiments indicated that both genetic and environmental factors play a role in phally expression (Jarne et al., 2010). In *C. clienta*, phally expression can be influenced by environmental conditions (Baur et al., 1993). A laboratory experiment revealed that juveniles from one population became more frequently euphallic than expected under complete genetic determination when they were raised under conditions of low food supply, while the density of individuals had no effect. However, juveniles from another population raised under different food and density conditions did not differ from the original population in frequency of aphally (Baur et al., 1993). In the slug *Deroceras laeve*, the development of male reproductive organs was inhibited by low temperatures and/or exposure to light (Nicklas & Hoffmann, 1981).

An increase in the frequency of uniparental reproduction, most probably self-fertilization, is assumed in populations with large proportions of aphallic or hemiphallic individuals. In fact, the lack of heterozygotes in otherwise polymorphic *C. clienta* and *Z. nitidus* populations indicates a uniparental breeding system (Baur & Klemm, 1989; Jordaens et al., 1998). With increasing number of aphallics or hemiphallics in a population the extent of sperm competition may also decrease.

In the slug *Deroceras laeve*, aphally appears to be a character entirely different from that in all other species (Jordaens et al., 2006; Pokryszko, 1987). This species has external sperm transfer between intertwined penises and the penis is needed for sperm receipt. Hence, aphallic and hemiphallic individuals are unable to receive sperm. These individuals are therefore restricted to reproduce uniparentally (Reise & Hutchinson, 2002). This means that the rate of self-fertilization in aphallic and hemiphallic individuals is one and that there is no frequency-dependent selection on aphally and therefore polymorphism cannot be maintained. Genetic drift and possibly

directional selection will then ultimatively lead to the fixation of aphallics or euphallics, depending on which of the two phally morphs has the higher relative fitness. Hence, there are only two stable states in *D. laeve*, purely aphallic and purely euphallic populations (Jordaens et al., 2006). In a laboratory breeding experiment, growth, egg production, and hatching success of eggs were compared between aphallic and euphallic individuals of *D. laeve* derived from 13 families (Jordaens et al., 2006). The reproductive organs represented 16.4% of the total body weight in aphallic and 18.8% in euphallic slugs and were highly variable among individuals, even of the same family. Interestingly, however, in contrast to sex allocation theory, aphallic individuals of *D. laeve* did not reallocate resources from the lost male function toward the female function by producing more eggs (Jordaens et al., 2006).

Apophallation, the rather bizarre behavior of penis amputation, could also contribute to aphally. Reciprocal and unilateral apophallations have been described in the Banana slugs *Ariolimax californicus* and *A. dolichophallus*, whereby the penis is chewed off by the partner at the end of copulation (Harper, 1988; Leonard et al., 2002, 2007). Apophallation was observed in 4 out of 121 copulations (2 unilateral and 2 reciprocal apophallations) in the course of a laboratory study on the mating behavior of *A. californicus* and *A. dolichophallus* (Leonard et al., 2002). However, as shown in *Ariolimax buttoni*, these slugs have an innate phally dimorphism, with both aphallic and euphallic individuals in the same populations, rejecting the hypothesis that aphally is a result of apophallation (Leonard et al., 2007). The adaptive significance of the occasionally occurring apophallation in *Ariolimax* is not yet known (Leonard et al., 2002; Reise & Hutchinson, 2002).

9.3.4 SEMELPARITY VERSUS ITEROPARITY

Semelparity refers to a life cycle characterized by a single reproductive attempt, followed by death. Death ensues because the extreme reproductive effort of semelparous species involves extensive drainage of resources (Stearns, 1992). In some species, however, new resources for reproduction are collected between the productions of successive clutches. Iteroparity refers to a life cycle characterized by several reproductive attempts distributed over two or more reproductive seasons. Iteroparous organisms have—compared to semelparous organisms—a moderate reproductive effort and continue to invest in the maintenance of somatic tissues during and after reproduction, which results in a substantial chance to survive and reproduce

again later. In this chapter, broad definitions of semelparity and iteroparity are used: semelparous gastropods reproduce during one reproductive season, after which they die, whereas iteroparous species reproduce in at least two reproductive seasons usually interrupted by hibernation or estivation.

Semelparity in stylommatophoran gastropods is not closely linked to annual species (Heller, 2001; Kuznik-Kowalska et al., 2013). The close dependence of growth on environmental factors, especially on climatic conditions, results in a huge geographic variation in age at sexual maturity within species, even though all individuals may be semelparous (e.g., in *T. pisana* with an annual or biennial lifespan; Baker & Vogelzang, 1988; Cowie, 1984). Heller (2001) examined characteristics of semelparous and iteroparous gastropods using a data set with reliable information on life strategies in 35 genera. Fifteen genera were semelparous and 20 iteroparous. Heller (2001) did not find any biogeographical pattern in semelparity among terrestrial gastropods, except that semelparous species appear not to occur in deserts. Deserts constitute a habitat with unpredictable abiotic conditions including pronounced year-to-year fluctuations in precipitation. In many other habitats, semelparous and iteroparous species coexist. An interesting finding is that most agricultural pest species are semelparous (Heller, 2001). It is also noteworthy that several iteroparous stylommatophoran species have a long lifespan and reproduce over several years (Heller, 1990; Schmera et al., 2015). For example, the clausilid *Cristataria genezarethana* has a life span of up to 16 years and reproduces over at least 5 years (Heller & Dolev, 1994), and the helicid *A. arbustorum* lives for up to 18 years and can reproduce over 14 years (Baur & Raboud, 1988).

9.4 MATING STRATEGIES

9.4.1 FINDING MATES

Mate finding in stylommatophoran gastropods depends on chemical cues (Chase, 2002). Mucus trail following is a common behavior to find potential mating partners (Cook, 2001). Gastropod trails are made of mucus that comes from a gland embedded in the foot (Chase, 2002). In most species, the mucus is released through a pore at the anterior end as the animal moves. Chase et al. (1978) reported that *Achatina fulica* follows conspecific trails but not those of *Otala vermiculata*. Moreover, mature snails that were maintained singly in containers for 30 days showed a greater tendency to follow conspecific trails than did snails that were housed in groups with

opportunities for mating. This suggests that trail following in *Achatina* is used to find mates. Wareing (1986) investigated trail following in the slug *Deroceras reticulatum* in an outdoor arena. Trails were followed in the same direction in which they were laid on 16 out of 17 occasions, and the follower slug usually courted the trail-producing individual after catching up with it. However, not all species that use trail-following respond to the direction in which the original trail was laid (e.g., *Limax pseudoflavus*; Cook, 1992).

Distant chemoreception is mediated via the olfactory epithelia of the cephalic tentacles (Gelperin, 1974). Some snail species may use airborne pheromones to find mates, either as complement to trail following or as an alternative (Chase, 2002). Experiments with a T-maze connected to an olfactometer revealed that individuals of *A. fulica* and *C. aspersum* are able to distinguish between pheromones of either species by moving to the direction from which air with pheromones from conspecifics came (Chase et al., 1978). In several species of the families Bradybaenidae, Hygromiidae, and Helicidae, a pheromone is released from the "head-wart," which protrudes between the two optical tentacles (Falkner, 1993; Takeda & Tsuruoka, 1979). However, the significance of this pheromone with respect to mate finding and choice is still unclear.

Aggregations of gastropods may also result from an attraction to food resources or other favorable features of the habitat. Indeed, mating in stylommatophoran gastropods often occurs on feeding grounds (Chase, 2002).

9.4.2 COURTSHIP

There is a bewildering variety of courtship behavior in terrestrial gastropods, yet some general patterns have been recognized (Davison & Mordan, 2007; Jordaens et al., 2009). In some species, courtship display begins with reciprocal tactile and oral contacts, continues with curving turns to reach the face-to-face copulation position and ends with simultaneous intromission of the penises (e.g., in *H. pomatia*, Lind, 1988; and in *A. arbustorum*, Hofmann, 1923; Locher & Baur, 1999). Both partners seem to play simultaneously the same role during courtship. In other species, the two partners play different roles during courtship. For example, in *S. putris*, an "active" individual mounts the shell of a "passive" partner after which penises are intromitted simultaneously (Dillen et al., 2009). In a laboratory experiment, active and passive individuals of *S. putris* did not differ in the number of eggs produced, egg weight, hatching success of eggs, and onset of egg laying (Dillen et al., 2010a). Within mating pairs active individuals were significantly smaller

than their passive partners. These findings are in agreement with the gender-ratio hypothesis, which states that mating behavior in hermaphrodites should be flexible and determined by the potential male and female fitness gain in each single mating (Anthes et al., 2006).

In species that mate by shell mounting, it is not always justified to appoint the "male" role (sperm donor) to the shell mounting individual and the "female" role (sperm receiver) to the inactive individual, because sperm are transferred reciprocally in some species, while in other species the shell mounter is not always the sperm donor, and in still other species have unilateral sperm transfer (Jordaens et al., 2009).

Courtship and mating duration ranges from a few to more than 36 h in stylommatophoran species (Baur, 1998; Reise, 2007). In most species actual intromission and sperm transfer is rather short compared with the extended courtship (Leonard et al., 2002; Tompa, 1984). In other species, however, courtship is much shorter than the actual copulation. For example, in the slug *Veronicella sloanii*, courtship lasted less than 2 minutes, copulation approximately 1 hour (Clarke & Fields, 2013).

Asami et al. (1998) reviewed the courtship and mating behavior of 17 stylommatophoran families with respect to the shell shape of the snails (i.e., depressed [low-spired or flat] vs. elongated [high-spired or tall] shells). Species with depressed shells in general show a symmetrical courtship behavior, mate face-to-face and have reciprocal penis intromission. By contrast, species with elongated shells show an asymmetrical courtship behavior during which one individual (the "male") mounts the shell of its partner (the "female"). This is followed by unilateral penis intromission (i.e., the "male" inserts his penis into the reproductive tract of the "female" and donates sperm). In some species, the first mating is followed by a second in whom the partners reverse roles (e.g., in *Partula*; Lipton & Murray, 1979). Asami et al. (1998) found only a few exceptions from these patterns. Davison et al. (2005) used an extended list consisting of 60 genera belonging to 28 families and basically confirmed the findings of Asami et al. (1998). More recently, however, Jordaens et al. (2009) showed that the relationship between shell shape and mating behavior is not as straightforward as previously assumed. Actually, there are several exceptions from the pattern that species with depressed shells mate face-to-face and reciprocally and species with elongated shells mate by shell mounting and unilaterally. Furthermore, Jordaens et al. (2009) showed that there is considerable intraspecific variation in courtship and mating behavior in several stylommatophoran species, and that mating position does not predict reciprocity of penis intromission and sperm exchange. Individuals of the slug *V. sloanii* mated reciprocally in

pairs, but also in a multipartner ring formation involving three individuals (Clarke & Fields, 2013). In this species, aggressive behavior during mating is manifested by non-mating individuals pushing themselves between mating pairs resulting in the withdrawal of the penis of the mating pairs and cessation of copulation (Clarke & Fields, 2013).

9.4.3 MATE CHOICE

Hermaphroditic land snails would greatly enhance their reproductive success by choosing large mates because female fecundity (number of clutches, clutch size, and egg size) is positively correlated with shell size (Baur, 1988a; Baur & Raboud, 1988; Wolda, 1963). However, mating has been reported to be random with respect to shell size in *Cepaea nemoralis* (Wolda, 1963), *A. arbustorum* (Baur, 1992a), and *S. putris* (Jordaens et al., 2005) and with respect to shell color and banding pattern in *C. nemoralis* (Schilder, 1950; Lamotte, 1951; Schnetter, 1950; Wolda, 1963). Furthermore, in their male role, hermaphroditic land snails are expected to show a preference for mating with virgin and other young snails to reduce the risk of sperm competition (see Section 9.5). However, individuals of *A. arbustorum* did not discriminate between virgin and non-virgin mating partners and did not adjust the number of sperm delivered to the mating status of the partner (Haeussler et al., 2014a). In *Achatina fulica*, which is protandrous over several weeks, adults capable of producing both sperm and eggs, were more favored as mating partners than young adults that produce only sperm (Tomiyama, 1996). Size-assortative mating has been observed among old adults of *A. fulica* (Tomiyama, 1996) and in the slug *V. sloanii* (Clarke & Fields, 2013). In contrast to the species listed above, *A. fulica* and *V. sloanii* both show indeterminate growth.

Courtship and mating duration may last more than 36 h in stylommatophoran gastropods and thus often exceed the period favorable for locomotor activity (conditions of high air humidity; Chung, 1987; Jeppesen, 1976; Lind, 1973, 1976). During courtship and copulation terrestrial gastropods are exposed to severe water loss and more susceptible to predation than single adults (Pollard, 1975). It has been suggested that because of the time-constrained activity and high costs of locomotion, the best strategy for a snail is to mate with the first mating partner available to minimize the risk of either ending up without any mating at all or to drying up during mating (Baur, 1992a). The random mating patterns recorded in stylommatophoran species do not necessarily imply random fertilization of eggs, because

multiple mating with different partners (see Section 9.5.1) and long-term allosperm storage offer opportunities for sperm competition (see Section 9.5). Furthermore, the structure and morphology of the sperm storage site (spermatheca), fertilization chamber (see Section 9.2.2), and the presence of a sperm digestion organ offer opportunities for sperm selection by the female function of the hermaphrodite (Baur, 1998, 2010).

Mate-choice tests with *A. arbustorum* from geographically isolated populations in Sweden and Switzerland revealed that snails preferred to mate with individuals from their population of origin, and pairs involving snails from two distant Swiss populations showed a reduced fertility, indicating effects of outbreeding depression (Baur & Baur, 1992a). In general, mating between closely related individuals can incur substantial fitness costs (i.e., inbreeding depression). However, effects of inbreeding and outbreeding are population-specific, depending among others on the history of the particular population (Thornhill, 1993). Therefore, one should be careful to draw general conclusions from individual studies. Individuals of *A. arbustorum* from a subalpine population mated randomly with respect to degree of relatedness, indicating a lack of inbreeding avoidance by selective mating (B. Baur & A. Baur, 1997). Snails that mated with full-sibs did not differ in number of eggs, hatching success of eggs, or number of offspring produced from those that mated with unrelated conspecifics. In another population of *A. arbustorum*, Chen (1993) found that eggs of inbred snails showed a lower hatching success (30.4%) than those of outbred snails (48.5%). Furthermore, inbred offspring reared in the garden had a higher mortality than outbred offspring reared in the same environment, but no difference was found when offspring from both groups were kept in the laboratory. This result supports the hypothesis that cross-fertilization in simultaneous hermaphrodites is maintained by inbreeding depression. It also shows that the extent of negative inbreeding effects varies between populations and environments in which the snails are kept (see Section 9.3.1).

In many species, courtship and mating behavior is very complex, demanding a high amount of coordination between partners. Hence, interspecific differences in mating behavior might cause effective reproductive barriers. For example, individuals of *D. rodnae* and *D. praecox* differ in the timing and duration of courtship (Reise, 1995). Particularly, the different timing of courtship behavior seems to act as an effective prezygotic isolation mechanism preventing interspecific crosses (Reise, 1995). In contrast, despite consistent differences in genitalia morphology and courtship behavior, interspecific spermatophore exchange can occur with a reduced success between the native slug *Arion rufus* and the invasive slug *Arion vulgaris*, indicating

an incomplete reproductive barrier (Dreijers et al., 2013). In fact, introgression of *A. rufus* with *Arion ater* and of *A. ater* with *A. vulgaris* has been demonstrated in Norway (Hatteland et al., 2015).

9.4.4 MODES OF SPERM TRANSFER

Individuals of many species transfer spermatozoa packed in a spermatophore, whose shape and size are species-specific and thus of taxonomic significance (Tompa, 1984). The spermatophore of the Cuban tree snail *Polymita picta* is equipped with spines (Reyes-Tur et al., 2015). It has been suggested that these spines might slow down spermatophore uptake into the digesting bursa copulatrix (Reyes-Tur et al., 2015). The spermatophore itself might be an adaptation of the sperm donor that allows a larger number of his sperm to escape digestion and to reach the spermatheca of the receiver.

Other species deliver spermatozoa as an unpacked sperm mass. Sperm transfer when penises are reciprocally intromitted is normally reciprocal in *A. arbustorum* (Baur et al., 1998), *C. aspersum* (Chase & Vaga, 2006; Rogers & Chase, 2001), *Euhadra subnimbosa* (Koene & Chiba, 2006), and *Polymita muscarum* (Reyes Tur & Koene, 2007). However, unidirectional sperm transfer may occur. Jordaens et al. (2005) reported occasional unilateral sperm transfer in *S. putris* despite reciprocal penis intromission (in 12 out of 87 pairings (14%) involved unilateral sperm transfer). In several species, the penises of both partners are protruded and entwined (in some Succineidae, Polygyridae, and Helicodiscidae [Jordaens et al., 2009], and in several slugs [Reise, 2007; South, 1992]). Sperm are then exchanged at the top of the penises. In this kind of mating, the penis is also used to take up the allosperm. Even in species that show penis entwining, sperm transfer is not always reciprocal (Reise, 2007). In 3 out of 15, apparently normal copulations (20%) of *D. rodnae* only 1 of the partners transferred a sperm mass (Reise, 1995).

Following sperm transfer the partners may quickly separate (Reise, 2007). In a variety of species, however, there is a period of immobility (e.g., lasting 0.5–9 h in *H. pomatia*; Lind, 1976), during which the spermatophore is transported in the reproductive tract of the recipient toward the bursa copulatrix, where it is eventually digested. During this period sperm leave the spermatophore. Depending on the location of the spermatophore in the female reproductive tract, sperm may reach the spermatheca (female sperm-storage site) or they may be transported into the bursa copulatrix where they are eventually digested (see Section 9.6.1).

9.5 SPERM COMPETITION

Sperm competition is the competition between the spermatozoa from two or more males to fertilize the eggs of a single female during one reproductive cycle (Parker, 1970). In stylommatophoran species, sperm competition occurs when the individuals mate with two or more partners and when they store viable sperm from these partners in the spermatheca.

9.5.1 MULTIPLE MATING

Evidence for multiple mating is available for several stylommatophoran species. For example, individuals of *H. pomatia* (Lind, 1988), *C. aspersum* (Fearnley, 1996), *C. nemoralis* (Murray, 1964; Wolda, 1963), and *A. arbustorum* (Baur, 1988b) have been observed to mate repeatedly with different partners in the course of a reproductive season. Research on mating strategies in the wild has been hampered by the notorious difficulty with which mating gastropods can be reliably observed in natural populations. Recently, the use of molecular techniques for paternity assignment has shown that multiple mating is widespread in stylommatophoran species, resulting in multiple paternity in egg batches (several "males" siring the offspring of a batch). The contribution of a particular "male" in fertilizing eggs can be influenced by competition with sperm from other males and by female manipulation (see Section 9.6.1). Estimates of paternity represent the number of genetic mating partners and are therefore estimates of the minimum mating frequency. Multiple paternity within batches has been demonstrated in *C. aspersum* (Evanno et al., 2005) and *A. arbustorum* (Kupfernagel et al., 2010; Kupfernagel & Baur, 2011) suggesting intensive sperm competition in these species. However, experimental studies on factors influencing sperm competition in stylommatophorans are so far restricted to a few species: *C. aspersum* (e.g., Chase & Vaga, 2006; Evanno et al., 2005; Garefalaki et al., 2010; Rogers & Chase, 2002), *S. putris* (Dillen et al., 2008, 2010a,b; Jordaens et al., 2007), and *A. arbustorum* (Baur, 1994b; Locher & Baur, 1999).

9.5.2 SPERM NUMBER

Sperm number, in some cases, is an important determinant for achieving successful fertilization in sperm competition. Theoretical models and empirical evidence from various studies suggest that, fundamentally, numerical

superiority is an adaptive strategy for sperm competition (Birkhead & Møller, 1998). In *S. putris*, individuals with different mating history transferred between 188,000 and 6392,000 sperm to their partners (Jordaens et al., 2005), whereas in individuals of *A. arbustorum* that copulated for the first time the number of sperm delivered ranged between 803,000 and 3969,000 (Baur et al., 1998). However, only a small portion of the sperm transferred may reach the female sperm-storage organ of the mating partner (see Section 9.2.2).

Adjusting the number of sperm delivered to the size of the (female) partner and to the expected intensity of sperm competition is assumed to be adaptive strategies (Birkhead & Møller, 1998). In *S. putris*, the number of sperm delivered during mating was highly variable but was not related to the size of the donor, the size of the recipient, the size difference between the two partners or mating duration (Jordaens et al., 2005). In another study on *S. putris*, however, the number of delivered sperm increased with donor and recipient body size (Dillen et al., 2010b). Individuals of *A. arbustorum* are not able to adjust sperm expenditure to the mating history (virgin or nonvirgin) of the partner (Baur et al., 1998). The number of sperm transferred was neither correlated with the size of the sperm recipient nor with that of the sperm donor. Furthermore, sperm number was not related to mating duration (Baur et al., 1998). Locher and Baur (2000a) examined the effect of increased sperm competition risk on male and female reproductive traits in *A. arbustorum*. Courtship behavior, spermatophore size and number of sperm delivered were not influenced by a higher sperm competition risk. However, snails constantly exposed to mucus trails of conspecifics deposited more egg batches than snails denied any cues from conspecific mucus trails (Locher & Baur, 2000a). Snails from different populations of *A. arbustorum* differed in the number of sperm delivered (Baminger et al., 2000; Minoretti & Baur, 2006).

The prevalence and consequences of autosperm depletion have so far received little attention in stylommatophorans, except in two species. Individuals of *A. arbustorum* required 8–21 days to replenish sperm and seminal fluid stores, delivering fewer sperm when intermating intervals were shorter (Hänggi et al., 2002; Locher & Baur, 1999). In *S. putris*, the number of sperm delivered increased with longer mating intervals, but decreased toward the end of the reproductive season indicating that the number of previous matings affects male resource allocation (Dillen et al., 2010b).

"Male" mate choice and, in particular, its manifestation in strategic sperm allocation therefore remains a poorly investigated phenomenon in stylommatophoran gastropods.

9.5.3 SPERM MORPHOLOGY

Sperm morphology is an important factor for fertilization success. Much interest has been focused on theory concerning the significance of the sperm size and quality (reviewed in Birkhead et al., 2009). Sperm size may influence the power and swimming speed as well as longevity because of changes in the energetic demands of longer or shorter flagella. In taxa with sperm storage organs, sperm length may determine the ability to reach the storage organs first and to move to the ovum from the storage organs once ovulation takes place. Furthermore, sperm-female interactions, for instance, between sperm length and the morphology and biochemistry of the female reproductive tract, may cause sperm diversity (Beese et al., 2009).

Stylommatophoran sperm are characterized by a small head with a very small but often complex nucleus (Thompson, 1973). The nucleus consists of densely packed chromatin and often has a pronounced helical surface sculpturing. The large interspecific variation in sperm morphology is frequently used as a taxonomic character (Healy, 1988, 1996; Luchtel et al., 1997; Thompson, 1973). Parivar (1981) described sperm dimorphism in the slug *A. ater* involving two different types of sperm, eupyrene and apyrene. The most obvious difference between the two sperm types is the lack of an acrosome in the apyrene form. Apyrene sperm are not capable of fertilizing eggs. Luchtel et al. (1997) suggested that apyrene sperm may have some physiological or endocrinological role in the gonad. There is, however, no further evidence for sperm dimorphism in any other stylommatophoran species. Yet, abnormal sperm (lack of acrosomes, modified centrioles, multiple flagella) occur in large numbers in several terrestrial slug species (e.g., *Milax gagates* and *Deroceras agrestis*; South, 1992). The function of abnormal sperm still remains to be clarified in stylommatophoran gastropods.

Information on the size of spermatozoa is summarized in Thompson (1973). Spermatozoa of stylommatophorans are among the longest of the Mollusca (e.g., 850 μm in *H. pomatia* and 1140–1400 μm (of which the head accounts for only 10 μm) in *Hedleyella falconeri*). Within- and among-species variation in sperm length and shape may reflect population- and species-specific differences in fertilization mode, allometry, and strength of post-copulatory sexual selection. In a comparative study considering 57 stylommatophoran species from Europe and South America, sperm length ranged from 101 μm in *Oxychilus navarricus helveticus* to 1341 μm in *Plagiodontes patagonicus* (Schmera et al., 2016). Sperm length increased with the shell size of the species and snails with oblong and globose shells had significantly longer sperm than snails with depressed shells and slugs.

Most interestingly, sperm length was also affected by the fertilization system: species with cross-fertilization had longer sperm than species with self-fertilization or a mixed breeding system, most probably a response to increased sperm competition risk in cross-fertilizing species. All these findings remained when phylogenetic relationships among the species were taken into account in the analyses (Schmera et al., 2016).

Within species, sperm morphology is assumed to be maintained by stabilizing selection, but it can respond to directional selection, including selection pressure from the female reproductive tract (Birkhead et al., 2009). Hence, if spatially segregated populations vary in strength or form of selection, sperm might be selected toward different optima across populations. Despite great interest in evolutionary diversification of sperm morphology and its implication on reproductive success, information on intraspecific variation in sperm length is restricted to a single stylommatophoran species, *A. arbustorum*. In this species sperm are monomorphic. Independent of adult shell size, sperm length differed among four populations (mean values of the populations: 878, 898, 913, and 939 μm) and—to a minor extent—even among individuals within populations (Minoretti & Baur, 2006). Individual snails showed consistent sperm length in successive matings (Minoretti & Baur, 2006). Mean sperm length of an individual, however, was not correlated with the number of sperm delivered in a spermatophore. Haeussler et al. (2014b) examined total sperm length and sperm head length in 23 *A. arbustorum* populations sampled across the distributional range of the species in Central and Northern Europe and found a difference of 11.0% of total sperm length between the shortest and longest population means. Differences among populations explained 62.9% of the variance in total sperm length, differences among individual snails within population 23.4% and differences within individual snail 13.7%. Interpopulation differences in total sperm length increased significantly with geographical distance between populations. Infection by parasitic mites (see Section 9.9) had a positive effect and longitude of the population sampled had a negative effect on total sperm length (Haeussler et al., 2014b). Thus, independent of the population examined, mite-infected individuals of *A. arbustorum* produced larger sperm than uninfected snails and total sperm length decreased from west to east. Sperm head length also varied among populations, but it was not influenced by any of the factors examined. In a subsample of 12 populations restricted to the mountains of Switzerland (elevational range 440–2485 m a.s.l.), total sperm length decreased with increasing elevation (Haeussler et al., 2014b). These findings suggest that selection pressures acting among populations may differ from those acting within. Stabilizing selection might

be a possible mechanism for producing the reduced variation observed in sperm length within a population.

Minoretti et al. (2013) used two complementary approaches (one-parent–offspring regression and full-sibling split design of offspring raised at three different temperatures: 11, 15 and 20 °C) to estimate heritability of sperm length in *A. arbustorum*. The breeding experiment revealed both environmental and—to a minor extent—genetic effects on sperm length. The results indicated that independent of shell breadth, sperm length was affected by temperature but not by family of origin.

The ability of sperm to stay and survive in the female storage organ may influence fertilization success, suggesting that optimal sperm morphology may maximize sperm longevity. The sperm storage organ (spermatheca) of stylommatophoran gastropods has a complex structure and it functions in the context of intense sperm competition (see Section 9.2.2). In *A. arbustorum*, sperm from different mating partners are stored in the spermatheca for months or even years before being used to fertilize eggs (Baur, 1988b; Kupfernagel et al., 2010). In this way, the processes of insemination and fertilization are uncoupled in space and time. Among the sperm stored, those with their heads in tight contact with the epithelial walls survive best (in *C. aspersum*; Chase & Darbyson, 2008). It has also been suggested that the beating of the flagella of sperm stored from the first mate could provide paternity assurance through increased resistance to incoming sperm from subsequent mates (Rogers & Chase, 2002), with longer sperm and larger number of sperm resulting in a stronger resistive force. Thus, longer spermatozoa might have a higher fertilization success than shorter spermatozoa.

Available literature on gonochoristic animals indicates that sperm-quality traits (proportion of live, morphologically normal spermatozoa, and motility of spermatozoa) affect fertilization success and that they are important in both sperm competition and cryptic female choice (Birkhead et al., 2009; Snook, 2005). Mean sperm velocity in *A. arbustorum* was neither influenced by the shell size of the snails, nor did it differ between two populations (Minoretti & Baur, 2006). However, mean sperm velocity differed among individual snails (range 52–112 μm/s). Furthermore, the percentage motility and longevity of sperm differed between snails from the two populations, but were not affected by shell size. No correlations were found between length, velocity, percentage motility, and longevity of sperm. Thus, individual snails differed in sperm quality. This interindividual variation may partly explain differences in fertilization success.

9.5.4 MATE MANIPULATION: DART SHOOTING

"Love darts" are hard "needles" that many snails and slugs use to pierce their partner during mating (Chase, 2007; Schilthuizen, 2005). Darts carry a gland product on their outside and enter this into the partner's hemolymph by stabbing. This gland product causes conformational changes in the female reproductive system, which impedes the entrance to the sperm-digesting organ (bursa copulatrix; see above) (Koene & Chase, 1998; Kimura et al., 2014). Whereas the sperm donor benefits from shooting a dart, the dart receiver may be disadvantaged because its skin is injured, infection risk may increase, and the process of sperm storage is altered (Landolfa et al., 2001; Rogers & Chase, 2002).

Dart shooting occurs in many ways, and the darts have species-specific shapes and sizes (Davison & Mordan, 2007; Koene & Schulenburg, 2005; Tompa, 1980a). Some species have a single dart, which stays in the skin of the partner and has to be rebuilt after copulation by the shooter (e.g., *C. aspersum*; Chung, 1987), in other species the dart remains attached to the body of the shooter and is retracted and reused (e.g., *Polymita muscarum*; Reyes-Tur & Koene, 2007). Still others have multiple darts (e.g., the slug *Trichotoxon heynemanni*; Schilthuizen, 2005). In *A. arbustorum*, dart shooting is optional (Baminger et al., 2000), but in other species is obligatory, except for virgins (Chung, 1987).

Dart shooting is best studied in *C. aspersum* (for a review see Chase, 2007). Dart shooting occurs when a snail quickly everts the basal tubercle of the dart sac out of its everted genitals. The dart is never propelled through the air, because it is firmly attached by its base to the tubercle until it is lodged in the partner's tissue. Occasionally, the dart does not hit the partner. A new dart is produced within 5–6 days after dart shooting. Chase and Vaga (2006) found that in mating *C. aspersum* each dart was shot independently, and each animal appeared to be interested only in getting off the best possible shot, probably one that penetrates deeply near the genital pore. The outcomes of the dart shots affect neither the probability that the courtship will culminate in copulation nor the size of the ensuing sperm donation (Adamo & Chase, 1988; Chase & Vaga, 2006). Instead, the dart transfers a substance that induces conformational changes in the female reproductive tract of the recipient, closing off the entrance to the gametolytic bursa copulatrix and thus most likely reduces sperm digestion (Chase & Blanchard, 2006; Koene & Chase, 1998). Thus, successful dart shooting more than doubles the number of donated sperm that are stored by the recipient (Rogers & Chase, 2001), and it significantly increases the relative paternity when a

dart shooter competes with an unsuccessful shooter (Landolfa et al., 2001; Rogers & Chase, 2002). In helicids (e.g., *C. aspersum* and *H. pomatia*), the dart is bladed and shot once into the partner, where it stays beneath the skin (Chase, 2007). In some species (*Euhadra subnimbosa*, *Helminthoglypta* spp.), however, mating snails stab their partner repeatedly with the same dart (more than 3000 times during 22 min courtship or 136 stabs/min; Koene & Chiba, 2006). The Cuban tree snail *Polymita picta* also uses its dart repeatedly, but with a lower stabing frequency (0.04–1.05 stabs/min; Reyes-Tur et al., 2015). Recent work explored the enormous diversity in dart structure and potential effects on the dart receivers. For example, species of the tropical snail genus *Everettia* possess a syringe-like dart that serves as a real injection needle (Koene et al., 2013). In *Euhadra quaesita*, the mucus of the dart suppresses subsequent matings in darted individuals (Kimura et al., 2013).

These findings indicate a function for the dart shooting behavior in the survival and storage of allosperm. Thus, an indirect cost might be the partial loss of control over fertilization by the female function. It follows that any defense against this cost should be expressed in the sperm-receiving organs, but not in courtship behavior or sperm transfer. Indeed, Koene and Schulenburg (2005) found evidence for the coevolution of the dart apparatus and the bursa tract diverticulum. Their phylogenetic analyses revealed that the length of the diverticulum increases as the size and complexity of the dart apparatus increases. One interpretation of these findings is that the female function is responding defensively to male-function manipulation by means of the dart (Chase & Vaga, 2006).

Dart shooting is perhaps the best studied sexually selected behavior in stylommatophorans. However, other so far less investigated behaviors or morphological structures may also influence the mating partners of stylommatophoran gastropods. For example, Reise et al. (2007) suggested that the secretion of the appending penial gland of slugs of the genus *Deroceras* has a similar manipulative function as those involved in dart shooting (see Section 9.2.6).

9.6 POST-COPULATORY PROCESSES

Females (or the female function in hermaphrodites) may influence the outcome of sperm competition by selective sperm use and cryptic female choice (Eberhard, 1996). Females might be able to discriminate between and differentially utilize the sperm of different males, a process referred to as "sperm choice." There are broad and narrow definitions of "sperm

choice;" some authors make it synonymous with "cryptic female choice" (see Eberhard, 2000; Kempenaers et al., 2000; Pitnick & Brown, 2000). Cryptic female choice has been defined as nonrandom paternity biases resulting from female morphology, physiology, or behavior that occur after coupling (Pitnick & Brown, 2000). This definition ascribes to sperm choice any biases in paternity owing to the way females handle sperm, regardless of the specific mechanism or evolutionary causes, and regardless of proximate control.

9.6.1 SELECTIVE ALLOSPERM STORAGE AND FERTILIZATION

In the context of cryptic female choice, the sites of sperm storage (spermatheca), fertilization, and sperm digestion (bursa copulatrix) are of major interest. The sites of sperm storage were examined in *A. arbustorum* that had mated successfully. In some snails, a part of the spermathecal tubules was filled with spermatozoa, while in other animals no sperm were found in the spermatheca (Haase & Baur, 1995). In these snails, sperm were found exclusively in the sperm-digesting bursa copulatrix. Bojat and Haase (2002) assessed the amount of allosperm stored in the spermatheca of *A. arbustorum* in relation to the structure of the spermatheca (number of spermathecal tubules) in 18 individuals that had copulated once. Snails differed in patterns of sperm storage: two individuals used 100% of their spermathecal tubules, two used 80%, three 75%, two 66.7%, one 50%, two 40%, three 33.3%, two 25%, and one used 20%. The main tubule always contained sperm (51–100% of the total amount of sperm stored, i.e., more than all lateral tubules combined). The amount of sperm stored was positively correlated with the number of spermathecal tubules. However, the amount of sperm stored was not correlated with the volume of the received spermatophore. These findings suggest that the female reproductive system of *A. arbustorum* may be able to influence fertilization by a selective digestion of sperm from certain mating partners.

Individuals from different *A. arbustorum* populations did not differ in the amount of sperm stored (Baminger & Haase, 1999). Furthermore, the amount of sperm stored was not correlated with population density. In *C. aspersum*, no correlation between the number of spermathecal tubules or the cumulative length of all tubules and the local population density was found (Koemtzopoulos & Staikou, 2007).

Viable allosperm indicated by fertilized eggs have been found 108 days after the last copulation in the tropical snail *Limicolaria flammea*

(Egonmwan, 1990), 520 days in *Limicolaria martensiana* (Owiny, 1974), 341 days in *Achatina fulica* and 476 days in *Macrochlamys indica* (Raut & Ghose, 1979) and 4 years in *C. nemoralis* (Duncan, 1975). However, sperm viability is not a simple function of time. In *A. fulica* and *M. indica*, the viability of sperm stored is influenced by the length of the estivation period (Raut & Ghose, 1982). Sperm viability decreased to 105 days in estivating *A. fulica* and to 150 days in *M. indica*.

In long-term sperm storage, the processes of insemination and fertilization are uncoupled in space and time. Chase and Darbyson (2008) showed that sperm stored with their heads in tight contact with the epithelial walls of the spermatheca survive best in *C. aspersum*. It has been suggested that attachment may provide an anchor to prevent sperm from being removed from the tubule, either by passive drift or as a consequence of flagellar beating by later arriving sperm (Rogers & Chase, 2002). Moreover, the beating of flagella of sperm stored from the first mate could provide paternity assurance through increased resistance to incoming sperm from subsequent mates (Rogers & Chase, 2002). However, there is so far no proof that sperm with their head in tight contact with the epithelial wall receive nourishment.

For a mechanistic understanding of the fate of received sperm and the involved patterns of postcopulatory sexual selection, it is necessary to distinguish allosperm from two (or more) donors in the storage organ. Kupfernagel et al. (2013) adjusted an immunocytochemical sperm-labeling technique to *A. arbustorum* to track the fate of 5-bromo-2′-deoxyuridine (BrdU)-labeled sperm in the female reproductive organs of the recipient. The proportion of sperm labeled among the sperm produced by the donor snails averaged 99.3% and labeled sperm could be consistently visualized in both the sperm storage and sperm digestion organ of all recipients examined. Combined with a traditional sperm staining technique, Kupfernagel et al. (2013) showed that the BrdU-labeling technique allows to distinguish between sperm from two males (one with labeled sperm, the other with unlabeled sperm) in the female reproductive tract of double-mated snails (Fig. 9.2). In the spermatheca illustrated in Fig. 9.2, 47% of the sperm stored were from the first mate and 53% from the second mate (R. Janssen, unpublished data). A preliminary analysis indicates that in the main tubule of most snails examined sperm from both partners were mixed, while in lateral tubuli frequently sperm from single partners occur (R. Janssen, unpublished data). In a control experiment, Kupfernagel et al. (2013) showed that repeated BrdU-application slightly retarded shell growth of juvenile snails, but did not influence their adult size, survival, number of sperm delivered and mating frequency. This immunocytochemical approach opens new gates

to investigate mechanisms and processes of postcopulatory sexual selection, including sperm transfer and both selective storage and utilization of sperm.

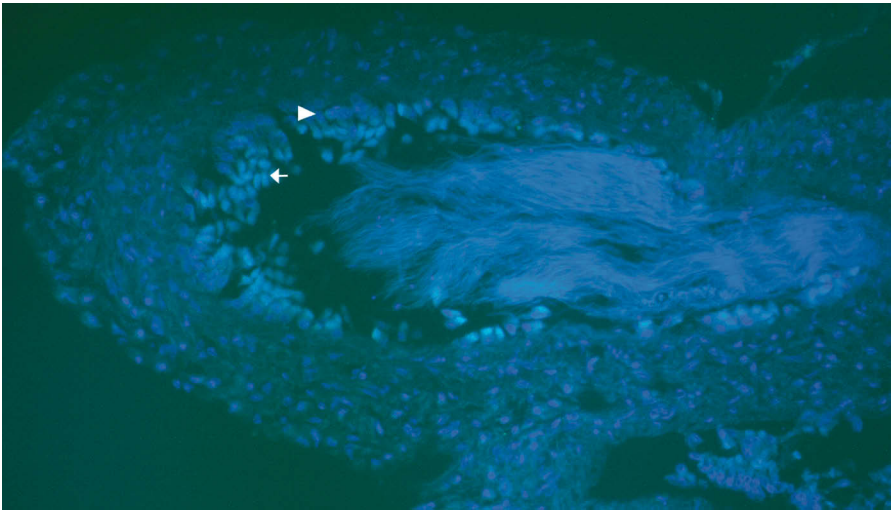

FIGURE 9.2 Overlaid images from a longitudinal section of a spermathecal tubule of a double-mated individual of *Arianta arbustorum*. The combined image (DAPI and FITC filters) shows the storage of unlabeled DAPI-stained sperm from the first partner (arrowhead) and BrdU-immunolabeled sperm from the second partner (arrow). Most of the sperm are attached with their heads to the epithelial (inner) tubule wall and their long tails are directed to the lumen of the tubule. The interval between the two matings was 18 days. (Staining and photography by Ruben Janssen and Bruno Baur).

9.6.2 PATTERNS OF PATERNITY

Sperm precedence is the differential sperm usage from consecutive matings (mating order effect). It is typically measured as the proportion of eggs fertilized by the second of two mates (the P_2 value). Sperm precedence (P_2) in doubled-mated *A. arbustorum* was influenced by the time between the two matings when the mating delay exceeded 70 days (one reproductive season). In the first brood of snails that mated twice within 70 days, P_2 averaged 0.34, indicating precedence of sperm from the first mate (Baur, 1994b). In contrast, P_2 averaged 0.76 in broods of snails that remated in the following season, indicating a decreased viability of sperm from the first mate. Analysis of long-term sperm utilization in 23 snails that laid 3–9 batches over 2 years revealed striking differences among individuals. Five snails (22%) exhibited precedence of sperm from the first mate throughout, eight snails (35%)

showed precedence of sperm from the second mate throughout, whereas 10 snails (43%) exhibited sperm mixing in successive batches. This indicates that different mechanisms might be involved in creating the observed inter-individual variation in sperm precedence.

Kupfernagel et al. (2010) assessed the level of multiple paternity and sperm utilization patterns in four natural populations of *A. arbustorum* using polymorphic microsatellite loci. Multiple paternity was detected in the offspring of all 26 mother snails examined with the contribution of at least two to six fathers. The four populations examined differed in the level of multiple paternity. Snails in the population with the highest density of adults showed the highest level of multiple paternity, whereas snails in the population with the lowest density exhibited the lowest value of multiple paternity. Highly skewed paternity patterns were found in the progeny of 15 (58%) of the 26 mother snails (Kupfernagel et al., 2010). In another natural population of *A. arbustorum*, multiple paternity was recorded in the offspring of all 19 mother snails examined with similarly skewed paternity patterns (Kupfernagel & Baur, 2011).

Janssen and Baur (2015) examined temporal variation in the level of multiple paternity in a natural population of *A. arbustorum* on an alpine grassland. The level of paternity was very similar throughout the reproductive season, with an average of four fathers siring each egg batch. However, not all fathers contributed equally to the fertilization of the eggs. There was a skew toward two main sperm donors siring most of the eggs of the batches. This suggests differences in sperm number and quality (higher competitive ability of sperm from certain mates) or selection by the recipient (cryptic female choice).

The highly skewed paternity patterns suggest an involvement of selective sperm use by the female role. However, there exists so far no experimental evidence for the occurrence of cryptic female choice in any stylommatophoran species. Yet, there are several lines of evidence indicating that the sperm recipient might influence paternity after multiple mating. First, in reciprocally mating individuals of *A. arbustorum*, some recipients directed the spermatophore together with sperm to the digesting bursa copulatrix and no allosperm were stored from these mating partners (Haase & Baur, 1995). Second, the amount of sperm stored was not correlated with the volume of the received spermatophore (Bojat & Haase, 2002), and thus with the number of sperm received (Baur et al., 1998). Third, the huge inter-individual variation in sperm precedence patterns found among individuals of *A. arbustorum* cannot be explained by sperm number or sperm quality alone (Baur, 1994b; see above). Fourth, the complex muscular network of

the tubules and its innervation indicate that the spermathecal muscles of *A. arbustorum* are capable of finely tuned movements with agonistic and antagonistic interactions (Bojat et al., 2001b,c). The muscular arrangement indicates that the action of muscles is more important for the extrusion of sperm from the spermatheca than for the uptake and distribution of sperm among the tubules. Bojat et al. (2001b) suggested that the distribution of allosperm among the tubules can only be accomplished by the ciliation of the spermathecal common duct.

Most probably, the observed patterns of multiple paternity in stylommatophoran gastropods are a result of both sperm competition and post-copulatory processes.

9.6.3 BENEFITS OF MULTIPLE MATING FOR THE FEMALE FUNCTION

In many animals, males are selected to mate as many times as possible to maximize their reproductive success (Bateman, 1948; Trivers, 1972). For females, in contrast, the advantage of multiple mating is not so obvious. The relatively small number of ova produced by a female could be fertilized by sperm from one or very few male ejaculates, especially when the female can store sperm or has a short reproductive period. Moreover, possible costs of mating should select against unnecessary matings. While females of some species mate only once during lifetime, multiple mating by females is generally very common. Several hypotheses have been suggested to explain the adaptive significance of multiple mating by females (Birkhead & Møller, 1998). Among them, the hypothesis of sperm replenishment is the most straightforward explanation for multiple mating by females. The underlying mechanism could vary: the sperm received from one male may not be enough to fertilize all the eggs produced by a female, the viability of sperm stored may decrease with time, or a mate may have transferred sperm of low quality. Another hypothesis predicts genetic advantages: multiple mating with different partners may lead to multiple paternity and thus increase the genetic variability among the offspring of a brood (Jennions & Petrie, 2000; Keller & Reeve, 1995). Furthermore, females may enjoy nutritional benefits from repeated matings by receiving nutrients with the spermatophore. These hypotheses are not mutually exclusive.

Chen and Baur (1993) examined reproductive traits over 2 years in individuals of *A. arbustorum* that copulated several times per year (snails kept in pairs), in individuals that copulated twice (once at the beginning of each

year) or once (at the beginning of the first year), and in individuals prevented from copulation (snails kept isolated). Copulations were not always reciprocally successful: 3 of 57 snails (5%) failed to produce fertile eggs, although their mates reproduced successfully. Similarly, 2 of 15 pairs (13%) failed to reproduce successfully. Snails allowed to mate repeatedly within each season tended to lay more eggs than snails that mated once per year. However, the number of hatchlings did not differ significantly between the two treatment groups because eggs laid by snails allowed to mate repeatedly had a lower hatching success. Snails that remated in the second year laid more eggs with a higher hatching success, and thus produced more hatchlings, than snails that mated only once at the beginning of the first year. Snails that were prevented from mating produced a few hatchlings (by self-fertilization) in the second year; their reproductive success was less than 1% of that of mated snails. These results suggest that multiple mating is also adaptive for the female function of *A. arbustorum* by increasing female fecundity and fertility and serving as a hedge against unsuccessful copulations.

In fact, in several stylommatophoran species, egg production is stimulated by mating behavior and/or substances derived from male ejaculates (Bride et al., 1991; Takeda, 1983). In *C. aspersum*, mating increases the synthesis and release of a dorsal body hormone essential for vitellogenesis, ovulation, and egg laying (Saleuddin et al., 1991). Whether this activation of the dorsal body is direct, under either neural or hormonal control, or indirect under gonadal influence, is not known (Saleuddin et al., 1991). Baur & Baur (1992b) examined experimentally whether extended courtship display or repeated copulation in the course of a reproductive season stimulates egg production in *A. arbustorum*. Clutch size decreased in successive egg batches of individuals that copulated once at the beginning of the reproductive season (a seasonal decrease in clutch size was also observed in *A. arbustorum* kept in field cages; Baur, 1990a). Repeated copulation, however, was found to increase clutch size, while courtship display did not affect egg production. Repeated copulation neither accelerated the onset of egg laying nor increased the hatching success of eggs. These results suggest that reciprocal intromission and/or receipt of a spermatophore, but not the long-lasting courtship behavior, stimulates egg production in *A. arbustorum* (Baur & Baur, 1992b). Hence, the female function of *A. arbustorum* may have multiple benefits from receiving additional sperm such as (1) reproductive assurance (Chen and Baur, 1993); (2) inbreeding avoidance (B. Baur & A. Baur, 1997; Chen, 1994); (3) increased genetic diversity among offspring (Kupfernagel et al., 2010); (4) the opportunity of cryptic female choice when receiving sperm from different mates (Baur, 1994b); (5) energy and nutrients

gained from resorbing sperm and spermatophore; and/or (6) stimulation of egg production.

9.7 SOCIAL FACILITATION

In a variety of animal species, individuals are sensitive to the presence of conspecifics in their close surroundings. Some aspects of their behavior, metabolism, growth, and/or reproduction may change in response to the number of conspecifics in their proximity, a phenomenon called "social facilitation." In stylommatophoran snails, social facilitation may occur when self-fertilizing individuals produce more offspring in the presence of conspecifics than in their absence. Information on social facilitation in stylommatophoran gastropods is so far restricted to a single species.

Baur and Baur (2000) kept individuals of the clausilid *B. perversa* singly or in pairs over two generations. All snails reproduced exclusively by self-fertilization (Baur & Baur, 2000; Wirth et al., 1997). Snails kept isolated throughout their life had a reduced lifetime fecundity (in the parent generation) and longevity (in both generations) compared with snails kept in pairs. Thus, the lower reproductive output of isolated snails is not attributable to self-fertilization per se. If inbreeding would be the only factor reducing the reproductive output of isolated snails, both groups should have similar reproductive outputs. This suggests that social facilitation may affect longevity in self-fertilizing *B. perversa* (Baur & Baur, 2000).

9.8 SEX ALLOCATION

Sex allocation theory in simultaneous hermaphrodites predicts that individuals have a fixed amount of reproductive resources to allocate to either of the sexual functions and that they trade off their allocation to either sex depending on environmental conditions (Charnov, 1982). Individuals of *A. arbustorum* invest a significant proportion of their energy resources into the female function (Baur, 1994; Baur & Baur, 1998). However, the investment in eggs also varies among populations and within reproductive season. Reproductive traits and nitrogen and carbon concentrations of eggs were assessed in eight populations of *A. arbustorum* distributed over an elevational gradient from 370 m to 2340 m above sea level in Switzerland (Baur & Baur, 1998). Egg dry mass, nitrogen concentration in eggs, clutch size and reproductive investment in an egg batch (clutch size x egg dry mass)

decreased with increasing elevation. In contrast, no elevational variation in carbon concentration was found in *A. arbustorum* eggs. Under laboratory conditions, individuals of *A. arbustorum* deposited four to seven egg batches within a reproductive season (Baur & Baur, 1997). Clutch size tended to decrease in successive batches. Egg size increased over the reproductive season in a lowland population, whereas no seasonal change was observed in a subalpine and an alpine population. In a field experiment maintaining individuals of *A. arbustorum* in cages at different elevations in the Swiss Alps, the reproductive investment per egg (measured as egg dry weight per 100 mg snail dry weight) increased with elevation from 0.57 at 1220 m to 0.95 at 2600 m (Baur & Raboud, 1988). Thus, one egg represents a higher investment for a mountain snail than for a valley snail.

Sex allocation theory predicts that both mating frequency and long-term sperm storage affect the relative allocation to male and female function in simultaneous hermaphrodites. Locher and Baur (2000b) examined the effect of mating frequency on male and female reproductive output in *A. arbustorum*. Resource allocation to gamete production (eggs vs. sperm and spermatophore) expressed as dry mass, nitrogen, or carbon content was highly female-biased (>95% in all estimates). With increasing number of copulations, the relative nitrogen allocation to the male function increased from 1.7% (one copulation) to 4.7% (three copulations). At the individual level, a positive correlation between the resources allocated to the male and female function was found, contradicting current theory of sex allocation. Snails that delivered many sperm also produced a large number of eggs. Similarly, Minoretti et al. (2011) found that female reproductive success (number of hatchlings emerging from the eggs laid by a snail) was positively correlated with male reproductive success (number of hatchlings sired by that snail) in *A. arbustorum*. Both female and male reproductive success were determined by the individual's activity and positively influenced by the snail's degree of genetic heterozygosity. It may be difficult to demonstrate the predicted trade-off in stylommatophoran snails when the resource allocation is highly biased in favor of one sexual function, and when the mating frequency is relatively low.

Locher and Baur (2002) examined the effect of nutritional stress (extremely restricted [25%], restricted [50%] and ample [100%] food supply) on the mating behavior and male and female reproductive output. Independent of the extent of nutritional stress, 10–12% of the resources taken up were invested in reproductive output (both sexual functions together) and 88–90% in maintenance (including feces and excretion). Courtship and copulation behavior was affected by nutritional stress. Snails with an

extremely restricted food supply did not mate. Snails kept under nutritional stress invested relatively more resources into the male than the female function. Nevertheless, the absolute reproductive output remained highly female biased (>95% in all experimental groups).

In hermaphroditic snails, a reduced protein and calcium supply might affect growth and alter the allocation to either sexual function. Wacker and Baur (2004) tested this hypothesis by maintaining subadult *A. arbustorum* on artificial diets composed of single compounds (particular amino acids, carbohydrates, fatty acids, minerals, vitamins) on an agar-based diet. Snails fed a high protein diet grew faster and reached adulthood earlier. Different calcium contents did not affect shell growth, but increased mortality when the calcium content of the food was low. Furthermore, diet-related differences in mating propensity were found (Wacker & Baur, 2004). A transplant experiment indicated that the type of natural substrate (soil type) can also influence the female reproductive output but not the male reproductive output in *A. arbustorum* (Baur et al., 2009).

9.8.1 TEMPORAL PATTERNS OF SEX ALLOCATION

Janssen and Baur (2015) examined temporal patterns of sex-specific reproduction in a natural population of *A. arbustorum*. Adult and premature individuals (snails in a short protandric phase) were collected on four occasions over the activity season. In their male function, individuals were active throughout the reproductive season, whereas egg production—the major task of the female function—was restricted to the first half of the season. Individuals that became sexually mature later in the reproductive season began to mate, but did not start to produce eggs before emerging from hibernation. These findings indicate that the reproductive behavior of *A. arbustorum* is adapted to seasonal characteristics.

In general, egg production in stylommatophoran snails is influenced by the photoperiod; decreasing day length results in a reduction and finally complete stop of egg laying (Gomot de Vaufleury, 2001). Thus, snails do not produce offspring shortly before hibernation and in this way save energy for overwintering. In temperate regions, winter mortality of juvenile snails is negatively correlated with their shell size (in *A. arbustorum*: Terhivuo, 1978; Andreassen, 1981; in *B. perversa*: Baur & Baur, 1991). Hatchlings emerging from eggs deposited early in the season have more time for growth until hibernation than those hatching from eggs deposited later (Baur, 1990b). Individuals of *A. arbustorum* are not able to hibernate in the egg stage.

9.9 PARASITE-MEDIATED CHANGES IN REPRODUCTION

Parasites are known to influence life-history traits and the behavior of their hosts (Moore, 2002). Parasite infection may induce both short-term physiological and evolutionary responses. As a result of co-evolutionary processes, reduced individual fecundity and survival of the host may lead to an increase in the reproductive effort (Gandon et al., 2002) and favor early reproduction in host populations. However, the effects of parasite pressure on host populations strongly depend on environmental conditions and on attributes of the parasite. Parasites frequently influence the activity and locomotion of their hosts, which may result in both reduced dispersal and foraging activity, and decreased predator avoidance (Goater et al., 1993). A reduced activity in turn can lead to a decreased growth rate.

In stylommatophoran snails, various nematode species are transmitted during mating (Morand, 1989; Morand & Baker, 1995; Morand & Hommay, 1990; Ribas & Casanova, 2002). High loads of parasites reduce egg production but do not affect survivorship of host snails.

The hematophageous mite *Riccardoella limacum* lives in the pulmonary cavity of stylommatophoran snails (Baker, 1970a,b; Fain & Van Goethem, 1986; Mienis, 1990). The mites feed on blood in the lung of their host, but frequently leave the pulmonary cavity through the respiratory pore and move on the host's soft body (Graham et al., 1996). Heavy infestations with *R. limacum* reduce growth rate and considerably delay reproductive development of the snail *C. aspersum* (Graham, 1994; Graham et al., 1996). These adverse effects can seriously influence the economy of commercial snail farming (Graham et al., 1996). In *A. arbustorum*, prevalence of mite infection ranged from 45.8% to 77.8% in four natural populations, while in seven other populations no infected snails were found (Baur & Baur, 2005). Intensity of infection also differed among the four host populations. Parasitic mites did not occur in snail populations situated at elevations of 1290 m or higher in the Swiss mountains (Baur & Baur, 2005). It has been hypothesized that the host's hibernation period at high elevation might be too long for mites and their eggs to survive. Haeussler at al. (2012) tested this hypothesis by allowing experimentally infected individuals of *A. arbustorum* to hibernate at 4°C for periods of 4–7 months. The intensity of mite infection decreased with increasing hibernation duration. A further experiment revealed that *R. limacum* survives the winter in the egg stage in the host (Haeussler et al., 2012). Thus, long periods of low temperature at high elevation may limit the occurrence of *R. limacum*.

Naturally infected *A. arbustorum* collected in the wild showed a decreased activity and a reduced reproductive output (number of eggs deposited) compared with uninfected snails (Schüpbach & Baur, 2008a). Winter survival was reduced in infected snails in two out of three populations. Furthermore, experimentally infected snails from an uninfected population showed a reduced winter survival compared to control snails. It has been assumed that the parasitic mite is transmitted during courtship and mating of the host. However, Schüpbach and Baur (2008b) presented experimental evidence that *R. limacum* can also be successfully transmitted via soil without physical contact among hosts. Further experiments revealed that parasitic mites show a preference to move on fresh snail mucus (Schüpbach & Baur, 2008b), and that in infected individuals of *A. arbustorum*, parasite load was affected by family origin and increased with increasing shell size of snails (Schüpbach & Baur, 2010a). Estimated heritability of parasite load was 0.63 (Schüpbach & Baur, 2010a). Another experiment showed that the transmission success of *R. limacum* is mainly affected by physical contacts among snails and slightly influenced by parasite intensity of the infected snails (Schüpbach & Baur, 2010b).

9.10 STRATEGIES TO INCREASE EGG AND HATCHLING SURVIVAL

9.10.1 OVIPARITY

The majority of stylommatophorans are oviparous with eggs laid singly or in batches; embryogenesis occurs after oviposition. All oviparous stylommatophorans thus far examined deposit individual eggs (i.e., each ovum is surrounded by its own egg shell or distinct jelly layer) and not egg masses or capsules, as occur in the freshwater pulmonates and in most marine prosobranchs (Tompa, 1976). Eggs of stylommatophorans are cleidoic; they contain all the nutrients and trace elements needed for a successful embryonic life and have direct development (Tompa, 1980b).

Parental investment may often be critical to the survival and growth of young, but the larger the investment per offspring, the lower the number of offspring that can be produced (Stearns, 1989). In many stylommatophoran species, egg size is negatively correlated with the number of eggs produced (Baur, 1994; Heller, 2001; Kramarenko, 2013), while hatchling size is positively correlated with egg size (e.g., in *C. nemoralis* and *A. arbustorum*; Baur, 2007; Wolda, 1963). Thus, a larger parental investment in single eggs

results in larger hatchlings. Large hatchlings, in turn, may enjoy an enhanced survivorship compared to small hatchlings. For example, large hatchlings of *Strophocheilus oblongus* more frequently survived immediate posthatching starvation than small ones kept under identical conditions (Tompa, 1984). However, hatchlings of *A. arbustorum* emerging from larger eggs have also a longer developmental period than those from smaller eggs (Baur, 2007).

In stylommatophoran gastropods, the among-species variation in egg size is pronounced ranging from 0.5 mm (in *Punctum pygmaeum* with an adult shell width of 1.5 mm; Baur, 1989) to 50 mm (in the giant South American snail *Strophocheilus (Borus) popelairianus* with a shell length of 15–23 cm; Bequaert, 1948). There is, however, a large variation in egg size even within species. In *A. arbustorum*, egg size varies among populations and in the course of the reproductive season (A. Baur & B. Baur, 1997, 1998). On mountain slopes, egg size of *A. arbustorum* decreased with increasing elevation of the population but this effect disappeared when the shell size of the parent snail was accounted for (Baur & Baur, 1998). Within batch eggs vary little in size (Baur, 1994).

Stylommatophoran eggs contain calcium carbonate which is used for the calcification of the embryonic shell and for the deposition of calcium reserves in the first calcium cells which differentiate during the embryonic life (Crowell, 1973; Fournié & Chétail, 1984). Developing embryos resorb calcium from their egg shells (Tompa, 1976), and in some species the hatchlings eat their own egg shell and those of unhatched siblings (see below). Protein concentrations in freshly laid eggs ranged from 14.2% of their dry weight in *H. pomatia* (Alyakrinskaya, 1981) to 25.5% in *A. arbustorum* (Baur, 1994) and 38.8% in *Sphincterochila zonata* (formerly *S. boissieri*) (Yom-Tov, 1971). In eggs of *A. arbustorum*, the protein concentration decreased in successive batches in two of three populations examined as did carbon concentration in one of the populations, indicating that the trade-off between batch size and egg size can be influenced by changes in the nutrient concentration and energy content of eggs (A. Baur & B. Baur, 1997). Furthermore, nitrogen concentration of eggs decreased with increasing elevation of the population, an effect that remained when parental snail size was corrected for (Baur & Baur, 1998).

There is also a huge intraspecific variation in number and size of egg batches produced by an individual snail (Heller, 2001; Kramarenko, 2013). This variation is mainly influenced by the shell size of the mother snail, the seasonality in climate including periods of drought, and by other factors such as intra- and interspecific competition (Baur, 1990; Baur & Baur, 1990; Carter & Ashdown, 1984; Wolda, 1967; Wolda & Kreulen, 1973). Snails of small size produce only a few eggs at any time, and deposit them singly

(e.g., six eggs in *Punctum pygmaeum* during an average life span of 170 days; Baur, 1989). Larger gastropods produce many more eggs during their lifetime: *Deroceras reticulatum* up to 500 eggs (Carrick, 1938), *A. arbustorum* 800 eggs (Baur & Raboud, 1988) and the veronicellid slug *Vaginulus borellians* over 1300 eggs (Runham & Hunter, 1970).

9.10.2 EGG RETENTION

As a modification of simple oviparity, the eggs may be retained within the female reproductive tract for periods of different length, resulting in a shorter time from laying to hatching (hereafter called egg retention). Egg retention is a simple form of parental care, which increases the survival of eggs. Egg retention allows successful reproduction in harsh environments such as alpine or dry regions, where the reproductive season is short and often interrupted by unfavorable weather. If the conditions for oviposition become favorable (e.g., the soil is moist or soft enough to allow a hole to be excavated for a nest), then the snails release their eggs immediately, as any oviparous snails do. For example, *L. martensiana* retains its eggs when it estivates during the dry season in Central Africa (Owiny, 1974). At the beginning of the rainy season, eggs and young are immediately deposited, ensuring them the best prospects of survival. In the clausilid *Balea (Pseudalina) fallax*, 60% of the adults collected in June retained 3–17 eggs with developing embryos, while in other months egg-retaining snails constituted less than 10% of the sample (Sulikowska-Drozd et al., 2012).

Although most stylommatophorans are oviparous, there is at least one species with egg retention or ovoviviparity in each of 30 of the approximately 80 families (Maltz & Sulikowska-Drozd, 2008; Sulikowska-Drozd et al., 2012; Sulikowska-Drozd, 2009; Tompa, 1979a). Variations in pattern of egg retention and development are virtually continuous, but a division into oviparous species, species with egg retention, ovoviviparous and viviparous species is generally accepted (Solem, 1972). Egg retention and ovoviviparity have evolved independently several times in stylommatophoran snails (Tompa, 1979a,b).

9.10.3 OVOVIVIPARITY AND VIVIPARITY

In ovoviviparous species, eggs are retained within the female reproductive tract for the entire embryonic period. Thus, ovoviviparity is the most

extreme form of egg retention. Hatching may occur just after oviposition, or young may hatch from the egg inside the female reproductive tract followed by birth (Baur, 1994a). In ovoviviparous species, the eggs are arranged in succession in the reproductive tract of the mother. The egg shell is resorbed by the parent or consumed by the embryo, which uses the calcium carbonate to build up its own shell.

Ovoviviparity occurs in several families of the Stylommatophora (Maltz & Sulikowska-Drozd, 2008; Sulikowska-Drozd, 2009). Partulidae are tree-living, ovoviviparous snails occurring on islands in the Pacific. In these snails, the egg shell is resorbed by the parent before birth (Murray and Clarke, 1966). The rock-dwelling pupillid *Lauria cylindricea* is ovoviviparous. In Israel, up to 100% of the adults contained eggs with embryos, usually four per individual (Heller et al., 1997). A reproductive adult weighed about 4.5 mg, of which 17–25% was embryo weight. Ovoviviparity allows terrestrial gastropods to persist in habitats otherwise unsuitable for oviparous species due to the lack of oviposition sites (e.g., *Pyramidula rupestris*, *B. perversa* on exposed rock walls; Baur, 1990), or highly variable environmental conditions including periods of drought. Desiccation of eggs causes most of the mortality in the tropical snail *L. martensiana* (Owiny, 1974). In this case, ovoviviparity could have evolved in response to selection pressures such as irregular starts of the rainy season (Peake, 1978). Heller (2001) found that ovoviviparity mainly occurs in small-sized snail species. Minute snails are constrained to low fecundity: they can produce only few ova per unit time. Under such conditions, ovoviviparity might be advantageous in that the (few) hatchlings can immediately feed and grow, fight off fungi and cope with periods of drought or avoid flooding (Heller, 2001).

Egg-retention and ovoviviparity also represent costs to the animals. Both clutch and hatchling size scale allometrically with adult shell size in land snails from Northwestern and Central Europe (Bengtsson & Baur, 1993). When the potentially confounding effects of shell size are removed (by considering the residuals from clutch size, mother shell volume and hatchling size, mother shell volume regressions), egg-retaining and ovoviviparous species produce significantly smaller clutches than oviparous species (Baur, 1994a). On the other hand, hatchling size does not differ between egg-retaining/ovoviviparous and oviparous species (Baur, 1994a). This indicates a cost of retaining eggs or young in the reproductive tract in terms of a reduced fecundity.

In some species, reproduction can either be oviparous, egg-retaining or ovoviviparous (Owen, 1965; Peake, 1978). For example, individuals of the clausilid *Lacinaria biplicata* are usually ovoviviparous, but under favorable

environmental conditions they lay eggs with well-developed embryos (Falkner, 1990).

In viviparous species, there is a transfer of nutritional material from the parent to the developing embryo, which is retained in the female reproductive tract until extrusion as free-living young. There are few reports on viviparity in stylommatophorans. In *Tekoulina pricei*, which occurs in the Cook Islands, five to seven embryos of increasing size were found in the uterine oviduct (Solem, 1972). However, details on the mechanism of nutrition are unknown. Similarly, based on size differences of embryos in the female reproductive tract, the two African snails *Pseudoveronicella zootoca* and *Pseudoveronicella pauliani* are considered as viviparous (Forcart, 1953). In *Achatinella*, the largest embryo found in the female reproductive tract was 92 times the size of a fertilized egg and 11 times that of the smallest embryo, suggesting a form of supplemental nutritive transfer (Solem, 1972).

9.11 OTHER BEHAVIORS THAT INCREASE OFFSPRING SURVIVAL

A snail may increase the survivorship of her offspring by an optimal choice of the oviposition site (Asami & Ohbayashi, 1999), by protecting the eggs against desiccation (Baur, 1988c), heat stress (Nicolai et al., 2013), predators, fungi and bacteria, and/or by providing additional food to the hatchlings (Alexander, 1974).

9.11.1 CHOICE AND PREPARATION OF OVIPOSITION SITES

Egg batches are usually deposited in damp and shaded places (Baur, 1988c). Covering batches with mucus is an important aspect of egg-laying behavior (Kuznik-Kowalska, 1999, 2005, 2006). The mucus reduces desiccation of eggs and functions also as an antibacterial agent (Berniyanti et al., 2007; Fiolka & Witkowski, 2004; Zhong et al., 2013). Numerous oviparous snail species deposit their eggs in excavations, which they dig in moist soil (Tompa, 1984). In *H. pomatia*, nest digging takes about 1 h at a site suitable for egg laying (Perrot, 1938). The cavity is lined with mucus (most probably to protect the eggs against bacteria and fungi). Egg laying lasts 35 h for a 70-egg clutch. After oviposition the nest is covered by soil and abandoned. Snails search actively for suitable oviposition sites. Not all nesting excavations that are initiated will be successfully filled with eggs; many holes are abandoned before one is finally considered suitable. Apart from

suitable soil structure, soil moisture content is important for the choice of the oviposition site. In choice tests, individuals of *A. arbustorum* preferred to oviposit in soil of high moisture content and thus reduced the risk of later egg desiccation (Baur, 1988c). The desert snail *S. zonata* (formerly *S. boissieri*) digs a 3–4 cm deep hole and lays its eggs in a mucid saccule that hangs in the cavity without touching the wall (Yom-Tov, 1971). The tropical snail *Archachatina (Calachatina) marginata* digs the 10–15 cm deep cavity for oviposition in the course of one night (Plummer, 1975). Sometimes it takes two nights to complete the preparation of the oviposition site and egg laying, most probably due to the hardness of the soil or to disturbance by other snails.

Individuals of the West African tree snail *Pseudachatina downesii* and those of other arboreal species lay their eggs in the axils of branches of tree upon which they live (Standen, 1917). Several species of tree-living snails lay their eggs in brood chambers made of leaves. In Celebes, tree snails of the genus *Cochlostyla* deposit their eggs in leaves rolled up into a cornucopia, stuck together with mucus, and lined with mucus (Sarasin & Sarasin, 1899). The Javan tree snail *Amphidromus purus* also forms nests for their eggs by plastering leaves together (Paravicini, 1921). Nest preparation and oviposition lasts 4–8 days in this species. Having finished egg laying (up to 234 eggs of 3-mm diameter are put in one nest), the envelope is sealed with mucus and the snail drops from the tree.

9.11.2 POST-LAYING CARE OF EGGS

There are few studies reporting on post-laying care of eggs in stylommatophoran gastropods. Several Endodontid species exhibit a most-sophisticated form of parental care. On the Cook Islands, the endodontid *Libera fratercula* and its congeners brood 4–6 eggs in the umbilicus of the shell, which forms a pouch-like cavity (Solem, 1969, 1976). In some species of this genus, eggs and newly hatched snails are retained by a temporary shell plate, which partly covers the umbilicus. This plate is broken away or absorbed by the mother to release the young under favorable environmental conditions. A kind of incipient parental care has been observed in laboratory breedings of the endodontid *Discus rotundatus*, *D. ruderatus*, and *D. perspectivus* (Kuznik-Kowalska & Pokryszko, 2007). All three species of *Discus* lay batches of 1–6 relatively large (diameter 1.5 mm), calcified eggs. Having completed oviposition, the parent crawls over the batch, covering it with mucus (Kuznik-Kowalska, 1999, 2005, 2006). In 7–28% of instances of

egg-laying, one to four eggs were observed to stick in the umbilicus during the mucus-covering process, and were subsequently carried by the parent for a variable period of time (in most cases 15 min–4 days). In some cases the eggs were carried till hatching (in 4.6% of the egg-laying of *D. ruderatus* and in 2.3% of *D. rotundatus*).

Stylommatophoran gastropods estivate during periods of drought, often exposed to wind and insolation. In these species, eggs carried on the shell would desiccate (Riddle, 1983). In terrestrial habitats, the low moisture content of the air may select against egg carrying behavior. Instead, it may select for strategies protecting eggs such as egg retention and ovoviviparity.

9.11.3 EGG CANNIBALISM OF HATCHLINGS

Hatchlings of various species of herbivorous stylommatophoran gastropods cannibalize eggs (Baur, 1992b; Desbuquois, 1997; Desbuquois & Madec, 1998; Desbuquois et al., 2000; Kuznik-Kowalska, 1999, 2005, 2006; Maltz & Sulikowska-Drozd, 2008; Shen, 1995). Emerging *A. arbustorum* first eat their own egg shells and then the eggs of unhatched siblings, including those with fully developed embryos (Baur & Baur, 1986). Egg cannibalism is restricted to the hatchling stage; the hatchlings' propensity for eating eggs decreases with increasing age (Baur, 1987a). Food choice tests showed that new hatched individuals of *A. arbustorum* with no prior feeding experience fed exclusively on eggs, four-day-old snails ate eggs and lettuce in equal proportions, while 16-day-old animals preferred lettuce (Baur, 1987a). Individuals older than 4 weeks fed exclusively on lettuce; even in the absence of vegetable food, individuals will starve rather than eat conspecific eggs. Cannibalistic hatchlings eat only conspecific eggs and do not discriminate between sibling and non-sibling eggs (i.e., eggs from neighboring batches; Baur, 1987b, 1988d, 1993).

The extent of intra-batch egg cannibalism depends on the hatchlings' propensity for egg cannibalism and on the hatching asynchrony of the young within a batch, which in turn is influenced by environmental factors (e.g., microhabitat differences in temperature and moisture; Baur & Baur, 1986; Baur, 1994c). Under unfavorable environmental conditions, hatching asynchrony increases and thus provides a few of the hatchlings with the opportunity for egg cannibalism. A cannibalistic diet during the hatchling stage results in accelerated growth and higher survival (Baur, 1990b,c, 1992b). Selection favors egg cannibalism if the potential reduction of the mother snail's total fecundity due to sibling cannibalism is compensated for by the

increased survivorship of her cannibalistic progeny. Thus, sibling cannibalism can be favored when the fitness benefits outweigh the fitness costs of this (otherwise counterintuitive) behavior (Baur, 1987b). In *A. arbustorum*, most egg batches are sired by 3–5 fathers (Kupfernagel et al., 2010; see above). Thus, within-batch cannibalism frequently relates to half sibs, which reduces the inclusive fitness costs (Baur, 1987b). Furthermore, not all eggs within a batch hatch; indeed, some eggs are not fertilized and in others the embryos die during development. Eggs that fail to hatch can serve as "trophic" eggs. Alexander (1974) suggested that if parents are unable to increase their investment in young through increasing egg size, an alternative strategy is to increase clutch size and allow some siblings to consume others (the icebox effect). The optimum clutch size can be found by calculating the clutch size leading to maximum brood productivity, taking into account the effects of sibling cannibalism and possible trade-offs. In *A. arbustorum* and several other stylommatophoran species, egg cannibalism can be considered a kind of facultative food provisioning that becomes most important under harsh environmental conditions.

9.12 CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

This review provides insight into the enormous variation in reproductive characters and behavioral patterns in stylommatophoran gastropods and indicates potential consequences for fertilization and sperm competition. Sexual selection plays an important role in hermaphrodite evolution (Schilthuizen, 2001). As long as sperm are cheap and eggs are the limiting factor, selection will favor those individuals with male reproductive systems that enhance the success of autosperm (i.e., their own, outgoing sperm), and with female genitalia that can select from the foreign, incoming allosperm (Schilthuizen, 2005). Although the need for compromises within a single gastropod can place limits on the strength of sexual selection (Greeff & Michiels, 1999), the abundance and diversity of extreme reproductive measures found in hermaphroditic animals suggest that sexual and sexually antagonistic selection are important factors for their evolution (Michiels, 1998). For example, sexual conflict may play a key role in the evolution of dart shooting in stylommatophoran gastropods (Chase, 2007). The dart shooter manipulates its partner to increase allosperm storage and paternity by the transfer of an allohormone, while the sperm (and dart) recipient tries to be selective in allosperm storage and use. Another sexual conflict concerns the allocation

of reproductive resources to the male and female function in hermaphroditic individuals (Locher & Baur, 2000b, 2002).

In stylommatophoran gastropods, almost all reproductive characters appear to be shaped by both natural and sexual selection (Baur, 2010), and there is increasing evidence that intersexual counter-adaptations may drive correlated reproductive character evolution in this group (Beese et al., 2006a; Davison et al., 2005; Koene & Schulenburg, 2005). Behaviors and traits to enhance survival of both eggs and hatchlings—the other important component of reproductive strategies—are shaped by natural selection. In an evolutionary context, two different solutions to the problem of high egg and hatchling mortality can be recognized in stylommatophoran species: reducing embryonic development time in the unprotected egg stage by egg retention and ovoviviparity and food provision to the hatchlings. Stylommatophoran gastropods produce no nurse eggs (unfertilized eggs as food provision) as marine prosobranch species do (Baur, 1992b). Instead, there is facultative egg cannibalism, which increases the survival probability of a reduced number of juveniles under less favorable conditions.

9.12.1 SUGGESTIONS FOR FUTURE RESEARCH

This review indicates that several stylommatophoran species are well suited for studies on the evolution of reproductive traits, sexual conflicts and sperm competition. However, the current knowledge is mainly based on a few model species (*C. aspersum*, *A. arbustorum*, *S. putris*). Consequently, future studies should consider a wider range of species. Furthermore, there are many topics that remain largely unexplored. Beyond studies on sexual conflicts the following aspects deserve attention:

- Patterns of sperm transfer and use are critically important in understanding the evolution of mating strategies in stylommatophoran species. Different (condition-dependent) frequencies of self-fertilization make this topic to a most challenging project. The use of molecular techniques allows estimates of the frequency of self-fertilization even in natural snail populations.
- The adaptive significance of variation in sperm characters such as length and swimming velocity is still not known. Careful studies of the morphology of the female reproductive tract with respect to allosperm storage and mating experiments using molecular markers for paternity analyses should prove to be particularly rewarding.

Furthermore, understanding the diversity of sperm morphology requires consideration of the complex functional demands on sperm. In stylommatophoran gastropods, sperm may perform a multitude of tasks including mobility to reach the storage organ and later the egg, transmission of paternal DNA, surviving prolonged storage within the spermatheca, and competing with sperm from other snails.

- In the immobile egg stage, embryos face the risks of desiccation, predation, parasitation, and pathogen infection. Which natural products in the egg shell or in the mucus coated around the eggs play a role in the defense and protection? More detailed investigations on protective compounds in eggs of terrestrial gastropods and in their mucus are urgently needed.
- Rigorous comparative studies across many species combined with phylogenetic reconstruction may help to gain a better understanding of reproductive strategies in hermaphroditic gastropods.

There is much to be learned in this most interesting animal group. Having two sexes makes hermaphrodites twice as interesting (Schilthuizen, 2005).

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- **adaptations**
- **gastropods**
- **spermatheca**
- **albumen gland**
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CHAPTER 10

PHYSIOLOGICAL FUNCTIONS OF GASTROPOD PEPTIDES AND NEUROTRANSMITTERS

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ABSTRACT

Among extracellular chemical messengers, peptides and neurotransmitters are involved in controlling almost every physiological process in gastropod molluscs. Dozens of novel peptides and structurally similar peptides to those present in vertebrates have been identified in gastropods. However, except for a few instances, there is limited information regarding the control of synthesis, release and mode of action for any single peptide or neurotransmitter in molluscs. Therefore, this review will focus on the functions of selected peptides and neurotransmitters in several gastropod models, namely *Aplysia*, *Lymnaea*, *Helisoma*, *Biomphalaria*, *Helix*, and *Achatina*. The physiological processes that are emphasized in some detail include reproduction, growth, hydromineral balance, and the control of muscular and ciliary activities.

The physiology of reproduction focuses mainly on the neuroendocrine regulation of the female reproductive system. The synthesis, processing, and the control of secretion of the egg-laying hormone and its related peptides from the neurosecretory bag cells of *Aplysia* is highlighted, as well as recent studies on the regulation of bag cell excitability. Vertebrate GnRH-related peptides have been characterized in several molluscan species but their role in the regulation of reproductive activities in gastropods is unclear. The effect of peptides and amines on the secretory activity of the albumen gland, a female accessory sex gland, is also covered. In gastropods, there are conserved neuronal populations in the central nervous system which control male mating behavior. Characterized peptides as well as some novel neuropeptides are thought to regulate mating behavior through their actions on neuronal activity and the musculature of the male reproductive system.

The regulation of growth and metabolism in aquatic and terrestrial snails is discussed with respect to peptides produced by neuroendocrine cells of the central nervous system. In *Lymnaea*, several insulin-related peptides have been identified in the putative growth-controlling light green cells. Similarly, neuroendocrine cells in other gastropods have also been shown to produce insulin-like peptides and the receptors for these peptides have been cloned and sequenced. There is a growing body of evidence suggesting that insulin-like peptides regulate soft tissue growth and metabolism in gastropods, as well as having other novel physiological functions.

Peptides or neurotransmitters can act directly on muscle cells to induce contraction or relaxation. Many novel cardioexcitatory and cardioinhibitory peptides have been isolated from molluscan hearts or nervous tissues using sensitive bioassays coupled with biochemical techniques. The regulation

of cardiac activity has important implications for the distribution of hemolymph in the body as well as contributing to locomotion and osmoregulation.

The characterization of various osmoregulatory peptides and neurotransmitters in freshwater and marine snails has provided insight into the physiological mechanisms that regulate hydromineral balance in gastropods. Finally, the function of serotonin in controlling ciliary beat frequency in embryos of freshwater pulmonates has an important adaptive function which allows embryonic snails to survive prolonged periods of hypoxia or anoxia.

10.1 INTRODUCTION

10.1.1 WHY STUDY GASTROPOD PEPTIDES AND NEUROTRANSMITTERS?

Among the Mollusca, the Gastropoda is the largest and most diverse class, containing an estimated 40,000–150,000 species which constitute about 85% of all described molluscs (Haszprunar and Wanninger, 2012; Ponder and Lindberg, 2008). Besides being the most numerous molluscan group, there are several important reasons why regulatory peptides and neurotransmitters are the subject of intensive research in this group. From a scientific standpoint, many gastropod molluscs have large, identifiable neurons, and their anatomically simpler nervous systems are highly amenable for the study of peptide structure and function. For example, the study of egg-laying-inducing neuropeptides in *Aplysia californica* and *Lymnaea stagnalis* has uncovered important cellular mechanisms involved in neuropeptide biosynthesis, processing, transport and release (for excellent reviews see Arch and Berry, 1989; Nagle et al., 1989a; Sossin et al., 1990a,b). Other studies in bivalves and in gastropods have identified novel peptides involved in shell repair and biomineralization (Mann et al., 2012; Werner et al., 2013; Zhao et al., 2012a).

Gastropod molluscs are of economic importance to humans as well. In many regions of the world, abalone, conch, escargot, periwinkles, and whelks are considered delicacies. They are also and provide income to local human populations that harvest them. Studies focused on peptides and neurotransmitters governing growth and reproduction may have direct applications to molluscan aquaculture and food security. In addition, many novel chemicals which have potential biomedical or commercial value have been discovered in gastropods (Cimino and Gavagnin, 2006; Benkendorff, 2010). For example, numerous peptide venoms from cone snails that modulate ion

channels have been isolated and identified as sources of novel medicinal or research chemicals (Dang et al., 2015; Vetter and Lewis, 2012).

In various regions of the world, terrestrial slugs and snails are major agricultural and horticultural pests. Grazing by slugs is known to damage or to reduce the yield of crops such as canola, soybeans, cereals, and legumes, whereas terrestrial snails such as *Helix aspersa* are a major pest in citrus orchards (Barker, 2002). Some freshwater pulmonates are intermediate hosts of trematode parasites which infect numerous mammalian species, including humans, and thus are of direct significance to human health. Freshwater pulmonates such as *Biomphalaria glabrata*, *Bulinus truncatus*, and *Oncomelania hupensis* are intermediate hosts of *Schistosoma* spp., whereas several species of freshwater snails can serve as hosts for other parasitic flukes such as *Fasciola hepatica* and *Clonorchis sinensis* (Roberts et al., 2013). Therefore, the study of peptides and neurotransmitters in these snails may lead to the development of strategies aimed at halting the transmission of diseases such as schistosomiasis and fasciolosis.

Ecologically, terrestrial and aquatic gastropods are an important part of numerous ecosystems as they have large influences over both animal and plant communities (Russell-Hunter, 1983). Because of their importance in both terrestrial and aquatic ecosystems and their sensitivity to pollutants, perturbation of chemical signaling molecules of the gastropod neuroendocrine system has been used as a bioindicator of environmental health (Lagadic et al., 2007; Oehlmann et al., 2007). Finally, many molluscan species are experiencing global decline so a better understanding of the peptides and neurotransmitters regulating physiological processes is important for the conservation and maintenance of biodiversity (Harley, 2011; Lydeard et al., 2004).

10.1.2 PEPTIDES

Animal cells can receive information from the external and internal environment in the form of light–dark cues, nutrients, temperature, water, and ion concentrations, and then process this information to coordinate cellular activities in the whole organism. The communication of information between animal cells is an essential activity that maintains homeostasis. The two major systems that maintain homeostasis in animals are the nervous and endocrine systems. The cells that belong to these two systems communicate with one another is through chemical signaling molecules such as peptides and neurotransmitters.

Peptides are the largest and most diverse group of extracellular signaling molecules, and probably represent an evolutionarily ancient form of chemical communication. For example, in the Cnidaria, peptides are utilized as the main form of chemical communication during development (Grimmelikhuijzen et al., 1996). In invertebrates, many peptide signaling molecules are synthesized and secreted from neurons where they can act on target cells as neurotransmitters, neuromodulators, or neurohormones (Altstein and Nässel, 2010; Kiss, 2011; Thorndyke and Goldsworthy, 1988). Peptides are generally grouped together based on the structural similarities of their primary amino acid sequences (e.g., RFamide-like peptides, insulin-like peptides, myomodulin-like peptides). However, peptides with structural similarities can often have different functions in the same animal. In addition, peptide nomenclature can sometimes be confusing as the peptide's name may not reflect its main physiological function as many peptides are assigned a name based on the functional assay that was originally used to characterize the peptide. In some cases, peptides may even have the same acronym (e.g., MIP-*m*olluscan *i*nsulin-related *p*eptide or *Mytilus i*nhibitory *p*eptide).

10.1.3 METHODS USED TO IDENTIFY AND ISOLATE MOLLUSCAN PEPTIDES

Since the publication of the Physiology series of "The Mollusca" (Volumes 4 and 5) more than 30 years ago, there have been enormous advances in peptide biochemistry which has enabled the isolation and identification of an unprecedented number of novel peptides from molluscs. In general, there are two different ways to isolate and identify peptides—the first is a "function first or forward approach", and the second is a "peptide first or reverse approach". The function first approach seeks to purify a peptide based on its physiological activity in a bioassay. Historically, the bioassay is the oldest method used to identify biologically active peptides. It is generally very reliable since it detects activity based on physiological function. Bioassays can be performed on whole animals (in vivo bioassay) such as the induction of egg-laying in *A. californica* by crude abdominal ganglion extracts (Kupfermann, 1967) or in vitro using isolated cells, tissues, or whole organs in culture.

The in vitro bioassays provide greater sensitivity and have high throughput compared to in vivo assays; however, they do not reflect the physiological complexity of the entire animal. An example of an in vitro bioassay that is highly successful in isolating novel myotropic peptides is the effect of peptide-containing tissue extracts on pieces of muscle attached to a sensitive

force transducer (Kuroki et al., 1990; Muneoka and Kobayashi, 1992). Typically, cell or tissue extracts are fractionated by chromatography (e.g., high-performance liquid chromatography, HPLC) and then the separated fractions are tested in the bioassay for physiological activity. Additional rounds of chromatography coupled with further bioassays are performed until a chromatographically pure peptide fraction is obtained for sequence analysis. The first molluscan neuropeptide isolated in such a fashion was the cardioexcitatory peptide FMRFamide (Price and Greenberg, 1977).

The peptide first or reverse approach typically seeks to identify the primary sequences of peptides in a cell or tissue extract, and then determine what their functions are. A commonly used approach is to identify molluscan peptides employs radioimmunoassay (RIAs) or enzyme-linked immunosorbent assays directed against the amidated C-terminus of peptides, since α-amidation is a common chemical modification of secreted peptides. These immunological detection methods are usually combined with chromatographic separation of cell or tissue extracts and have the ability to identify groups of related peptides in different molluscan species or from different animal pkyla (Greenberg and Price, 1992; Nassel, 1999).

During the 1980s, molecular methods such as the cDNA cloning of precursor proteins enabled the identification of genes encoding the deduced amino acid sequence of peptide precursors (Nambu et al., 1983; Scheller et al., 1982; Taussig and Scheller, 1986). For example, the first insulin-like peptide (ILP) in invertebrates was isolated from the neuroendocrine light green cells (LGCs) of the pond snail *L. stagnalis* using a subtractive hybridization approach (Smit et al., 1988). Furthermore, since the vast majority of peptides bind to cell surface G-protein-coupled receptors (GPCRs), another frequently used strategy involves the cloning of orphan GPCRs, then expressing these receptors in a heterologous expression system. Peptide extracts or purified peptides are then screened for their ability to bind and activate the expressed receptors. A novel cardioactive peptide has been isolated from *L. stagnalis* using this approach (Tensen et al., 1998).

More recently, HPLC or capillary electrophoresis coupled with sensitive techniques such as mass spectrometry allows for the identification of peptides from complex tissue mixtures or from cell releasates in vitro (Croushore et al., 2012; Nemes et al., 2011). Moreover, during the last decade, technical improvements to massively parallel or next-generation sequencing techniques (e.g., Illumina, Roche 454, PacBio) have allowed the entire transcriptome of the cell or tissue to be rapidly sequenced and then in silico predictions of function can be inferred (Puthanveettil et al., 2012; Senatore et al., 2015). Sequences can be matched against a reference genome, if one is available,

or in the case of less studied species, de novo transcriptome assembly is undertaken. In addition, these next generation sequencing methods allow for the quantitation of transcript levels in tissues, and even single cells, under different experimental conditions allowing one to infer function (Chu et al., 2014). Antibodies may also be generated against specific peptide fragments derived from proteomic or RNA sequencing studies, and then localized in cells by immunohistochemistry or used in functional assays (De Haes et al., 2014).

This chapter deals with the physiological functions of selected peptides in gastropod molluscs, as well as the contribution of some classic neurotransmitters such as serotonin and dopamine as it relates to selected physiological processes in gastropods. Since it is nearly impossible to describe all the peptides and neurotransmitters regulating the diverse physiological processes in gastropods, we will focus our attention to those peptides and neurotransmitters where there is a substantial body of evidence pertaining to specific physiological functions, namely reproduction, growth, cardiac function, osmoregulation, and ciliary activity.

10.2 REGULATION OF REPRODUCTION

Reproduction is a process that is essential for the continuation of a species and contributes to genetic diversity in sexually reproducing organisms. The Gastropoda have radiated into varied and diverse habitats which include trees, deserts, lakes, rivers, estuaries, marine intertidal zones, pelagic, and benthic habitats. There is a staggering array of reproductive strategies used by gastropod molluscs, and the organization of the gastropod reproductive system reflects this considerable diversification (Tompa et al., 1984). In gastropods, the Archaeogastropods and Neogastropods are gonochorists. Fertilization occurs externally in Archaeogastropods, whereas in Neogastropods it is internal. By contrast, the pulmonates and opisthobranchs are hermaphroditic, and fertilization occurs internally after the exchange of gametes with conspecifics. Synchronized gamete release is essential for species with external fertilization, hence the maturation of the gonads as well as the accessory sex organs must be synchronized to environmental cues (e.g., photoperiod or temperature), which trigger gamete release. On the other hand, courtship and mating behaviors contribute to successful copulation with conspecifics and are generally associated with species where fertilization is internal.

The neuroendocrine control of egg-laying has been studied extensively in two species of gastropods, the sea hare *A. californica* and the pond snail *L.*

stagnalis. In these two gastropods, and likely in others, egg-laying involves a series well defined physiological processes including ovulation, packaging the fertilized eggs into the egg string or egg mass, and finally oviposition. In addition, specific stereotyped behaviors have been documented during oviposition, which are controlled by neuropeptides (Geraerts et al., 1988). The peptidergic control of egg-laying behavior in *A. californica* will be used as an example, since this the cell and molecular biology of the neuroendocrine system is well understood.

10.2.1 NEUROPEPTIDES REGULATING FEMALE REPRODUCTION IN APLYSIA

Egg laying in *A. californica* and *L. stagnalis* consists of a series of stereotyped behaviors including ovulation, the packaging of fertilized eggs into the egg string/mass, and oviposition. The egg-laying hormone (ELH) and ELH-related peptides such as califin in *Aplysia*, as well as the caudodorsal cell hormone (CDCH) in *Lymnaea* are well studied peptide hormones controlling egg-laying. In particular, there is a long history of investigation on the regulatory actions of ELH and its related peptides on reproduction in *A. californica* that began almost 50 years ago and continues to this day.

10.2.2 THE BAG CELLS AND ATRIAL GLAND

In *Aplysia*, two distinct secretory centers are known to regulate egg-laying behavior. The first center is the bag-cell neurons that are located around proximal region of the pleuro-abdominal connectives on rostral side of the abdominal ganglion (see Fig. 10.1). The second center is the atrial gland, an accessory sex gland that is located on the large hermaphroditic duct (Hadfield and Switzer-Dunlap, 1984). The bag cells are clusters of neurosecretory cells consisting of around 400 cells in the left and right portions of the abdominal ganglion (Coggeshall et al., 1966). Axon terminals of the bag cells are found in the connective tissue sheath covering the pleuro-abdominal connectives, as well as in the space between the surface of the abdominal ganglion and the ganglionic sheath. Neurosecretory material enters the systemic circulation via the caudal artery (Chiu and Strumwasser, 1981; Kaczmarek et al., 1979).

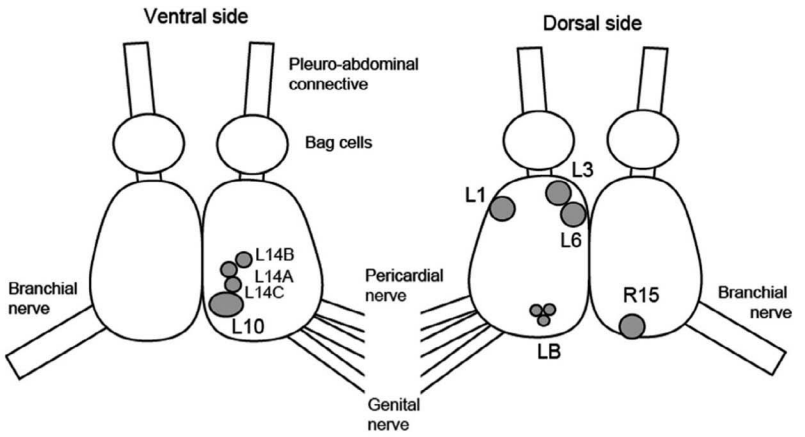

FIGURE 10.1 Ventral and dorsal view of the abdominal ganglion of *Aplysia* showing the location of the neurosecretory bag cells, bursting neuron R15, neurons L1, L3, L6, L10, neurons L14A/B/C, neuronal cluster LB and LC.

The function of the bag cells in *A. californica* was first demonstrated by Irving Kupfermann in the late 1960s and early 1970s. Injection of a crude homogenate of the bag cells into sexually mature *A. californica* induced egg-laying (Kupfermann, 1967, 1970). Brief electrical stimulation to the abdominal ganglia induces depolarization of the bag cells, initiating the synchronized discharge of action potentials, the afterdischarge, which continues for more than 20 min (Kupfermann, 1970). The afterdischarge of the bag cells can also be recorded in vivo with extracellular electrodes placed in the vicinity of the bag cells (Pinsker and Dudek, 1977; Dudek et al., 1979). Thus, the afterdischarge of the bag cells is an important event for the initiation of egg-laying.

It has also been recognized that the freshly deposited egg strings of *A. californica* release factors that promote egg-laying in conspecifics (Nagle et al., 1985). The source of these egg-laying stimulatory factors was determined to be an accessory sex organ, the atrial gland, an exocrine organ located on the reproductive tract. Several peptides with egg-laying activity have been isolated from the atrial gland such as califin and Peptide-A (Nagle et al., 1985; Rothman et al., 1986). Califin and Peptide-A are packaged into the secretory granules of the glandular cells of the atrial gland, and trigger egg-laying in *A. californica* when injected into mature animals. However, the involvement of the atrial gland in the regulation of egg-laying behaviors is not fully understood since the gland secretes peptide hormones into the reproductive tract but not into the hemolymph.

10.2.3 IDENTIFICATION OF ELH

The first attempt to identify the egg-laying inducing factor was made by analyzing the secretion of peptides from the bag cells. To this end, bag cells were incubated with [^{3}H]-leucine which was incorporated into newly synthesized proteins and peptides. Depolarization of the bag cells with K^+-rich solution induced secretion of radiolabeled peptides of approximately 6000–7000 Da in a Ca^{2+}-dependent fashion (Arch, 1972a). Pulse-chase labeling with [^{3}H]-leucine revealed that most newly synthesized proteins were initially present as larger protein around 25,000 Da, and then the quantity of radioactively labeled proteins increased in a 6000–7000-Da peptide (Arch, 1972b). Moreover, the egg-laying activity of the peptide extract of the bag cells coeluted with the 6000–7000 Da material from gel filtration chromatography (Arch et al., 1976a). These results strongly suggested that the 6000–7000-Da peptide was the ELH of *Aplysia*.

The bag cells project their nerve endings into the space between the surface of the abdominal ganglion and the overlying connective tissue sheath. Since this extraganglionic space is connected with the systemic circulation via the caudal artery, it is likely that ELH released in this space is delivered to various parts of the abdominal ganglion. To determine if the bag cells release ELH peptide during the afterdischarge, the bag cells were pulse-labeled with radioactive amino acid which was followed by electrical stimulation and collection of releasate. It was clearly demonstrated that the radioactive label and egg-laying activity coeluted in a peak of 6000–7000 Da (Mayeri et al., 1985). Subsequent purification of ELH from bag cells extracts was accomplished using HPLC and bioassay of the fractions possessing egg-lying inducing activity. A peptide of 36 amino acids was identified after automated Edman degradation (Chiu et al., 1979).

10.2.4 PHYSIOLOGICAL FUNCTIONS OF ELH

The physiological actions of ELH are diverse. Application of ELH-containing extracts to isolated pieces of ovotestis in vitro induces the release of oocytes into the bathing media (Dudek and Tobe, 1978; Dudek et al., 1980; Rothman et al., 1983b). ELH appears to have a direct action on the ovotestis by inducing ovulation. Another important action of ELH is the modification of the neuronal activities in various neurons in the central ganglia (see Table 10.1). Perfusion of ELH into the isolated abdominal ganglion from the caudal artery initiates discharge in the silent motoneuron, the LC cell,

which regulates muscle tone in in the gill, pericardial region, and vasculature (Mayeri et al., 1979a,b). ELH also increases the frequency of discharge in the bursting pacemaker neuron R15. Neuron R15 is itself a peptidergic neuron that releases R15α peptide as well as other peptides which are thought to be involved in cardiovascular function and osmoregulation (Weiss et al., 1989). Immunohistochemical localization of R15α peptide-containing nerve processes revealed that R15 peptides are distributed in the in the auricle, pericardium and arteries (Alevizos et al. 1991a,b) as well as in the large hermaphroditic duct (Alevizos et al., 1991d). Moreover, R15α peptide is myoactive on those tissues (Alevizos et al., 1991d). Thus, R15 affects transport of the egg-strings by regulating the contraction of the muscular tissues of the large hermaphroditic duct. The left lower quadrant cells in the abdominal ganglion also mediate the regulation of the arterial system by the bag cells (Ligman and Brownell, 1985). The increase in cardiovascular activity during egg-laying may help control locomotor activity by altering the hydrostatic pressure and redistributing hemolymph in the body.

TABLE 10.1 The Actions of ELH on the Neurons and Neural Clusters in the Abdominal Ganglion of *Aplysia* (Source: Mayeri, E. *Fed. Proc.* **1979,** *38*, 2103–2108).

	Neurons	**Functions**	**Response to ELH**	**End response**
Neurons	L3/L6	Bursting pacemaker neuron	Prolonged inhibition	
	R1/L1	Mechanoreceptor neuron	Transient excitation	Unknown
	L10	Cardiovascular motoneuron	Transient inhibition followed by prolonged excitation	Increase in cardiac output
	L14A/B/C	Ink-gland motoneurons	Slow inhibition	Suppression of ink release
	R15			Salt and water balance
Neuronal clusters	LB/LC	Motoneurons on gill, siphon, and vasculature	Prolonged excitation	Regulation of muscle tone

The left upper quadrant (LUQ) cells express two neuropeptide precursors, LUQ-1 that encodes bradykinin-related peptides and L5–67 that encodes ACEP-1-related peptide. LUQ-1 peptides are found in the nerve processes innervating the ovotestis, whereas L5–67 peptide is found in axonal processes projecting to the heart. Although the physiological actions

of bradykinin- and ACEP-1-like peptides are not fully understood in *Aplysia*, it is likely that these peptides are involved in the regulation of egg-laying behaviors because bag cell discharge negatively regulates the discharge of the LUQ cells. These diverse actions of ELH on neural activity are fundamental to the complex, integrated behaviors that regulate egg-laying.

10.2.5 ELH-ASSOCIATED PEPTIDES IN APLYSIA

Although purified ELH mimics various actions induced by bag cell afterdischarge or by the application of the bag cell extract to the abdominal ganglion, it does not fully reproduce specific effects on other neurons such as the inhibition of the left-upper quadrant (LUQ) cells. Therefore, the bag cells release other signaling molecules together with ELH during the afterdischarge. One group of peptides that was identified was α-, β-, and γ-bag cell peptides (BCPs). The β-BCP and γ-BCP share the common Arg-Leu-Arg-Phe (RLRF) sequence with the α-BCP (see Table 10.2).

The specific functions for some of the BCPs have been reported. For instance, the α-bag cell peptide (α-BCP) is known to inhibit neuronal activity of the LUQ cells (Rothman et al., 1983a; Sigvardt et al., 1986). Another important function of the BCPs is auto-excitation of the bag cells (Brown et al., 1989). All the BCPs depolarized membrane potential in the neurites of the bag cells with the following rank potency order, α-BCP > β-BCP > γ-BCP. In addition, the BCPs modulate intracellular cAMP levels in the bag cells by changing the activity of adenylate cyclase in the cells (Redman and Berry, 1992). Interestingly, the effects on intracellular cAMP levels is temperature-dependent, namely, α- and γ-BCP reduced cAMP at 15°C, whereas at 30°C, it increased cAMP (Loechner et al., 1990). β-BCP, as well as the RLRF peptide (the common structure shared by the three BCPs), increased intracellular cAMP in the bag cells at both low and high temperatures (Berry et al., 1994). Since the inhibitory actions of the α- and γ-BCPs on cAMP are GTP-dependent and pertussis toxin-sensitive, this indicated the involvement of the inhibitory GTP-binding protein (Gi) (Redman and Berry, 1993). Although it is unclear whether the increase in cAMP is mediated by the stimulatory GTP-binding protein (Gs), temperature-dependent switching between Gi and Gs is a suggested mechanism in the bag cells. These temperature-dependent modulations of bag cell activity may be involved in the initiation of egg-laying behavior which is associated with changes in seasonal temperature.

TABLE 10.2 Structures of Egg-laying Hormone and Related Peptides Found on the ELH-related Peptide Precursors in Molluscs.

Source animal	Peptide name	Structure
Aplysia	ELH	ISINQDLKAITDMLLTEQIRERQRYLADLRQRLLEKa
	Califin (large)	ISINQDLKAITDMLLTEQIQARRRCLDALRQRLLDLa
Lymnaea	CDCH-1	LSITNDLRAIADSYLYDQNKLRERQEENLRRRFLELa
	CDCH-II	SITNDLRAIADSYLYDQHKLREQQEENLRRRFY-ELSLRPYPDNLa
Peptides found on the ELH and CDCH precursors		
Aplysia	Peptide A	IFVPNRAVKLSSDGNYPFDLSKEDG AQPYFMTPRLRFYPI
	α-BCP	APRLRFYSL
	β-BCP	RLRFH
	γ-BCP	RLRFSD
Lymnaea	α-CDCP	EPRLRFHDV
	β_1-CDCP	RLRFH
	β_2-CDCP	RLRAS
	β_3-CDCP	RLRFN
Aplysia	δ-BCP	DQDEGNFRRFPTNAVSMSADENSPFDL SNEDGAVYQRDL
Lymnaea	Calfluxin	RVDS---ADESNDDGFD
Aplysia	Califin (small)	DSDVSLFNGDLLPNGRCS
	Acidic peptide	SSGVSLLTSNKDEEQRELLKAISNLLD

All the BCPs also induce depolarization of the cell membrane in cultured bag cells. The depolarization response induced by the BCPs can be desensitized by repeated BCP application. During desensitization there is an augmentation of K^+-current via the delayed voltage-gated potassium channel (Loechner and Kaczmarek, 1990, 1994). Interestingly, the cultured bag cells that had been previously desensitized with α-BCP responded to β-BCP by depolarization, suggesting that the two peptides bind to different bag cell autoreceptors from one another (Loechner and Kaczmarek, 1994).

Immunohistochemistry using anti-ELH and anti-α-BCP antibodies demonstrate the localization of ectopic bag cells in the cerebral and right pleural ganglia, respectively (Painter et al., 1989; Chiu and Strumwasser, 1984). The electrical properties of the α-BCP-immunopositive cells in the central ganglia resemble that of the bag cells in the abdominal ganglion (Brown et al., 1989). The α-BCP-containing cells send their processes to the

neuropile, but not to the connective tissues surrounding the ganglia (Brown et al., 1989). In fact, burst discharge of the α-BCP-containing cells initiates the burst discharge in the bag cells with a 10-s delay, and vice versa, suggesting a functional coupling between the α-BCP-containing cells with the bag cells (Brown and Mayeri, 1989). Furthermore, retrograde labeling (Shoppe et al., 1991), as well as the detection of α-BCP by mass spectrometry (Hatcher and Sweedler, 2008), supports this functional connection. Since sensory inputs are integrated in the cerebral and pleural ganglia, it is likely that sensory stimuli including temperature, photoperiod, and chemical cues (e.g., pheromones) may trigger burst discharge of the α-BCP-containing cells in the head ganglia, which then initiates afterdischarge in the bag cells.

The *Aplysia* atrial gland is known to produce the peptide califin and Peptide A. Califin consists of a large subunit (36 amino acids) and small subunit (18 amino acids) joined by a disulfide bond. The N-terminal 19 amino acids of califin's large subunit are identical to that of ELH. Peptide A is composed of 34 amino acids and shares a common C-terminal Arg-Leu-Arg-Phe sequence to that of α-BCP. Both califin and Peptide A induce egg-laying when injected to sexually mature *A. californica* and activate putative receptors for ELH and α-BCP, respectively.

It is clearly established that ELH and ELH-associated peptides govern various aspects of female reproduction from ovulation to oviposition in *Aplysia*. However, it is unlikely that these peptides induce gonadal maturation in the animal since injection of ELH and BCPs into animals outside the breeding season does not induce gonadal maturation, nor egg-laying. Information on the extracellular signaling molecules that induce gonadal maturation in *Aplysia* is largely unknown (reviewed by Hadfield and Switzer-Dunlap, 1984).

10.2.6 INTRACELLULAR MECHANISMS CONTROLLING AFTERDISCHARGE IN THE BAG CELLS

The bag cell afterdischarge is triggered by a brief electrical stimulation and is essential for the prolonged release of ELH. What is happening in the bag cell to initiate the afterdischarge? Electrical stimulation depolarizes the bag cell membrane and induces the opening of voltage-gated Ca^{2+}-channels resulting in Ca^{2+} influx across the cell membrane. The local increase in Ca^{2+} in the vicinity of cell membrane causes the opening of Ca^{2+}-sensitive, nonselective cation channels. The elevation of internal Ca^{2+} induces sustained depolarization of membrane potential, which results in the prolonged discharge of the bag cells.

Although the afterdischarge is essential for triggering ELH release, the time course of ELH release is somewhat different from that of discharge (Loechner et al., 1990). The frequency of discharge is maximal within a few minutes, then it decreases gradually and terminates in 15–20 min. By contrast, ELH release is maximal after 15–20 min, then decreases to basal levels as the discharge is terminated. In fact, extracellular Ca^{2+} influx via the plasma membrane is not necessary for ELH-release (Wayne and Frumovitz, 1995). For instance, depletion of extracellular Ca^{2+} by the Ca^{2+} chelator BAPTA, reduced the duration of afterdischarge, while it had little effect on the ELH-secretion (Wayne et al., 1998). Any treatments that increase intracellular Ca^{2+} levels including Ca^{2+} mobilization from intracellular stores or Ca^{2+}-influx by sustained depolarization of membrane potential induces ELH-release without afterdischarge (Wayne et al., 2004). Recent evidence suggests the importance of Ca^{2+} buffering by intracellular Ca^{2+} stores and mitochondria for ELH release (Groten et al., 2013; Hickey et al., 2010, 2013).

The molecular mechanisms that induce the afterdischarge and prolonged release of ELH from the bag cell are complex and are not yet fully understood. However, studies by the Greengard and Kaczmarek labs demonstrated that several protein kinases mediated these phenomena. For instance, the fact that some calmodulin (CaM) inhibitors such as TFP and W-7 inhibit the afterdischarge, and ELH-secretion induced by electrical stimulation of the bag cells (DeRiemer et al., 1985) suggests the involvement of CaM-dependent enzymes. One of the enzymes is CaM-dependent kinase (CaM-kinase). In the CaM-free soluble fraction prepared from the bag cells, phosphorylation of a 51-kDa protein is augmented in the presence of CaM and Ca^{2+} (DeRiemer et al., 1984). Although the entity of the 51-kDa protein is unclear, phosphorylation by CaM-kinase seems to modulate the activity of nonselective cation channels because a CaM-kinase inhibitor attenuated the spike-broadening of action potentials observed in the afterdischarge (Hung and Magoski, 2007).

The involvement of PKC in the afterdischarge is also suggested by the fact that action potential during the afterdischarge from the cultured bag cell is augmented by a PKC activator, TPA, whereas it is reduced by a PKC inhibitor, H-7 (Conn et al., 1989). Using various preparations such as whole-cell or excised patch clamp, it was demonstrated that PKC-dependent protein phosphorylation increases the open probability of a nonselective cation channel, which promotes Ca^{2+} influx (Gardam and Magoski, 2009; Tam et al., 2011). Single channel-containing patch clamping suggests a direct interaction between the PKC and the nonselective cation channel (Magoski and Kaczmarek, 2005; Magoski et al., 2002; Wilson et al., 1998).

PKC augments Ca^{2+} influx to the bag cells in different ways. In addition to the Ca^{2+}channel in the plasma membrane, the bag cells have vesicular pools of distinct Ca^{2+} channels (Strong et al., 1987; Wayne et al., 1999; White and Kaczmarek, 1997). The Ca^{2+} channel in the plasma membrane, classified as Apl Ca_v1, has a smaller Ca^{2+}-conductance and constitutively locates in the plasma membrane. By contrast, the Ca^{2+} channel in the vesicular pool, classified as Apl Ca_v2, has a larger Ca^{2+}-conductance and translocates to the plasma membrane, when the cells are excited. The discharge in the bag cells promotes translocation of Apl Ca_v2-containing vesicle through the activation of PKC. Apparently, it results in the increase of Ca^{2+} influx because the number of the functional Ca^{2+} channels is increased in the plasma membrane (Zhang et al., 2008b). Actin filaments appear to mediate channel-containing vesicle translocation and insertion to the plasma membrane because actin inhibitors disrupt vesicle fusion to the membrane, as well as the increase in the Ca^{2+} influx (Groten et al., 2013).

Conversely, PKC is also involved in the desensitization and refractory period of the bag cells. It is known that an injection of inositol trisphosphate (IP_3) into the cultured bag cell augmented the outward K^+-current. Since Ca^{2+} imaging with Fura-2 demonstrated that injected IP_3 mobilized intracellular Ca^{2+}, it is likely that K^+ efflux through the Ca^{2+}-sensitive K^+ channel is augmented. PKC-dependent phosphorylation appears to positively regulate the K^+ efflux, since a PKC activator augmented the Ca^{2+}-sensitive K^+-current, which results in the hyperpolarization of membrane potential and attenuation of the action potential (Zhang et al., 2002). These results suggest that activation of PKC promotes afterdischarge by increasing Ca^{2+} influx, whereas it attenuates the discharge by increasing K^+ efflux. Thus, PKC mediates both of the initiation and cessation of egg-laying.

The aforementioned results demonstrate that PKC plays important roles for the induction of the afterdischarge by elevating intracellular Ca^{2+} levels. How is PKC is activated in *Aplysia* bag cells? The hydrophilic IP_3 that mobilized Ca^{2+} from intracellular Ca^{2+} stores and hydrophobic diacylglycerol are important factors for the activation of PKC. These substances are generated by degradation of phosphatidyl inositol phosphate (PIP_2) catalyzed by phospholipase C (PLC). PLC is activated by interaction between ligand-bound GPCR and GTP-binding protein (Gq).

When the bag cells are labeled with [^{3}H]-inositol, incorporation of [^{3}H]-inositol into both of the soluble and membrane fractions of the cell are increased following the afterdischarge (Fink and Kaczmarek, 1988). Incorporation of [^{3}H]-inositol into the soluble fraction indicates IP_3 synthesis, while that into the membrane fraction reflects de novo synthesis of PIP_2 for

replenishment. FMRFamide is one of the endogenous ligands that activate PLC in the bag cells, since the peptide modulates IP_3 production in various molluscan tissues (Falconer et al., 1993; Willoughby et al., 1999b). In fact, FMRFamide is known to terminate the afterdischarge in the bag cells (Fisher et al., 1993).

During the afterdischarge, a transient increase in intracellular cAMP levels also occurs (Kaczmarek et al., 1984). The cAMP concentration reached a peak around 2 min after the initiation of discharge, and returned to basal levels in 8 min. In cultured bag cells, the membrane-permeable cAMP analog 8-benzylthio-cAMP increased the inward Ca^{2+}-current through the attenuation of the delayed and transient K^+-currents. In the whole-mount cell patch on the cultured bag cell, forskolin, an adenylate cyclase activator, and theophylline, a phosphodiesterase inhibitor, showed similar effects (Strong and Kaczmarek, 1986). As mentioned above, BCPs released from the bag cells areknown to elevate cAMP level in the cell by activating adenylate cyclase.

To investigate the PKA-dependent phosphorylation during the afterdischarge, a crude membrane preparation of the bag cells was incubated with γ-^{32}P-ATP and analyzed by SDS-PAGE and autoradiography. Phosphorylated proteins with molecular weight at 33 kDa (BC-I) and 21 kDa (BC-II) were detected and markedly augmented by cAMP, suggesting phosphorylation by PKA (Jennings et al., 1982). Of the two proteins, BC-II seemed to be the bag cell specific protein, because it was not found in other ganglia. When isolated bag cells were labeled with $Na_2H[^{32}P]O_4$ and then analyzed by SDS-PAGE/autoradiography at different times after the initiation of afterdischarge, radiolabeled BC-I and BC-II was detected at 20 min (but not at 2 min) following the afterdischarge. Such a delayed PKA-dependent phosphorylation during afterdischarge, together with the cAMP-dependent increase in K^+-current in the bag cell suggests that cAMP mediates termination of prolonged discharge of the bag cells. Unfortunately, the structure and function of the radiolabeled proteins are not known.

Using an excised inside-out patch clamp of the bag cells, Magoski's lab postulated a model in which the cation channel makes a complex with PKC and protein phosphatase (Magoski, 2004). In this hypothesis, PKC-dependent phosphorylation of the channel increases the open-probability of the cation channel in the initial phase of afterdischarge, while the protein phosphatase turns the channel back to the resting state by removing phosphate from the channel. On the other hand, in the late phase of the discharge, PKC can be replaced with PKA, and then PKA-dependent phosphorylation of the

channel reduces the open-probability, which results in the termination of the discharge.

10.2.7 THE ELH PRECURSOR

The structure of the ELH gene was identified in the early 1980s. Messenger RNAs were prepared from the bag cells, abdominal ganglia, and digestive gland, and then cDNAs were synthesized by reverse-transcription. Using the cDNA as a probe, a genomic DNA-library of *A. californica* was screened and clones that exclusively hybridized with cDNA from the bag cells were isolated (Scheller et al., 1982). By analyzing the nucleotide sequences of the positive DNA fragments, the amino acid sequence of the ELH precursor protein was determined (Scheller et al., 1983). The precursor protein for ELH consisted of 271 amino acids, including N-terminal signal peptides. The ELH sequence was found at the C-terminal region of the precursor, which is followed by a C-terminal acidic peptide (AP). At the N-terminal region, α-, β-, δ-, and γ-BCPs were aligned in tandem. Thus, egg-laying associated peptides in the bag cell were encoded on the same precursor protein.

There are several variants of precursors encoding for ELH-related peptides such as Peptide-A and califin (Scheller et al., 1983). For instance, one precursor cDNA has a nucleotide deletion which results in the deletion of 83 amino acids in the N-terminal region of the ELH-precursor. As the result, this variant does not have β-, γ-, and δ-BCPs found in the ELH-precursor but encodes Peptide-A. The Peptide-A precursor also includes the ELH-related peptide, califin, at its C-terminal region. The nucleotide sequence of another variant encoding the Peptide-B precursor is similar to that of the Peptide-A precursor. However, the encoded precursor protein is much shorter than that of the Peptide-A precursor because a deletion of a nucleotide in the Peptide-B precursor causes a frame shift, resulting in a stop codon in the middle of the precursor protein. As a result, Peptide B does not include the califin-coding region. The ELH precursor is expressed in the bag cells, whereas the Peptide-A and Peptide-B precursors are expressed in the atrial gland (Nagle et al., 1986; Scheller et al., 1982).

The *Aplysia* ELH-gene consists of three exons (Mahon et al., 1985a). The short exons, exon I and II, are connected by a splicing consensus nucleotides (GT), while exon II and III are connected by an intron-spanning 5.3-kb sequence. The entire length of the open-reading frame for all the ELH precursors is located within the confines of exon III. Thus, alternative splicing produces mRNA variants with different 5′-UTRs.

Besides *A. californica*, the ELH precursor is conserved among the other *Aplysia* species including, *A. parvula*, *A. punctate*, *A. brasiliana*, *A. dactylomela*, *A. oculifera*, *A. juliana*, and *A. vaccaria* (Li et al., 1999; Nambu and Scheller, 1986). By contrast, Peptide-A and -B precursors are not found in these marine snails. It is likely that the Peptide-A and -B precursor genes evolved after the divergence of *A. californica* and other *Aplysia* species from their common ancestor (Nambu and Scheller, 1986).

10.2.8 PROCESSING OF THE ELH PRECURSOR AND RELEASE OF ELH

As with other precursor proteins for short neuropeptides, the ELH-precursor protein is subject to posttranslational modifications (PTMs) before the bioactive mature peptide is produced. The processing and cleavage of the polypeptide chain at appropriate sites is fundamental to the PTM of ELH-precursor (Arch et al., 1976b). Processing enzymes, such as furin (Nagle et al., 1993), prohormone convertase PC2 (Ouimet et al., 1993), as well as an amidating enzyme (Fan et al., 2000), have been cloned in *Aplysia* and were demonstrated to be expressed in the bag cells (Chun et al., 1994; Nagle et al., 1993; Ouimet et al., 1993). In fact, ELH and BCPs have consensus sites for cleavage by processing enzymes such as prohormone convertase 2 and furin (Sossin et al., 1990a,b). Cleavage of the ELH-precursor by furin generates the N-terminal fragment including BCPs and C-terminal fragments including ELH. Using a radiolabeled ELH precursor, localization of the ELH-containing fragment and the BCP-containing fragment in the bag cells were detected by electron microscope autoradiography (Sossin et al., 1990a,b). In the soma of the bag cell, the ELH-containing fragment was packaged into small, immature dense-core vesicles (DCVs) as it passed though the trans-Golgi network. These vesicles are then subjected to the PTM pathway and transported toward the axon terminal in the ganglionic space. By contrast, the BCP-containing fragment was packaged into a larger immature DCV and transported elsewhere in the cell. Therefore, the axon terminals located on the abdominal ganglion mainly release ELH, rather than BCPs as the bag cells are undergoing discharge (Fisher et al., 1988; Jung and Scheller, 1991).

Interestingly, the axon terminals on the pleuro-abdominal connectives contained two classes of vesicles, namely, one containing the BCP fragment and the other containing the ELH fragment (Sossin et al., 1990a,b). Thus, both the ELH and BCPs are released from nerve endings on the pleuro-abdominal connectives. A similar distribution of BCP- or ELH-containing

vesicles is also observed by immunoelectron microscopy using antibodies that recognize the N-terminal and C-terminal fragments, respectively. These results suggest that the bag cells release different combinations of peptides in the pleuro-abdominal connectives and in the abdominal ganglion. The ELH-containing vesicles are released into the peripheral circulation, whereas the BCPs are secreted onto other bag cells or nearby neurons and have neurotransmitter-like effects. The differential sorting and release of ELH and the BCPs is important in coordinating the stereotyped set of behaviors involved in egg-laying.

The axon terminals of the bag cells in the pleuro-abdominal connective make synaptic contacts and receive neural input that triggers the afterdischarge. When the axon terminals are depolarized, co-release of BCPs with ELH, augments the excitability in the axon terminals of other nearby bag cells, and induces prolonged discharge of action potentials. This auto-excitatory action of BCPs, together with the electrical coupling of the bag cells, triggers the synchronized afterdischarge in the cells. The afterdischarge then propagates to the axon terminals located on the surface of the abdominal ganglion and initiates massive release of ELH into the ganglionic space (Jung and Scheller, 1991).

Synthesis and processing of ELH-precursor is modified by the neuronal activities in the bag cell. The involvement of depolarization-sensitive Ca^{2+} influx in neurites of the bag cells is suggested, because high K^{+}-solution augmented the incorporation of ^{3}H-leucine in the ELH-precursor. Incorporation of radiolabeled leucine was not observed in bag cells without neurites, and it was sensitive to Ca^{2+}/Mg^{2+} concentration. Moreover, elevation of intracellular cAMP in the bag cells also augmented radiolabeling of the ELH-precursor.

On the other hand, Azhderian and Kaczmarek (1990) reported that the afterdischarge of the bag cells reduced the amount of ELH-precursor. Since the reduction of the precursor was observed in the presence of the translation inhibitor, anisomycin, it was suggested that increased precursor processing occurred rather than decreased de novo synthesis (Bruehl and Berry, 1985). Precursor processing was augmented via elevation of bag cell cAMP levels as was previously shown. During the afterdischarge, it is likely that the bag cells initiate the processing of preexisting ELH-precursors, and then promote de novo synthesis of the ELH precursor to replenish the depleted precursor pool.

As is the case with other eukaryotic cells, protein synthesis in the bag cells is regulated by a cap-dependent initiation cascade. During this process, phosphorylated eukaryotic initiation factor 4E (eif-4E) and the 40S subunit

of ribosome bind to the 5′-cap end of mRNA, and then scans on the mRNA to find the methionine initiation site. The 60S ribosomal subunit then binds to the complex at the initiation methionine site and polypeptide synthesis begins. However, in *Aplysia* bag cells Lee and Wayne (1998) reported that de-phosphorylation of eif-4E is dominant during the afterdischarge, which results in a reduction of protein synthesis. Dyer et al. (2003) suggested the involvement of a novel cap-independent internal ribosome entry system in ELH-precursor synthesis as a mechanism by which the bag cells promote the synthesis of ELH-precursor during the afterdischarge.

10.2.9 LOCAL SYNTHESIS OF ELH

It is generally accepted that the neuronal soma is the site at which all protein synthesis that is essential to maintain neural activity occurs. However, the axon terminal also has ability to synthesize proteins. For instance, in situ hybridization for ovulation hormone precursor mRNAs demonstrated the localization of some neuropeptide mRNAs in the axon hillock and in the axons of the caudodorsal cells of *L. stagnalis* (Van Minnen, 1994), suggesting the transport of mRNA toward the nerve endings. In fact, in *L. stagnalis* polysomes can be found in the nerve endings by in situ hybridization (Spencer et al., 2000). Furthermore, injection of ovulation hormone precursor mRNA into the isolated neurites resulted in the de novo synthesis of ELH (Van Minnen et al., 1997). In *Aplysia* bag cells, reverse transcription-PCR using mRNA isolated from the axon terminal of the bag cells amplified cDNA encoding ELH precursor (Lee et al., 2002). It was demonstrated that the afterdischarge of the bag cell induces local synthesis and processing of ELH-precursor in the neurites, which contributes to the massive release of ELH from the bag cells in vivo (Lee and Wayne, 2004).

These results demonstrate that machinery for protein synthesis is present in the nerve endings. However, the mechanism that enables these axonal proteins to be released into the extracellular space is still unknown. Electron microscopy demonstrated that polysomes for local protein synthesis are located in the axoplasm of cultured neurons of *Lymnaea*, but not associated with membranous structures, such as the Golgi apparatus, mitochondria, and small vesicles located in the axon terminals (Spencer et al., 2000). Therefore, it is assumed that synthesized polypeptides remain in the axoplasm. A plausible explanation may be the existence of a bacterial Sec translocase-like protein (Du Plessis, 2011) that is functional in the axon terminal and translocates axoplasmic proteins to the extracellular space.

10.2.10 REPRODUCTIVE PEPTIDES IN OTHER MOLLUSCS

In the freshwater snail *L. stagnalis*, there are functionally equivalent neurosecretory cells to the bag cells *of A. californica*, called the CDCs (see Fig. 10.2). The CDCs are located in in two clusters of 50–100 cells on the dorsal side of the left and right cerebral ganglia (Wendelaar-Bonga, 1970, 1971a). The CDCs project their axons to the neurohemal area, the cerebral commissure, and release their secretory products in this region (Schmidt and Roubos, 1987, 1989). A similar anatomical arrangement of

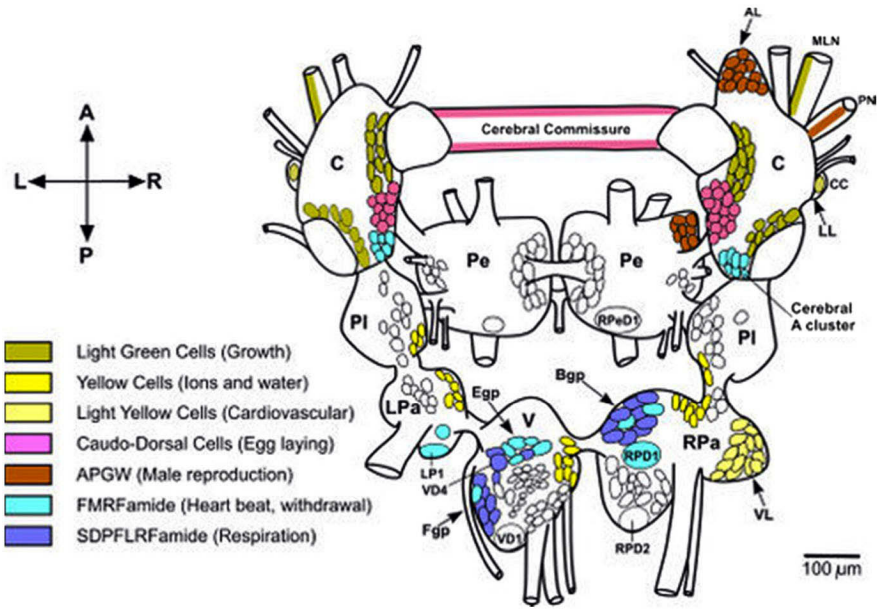

FIGURE 10.2 Map of peptidergic neurons in the CNS of *Lymnaea*. The cerebral commissure is the neurohemal organ of the CDCs. The median lip nerves (MLN) are the neurohemal organ of the LGCs. The anterior lobe (AL) APGW neurons project along the right penis nerve (PN) and innervate the penis complex. Note that the cluster of Yellow Cells that lie on the right side of the visceral ganglion are a special group of cells whose axons project along the distal processes of the intestinal nerve to innervate the pericardium and the reno-pericardial canal (see text). The cerebral A cluster cells are an example of whole-body withdrawal response motoneurons. Abbreviations: C, cerebral ganglion; CC, Canopy Cell; B/E gp, B/E group; LL, lateral lobe; LP1, left parietal 1; LPa, left parietal ganglion; Pe, pedal ganglion; Pl, pleural ganglion; RPa, right parietal ganglion; RPD1/2, right parietal dorsal 1/2; RPeD1, right pedal dorsal 1; V, visceral ganglion; VD1, visceral dorsal 1; VD4, visceral dorsal 4; VL ventral lobe. (Source: Benjamin, P. R., Kemenes, I. Lymnaea Neuropeptide Genes. *Scholarpedia*, 8(7), 11520). (Used with permission.)

neurosecretory cells involved in egg-laying is present in the planorbid snail *Helisoma duryi* (Khan et al., 1990; Saleuddin et al., 1990), as well as in other gastropods (Ram et al., 1998). Ablation of the CDCs in adult *L. stagnalis* results in a cessation of egg-laying which could be restored by injection of a crude extract of the cerebral commissure (Geraerts and Bohlken, 1976). The ovulation-inducing hormone synthesized and secreted by the CDCs was referred to as CDCH, also referred to as ovulation hormone. Like the *Aplysia* bag cells, the *Lymnaea* CDC somata are located near the surface of ganglia and are large (~90 μm in diameter) neurosecretory cells (Boer, 1965; Joosse, 1964). This makes the CDCs an experimentally tractable system for studying neuropeptide synthesis and secretion using electrophysiological, molecular and proteomic techniques.

Various environmental factors are known to stimulate the activity of the CDCs including, adequate nutrition (Ter Maat et al., 1982), long-day photoperiods (Dogterom et al., 1983), and a clean, oxygenated water stimulus (Ter Maat et al., 1983). Once the CDCs are induced to fire, they undergo a bout of electrical bursting activity which is followed by the secretion of CDC neurosecretory products, and finally egg-laying (De Vlieger et al., 1980; Ter Maat, 1992). Thus, the pattern of electrical activity and secretion of CDC neuropeptides is similar to the electrical events seen in the *Aplysia* bag cells that lead to ELH secretion. The CDCH is a 36 amino acid peptide and shares 44% sequence homology to *Aplsyia* ELH (Ebberink et al., 1985). Based on biochemical and molecular cloning studies (Geraerts et al., 1988; Li et al., 1992; Vreugdenhil et al., 1988), the CDCs are known to produce neuropeptides that are part of as small multigene family. Two genes, CDCH-I and CDCH-II, encode preprohormones from which ovulation hormone or CDCH (now referred to as CDCH-I) and CDCH-II are derived from, respectively. CDCH-II has been biochemically purified from cerebral commissure extracts and is a neuropeptide of 44 amino acids and is nearly identical to the N-terminus of CDCH-I except for a truncated amino acid but it differs from CDCH-I by having a C-terminal extension of nine amino acids (Li et al., 1992). The function of CDCH-II has not been demonstrated but it is believed to play a role in egg-laying behavior as it is colocalized with CDCH-I in the same neurosecretory granules (Van Heumen and Roubos, 1991). In addition to CDCH-I, nine other peptides are encoded on the CDCH-I preprohormone gene (Hermann et al., 1997; Li et al., 1994a–c; Vreugdenhil et al., 1988), including calfluxin, a peptide that stimulates calcium influx into the albumen gland (AG) (Dictus and Ebberink, 1988; Dictus et al., 1987), and four types of CDC peptides (α-, β-, γ-, and δ-CDCPs). All the CDCPs are auto-excitatory on the CDCs; however, only specific combinations of CDCPs together

with CDCH are capable of inducing characteristic CDC discharges in vitro (Brussaard et al., 1990). Furthermore, injection of β3-CDCP and α-CDCP into intact *L. stagnalis* stimulated electrical activity in the right pedal motoneurons which are involved in coordinating the stereotypical turning behavior during oviposition (Hermann et al., 1997). These physiological studies suggest that the N-terminal peptides on the CDCH-I precursor are involved in controlling neuronal activity in the CNS, whereas the peptides at the C-terminal region (e.g., CDCH-I) act as true hormones as they are transported in the hemolymph to distant organs in the periphery. Analysis of the spatiotemporal dynamics of peptide secretion by the CDCs using mass spectrometry showed a large depletion of CDC-derived peptides from the cerebral commissure during an electrical discharge as predicted by electrophysiological and cell biology experiments (Jiménez et al., 2004). In addition, two novel peptides of unknown function that were not encoded on the CDCH-I precursor were identified, suggesting that CDCs produce other peptides which may be involved in coordinating ovulation or egg-laying behaviors.

The activity of the two major reproductive centers *in L. stagnalis*, the CDCs and dorsal bodies (DBs) is known to modulated by infection with the schistosome *Trichobilharzia ocellata*, a parasite that causes swimmer's itch. Parasitic platyhelminths are well known to modulate the immunological and metabolic activities of their hosts, an adaptive trait that is thought to shift host resources toward parasite multiplication. It is thought that a factor secreted from *T. ocellata* causes the release of a peptide known as schistosomin from the CNS of *Lymnaea.* Schistosomin is then secreted into the hemolymph where it acts as an antigonadotropin to shift resources away from host reproduction (Schallig et al., 1992). Infection of *L. stagnalis* by *T. ocellata* inhibits reproduction by acting as an antagonist to calfluxin's action on the AG (De Jong-Brink et al., 1988) and blocks the action of the putative dorsal body hormone (DBH), which controls vitellogenesis (Hordijk et al., 1991a). Furthermore, application of hemolymph from parasitized snails to the CDCs inhibited their electrical activity suggesting that schistosomin has central effects on the CDCs as well its peripheral actions on the female reproductive system (De Jong Brink et al., 1992). Schistosomin is a 79-amino-acid peptide containing eight cysteine residues which are predicted to form four disulfide bonds (Hordijk et al., 1991b). Antibodies raised against schistosomin stain the neurosecretory LGCs, which are known to produce a hormone that regulates growth in *L. stagnalis*. In *Biomphalaria glabrata*, a schistosomin-like peptide was also identified which showed 62–64% amino acid sequence

identity to *L. stagnalis* schistosomin (Zhang et al., 2009). However, based on mRNA and protein expression studies, schistosomin peptide appears to be most abundant during embryonic development and in juvenile snails. In addition, schistosomin expression does not change between parasitized versus non-parasitized snails, suggesting that in *B. glabarata*, schistosomin does not play a direct role in parasite-mediated shifts in metabolic activities in the adult snail (Zhang et al., 2009).

In *L. stagnalis*, other CNS neuropeptides that can be altered by infection with *T. ocellata* have been identified. For example, *Lymnaea* neuropeptide Y (LyNPY) is upregulated during parasitic infection with *T. ocellata*, and experimentally elevating in vivo NPY concentrations in snails suppressed egg-laying and inhibited growth (De Jong Brink et al., 1999). Restoring basal levels of NPY using implanted peptide-containing capsules restored both reproductive activity and growth. In addition, double labeling immunohistochemistry for LyNPY and CDCH demonstrated that NPY-positive axons are closely associated with CDCH-positive axons. Collectively, these data suggest that *in L. stagnalis*, infection with *T. occellata* upregulates NPY titers in the snail acting to suppress both reproduction and growth. Parasitic infection also upregulates FMRFamide and LFRFamide-related peptides which is thought to suppress host metabolism and allow the parasite to exploit the energy resources of the snail (Hoek et al., 1997, 2005).

ELH-like and CDCH-like peptides have been reported in other gastropods as well as some bivalves. In whelks, injection of CNS extract induces the laying of egg capsules (each capsule contains about 50 eggs) in *Busycon canaliculatus* and *B. carica* (Ram, 1977; Ram et al., 1982). The egg-laying-inducing activity of the brain extracts is abolished by protease treatment and the putative egg-laying peptide in CNS extract coelutes with *Aplysia* ELH using gel-filtration chromatography. This suggests that the nervous system of *B. canaliculatus* and *B. carica* contains a peptide that stimulates egg-laying and has a similar molecular mass as *Aplysia* ELH. However, the sequence of *Busycon* ELH has not been determined.

The distribution of ELH-related peptides or α-CDCP-related peptide has been detected in the CNS of several prosobranch gastropods by immunohistochemistry. For example, in *B. canaliculatum*, immunoreactive neurons are widespread in in the CNS, and in particular, there are clusters of cells near the medial margins of the cerebral ganglia that stain strongly with both anti-α-CDCP and anti-ELH (Ram et al., 1998). In the abalone, *Haliotis rubra*, the gene encoding for an abalone ELH has been cloned (Wang et al., 1998). Antibodies raised against authentic abalone ELH (aELH) and used

in immunohistochemistry stain neurosecretory cells in the brain, neurites in the neuropil, and the connective tissue sheath of the cerebral, pleuropedal, and visceral ganglia in *H. asinia* (Saitongdee et al., 2005). Interestingly, follicular and granular cells in the ovary also stain positive for aELH as well as the cytoplasm of mature oocytes. The authors suggest that the ovary may be a local source of ELH in *H. asinia.* Also, injection of aELH into juveniles caused early sex differentiation, whereas injection of aELH into ripe adults of *H. asinia* induced spawning in both sexes, suggesting an ELH-like peptide is involved in the maturation of the reproductive system and egg-laying (Nuurai et al., 2010).

Recently, by analyzing genome databases or by transcriptome analysis, several ELH-related peptide precursors have been identified in the owl limpet, *Lottia gigantea* (Veenstra, 2010) and two bivalves, *Crassostrea gigas* (Stewart et al., 2014) and *Pinctada fucata* (Matsumoto et al., 2013). The structures of ELH-related peptides encoded on the precursor proteins share similarities with *Aplysia* ELH. However, the overall structures of the precursors are somewhat different from the *Aplysia* ELH precursor. For example, two distinct ELH-related peptides are found on the same precursor in *L. gigantea*, while two distinct ELH-related peptides are found on precursor protein in *C. gigas*. None of the precursors contained BCP-related peptides; therefore, the interaction between ELH peptides and BCPs that coordinate egg-laying behavior in *Aplysia* is unlikely to occur in in the life history of *L. gigantea* and in bivalves. The elucidation of the physiological functions of ELH- and CDCH-related peptides in gastropods other than *Aplysia* and *Lymnaea* is an important issue that needs further research.

10.2.11 GONADOTROPIN-RELEASING HORMONE

Gonadotropin-releasing hormone (GnRH) is 10-mer peptide that plays central roles in regulation of reproductive activities in vertebrates. In mammals, GnRH is synthesized by neurosecretory cells in the hypothalamus and secreted in the capillaries of the median eminence of the pituitary gland. The secreted GnRH is transported to the anterior pituitary and promotes the synthesis and secretion of the gonadotropins, follicle-stimulating hormone, and luteinizing hormone in the parenchymal cells (Iversen et al., 2000; Schally, 1978). Structurally related peptides to GnRH have been found in invertebrates, including molluscs.

10.2.12 STRUCTURES OF GnRH AND GnRH-RELATED PEPTIDES

The structural sequence of the GnRH was first determined in mammals (Baba et al., 1971; Matsuo et al., 1971). Since then, more than 20 kinds of GnRHs have been found in chordates (Okubo et al., 2008; Tsai, 2006). In addition, GnRH-related peptides have been identified in invertebrates such as molluscs and annelids (Sun et al., 2012a,b). There are clear differences in the basic structures of vertebrate GnRHs and invertebrate GnRH-related peptides. For instance, all of vertebrate GnRHs share the N-terminal pyroglutamine and C-terminal Pro-Gly-NH_2. By contrast, the invertebrate GnRHs have insertions of two amino acids between the N-terminal pyroglutamine and penultimate histidine, and the C-terminal glycine residue found in vertebrate GnRHs is missing. As the result, invertebrate GnRHs are 11 mer peptides. An exception is octopus GnRH which consists of 12 amino acids since it has a C-terminal glycine like the vertebrate GnRHs.

10.2.13 LOCALIZATION AND FUNCTION OF MOLLUSCAN GnRH-RELATED PEPTIDES

Localization of GnRH-like peptide in the central and peripheral nervous systems was demonstrated by immunohistochemistry in the chiton (Amano et al., 2010a), oyster and mussel, abalone (Amano et al., 2010b; Nuurai et al., 2014), freshwater snail (Goldberg et al., 1993; Young et al., 1999), sea hare (Zhang et al., 2000), and octopus (Di Cosmo and Di Cristo, 1998). In most cases, GnRH-like peptides were found in the cerebral, pleural, and pedal ganglia of the central nervous system, while numerous GnRH-positive nerve processes were detected in the peripheral nervous system. However, some of these studies were conducted with antibodies that recognize vertebrate-type GnRH or by using vertebrate bioassays which tested the actions of vertebrate-type GnRH. Considering the differences in the structures of GnRHs between vertebrates and invertebrates, careful consideration may be necessary for the interpretation of the results.

The first report suggesting the existence of GnRH-like peptide in gastropods appeared in 1980. Application of mammalian GnRH to the abdominal ganglion of *A. californica* augmented the bursting activity of the right-upper quadrant (RUQ) neurons (Seaman et al., 1980). The initial investigations on the distribution of GnRH-like peptides in nervous and reproductive tissues in *Aplysia* were conducted by immunohistochemistry or RIA with different

antivertebrate GnRH antibodies. Although it is practical and convenient to use neuropeptide antibodies to explore structurally related peptides in different animals, careful interpretation of the data are necessary. For instance, some antibodies detect the presence of GnRH-like peptides in hemolymph (Zhang et al., 2000), whereas others detect it in the central nervous system (Tsai et al., 2003). Moreover, subsequent molecular cloning studies revealed that the structure of *Aplysia* GnRH-related peptide is somewhat different from vertebrate GnRHs at both of the N-terminal and C-terminal regions (Zhang et al., 2008a). Thus, some anti-GnRH antibodies may have recognized potentially different substances in *Aplysia* tissues. Subsequent studies using immunohistochemistry with an antibody against authentic *Aplysia* GnRH-related peptide, as well as in situ hybridization, demonstrated that neurons in the cerebral and pedal ganglia contain GnRH-related peptides (Jung et al., 2014; Sun and Tsai, 2011; Zhang et al., 2008a). By contrast, no GnRH-like immunoreactivity was detected in the reproductive organs, although the RT-PCR detected the expression of precursor GnRH mRNA.

Functional studies on the *Aplysia* GnRH suggest that the peptide does not directly play a role reproduction. Since GnRH-containing nerve endings are not found in reproductive organs such as ovotestis, it is assumed that GnRH release from the neurons in CNS may have hormonal action on the reproductive organs. However, repetitive injection of GnRH into sexually immature *Aplysia* failed to induce gonadal maturation or egg-laying behavior (Tsai et al., 2010). Moreover, in vitro application of GnRH on the abdominal ganglion reduces the number of bag cells exhibiting afterdischarges. These results suggest that GnRH acts as a negative regulator of reproduction in *Aplysia* as opposed to its stimulatory role in vertebrates.

Interestingly, *Aplysia* individuals that received an injection of GnRH showed some unique behaviors such as frequent parapodia opening and reduced detachment from rigid substrates (Tsai et al., 2010). In this context, it is noteworthy that GnRH neurons in the pedal ganglion send their axons through the P1, P6, and P9 nerves. These nerve bundles which emanate from the pedal ganglion project onto the foot and parapodial region. Therefore, it is likely that GnRH neurons in the pedal ganglion mediate neural regulation of the parapodia and foot muscles.

In pulmonates, the presence of a GnRH-like peptide was first reported in *Helisoma trivolvis* by immunostaining and RIA with an anti-GnRH antibody, as well as its gonadotropin-releasing activity on fish pituitary cells (Goldberg et al., 1993). Young et al. (1999) found that neurons immunopositive to antimammalian GnRH antibody were diffusely distributed in all the circumesophageal ganglia. Immunopositive nerve fibers were also found in

the reproductive organs such as penial complex, vas deferens, oviduct and ovotestis, as well as in the neuropile in the ganglia. A similar localization of GnRH-related peptide occurs in *L. stagnalis*. Although detailed physiological investigations regarding the function of GnRH in pulmonates is lacking, the localization of GnRH in the central and peripheral nervous systems suggest that GnRH may be involved in controlling male reproductive behavior by affecting the muscles of the penial complex and vas deferens.

In prosobranch gastropods, Amano et al. (2010a,b) found GnRH-like peptide-containing neurons in the cerebral ganglion of *Haliotis discus hannai* whereas, Nuurai et al. (2014) found GnRH-immunopositive neurons in both of the cerebral and pleuro-pedal ganglia of another abalone, *Haliotis asinine*. Since immunopositive nerve fibers were not found on the mature gonad, the authors speculate that *Haliotis* GnRH has hormonal action on this tissue. Nevertheless, GnRH may mediate the initial phase of oocyte maturation because oocytes in the early stage of maturation in the ovary of *H. asinine* are immunopositive to anti-lamprey GnRH antibody (Nuurai et al., 2010). Although it was reported that repetitive injections of salmon GnRH analogue induced maturation of the gonad in Hawaiian limpet, *Cellana* (Hua et al., 2013), the functional significance of GnRH in prosobranchs reproduction remains unclear.

10.2.14 PHYLOGENETIC RELEVANCE OF GnRH AND GnRH-RELATED PEPTIDES

The structures of GnRH-related peptides in protostomes, including molluscs, show resemblance to those of insect corazonin or adipokinetic hormone (AKH). Corazonin is a cardioactive peptide that regulates heartbeat in insects (Veenstra, 1989), while AKH is a peptide hormone that regulates energy metabolism during insect flight (Stone et al., 1976). The relationship between corazonin/AKH and GnRH has also been suggested in *Drosophila* where the endogenous ligand to the GnRH-like receptor is AKH (Park et al., 2002).

Different analyses based on the similarity in amino acid sequences of receptors for corazonin, AKH, and GnRH place the molluscan GnRH receptors with insect AKH receptors or corazonin receptors, but not with vertebrate GnRH receptors (Hauser et al. 1998; Hauser and Grimmelikhuijzen, 2014; Roch et al., 2011). GnRH-like peptides appear to be widespread in metazoans as GnRH-like immunoreactivity is present in the diffuse nerve net of a sea pansy, suggesting that GnRH-related peptides have evolved in

animals with the first primitive nervous systems (Anctil, 2000). With respect to GnRH receptor evolution, the ancestral GnRH receptor is thought to have emerged before the divergence of deuterostomes and protostomes. As the two animal groups diverged, the ancestral GnRH receptor also split into the AKH/corazonin receptor trait in protostomes and the GnRH receptor trait in the deuterostomes (Hauser and Grimmelikhuijzen, 2014). As the divergence of the Mollusca and Arthropoda occurred, the AKH/corazonin receptor then diverged again into the corazonin receptor trait and the AKH receptor trait. Peptide ligands for the receptors also followed a similar pathway of molecular evolution, where the ancestral GnRH diverged into corazonin, AKH, and GnRH. In molluscs, the endogenous ligand for the corazonin receptor is a GnRH-related peptide (Hauser and Grimmelikhuijzen, 2014).

10.2.15 CONTROL OF FEMALE ACCESSORY SEX ORGANS

Gastropod molluscs possess morphologically elaborate reproductive systems with unique accessory sex glands. The neuroendocrine regulation of the secretory activities of various accessory sex glands has been studied mainly in pulmonates and opisthobranchs. We will focus our attention to the AG as it is the best studied accessory sex gland and it is known to be regulated by peptides and neurotransmitters. The AG is an exocrine gland that secretes a viscous perivitelline fluid that coats each fertilized egg as it proceeds along the reproductive tract and is packed into an egg mass/string. It is the main source of nutrition to the developing embryos in the egg mass (Duncan, 1975; Geraerts and Joosse, 1984). In addition, several novel proteins have been isolated from gastropod AGs, including antibacterial proteins (Kamiya et al., 1986), neurotoxins (Dreon et al., 2013), and peptide pheromones (Cummins and Bowie, 2012).

The effect of neuroendocrine regulators of the pulmonate AG has been investigated using biochemical techniques or bioassays that measure the two main classes of substances synthesized by the AG, a large molecular weight polysaccharide known as galactogen, and proteins (for reviews see Cummins et al., 2006; Dreon et al., 2006; Geraerts and Joosse, 1984; Runham, 1983). The neuroendocrine control of the pulmonate AG has historically been studied using organ culture techniques that measure the incorporation of radiolabeled monosaccharide precursors into large molecular weight polysaccharides (Goudsmit and Ashwell, 1965; Wijdenes et al., 1983). Putative factors from the brain and endocrine dorsal bodies are known to stimulate the synthesis of AG polysaccharides, however, some of

these factors have not been fully purified and characterized (Goudsmit, 1975; Miksys and Saleuddin, 1985, 1988; Wijdenes et al., 1983). In *L. stagnalis*, a novel 14-residue peptide named calfluxin has been chemically identified as a peptide that is processed from the CDCH precursor protein (Dictus and Ebberink, 1988; Dictus et al., 1987). Calfuxin stimulates the influx of extracellular calcium and mobilizes calcium from internal stores as demonstrated by intracellular calcium deposition in the AG of *L. stagnalis* (Dictus et al., 1988). The rise in AG intracellular calcium levels is presumably linked to secretion of macromolecules from the AG, although this has not been shown. In *H. duryi*, in vitro protein secretion by the AG can be induced with a peptide-containing extract of the CNS (Morishita et al., 1998).

In addition to neuroendocrine regulation via peptides, the AG and reproductive tract of gastropods is innervated by catecholaminergic axons (Croll, 2001; Croll et al., 1999; Hartwig et al., 1980; Kiehn et al., 2001). For instance, the AG of planorbid snails has intrinsic catecholamine-positive cell bodies as well as extrinsic catecholaminergic innervation by varicosities from the CNS (Brisson, 1983; Brisson and Collin, 1980; Kiehn et al., 2001). In *H. duryi*, a large glycoprotein, *H. duryi* AG protein (HdAGP), is the major secretory protein synthesized and secreted by the AG (Mukai et al., 2004b). The in vitro secretion of HdAGP can be induced by treatment with dopamine (Saleuddin et al., 2000). Dopamine binds to and activates a D1-like dopamine receptor on the AG membrane which then elevates intracellular cAMP (Mukai et al., 2004a) and calcium levels (Kiehn et al., 2004), resulting in the release of HdAGP. Catecholamine localization in the AG demonstrates intense staining around the carrefour, a small sac-like structure into which secretory material from AG flows (Kiehn et al., 2001). The carrefour has sensory cells lining its lumen and it is where each fertilized egg receives an equal coating of perivitelline fluid. The neural stimulation of the AG by dopamine is thought to trigger the precise release of perivitelline fluid from the AG cells into the carrefour to ensure each egg receives a coating of perivitelline fluid (Mukai et al., 2004a).

In *B. glabrata*, dopamine content in the AG was highest when perivitelline fluid secretion was expected to occur (Boyle and Yoshino, 2002), and application of dopamine to the AG stimulates in vitro protein secretion from the AG (Santhanagopalan and Yoshino, 2000). In *B. glabrata*, serotonin also plays a role in reproduction as culturing snails in water containing serotonin increases egg production (Manger et al., 1996), and addition of serotonin to the AG in vitro stimulates protein secretion (Santhanagopalan and Yoshino, 2000).

10.2.16 REGULATION OF MALE MATING BEHAVIOR

Although pulmonates such as *L. stagnalis* are hermaphroditic, during mating one animal acts as the donor "male" and the other as the recipient "female." Prior to mating, there is a distinct courtship behavior that occurs when the male actively mounts the shell of the female snail. In *L. stagnalis*, male mating behavior involves the extrusion of the preputium and the penis which is followed by probing for the female genital opening. The male snail then assumes the copulation position on the female shell rim and inserts the penis into the female genital opening (intromission) and then the transfer of semen occurs. Lastly, retraction of the penis complex occurs which may be followed by reversal of role where the donor male now becomes the recipient female (Runham, 1983).

In addition to the extensive studies on the peptidergic control of the female reproductive system, a growing body of evidence suggests that peptides are also involved in controlling distinct aspects of male reproductive behavior. For example, in *L. stagnalis*, mating behavior is known to be regulated by the action of numerous neuropeptides that are produced in a specific region of the brain. In *L. stagnalis*, the gene for a vasopressin-like peptide, named Lys-conopressin has been cloned from the nervous system (Van Kesteren et al., 1992a,b). *L. stagnalis* preproconopressin has a similar organization to its vertebrate counterparts having a signal peptide, and a mature peptide flanked by dibasic residues at the C-terminus. Furthermore, the snail neurophysin (carrier protein of conopressin) domain shares 49% sequence identity to human vasopressin neurophysin, including all the conserved cysteine residues, suggesting that the tertiary structure of *L. stagnalis* neurophysin assumes a similar conformation to that of vertebrate neurophysins (van Kesteren et al., 1992b).

In situ hybridization and immunohistochemical analyses of conopressin expression demonstrated that conopressin is expressed in clusters of neurons in the right cerebral ganglion, a region known to control male reproductive behavior, as well as staining nerve fibers innervating the penis and vas deferens (van Kesteren et al., 1995). Application of conopressin to electrically stimulated CDCs in vitro rapidly hyperpolarized the membrane potential indicating that conopressin exerts an inhibitory effect on neurosecretory cells controlling ovulation and egg-laying behavior in *L. stagnalis*. In contrast to its inhibitory effect on female reproduction, conopressin had a dose-dependent stimulatory effect on smooth muscle contractions of the anterior vas deferens, suggesting a functional role in the regulation of male reproductive behavior (van Kesteren et al., 1995). In mammals, oxytocin/

vasopressin-related peptides are well known to regulate sexual behavior (Smock et al., 2008; Veenema and Newman, 2008), and recent studies in invertebrate systems also demonstrate that these peptides have similar functions as it relates to the modulation of sexual behavior (Garrison et al., 2012; Gruber, 2013). Collectively, these studies on the behavioral effects of oxytocin/vasopressin-related peptides suggest a conserved function for oxytocin/vasopressin-related peptides in diverse animal phyla.

In *L. stagnalis*, subsequent studies revealed that conopressin was co-localized with APGWamide in a subset of neurons in the right anterior lobe of *L. stagnalis* and then are transported to the penis complex (preputium and penis with retractor muscles) and the vas deferens via the penis nerve (Van Golen et al., 1995a). These two peptides were structurally identified by HPLC and immunoassays in the penis complex and co-localized by immunohistochemistry to some axon bundles innervating the vas deferens as well. Although conopressin had no effect on the contractions of the penis retractor muscle, it had potent excitatory effects on the total number and frequency of contractions of the anterior vas deferens (Van Golen et al., 1995a). On the other hand, APGWamide had a dose-dependent inhibitory effect on the posterior vas deferens and inhibited conopressin-induced contractions of the anterior vas deferens. The coordinated actions of these two peptides are thought to facilitate the transport of semen during copulation. Although APGWamide-like immunoreactivity is widespread in the central nervous systems of *L. stagnalis*, *A. californica*, and *H. aspersa*, there appears to be a conserved anatomical region of the brain, namely clusters of peptidergic neurons in the right cerebral ganglion that control male reproductive behaviors (Croll and Van Minnen, 1992; de Lange and van Minnen, 1998; Fan et al., 1997; Griffond et al., 1992; Koene et al., 2000). In addition to APGWamide and conopressin, other peptides have been identified from neurons in the right cerebral ganglion of *L. stagnalis*. For example, FMRFamide-related peptides in are present in the penis nerve and modulate the activity of the penis retractor muscle (Van Golen et al., 1995b). The FMRFamide gene undergoes alternative splicing resulting in two transcripts coding for the tetrapeptides (FMRFamide FLRFamide) and the heptapeptides (GDPFLRFamide and SDPFLRLamide) which are expressed in neurons of the right cerebral ganglia, and in the B-group neurons of the right parietal ganglion and a few neurons in the right pleural ganglion (Van Golen et al., 1995b). The tetrapeptides induce rapid contractions of the penis retractor muscle whereas the the heptapeptides cause relaxation (Van Golen et al., 1995b). Therefore, the tetrapeptides have distinct effects from the heptatpeptides and are likely involved in the modulation of the penis retractor

muscle during the distinct phases of male mating behavior. In the terrestrial snail *H. aspersa*, parts of the reproductive system such as the hermaphroditic duct (ovotestis duct), seminal vesicle and fertilization pouch-spermathecal complex are innervated by branches of the intestinal nerve (Geoffroy et al., 2005). FMRFamide causes the relaxation of the muscles of the hermaphroditic duct and is thought to be involved in the regulation of ejaculation which is important for the proper transfer of sperm to the mating partner.

Four myomodulin-related peptides have also been identified from a cluster of neurons in the right cerebral ganglion of *L. stagnalis*, and each of these myomodulin isoforms have distinct but overlapping functions on the penis retractor muscle (Van Golen et al., 1996). It is clear from the physiological biochemical studies on male mating behavior in *Lymnaea* that there are multiple neuropeptides with distinct functions which are released from the penis nerve. The various secreted peptides permit a range of contraction and relaxation states and thus provides a mechanism for the fine-tuning of the penis retractor muscle (and other male accessory organs) to coordinate complex mating behaviors.

10.3 REGULATION OF GROWTH AND METABOLISM

The presence of a shell is a major diagnostic feature of the Mollusca and the majority of gastropods possess a shell or a reduced/absent shell as in the terrestrial slugs and some marine gastropods. When discussing the regulation of growth in shelled gastropods, there are two distinct aspects to consider, the growth of the soft body parts, and the growth of the shell. Growth of the soft body tissues occurs through increases in the weight of organic material mainly by the synthesis of various macromolecules (Wilbur, 1964; Wilbur and Saleuddin, 1983). In gastropods, new shell growth occurs at the shell edge via secretion of the periostracum, the outermost organic layer of the shell. Calcium carbonate crystals are then deposited on the periostracum, resulting in an increase in shell growth. In general, shell growth occurs simultaneously with body growth to provide space for the proportional growth of the soft body tissues.

The shell is a product of biologically controlled mineralization or biomineralization, and it is secreted by the underlying mantle epithelium. The mantle is composed of three main regions: the inner epithelium which is in contact with the environment (e.g., water for a shelled, aquatic gastropod), underlying internal tissues such as muscle, connective tissue including nerve

fibers, and the outer epithelium which secretes proteins, carbohydrates, lipids, and ions that are required for shell formation (Marin et al., 2012).

In gastropods, the anterior portion of the mantle has a fold known as the periostracal groove, which possesses specialized cells that secrete the outermost organic covering of the shell called the periostracum. This tough proteinaceous periostracal layer functions and serves as an organic matrix for shell deposition (Saleuddin and Petit, 1983). The periostracum is secreted as a fluid containing soluble tyrosine-rich precursors and fibrous proteins, which becomes hardened and insoluble in the extracellular environment via sclerotization and quinone-tanning.

The inorganic portion of the shell is mainly composed of two polymorphs of calcium carbonate, calcite, or aragonite. The calcium used in shell formation is taken up from the environment in aquatic gastropods and from dietary sources in terrestrial gastropods. The carbonate ions are supplied from the reaction of carbon dioxide and water in the animal, or via the uptake of bicarbonate from the external medium. Free calcium and carbonate are then transported by the hemolymph to calcification sites along the mantle epithelium. During the last decade, significant advances have been made toward the identification of various macromolecules involved in molluscan biomineralization, especially in commercially important gastropods and bivalves (Aguilera et al., 2014; Marie et al., 2012; O'Neill et al., 2013). Despite these advances in the identification and characterization of shell matrix proteins and the genes encoding them, we know very little about how these unique biomineralization proteins are regulated by nervous and/or endocrine mechanisms.

Classic endocrinological extirpation and re-implantation studies in *Crepidula fornicata* suggested that the cerebral ganglia produced a factor that stimulated body growth (Lubet, 1971; Lubet and Silberzahn, 1971). The putative growth-stimulating factors from the cerebral ganglia were hypothesized to stimulate growth via nerves as well as hormones. However, the putative growth-stimulating factor was never identified in *C. fornicata.* In *L. stagnalis*, paired groups of neurosecretory cells in the cerebral ganglia, the LGCs, were determined to be the source of a putative growth hormone as extirpation of the LGCs from growing juveniles resulted in reduction of body growth (Geraerts, 1976). Functionally similar neurosecretory medial cells in the slug *Deroceras reticulatum* (Widjenes and Runham, 1977), and the mediodorsal cells (MDCs) of the freshwater snail *H. duryi* were also demonstrated to be involved in the regulation of body growth (Kunigelis and Saleuddin, 1985; Saleuddin and Kunigelis, 1984).

In addition to causing cessation of growth, LGC extirpation in *L. stagnalis* results in a secondary accumulation of glycogen stores and higher glucose levels in the hemolymph (Dogterom, 1980; Geraerts, 1976). Extirpation of the LGCs also diminishes incorporation of protein into periostracum formation, whereas shell matrix formation remains unaffected (Dogterom and Jentjens, 1980). The putative growth hormone from the LGCs affects calcium transport primarily at the mantle edge as LGC removal decreased radiolabeled calcium incorporation in this region of the mantle by 30% when compared with control animals (Dogterom et al., 1979). Furthermore, LGC ablation also caused reduction of a putative calcium-binding protein in the mantle edge (Dogterom and Doderer, 1981). Based on studies in *L. stagnalis*, the growth hormone from the LGC is thought to exert its effect on shell growth by increasing the synthesis of periostracal proteins and maintaining high concentrations of calcium at the mantle edge which is required for calcification.

Initial attempts to characterize the chemical nature of the *L. stagnalis* growth hormone relied on in vivo bioassays that involved the injection of peptide-containing extracts of the median lip nerves (neurohemal area of the LGCs), then measuring ornithine decarboxylase activity or cAMP production at the mantle edge, or the incorporation of $^{45}Ca^{2+}$ at the shell edge (Dogterom and Robles, 1980; Ebberink and Joosse, 1985). These bioassays were generally unreliable because they showed extreme variability and relatively low specificity to be of use in screening crude extracts for growth hormone activity (Joosse, 1988). However, Ebberink and Joosse (1985) were able to partially purify a hydrophobic peptide of approximately 1000 Da that stimulated calcium incorporation into the shell and increased cAMP levels in the mantle edge. However, no further biochemical characterization of this 1000-Da peptide has been achieved.

Vertebrate peptides have also been reported in the LGCs of *L. stagnalis*. Immunohistochemical staining of the CNS using antibodies against various vertebrate peptides have shown the presence of somatostatin-like immunoreactivity in the LGCs and the median lip nerves (Grimm-Jørgensen, 1983; Schot et al., 1981). However, vertebrate somatostatin did not have any growth-promoting activity in *L. stagnalis* (Grimm-Jørgensen, 1985). ILPs have also been reported in the LGCs of *L. stagnalis* and the release of these ILPs could be induced in vitro (Ebberink et al., 1987).

As molecular cloning techniques became widely available in the 1980s, they were applied to molluscan nervous systems to identify and clone neuropeptide genes regulating numerous physiological processes. Using a subtractive hybridization approach, Smit et al. (1988) searched for mRNAs

specifically expressed in the LGCs of *L. stagnalis* and identified a cDNA clone that encoded the precursor of an insulin-like molecule which was named MIP, now called MIP-I. The pre-proMIP-I possessed both A and B chains and a C-peptide; therefore, it resembled the overall structure of mammalian insulin precursors. The A and B chains of MIP-I had approximately 50% and 18% sequence identity, respectively, when compared with vertebrate insulin chains but MIP-I possessed no sequence homology to vertebrate C peptide (Smit et al., 1988).

Subsequent molecular biological and peptide isolation studies by the Amsterdam researchers found that there were seven MIPs in *L. stagnalis*, named MIP-I to VII (Li et al., 1992a,b, Smit et al., 1991, 1992, 1993c, 1996). MIP IV and VI are considered pseudogenes since they are not transcribed in *L. stagnalis* (Meester et al., 1992). Sequence analyses of the MIPs revealed that their surface residues and N-termini were highly divergent compared with human insulin thus, MIPs were not expected to have similar biophysical properties in terms of receptor binding, the ability to form hexamers and other molecular interactions (Geraerts et al., 1992).

Although *L. stagnalis* MIP-I did not share high amino acid sequence identity with human insulin, the position of the cysteine residues in the A and B chains and the hydrophobic core amino acids were found to be conserved, suggesting that the MIPs have a similar overall organization of the A and B chains and the C-peptide to vertebrate insulins (Smit et al., 1988). MIP-I and all subsequent MIPs identified have conserved cysteine residues (see Fig. 10.3) in their A- and B-chains which are predicted to form interchain disulfide bonds. The MIPs also possess an extra pair of conserved cysteine residues in the A-chain, suggesting that the MIPs form an intrachain disulfide bond. Furthermore, the sequences of the C-peptides of the MIPs are highly conserved in *L. stagnalis* suggesting that they might have important physiological functions in this snail (Smit et al., 1998). The MIPs, however, are not considered true insulin molecules because their surface residues, which are important for receptor recognition, solubility, conformation, and intermolecular interactions, are completely divergent (Geraerts et al., 1992; Roovers et al., 1995; Smit et al., 1998).

The various MIP genes appear to be differentially expressed in the brain of *L. stagnalis*. Using oligonucleotide probes to specific MIP transcripts, the LGCs were shown to co-express MIP I, II, III, V, and VII (Meester et al., 1992; Smit et al., 1996). MIP VII appears to be exclusively expressed in neurons of the buccal ganglia, suggesting that it may be involved in the regulation of feeding (Meester et al., 1992). Furthermore, two types of LGCs could be distinguished with in situ hybridization, type A cells express all

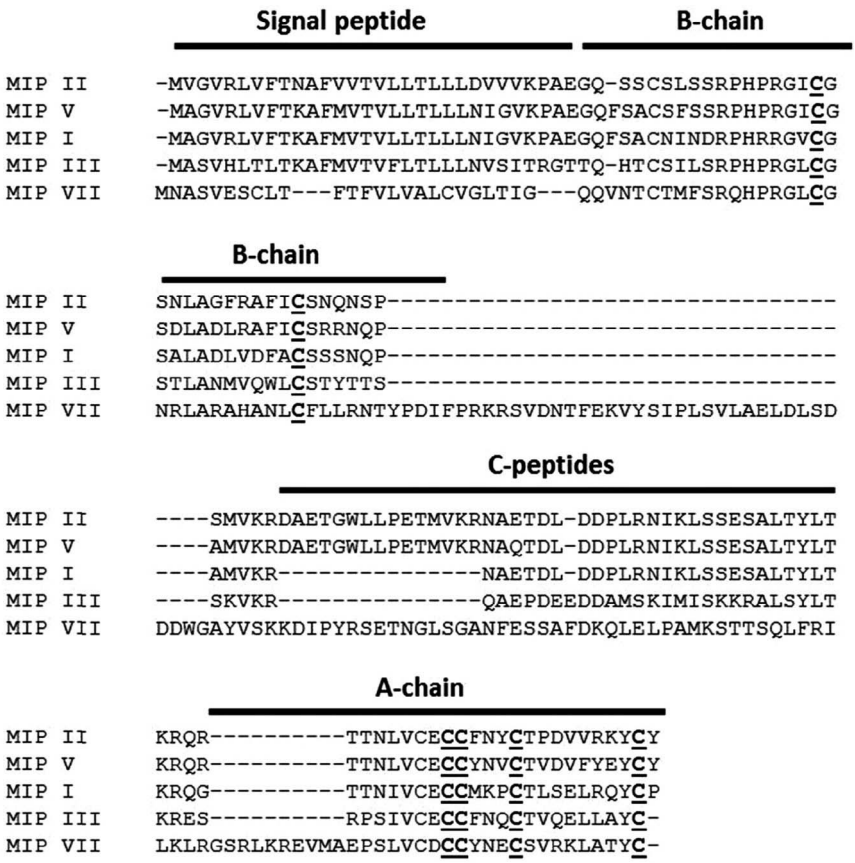

FIGURE 10.3 Clustal W sequence alignment of the five functionally transcribed molluscan insulin-related peptides (MIPs) from *Lymnaea stagnalis* showing the regions of the signal peptide, A- and B-chains, and the C-peptides. Two pairs of conserved cysteine residues (bolded and underlined) in the A- and B-chains, respectively, are predicted to form interchain disulfide bonds, and a third pair of cysteine residues in the A-chain is predicted to form an intrachain disulfide bond

MIP transcripts, whereas type B cells generally do not express or weakly express MIP I (Meester et al., 1992). MIP III is strongly expressed in type B cells, whereas MIP II and V are moderately expressed in both cell types (Meester et al., 1992). The physiological significance of differential MIP gene expression is unknown but may be linked to stimuli from outside the animal as well as signals within the animal. For example, ablation of the lateral lobe, a specialized neuron on each side of the cerebral ganglia which is thought to regulate the activity of the LGCs, accelerates growth

and enhances expression of MIP I, II, II, and V (Meester et al., 1992). On the other hand, snails subjected to starvation show a reduction in MIP II and V (Meester et al., 1992). Therefore, expression of the different MIP genes appears to be regulated independently, suggesting that some MIPs have overlapping function as well as potentially distinct functions.

In vertebrates, insulin binds to its receptor on target cells and stimulates specific intracellular signaling cascades linked to the activation of tyrosine kinases (Boucher et al., 2014; Du and Wei, 2014). Several insulin receptors have been cloned in gastropod molluscs, including *L. stagnalis* (Roovers et al., 1995), *A. californica* (Jonas et al., 1996), and *B. glabrata* (Lardans et al., 2001). In addition, the presence of insulin receptors in other gastropod nervous systems has been demonstrated by immunohistochemistry (Saavedra et al., 1989; Sonetti and Bianchi, 1993), radioreceptor autoradiography (Kerschbaum et al., 1993), or the production of known intracellular messengers linked to insulin receptor activation (Sossin et al., 1996a,b). However, binding of specific gastropod ILPs to their cognate receptors has not been demonstrated.

10.3.1 INSULIN-LIKE PEPTIDES AS REGULATORS OF GROWTH AND METABOLISM IN GASTROPODS

Since the discovery of ILPs in the LGCs of *L. stagnalis* (Smit et al., 1988), several labs have demonstrated the presence of ILPs in the nervous systems of gastropods. Using antibodies against mammalian insulin, the growth-controlling MDCs of the freshwater pulmonate *H. duryi* (Khan et al., 1992) and the mesocerebral neurosecretory cells of the terrestrial pulmonate *H. aspersa* were shown to contain ILPs (Gomot et al., 1992). In addition, immunohistochemical approaches using antibodies raised against specific peptide fragments deduced from molecular cloning demonstrate the presence of *L. stagnalis* pro-MIP-like immunoreactivity in the dorsal cells and other neurons of the CNS of freshwater snail *Planorbarius corneus* (Sonetti et al., 1992), whereas in the marine gastropod *A. californica*, the F and C clusters of the cerebral ganglia show intense staining for *Aplysia* insulin (AI) with an antibody raised against a portion of the predicted sequence of the C-peptide (Floyd et al., 1999).

In the freshwater pulmonate *H. duryi*, a circadian fluctuation of insulin-like immunoreactive material in the hemolymph was detected with a mammalian insulin RIA (Sevala et al., 1993a). Peaks of insulin-like material were detected during the photophase, which preceded daily increases

in shell deposition rates, suggesting ILPs may be involved in shell growth in *H. duryi* (Sevala et al., 1993a). To further investigate the possible role of ILPs in the regulation of growth, Sevala et al. (1993b) demonstrated that partially purified ILPs in *H. duryi* stimulated in vitro protein synthesis in mantle tissue explants. In the related planorbid snail *B. glabrata*, application of bovine insulin to Bge cell lines increased the rate of amino acid incorporation (Lardans et al., 2001).

ILPs appear to play a role in growth and metabolism in stylommatophorans as well as basommatophorans. In *H. aspersa*, removal of the mesocerebral green cells, a group of insulin-positive neurosecretory cells, causes a cessation of growth and the accumulation of glycogen in the epithelium of the mantle edge (Gomot et al., 1992). Immunohistochemical and Western blotting studies in *Otala lactea* demonstrated the presence of ILPs in the cerebral ganglia and other parts of the CNS, as well as the gut (Abdraba and Saleuddin, 2000a,b). Both crude and partially purified ILPs from brain extracts applied to an mantle collar explants in vitro , a bioassay for growth, stimulated total protein synthesis (Abdraba and Saleuddin, 2000b). These studies in aquatic and terrestrial pulmonates suggest that ILPs stimulate cellular protein synthesis and have functional characteristics of a growth hormone.

In the opisthobranch *A. californica*, injection of extracts derived from the upper labial and anterior tentacular nerves, the neurohemal regions of ILPs in this animal, decreased glucose concentration in the hemolymph but did not affect feeding behavior (Horn et al., 1998). Furthermore, expression of AI mRNA decreased when the animals were starved, whereas injections of highly purified AI caused a reduction in hemolymph glucose concentration (Floyd et al., 1999). Therefore, ILPs appear to function as metabolic regulators of carbohydrate metabolism in *A. californica*, similar to the functions of insulin in vertebrates. The role of ILPs as a potential growth regulator in in *Aplysia* is not known. Taken together, the studies in *Aplysia*, *Lymnaea*, *Helisoma*, and *Helix* strongly suggest a conserved function of ILPs in influencing growth and metabolic activities in molluscs.

10.3.2 NOVEL FUNCTIONS OF ILPs IN GASTROPODS

ILPs in gastropods have also been reported to have other functions not directly related to carbohydrate metabolism and growth. For instance, in *L. stagnalis* neuronal extracts containing MIP were purified by HPLC, and then applied to cultured neurons in vitro. Purified MIP stimulated neurite outgrowth suggesting that ILPs may play a role in neuronal development

(Kits et al., 1990). ILPs have also been reported to be important in the formation of long-term memory as it relates to conditioned taste aversion behavior in *L. stagnalis* (Mita et al., 2014). Indeed, insulin is increasingly recognized as an important signaling molecule in the brain which regulates neuronal function in both vertebrates and invertebrates (Derakhshan and Toth, 2013; Liu et al., 2014).

Recently, a novel function of ILPs has been reported in two fish-hunting cone snails, *Conus geographus* and *Conus tulipa*. The ILPs in these two snails are closely related to insulin in their fish prey. The cone snail insulin is released together with other toxins into the water and induces hypoglycemic shock in fishes, thereby facilitating prey capture (Safavi-Hemami et al., 2015). In *A. californica*, application of bovine insulin to isolated bag cells induces ELH secretion in vitro without triggering action potentials (Jonas et al., 1997). Vertebrate insulin appears to bind to and activate an insulin receptor on the bag cells resulting in the mobilization of calcium from a unique intracellular calcium store. Therefore, ILPs in *Aplysia* can communicate with the neuroendocrine system controlling egg-laying behavior and potentially influence reproductive processes as well carbohydrate metabolism.

10.3.3 REGULATION OF GROWTH HORMONE-PRODUCING CELLS

In *L. stagnalis*, the MIP-producing LGCs appear to be capable of detecting extracellular glucose in vitro as concentrations of 1 mM glucose or higher evoked long-lasting spiking activity (Kits et al., 1991). Thus, the LGCs are functionally somewhat similar to mammalian pancreatic β-cells in that they can detect changes in extracellular glucose concentrations. However, the cellular mechanism for glucose uptake by the LGCs is different than the β-cells, and the secretion of specific MIPs in response to increased glucose has not been shown. Other neuroactive agents have been demonstrated to affect the electrical excitability of the LGCs. The catecholamine dopamine (Stoof et al., 1984; De Vlieger et al., 1986), and the peptides FMRFamide and APGWamide (van Tol-Steye et al., 1997, 1999) cause a hyperpolarization of the LGCs. Dopamine, FMRFamide, and APGWamide appear to converge on a potassium channel and activate an S-like potassium conductance via different G proteins.

In freshwater pulmonates, there is a close physiological interdependence between body growth and reproduction since these two processes require

considerable energy investments. For example, experimental or parasite-induced castration markedly increases shell growth compared to reproducing snails which grow at slower rates (Geraerts and Mohammed, 1981; Miksys and Saleuddin, 1987). Hemolymph from parasitized snails or application of the neuropeptide schistosomin reduced the excitability of the CDCs, whereas the same treatments had the opposite effect on LGC excitability (Hordijk et al., 1992). Furthermore, differential mRNA screening of parasitized versus nonparasitized snails showed that several genes in the CNS coding for various peptides are differentially expressed (Hoek et al., 1997). Application of LFRFamide (an FMRFamide-related peptide) to the LGCs or the CDCs inhibits their electrical activity (Hoek et al., 2005). It has also been shown that there is a close morphological association of NPY (neuropeptide Y)-positive axons and the axons from the LGCs, suggesting that a *Lymnaea* NPY-like molecule (LNPY) may regulate growth. For example, implantation of slow-release pellets containing LNPY inhibited both growth and reproduction in a dose-dependent fashion (De Jong-Brink et al., 1999). It is thought that parasites manipulate the host snail's neuroendocrine system by suppressing metabolism and reproduction and exploiting the host's energy reserves for their own use.

The LGCs in *L. stagnalis* receive input from other regions of the CNS such as the lateral lobes (Geraerts, 1976; Roubos et al., 1980) and from sensory cells located near in the epidermis near the base of the tentacle which can perceive environmental stimuli (Roubos and Wal-Divendal, 1982). The precise cellular mechanisms by which these cells regulate the LGCs are unknown.

10.4 REGULATION OF CARDIAC ACTIVITY AND CIRCULATION BY NEUROPEPTIDES

10.4.1 STRUCTURE OF THE CIRCULATORY SYSTEM IN MOLLUSCS

The basic architecture of the molluscan heart consists of a pair of auricles that collect blood from the kidney and gill, and a single ventricle that pumps the blood out to the sinus. There are flaps between the auricle and ventricle (auricloventricular valve) that prevent back flow from the ventricle to the auricle. The heart is nestled in the pericardial cavity located on the dorsal side of the body. There are well developed arteries and veins connected to the heart, although there are no direct connections between vessels (Jones,

1983). Hemolymph pumped out from the ventricle flows toward the sinus surrounding tissues and organs through the arteries and aortae. Hemolymph then leaks out to the hemocoel through lacunae on the sinus wall. Hemolymph is collected to the auricle after it passes through the gill or kidney. Thus, the molluscan circulatory system is considered semi-open.

In gastropods, it is believed that ancestral form of the heart consisted of a pair of auricles, each of them connected to the right and left gills, respectively. One ventricle is connected to the auricle and receives blood supply from both of the auricles. However, most gastropods (except for the Archaeogastropods such as limpets) lost the auricle and gill on the left side as a result of torsion. Therefore, in the hypsobranchia, including neogastropods, the heart consists of single auricle connected to the ventricle. This basic architecture of the heart is unchanged in opisthobranchs and pulmonates that have not undergone torsion.

An important function of the circulatory system in molluscs is the transport of hemolymph, which contributes to the delivery of nutrients and O_2 to the cell as well as to the removal of waste and CO_2 from the cell. Moreover, the circulatory system in molluscs is also involved in locomotion. For example, the redistribution of hemolymph is known to be important for the extension of the foot in bivalves. In *Aplysia*, the anterior aorta is the major artery supplying hemolymph to the CNS, buccal mass, the genital organs, opaline gland, and other somatic tissues at the anterior end of the animal. The hydrostatic pressure generated by the redistribution of hemolymph is the motive force for the extension of various body parts. For instance, closure of the sphincter muscle in the region of the abdominal aorta introduces hemolymph in the posterior part of the body and is associated with an increase in heartbeat, and resultant hemolymph accumulation in the head region (Sasaki et al., 2004; Skelton et al., 1992). Concomitant relaxation of body wall in the anterior region results in the extension of the head forward. Thus, the cardiovascular system is important for locomotion in soft-bodied animals. The regulation of aortic blood flow is known to be under control of classical transmitters as well as peptides.

Another function of the circulatory system is in urine formation. In some gastropods, a portion of the hemolymph in the heart spills out to the pericardial cavity through the wall of heart or crista aortae (Andrews, 1988). The pericardial cavity is connected to the kidney via the renal-pericardial pore and hemolymph in the cavity drains into the kidney. Absorption and secretion of salts, nutrients and wastes occurs in the kidney, and, finally, urine is excreted to the outside through the renal pore. Thus, in some gastropods, the heart is the proposed site of filtration.

The heartbeat of the molluscs is generated by a myogenic pacemaker and the beating amplitude and frequency are regulated by both excitatory and inhibitory neural inputs. The following sections will summarize how neuropeptides are involved in the neural regulation of cardiac activity. For more information, refer to other excellent reviews (Kodirov, 2011; Skelton et al., 1992).

10.4.2 REGULATION OF HEARTBEAT BY NEUROPEPTIDES

In *Aplysia*, heart rate changes in response to various external and internal stimuli such as feeding (Dieringer et al., 1978), food-induced arousal (Koch et al., 1984), sensitizing stimuli (Krontiris-Litowitz, 1999), and hypoxia (Pinsker et al., 1974). The neural regulation of the cardiovascular system has been well studied in this gastropod (Koester et al., 1974; Liebeswar et al., 1975; Mayeri et al., 1974; Rozsa, 1979). In *Aplysia*, for instance, heart excitatory neurons such as RB_{HE}, are serotonergic, and heart inhibitory neurons such as RB_{HI} are cholinergic. On the other hand, some neurosecretory cells that release peptides are also known to be involved in the regulation of the cardiovascular system.

In the abdominal ganglion of *Aplysia*, the R3-R13 neurons located in the RUQ region, as well as the R14 and R15 neurons of the caudal side of the ganglion, project axons toward the cardiovascular region, including the gill and efferent veins, anterior and gastroesophageal aortae, ganglionic artery dorsal, and mantle arteries, auricle and the auricloventricular valve (Rittenhouse and Price, 1986a,b; Skelton and Koester, 1992). These neurons are involved in the regulation of heartbeat and the contraction of the aorta (Rozsa et al., 1980; Sawada et al., 1981).

R3–14 neurons appear to be neurosecretory cells because of their whitish appearance, the presence of DCVs and blunted nerve-endings (Coggeshall et al., 1966). In fact, R3–14 neurons synthesize a 12-kDa-protein (Aswad et al., 1978). Nambu et al. (1983) cloned a cDNA expressed specifically in R3–14 cells, and predicted that the 12-kDa-protein is a neuropeptide precursor consisting of 108 amino acids (Nambu et al., 1983). The predicted precursor is posttranslationally cleaved into three fragments since the precursor has two dibasic processing sites. Of the three fragments, the one consisting of 43-amino acids was purified from the abdominal ganglion and its structure was confirmed (Campanelli and Scheller, 1987; Knock et al., 1989; Nagle et al., 1989a,b). Although the effective concentration used in bioassays is relatively high (over 10^{-6} M), the peptide referred to as histidine-rich basic peptide (HRBP), augments heartbeat of *A. californica* (Campanelli and

Scheller, 1987). Precursor genes for LYCPs (light-yellow cell peptides) in *Lymnaea stagnalis* (Smit et al., 1993a) and HSD-1/-2 in *Helix lucorum* (Bogdanov et al., 1996) also encode HRBP-related peptides. On the other hand, the R15 neuron releases two distinct peptides, R15α1 and R15α2. R15α2 peptide-containing nerve processes are distributed in the arterial system (Skelton and Koester, 1992) and have moderate cardioexcitatory action on the *Aplysia* heart (Alevizos et al., 1991b).

Morishita et al. (1997) isolated a D-tryptophan-containing cardioexcitatory Asn-D-Trp-Phe-NH_2 (NdWFamide) from *Aplysia kurodai* heart (Morishita et al., 1997). Immunohistochemistry with specific anti-NdWFamide antibody revealed that some neurons in the RUQ region of the abdominal ganglion contain NdWFamide. Based on cell location and cell size, NdWFamide-containing neurons include some of the R3–14 cells, as well as other smaller white cells in the RUQ region (Morishita et al., 2003c). Moreover, the distribution of NdWFamide-containing nerve processes in the cardiovascular region overlaps with axons of the R3–14 neurons. Therefore, it is likely that NdWFamide mediates the physiological action of the RUQ neurons including some R3–14 neurons.

Besides the aforementioned neuropeptides, many peptides such as FMRFamide, SCP_B, achatin-1, and ACEP-1 are also involved in the regulation of heartbeat in molluscs. In the following section, we will focus on some selected neuropeptides and summarize how they are involved in the regulation of heartbeat.

10.4.3 FMRFAMIDE

FMRFamide consists of four amino acids (Phe-Met-Arg-Phe) with a C-terminal amide and it was originally purified from the ganglia of the bivalve, *Macrocallista nimbosa* (Price and Greenberg, 1977). The discovery of FMRFamide by Greenberg and his colleagues was a landmark publication that opened up new avenues in peptide research and further strengthened the concept that peptides play crucial roles in metazoan neural communication. Immunohistochemical staining with anti-FMRFamide antibody has demonstrated the distribution of FMRFamide in the central and peripheral nervous systems in numerous animal groups (Grimmelikhuijzen, 1983). In fact, various neuropeptides having a C-terminal RF-NH_2 structure have been isolated in other animal phyla including Cnidaria, Platyhelminthes, Arthropoda, and Chordata (Grimmelikhuijzen and Graff, 1985; Johnston et al., 1995; Schneider and Taghert, 1988; Ukena et al., 2002). It is thought that

the RFamide-related peptides belong to an ancestral peptide family because the C-terminal Arg-Phe-NH_2 structure was conserved during the diversification and molecular evolution of neuropeptides.

FMRFamide and structurally related peptides (FMRFa-RP), FLRFamide have been identified in many molluscan groups. Moreover, longer forms of FMRFamide such as GDPFMRFamide and *Mytilus*-FFRFamide are also found in bivalves, pulmonates and cephalopods (Fujisawa et al., 1992; Lopez-Vera et al., 2008; Price et al., 1987). FMRFamide augments the amplitude and frequency of the isolated heart of *Mercenaria* (threshold = 10^{-9}–10^{-8} M) (Price and Greenberg, 1979), *L. stagnalis*, *H. aspersa* (around 10^{-7}–10^{-6} M) (Price et al., 1990), *Achatina fulica* (threshold = 10^{-10}–10^{-9} M) (Koch et al., 1993), *Rapana thomasiana* (threshold = 10^{-9}–10^{-8} M) (Fujiwara-Sakata and Kobayashi, 1992), and *Archidoris montereyensis* (around 10^{-6}–10^{-4} M) (Wiens and Brownell, 1995). FMRFamide-related peptides such as pQDP-FLRFamide and SDPFLRFamide are also cardioactive on *Helix*, *Lymnaea*, and *Achatina*. By contrast, the cardioexcitatory action of FMRFamide on *Aplysia* heart is unclear (Harris et al., 1995).

The signal transduction pathway mediating the cardioexcitatory action of FMRFamide is rather complex. Unlike small cardioactive peptide B (SCP_B) and serotonin, FMRFamide does not elevate intracellular cAMP levels in *Helix* heart (Reich et al., 1997a). However, in *L. stagnalis*, the cardioexcitatory action of FMRFamide and related peptides correlate with increases in intracellular cAMP (Willoughby et al., 1999a). In the optic lobe of a squid, *Loligo pealei*, FMRFamide activated adenylate cyclase in a GTP-dependent manner (Chin et al., 1994). Thus, one possible signaling pathway is that FMRFamide activates adenylate cyclase by activating the Gs subunit of a G-protein. Another possible pathway is via the elevation of inositol phosphates by activating PLC. FMRFamide promotes generation of inositol trisphosphate in molluscan hearts (Bayakly and Deaton, 1992; Ellis and Huddart, 2000; Willoughby et al., 1999b), which triggers Ca^{2+}-mobilization from intracellular stores. In addition, it is suggested that activation of protein kinase C is involved in the cardioexcitatory action of FMRFamide.

In both of these signaling pathways, FMRFamide binds to a 7-transmembrane spanning GPCR. FMRFamide receptors in molluscs are predicted by annotation of genome database sequences (e.g., accession number: XM_005110697 for *C. gigas*, XP_005110754.1 for *Aplysia*). However, the biochemical and physiological characterization of these receptors is lacking at this time.

In addition to the metabotropic receptor system, FMRFamide modulates ion channel activities on cardiac muscle cells. In *L. stagnalis*, FMRFamide

increases the open-probability of Ca^{2+}-channels in isolated cardiac muscle cells, which gradually decreases during repetitive application of the peptide (Brezden et al., 1999). In the heart muscle cells of the squid *Loligo forbesii*, FMRFamide shows opposing actions on L-type Ca^{2+}-channels in distinct cardiac cell types(Chrachri et al., 2000). As is in *Lymnaea* heart cells, FMRFamide increased Ca^{2+}-current through an L-type Ca^{2+}-channel in the type-II heart cells. By contrast, the peptide decreased Ca^{2+}-current in the type-I heart cells through a pertussis toxin-sensitive G-protein. It can be argued that the modulatory actions of FMRFamide on molluscan heart cells are mediated by G-proteins, rather than the direct action on a channel protein.

FMRFamide is known to modulate sodium channels by binding to a channel protein. FMRFamide-gated Na^{+}-channels have been identified in the nervous tissues of *H. aspersa* (Lingueglia et al., 1995), *H. trivolvis* (Jeziorski et al., 2000), and *A. kurodai* (Furukawa et al., 2006) (accession number: X92113 for *H. aspersa*, AF254118 for *H. trivolvis* and AB206707 for *A, kurodai*, respectively). The FMRFamide-gated channel is an amiloride-sensitive Na^{+}-channel. It has two transmembrane domains and functions as tetramer (Coscoy et al., 1998), whereas the ligand-binding site is located near the first transmembrane region (Cottrell et al., 2001). This was an important discovery in the field of peptide and ion channel research as it demonstrated the presence of a ligand-gated ion channel that is activated by direct binding of a neuropeptide.

The structures of FMRFamide precursors have been cloned in several molluscan species. For instance, the precursor protein for FMRFamide in *Aplysia* consists of 552 amino acids and 28 copies of the sequence FMRFGKR repeats on the precursor (Taussig and Scheller, 1986). A single copy of FLRFGKR sequence was found in the N-terminal region of the precursor. In *Mytilus edulis*, the cloned precursor consists of 403 amino acids. All the FMRFa-RPs identified from *Mytilus* (FLRFamide and *Mytilus*-FFRFamide) are encoded on this precursor, together with 16 copies of FMRFamide (Favrel et al., 1998). Therefore, when the precursor proteins are subjected to PTMs, all the FMRFa-RPs are generated as a minor component of mature peptides. By contrast, in *Lymnaea*, tetra- and penta-FMRFa-RPs including authentic FMRFamide are encoded on the precursor distinct from that encoding longer hepta-FMRFa-RPs. The *L. stagnalis* FMRFamide precursor gene consists of five exons. All the precursors are generated by alternative splicing from the single precursor gene (Santama and Benjamin, 1995). For instance, precursor 1 which encodes the tetrapeptides (e.g., FMRFa, FLRFa, and others) is generated by the combination of exons I and II, whereas precursor II that encodes

longer FMRFamide-RPs (e.g., GDPFLRFa, SDPFLRFa) is generated by exons I, III, IV, and V (see Fig. 10.4). As shown by in situ hybridization and mass spectrometry, the heart exciter neuron, Ehe, expresses precursor 1 and expresses the tetrapeptides, whereas the visceral white interneuron

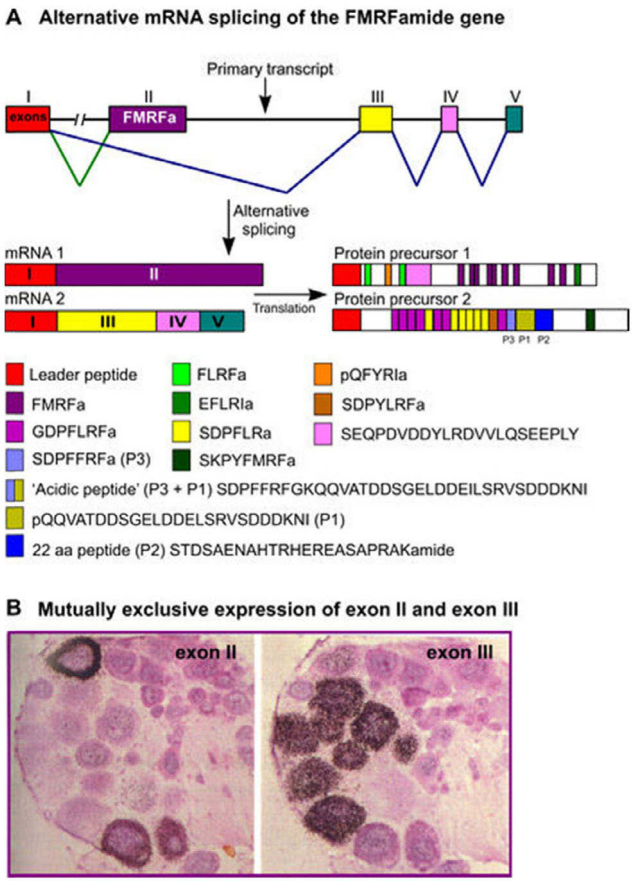

FIGURE 10.4 Alternate mRNA splicing of the FMRFamide gene. (A) Two mRNA variants are spliced from the primary transcript. Protein precursor 1 encodes 5 different peptides, including multiple copies of the tetrapeptides, FMRFamide and FLRFamide. Post-translational processing of QFYRIamide converted Q into pQ. Protein precursor 2 encodes 7 peptides including multiple copies of the heptapeptides, SDPFLRFamide and GDPFLRFamide. Post-translational cleavage of the "acidic peptide" resulted in two further peptides P3 and P1. Only peptides that were confirmed by sequencing and mass spectrometry are included in the list of peptides. (B) In situ hybridization of the alternatively spliced transcripts shows the mutually exclusive mRNA expression at the single neuron level. The same neurons can be identified in these adjacent sections of the visceral ganglion. (Source: Benjamin, P. R., Kemenes, I. Lymnaea Neuropeptide Genes. *Scholarpedia*, 8(7), 11520). (Used with permission.)

expresses precursor 2 (heptapeptides) in the visceral ganglion, suggesting that alternative splicing generates two mutually exclusive neuronal populations (Santama and Benjamin, 2000). Therefore, the *Lymnaea* FMRFamide-related peptides demonstrate how alternative splicing generates peptide diversity and how the cell-specific expression of these different peptides is capable of controlling cardiac function.

10.4.4 THE SMALL CARDIOACTIVE PEPTIDES

As described above, the ganglia of gastropod molluscs contain several cardioactive substances. In *H. aspersa*, three cardioexcitatory and one cardioinhibitory substance was separated by size-exclusion chromatography (Lloyd, 1982, 1978). The low molecular weight cardioexcitatory substance appeared to be serotonin because the substance coeluted with serotonin, and methysergide, a serotonin antagonist, blocked its cardioexciatory action on the *Helix* ventricle. On the other hand, the cardioinhibitory substance was acetylcholine (Ach) since it coeluted with Ach, and its physiological effect was diminished by treatment with cholinesterase. The other two cardioexcitatory substances seem to be peptides because protease treatment diminished cardioexcitatoy actions of the substances on the heart. The cardioexcitatory peptide with larger molecular weight (6000–8000 Da) was named as large cardioexcitatory peptide (LCP), while the other with low molecular weight (ca. 1500 Da) was named as small cardioexcitatory peptide (SCP).

An SCP-like substance was also found in the peptide-containing extract of the central ganglia of *A. brasiliana* (Morris et al., 1982) and *L. stagnalis* (Geraerts, 1981), whereas no LCP-like substances were found in *Aplysia*. Using a peptide extract from the esophagus and crop of *A. californica*, peptides with SCP-like activity were separated by size-exclusion, ion exchange and reverse-phase column chromatography and resulted in the isolation of two distinct SCP-like peptides, SCP_A and SCP_B, respectively. Analysis by fast-atom bombardment mass spectrometer revealed that the structure of SCP_B was Met-Asn-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2. The structure of SCP_A was determined as Ala-Arg-Pro-Gly-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2 (Lloyd et al., 1987).

In *H. aspersa*, SCP_B is identical to that of *Aplysia* SCP_B, although that of *Helix* SCP_A is slightly different from that of *Aplysia* SCP_A (Price et al., 1990). Nevertheless, all of the SCPs share the same C-terminal YLAFPRM-NH_2 structure. SCP-related peptides are also present in other molluscs, including the freshwater pulmonates, *L. stagnalis* and *H. trivolvis*, the

bivalves *Mercenaria mercenaria*, *Crassostrea virginica* and *Mytilus edulis* (Candelario-Martinez et al., 1993; Fujisawa et al., 1993), and the cephalopod, *Octopus vulgaris* (Kanda and Minikata, 2006). The structures of various molluscan SCPs are listed in Table 10.3. Molecular cloning of the SCP precursors in *Aplysia* and *Lymnaea* has shown that SCP_A and SCP_B are encoded in tandem in the N-terminal region of the same precursor (Mahon et al., 1985a; Perry et al., 1999).

TABLE 10.3 Structures of cardioactive peptides referred in this review

Peptides		Structure	Animals
FMRFa-RP		FMRFa	***Lymnaeastangalis***
		FLRFa	
		QFYRIa	
		EFLRIa	
		GDPFLRFa	
		pQDPFLRFa	
		SDPFLRFa	
SCP-RP	A	SGYLAFPRMa	***Helix pomatia***
	B	MNYLAFPRMa	
	A	SGYLAFPRMa	***Lymnaeastagnalis***
	B	pQNYLAFPRMa	
	A	SQGYLAFPRMa	***Helisomatrivolvis***
	B	SGYLAFPRMa	
	A	PGYLAFPRMa	***Aplysiacalifornica***
	B	NMYLAFRPMa	
		SQPYIAFPRMa	***Littorinalittorea***
		NYLAFPRMa	***Thais heamastoma***
	A	IAMSFYFPRMa	***Mercenariamercenaria***
	B	YFAFPRQa	
		APNFLAYPRLa	***Mytilus edulis***
	A	AMSFYFPRMa	***Dinocardiumrobustum***
	B	YFAFPRQa	
ACEP-RP	ACEP-1	SGQSWRPQGRFa	***A. californica***
	LyCEP	TPHWRPQGRFa	***L. stagnalis***
	LUQIN	APSWRPQGRFa	***A. californica***

TABLE 10.3 *(Continued)*

HRBP-RP	HRBP	pQVAQMHVWRAVN-HDRNHGTGS-GRHGRFLIRN-RYRYGGGHLSDA	***A. californica***
	LYCP	SLAQMYVGNHHF-NENDLTSTHGGSRRWS-NRKHQSRIYTGAQLSEA	***L. stagnalis***
	HDS-1	NTAQWLQGRQHFN-NYDHESNRRFSH-PRYNIRNNGRGVIAEA	***Helixlucorum***
R15α peptide	R15α peptide-2	MGSGGLKLHCQVH-PANCPGGLMVT	***A. californica***
	VD1/RPD2 α-peptide	DMYEGLAGRCQHH-PRNCPGFN	***L. stagnalis***
DAACP	Achatin-1	GdFGD	***Achatinafulica***
	fulicin	FdNEFVa	***A. fulica***
	NdWFa	NdWFa	***Aplysiakurodai, L. stagnalis,***
Cardioinhibitory peptide			
	Helix HCIP	VFQNQFKGIQGRFa	***Helix aspersa***
	APGWamide	APGWa	***H. aspersa***
	CARP	AMPMLRFa	***Rapanathomasiana***
	FRFamide	GSLFRFa	***A. fulica***
		SSLFRFa	***A. fulica***

ACEP: *Achatina* cardioexcitatory peptide, DAACP: D-amino acid containing peptide, HRBP: Histidine-rich basic peptide, SCP: Small cardioactive peptide.
Note that "a" represents C-terminal amide, while "d" represents D-amino acid.

SCPs increase both the frequency and amplitude of the heartbeat in *A. californica* (Cawthorpe et al., 1985) and in *H. aspersa* (Lesser and Greenberg, 1993). One of the remarkable features of the SCPs is its wide effective concentration range on the heart. For example, in the isolated and perfused preparation of the *Aplysia* heart, the threshold concentration of SCP_B was as low as 10^{-12} M whereas the maximum action was obtained at concentration above 10^{-7} M (Cawthorpe et al., 1985). Application of SCP concentrations of higher than 10^{-10} M required more than 20-min before the heartbeat returned to basal. SCPs augment intracellular levels of cAMP in the myocardial

fibers of *Aplysia* (Cawthorpe et al., 1985) and in *Helix* (Reich et al., 1997a) through the activation of adenylate cyclase. In the dispersed myocardial cells of *Helix*, SCP-dependent elevation of cAMP promotes phosphorylation of a 53-kDa protein (Reich et al., 1997b), although the chemical characterization of the protein is not known.

Immunohistochemistry using anti-SCP_B antibody demonstrates SCP-staining neurons in the central and peripheral nervous systems in several gastropods including *Aplysia*, *Helix*, and *Lymnaea.* In *Aplysia*, the most prominent signals were found in the buccal ganglia, although a few neurons in the other ganglia also react with anti-SCP antibody (Lloyd et al., 1985). The B1 and B2 motoneurons that regulate the motility of buccal mass as well as many small neurons in the ganglion are immunopositive. Therefore, it is likely that in addition to cardiac regulation, SCPs are involved in the regulation of feeding behavior.

In the abdominal ganglion, SCP-containing nerve processes are abundant in the neuropil, connectives and the nerve bundles that emanate from the ganglia. However, only a few small neurons are immunoreactive to the anti-SCP antibody in this ganglion. In addition, the distribution of SCP-containing nerve processes is quite diffuse in the cardiovascular region (Lesser and Greenberg, 1993). Thus, despite of the potent action on the molluscan hearts, it is currently unknown which SCP-positive neuron regulates heartbeat. Nevertheless, the fact that SCPs are effective on the heart at low concentrations, even trace amounts of SCPs released from those nerve endings may have action on the myocardium.

10.4.5 ACHATINA CARIDOEXCITATORY PEPTIDE-1

Achatina cardioexcitatory peptide (ACEP)-1 is an undecapeptide having C-terminal RF-NH_2 structure (Ser-Gly-Gln-Ser-Trp-Arg-Pro-Gln-Gly-Arg-Phe-NH_2). ACEP-1 was originally isolated from the auricle of *A. fulica* using HPLC fractionation and a bioassay measuring ventricular contraction (Fujimoto et al., 1990). Although ACEP-1 was isolated from the auricle it has no effect on the auricle itself, whereas it augments the beating amplitude of the *Achatina* ventricle. Fujiwara-Sakata and Kobayashi (1994) subsequently examined the localization of ACEP-1 in *Achatina* by immunohistochemistry. ACEP-1-like immunoreactivity is localized to the left and right pedal nerve large neurons (L- and R-PeNLN), ventral visceral large neuron (v-VLN), and ventral right parietal large neuron (v-RPLN). Moreover, other large neurons in the visceral ganglion send ACEP-1-containing nerve

processes toward the atria through the intestinal nerve. In addition, dense ramifications of ACEP-1 containing nerve endings are seen in the auricle. It is likely that ACEP-1 released from the auricle acts in a paracrine fashion on the ventricle.

ACEP-1-related peptides have also identified in *A. californica* and *L. stagnalis*. In *Aplysia*, biochemical identification of ACEP-1-related peptides (LUQIN) was preceded by the molecular cloning of its precursor. Shyamala et al. (1986) found that the L5 neuron in the LUQ region of the abdominal ganglion in *Aplysia* was immunoreactive to anti-FMRFamide antibody (Shyamala et al., 1986). When one of the cDNA clones designated as L5–67, which was derived from the mRNAs specifically synthesized in the L5 neuron was sequenced, it was found that the predicted protein included the Arg-Phe-Gly-Lys-Arg sequence in the N-terminal region. The mature form of the novel RFamide peptide (LUQIN) was subsequently purified from LUQ cells and its structure was determined as Ala-Pro-Ser-Trp-Arg-Pro-Gln-Gly-Arg-Phe-NH_2 (Aloyz et al., 1995). LUQIN share the same C-terminal seven amino acids with ACEP-1 and alternative splicing generates another form of the LUQIN precursor N-terminal region including the first 45 amino acids which are identical to the original L5–67 propeptide (Angers and DesGroseillers, 1998). In situ hybridization analysis localized the expression of the LUQIN precursor in the identified neurons L2, L3, L4, and L6 in the LUQ region of the *Aplysia* abdominal ganglion (Giardino et al., 1996; Landry et al., 1992). Tissue contents of the LUQIN precursor estimated by immunoreactivity to anti-LUQIN precursor antibody detected significant immunoreactivity in the abdominal, pleural, pedal, and cerebral ganglia.

In the freshwater snail *L. stagnalis*, a novel cardioexcitatory peptide was isolated by screening for ligands that bound to some previously cloned GPCRs (Cox et al., 1997; van Kesteren et al., 1995; Tensen et al., 1998). Endogenous ligands from ganglionic extracts were screened for their ability to bind to cloned receptors that were expressed in *Xenopus* oocytes. From this heterologous screening procedure, Tensen et al. (1998) identified of a novel ACEP-1-related peptide which bound to clone GRL 106 and was named *Lymnaea* cardioexcitatory peptide (LyCEP). ACEP-1, LUQIN, and LyCEP have cardioexcitatory action on *A. fulica* and *L. stagnalis,* respectively (Tensen et al., 1998). Unlike ACEP-1, LyCEP augments beating frequency of *Lymnaea* auricle. However, ACEP-1 and related peptides appear to be multifunctional. For instance, cholinergic motoneuron B10 in buccal ganglion of *A. fulica* is colocalized with ACEP-1. ACEP-1 is thought to function as a peptide co-transmitter and regulates the radula protractor muscle. ACEP-1 also functions to increase the phasic contractions of the

penis retractor muscle and buccal muscle that was previously excited by electrical stimulation. In *L. stagnalis*, LyCEP appears to have reproductive functions as well. LyCEP-immunopositive nerve endings are found in the vicinity of the CDCs and the LyCEP receptor is also expressed in some CDCs. LyCEP is known to decrease the electrical discharge of CDCs and may regulate feeding behaviors by inducing the spontaneous discharge of buccal neuron B4 (Tensen et al., 1998).

10.4.6 CARDIOACTIVE PEPTIDES CONTAINING D-AMINO ACIDS

Amino acids, except for glycine, have an asymmetrical carbon atom in their molecules. Accordingly, there are stereoisomers, D-type and L-type, for each amino acid. Nevertheless, peptides and proteins found in living organisms usually consist of L-amino acids. Although it is a rare phenomenon, several cardioactive peptides found in molluscs possess a D-amino acid residue in their structure. Achatin-1 (G_{D-}FAD), fulicin (Phe-D-Asn-Glu-Phe-Val-NH_2) and NdWFamide are examples of such D-amino acid containing cardioactive neuropeptides in molluscs (Kamatani et al., 1991; Ohta et al., 1991; Morishita et al., 1997). A common structural feature shared by those D-amino acid-containing peptides is that the D-/L-conversion occurs on the N-terminal penultimate amino acid. Other peptides such as fulyal from *A. fulica*, FLRFamide-related peptide from *M. edulis*, and crustacean cardioactive peptide (CCAP)-related peptide from the Roman snail, *Helix pomatia*, are other examples of D-amino acid containing neuropeptides, although it is unclear if they modulate heartbeat these animals (Fujisawa et al., 1992; Minakata, 1996; Yasuda-Kamatani et al., 1997).

Achatin-1 is a tetrapeptide containing D-phenylalanine identified in the African giant snail, *A. fulica* (Kamatani et al., 1991). The same peptide was also purified from the *Achatina* aorta by a different group (Fujimoto et al., 1991). Structurally related peptides to achatin-1 are found in *Octopus* and these peptides also have cardioexcitatory action on the isolated systemic heart (Iwakoshi et al., 2000). An achatin-1-like peptide has also been identified in *Aplysia*, however, a cardioexcitatory action of *Aplysia* achatin-1 on the heart has not been reported (Bai et al., 2013).

Achatin-1 has a direct action on the *Achatina* heart augmenting the beating amplitude and frequency (Fujimoto et al., 1990). Immunostaining using specific anti-achatin-1 antibody demonstrated dense ramifications

of achatin-1-containing nerve endings in the atria. However, physiological experiments reveal that achatin-1 is only effective on the ventricle but not on the atria, indicating that ahcatin-1 probably acts in a paracrine fashion. Achatin-1 also has an indirect action on cardiac regulation. Achatin-1 augments the excitability of the heart exciter neuron, PON, in the visceral ganglion of the snail (Takeuchi et al., 1995). In situ hybridization localized the expression of achain-1 precursor in some neurons in medial region of right and left hemisphere of the pedal ganglion of *Achatina*, although morphological and chemical characterization of the neurons is still lacking (Satake et al., 1999).

Fulicin is another D-amino acid containing neuropeptide isolated from the ganglia of *A. fulica* (Ohta et al., 1991). Recently, structurally related peptides were identified in *Aplysia*. Interestingly, *Aplysia* fulicin consists of all L-amino acids. Fulicin at 10^{-6} M clearly increases beating amplitude but not the frequency of *Achatina* ventricle. Fulicin may also be involved in the regulation of reproduction, because it has a more potent effect on the penis retractor muscle and the oviduct of the snail (Fujisawa et al., 2000). Fulicin-containing neurons have been localized to the left and right parietal ganglion of *Achatina* by in situ hybridization (Satake et al., 1999).

NdWFamide is a potent cardio-excitatory peptide in *Aplysia*, *Lymnaea* and the terrestrial snail, *Euhadora congenita*. The peptide augments both of the beating rate and amplitude of the auricle and ventricle in these snails (Morishita et al., 2003b,c). Presumably, *A. californica*, *Limax valentianus*, *Tritonia*, and *L. gigantea* have NdWFamide or structurally related peptides because precursor cDNAs encoding precursor of the peptide have been identified in these molluscs (Matsuo et al., 2011).

FMRFamide and SCP_B are also cardioactive on the heart of *A. kurodai* (Harris et al., 1995; Lloyd et al., 1985). However, FMRFamide is effective at relatively higher concentrations than NdWFamide. Although SCP_B is effective on the heart at the lower concentrations than NdWFamide, this effect is not dose-dependent (Morishita et al., 2001). In this context, NdWFamide is the most dominant mediator that regulates heartbeat in *A. kurodai*. Moreover, of the three cardioactive peptides, NdWFamide alone induced contraction in the arteries of *A. kurodai* (Morishita et al., 2001). Therefore, NdWFamide may have diverse action on the cardiovascular system.

As described earlier, immunostaining with specific anti-NdWFamide antibody localized the peptide in some of the R3–14 neurons and white cells in RUQ region of the abdominal ganglion (Morishita et al., 2003c). NdWFamide containing nerve processes emanating from these neurons

project onto the cardiovascular region including the branchial and kidney vein, and the pericardial cavity via the branchial nerve.

In *A. kurodai*, the abdominal aorta supplies hemolymph toward the digestive gland which is located in the tail region. The aorta has a sphincter that regulates blood flow through the aorta. Sasaki et al. (2004) reported that NdWFamide reduces the blood flow to the digestive gland by contracting the sphincter muscle. If NdWFamide induces both the contraction of sphincter and augments heartbeat at the same time, it may promote the accumulation of hemolymph to the head region, generating hydrostatic pressure that contributes to the forward extension of the head.

Unlike SCP_B and serotonin, NdWFamide has no effect on the intracellular concentration of cAMP in the ventricle of *Aplysia* (Morishita, F., unpublished observations). By whole-cell patch-clamp recording, Kanemaru et al. (2002) demonstrated that NdWFamide augmented a high voltage-activated inward Ca^{2+}-current across the plasma membrane of dispersed myocardial cells of *Aplysia*. Since the increase in Ca^{2+}-current is blocked by the nifedipine and GTP-γS, it was suggested that NdWFamide activates an L-type Ca^{2+}-channel through the mediation of certain GTP-binding proteins.

NdWFamide is highly resistant to digestion by peptidase (Morishita et al., 2003a). Arguably, the unique molecular shape of NdWFamide prevents the peptide from fitting into the binding pocket of the peptidases. Although the *Aplysia* CNS does possess deamidase-like activity that inactivates NdWFamide by removing C-terminal amide (Morishita et al., 2003a), the molecular mechanism that inactivates NdWFamide is not fully understood.

Generally, the D-configuration of amino acids is essential for the bioactivity of D-amino acid-containing peptides (Fujita et al., 1995; Kim et al., 1991; Morishita et al., 2003a). In achatin-1, structural analysis demonstrated that replacement of D-phenylalanine to L-phenylalanine greatly modifies the molecular shape of the peptide (Ishida et al., 1992; Kamatani et al., 1990). For NdWFamide, the D-configuration of tryptophan, as well as the C-terminal amide, is essential for the cardioexcitatory action on the heart because an analog peptide with L-tryptophan, or the one without C-terminal amide, markedly reduces bioactivity. Structure-modeling by analysis with nuclear magnetic resonance (Yokotani et al., 2004) predicted that the molecular shape of NdWFamide is compactly folded by two intramolecular interactions, namely, the one between the phenyl ring on the phenylalanine residue and the amide moiety on the side chain of the N-terminal asparagine, and the other between the indole ring on the D-tryptophan residue and the C-terminal amide on the phenylalanine. Apparently, the aforementioned

analog peptides disrupted the native molecular shape of the peptide which resulted in diminished bioactivity.

Precursor cDNAs for achatin-1, fulicin and NdWFamide have been identified in some species of molluscs (Morishita et al., 2012; Satake et al., 1999; Yasuda-Kamatani et al., 1995). In all the precursor cDNAs encoding the precursors for D-amino acid neuropeptides, the D-amino acid residue is encoded by the codon for the corresponding L-amino acid. Since transfer RNA specifically binds to L-type amino acids and carries it to the ribosome for protein synthesis, the precursor protein for the D-amino acid-containing neuropeptides is synthesized with all L-amino acids on ribosomes, and then the D-/L-conversion of an amino acid in neuropeptides takes place during PTM of the precursor.

Peptidyl-aminoacyl-L-/D-isomerase which catalyzes the L-/D-conversion of an amino acid has been partially characterized in the spider, platypus and leaf frog (Jilek et al., 2011; Torres et al., 2007). The L-/D-conversion of amino acids in neuropeptides by this enzyme is a slow reaction which requires long incubation times (hours) to obtain a detectable end product. Using sophisticated mass-spectrometry, Song and Liu (2008) detected NWFamide, the diastereomer of NdWFamide in *Aplysia* abdominal ganglia extracts. It was suggested that NWFamide is the precursor to NdWFamide which undergoes post-translational modification in *Aplysia* abdominal ganglion (Song and Liu, 2008). Characterization of a peptidyl-aminoacyl-L-/D-isomerase has not yet been reported in molluscs.

10.4.7 CARDIOINHIBITORY PEPTIDES

In molluscs, several neuropeptides are known to have inhibitory actions on the heart. A tetrapeptide, APGWamide (Ala-Pro-Gly-Trp-NH_2) at 10^{-8} M order terminates regular beating of isolated ventricle of *H. aspersa* (Reich et al., 1997a). When Price et al. (1990) examined the cardiac actions of several SCP-related peptides, they found that some peptides terminated the heartbeat of the snail. However, transient, but marked increases in the beating amplitude preceded the termination of heartbeat (Price et al., 1990). Therefore, the possibility remains that termination of heartbeat reflects the systolic arrest of the heart. Baud et al. (1998) isolated a tridecapeptide from *H. aspersa* which shares the same C-terminal tetra-peptide with ACEP-1 of *Achatina*. The authors named the peptide as *Helix* cardioinhibitory peptide (HCIP) because it reduced the beating frequency of the heart. However, HCIP also augmented the beating amplitude. Considering the fact that cardiac muscle

has a longer refractory period than other muscles, prolonging heartbeat intervals via HCIP action could be a consequence of elevated heart muscle excitability.

On the other hand, Walker and colleagues examined the action of RFamide peptides on isolated perfused heart of *A. fulica*, and found that FMRFamide and FLRFamide had an excitatory action, whereas SSLFRFamide and GSLFRFamide had an inhibitory action. This is an interesting result in view of structure-activity relationship of peptides because the difference in peptide sequence reversed its physiological action on the heart. However, since the two cardioinhibitory peptides are not endogenous in *Achatina*, it is unclear if *Achatina* has a cardioinhibityory RFamide.

Catch-relaxing peptide (CARP), a myomodulin-related peptide isolated from *Mytilus*, inhibited the heartbeat in the isolated heart of a whelk, *R. thomasiana* (Fujiwara-Sakata et al., 1992), suggesting that myomodulin-related peptide is cardioinhibitory peptide in the whelk. However, the cardioinhibitory action of myomodulin may not be universal because the peptide has cardioexcitatory effects on the heart of a nudibranch (Wiens and Brownell, 1995).

The inhibition or termination of heartbeat may play an important physiological role during hibernation or during prolonged defensive closure of the shell in molluscs. It is likely that many peptidergic signaling molecules mediate the inhibitory regulation of the molluscan heart.

10.5 REGULATION OF HYDROMINERAL BALANCE AND RENAL FUNCTION

The majority of studies involving peptides or neurotransmitters that regulate hydromineral balance in molluscs have been done on gastropods. In general, most strictly marine gastropods are isosmotic (but not isoionic) with respect to the environment, however, they do show considerable neuroendocrine control over body volume and ionic composition of hemolymph. In contrast, all freshwater pulmonates are hyperosmotic regulators whereas the terrestrial pulmonates live in environments where the availability of water may change dramatically over time.

Freshwater snails live in an environment that is hypotonic relative to their hemolymph and tissues, therefore water is continually entering the animal's body via surfaces directly exposed to the water, and major ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, and HCO_3^-) are continually being lost to the environment. To counterbalance osmotic influx and solute losses, water entering the snail

by osmosis is removed by the kidney which excretes a hypotonic urine (Potts, 1967), whereas ions are actively taken up from the external medium and via the consumption of food (Burton, 1983). The following sections will deal with these two processes and the peptides that regulate them.

In most molluscs, the formation of prourine is thought to occur by ultrafiltration of the hemolymph (Potts, 1967). The site of ultrafiltration in pulmonates is believed to be the kidney as ultrastructural studies have shown the presence of podocyte-like processes in the kidney sac as well as septate junctions between kidney epithelial cells (Khan and Saleuddin, 1979a,b; Matricon-Gondran, 1990; Newell and Skelding, 1973a,b; Wendelaar-Bonga and Boer, 1969). The neuroendocrine control of osmoregulation has been studied mainly in the freshwater pulmonates *L. stagnalis* and *H. duryi.* In the visceral and left parietal ganglia of *H. duryi*, there are two identified neurosecretory cell types that are respond to osmotic stress, the type 2 and type 3 cells, as identified by PAF staining (Khan and Saleuddin, 1979a,b). Based on ultrastructural studies, type 2 cells show increased synthetic activity when snails are under hypoosmotic stress (distilled water). A functionally similar response to hypoosmotic stress is seen in the dark green cells (DGCs) of *L. stagnalis* which are mainly located in the pleural and parietal ganglia (Wendelaar-Bonga, 1971b, 1972).

In *H. duryi*, hypoosmotic treatment causes the extracellular spaces between kidney epithelial cells to become wider, whereas under hyperosmotic stress the spaces between cells become reduced (Khan and Saleuddin, 1979a,b). This expansion of intercellular spaces suggests increased urine production to excrete the excess water that has entered the body by osmosis. Extracts of visceral ganglia obtained from hypoosmotically stressed snails, and then applied to kidneys from snails acclimated to isosmotic medium, resulted in a widening of the extracellular spaces. These data suggest that the type 2 cells in *H. duryi* produce a diuretic factor that acts on the kidney epithelium to control paracellular transport (Khan and Saleuddin, 1979a,b). In contrast, the type 3 cells show increased synthetic activity under hyperosmotic stress when snails are placed in isosmotic medium, suggesting they produce an antidiuretic factor. Khan and Saleuddin (1981) further demonstrated that brain extracts from hyposmotic snails induced structural changes in the septate junctions of the kidney epithelial cells and suggest that a brain factor might act on the septate junctions of the kidney to alter paracellular permeability.

The identity of the diuretic and antidiuretic factor(s) in *H. duryi* are presumed to be peptides based on the presence of many electron-dense, membrane-bound vesicles which is typical of neuropeptide synthesizing

cells (Khan and Saleuddin, 1979a). At the time these studies were conducted, the identity of the peptide(s) in the type 3 cells was unknown. Subsequent immunogold labeling studies, however, showed neurosecretory granules in the type 3 cell stained positive for FMRFamide-like peptides (Saleuddin et al., 1992). FMRFamide-immunoreactive axons in the intestinal nerve project to and innervate the kidney smooth muscle cells and epithelium. In addition, *H. duryi* kidneys incubated in vitro with FMRFamide showed an increase in wet weight, indicating that this peptide (or a related peptide) might have an antidiuretic function in *H. duryi* (Saleuddin et al., 1992). When the concentration of FMRFamide-related peptides was measured by RIA in two major osmoregulatory organs, the kidney and mantle, higher concentrations of FMRFamide-like peptides were found in both these tissues under hyperosmotic stress (Madrid et al., 1994).

During the embryonic development in gastropods, one of the earliest neuronal elements to form are neurons that produce FMRFamide (Croll, 2000; Croll and Voronezhskaya, 1996). In the embryos of the freshwater snails *L. stagnalis* and *H. trivolvis*, hyperosmotic conditions are thought to elevate FMRFamide levels and induce developmental irregularities such as hydropia as well as earlier synthesis of serotonin in the visceral ganglia (Chaban and Voronezhskaya, 2008). This suggests that FMRFamide may also have an osmoregulatory role in developing embryos as it does in adult snails. A role for serotonin in osmoregulation has been suggested in in *H. duryi*. The kidney of *H. duryi* is innervated by serotonergic fibers from the visceral ganglion, suggesting that serotonin may regulate some aspect of kidney function (Khan et al., 1998). Depleting serotonin levels in the mantle and kidney with selective neurotoxins resulted in a significant reduction of FMRFamide-like peptides in these tissues. Therefore, serotonin may be involved in the synthesis and/or release of FMRFamide-like peptides in osmoregulatory organs of *H. duryi*.

The role of peptides in hydromineral balance has been studied extensively in *L. stagnalis*. Removal of the cerebral ganglia in this snail resulted in a reduction of Na^+ and Cl^- concentrations in the hemolymph, suggesting the presence of a presumptive ion transport stimulating factor from the cerebral ganglia (De With and van der Schors, 1986). Injection of a crude peptide extract from the cerebral ganglia into intact snails stimulated Na^+ uptake from the external medium as measured by $^{22}Na^+$ uptake (De With and van der Schors, 1986). To aid in the purification of a putative sodium-influx stimulating peptide (SIS) in *L. stagnalis*, a bioassay that measured ion transport across the membrane using *Lymnaea* skin attached in an Ussing chamber was developed (De With et al., 1988). Brain extracts or median lip

nerve extracts containing SIS peptide were capable of increasing the potential difference and short circuit current across skin epithelium. Using this SIS peptide bioassay, a 76-amino acid peptide was biochemically purified from *Lymnaea* CNS by HPLC and Edman sequencing (De With et al., 1993).

The cloned precursor cDNA of SIS peptide encodes a precursor protein which consists of 100 amino acids, including an N-terminal 24 amino acid signal peptide. In addition, six cysteine residues are thought to form three disulfide bonds which contribute to the peptide's tertiary structure (Smit et al., 1993b). Synthetic SIS peptide stimulates ion transport across the skin epithelium and is localized to specific neurons of the visceral and right parietal ganglia, and in axons of the intestinal nerve and right internal pallial nerve (De With et al., 1993). SIS peptide-positive nerve endings are abundant in the cardiovascular and renal regions of *Lymnaea*, suggesting that SIS peptide is involved in regulating cardiac and renal activities (Boer et al., 1992). Based on the pattern SIS peptide localization by immunohistochemistry and in situ hybridization, SIS peptide is believed to be synthesized and secreted by the neurosecretory Yellow Cells (YCs) in the CNS and regulates ion uptake across the integument, and perhaps in the renopericardial system as well (Boer et al., 1992; De With et al., 1993; Smit et al., 1993a).

When *L. stagnalis* is acclimated in water with low osmolarity, the YCs become electrically active and the numbers of neurosecretory granules in the neurons stained with paraldehyde fuchsin (PAF) are greatly reduced, suggesting the peptide is being released into the extracellular space (Soffe and Slade, 1979). These results collectively demonstrate that in *L. stagnalis*, hyposomotic stress causes the release of SIS peptide from the yellow cells resulting in Na^+ uptake in osmoregulatory tissues.

In addition to SIS peptide, the monoamine serotonin also increases short circuit current and electrical potential difference in *L. stagnalis* skin preparations via an intracellular cAMP signaling pathway (De With et al., 1988, 1993). Serotonin is an abundant neurotransmitter in the CNS of *L. stagnalis* (Audesirk, 1985; Croll and Chiasson, 1989; Marois and Croll, 1992) and other gastropods (Bernocchi et al., 1998; Croll, 1987, 1988, 2000; Salimova et al., 1987), and it has been implicated to play a role in osmoregulation in *H. duryi* (Khan et al., 1998). Although the mantle and renopericardial system of gastropods has been implicated in ion uptake, the role of specific transmitters regulating ion uptake in these tissue has not been demonstrated. It would be interesting to test if other known neuroactive substances in the gastropod nervous system are capable of stimulating active ion uptake from freshwater similar to the mechanism used in the gills of freshwater fishes. In this regard, the development of an in vitro model using kidney or mantle epithelial cells

cultured on a solid support with distinct apical and basal sides would be of exceptional utility (Wood et al., 2002). These "surrogate" kidney or mantle models could be used as bioassays to screen for peptides and other chemical mediators regulating ion transport across osmoregulatory tissues in snails.

The role of peptides in the control of osmoregulation in marine gastropods has been studied mainly in the opisthobranch, *A. californica*. Injections of identified neuron R15 from the abdominal ganglion into the hemocoel of intact animals resulted in the rapid uptake of water in the recipients, whereas an extract of other neurons such as R2 and R14 in the abdominal ganglion failed to increase body weight (Kupfermann and Weiss, 1976). The putative osmoregulatory factor from neuron R15 was estimated to be a peptide with a molecular mass of 1500 Da (Gainer et al., 1977). R15 is a bursting pacemaker neuron whose firing is inhibited when the animal is subjected to hyposmotic stress, suggesting that R15 may produce an antidiuretic peptide (Bablanian and Treistman, 1983). The peptides synthesized by neuron R15 were purified and isolated by Weiss et al. (1989). Two neuropeptides named R15α1 (38 amino acids) and R15β (28 amino acids) were purified from R15 extracts and stimulated weight gain when injected into intact *Aplysia californrnica*.

The response of neuron R15 to changes in seawater osmolarity appear to be mediated through the osphradium, a chemosensory organ (Skinner and Peretz, 1989). Furthermore, osmoregulatory responses to dilute media appear to decline with age. However, in the related marine snail *A. brasiliana*, which does not regulate its hemolymph osmolarity like *A. californica*, no such response to dilute media was found in the osphradium–R15 pathway (Scemes et al., 1991).

The kidney of *A. californica* is innervated by cholinergic branches of neuron L10, and a group of five peptidergic neurons (L2–L6) in the LUQ within the abdominal ganglion (Angers et al., 2000; Koester and Alevizos, 1989). Specifically, L10 and a subset of LUQ neurons, cells L2–4, 6, and L5, send branches to the renal pore which is a sphincter that controls urine efflux. Electrical input from L10 stimulates the opening of the renal pore whereas the LUQ neurons control its closing. Two transcripts have been cloned by differential screening of an abdominal ganglion cDNA library and code for the putative neuropeptide precursors, L5–67 and LUQ-1 (Shyamala et al., 1986; Wickham and DesGroseillers, 1991). L5–67 and LUQ-1 mRNAs are expressed in cells L2–4, 6, and L5 (Landry et al., 1992). The mRNAs and peptides are present in the axons innervating the region surrounding the renal pore as well as other parts of the kidney.

In the LUQ neurons, the L5–67 transcript is processed to produce a decapeptide LUQIN which shares sequence similarity to ACEP-1 (*Achatina* cardioexcitatory peptide 1) and LyCEP (*Lymnaea* cardioexcitatory peptide). A second peptide having 89 amino acids, proline-rich mature peptide (PRMP) is also processed from the L5–67 transcript (Aloyz and DesGroseillers, 1995). The LUQ-1 mRNA directs the production of a bradykinin-like peptide which is present in L5 and some neurons of the CNS (Giardino et al., 1996; Landry et al., 1992). It would be interesting to determine the identity of the neuropeptides responsible for the closing of the renal pore and if these peptides may have other osmoregulatory functions in *A. californica*. These results suggest that neuropeptides mediate some aspects of osmoregulatory function in *A. californica*, although the precise cellular mechanisms are not fully understood.

Recent advances in next-generation DNA sequencing technology have enabled investigators to sequence and mine data from the genomes of numerous molluscs more rapidly than ever before. The next-generation sequencing techniques can also be used to obtain entire mRNA sequences transcribed in particular cells or tissues treated under different experimental conditions or exposed to different environments. Using this technique, several papers have reported changes in mRNA expression level in response to applied osmotic stress in some molluscs (Eierman and Hare, 2014; Lockwood and Somero, 2011; Zhao et al., 2012b). Although changes in neuropeptide gene expression does not in itself demonstrate function, it may allow for the identification of specific target molecule(s) and elucidation of neuroendocrine pathways involved in the regulation of molluscan osmoregulation.

10.6 REGULATION OF EMBRYONIC CILIARY ACTIVITY

10.6.1 RESPIRATORY RESPONSE TO HYPOXIA BY FRESHWATER PULMONATE EMBRYOS

The control of respiratory behavior in adult gastropods has been studied extensively in the freshwater pulmonate *L. stagnalis* where identified central pattern-generating neurons are responsible for coordinating respiratory behaviors (for reviews see Lukowiak et al., 2006, 2014). We will not address respiratory behavior in adult snails as it have been covered elsewhere in the context of operant conditioning of respiratory behavior in response to hypoxia. In this section, we will discuss the behavioral response

of embryonic snails to environmental hypoxia (reduced oxygen concentration) as this response is known to serve a respiratory function and is regulated by specific neurotransmitters.

Environmental conditions such as pH, salinity and oxygen tension are generally more variable in freshwater in comparison with the marine environment. For example, oxygen concentrations in freshwater can undergo dramatic fluctuations depending on temperature, the amount of dissolved nutrients and ions, and the presence of organisms consuming dissolved oxygen in water. The embryos of gastropods are generally encased in a gelatinous egg mass which is affixed to underwater substratum and are therefore subjected to ambient environmental conditions. Unlike marine gastropods which have a free-swimming larval stage which can escape unfavorable environmental conditions, freshwater snails undergo all their larval stages within the egg mass and develop directly into juvenile snails (Morrill, 1982). The embryos of freshwater snails and likely those of other aquatic organisms that undergo encapsulated development within egg masses have evolved adaptive mechanisms which allow them to survive periods of environmental hypoxia.

Aquatic gastropods that develop in egg masses are surrounded by a viscous capsular fluid which is derived mainly from the albumen gland. Developing embryos display a characteristic ciliary-driven rotational behavior within the egg mass which is thought to enhance the mixing of diffusion-limiting processes in the capsular fluid (Hunter and Vogel, 1986; Moran and Woods, 2007). For instance, the embryos of the freshwater snail *Helisoma trvivolvis* display two characteristic rotational behaviors, a slow basal rotation which is due to constitutive ciliary beating, and periods of transient accelerations called surges. Under normoxic conditions, the ciliary-driven rotation is predicted to function similar to a laboratory "stir-bar" which enhances the mixing of oxygen in the capsular fluid. Mixing of the capsular fluid is hypothesized to maintain a constant diffusion gradient of environmental oxygen to the developing embryo (Goldberg et al., 2008). In support of this hypothesis, egg masses kept in hypoxic pond water have a faster basal rotation rate and increased frequency of rotational surges as compared to egg masses maintained under normoxia (Kuang et al., 2002).

In *H. trivolvis*, ciliary-driven rotational behavior is mediated by a pair of embryonic neurons named embryonic neurons C1 (ENC1s). The ENC1s are specialized serotonergic sensorimotor neurons that sense environmental oxygen and synapse with target pedal ciliary cells in the embryo to control rotational behavior. Since the ENC1s develop well before the formation of

the CNS neurons, they are implicated to play an important role in normal embryonic development and survival as intact ENC1s are necessary for the response to environmental hypoxia (Kuang et al., 2002). Furthermore, laser treatment of the ENC1s is known to perturb their activity and cause a transient increase in those cilia that are in close anatomical association with the ENC1s (Kuang and Goldberg, 2001). Application of serotonin to egg masses causes a marked increase of embryonic rotation rate and ciliary beat frequency, whereas serotonin-induced increases in rotational behavior and cilio-excitatory response is suppressed by mianserin, a serotonin receptor antagonist (Diefenbach et al., 1991; Goldberg et al., 1994; Kuang and Goldberg, 2001; Kuang et al., 2002). Collectively, these studies suggest that the ENC1s in *H. trivolvis* respond to hypoxia by releasing serotonin at the embryonic ciliary cells which results in increased embryonic rotational behavior and enhances the mixing of oxygen in the capsular fluid.

The ciliary beat frequency is believed to be controlled by the constitutive activity of nitric oxide in embryonic ciliary cells of *H. trivolvis*. Inhibition of nitric oxide production using nitric oxide synthase inhibitors (e.g., L-NAME and 7-NI) reduced embryonic rotational rate, whereas providing NO donors (SNAP and SNP) increased embryonic rotation rate twofold (Cole et al., 2002). Furthermore, ciliary cells bathed in the presence of NOS inhibitors abolished the cilio-excitatory response to serotonin, indicating that a functional nitric oxide signaling system plays a permissive role in regulating the cilio-excitatory response to serotonin (Doran et al., 2003). In *H. trivolvis*, two serotonin receptor subtypes have been cloned which have been named HT_{1Hel} and $5HT_{7Hel}$. Activation of these serotonin receptor subtypes is thought to regulate ciliary beating via the activation of protein kinase C (PKC) and the release of calcium from a caffeine-sensitive intracellular store (Christopher et al., 1999; Doran and Goldberg, 2006; Doran et al., 2003, 2004).

In addition to playing a central role in regulating basal ciliary beating and rotational response to hypoxia, serotonin is thought to play an important protective function in response to long-term anoxia in *H. trivolvis.* Long-term anoxia induces a transient increase in embryonic rotation rate in egg masses exposed to anoxic pond water, a response similar to that seen with hypoxia treatment. Basal rates of embryonic rotation under anoxia were maintained for up to 13 h after which the embryos perished (Shartau et al., 2010). However, when embryos were cultured in anoxic pond water in the presence of serotonin, their embryonic rotation rates persisted for up to 40 h leading to markedly prolonged survival (Shartau et al., 2010). The authors

suggest that anoxia or long-term hypoxia suppresses metabolic activities at the expense of development and growth since embryonic development is delayed in hypoxic embryos. Thus, ENC1 serotonin release is proposed to have a dual role in *H. trivolvis* embryos. Serotonin is proposed to control embryonic rotational behavior under normoxia and hypoxia via the activation of 5-HT_{7Hel} receptors which are present in embryonic ciliated cells. An additional function of serotonin might be to increase ATP production via increased glycogenolysis, thereby enhancing energy production to sustain rotational behavior during periods of hypoxia.

To determine if the aminergic control of embryonic rotational response to hypoxia is an evolutionarily conserved response of other freshwater snails, Goldberg et al. (2011) studied the embryos of *L. stagnalis*. In addition to having pedal and dorsolateral ciliary bands as in *H. trivolvis*, only *L. stagnalis* has a specific ciliated apical plate region during embryonic development. The ciliated apical plate is innervated by transient apical catecholaminergic neurons which contain dopamine and are primarily responsible for driving embryonic rotational behavior (Voronezhskaya et al., 1999). Both serotonin and dopamine increase the rate of embryonic rotation in vivo (Goldberg et al., 2011), however, serotonin is more potent than dopamine in stimulating ciliary beat frequency of isolated ciliary patches in vitro. Furthermore, the rotational response to hypoxia was blocked by the dopamine receptor antagonist SKF83566 but not by mianserin, suggesting that the rotational response to hypoxia in *L. stagnalis* is mediated by a dopamine D1-like receptor. Although the major neurotransmitter regulating embryonic rotational behavior in *L. stagnalis* is dopamine, the basic neural mechanism underlying the ciliary response to hypoxia is similar to that seen in *H. trvolvis*, suggesting that transient increases in ciliary beating may be an evolutionarily conserved response in freshwater snails to maintain sufficient oxygen supply to the embryos.

10.7 CONCLUSIONS

Peptides are the most diverse extracellular chemical messengers in animals. In gastropods, there are likely dozens of peptides yet to be fully characterized at a functional level in any single animal. Most gastropod peptides that have been studied in significant functional detail have been done using the

sea hare *A. californica* and the pond snail *L. stagnalis*. These two molluscan models have provided a great deal of information regarding selected peptides and their functions in various aspects of reproduction, growth, cardiac function, and osmoregulation, particularly at the cellular and molecular levels. In addition, classical neurotransmitters commonly work together with peptides to exert precise control over key physiological processes. We know many pieces of interesting pieces of information about the function many molluscan peptides and neurotransmitters. However, most of this information is derived from in vitro studies that are conducted on isolated preparations. Therefore, a major challenge in the future will be to determine how these signaling molecules function in the context of whole animal physiology. As current proteomic and next-generation sequencing techniques continue to improve, more data from the proteome and transcriptome of model and nonmodel molluscs will become available. This should open the door for greater widespread use of techniques such RNA interference in molluscs, and perhaps the future implementation of methods such as CRISPR (clustered regularly interspaced short palindromic repeat) to alter gene function. Finally, better functional assays will need to be developed to study all these newly sequenced peptides and link them to in vivo physiology.

KEYWORDS

- **Gastropoda**
- ***Aplysia***
- ***Lymnaea***
- ***Helisoma***
- ***Biomphalaria***
- ***Helix***
- ***Achatina* neuropeptides**
- **egg-laying hormone**
- **schistosomin**
- **APGWamide**

- **FMRFamide**
- **molluscan insulin-related peptide**
- **sodium influx-stimulating peptide**
- **gonadotropin-releasing hormone**
- **cardioexcitatory peptides**
- **cardioinhibitory peptides**
- **bag cells**
- **R-15**
- **caudodorsal cells**
- **light green cells**
- **yellow cells**
- **atrial gland**
- **albumen gland**
- **neurotransmitters**
- **dopamine**
- **serotonin**
- **animal behavior**
- **reproduction**
- **osmoregulation**
- **cardiac activity**
- **growth**
- **ciliary activity**
- **neuropeptide synthesis**
- **neuropeptide processing**
- **neuropeptide secretion**
- **identifiable neuron**
- **neurosecretory cell**
- **neuroendocrine**
- **signal transduction**
- **G protein-coupled receptors**
- **D-amino acid peptide**

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