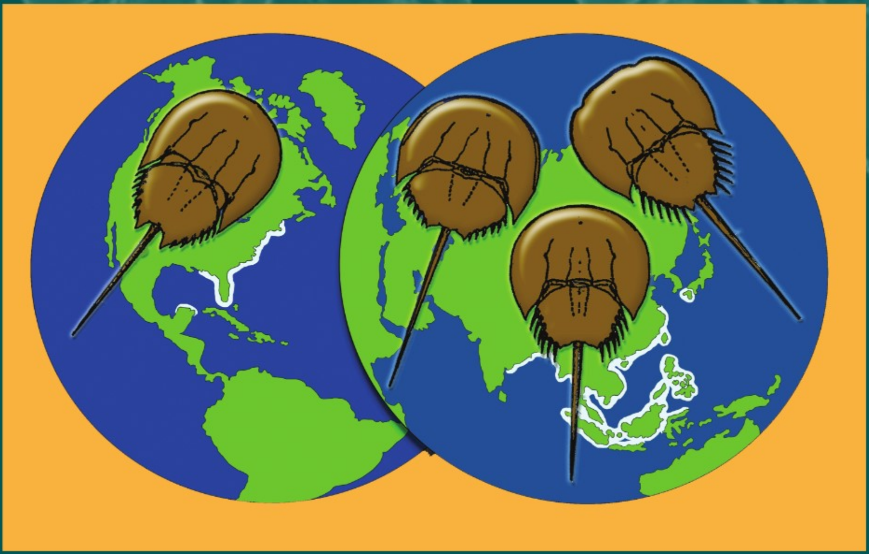
Biology and Conservation of Horseshoe Crabs



John T. Tanacredi • Mark L. Botton • David R. Smith
Editors

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Foreword by Sylvia A. Earle

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We dedicate this book to Drs. Carl N. Shuster, Jr. and Koichi Sekiguchi for their life-long contributions to the biology and conservation of the magnificent horseshoe crab.

Foreword

Horseshoe crabs, those mysterious ancient mariners, lured me into the sea as a child along the beaches of New Jersey. Drawn to their shiny domed shells and spiked tails, I could not resist picking them up, turning them over and watching the wondrous mechanical movement of their glistening legs, articulating with one another as smoothly as the inner working of a clock.

What was it like to be a horseshoe crab, I wondered? What did they eat? Did they always move around together? Why were some so large and others much smaller? How old were they, anyway? What must it feel like to live underwater? What else was out there, down there, in the cool, green depths that gave rise to such intriguing creatures? The only way to find out, I reasoned, would be to go into the ocean and see for myself, and so I did, and more than 60 years later, I still do.

When Dr. John Tanacredi, perhaps the horseshoe crab's best friend, invited me to come to Dowling College to participate in the first international conference about these "living fossils," I was pleased to join more than 150 scientists from 10 countries to explore the past, present, and future of the creatures that have inspired in all who attended an unabashed admiration, relentless curiosity, and growing concern about their recent sharp decline. All of us not only learned from our shared experiences, but were thrilled and deeply inspired by the many youngsters whose artistic interpretations of the life and times of horseshoe crabs were displayed at the conference.

Excitement at the conference about new discoveries was tempered by an undercurrent of concern about the possible loss of the four species of horseshoe crabs presently maintaining a tenuous hold on life in parts of the Atlantic, Pacific, and Indian oceans. They are, after all, symbols of survival on a geological scale. Fossil remains of species from four million centuries ago appear remarkably similar to the craggy, golden-brown animals that now clamber high on moist, sandy beaches to deposit masses of eggs on the tides of early summer.

Gone are the ammonites, the trilobites, and numerous other once abundant creatures with whom they shared ancient oceans. The Atlantic Ocean has opened, closed, and formed again during their time. Ice ages have come and gone as the planet repeatedly cooled, warmed, and cooled again. Horseshoe crabs were witnesses to the arrival and departure of great ocean-dwelling reptiles and terrestrial dinosaurs and remained through waves of extinction and of numerous new arrivals: insects, spiders, birds, mammals, salamanders and frogs, trees, flowers, and eventually, *Homo sapiens*.

If a population of living pterodactyls or *Tyrannosaurus rex* turned up alive and well in some remote part of the planet, it is likely that supreme efforts would be mobilized to protect them while we took advantage of the opportunity to gain new insights about them, and through them, something about the nature of life as it was during the Jurassic Era. Horseshoe crabs preceded such creatures by many millions of years and miraculously, some of them are still around. With living creatures rather than fossilized remains, we have a chance to discover something of the origins of various other life forms, learn secrets of survival during major shifts in planetary temperature, chemistry, food webs, and much more.

The four species of horseshoe crabs, the only living representatives of an entire Class of organisms, have already provided scientists with a window into the past as well as yielding valuable insight concerning the chemistry of life, the nature of vision, and some of the intricacies of nervous system development. Their unique blood, eyes, genetic material, and basic structure continue to provide priceless knowledge that far outweighs their value when killed as a commodity. Direct predation by people, incidental killing of large numbers trapped in nets and trawls, and vast depletion of their shoreline habitats, from beaches to productive offshore waters, have combined in the past few decades to threaten these amazingly durable animals with extinction.

There is time, but not a lot, to take actions to protect these venerable but vulnerable animals. Under the UNESCO World Heritage Program, it may be possible to win support for horseshoe crabs as the first "World Heritage Species." Designating protected areas along critical coastal nesting areas would certainly help, as well as providing rational guidelines for when and how – and how many – might be extracted for scientific, medical, or other uses. It will be difficult to explain to future generations that we valued these amazing creatures more for use as bait and fertilizer than we did as an on-going source of knowledge – and inspiration.

If for no other reasons, it makes sense to protect these amazing animals out of respect for what they represent as planetary "elders." Having endured so much for so long, it would be a terrible legacy for humankind if the horseshoe crabs' astonishingly capacity for survival would come to an end on our watch.

The 2007 International Horseshoe Crab Conference and this volume are promising signs that knowing leads to caring, and with caring, there is hope that horseshoe crabs – and humankind – will share an enduring future.

Sylvia A. Earle

Preface

In this book, you will find the proceedings of a ground-breaking international symposium on the science and conservation of a remarkable group of living species, the horseshoe crab. Earth has only four extant horseshoe crabs: *Limulus polyphemus* in North America; *Tachypleus tridentatus; T. gigas;* and *Carcinoscorpius rotundicauda* in southeast Asia. In past decades, these animals were viewed as a natural history curiosity at best or reviled as enemies of shellfish. Today, we know them as flagship species and invaluable to human health. Their blood provides a chemical known as *Limulus* amebocyte lysate or LAL that clots in the presence of minute quantities of bacterial endotoxin and is used to ensure that pharmaceuticals and surgical implants are free of bacterial contamination. Current research points to the promise of horseshoe crab blood in the discovery of new treatments for human disease. Horseshoe crabs are integral to the food web of coastal marine ecosystems and to the economies of coastal communities.

Horseshoe crabs are well known as "living fossils" with a geological history covering hundreds of millions of years and an ancestry reaching back 455 million years to the doorstep of the Cambrian. In spite of this longevity, each species now faces common and growing threats to their survival. Loss of essential spawning habitat due to erosion and shoreline development, coastal pollution, and overfishing all threaten horseshoe crab populations. Biologists and conservationists working in coastal areas throughout North America and Southeast Asia have become increasingly concerned by declines observed in all species of horseshoe crab. A consensus emerged that a structured forum was needed where those working on horseshoe crab conservation could meet, exchange ideas, and plan future research and conservation strategies. In the fall of 2005, a planning committee was formed and selected Dowling College to host the first International Symposium on the Science and Conservation of Horseshoe Crabs (ISSCHC 07).

The symposium, which was held during June 11–14, 2007, brought together a diverse group of researchers, ecologists, managers, and educators who took full advantage of the rare opportunity to interact. Participants included biologists who discover processes underlying behavior and population dynamics, medical researchers who mine deep into the pharmaceutical applications, resource managers who work to ensure healthy and viable populations, and educators who creatively share fascinating details about horseshoe crabs with the broader public and future generations. Those at the symposium were enlightened by the display of children's art work from around world organized by ERDG, a non-profit organization. The display reminded the attendees of their own youthful excitement as they first encountered and explored these alien-looking creatures.

In addition to its many ecological roles, the horseshoe crab is an international ambassador. The global distribution of the four horseshoe crab species links the eastern and western hemispheres. The symposium logo designed by Dr. Carl N. Shuster Jr. reflects this interconnectedness by showing the two hemispheres and the four species side-by-side facing the same future. And so it was at the symposium, where participants from around the world gathered to share knowledge, present research results, and identify conservation challenges facing horseshoe crabs. Over 150 scientists, researchers, and students from 10 countries presented over 45 papers and 40 posters. And it is in the pages of this book where that shared international knowledge and experience is presented as a beginning in our collective efforts to conserve the world's horseshoe crab species.

Science helping to advance conservation; Conservation helping to prioritize science was chosen as the symposium theme to emphasize that effective conservation depends on science-based resource management. Using biological knowledge as a foundation, populations can be effectively conserved through management of habitat and human use. This theme of bridging biological science and conservation served to guide the symposium program and the organization in this book.

This book is organized, as the title implies, into two main sections: Biology and Conservation. The Biology section is divided into two subsections: (1) Populations and Habitats and (2) Physiology, Reproduction, and Development. The Conservation section is divided into three subsections: (1) Commercial Use and Management of Populations and Habitat, (2) Culture and Captive Breeding, and (3) Public Awareness and Community-based Conservation. Throughout the book, the reader will find results of new studies, and authoritative reviews on the science and conservation of all four of the world's horseshoe crab species. The chapters in this book were drawn from the invited oral presentations at the symposium, with a number of additional chapters chosen from among the contributed poster presentations to achieve greater balance among topics or geographic regions.

During the symposium, a petition with over 200 signatures was prepared to be given to the Director of UNESCO asking that the very first officially designated "World Heritage Species," an invertebrate, be assigned to "Horseshoe Crabs" to afford them added protection and awareness, under the World Heritage Program of the United Nations.

The Editors gratefully acknowledge the work and contributions of our fellow members of the ISSCHC 07 Planning Committee: Jim Berkson, Jane Brockmann, Ruth Carmichael, Anil Chatterji, Chang-Po Chen, Annie Christianus, Glenn Gauvry, Tomio Itow, Jack Levin, Mike Millard, Mike Oates, Martin Schreibman, Carl Shuster, and Jaime Zaldívar-Rae. Glenn Gauvry and Mariko Sai helped to encourage the participation of many of the attendees from southeast Asia, and they were extremely helpful in coordinating the submission and review of their manuscripts. Dowling College with the Great South Bay in its backyard provided a welcoming facility for the first, of what we hope will be many, International Symposia on the Science and Conservation of Horseshoe Crabs. Communication among symposium attendees was greatly facilitated by the expert translation services provided by Andrew Meehan-Migita and associate. Valerie Royall graphically designed the symposium logo based on Carl Shuster's conceptual drawing. We also thank Drs. Gregory Lewbart, Cheryl Morrison, Meredith Bartron, and Bob Loveland for their helpful manuscript reviews. MLB is grateful to Fordham University for providing him with a Faculty Fellowship during the fall 2007 semester, when much of the preparation of this book took place. DRS is grateful to USGS Leetown Science Center for administrative support during the planning of the symposium.

JTT extends very special thank you to Ms. Traci D'Alessio, the Dowling College HSC Conference Coordinator and Graduate Assistant whose patience, professionalism and organizational skills were instrumental in making this conference such a success; others at Dowling College that we extend a special thank you to are Tom Franza, Charles McCabe, Roxann Hristovsky from the IT Division for their expert assistance; Lou Siegel, Erik Paulson, Dr. Linda Ardito, Dr. Paul Abramson, Dr. Yu-wan Wang, the Director of International student services at Dowling College whose abundant grace and intelligence, provided invaluable travel and translation service to the international participants, Ms. Henna M. Wang, the Dowling Institute (Horseshoe Crab Central), Sixto Portilla, Lauren Macri, Deborah Wynne, Maria Lovejoy, Tom Downs, Mark Carattini, Food Services by Lessings Inc., volunteers, Joanne Cardinali, Jeannine Lyn Orlando, and Dowling College Earth and Environmental Science students, Farah LaRonde, Sherri Eisenberg and Russell Ainbinder; all whose help made this conference and its proceedings possible JTT would also care to extend a heartfelt "thank you" to New York State Assemblywoman Ginny Fields who continuously supported the efforts to preserve and protect horseshoe crab habitat and populations on Long Island.

Lastly, the Editors wish to extend our heartfelt thanks to the publishers of this work, Springer, Ltd. most notable to Melinda "Lindy" Paul who got this off the ground and to Janet Slobodien who has through her guidance, foresight, review, and overall support has allowed these proceedings to be completed. As with any such undertaking, mistakes or omissions are ours only to bear.

Oakdale, New York — John T. Tanacredi
New York City, New York — Mark L. Botton
Kearneysville, West Virginia — David R. Smith

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A few words . . .

One of my first official acts as President of Dowling College was to greet the participants in the International Symposium on the Science and Conservation of Horseshoe Crabs (ISSCHC) 2007 Conference held on the Dowling campus.

Throughout my professional career, whether as an elected official, practicing attorney, or President of an academic institution, I have been guided by the maxim of the Nobel Laureate Rene Dubos: “Think globally; act locally.” The ISSCHC-07 Conference brought that concept to fruition. Our campus hosted scientists, students, and concerned individuals from around the world – a truly global event.

I would like to offer a special note of appreciation to Dr. John Tanacredi, Chair of the Department of Earth and Marine Sciences at Dowling College, and member of the ISSCHC-07 planning committee whose initiative brought the conference to the Dowling venue. The planning committee valiantly worked to raise the necessary funds to hold this symposium, and their collective energy spawned the participation of some 150 scientists and students from 10 countries.

The international perspective on horseshoe crabs brought home to those of us living on Long Island the need for constant vigilance in order to conserve these amazing animals. If we are to preserve these globally significant species, there must be a continual search for answers to the questions needing attention. I am proud that Dowling has been part of this effort.

Robert J. Gaffney

President
Dowling College

Thank You to the Sponsors of the 2007 International Symposium on the Science and Conservation of Horseshoe Crabs

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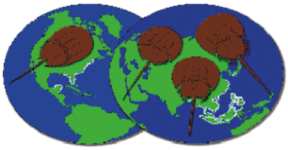

International Symposium on Science & Conservation of Horseshoe Crabs ISSCHC

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Part I
Biology

Part IA
Populations and Habitats

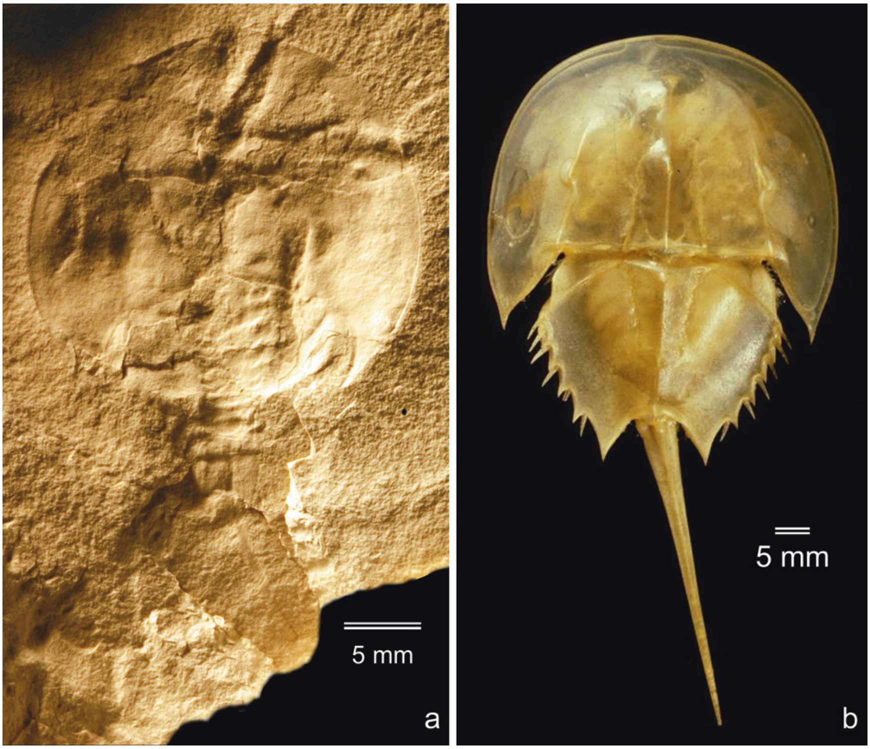

Fig. 1a The oldest known fossil horseshoe crab, *Lunataspis aurora* and its living relative, *Limulus polyphemus*. (courtesy of D. Rudkin and G. Young)

Limits on the Global Distribution of Horseshoe Crabs (Limulacea): Lessons Learned from Two Lifetimes of Observations: Asia and America

Koichi Sekiguchi and Carl N. Shuster Jr.

Abstract This paper is based on the premise that understanding the natural history and ecology of the horseshoe crabs (Limulacea) is paramount to conservation of the species. Our objective has been to select those large-scale, global parameters that help to define the opportunities for, and the limits on, their distribution. Essentially, we have selected the big-picture type of conditions pertinent to considerations of the conservation of the species. This has led to an examination of potential biotic and environmental parameters. Of these we have selected eight, four in each category. The biotic aspects are: *who* they are (taxonomically), whether they are *living fossils*, *what* they are (ecologically), and the significance of *discrete populations* in conservation of the species. Four large-scale environmental parameters that limit their global distribution are *geologic* (estuarine environments, continental shelves as avenues of distribution, ocean deeps as deterrents), *temperature* which sets boundaries, *tidal types* that define predominant potential spawning sites, and the influence of *benthic currents*.

1 Introduction

If understanding horseshoe crabs, their natural history and ecology, is the basis for considering their conservation, then we have accumulated the right background. We have been fortunate in having major scientists as our mentors and access to information compiled by the many scientists that preceded us. We began with studies on developmental stages of horseshoe crabs. Later, research included specimen and data-collecting missions to many Indo-Pacific habitats for three species (*Tachypleus tridentatus*, *T. gigas*, and *Carcinoscorpius rotundicauda*) (KS) and from Dauphin Island, Alabama to Maine, USA (*Limulus polyphemus*) (CS), and several mutual rendezvous on the shores of Delaware Bay.

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When we began our studies in the 1930s and 1940s, respectively, virtually no one else was broadly interested in their natural history and ecology. Also, we are of the generation of students whose professors expected them to have an understanding of who knew what, where, and when about the subject, and, when pertinent, how and why. From that beginning we also have been fortunate to have witnessed and participated with many others in learning more about horseshoe crabs. For all those reasons, we have had scientifically exhilarating careers; ones that have bridged the gaps between the leaders of yore in the study of horseshoe crabs with those of the present.

2 The Nature of the Limulacea

The Limulacea are an ancient group of aquatic merostome arthropods. Their existence through millions of years with relatively few species, exemplifies their designation as ecological generalists. They inhabit coastal embayments and breed on intertidal shores within global constraints such as the oceanic abyss and certain tidal regimes.

2.1 They Are Ancient

Horseshoe crabs are merostomes, those creatures with their legs arranged around their ventral mouths. They are aquatic grade chelicerates (Shuster and Anderson 2003), closely related to trilobites, that occurred earlier in geologic time than the arachnids. Only a few species of horseshoe crabs have existed during any geological period throughout time (Shuster and Anderson 2003). Their conservative exoskeleton and internal organs (Sekiguchi 1988) date back at least to the Carboniferous Period, 245 million years ago. An older date of 445 million years was reported at this symposium by David Rudkin and later published (Rudkin et al. 2008). The exoskeleton is comprised of three articulating sections: the *prosoma* (a cephalic/thoracic shield containing most of the organ systems and eight pairs of variously modified legs) that articulates by a piano-type hinge with the *opisthosoma* (the mid-piece bearing the branchial appendages) from which the long spike-like *telson* swivels on an almost universal joint (Shuster 2001). In reflecting upon the horseshoe crab body as a machine, it is remarkable that of the many thing it can do it cannot back up – it is unable to reverse its tracks.

Taxonomically the four extant species (Figs. 1 and 2) are assigned to three genera in two families in the Superfamily Limulacea:

Family Limulidae
    Limulus polyphemus (Linnaeus)

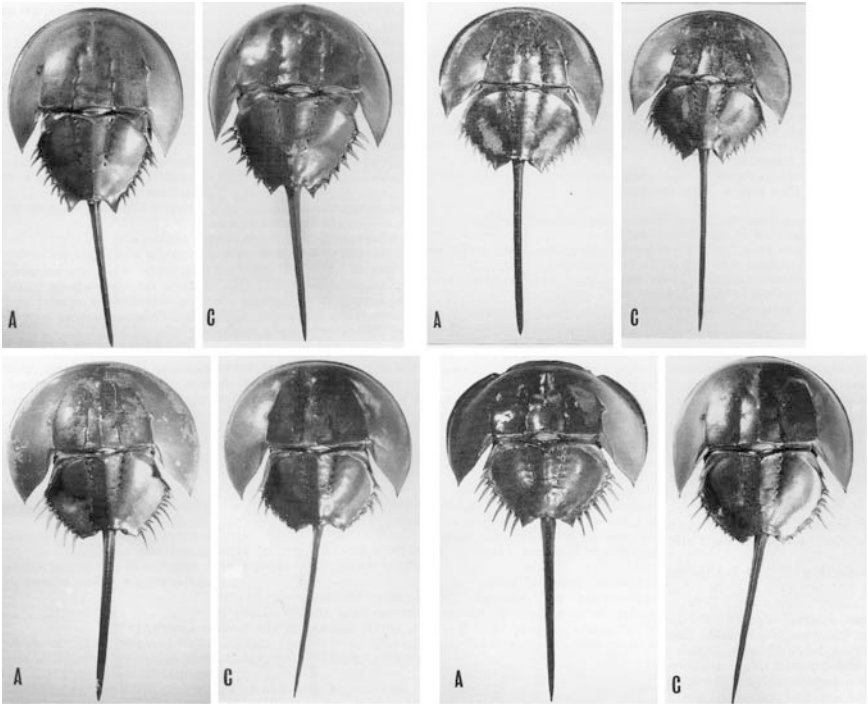

Fig. 1 Dorsal aspects of the adults of the four extant species (A = males; C = females): *Top row* (*left* to *right*): *Limulus polyphemus* and *Carcinoscorpius rotundicauda*; *Bottom row* (*left* to *right*): *Tachypleus gigas* and *Tachypleus tridentatus* (Yamasaki 1988)

Family Tachypleinae
  Tachypleus tridentatus (Leach 1819)
  Tachypleus gigas (Muller 1785)
  Carcinoscorpius rotundicauda (Latreille 1802)

Yamasaki (1988) proposed renaming *C. rotundicauda* as *Tachypleus rotundicauda* (Latreille 1802) n. comp. – with which we agree, but this has to be brought to the International Commission on Zoological Nomenclature for adjudication. Meanwhile, *Carcinoscorpius,* an apt descriptor, is in general use.

2.2 Are They Living Fossils?

Extant horseshoe crabs have been labeled "living fossils" for a long time. This appellation is not exactly true – there are no known fossils of any of the four extant species. The concept most likely had its basis in the constancy of the typical horseshoe crabs three-piece exoskeleton (prosoma, opisthosoma, and telson) since the mid-Paleozoic Era. This lengthy history makes a strong

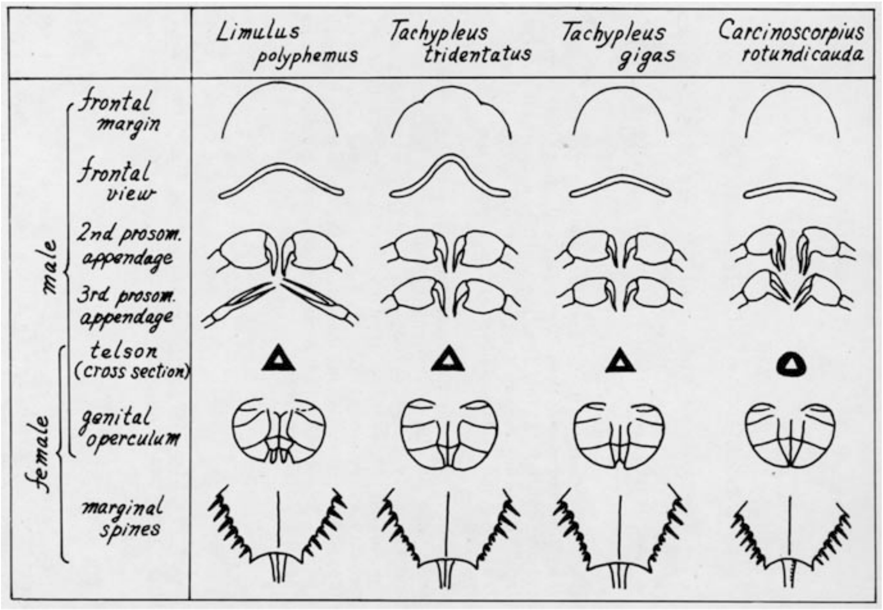

Fig. 2 Distinguishing morphological characteristics of the four species of extant horseshoe crabs (Sekiguchi and Nakamura 1979)

positive statement – that the three-piece body architecture did the job millions of years ago and, with minor various, is still performing adequately. Their physiology has changed, however, as indicated by the genetic and habitat differences in populations. The combination of a static body form and an adaptable physiology is what probably enabled xiphosuran species to exist in different environmental conditions during that time span.

Two anatomically well-defined fossilized species illustrate the basic body form: (1) *Limulus coffini* (Fig. 3) – found in a concretion formed some 80 million years ago during the Cretaceous in the shallow seas where Colorado now exists (Reeside and Harris 1952), and (2) *Mesolimulus walchi* (Fig. 4) – preserved in the Solnhofen limestones laid down during the Upper Jurassic in the shallow sea that once covered central Europe about 150 million years ago. If these two species could be restored to their original form, it is unlikely that they would be any more anatomically different from the four extant species than the four are from each other. Indeed, if there were more of the single fossil on which *L. coffini* was based, so that a better identification could be made, it might qualify as an ancient race of *L. polyphemus*. If so, we would have a factual basis for the concept of a "living fossil" – at least for about 80 million years, well within the divergence of the American species from the three Indo-Pacific species 135 million years ago (Shishikura et al. 1982).

Fig. 3 A comparison of the opisthosomas of *Limulus coffini* (*left*) and *Limulus polyphemus* (*right*) (mold of the fossil, *L. coffini*, courtesy of Dr. J. B. Reeside)

2.3 They Are Environmental Generalists

A major research emphasis in Japan compared the natural history and ecology of all four species (summarized by Sekiguchi 1988). The Limulacea are essentially estuarine dwellers. They are fully capable, however, of excursions onto the continental shelf in search of prey and perhaps also to redistribute their populations when they become too dense or their local habitat is threatened. But they do not live or thrive everywhere – their distribution is often interrupted and, even within their ranges, their populations vary in numbers. Yet all species have extensive geographic ranges: *Limulus polyphemus*, ranges from 21° N to 44° N and 68° W to 90° W (Fig. 5). *Tachypleus tridentatus* ranges from 12° N to 31° N and 90° E to 125° E, *Carcinoscorpius rotundicauda* from 91° E to 118° E, and 6° S to 20° N, and *T. gigas* from 91° E to 117° E and 6° S to 20° N (Fig. 6). Despite the frequent overlapping of their distributions and habitats, the ecological niches of the three Indo-Pacific species are not directly competitive (Sekiguchi 1988). The extant species exhibit discrete populations – morphometrically and genetically. They probably also exist in physiological races (Shuster 1955, 1979). Due to these characteristics and their ability to cope with wide-ranging environmental conditions, especially salinity and temperature (Towle and Henry

Fig. 4 An internal mold of *Mesolimulus walchi* from the private collection of H. Leich, Germany. It is an excellent example of a positive-negative impression; prosomal width = 5.5 cm (Shuster and Anderson 2003)

2003), horseshoe crabs qualify as environmental generalists (Loveland et al. 1997, Eldredge 1991).

Because they are ecological generalists and because their distribution and activities are often described to general terms, we cannot over emphasize the fact that the details in many studies are essentially only accurate for the place, time, and environmental conditions that existed at the time of the study. We tend to generalize, but at the same time we are mindful that horseshoe crabs do not always do what they are expected to do – hence their behavior may fall outside a generality under certain circumstances.

2.4 Significance of the Discreteness of Populations

When morphometric data (Fig. 7) on nearby and on widely separated populations of *Limulus polyphemus* were obtained it led to the deduction that there were discrete populations (Shuster 1955, 1979). The discovery that morphometric

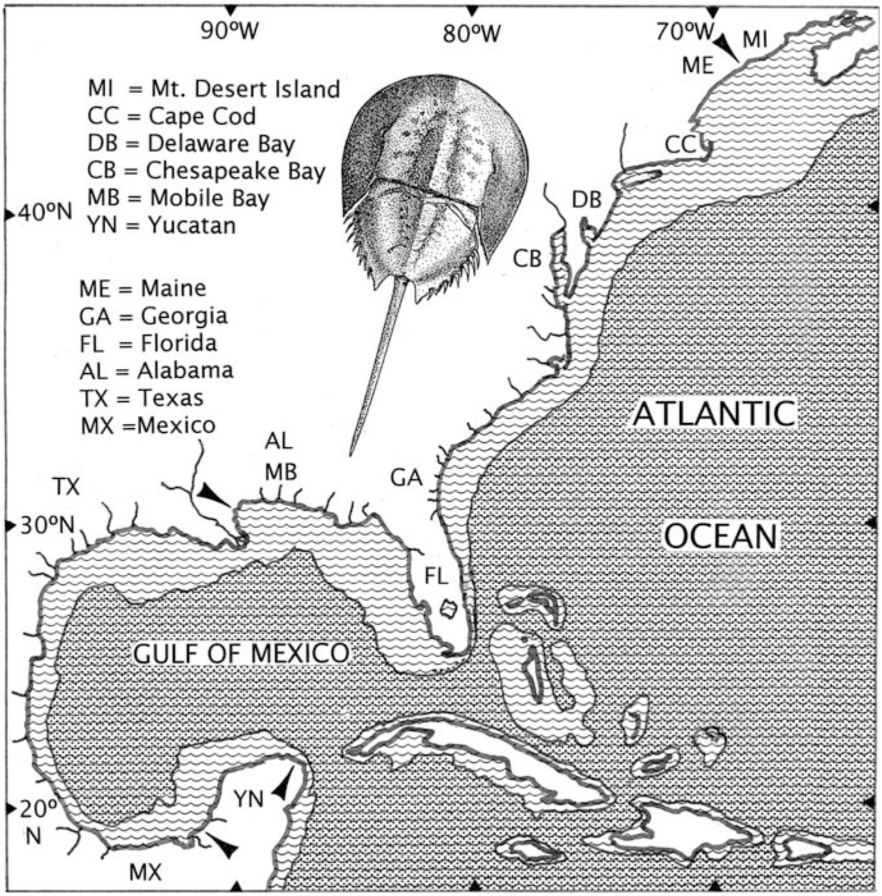

Fig. 5 Breeding populations of the American horseshoe crab, *Limulus polyphemus*, are distributed, intermittently, on the shore of Yucatan and from the Mississippi delta eastward to the Florida Keys and then along the Atlantic coast, Florida to Maine (from about 21°N to 44°N), with occasional strays reported in Texas and Canadian waters. Except for a narrow continental shelf along the southeast coast of Florida, the shelf is broad elsewhere

discrete populations of *L. polyphemus* displayed a latitudinal gradient of smaller-sized adults from Georgia southward and northward from Cape Cod was confirmed by Riska (1981). Similar data (Fig. 8) are available for the three Indo-Pacific species (Yamasaki 1988).

Temperature tolerance (Mayer 1914) and morphometric discreteness led Shuster (1955, 1979) to deduce that these were physiological races (Shuster 1955, 1979). Mayer had reported differences in temperature tolerance of crabs from two populations of *Limulus*. Crabs from Marquesas Keys, Florida, could not withstand the colder temperatures in which crabs from Woods Hole, Massachusetts lived, and, conversely, the northern crabs could not survive in

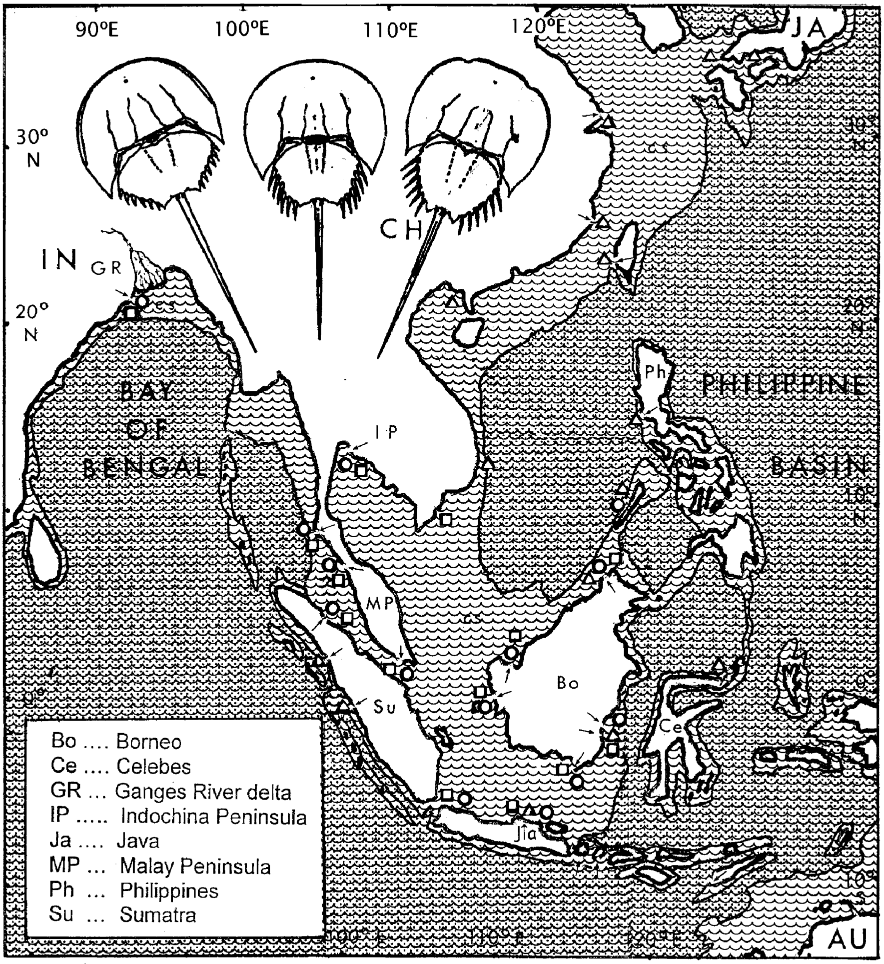

Fig. 6 The continental shelf (cs) is the avenue of distribution for horseshoe crabs. The three Indo-Pacific species are widely distributed with their ranges overlapping in many locations (modified from Anderson and Shuster in Shuster, Barlow and Brockmann 2003, based on Sekiguchi 1988). One or more species were found near most research sites (*small arrows*); *triangles* = *Tachypleus tridentatus* (dorsal view to right in masthead); *squares* = *Tachypleus gigas* (middle of masthead), and *circles* = *Carcinoscorpius rotundicauda* (*left* in masthead); JA = Japan CH = China, IN = India, and AU = Australia

the warmer southern water temperatures. Maximum activity in the Florida crabs occurred at 41°C and at 38°C for the northern crabs. Reynolds and Casterlin (1979) described a range of thermoregulatory behavior in juvenile *Limulus* (prosomal widths from 20 to 50 mm) from the northeastern coast of Gulf of Mexico. They demonstrated that locomotor activity was likely to be optimum in the range of 25°–30°C; temperature, in one way or another, is a major factor in adult size and in activity.

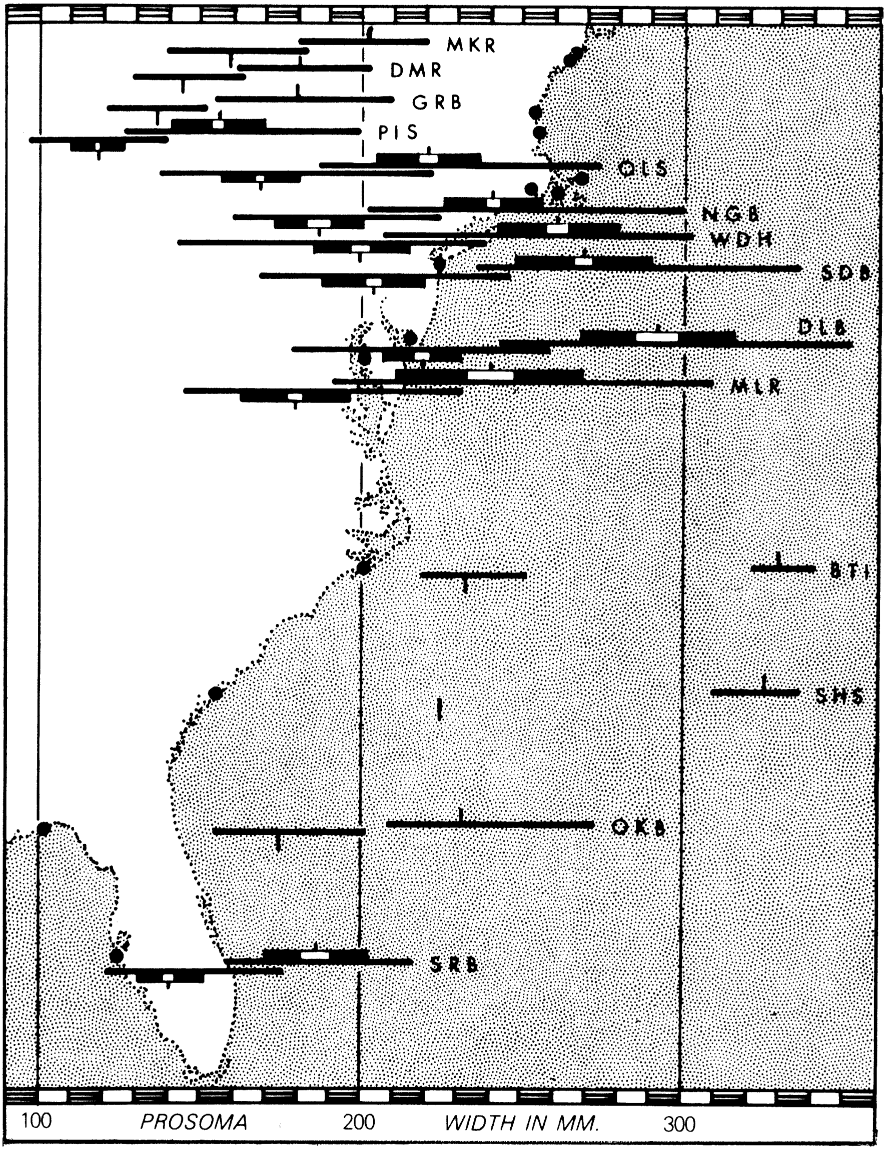

Fig. 7 Populations of *Limulus polyphemus* are morphometrically discrete (Shuster 1955, 1979). Graphic representation of the prosomal widths of adults in millimeters (after technique of Hubbs and Hubbs 1953): range in prosomal width (*horizontal line*); mean (*vertical spike*), one standard deviation (*wide bar*); two standard errors of the mean (*white portion of each bar*); the female dimensions are greater than those of the males. SRB = Sarasota Bay, Florida (27°30′ N); OKB = Ochlockonee Bay, FL; SHS = St. Helena Sound, South Carolina; BTI = Beaufort Inlet, North Carolina; MLR = Miles River, Chesapeake Bay, Maryland; DLB = Delaware Bay, New Jersey; SDB = Sandy Hook Bay, NJ; WDH = Woods Hole, Massachusetts; NGB = Narragansett Bay, Rhode Island; OLS = Pleasant Bay, Cape Cod, MA; PIS = Plum Island Sound, MA; GRB = Great Bay, New Hampshire; DMR = Damariscotta River, ME; MKR = Medomak River, ME (44° N)

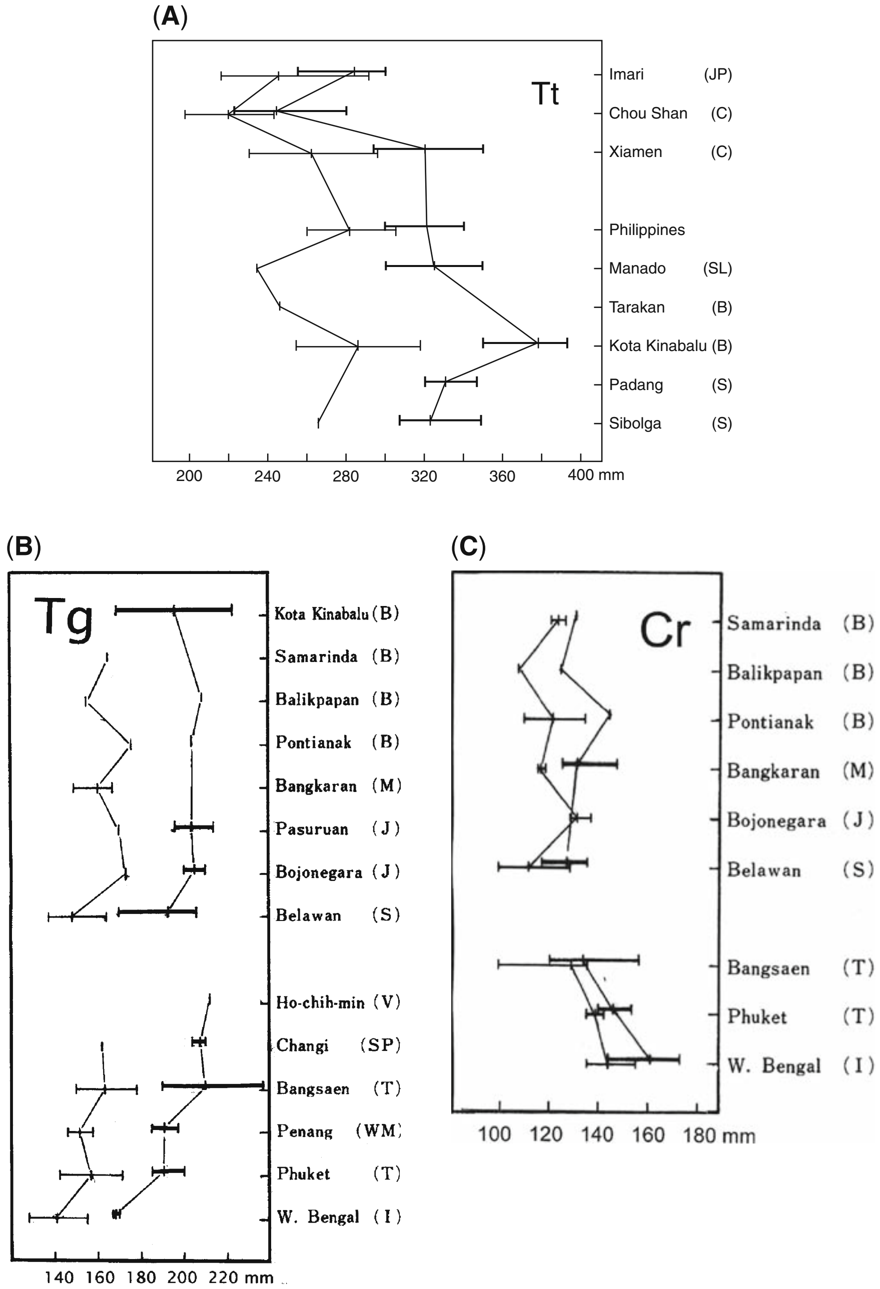

Fig. 8 Populations of the Indo-Pacific species of horseshoe crabs can be defined by their prosomal widths. (**A**) *Tachypleus tridentatus* (Tt). The males (*left-hand* portion of graphs) are smaller than the females; maximum, mean, and minimum widths are shown. Countries are abbreviated as B = Borneo; C = China; JP = Japan; S = Sumatra; SL = Sulawesi (Celebes).

Horseshoe crabs are not gregarious arthropods. Their distribution appears to be closely linked to abundance of the crabs in a population – increasing numbers probably are responsible for increased searches for food. As the crabs get larger, they appear to congregate less and less, especially by sex, except when feeding or breeding. Going their separate ways may be, in part, to escape the possibility of cannibalism by the larger crabs attacking molting crabs.

2.5 Genetic Considerations

Genetic studies substantiate the existence of discrete populations and lend credence to the physiological races concept. Selander et al. (1970) established that electrophoretic variations between four populations of *Limulus polyphemus* were comparable to those of other animals and concluded that the stabilization of their external morphology for at least 200 million years was due to the temporal uniformity of the organic environment and/or genetic homeostasis. Sokoloff (1978) studied eye color and concluded that there were physiological races of *Limulus*. Microsatellite DNA analyses of several *Limulus* populations, from the Gulf of Mexico to New England (Saunders et al. 1986), revealed distinct populations. Pierce et al. (2000) showed that the smaller-sized *Limulus* from the upper Chesapeake Bay were also genetically different from the larger-sized Delaware Bay horseshoe crabs. The latest evidence for genetic differentiation in *Limulus* was provided by a large-scale study of populations throughout their range (King et al. 2003). The extent of the available genetic information leaves little doubt as to the physiological differences of the populations.

Other evidences of relationships between the extant species were demonstrated in a series of developmental and biochemical studies by Sekiguchi and colleagues (1988). Experimental hybridization using reciprocal fertilization of gametes produced viable, swimming larvae only in crosses between *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda*; reciprocal crosses between the three Indo-Pacific species and *Limulus* were non-fertile (Sugita et al. 1988). The diploid number of chromosomes is 26 (*T. tridentatus*), 28 (*T. gigas*), 32 (*C. rotundicauda*), and 52 (*L. polyphemus*) (Iwasaki et al. 1988). Extensive biochemical and immunological studies on the hemocyanin also yielded specific relationships among the species (Sugita et al. 1988). Serological correspondence studies yielded immunological distances that placed the separation of the Indo-Pacific species and *Limulus* at some 130 million years ago while *T. gigas* separated from the other two about 20mya and *T. tridentatus* and *C. rotundicauda* about 10 mya (Shishikura et al. 1982).

Fig. 8 (continued) (**B**) *T. gigas* (Tg), (**C**) *Carcinoscorpius rotundicauda* (Cr). Countries and cities are abbreviated as B = Borneo; I = India; J = Java; M = Madura; S = Sumatra; SP = Singapore; T = Thailand; V = Vietnam; and WM = West Malaysia. “Samarinda” = a coastal site near Samarinda. Modified from Yamasaki et al. (1988)

2.6 Physiological Considerations

The physiological bases for coping with widely ranging environmental conditions were summarized by Towle and Henry (2003). *Limulus* behaves as an osmotic conformer in salinities above 23 ppt and an osmotic regulator in lower salinities. Horseshoe crabs cope with limited oxygen supplies and, particularly during the spawning season respond to a wide range of temperatures. These studies were pioneered by Charlotte Mangum at the College of William and Mary. Although the physiological responses of different populations to the environmental variables were not compared, it can be assumed that differences exist, at least for temperature tolerance (Mayer 1914).

On the basis of the physiological information, we conclude that the conservative retention of an ancient external morphology masks an up-to-date physiology. Because discrete populations have their own set of environmental tolerances – probably due the wide geographical ranges of the species – the separate populations must be the unit of consideration, rather than the species as a whole, when considering conservation measures. Another overall interpretation of this information is that each major population of *Limulus*, and presumably those of the three Indo-Pacific species, has its own distinct range of environmental adaptability that is less than that for the species as a whole.

3 The Large-Scale Limitations on Distribution

There are at least four large-scale, global, environmental parameters that, spatially and temporally, set limits on the distribution of horseshoe crabs. Of these, continental geomorphology and temperature regimes are two major constraints on the widespread distribution of horseshoe crabs. Basically, the continental shelves define the areas available to horseshoe crabs while low temperatures limit their northernmost occurrence. Tidal types and benthic currents also play a role in the distribution of horseshoe crabs.

3.1 Geology Sets Boundaries

The geologic record strongly suggests that horseshoe crabs have always been shallow water creatures. Doubtless the habitats that we associate with the Limulacea – estuaries, shallow seas, and continental shelves – have always existed some where throughout geologic time, changing only in distribution, quantities, and qualities. But there were always some shallow water habitats where horseshoe crabs could exist, even when the low-salinity region of estuaries and continental shelves comprised the geologic boundaries.

3.2 Estuaries

In recent geologic history estuaries are the breeding and nursery grounds for horseshoe crabs. Perhaps shallow water, intertidal areas have always been their habitats. It seems as if horseshoe crabs evolved adaptations to the relative low-energy levels and the salinity ranges and certain beach conditions. However, we surmise that breeding sites and conditions may not have always been as they are today.

Coastal embayments provide low-energy areas critical in the development of the eggs into larvae. At first glance it may seem that these calmer waters are most important because less stranding would occur during the spawning activity than might occur in rougher waters. But, this is a minor situation. The real reason is that horseshoe crabs cannot spawn in rough surf. Indeed, when waves striking a beach are higher than one foot all or most *Limulus* spawners are swept off the beach (Shuster 1955, 1958).

Populations of *Limulus* exist in wide ranges of salinities with limiting salinities as low as 8–9 parts per thousand, as in the upper Chesapeake Bay, and their eggs can develop in full-strength seawater and hypersaline conditions (Jegla and Costlow 1982, Ehlinger et al. 2003, Ehlinger and Tankersley 2005). Those beaches with an appropriate sediment size, moisture and oxygen content, and warmth are ideal incubators of horseshoe crab eggs (Brady and Schrading 1983). But when did horseshoe crabs begin to use the beaches? It is tempting to surmise that beach egg-laying was less likely until the opisthosomal segments fused and a deep vault was formed during the Devonian some 380 million or so years ago. This created a chamber in which the book gills could retain moisture for a few hours. However, small-sized species such as *Paleolimulus* and *Euproops* probably laid their eggs in superficial surface materials such as matted plant debris. Probably it was not until the larger-sized crabs evolved that beaches came into their own as incubators.

3.3 Continental Shelves

The advent of extensive exploration of the continental shelf by horseshoe crabs must have occurred when they were large-sized, possibly at least as large as 15 cm in prosoma width, and when their populations were so large that they tended to exhaust local concentrations of food resources. Also, if ancient horseshoe crabs were like those of today, they seem to avoid each other except to congregate to spawn or to feed. Thus, shallow water zones, where oceans meet land, became important sources of food. They also served as avenues of distribution, from one estuary to another. Interconnected coastal waterways probably also served as limited avenues of distribution. Given the distance of at least 50 statute miles (57.5 nautical miles) that *Limulus* moves in and about Delaware Bay (Swan 2005), the combination of larger-sized crabs and continental shelves was (are) important in their distribution.

3.4 Latitudinal Limitations

Limulus does not extend south of Yucatan, in comparison with the three Indo-Pacific species that coexist on both sides of the equator (Fig. 6). Why *Limulus* does not exist south of Yucatan may be due to a lack of embayments with suitable spawning and juvenile nursery habitats. Other possible conditions may also create barriers, including the lack of a more southern continental shelf avenue. Although the Indo-Pacific species straddle the equator and fossil horseshoe crabs have been reported from Australia, the extant species no longer exist as far south due to the lack of a continental shelf connection and the fossil species are all that remain in Australia.

All extant species of horseshoe crabs are limited in their northern range, suggesting that the intensity and length of winter temperatures form the northern boundary. Indeed, that kind of temperature regime, in effect, controls the distribution of and the migrations of many marine species. Parr's (1933) graph illustrated the fact that local seasonal temperature regimes are markedly different in the surface water temperature along the Atlantic coast of the United States. Reference to his graph demonstrates that local populations of *Limulus* exist within lesser temperature extremes than the range represented by the species as a whole. Many migrating species of fish cannot negotiate the band of rapidly changing water temperatures that develops during the months of December through March in the vicinity of Cape Hatteras, North Carolina – the drastic change within a few miles creates a thermal barrier. This band weakens as the water warms northward in April and June. A lesser temperature barrier forms during June through October at Cape Cod, Massachusetts. Such seasonal changes in the temperature regimes govern migrations of an important predator of *Limulus,* the juvenile loggerhead turtle (Keinath 2003).

An interpretation of the impacts of the last Great Ice Age upon the four extant species, especially upon *Limulus*, suggests what may happen to the Limulidae during periods of global warming. If the distribution of *Limulus* in the past was limited by low temperatures as it is today, then at the peak of the last ice age some 13,000 years ago *Limulus* may have been no further north than Florida. With rising sea level and water temperatures, *Limulus* may extend its range into Canadian waters.

3.5 Significance of Tidal Type

Our observations suggest that there is a general correlation between the occurrence of spawning sites and the tidal type. For example, populations of *Limulus* exist on the northern shores of Yucatan and along the Florida coast to at least Dauphin Island to the west of the entrance to Mobile Bay, and possibly to the Mississippi delta. However, there are no spawning populations in the western portion of the Gulf of Mexico. Because all other environmental parameters, food, hydroclimate, sandy beaches, etc. are suitable but the tides are not, it has been deduced that the tides, the lack of semi-diurnal tides with sufficient amplitude, are the limiting factor (Shuster 1979). The world-wide distribution of semi-daily, irregular semi-daily, irregular diurnal, and regular diurnal tides delineated by Doty (1957) is a good visual reference. This appears to be true for the Indo-Pacific species. Generally, spawning areas are restricted to those locations where the tides are diurnal (regular and irregular). Yet horseshoe crabs have a wide ability to adapt, a characteristic that may be the reason that they have existed for millennia. Such an adaptation is illustrated by the studies of Ehlinger et al. (2003) and Ehlinger and Tankersley (2003) on how *Limulus* exists in a microtidal, hypersaline lagoon on the east coast of Florida.

3.6 Utilizing Currents

The most rapid mode of horseshoe crab locomotion is scuttling wherein the walking legs, tip-toeing, elevate the ventral surface of a crab slightly off the bottom. Velocity is provided by the flow of water created by the flexing of the operculum and branchiae (book gills) and by benthic currents. Several kinds of observations provide the evidence for their riding the currents, i.e., going with the flow

- in clear shallow waters, especially when emerging from a resting burrow during a flooding tide,
- moving with surge currents (Rudloe and Herrnkind 1976),
- during their approach over intertidal flats to spawning beaches,
- from an experiment with juveniles in a flow tank (Luckenbach and Shuster 1997, Preliminary study on the behavior of juvenile *Limulus* in a flume tank. Unpublished manuscript, cited in Anderson and Shuster 2003), and
- from observations by Oates (2005, Personal communication from benthic video observations on *Limulus* in Delaware Bay) by use of a benthic sled using optical sensors.

4 Conservation Considerations

Because this discussion focuses on global limitations to horseshoe crab distribution, a key question is whether conservation strategies are best implemented on a global or local scale. We suggest that conservation of a species may be, in large part, dependent upon the extent to which local, discrete populations are conserved. In other words, conservation of local, discrete populations adds up to the global conservation of a species. Although diversity within and among species may buffer or otherwise enhance their geologic longevity (Shuster and Anderson 2003), this does not appear to be the major concern of conservationists. The ecological role of horseshoe crabs at Delaware Bay has become famous, scientifically and politically, due to the obligatory diet of migratory shorebirds on horseshoe crab eggs (Botton and Shuster 2003). But this is only one of their roles as a multiple-use resource (Berkson and Shuster 1999). Are all the uses equally important? What are some of the ecological considerations?

4.1 Species Diversity

Apparently there have always been only a few species of horseshoe crabs living at any specific geologic time (Shuster and Anderson 2003). That geologic history suggests that the four extant species are probably about right for the long haul into the future. If so, then all four are candidates for our consideration.

4.2 Physiological Diversity

Horseshoe crabs, as a taxonomic group and for millions of years, have demonstrated a kind of conservation. This is evidenced in their tri-part skeletal anatomy that has existed for millions of years. This has sufficed to enable them to do whatever is physically necessary to survive, except perhaps to back up. Perhaps more importantly, they have combined the conservative anatomical trait with an open-ended physiology. On this, geneticists have provide convincing data based on DNA and other analyses that American horseshoe crabs are still evolving throughout their extensive geographical range – this is plausibly also true for the Japanese, Chinese, and Indian species. Presumably this is in response to local ecological factors. In looking at the data amassed by King et al. (2003), the richness of the genetic picture suggests a reason why horseshoe crabs are physiologically robust: no matter what the environmental trend, there was and probably is a gene pool in at least one population that has or will carry the lineage along to the next geologic epoch.

Horseshoe crabs may have always been associated with tidal waters, particularly in estuarine habitats. They have grown to large sizes in temperature waters suggesting that they extended their ranges either northward from primeval warmer climes or to the south and north from temperate areas. In support of the last point, it is at the extremes of their distribution that they are smaller is size and where the genetic picture suggests that adaptive processes are most pronounced. Thus, examination of horseshoe crabs may reveal more interesting data where they exhibit extremes. This may also apply to comparison of areas where they are few or great in numbers. It might be that studies on the extremes, in range or in numbers, may yield more information than studies on average populations, as in studies of spawning behavior.

Horseshoe crabs have adequate motility to range widely. In a few thousand years they moved from a southern area, possibly in the region of Florida/Georgia, to Maine after the last great ice age (13,000 years ago). Despite their mobility and distribution, however, they have developed discrete populations. This was been demonstrated by morphometric indices (Shuster 1955, 1982, Riska 1981) and confirmed genetically (Saunders et al. 1986, Pierce et al. 2000, King et al. 2005).

4.3 Ecological Role

There is little doubt that horseshoe crabs, due largely to their ultimate large size, longevity, and abundance can be one of the major species in the ecology of coastal areas. They are a source of food for other species, are voracious feeders, and may disturb vast areas of benthic and beach habitats.

4.3.1 As Prey

Several species of birds feed on the eggs and stranded crabs worldwide. But the feeding on the eggs by migratory shorebirds all along the coast of North America, especially the great concentration at Delaware Bay that has attracted the most attention. But the shorebirds are competing with small fish, shrimp, and other benthic dwellers for the eggs (Botton and Shuster 2003). Then there are the predators on horseshoe crabs: fishes (small fish and sharks), the loggerhead turtle; birds (migratory shorebirds on eggs; gulls on eggs and crabs); alligators; pigs and raccoons (deSylva et al. 1962, Shuster 1982, Spraker and Austin 1997). Are any of these groups of feeders more important?

4.3.2 Voracious Predators

Horseshoe crabs are voracious predators on a large number of prey organisms (Botton 1984a, Botton and Shuster 2003). They are certainly competitors with other estuarine organisms (skates, rays, etc.) and with man (soft-shelled clam, razor clam, quahaug, surf clam, blue mussel).

4.3.3 As Excavators

Horseshoe crabs typically burrow, shallowly in the substrate when resting, feeding, or spawning (Shuster 1955, 1982, Botton 1984b, Kraeuter and Fegley 1994). The multitude of tracks and pits left on a beach after a heavy spawning event during calm weather looks like the aftermath of a miniature tanks battle. Whether the disturbance of beach sediments or the benthic excavations are significant factors in erosion or affecting ecological parameters has not been examined.

5 Conclusions

The conservative skeletal anatomy of horseshoe crabs, combined with genetic physiological diversity, has resulted in adept environmental generalists. This has enabled them to adapt to a broad range of environmental conditions and to survive for eons. The morphometric and genetic demonstrations that horseshoe crabs exist in discrete populations strongly indicate that any conservation program must recognize the uniqueness of each population as a potential physiological subspecies. We also need a better understanding of the ecological impact of horseshoe crabs.

Understanding horseshoe crabs and their habitat is a key to horseshoe crab conservation, one reason why sessions in this symposium have been devoted to the biology of horseshoe crabs and to their habitat requirements, management, and restoration. Because horseshoe crabs exist in discrete populations, conservation or management programs plans must consider not only the species but its separate populations and their habitats. Although horseshoe crabs have been primarily residents of estuaries and tidal streams, horseshoe crabs have moved from one estuary to another via continental shelves, especially after the last Great Ice Age.

Perhaps the best way to underpin a worldwide horseshoe crab conservation effort is to analyze as many populations as possible, morphometrically and genetically, and their numbers and extent of habitat. Basically, while the broad picture may be the same, what occurs in one habitat for one population under certain environmental conditions may never be observed exactly again; thus, the value of many observations. One reason why Delaware Bay has been so attractive to us and many others is due to its extraordinary large population of horseshoe crabs. This provides a stark contrast to habitats that support lesser numbers of crabs.

Our discussion has dealt with natural barriers to the distribution of horseshoe crabs. We have not included other environmental parameters due to man-induced stresses on the crabs, such as the multiple commercial uses of the crabs, changes in ecosystems, coastal construction, or pollution. Thus, because man can restrict or eliminate suitable habitat and ecosystems, we may create environmental conditions that horseshoe crabs may not be able to successfully adapt to or avoid. Therefore, future attention to favorable habitat conservation and preservation could be as large or larger problem than just managing horseshoe crab populations.

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Horseshoe Crabs – An Ancient Ancestry Revealed

D.M. Rudkin and G.A. Young

Abstract The fossil record of the basic xiphosurid horseshoe crab body plan has been extended back to the Late Ordovician Period, about 445 million years ago, demonstrating an origin that lies outside of the paraphyletic 'synziphosurines.' Horseshoe crab body fossils are exceptionally rare and are found mostly in shallow coastal and marginal marine Konservat-Lagerstätten deposits. Their sporadic occurrences document a post-Cambrian history of low overall diversity with a modest morphological and taxonomic peak in the Late Paleozoic Era. Survival of a single xiphosurid lineage through the end-Permian mass extinction events was followed by a minor secondary radiation during the Triassic Period. The Jurassic to Recent fossil record of horseshoe crabs is relatively impoverished in both taxa and known occurrences. Overall, the rarity of fossil xiphosurids reflects both taphonomic biases inherent in the unusual conditions required for preservation of their non-biomineralized exoskeletons and complex ecological factors related to a long-term association with shallow marginal aquatic habitats. Focused paleontological investigations should yield additional fossil horseshoe crab discoveries that will in turn inform research on their phylogeny, morphological stasis, and ecological persistence.

1 Introduction

Within the impressive diversity and ecological range of extant euchelicerate arthropods, only the xiphosurid horseshoe crabs retain a primitively obligate aquatic habit harking back to their distant genealogical roots. These animals have gained near iconic status as 'living fossils,' and while the popular basis for this view seems rooted mostly in a simple perception of horseshoe crabs as decidedly prehistoric-looking, rare fossil representatives of this morphologically conservative lineage document a history that does indeed extend back

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through the Cenozoic and Mesozoic eras, and deep into the Paleozoic. Recent fossil discoveries show that the earliest accepted horseshoe crabs, strikingly similar in overall appearance to modern forms (Fig. 1), lived some 445 million years ago during the Late Ordovician Period.

Much of the general paleontological background to the horseshoe crab story has been admirably covered in earlier publications, especially in Bergström (1975), Selden and Siveter (1987), Anderson and Selden (1997), Anderson and Shuster (2003), Shuster and Anderson (2003), and numerous references cited therein. Here we present an updated and highly selective overview of the geological history of body fossils of horseshoe crabs and their kin, accompanied by illustrations of a few key representative examples. Our emphasis is on the early part of the record, from the appearance of what are now thought to be the oldest known members of the Xiphosurida about 445 million years ago to

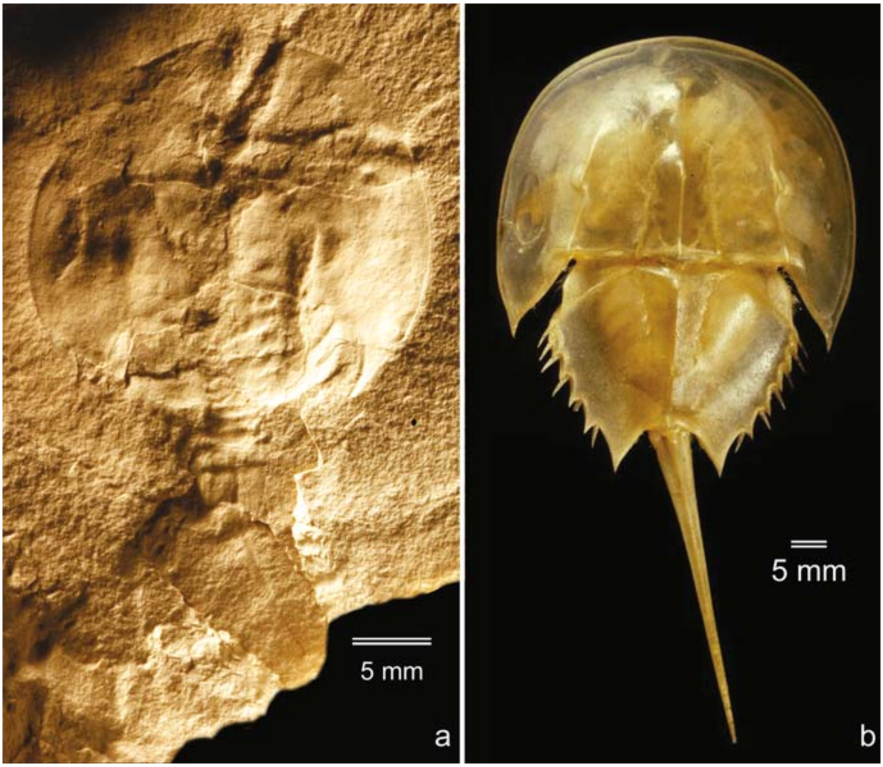

Fig. 1 Temporal end-members of the xiphosurid phylogeny showing highly conserved basic body plan of large crescentic prosomal shield with lateral compound eyes, fused or partially fused 'thoracetron,' and narrow elongate telson spine. (**a**) The oldest fossil horseshoe crab, *Lunataspis aurora* (Manitoba Museum I-3989), Late Ordovician (William Lake Lagerstätte), Manitoba; (**b**) juvenile specimen of the extant Atlantic horseshoe crab species, *Limulus polyphemus*, USA

the end of the Paleozoic Era roughly 200 million years later. We touch relatively briefly on the Mesozoic and Cenozoic portions of the story. For non-paleontologists, we preface our account with a condensed review of geological time and the history of life, mentioning in passing a few preservational and ecological issues that influence the quality and nature of the horseshoe crab record. We conclude with some speculations about how future paleontological investigation may reveal further insights into the early ancestry and ecology of these venerable and resilient members of the most speciose animal phylum on Earth. The following taxonomic hierarchy and nomenclatural framework is implicit throughout:

Phylum Euarthropoda
Chelicerata (including stem-group forms + Pycnogonida)
Euchelicerata (Xiphosura (including 'synziphosurines'†) + Eurypterida† + Arachnida)
Order Xiphosurida (the true horseshoe crabs)
[† = extinct]

For the sake of simplicity, the informal term 'xiphosurid' is used hereafter to designate members of the Xiphosurida.

2 Geological Time, the Fossil Record, Biomineralization, Taphonomy, and Konservat-Lagerstätten – A Paleontological Primer

Earth is approximately 4.6 billion years old. The first 87% of the history of the planet falls within an interval of time known as the Precambrian 'supereon,' and although life was established on Earth by at least 3.6 billion years ago, the Precambrian fossil record is essentially one of microbial remains. Large, multicelled eukaryotes did not appear until after 600 million years ago. The Phanerozoic Eon (Fig. 2) spans just the last 542 million years of geological time, incorporating virtually the entire fossil record of true multicellular animals – the 'eumetazoa.' Phanerozoic time is in turn partitioned into three eras of unequal length, with intervening boundaries corresponding to two episodes of global mass extinction. The oldest and longest, the Paleozoic Era, commenced at the beginning of the Cambrian Period (542 million years ago) and terminated 251 million years ago at the close of the Permian Period and the start of the Triassic, the initial period of the Mesozoic Era. The Mesozoic Era concluded at the end of the Cretaceous Period, 65 million years ago, whence began the Cenozoic Era in which we continue today. The divisions between the Paleozoic and the Mesozoic, and the Mesozoic and the Cenozoic, both represent the culmination of major mass extinctions in which global biodiversity, especially in the seas, was severely depleted.

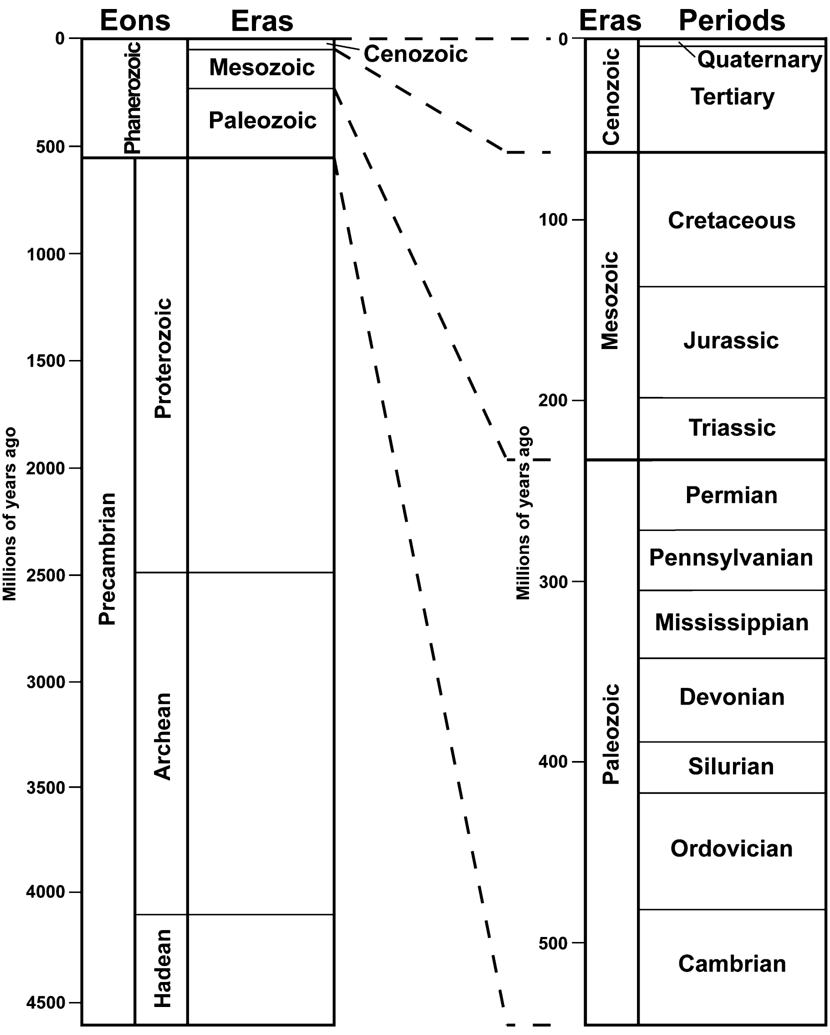

Fig. 2 Simplified geological time chart showing divisions of the Phanerozoic Eon and key absolute age dates mentioned in this chapter; note that on this version the internationally recognized Carboniferous Period (cited in the text) is divided into a *lower* and an *upper* interval and labeled, respectively, as the Mississippian and Pennsylvanian periods (following standard terminology in the USA)

The conventional Phanerozoic fossil record, which comprises our primary macroscopic record of life on this planet, is overwhelmingly dominated by fragmentary shelly remains of small, aquatic animals. This is a straightforward probabilistic consequence of preservational bias (Kidwell 2001; Allmon 2001). Hard, decay-resistant ‘skeletal’ components of abundant bottom-dwelling organisms that lived in environments with repeated or rapid sediment input (and hence burial opportunities) were far more likely to endure the largely destructive rigors of fossilization. Organisms entirely lacking mineralized hard parts (up to 79% of modern marine biotas – see Allison and Briggs 1991), or those that inhabited environments where sediments were continually recycled or not deposited at all, are correspondingly rare as fossils. Phanerozoic epeiric seas, fluvial-deltaic systems, and open marine shallow-shelf environments were characterized by relatively large areal extent, high sedimentation rates, and long-term geological stability, so their deposits are major repositories of fossil remains; shoreline, lagoon, and lacustrine environments, which are not as well represented by sedimentary deposits, contribute proportionally less to the record (Martin 1999).

By all accounts, members of the biomineralizing (shelly) invertebrate marine benthos were clearly favored for preservation, but almost always without direct evidence of their soft components, and fossils of entirely non-mineralized organisms are extremely uncommon. It is the study of such soft-tissue rarities and the circumstances responsible for their exceptional preservation that 'fleshes out' the fossil record, providing, among other things, unique insights into non-mineralized anatomy, better estimates of original biodiversity, and a clearer grasp of ecological relationships. Taphonomy is the branch of paleontology concerned with preservational pathways: the biological, chemical, and physical processes – destructive and constructive – that acted upon organisms from the time of death until they were locked into the fossil record (Martin 1999). Knowledge of taphonomic processes and pathways is vital to understanding the biases inherent in the fossil record.

Fossil 'Konservat-Lagerstätten' deposits are paleontological treasure troves that represent rare and highly unusual burial conditions in which destructive taphonomic processes were minimized or interrupted at an early stage (Seilacher et al. 1985; Briggs 2001; Bottjer et al. 2002). This allowed for the exceptional preservation of intact soft-bodied or non-biomineralizing organisms and of the otherwise labile tissues of shelly or hard skeleton-bearing forms. As a consequence, world-renowned Konservat-Lagerstätten (hereafter shortened to Lagerstätten; singular: Lagerstätte) such as the Burgess Shale, Mazon Creek, Bear Gulch, and the Solnhofen Limestone, yield some of the most spectacular, interesting, and informative of fossils. Such sites also provide a wealth of information on ancient biodiversity, anatomy, and ecology (Bottjer et al. 2002; Selden and Nudds 2004). Complete body fossils of xiphosurid horseshoe crabs, all with sclerotized but non-biomineralized exoskeletons (Stankiewicz and Briggs 2001; Shuster and Anderson 2003; Gupta et al. 2007), are very uncommon and come almost exclusively from a few Lagerstätten representing deposition in shallow to very shallow marine coastal environments, including lagoonal and estuarine settings. This in turn reflects a longstanding ecological association (for at least parts of their life cycle) of horseshoe crabs and marginal and shoreline habitats, with possibly sporadic incursions into freshwater systems (Anderson and Shuster 2003). The near-exclusive occurrence of intact xiphosurid fossils in unusual marginal marine deposits does not imply that the living animals were permanently limited to very shallow coastal habitats, but rather that they stood very little chance of being fossilized in the more ‘normal marine’ range of their environments. The same apparently holds for the preservation of individual dorsal exoskeleton elements such as prosomal shields (which are more heavily sclerotized than ventral appendages), either as molt remains or as parts of disarticulated carcasses. Initial requirements for preserving xiphosurid remains (in situ or minimally transported) were similar to those for other unmineralized organisms and included various combinations of rapid, gentle burial in fine-grained sediment, lack of dissolved oxygen (anoxia), exclusion of scavengers, hyper- or hyposalinity, water column stratification, microbial stabilization and appropriate pore-water chemistry (Allison and Briggs 1991; Martin 1999). These conditions may have been more prevalent in lagoonal, estuarine, and restricted shoreline settings than on the open marine shelf.

The overall rarity of xiphosurid fossils is almost certainly not a function of low original population numbers (except, perhaps, during their earliest evolutionary history). It arose, rather, through the complex taphonomic interplay between intrinsic anatomical attributes, autecology, local sedimentation, and geochemistry and much larger scale processes, such as plate tectonics, that have affected the extent and spatial distribution of entire ecosystems through the Phanerozoic (Kidwell 2001).

3 The Fossil Record of Horseshoe Crabs

Our examination of the fossil record of horseshoe crabs starts from the geologically oldest Paleozoic (Late Ordovician) occurrence and moves stratigraphically up through youngest (Cenozoic) fossils (see Fig. 2). A short overview of relevant arthropod fossils that appeared in the so-called Cambrian Explosion, beginning about 540 million years ago, sets the stage.

3.1 Cambrian Context

An extraordinary burst of metazoan evolution in Early and Middle Cambrian seas saw the appearance of a bewildering array of arthropods and arthropod-like animals, almost certainly derived from cryptic ancestral lineages established in the Late Neoproterozoic (Briggs and Fortey 2005; Waloszek et al. 2005). Among these, the trilobites, one of the most familiar of all extinct animal groups (Fortey 2000), rose rapidly to prominence as diverse and abundant members of the biomineralized or ‘shelly’ marine biota (Fig. 3a). Indeed, the Cambrian is often referred to as the ‘Age of Trilobites’ for the profusion of their pervasively calcified exoskeletons in marine sedimentary rocks of this period. In the same explosive radiation, a number of non-biomineralized arthropod

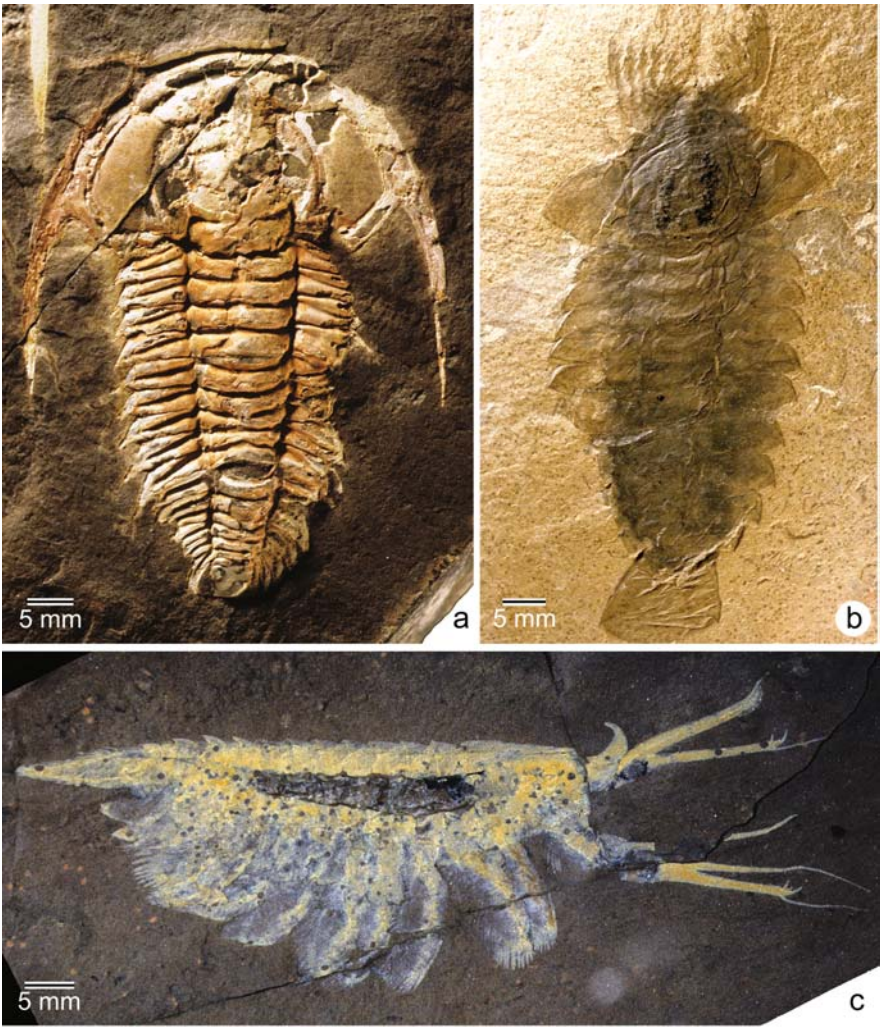

Fig. 3 Representative Cambrian arthropods. (**a**) Trilobite, *Eoredlichia takooensis* (Royal Ontario Museum 56928), Early Cambrian, Australia; (**b**) 'arachnomorph' arthropod, *Sanctacaris uncata* (Royal Ontario Museum 43502), Middle Cambrian (Burgess Shale Lagerstätte), British Columbia; (**c**) 'great appendage' arthropod, *Leanchoilia superlata* (Royal Ontario Museum 90-1484 – photo by D. H. Collins), Middle Cambrian (Burgess Shale Lagerstätte), British Columbia

groups with a mosaic of confusing chelicerate-like character suites also entered the scene (Waloszek et al. 2005, 2007). Especially well represented (along with their trilobite cousins) in the Chengjiang, Burgess Shale, and Orsten-style Lagerstätten, even the most exquisitely preserved of these taxa have historically proven difficult to accommodate within conventional arthropod classifications based upon features of extant crown-groups. Attempts to reconcile their interrelationships and broader affinities have resulted in proposals that place chelicerate-like forms together with trilobites and many so-called 'trilobitomorphs' in higher level taxa variously labeled Arachnomorpha or Arachnata (Selden and Dunlop 1998; Cotton and Braddy 2004; Hendricks and Lieberman 2008), although these views have not remained unchallenged (Scholtz and Edgecombe 2005, 2006). A number of Cambrian 'arachnomorphs' have been proposed as the earliest euchelicerate arthropods or as euchelicerate ancestors (e.g., Briggs and Collins 1988; Wills 1996), based almost entirely on contentious interpretations of anterior appendages (Fig. 3b). Another incomplete and poorly preserved Cambrian fossil, *Eolimulus alata*, was even referred to the Xiphosurida (Bergström 1968, 1975 – but see Selden and Dunlop 1998). Careful reappraisals have since resulted in the rejection of such claims and these new studies, incorporating evidence from fossils (including recently discovered material) and from molecular and developmental studies on living organisms, are beginning to offer a somewhat clearer, although still controversial, picture of early arthropod relationships (e.g., Edgecombe et al. 2000; Pisani et al. 2004; Giribet et al. 2005; Budd 2008). There is now a growing acceptance that stem-group chelicerates are represented in Early to Middle Cambrian Lagerstätten by certain 'great appendage' arthropods (such as *Leanchoilia* from the Burgess Shale; Fig. 3c) (Chen et al. 2004; Waloszek et al. 2007). Cambrian representatives of the Pycnogonida (sea spiders), sister group to the Euchelicerata (Siveter et al. 2004), have now been described (Waloszek and Dunlop 2002), but to date no fossil arthropod possessing a suite of unequivocal euchelicerate crown-group characters has been found in rocks of Cambrian age (Dunlop and Selden 1997; Selden and Dunlop 1998).

3.2 Post-Cambrian Paleozoic Developments

In contrast to those of the Cambrian Period, sedimentary sequences of Ordovician age contain relatively few fossil Lagerstätten deposits. Consequently, the record of soft-bodied organisms and non-biomineralizing arthropod groups (including the chelicerates and their relatives) falls off dramatically, especially over the earlier part of the Ordovician. The picture improves for the Late Ordovician, with the appearance and initial diversification of the oldest representatives of the Eurypterida ('sea scorpions' – see Fig. 4a), an undoubted euchelicerate crown-group (Tetlie 2007; Tetlie and Cuggy 2007). Eurypterids (extinct since the end of the Permian Period) had long been grouped with xiphosurans in the Merostomata (e.g., Størmer 1952) based primarily on a shared aquatic habitat, but subsequent work (Kraus 1976; Selden and Siveter 1987) has shown 'merostomes' to be a paraphyletic grade. Some analyses support a much closer connection between Eurypterida and terrestrial Arachnida via the scorpions (Fig. 4b), whose oldest Silurian fossil representatives were also aquatic

Fig. 4 Post-Cambrian euchelicerate arthropods. (**a**) Eurypterid, cf. *Eurypterus* species (Royal Ontario Museum 53629), Early Silurian (Eramosa Lagerstätte), Ontario; (**b**) scorpion, undescribed taxon (Royal Ontario Museum 53248), Early Silurian (Eramosa Lagerstätte), Ontario

and possibly marine (Dunlop and Selden 1997; Selden and Dunlop 1998; but see Kamenz et al. 2008 for another recent view). Eurypterids, along with a mixed bag of other extinct Paleozoic aquatic euchelicerate forms, possessed a generally long, tapering opisthosomal region made up of 9–13 freely articulated segments followed by a telson of highly variable morphology. Some of the post-Ordovician non-eurypterid fossils bear a superficial resemblance to xiphosurid horseshoe crabs and have been considered their potential ancestors (see discussion of 'synziphosurines' below).

Far more relevant to this account, however, is the recent description of genuine horseshoe crab fossils of startlingly 'modern' appearance (Rudkin et al. 2008) from two newly discovered Lagerstätten in Upper Ordovician rocks of central Canada (Young et al. 2007). Named *Lunataspis aurora*, this rare form features a proportionally large crescentic prosomal shield bearing lateral compound eyes, anterior opisthosomal tergites fused into a broad rigid plate, and a xiphous (lanceolate) tail spine.

The fossils (Fig. 5) are small and occur as moderately convex molds of the dorsal exoskeleton, some even with adhering patches of translucent organic cuticle; less heavily sclerotized ventral structures, such as appendages, are not well preserved in specimens discovered to date. *Lunataspis* co-occurs with

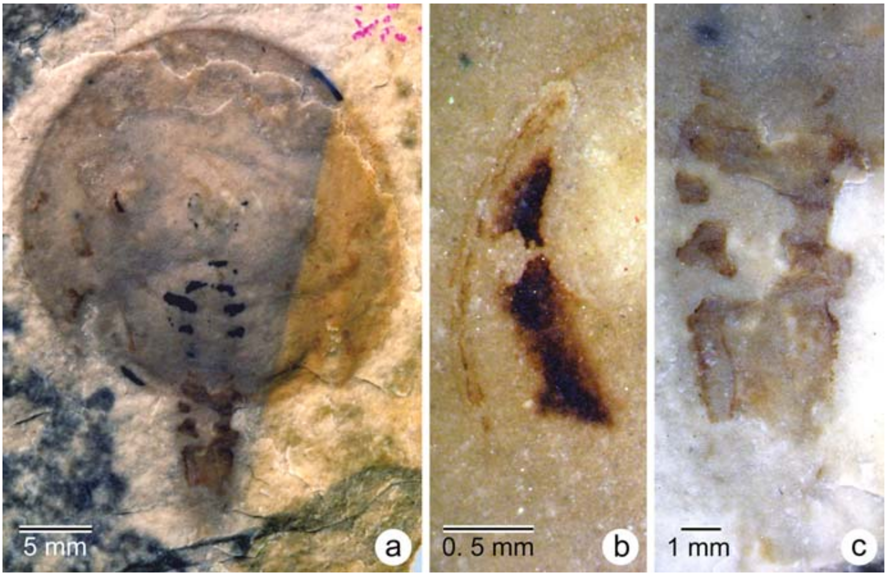

Fig. 5 Oldest known fossil xiphosurid horseshoe crab, *Lunataspis aurora* (Late Ordovician Airport Cove Lagerstätte), Manitoba. (**a**) Holotype specimen (Manitoba Museum I-4000A) showing general outline of articulated dorsal exoskeleton – dark patches represent secondary mineral deposits associated with marginal rim and axial furrows of prosomal shield, and with paired structures beneath mesosoma; (**b**) close-up view of pigmented cuticle on visual surface of left lateral eye; (**c**) close-up view of metasoma and anterior of telson showing preserved cuticle with reticulate pattern

diagnostic Late Ordovician microfossils and a variety of soft-bodied, weakly sclerotized, and shelly macrofossils in demonstrable shoreline and near-shore marine paleoenvironments (Young et al. 2007). The key morphological development that distinguishes *Lunataspis* from virtually all other Ordovician, Silurian, and Devonian horseshoe crab-like animals is the fusion of opisthosomal segments to form a bipartite 'thoracetron' composed of a broad anterior mesosoma followed by a short, narrow metasoma (Rudkin et al. 2008 – see Fig. 5). A one- or two-piece thoracetron is the signature attribute of the Xiphosurida (Anderson and Selden 1997). Prior to the discovery of *Lunataspis*, the oldest fossil forms known to possess this feature came from rocks of latest Devonian and Early Carboniferous age (about 350–360 million years old) (Anderson and Selden 1997; Moore et al. 2007) and paleontologists had looked to the promising Silurian and Devonian Lagerstätten record for likely ancestors to thoracetron-bearing xiphosurids (a previous account of a putative Early Ordovician stem-group xiphosuran has been rejected – see Moore and Braddy 2005). To date, the only available candidates known from this interval comprise an odd assortment of fossil chelicerates grouped together in a taxon known as the Synziphosurina (Eldredge 1974). With one or two notable exceptions (e.g., Moore et al. 2005a) Silurian (Fig. 6) and Devonian 'synziphosurines' are

Fig. 6 ‘Synziphosurine’ chelicerate, *Bunaia woodwardi* (Royal Ontario Museum 53886), Late Silurian (Bertie Lagerstätte), Ontario, showing freely articulated opisthosomal segments behind prosomal shield

generally poorly preserved forms, and all possess 9–11 freely articulated opisthosomal segments posterior to a semi-circular prosomal shield. Although vaguely horseshoe crab-like in some respects, they are now considered to represent a paraphyletic assemblage of stem-group xiphosurans (Anderson and Selden 1997) none of whose members lie directly along the lineage leading to modern forms. The only known post-Devonian ‘synziphosurine’ was recently described from the famed Bear Gulch Lagerstätte (Montana, USA) of Early Carboniferous age, in a biota that also includes two species of xiphosurid horseshoe crabs (Moore et al. 2007).

With the description of *Lunataspis* from Late Ordovician shallow marine deposits, the record of the xiphosurid (thoracetron-bearing) body plan is pushed back almost 100 million years beyond the previous tie-point (Late

Devonian–Early Carboniferous), which in turn drives the search for ancestral forms even deeper into the Early Paleozoic. This temporal extension also begs the question of why no similar fossils have yet been recovered from Silurian or Devonian rocks. It cannot be due entirely to a lack of appropriate preservational conditions (taphonomic bias), because eurypterids and 'synziphosurines' (with similarly non-biomineralized cuticles) occur in a number of well-documented shallow marine Lagerstätten from this interval. In fact, despite their overall rarity, both of those chelicerate groups achieve maximal diversity through the Siluro-Devonian (Moore et al. 2005b; Tetlie 2007). Possible explanations for the relative paucity of early xiphosurid fossils may include more limited dispersal abilities (restricted ecological and geographic range) and lower population numbers than their eurypterid or 'synziphosurine' cousins.

It was during the Carboniferous Period, between about 300 and 360 million years ago (Fig. 2), that horseshoe crabs achieved their own modest heyday of diversity. Eight or nine valid genera, representing two divergent xiphosurid subgroups, are currently listed from about a half-dozen Carboniferous Lagerstätten of wide paleogeographic distribution. The Bellinurina, on the one hand, was a distinctive group of generally small, variably spinose or highly effaced forms (Fig. 7a) that did not survive beyond the Early Permian (Anderson and Selden 1997). Their peculiar morphologies and sedimentological associations have led some workers to suggest they were narrowly specialized for life in a range of brackish, freshwater, and even semi-terrestrial habitats associated with Late Paleozoic coal swamps (Fisher 1979; Racheboeuf et al. 2002; Crônier and Courville 2005), but there are serious challenges to many of these propositions (Anderson 1996; Anderson and Shuster 2003). A second less morphologically

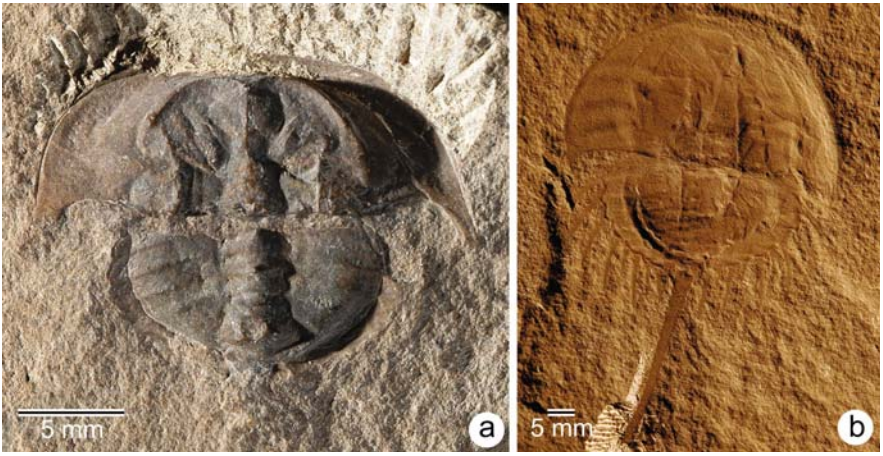

Fig. 7 Representative Carboniferous xiphosurid horseshoe crabs. (**a**) Bellinurina, *Euproops danae* (Royal Ontario Museum 5827), Late Carboniferous (Mazon Creek Lagerstätte), Illinois; (**b**). Limulina, *Paleolimulus longispinus* (Royal Ontario Museum 58752), Early Carboniferous (Bear Gulch Lagerstätte), Montana

diverse group, with generally larger size and strong ties to marine and marginal environments, includes the Limulina (Fig. 7b), the lineage in which all subsequent Mesozoic and Cenozoic forms (including extant species) are currently classified (Anderson and Selden 1997).

Species of one limuline and three bellinurine genera have been recorded from a single Late Carboniferous *Lagerstätte* (Bickershaw, UK), representing an unusually high level of xiphosurid co-occurrence (Anderson et al. 1997). Other Carboniferous sites typically contain only one or perhaps two xiphosurid taxa, but specimens may be locally abundant (hundreds to thousands of individuals; e.g., Mikulic 1997) or exceptionally well preserved (Babcock et al. 2000; Racheboeuf et al. 2002), and samples such as these provide vital information on ventral anatomy, growth and development, paleoecology, and taphonomic processes. At least one Carboniferous limuline, *Paleolimulus signatus*, apparently possesses a bipartite thoracetron, with a broad anterior sclerite and a short, narrow posterior one (Babcock et al. 2000), comparable (but not necessarily homologous) to the opisthosomal condition described in *Lunataspis* (Rudkin et al. 2008).

The record for the Permian Period shows a marked decrease in xiphosurid diversity and distribution, with a single bellinurine (Malz and Poschmann 1993; Anderson 1996) and two limuline genera (Ewington et al. 1989; Babcock et al. 2000; Allen and Feldmann 2005) described from just a few scattered sites. This may be a consequence, in large part, of the relative paucity of suitable habitats or surviving Lagerstätten deposits on a worldwide scale. The Permian was a time of dramatic changes in global climate, sea level, ocean chemistry, and volcanic regimes, as plate tectonic movements brought about the final assembly of the supercontinent Pangea. These changes heralded the most severe extinctions in the entire half-billion year history of eumetazoans, marking the close (Fig. 2) of both the Permian Period and the Paleozoic Era, 251 million years ago (Erwin et al. 2002).

3.3 The Mesozoic and Cenozoic Records

Following the unprecedented end-Permian losses in global biodiversity (which included the final demise of both trilobites and eurypterids, among many, many others), the fossil record shows increasing rates of faunal recovery in the aquatic realm through the Triassic Period (Yin et al. 2007). The limuline horseshoe crab lineage not only survived the so-called 'Great Dying,' but actually contributed in a small way to this early Mesozoic re-radiation. In their graphic plot of diversity, Moore et al. (2007, text-Fig. 2) indicate a total of seven limuline genera for the Triassic, but most of these are monospecific and based on one or a few specimens. Only during the Carboniferous Period was this number of taxa exceeded, when both bellinurines and limulines contributed to maximal xiphosurid genus-level diversity. Two limuline species described from separate

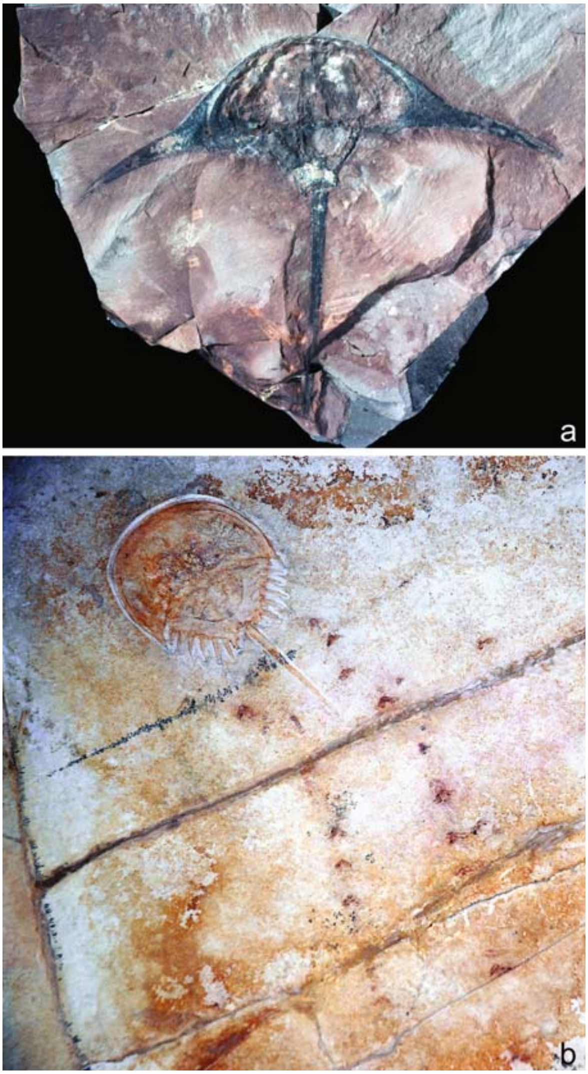

Fig. 8 Representative Mesozoic horseshoe crabs. (**a**) *Austrolimulus fletcheri* (Australian Museum F.38274, with permission), Triassic, Australia – specimen measures 180 mm across genal spines; (**b**) *Mesolimulus walchi* 'dead in its tracks' (Royal Ontario Museum 53253), Jurassic (Solnhofen–Eichstätte Lagerstätte), Germany – maximum width of prosomal shield is approximately 80 mm

Australian Triassic localities are noteworthy because they display highly unusual prosomal shield morphologies. The most bizarre of these, *Austrolimulus fletcheri* (Riek 1955, 1968 – see Fig. 8a), is further distinguished by possession of a thoracetron with a broad anterior component and a shorter, narrower posterior section. Curiously, both Australian forms are supposed to come from

Triassic strata interpreted to be of undoubted freshwater origin (Pickett 1984). The same contention applies to at least one other odd Triassic form (*Psammolimulus gottingensis* – see Meischner 1962), as well as several Carboniferous bellinurines, and this remains a challenge to reconcile with the ecological ranges of other fossil horseshoe crabs and of the living species.

Following this secondary comeback in the Triassic, limuline diversity apparently declined again abruptly. For the balance of the Mesozoic and right through the Cenozoic Era (Fig. 2), their fossil record is restricted to a very few sites yielding a total of just six genera (including the three familiar living ones), all of more-or-less similar morphology. Despite a dearth of occurrences for the entire post-Triassic, the best known of all fossil horseshoe crab species comes from this interval, in the renowned Solnhofen–Eichstätte Lagerstätte of Late Jurassic age in Germany. Images of extraordinary specimens of *Mesolimulus walchi* (Demarest), frozen in death at the end of their meandering or spiral trackways, have appeared in so many texts and popular paleontological publications that they have become virtual emblems of the fossil record (Barthel et al. 1990 – see Fig. 8b). Numerous other specimens of complete *Mesolimulus* individuals have been collected from the Solnhofen–Eichstätte Plattenkalks, many of which reveal features of the ventral side in superb detail. A single new specimen of *Mesolimulus* recently described from the slightly older Nusplingen Lagerstätte, about 350 km southwest of Solnhofen, is even more remarkable in that it preserves the prosomal musculature (and associated microbes!) in exquisite microscopic detail (Briggs et al. 2005). Species of both Triassic and Cretaceous age (Woodward 1879; Vía 1987) have also been assigned to *Mesolimulus*, but it remains the only genus so far recorded from Jurassic rocks. Its overall morphology is sufficiently similar to the living horseshoe crabs that *Mesolimulus* has been placed along with them in the same family, Limulidae, as have several Triassic genera and two forms known exclusively from overlying Cretaceous strata (Riek and Gill 1971; Pickett 1984; Vía 1987). The scant record for horseshoe crabs in that final period of the Mesozoic Era also includes one species (based on a single fragmentary specimen) referred to the extant genus *Limulus* (Reeside and Harris 1952).

A global mass extinction about 65 million years ago marks the termination of the Cretaceous Period and the end of the Mesozoic (Fig. 2). Best known for its association with a major extraterrestrial bolide impact, extensive volcanism, and the demise of the non-avian dinosaurs, the so-called K/T boundary event has captured wide public attention (Alvarez 1998; Chatterjee et al. 2006; Ocampo et al. 2006). Horseshoe crabs clearly managed to survive the related ecological turmoil, but their subsequent fossil record does not show any overall improvement in absolute abundance or quality of preservation. In fact, the Cenozoic portion of their history, covering all of the most recent 65 million years, is woefully impoverished, with just a single reasonably well-documented fossil occurrence in the Miocene of Germany: *Tachypleus decheni* (Störmer 1952). This continues to be largely a reflection of low preservation potential arising from lack of pervasive exoskeletal mineralization, the same taphonomic issue that plagues the entire pre-Cenozoic xiphosurid record. But there may be other factors at work as well, particularly ecological ones: shifts in habitat preference or availability, diversification and expansion of predator and scavenger species, loss or reduction of environments with specific physical and chemical parameters favoring preservation, and so on.

The net effect of this poor record is that we do not yet have a clear picture of exactly how the four living species, allocated to three genera, were derived from Mesozoic lineages (Fisher 1982, 1984). Molecular studies on extant populations (Avise et al. 1994; Xia 2000) have provided some clues to their genealogical relationships, but still leave open many questions about their disjunct distributions.

4 Paleontological Postscript

We still do not know the reasons for the enduring success and hence remarkable longevity of the horseshoe crab body plan. Why has the more-or-less ‘original’ xiphosurid morphology remained effectively unchanged from the Ordovician to the present (Fig. 1), while various elaborations on this basic theme fell by the wayside? Why have horseshoe crabs persisted through successive waves of global extinction while ‘great appendage’ arthropods, trilobites, and eurypterids have all succumbed? One might ask the same questions about two other groups of chelicerates (sensu lato), for both the pycnogonid sea spiders and the scorpions have maintained what one might call ‘relict’ body plans over comparable spans of time. The fossil record of sea spiders and scorpions is also pretty dismal due to essentially the same factors that contribute to the rarity of horseshoe crabs – lack of pervasive biomineralization and association with environments not particularly conducive to preservation. Indeed, their remains are also found in just a very few Lagerstätten deposits. Interestingly enough, scorpions made the ecological transition to an exclusively terrestrial existence about 350 million years ago while the sea spiders have remained entirely marine – and both groups are far more diverse at the species level than the modern horseshoe crabs. Locking into a genetically constrained body plan early in the history of a lineage because it works (doing it right the first time) cannot be the whole explanation to survival as a ‘living fossil.’ Ecological functionality (including reproductive behavior, trophic strategies, etc.) must be at least as important as anatomy, and it is entirely possible for an organism to make major shifts in life habits without making conspicuous changes to external morphology (witness the scorpions).

Focusing future paleontological efforts on the search for more shallow marine and marginal aquatic Lagerstätten deposits of Cambrian and Ordovician ages should in all likelihood reveal new insights on the origin of xiphosurids and their early evolution and ecology (Rudkin et al. 2008). Significant temporal gaps in the horseshoe crab record (Siluro-Devonian, Permian, Late Mesozoic–Cenozoic) may also be filled with the discovery of additional taphonomic ‘windows.’

Trace fossils (tracks, trails, and burrows) assignable to the benthic activity of ancient xiphosurids have not been considered in this chapter, but these remain a source of valuable paleontological information on behavior and ecology, especially in sedimentary sequences where body fossils are not generally preserved (Seilacher 2007 and references therein). Other intriguing topics approachable through new fossil discoveries and careful interpretation of the geological record include evolutionary size trends in horseshoe crabs, ancient dispersal patterns and paleogeography, and community interaction and integration (predator/prey status, etc.) through time (e.g., Anderson and Shuster 2003). Each has a bearing on the diversity, distribution, and ecological importance of modern horseshoe crabs and as such can ultimately contribute to our better understanding and appreciation of these extraordinary 'living fossils.'

One thing remains certain above all else – after having survived a total of five major extinction intervals over a history now traceable back through 445 million years, the horseshoe crab lineage may now be closer to disappearing than ever before . . . literally at the hands of our own species (Tanacredi 2001; Walls et al. 2002).

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The Ecological Importance of Horseshoe Crabs in Estuarine and Coastal Communities: A Review and Speculative Summary

Mark L. Botton

Abstract Beyond their commercial importance for LAL and bait, and their status as a living fossil, it is often asserted that horseshoe crabs play a vital role in the ecology of estuarine and coastal communities. How would the various ecological relationships involving horseshoe crabs be affected if these animals were no longer abundant? Attempts to understand and generalize the ecological importance of horseshoe crabs are hampered by several constraints. We know relatively little about the ecology of juvenile horseshoe crabs. Most ecological studies involving adult *Limulus polyphemus* have been conducted at only a few locations, while much less is known about the three Indo-Pacific species. Furthermore, we are attempting to infer the ecological importance of a group of animals whose numbers may have already declined significantly (the so-called "shifting baseline syndrome"). Horseshoe crab shells serve as substrate for a large number of epibionts, such as barnacles and slipper limpets, but the relationships between these epibionts and horseshoe crabs appear to be facultative, rather than obligatory. Horseshoe crabs are dietary generalists, and adult crabs are ecologically important bivalve predators in some locations. The most notable predator–prey relationship involving horseshoe crabs is the migratory shorebird–horseshoe crab egg interaction in Delaware Bay. After hatching, the first and second instars are eaten by surf zone fishes, hermit crabs, and other predators. Virtually nothing is known about predator–prey relationships involving older juveniles, but adult *L. polyphemus* are important as food for the endangered loggerhead turtle, especially in the mid-Atlantic region.

1 Introduction

As with discussions about the significance of preserving global biodiversity, the rationale for conserving horseshoe crabs is often condensed into monetary terms. On these grounds, the considerable economic value of horseshoe crabs for lysate,

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bait, and ecotourism makes a very forceful case for the need for sustainable horseshoe crab populations (e.g., Berkson and Shuster 1999; Manion et al. 2000). However, just as the preservation of ecological diversity has ecological benefits that are not easy to transpose into dollars, horseshoe crabs may have underappreciated roles in the ecosystem that need to be part of the discourse about their value.

The objective of this chapter is to review the ecological role of horseshoe crabs in estuarine and coastal environments with the goal of better understanding some of these additional ecological benefits. One way of envisioning the ecological roles (as suggested to me by Jane Brockmann) is to project how the functioning of ecosystems would differ if horseshoe crabs were absent or extremely rare. To accomplish this analysis, I subdivide the ecological role of horseshoe crabs into three parts: the importance of horseshoe crabs as predators, their importance as prey, and their importance as hosts for epibionts.

In reviewing the literature, it became clear that there were constraints to the inferences that could be made about the ecological importance of horseshoe crabs. One reason for this is that most of the pertinent studies in the literature have been about *L. polyphemus*, with far fewer reports about *Tachypleus tridentatus*, *T. gigas*, and *Carcinoscorpius rotundicauda*. Of the ecological studies with *L. polyphemus*, most have been conducted on populations in the middle Atlantic and southern New England. There is also relatively little published information about the feeding ecology of juvenile horseshoe crabs and virtually nothing about the importance of predation on juveniles.

It is worth emphasizing that other aspects of horseshoe crab behavior, such as the correlation between spawning intensity and the lunar and tidal cycle, are highly variable across the species' range. For example, Barlow et al. (1986) found that spawning intensity in Cape Cod, Massachusetts was timed to coincide with the higher of the two daily high tides and that the highest peaks occurred on the spring tides (near the new moon and full moon). By contrast, in Delaware Bay the spring tide peaks may be disrupted by wind events and/or cold fronts that force the animals offshore until the return of favorable conditions (Shuster and Botton 1985, Smith and Michels 2006), and spawning in the Indian River Lagoon, Florida showed no relationship at all to the lunar cycle (Ehlinger et al. 2003). Given the extreme plasticity in spawning behavior, I would expect that there is geographic variation in feeding behavior and other aspects of the ecology as well. So, there is considerable danger in trying to generalize from the relatively few ecological studies of *L. polyphemus* in locations such as Delaware Bay and Cape Cod to other portions of its range, much less to the three other species.

We are in the somewhat tenuous position of extrapolating the ecological importance of a group of animals whose abundance has already declined substantially, what Pauly (1995) has referred to as the "shifting baseline syndrome." In other words, we risk misinterpreting the current situation as the norm, when in fact horseshoe crabs may have already been reduced to a small fraction of their historic abundances. For example, peaks in the Delaware Bay population that occurred during the nineteenth century may be imperfectly documented (Shuster 2003), but, in the writer's opinion, are suggestive of abundances far greater than any living scientist has witnessed. An even more extreme situation exists with *T. tridentatus* along the Seto Inland Sea in Japan, which have been reduced over the past century to the extent that some local populations are extinct (Tsuchiya 2009). The shifting baseline concept extends beyond the population level, in that the ecological importance of horseshoe crabs as predators, for example, is related to the collective impact of the population, so simply knowing what individual crabs eat is not enough to deduce the importance of these predators to the community.

2 Horseshoe Crabs as Predators

2.1 Predation by Adults

Adult horseshoe crabs are omnivorous, feeding on a wide variety of benthic invertebrates, including bivalves, polychaetes, crustaceans, and gastropods. The functional morphology of feeding and the anatomy and physiology of the digestive system has recently been reviewed by Botton and Shuster (2003), so this contribution emphasizes the ecological importance of horseshoe crab predation on estuarine and coastal benthic communities. *L. polyphemus* on the mid-Atlantic continental shelf primarily feed on bivalves including blue mussels (*Mytilus edulis*) and surf clams (*Spisula solidissima*) (Botton and Haskin 1984; Botton and Ropes 1989). When crabs encounter patches of preferred prey such as thin-shelled bivalves, they may feed extensively, literally packing their guts with shells (Fig. 1). Prey selection has been demonstrated in laboratory feeding experiments (Botton 1984a), where crabs exhibited a clear preference for thin-shelled bivalves (e.g., dwarf surf clams, *Mulinia lateralis* or soft-shell clams, *Mya arenaria*) when given a choice between these and thicker shelled species,

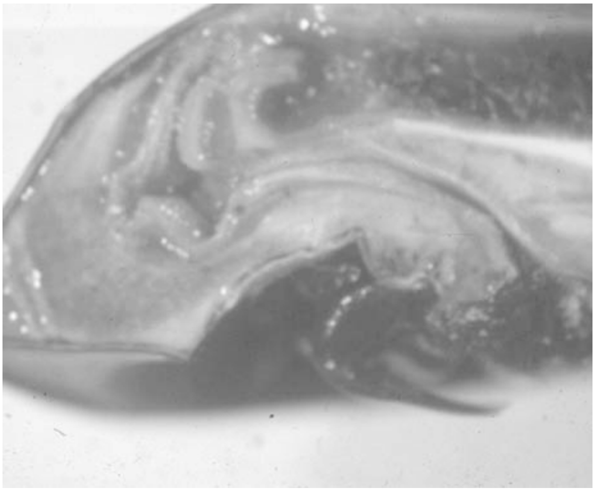

Fig. 1 A longitudinal section through a horseshoe crab showing the anterior portion of the digestive system (mouth, proventriculus or gizzard, and intestine). Note the presence of broken blue mussel shells (*Mytilus edulis*) in the intestine. Courtesy of Carl N. Shuster

including quahogs (*Mercenaria mercenaria*) and gem clams (*Gemma gemma*). Bivalves were the most important macrobenthic prey in the stomachs of adult *L. polyphemus* feeding on a Delaware Bay tidal flat during the breeding season (Botton 1984a), and this was true as well for *T. gigas* from India (Debnath et al. 1989). Both of these studies found that many animals contained sand, plant detritus, and minute food items such as Foraminifera and nematodes. Botton and Shuster (2003) suggested that when preferred prey are rare, horseshoe crabs may ingest sediment rather indiscriminately, which would account for the presence of such minutely sized food items. The horseshoe crab digestive system contains the enzyme cellulase (Debnath et al. 1989) demonstrating that the plant detritus may be nutritionally useful.

The ecological importance of adult horseshoe crabs as predators has primarily been studied on intertidal sand bars and mud flats during the breeding season. Horseshoe crabs withdraw to these areas after spawning on the beaches at high tide, and while they await the return of the tide, they dig pits in search of food or to avoid desiccation. Predator exclusion techniques (caging experiments) have been used to assess the ecological importance of predation by adult horseshoe crabs in these habitats. The first of these studies was conducted in Massachusetts in response to assertions from commercial fishermen that predation was causing enormous destruction to planted soft-shell clams. Plots that were caged to exclude predators had significantly higher clam survival than uncaged plots (Smith and Chin 1951; Smith et al. 1955). Many of the young-of-the-year clams within the caged areas achieved sizes of more than 20 mm by September, but no clams above 10 mm survived in the uncaged areas. Botton (1984b) demonstrated similar impacts of predation in an exclusion study on Delaware Bay tidal flats. There was substantial recruitment of small *M. lateralis* and *M. arenaria* into both caged and uncaged plots in June 1978. By August, there were few clams above 4 mm outside the predator exclosures, whereas *Mulinia* and *Mya* protected from predation grew to lengths of 15–24 mm. Aside from these dramatic effects on bivalve survival and growth, Botton (1984b) found that the exclusion of predators led to significant increases in total invertebrate abundance, biomass, and species diversity (average number species per core) than unprotected sediments.

In any predator exclusion study, it is difficult to ascertain the extent to which the target predator caused the response. Smith and Chin (1951) noted that the tidal flats were riddled with horseshoe crab pits and also found shell fragments of *Mya* and other bivalves in horseshoe crab guts and fecal pellets. However, other clam predators such as the green crab (*Carcinus maenas*) were also excluded by certain caging treatments. Botton's (1984b) inference that horseshoe crab predation was responsible for most of the effects seen in his caging experiment was corroborated by a study of gut contents and aquarium feeding trials, which confirmed that horseshoe crabs in this habitat preferentially consumed thin-shelled bivalves such as *Mulinia* and *Mya* (Botton 1984a). Furthermore, a manipulation using a top-only (floating mesh) design that was specifically designed to exclude predatory gulls and shorebirds showed that bird exclusion had minimal effects on the benthic community in comparison to the full cages (Botton 1984c).

The potential impacts of horseshoe crabs as predators are intertwined with their effects as sediment disturbers, and predator exclusion studies cannot readily differentiate between the importance of sediment disturbance versus predation. In the case of horseshoe crabs, both effects may be relevant. Kraeuter and Fegley (1994) found that portions of a Delaware Bay tidal flat that were subjected to horseshoe crab burrowing were disturbed to a mean depth of 11.1 cm, whereas areas that were caged to exclude horseshoe crabs were reworked (by waves and currents) to just 3.2 cm. The intense sediment reworking by horseshoe crabs could impact prey by crushing them or bringing them to the surface, where they may be consumed by predators such as gulls. The most common invertebrate on these tidal flats is the gem clam, *G. gemma*, which has a hard shell that makes it resistant to predation and disturbance. Comitto et al. (1995) studied the recolonization of *Limulus* pits and found that *Gemma* generally recovered within 1 day after disturbance events. A significant amount of sediment disturbance by horseshoe crabs also occurs during egg deposition (Jackson et al. 2005; Nordstrom et al. 2006; Smith 2007), and this may be an extremely important mechanism by which eggs in deep sediments are moved to the sediment surface where they are accessible to foraging shorebirds (see Section 3.1.1).

In summary, it is clear that dense aggregations of adult *L. polyphemus* have ecologically significant impacts on invertebrate populations on nearshore mud flats and sand bars, related to feeding and/or sediment disturbance. With the onset of cooler temperatures, the crabs disperse into deeper waters where much less is known about their ecological importance. There is virtually no available data on the feeding ecology of *L. polyphemus* in northern New England, or anywhere south of Virginia, and the paucity of information on the three Asian species is also noteworthy.

2.2 Predation by Juveniles

The trilobite larvae subsist on the yolk; independent feeding does not begin until the second instar stage. The digestive system in juvenile crabs is fully differentiated into discrete regions of mouth, esophagus, proventriculus, intestine, rectum, and anus (Shuster 1948). However, the size and crushing ability of the feeding appendages (see Botton and Shuster 2003) constrains the choice of foods in the smallest size classes of horseshoe crabs. Not surprisingly, then, the diets of these animals shift as they grow larger.

The diets of various sizes of *T. gigas* in the Bay of Bengal over a year-long period were reported by Chatterji et al. (1992). Important prey included mollusks, insects and crustaceans, and polychaetes. Decayed organic material, sand, and plant detritus were highest from July to October, coinciding with the period of time when preferred molluscan species were lowest. Crabs < 120 mm carapace length (CL) consumed less food than crabs > 141 mm CL. Zhou and Morton (2004) enumerated the gut contents of nine juvenile *T. tridentatus* between 8.5 and 67 mm prosoma width (PW) and two *C. rotundicauda* between 50 and 52 mm PW from tidal flats in Hong Kong. Based on this limited sample

Table 1 Estimated maximum percentage of potential prey items in the mixed diet of juvenile horseshoe crabs at Nauset Beach, Massachusetts based on stable isotope signatures

	Instar number								
Prey category	2	3	5	6	7	8	9	10	11
Bivalves	ND	ND	11	22	20	22	16	11	17
Gastropods	ND	ND	14	27	24	27	20	13	21
Crustaceans/polychaetes	ND	ND	44	56	63	79	87	84	95
Benthic POM	21	< 100	100	100	99	74	77	64	70
Suspended POM	100	< 100	23	42	34	31	18	9	16

POM = particulate organic material, ND = not detected.
Modified from Gaines et al. (2002).

size, the diets of the two species were similar, and insect larvae were the most common food item. Polychaetes, oligochaetes, small crabs and other crustaceans, and thin-shelled bivalves were also eaten.

Gaines et al. (2002) and Carmichael et al. (2004) used stable isotopes to study the feeding ecology of juvenile *L. polyphemus* from Massachusetts. Comparisons of nitrogen and carbon isotopic signatures of horseshoe crab tissues with potential prey in the estuary are useful in deducing the trophic position of the animals in the food web. The nutrition of second and third instars was largely derived from benthic and suspended particulate organic material, originating from phytoplankton, macroalgae, and salt marsh (*Spartina alterniflora*) production in second instars, but mainly from *Spartina* in third instars (Gaines et al. 2002). Gradually, the older juveniles became progressively more reliant on crustaceans, polychaetes, and mollusks (Table 1). The stable isotope technique also enabled Carmichael et al. (2004) to trace back the locations where juvenile crabs had been feeding to sub-estuaries that differed in nitrogen loading.

3 Horseshoe Crabs as Prey

3.1 Eggs, Trilobite Larvae, and Juveniles

3.1.1 Predation by Birds

The importance of horseshoe crab eggs as food for migratory shorebirds in Delaware Bay has now been very well established and is reviewed by Mizrahi and Peters (2009). Early evidence linking horseshoe crab eggs and shorebirds was based primarily on observational data (e.g., Botton et al. 1994), further supported by Tsipoura and Burger (1999) who found that shorebird stomach contents were dominated by *Limulus* eggs. There are strong relationships between the density of horseshoe crab eggs and the abundance of red knots along the Delaware Bay shore (Karpanty et al. 2006). Perhaps most persuasively, Haramis et al. (2007) showed that free-ranging shorebirds collected from Delaware Bay had $\delta 15$ N signatures that were identical with the $\delta 15$ N signatures of captive shorebirds that were fed a diet of horseshoe crab eggs alone.

Shorebirds feed on prodigious quantities of horseshoe crab eggs during their stopover on Delaware Bay. Castro et al. (1989) estimated a daily consumption of 8,300 eggs by sanderlings (*Calidris alba*). Haramis et al. (2007) estimated that red knots (*C. canutus*) eat an average of 18,350 eggs/day during the period of maximum feeding, with a peak daily consumption of 23,940 eggs, while ruddy turnstones (*Arenaria interpres*) ate an average of 13,300 eggs/day with a peak daily consumption of 19,360 eggs. These remarkable daily rations are required to achieve the increases in body mass that are needed for the northbound flight to the Arctic breeding areas. Shorebirds foraging on eggs at the sediment surface feed by pecking at rapid rates, ranging from 1.2 pecks/s for ruddy turnstones to 2.4 pecks/s for sanderlings (Gillings et al. 2007).

Haramis et al. (2007) deduced that red knots need to eat about 5,000 eggs in order to gain 1 g of mass. Since each red knot gains about 80 g mass during its stay in Delaware Bay, it follows that each bird would need to eat roughly 400,000 eggs. Hence, a population of 40,000 red knots would need to eat approximately 16 billion eggs. At the present time, only a minority of Delaware Bay beaches have sufficient densities of crab eggs to attract flocks of red knots (Karpanty et al. 2006), and the inability of red knots to find adequate food throughout the bay may be having serious effects on their survival (Baker et al. 2004).

Clearly, horseshoe crab eggs are vital for shorebirds in Delaware Bay, but does bird predation have a significant impact on the total numbers of horseshoe crab eggs deposited by female crabs? Gillings et al. (2007) demonstrated that shorebirds feeding on horseshoe crabs eggs in sand-filled trays could cause short-term depletion of surface eggs. However, for the large number of shorebirds that feed in the swash zone (Botton et al. 1994), eggs are continually being replenished by wave action (Nordstrom et al. 2006), and wave periods (ca. 3.2–10.6 s) are probably sufficiently rapid to neutralize the effects of shorebird foraging on surface egg density. Moreover, the deep sediments that contain most of the fresh egg clutches are extensively reworked by successive spawning events during both of the two daily high tides. It is this process of exhumation that ultimately brings most of the eggs toward the surface where they are available to birds (Smith 2007), because wave action during typical spring conditions in Delaware Bay causes much less disturbance to the sediment than the spawning process itself (Jackson et al. 2005). These processes would indicate that depletion of surface eggs by foraging shorebirds, as suggested by the work of Gillings et al. (2007), may only apply to those birds feeding on dry sand above the swash zone.

I would propose that shorebird predation on horseshoe crab eggs in Delaware Bay is far more important to the birds than it is as a potential limiting factor for the crabs. Eggs that are left on the surface litter lines by the receding tide (as described by Nordstrom et al. 2006) will desiccate and die if they are not consumed by predators. Moreover, the density of eggs in the upper 5 cm of the beach is generally 10% or less than the egg densities at depths of 5–20 cm (Botton et al. 1994). In addition, the annual peaks in horseshoe crab spawning abundance do not always coincide with the peak of the shorebird migration, because cold water temperatures and/or unfavorable wind and wave conditions can cause the crabs to delay spawning (Shuster and Botton 1985, Smith and Michels 2006). Eggs deposited in June have a smaller risk of predation, since most shorebirds depart the bay shore by the end of May (Clark et al. 1993).

Beyond the Delaware Bay area, the quantitative importance of bird predation on horseshoe crab eggs is largely unproven, although the interaction has been noted in locations such as Plymouth Bay, Massachusetts (Mallory and Schneider 1979), Jamaica Bay, New York (Riepe 2001), Sandy Hook Bay, New Jersey (Loveland and Botton unpublished), and Great Bay, New Jersey (Sullivan 1986). On Cape Cod, Massachusetts beaches, densities of horseshoe crab eggs are generally $<10{,}000\ \mathrm{m}^{-2}$, and mainly occur at depths <5 cm (James-Pirri et al. 2005). It is questionable whether such low egg densities would make it profitable for shorebirds to specialize on this resource, nor is it known whether bird predation is a major cause of mortality for crab eggs.

3.1.2 Predation by Fish and Crustaceans

There is very little quantitative information about the importance of fishes and benthic predators to horseshoe crab eggs and larvae. Feeding by the sand shrimp, *Crangon septemspinosa*, juvenile blue crabs (*Callinectes sapidus*) and spider crabs (*Libinia* sp.), and by various surf zone fishes such as eels, catfish, juvenile striped bass, white perch, killifish, weakfish, and Atlantic silversides has been observed (Warwell 1897; Perry 1931; deSylva et al. 1962; Price 1962; Spraker and Austin 1997; Botton and Loveland unpublished).

In Delaware Bay, New Jersey, killifish (*Fundulus heteroclitus*) and Atlantic silversides (*Menidia menidia*) are the two most common fishes caught near tidal creeks where horseshoe crabs frequently spawn (Botton and Loveland, unpublished). In August 2001, horseshoe crab eggs or trilobites occurred in the stomachs of 95% of the killifish and 96% of the Atlantic silversides (Table 2). Individual fish had up to 34 eggs and 51 trilobites in their stomach. *Menidia* consumed more eggs than *Fundulus*, although the difference was non-significant (2-tailed *t*-test, $t_{43} = 0.35$). Conversely, *Fundulus* consumed more trilobite larvae than *Menidia*, with the difference being marginally significant ($t_{43} = 1.94$, $p = 0.06$). Overall, *Fundulus* consumed an average of 16.9 horseshoe crab eggs and larvae, and *Menidia*, 11.5 ($t_{43} = 1.34$, $p = 0.19$). A previous study on *Menidia* indicated that trilobites, which were abundant in the guts just after

Table 2 Predation by killifish (*Fundulus heteroclitus*, $n = 20$) and Atlantic silversides (*Menidia menidia*, $n = 25$) on horseshoe crab eggs and trilobite larvae, based on a sample of fish collected on August 22, 2001, from a tidal creek at Kimbles Beach, Delaware Bay, NJ

		Frequency of occurrence			Mean (SD) per gut		
Species of fish	Mean TL	Eggs	Trilobites	Total stages	Eggs	Trilobites	Total stages
Killifish	53.7	30%	95%	95%	2.8 (7.6)	14.1 (11.7)	16.9 (15.1)
Atlantic silversides	79.2	56%	92%	96%	3.5 (5.0)	8.0 (8.6)	11.5 (11.0)

TL = total length, SD = standard deviation.

midnight (0015 hours), were completely evacuated by 0645 hours (Spraker and Austin 1997). If similar gut passage times apply to the fish collected from Delaware Bay that would indicate the potential for Atlantic silversides and killifish to have a daily ration of about 46 and 68 *Limulus* daily, respectively.

Hermit crabs (*Pagurus longicarpus*) are abundant on Delaware Bay tidal flats throughout the spring and summer, generally occupying shells of mud snails (*Ilyanassa obsoleta*) or Atlantic oyster drills (*Urosalpinx cinerea*). We sampled the hermit crab population by taking a series of 15 replicate 1 m^2 quadrats on each of the first 5 sand bars on the intertidal flats, a distance approximately 200 m offshore. This region includes about 80–90% of the young-of-the-year horseshoe crab population on the flats (Botton et al. 2003). About 75% of the hermit crabs were in the small size class (total claw length 3.0–4.9 mm), and nearly the entire remainder were medium sized (5.0–7.9 mm). Total hermit crab densities ranged from about 2.7 to 7.2 animals m^{-2} on July 20, and about 1.7 to 4.1 m^{-2} on July 27–28 (Fig. 2). By comparison, Botton et al. (2003) enumerated about 10 trilobites m^{-2}, 2–3 second instars m^{-2}, and < 1 third instar m^{-2} on the same tidal flats at the same time that these hermit crab surveys were conducted.

Behavioral observations of predation by *P. longicarpus* on *Limulus* trilobites and second instars in aquaria indicated that the hermit crabs attacked the ventral surface, using their claws to pick out portions of the legs and gills. Most of the chitinous tissue, including virtually all of the prosoma and opisthosoma, was not ingested. We therefore felt that stomach contents would not be a reliable measure of hermit crab predation. Instead, we tested individual hermit crabs in aquaria with known numbers of trilobites or second instars, sieved the sand after an 8–12 hour feeding interval, and then counted the remaining horseshoe crabs. The percent recovery of horseshoe crabs in the absence of a hermit crab predator was 99% (average of 52 replicates), indicating that there were no other significant losses that took place during the procedures.

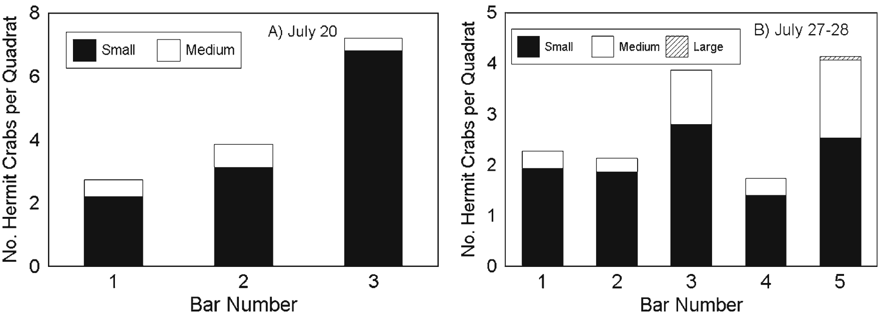

Fig. 2 Mean number of hermit crabs across a Delaware Bay intertidal sand flat, July 1999. Bar 1 is closest to shore and bar 5 is approximately 200 m from shore. Fifteen replicate 1 m^2 quadrats were taken on each of the five sand bars on each date. Hermit crabs were grouped into three size classes based on total claw length (i.e., combined length of the dactyl and propodus): small (3.0–4.9 mm), medium (5.0–7.9 mm), and large (> 8.0 mm)

Hermit crabs preyed on both trilobites and second instars. Among the small hermit crabs, 76.7% ate at least one trilobite, with an average predation rate of 0.33 per hour. Fourteen of 15 medium hermit crabs fed on at least one trilobite (93.3%), and the average predation rate was about three times higher, 1.12 per hour. Among the large hermit crabs, 13 of 15 fed on at least one trilobite (86.7%) and the average predation rate was 1.07 per hour. There was a statistically significant difference in predation rate between the small and both the medium and large size classes, but no difference between the medium and large size classes (1-way ANOVA, $F_{2,57} = 79.7$, $p < 0.001$ followed by post hoc Bonferroni multiple comparisons test). In 94 feeding trials in which small hermit crabs were put in aquaria with second instars, 85.1% consumed at least 1 second instar, with an average predation rate of 0.41 per hour (Fig. 3). Among medium hermit crabs ($n = 61$), 98.4% ate at least 1 second instar and the average predation rate was about doubled (0.83 per hour). Virtually all (98.0%) of the large hermit crabs fed ($n = 50$), and the average predation rate was 0.91 per hour. The predation rate by small hermit crabs was significantly smaller than both the medium and large size classes, but there was no difference in feeding rates between the medium and large size classes (1-way ANOVA, $F_{2,204} = 16.88$, $p < 0.001$). There were no statistically significant differences in predation rates on trilobites versus second instars for any of the three size classes of hermit crabs.

The net impact of hermit crab predation can be approximated by multiplying the predation rates for each size class by the densities of each size class on the intertidal flats (Table 3). Although the feeding rate per individual was lowest among small hermit crabs, they had the greatest numerical impact on horseshoe

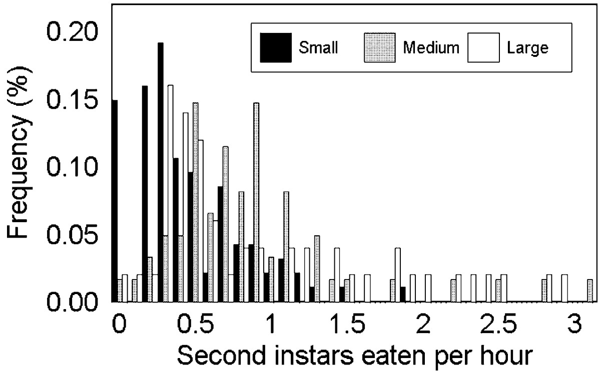

Fig. 3 Predation by hermit crabs on second instar horseshoe crabs based on aquarium feeding trials conducted on 15 dates between August and October 1999. Aquaria were 29 × 18 cm, and had a 1 cm layer of clean sand covered by 1.5 cm of filtered bay water. Hermit crabs were grouped into three size classes as defined in Fig. 2: small ($n = 94$), medium ($n = 61$), and large ($n = 50$). Second instars (initial $n = 30$ or 40) were given 30 minutes to acclimate and burrow before the hermit crab was added and allowed to feed for 8 or 12 hours. Feeding rates were normalized to the number of second instars eaten per hour; since feeding rates in 8 or 12 hour trials were not significantly different, the data were pooled

Table 3 The net impact of hermit crab predation on trilobites and second instars on intertidal sand flats in Delaware Bay, NJ

	Mean predation rate (no./day)		July 20			July 27–28		
Hermit crab size class	Trilobites	Second instars	Hermit crab density	Predation rate on trilobites × hermit crab density	Predation rate on Second instars × hermit crab density	Hermit crab density	Predation rate on trilobites × hermit crab density	Predation rate on second instars × hermit crab density
Small	8.00	9.95	4.04	32.36	40.23	2.11	16.85	20.96
Medium	26.80	19.84	0.56	14.89	11.02	0.71	18.94	14.02
Large	25.60	21.94	0.00	0.00	0.00	0.01	0.34	0.29

crabs on July 20 because they were much more numerous than medium and large hermit crabs. On July 27–28, medium hermit crabs had a slightly larger impact on trilobites, but the reverse was true for second instars.

A better understanding of the quantitative importance of predation on young-of-the-year and juvenile horseshoe crabs is needed if we are to have a better understanding of recruitment processes in natural populations (Botton et al. 2003; Grady and Valiela 2006). The limited amount of information from Delaware Bay, NJ, and Pleasant Bay, MA, suggests that there is very high mortality from eggs through the end of the first summer of life (Carmichael et al. 2003; Botton et al. 2003). There is also a growing interest in the feasibility of utilizing aquaculture as a means of enhancing stocks of horseshoe crabs. Fertilized eggs would be reared in the laboratory for eventual release into the field as juveniles (e.g., Mishra 2009, Schreibman and Zarnoch 2009). For this to be successful, we must have much more information about the causes and magnitude of stage-specific mortality. However, we currently lack data on the causes and magnitude of natural mortality for horseshoe crabs between the early instars and maturity.

4 Adult Horseshoe Crabs as Prey

Mortality of adult horseshoe crabs, caused by natural factors and fisheries, does not have the same importance to the population growth rate as does the survival of juveniles (Grady and Valiela 2006). The large size and dense exoskeleton of adult horseshoe crabs certainly protects them against most predators, and adult horseshoe crabs are not consumed with any regularity by most known predators (Botton and Shuster 2003). However, a number of interesting examples of adult mortality are known which may be important to particular populations of horseshoe crabs. Reid and Bonde (1990) and Ehlinger (personal communication) have observed large American alligators (*Alligator mississippiensis*) eating adult *L. polyphemus* on multiple occasions in the Indian River Lagoon, Florida. Adult horseshoe crabs are a significant component of the stomach contents of loggerhead turtles (*Caretta caretta*) in lower Chesapeake Bay (reviewed by Keinath 2003). Interestingly, the diet of loggerhead turtles in Virginia shifted from horseshoe crabs in the early to mid-1980s to blue crabs (*C. sapidus*) in the late 1980s, and then more recently to finfish (Seney and Musick 2007). These shifts in stomach contents were probably triggered by declines in the horseshoe crab and blue crab fisheries, respectively; moreover, the declines in horseshoe crabs and blue crabs may be linked with the decrease in the number of sea turtles in the lower Chesapeake Bay over the past two decades. Using aerial and net surveys, Spotila et al. (2007) found that sea turtle densities in lower Delaware Bay were comparable to the lower Chesapeake Bay. It has not been determined whether loggerheads in Delaware Bay feed extensively on horseshoe crabs, though this would seem a likely possibility given the high density of horseshoe crabs in this location. It is uncertain whether there are other species of sea turtles that feed on horseshoe crabs; Barleycorn and Tucker (2005) observed a Kemp's Ridley turtle (*Lepidochelys kempii*) eating a horseshoe crab in Charlotte Harbor (Gulf of Mexico), Florida.

Adult horseshoe crabs are frequently stranded on beaches during spawning, especially in relatively high-energy estuaries such as Delaware Bay (Botton and Loveland 1989). Predation by large gulls on stranded individuals has been implicated as a significant cause of mortality among stranded adults. Herring gulls (*Larus argentatus*) and great black-backed gulls (*Larus marinus*) frequently eat live upside-down horseshoe crabs by tearing out their gills and legs in order to access the eggs and other viscera (Botton and Loveland 1993). The only report of bird predation on any of the three Asian species is from Debnath and Choudhury (1988), who found that crows (*Corvus spelendens*) ate *T. gigas* in India.

5 Horseshoe Crabs as Hosts for Epibionts

Horseshoe crabs carapaces frequently serve as a substrate for encrusting invertebrates and algae. These associations are neither parasitic nor commensal, and are better described by the term epibiosis (Wahl 1989): a non-symbiotic, facultative association between the substrate organism and sessile animals (epizoans) or algae (epiphytes). Bryozoans, barnacles, tube-building polychaetes, and sessile mollusks such as mussels, oysters, and slipper limpets are among the more conspicuous epibionts on the three species of horseshoe crabs that have been studied, namely *T. gigas* (Key et al. 1996; Patil and Anil 2000), *C. rotundicauda* (Key et al. 1996), and *L. polyphemus* (Allee 1922; Botton and Ropes 1988; Dietl et al. 2000; Botton and Shuster 2003; Grant 2003). Typically, epibionts are generalists in their choices of substrates (Wahl and Mark 1999), and all of the epibionts that attach to horseshoe crabs are widespread on other types of hard substrate.

Epibionts living on horseshoe crabs are examples of sessile organisms living on moving substrates (Key et al. 1996). As such, their ecology may differ from their conspecifics that are attached to rocks and other non-moving objects. For example, sessile organisms living on mobile substrates may have enhanced gene dispersal, and filter-feeders may have better feeding opportunities because water flow may be enhanced (Wahl 1989). But because horseshoe crabs are so highly migratory, any organisms living on them must be able to survive the same range of temperatures and salinities as their host. They are also subjected to aerial exposure when the horseshoe crabs come ashore to spawn. When horseshoe crabs become stranded on a beach (Botton and Loveland 1989), the combination of desiccation and high temperature frequently kills epibionts such as the blue mussel (*M. edulis*) and slipper limpet (*Crepidula fornicata*) even though the horseshoe crab is still alive (personal observations).

For the most part, the associations of horseshoe crabs and epibionts cause little or no damage to the host (Key et al. 1996). Blue mussels sometimes attach to the walking legs and book gills, potentially impeding their function (Botton 1981). The ability of the crabs to obtain sensory input from their lateral eyes may be impeded by overgrowth (Wasserman and Cheng 1996), but an even more severe impact on vision is caused by bacterial and green algal infections that cause the loss of ommatidia, which apparently makes it less likely for males to visually locate females prior to amplexus (Duffy et al. 2006). These and other infestations of horseshoe crabs are discussed by Nolan and Smith (2009).

Epibionts have only been reported to occur on adult horseshoe crabs, in part because the frequency of molting (Sekiguchi et al. 1988) precludes the establishment of a fouling community on juveniles. In contrast, adult crabs generally do not molt after reaching sexual maturity (Botton and Ropes 1988). The smooth, glossy carapaces of juvenile and recently molted “young” adults normally lack epibionts, and the lateral eyes are clear (Duffy et al. 2006). The absence of epibionts in juveniles and young adults may be related to the secretion of a mucus with anti-fouling properties (Patil and Anil 2000; Harrington and Armstrong 2000).

6 Summary and Conclusions

Predation and sediment disturbance by adult horseshoe crabs can be ecologically significant phenomena where the animals are concentrated in large numbers. A good demonstration of the importance of considering the size of the horseshoe crab population in inferring the magnitude of an ecological process is the study by Smith (2007), who showed that the quantity of *L. polyphemus* eggs exhumed from beach sediments by horseshoe crab digging was dependent on the number of spawners. It is reasonable to assume that there are similar density-dependent relationships between horseshoe crabs and sediment disturbance on intertidal flats (Kraeuter and Fegley 1994) and between horseshoe crab predation and the effects on benthic infauna (Botton 1984b). To date, the ecological impacts of horseshoe crabs as sediment disturbers and predators have been evaluated only where crabs are most numerous, most notably Delaware Bay. There is a need for similar studies elsewhere, and in particular, for the three Asian species. The greatly diminished number of horseshoe crabs in Japan and elsewhere in southeast Asia limits our ability to infer the importance that these animals may have had in previous generations (Pauly 1995).

Since the review by Botton and Shuster (2003), there have been several papers adding to our understanding of the distribution and feeding ecology of juvenile horseshoe crabs (Botton et al. 2003; Zhou and Morton 2004; Carmichael et al. 2004), and advances on raising horseshoe crab juveniles in captivity (Carmichael et al. 2009, Schreibman and Zarnoch 2009) also give important insights into the trophic position of juvenile crabs. Stable isotope techniques have revealed that juvenile horseshoe crabs show dramatic ontogenetic shifts in their choices of food, and may move between different areas within an estuary (Gaines et al. 2002; Carmichael et al. 2004). As with adults, understanding the significance of juvenile horseshoe crabs in the flow of energy through the ecosystem depends on knowing their abundance as well as their individual feeding habits. We are beginning to understand that juvenile horseshoe crabs may be abundant in subtidal areas (Burton et al. 2009), where little is known about their role as predators. Studies coupling juvenile abundance and feeding would contribute much to our understanding of horseshoe crab ecology.

The nutritional significance of the horseshoe crab eggs for migratory shorebirds in Delaware Bay has been extensively documented (Botton et al 2003; Mizrahi and Peters 2009), but it is not clear whether horseshoe crab eggs are more than incidental food for birds in other coastal areas. Although shorebirds consume vast number of eggs during their stopover, I suggest that because shorebirds feed mainly on eggs at the sediment surface, they are eating eggs that would probably desiccate and die if they were not eaten. I do not believe that we understand enough about the numerical relationships between egg abundance, juvenile survival, and adult recruitment to infer that bird predation in Delaware Bay is presently limiting the size of the horseshoe crab population, but I would consider this very unlikely.

Loggerhead turtles (*C. caretta*) may be the only predator with a substantial consumption of adult horseshoe crabs, and it is notable that temporal shifts in their food preference from *Limulus* to blue crabs and fishes coincided with a decreased population of horseshoe crabs in Virginia (Seney and Musick 2007). We do not yet know whether significant consumption of horseshoe crabs by sea turtles occurs outside of the lower Chesapeake Bay. Because sea turtles are endangered, a better understanding of their interactions with horseshoe crabs is needed. Apart from some limited information about predation by fish (Table 2) and hermit crabs (Fig. 3 and Table 3), there is virtually no data on predation rates on trilobites and second instars. Even less is known about predation and other ecological factors affecting the survival of older juveniles. These are clearly vital gaps in our understanding of horseshoe crab ecology, with potentially important implications for understanding recruitment dynamics (Grady and Valiela 2006).

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Relationships Between Sandpipers and Horseshoe Crab in Delaware Bay: A Synthesis

David S. Mizrahi and Kimberly A. Peters

Abstract Recent and dramatic declines have been documented in several shorebird populations that stage in Delaware Bay during spring migration. As a result, considerable attention has been given to issues such as the reliance of sandpipers on Delaware Bay American horseshoe crab (*Limulus polyphemus*) eggs for refueling and how to best manage the horseshoe crab fishery to insure adequate resources for migratory shorebirds. In this chapter, we synthesize over 25 years of shorebird research and monitoring data in Delaware Bay to support the premise that horseshoe crab eggs are an essential element for migrating sandpipers during northbound passage through the bay. We then discuss long- and short-term trends in American horseshoe crab populations resulting from changes in demand and harvest regulations, and how this has affected shorebird population viability. Regulatory actions have led to recent increases in some demographic elements of the Delaware Bay crab population, but such changes have not yet translated into increased crab egg availability or population recovery indices in shorebirds. Because reduced availability of horseshoe crab eggs has severe consequences for migratory sandpipers at the individual and population levels, current conservation strategies that include harvest reductions on American horseshoe crabs in the Delaware Bay region must persist into the foreseeable future to insure the recovery of horseshoe crab populations and the long-term health of migratory sandpipers in Delaware Bay.

1 Introduction

Migrating animals have evolved complex and highly integrated behaviors and physiologies to overcome the considerable challenges they face during migration (Gauthreaux 1982; Berthold 1996; Dingle 1996). Birds are well known for the annual migrations, some of which are astounding marathons that carry

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individuals between the extreme ends of the earth (Baker 1978). Regardless of their journey's length or destination, individuals must stop along the migration route to rest and replenish energy stores (Moore et al. 1993).

The availability of suitable "stopover" habitats along the migration route that can provide the food resources necessary for birds to accumulate energy quickly and safely is essential to their ability to complete migration successfully (Moore et al. 1995). Destruction or degradation of a stopover habitat may compromise a bird's ability to reach its goal and for individuals migrating to the breeding grounds, this could affect nesting success and long-term population viability (Terborgh 1992; Skagen 2006). Rapid, human-induced alterations in global landscapes are having considerable consequences for migrating birds, especially those that travel long distances, because they likely encounter multiple perturbations en route to their destinations (Wilcove 2008).

Recent declines in American horseshoe crabs (*Limulus polyphemus*) have been acknowledged (Davis et al. 2006), resulting from dramatic increases in their harvest, especially in Delaware Bay. This has raised concern about the current status of horseshoe crabs, primarily because northward migrating sandpipers time their passage through Delaware Bay to coincide with major horseshoe crab spawning periods (Myers 1986). Upon arriving in the bay, sandpipers consume large quantities of crab eggs to replenish spent energy stores and prepare for their final flight to sub-Arctic- and Arctic-breeding grounds (Morrison 1984). Recent and alarming declines in some sandpiper populations that stage in the Delaware Bay during spring migration also have been documented (Morrison et al. 2001, 2006), suggesting an intrinsic and critical link between sandpiper population viability and horseshoe crab egg availability (Baker et al. 2004; Morrison et al. 2004; Niles et al. 2007).

Considerable contention has arisen regarding the extent of declines in horseshoe crab populations, the reliance of sandpipers on Delaware Bay horseshoe crab eggs, and how to best manage the horseshoe crab fishery to insure its long-term viability. In this chapter, we synthesize over 25 years of sandpiper research and monitoring data in Delaware Bay. We present results from these efforts in the context of migratory bird life history, ecology, behavior, and physiology to develop and support the premise that horseshoe crab eggs are an essential element for migrating sandpipers during passage through Delaware Bay. We then discuss recent declines in horseshoe crab egg availability in Delaware Bay and the negative consequences of these declines for migratory sandpipers at the individual and population levels.

2 Sandpiper Migration Systems

Sandpipers and their allies (Family Scolopacidae) are among the most migratory in the animal kingdom, often engaging in spectacular journeys between wintering grounds in the Southern Hemisphere and high latitude breeding areas in the Northern Hemisphere (Morrison 1984; Myers et al. 1987; Piersma and Baker 2000). Migration strategies of several species are typified by a series of non-stop, long-distance flights, each of which can be thousands of kilometers in length and take several days to complete (Morrison 1984; Wilson and Barter 1998; Battley et al. 2005; Piersma et al. 2005).

Interspersed between migration bouts are extended stopovers (Myers 1983; Cramp and Simmons 1983), which may constitute as much as 90% of the time individuals spend on the migration route (Hedenström and Alerstam 1997). During these intervening periods, individuals must accumulate energy stores, primarily as adipose tissue (Zwarts et al. 1990; Piersma and Jukema 1990; Gudmundsson et al. 1991; Hedenström and Alerstam 1998), to fuel subsequent legs of the migration, in many cases increasing their body mass 50–100% (Piersma and Jukema 1990; Piersma and Gill 1998; Battley et al. 2001; Baker et al. 2004). Rapid accumulation of energy stores is accomplished in several ways: eating more (i.e., hyperphagia), selecting diets with high lipid content, and increasing the assimilation efficiency of ingested food (Blem 1990; Stiles 1993; Bairlein and Gwinner 1994; Biebach 1996; Bairlein 1999). All of these mechanisms have been reported for migrating sandpipers (Zwarts 1990; Zwarts et al. 1990; Gudmundsson et al. 1991; Tsipoura and Burger 1999; Kvist and Lindström 2003; van Gils 2004; Battley et al. 2005).

Although adipose fat is the primary fuel for migratory flight (Ramenofsky 1990; Biebach 1996; McWilliams et al. 2004), long-distance migrants also deposit protein to build up flight muscle, heart and lung tissue that supports endurance flight (Piersma 1990; Piersma et al. 1999). Furthermore, many long-distance migrants are known to reduce the size of organs involved in metabolism (e.g., stomach, intestine, liver) just before initiating migration bouts (Jehl 1997; Piersma 1998; Piersma and Gill 1998; Biebach 1998; Piersma et al. 1999; Battley et al 2000). These must be rebuilt immediately upon arrival at a stopover or breeding site to accommodate the extensive feeding necessary to meet energy accumulation schedules (Piersma and Lindström 1997; Piersma et al. 1999).

Gudmundsson et al. (1991) suggest that Arctic-breeding sandpipers that complete migration in just a few long-distance flights punctuated by en route stopovers should adhere to a strategy that minimizes the time spent migrating (i.e., active flight and stopover periods). Timely arrival on the breeding grounds is paramount for Arctic-breeding sandpipers because of the short summer season typical of high latitudes (Drent et al. 2003). Optimal migration theory (Alerstam and Lindström 1990; Alerstam and Hedenström 1998) posits that "time-minimizing" species should select stopover sites that maximize energy intake and depart from sites when intake rates fall below what they will likely encounter at subsequent stopover locations. Frequently, time-minimizers will accumulate energy stores in excess of what is needed to reach the next stopover location (i.e., overloading) at high-quality stopover sites (i.e., those with high fuel deposition rates), allowing them to bypass low-quality sites (Gudmundsson et al. 1991; Lindström and Alerstam 1992). Overloading also may serve as insurance against sub-optimal fuel deposition conditions encountered en route. This excess accumulation can be especially important for sandpipers upon arrival on their Arctic-breeding grounds, when arthropod prey availability is low (Davidson and Evans 1989) and adverse weather conditions can cause high levels of mortality (Boyd 1992). Surplus energy stores are also used to reconstitute the metabolic and reproductive machinery reduced to facilitate migration (Farmer and Wiens 1999; Morrison and Hobson 2004; Morrison et al. 2005) and in some cases are mobilized for egg production in females (Morrison and Hobson 2004).

3 Delaware Bay: A Globally Important Sandpiper Stopover Site

The entirety of constraints faced by Arctic-breeding sandpipers during northward migrations and the intervening optimizing strategies shaping their behavior suggest that stopover site selection is a key component for successful breeding. Importantly, a disproportionately large number of sandpipers destined for Arctic-breeding grounds use a relatively small number of stopover sites (Myers 1983). These sites typically support hundreds of thousands to millions of individuals that represent hemispherically and globally significant portions of species populations (Myers et al. 1987). In North America, the most noteworthy are Gray's Harbor and the Copper River Delta on the Pacific coast, Cheyenne Bottoms along the mid-continental flyway, and Delaware Bay on the mid-Atlantic coast (Senner and Howe 1984; Myers et al. 1987).

In the Western Atlantic, Delaware Bay is the last major stopover for several sandpiper species before flights to the breeding grounds (Myers et al. 1987), and alternative sites nearer the breeding grounds are unknown (Baker et al. 2004). Past estimates suggest that historically between 400,000 and 1,000,000 individuals passed through the bay annually during spring migration (Wander and Dunne 1981; Clark et al. 1993), although current estimates are lower (Clark personal communication). Significant proportions of the world's Semipalmated Sandpipers (*Calidris pusilla*), Western Hemisphere's Red Knots (*C. canutus rufa*), North America's Ruddy Turnstones (*Arenaria interpres*), and the Atlantic coast's Sanderlings (*C. alba*) pass through Delaware Bay during spring migration (Myers 1983; Morrison 1984).

Consequently, Delaware Bay has been designated as a hemispherically important site by the Western Hemisphere Shorebird Reserve Network, a "Wetland of International Importance" by the Ramsar Convention, and a globally significant Important Bird Area by BirdLife International. Three of the four primary species (i.e., Red Knot, Ruddy Turnstone, Sanderling) are listed as species of high conservation concern in the U.S. Shorebird Conservation Plan, while Semipalmated Sandpiper is listed as a moderate conservation concern species (Brown et al. 2001). Additionally, Red Knot is a candidate species for endangered species listing by the U.S. Fish and Wildlife Service.

4 The Importance of Horseshoe Crab Eggs to Migrating Sandpipers

Sandpipers arrive in Delaware Bay in late April through mid-May, many after completing non-stop transoceanic flights from northeastern South America (Morrison 1984), and depart from mid-May through the first week of June (Clark et al. 1993). Generally, they arrive in near-lean mass condition and deposit fuel stores over a 10–21 day stopover period (Atkinson et al. 2007; Mizrahi unpublished data).

The main attraction for sandpipers passing through Delaware Bay during spring migration appears to be the abundant and predictable eggs produced by the largest spawning population of American horseshoe crab (Shuster and Botton 1985; Myers 1986; Castro and Myers 1993; Tsipoura and Burger 1999; Gonzalez et al. 2001). Here we present several lines of ecological, physiological, and behavioral evidence that suggest an intrinsic link between migrant sandpipers and crab eggs.

4.1 Spatiotemporal Relationships

Perhaps the most obvious link is the uncanny arrival of sandpipers during the peak spawning period of horseshoe crabs (Shuster and Botton 1985; Myers 1986; Botton et al. 1988; Botton et al. 2003). Given appropriate water temperatures and tidal phase, Delaware Bay horseshoe crabs begin spawning on sandy beaches and creek mouths in late April through early May, just when the earliest arriving sandpipers are making landfall in Delaware Bay (Botton et al. 2003). As crab spawning activity increases through May, there is a coincidental increase in sandpiper numbers. In some years, spawning activity peaks as sandpipers are completing their stopover.

A second line of evidence is related to the spatial relationships between horseshoe crab eggs and sandpiper feeding areas. Egg availability within the Bay is not uniform in space or time, either within or among seasons (Botton et al. 1994; Smith et al. 2002a, 2002b), and this appears to have a direct affect on the distribution and abundance of sandpipers. Botton et al. (1994) reported that horseshoe crab eggs were the most abundant food item found on Delaware Bay beaches and that sandpiper numbers increased coincidently with increasing egg density. Sandpiper aggregations were most apparent at beach sites with distinct shoreline discontinuities (e.g., creek mouths, jetties) that tend to trap and concentrate free-floating eggs.

Karpanty et al. (2006) also found that the number of horseshoe crab eggs was the most important determinant of beach use by Red Knots. They found that the relative proportion of crab egg biomass (i.e., as a proportion of total prey biomass) was similar at sites used by Red Knots and randomly selected sites (i.e., 91 and 94%, respectively). However, overall egg biomass was more than three times greater at sites used by Red Knot compared with random sites. Red Knot habitat use appeared to shift from emergent marsh and peat-beaches to sandy beaches, especially around the high tide period. Greater numbers of loose eggs are available in beach swash zones during rising versus falling tides (Nordstrom et al. 2006) and birds appear to respond with greater foraging activity during this period (Burger et al. 1997).

4.2 Ecophysiological Relationships

Although a variety of prey may be available in Delaware Bay (e.g., bivalves, small crustaceans, polychaete worms, insect larvae), sandpipers exhibit a strong preference for horseshoe crab eggs (Myers 1986; Castro and Myers 1993). Free-ranging sandpipers depleted 80% of horseshoe crab eggs presented in feeding trials and, regardless of egg density, did not exhibit a give-up threshold (Gillings et al. 2007). In a diet selection study, Tsipoura and Burger (1999) found that horseshoe crab egg membranes constituted the majority of gut contents in all species they sampled. Additionally, depletion of undesiccated eggs in the wrack and swash zones is nearly complete during daily tidal fluctuations (Fraser personal communication).

These results are not surprising. When horseshoe crab eggs are present in surface sediments, search and ingestion time was near-instantaneous (Gillings et al. 2007). Their nutritional constituents are easy to digest and assimilation rates can be as high as 70% (Castro et al. 1989; USFWS 2003), resulting in the highest energy accumulation rates in Red Knots worldwide (Piersma et al. 2005) and among the highest recorded in vertebrates (i.e., 5–7 times greater than basal metabolic rate, Atkinson et al. 2007). Plasma triglyceride and phospholipid levels measured in Semipalmated Sandpipers during stopovers in Delaware Bay are greater than at any stopover along the Atlantic coast or on Caribbean wintering grounds prior to spring migration initiation (Lyons et al. 2008) and among the highest observed in migrating sandpipers (Mizrahi et al., in preparation). Circulating levels of these metabolites can be used to infer fattening rates (Jenni-Eiermann and Jenni 1994, 2003) as they are involved in lipogenesis and adipose tissue deposition (Ramenofsky 1990).

This is principally because horseshoe crab eggs are rich in lipids comprised of 16- (stearic acid) and 18-carbon (palmitic acid) fatty acids and an 18:1 unsaturated fatty acid (oleic acid) (Haramis unpublished data). Fatty acids are the primary energy substrates for long-distance migratory flight (Jenni and Jenni-Eiermann 1998; Jenni-Eiermann and Jenni 2003; McWilliams et al. 2004) for several reasons. Fatty acids are twice as energy dense, per unit dry matter, as carbohydrates and protein. Fatty acids also are relatively anhydrous, thus can be stored with a minimum of additional water and weight. Weight can be an important constraint for migrants (McWilliams et al. 2004).

Haramis (unpublished data) found that fatty acid composition of adipose tissue from heavy Red Knots collected in Delaware Bay in late May were closely matched in type and concentration with those contained in horseshoe crab eggs, suggesting direct deposition of crab egg constituents to fat depots (McWilliams et al. 2004; Pierce and McWilliams 2005). Red Knot adipose tissue also contained two "essential" fatty acids (i.e., not synthesized by the organism but obtained through food intake), eicosapentaenoic acid and docosahexaenoic acid, both of which are constituents found in horseshoe crab eggs.

Using stable isotope methods, Haramis et al. (2007) provide several lines of evidence that support the thesis of a strong dietary link between migrating sandpipers and horseshoe crab eggs. They found that as body mass in free-ranging Red Knots and Ruddy Turnstones staging in Delaware Bay increased, their plasma $\delta^{15}N$ values increased asymptotically. This same pattern was exhibited by Red Knots and Ruddy Turnstones fed ad libitum horseshoe crab eggs during 32-day pen trials. Mean $\delta^{15}N$ asymptotic values in both free-ranging and captive birds were not significantly different, and as mass increased in both groups, these values converged on the mean $\delta^{15}N$ value found in horseshoe crab eggs (see Figure 2, p. 370, Haramis et al. 2007). In contrast, mean $\delta^{15}N$ values of local blue mussels (*Mytilus edulis*) and coquina clams (*Donax variabilis*), known bivalve prey of migrating sandpipers, were well below values for horseshoe crab eggs and plasma levels in free-ranging and captive sandpipers. Finally, Haramis et al. (2007) found that test subjects in pen trial exhibited mass gain rates consistent with those found in free-ranging knots and turnstones. They posit that low $\delta^{13}C$ fractionation values observed during feeding trials suggest that dietary lipids are rapidly assimilated and likely converted to adipose tissue with little biochemical alteration (Jenni and Jenni-Eiermann 1998; Jenni-Eiermann et al. 2002; McWilliams et al. 2004).

These relationships were evident in a similar study conducted with Semipalmated Sandpipers (Mizrahi et al., in preparation). That is (1) plasma $\delta^{15}N$ increased asymptotically in free-ranging individuals and captive birds fed exclusively on horseshoe crab eggs, (2) plasma $\delta^{15}N$ asymptotic values of each group were not significantly different, and (3) both groups gained mass rapidly and at similar rates, converging on the $\delta^{15}N$ values of horseshoe crab eggs as mass increased.

4.3 Alternative Food Resources?

Many sandpiper species respond to diminishing availability of preferred prey by switching to alternative prey (Zwarts and Drent 1981; Beukema 1993). In these cases, however, physiological condition can be severely compromised in the absence of suitable alternative prey (Goss-Custard et al. 2003). Botton et al. (1994) found relatively few macro invertebrates (i.e., > 1 mm, mostly small oligochaetes, nematodes, and insect larvae) on the Delaware Bay beaches they sampled and densities rarely exceeded 200 m^{-2}. Tsipoura and Burger (1999) found that some sandpiper species consumed various worm and insect prey, however, nutritional analyses and comparisons of profitability for migrating sandpipers have yet to be fully investigated.

Bivalves are generally favored by Red Knots in other parts of the world (Piersma et al. 1993), and certain species, such as *Gemma gemma*, can occur in high densities in Delaware Bay (e.g., Botton 1984). However, this and several other bivalves (e.g., *Mercenaria* spp., *Anomia* spp.) are not typically consumed by shorebirds (Skagen and Oman 1996) either because they are too large or their shells are too hard (Botton 1984). The availability of suitable bivalve prey (e.g., small *Mytilus* spp., small *Mya* spp.) is temporally and spatially patchy in Delaware Bay and nearby coastal areas (Sitters et al. 2005; Haramis et al. 2007), and densities do not appear high enough to allow a large numbers of staging sandpipers to meet their fuel deposition needs (Escudero and Niles 2001; Haramis et al. 2007). Even if suitable bivalve fauna were available at high densities in Delaware Bay or coastal sites in close proximity, the ability of Red Knots to double their mass in 3 weeks, as they do eating horseshoe crabs eggs (Baker et al. 2004), is unlikely. Red Knots feeding on blue mussels and gastropods (*Littorina* spp.) during 3-week spring stopovers in Iceland had half the fuel deposition rates as Delaware Bay knots (Alerstam et al. 1992).

Bivalves and other hard-shelled prey are less profitable than horseshoe crab eggs to consume for a variety of reasons related mostly to their shells. Shells must be crushed in the gizzard and are bulky, resulting in digestive constraints to intake rates and low digestive efficiency compared to soft-bodied prey (van Gils et al. 2003, 2005). Thus, time-minimizing migrants should select prey that minimizes digestive constraints (e.g., horseshoe crab eggs; van Gils et al. 2005). Furthermore, the gizzard, along with other digestive organs, undergoes dramatic reductions in size prior to long-distance migration bouts (Piersma et al. 1999). A predominantly bivalve diet would require Delaware Bay Red Knots to fully restore gizzard function to facilitate the processing of shells, which may be too costly for individuals constrained by the need to reach the Arctic quickly (van Gils 2005). van Gils et al. (2006) documented a negative relationship between gizzard size and mortality rates in Red Knots (*C. canutus islandica*) feeding on cockles in the Dutch Wadden Sea, implying that individuals with small gizzards were less able to obtain the energy needed from their food. Finally, the high protein-to-calorie ratio of bivalves can reduce the potential for fattening (Bairlein 1998; McWilliams et al. 2004).

5 Recent Changes in Egg Availability

The quantity of horseshoe crab eggs available to sandpipers migrating through Delaware Bay has been reduced in recent decades, likely due to the increased harvest of adult horseshoe crabs. During the 1990s, demand for horseshoe crabs for use as bait increased to accommodate the American eel (*Anguilla rostrata*) and rapidly expanding whelk (family Melongenidae) pot fisheries (ASMFC 1998 and addenda [2004a; 2006]). The eel fishery targets female crabs, whereas the whelk fishery uses both male and female crabs (ASMFC 2004b). The maximum Delaware Bay area (DE, NJ, MD, PA, and VA) horseshoe crab harvest in modern record occurred in the mid-1990s and was estimated at just under 2 million crabs landed annually (ASMFC 2004b), although historical records indicate that larger harvests may have been carried out in the late 1800s (e.g., 4 million in 1880, Kreamer and Michels, 2009). The current Delaware Bay estimate of horseshoe crab abundance reported by the Horseshoe Crab Technical Committee is 13.3 million crabs (ASMFC 2006).

Trawl surveys, landing reports, and egg density surveys indicate that horseshoe crab populations in Delaware Bay may have been affected by the rapid changes in harvesting activity that took place between 1990 and 2000 (Botton et al. 1994; USFWS 2003; Davis et al. 2006; Niles et al. 2007). For instance, surplus production models of fishery-dependent and -independent survey data from several different sources indicate that the relative biomass (B/B_{MSY}) of horseshoe crabs in the bay steadily declined from the mid-1990s to 2003 at an annual rate of approximately 7% (Davis et al. 2006). The models were recently updated and unpublished results indicate that biomass has been increasing since 2003 and may be approaching levels similar to the mid-1990s (D. Smith, personal communication). However, such trend analyses are tenuous at best, as the majority of abundance estimates derived from long-term sources are highly variable and occasionally conflict, (Davis et al. 2006; Smith et al. 2006) and statistically robust surveys that focus exclusively on horseshoe crabs have only recently been implemented. Furthermore, there is some concern about the use of the surplus production modeling technique to determine these trends because of assumptions inherent in the models (Brust et al. 2006). Current data from the most robust crab surveys (e.g., Delaware Bay spawning survey, Michels et al. 2008; offshore benthic trawl survey, Hata 2008; baywide tagging study, D. Smith, personal communication) do, however, indicate that male and juvenile crabs have been increasing at an appreciable rate in recent years. Although these same surveys suggest that female crab numbers have stabilized, return to former population levels is not yet apparent.

Other indices of horseshoe crab abundance (e.g., egg density, landings) also support the hypothesis that crab populations have been affected by changes in harvesting over the last two decades. For instance, horseshoe crab egg densities on New Jersey's Delaware Bay beaches fell from average counts of 40,000 eggs m^{-2} in the early 1990s (Niles et al. 2007) to approximately between 3,000 and 4,000 eggs m^{-2} in 2005–2007 (Kalasz et al. 2008), and data from 2007 reflect the lowest egg densities recorded in New Jersey in 8 years (Kalasz et al. 2008). Although average egg densities bay-wide and on Delaware beaches appear to be stable or increasing over the last 3 years, estimates have been driven primarily by increases in Mispillion Harbor, where the highest egg densities in the bay are recorded (Kalasz et al. 2008). Caution should be exercised when comparing egg densities between New Jersey and Delaware due to methodological differences in egg sampling employed in the two states (Kalasz et al. 2008). It is also difficult to assess temporal trends in egg densities due to the extreme variability in estimates among years, both for individual beaches and for the bay as a whole (Smith et al. 2002a, b, Pooler et al. 2003, Kalasz et al. 2008). The New Jersey Division of Fish and Wildlife (DFW) and Delaware DFW initiated a study in 2008 that aims to determine the comparability of egg density data across the two states (Kalasz et al. 2008), and should lead to clearer understanding of general trends in the bay.

Landing surveys indicate that the mean number of crabs per catch and mean catch per unit effort have also declined since the 1990s (USFWS 2003). However, these harvest indices have in part been driven by regulatory actions (see below) and have stabilized at low levels in recent years. Unfortunately, few data are available to assess the status of the Delaware Bay horseshoe crab population prior to 1990.

The density of spawning horseshoe crab females is also known to increase the process of bioturbation, through which buried eggs are released to the beach surface through intraspecific interference by competing females (Jackson et al. 2002; Smith 2007). Smith (2007) used a spatially explicit model to predict the percentage of horseshoe crab nests disturbed as a function of adult densities. The model predicted that the percentage of nests disturbed would increase linearly up to twice the 2004 spawning crab densities and asymptote at approximately 70% of nests. According to the model, the proportion of nests disturbed during peak spawning (i.e., second spring tide) in 2004 was approximately 45%, and at one-third this spawning level would be approximately 20%. It was also assessed that a minimum of roughly 5–9% of these disturbed eggs would be exhumed to the surface (i.e., 0–5 cm) through various processes (Smith 2007) and thus be available for consumption by most shorebirds. It is currently unclear what threshold densities of nest densities and nest disturbance levels are necessary to support target shorebird populations. However, it is known that this redistribution of eggs is crucial for sandpipers, as they are highly dependent upon eggs available near or at the surface (Tsipoura and Burger 1999). Furthermore, bioturbation by horseshoe crabs may also have indirect effects that can affect shorebirds. Through feeding and substrate agitation, crabs can disturb the benthic community, oxygenate substrates, and resuspend previously unavailable nutrients (Odell et al. 2005). Loss of these secondary processes could affect the availability of alternative resources for migrating sandpipers.

Concerns about the accelerated harvest and potential overexploitation of horseshoe crabs in Delaware Bay, chiefly due to their possible effects on migrating sandpipers, led to several regulatory actions at the regional and state levels to protect stock levels. The Atlantic States Marine Fisheries Commission (ASMFC) adopted a Fishery Management Plan for horseshoe crab in 1998 (ASMFC 1998), followed by addenda that established quota systems in 2000, 2001, 2004, and 2006. Legislative restrictions led to a 76% coast-wide reduction in bait landings between 1998 and 2005 (ASMFC 2006), and some surveys indicated that there was a 63% drop in landings in New Jersey and Delaware between 2003 and 2004 alone (Smith et al. 2009). In 2006, the state of New Jersey enacted a two-year moratorium on harvesting horseshoe crabs. Recently, the state passed legislation to further restrict the harvest, landing or possession of horseshoe crabs until the *rufa* Red Knot population reaches target recovery goals.

To date, changes in harvest regulations imposed since 1998 do not appear to have translated into increased bay-wide egg densities (Niles et al. 2007; Kalasz et al. 2008), although recent data from Delaware beaches are encouraging and suggest some site-specific increases. It is less clear how regulatory actions have affected the adult horseshoe crab population. Statistically robust surveys that focus exclusively on horseshoe crabs have only recently been initiated, including a 30-ft offshore trawl survey that started in 2001 (Hata and Berkson 2003). According to spawning surveys, which were redesigned in 1999 to provide better data for long-term trend analysis (Smith and Michels 2006), horseshoe crab activity on New Jersey and Delaware beaches has stabilized since 1999 (Smith et al. 2002b). However, most other available population estimates based on longer-term data sets show a continuous decline between 1995 and at least 2003 (Davis et al. 2006). Overall, 2003 biomass estimates from several sources suggested that levels remain less than 56% of those recorded in 1995 (Davis et al. 2006). This lack of population response may indicate a lag effect due to horseshoe crab age-to-recruitment, which is approximately 9–11 years (Shuster and Sekiguchi 2003).

6 Consequences for Sandpipers

6.1 Physiological Condition

The reduction in Delaware Bay horseshoe crab biomass has raised concern over future egg availability for migrating sandpipers as well as crab stock availability. Using a surplus modeling approach, Davis et al. (2006) predicted that under 2003 harvest levels (1356 tons), population recovery to B_{MSY} (spawning biomass that would produce maximum sustainable yield) would take at least 4 years, and four of seven models examined predicted that recovery would not reach B_{MSY} within 15 years. For sandpipers, such reductions in horseshoe crab biomass could negatively affect the quantity and availability of eggs needed for refueling.

Based on the equations of Kersten and Piersma (1987), and taking into account daily energy expenditure (Castro and Myers 1988) and modified assimilation rates based on Castro et al. (1989), the Delaware Bay Shorebird Technical Committee (USFWS 2003) estimated that a minimum of approximately 300 metric tons of eggs would be necessary to sustain current populations of six species of migratory sandpipers using the bay during spring migration (Ruddy Turnstone, Red Knot, Sanderling, Semipalmated Sandpiper, Dunlin [*C. alpina*], Short-billed Dowitcher [*Limnodromus griseus*]). Using this estimate, they calculated that 30,000–70,000 eggs m^{-2} would be needed to sustain all sandpipers passing through the bay (using an estimate of 423,000 individuals of the six primary species using the bay). This is likely a conservative estimate of densities needed to accommodate migratory sandpipers, as it does not take into account all species using the egg resource (e.g., Least Sandpiper, *C. minutilla*; gulls, family Laridae) and uses a low-end estimate of total sandpipers using the bay (i.e., estimates range from 400,000 to 1,000,000 individuals; Myers et al. 1987; Clark et al. 1993). The egg estimate also does not take into account the effects of intra- and interspecific interference competition on intake rates and resource accessibility (Stillman et al. 2002), or potential age differences in foraging efficiency (Caldow et al. 1999), which would likely be strongest when competitor densities are high and prey scarce (Caldow et al. 1999; Triplet et al. 1999). In Delaware Bay, competition between gulls and sandpipers for foraging space has been demonstrated in areas where horseshoe crab eggs accumulate (Burger et al. 2007). Finally, the estimate does not explicitly take into account the effects of horseshoe crab density on the vertical distribution, and thus availability of crab eggs, to sandpipers.

According to functional response models, Red Knot peck success is twice as high for surface eggs than for buried eggs, so that probing for buried eggs is not profitable unless they are present in extremely high densities (Gillings et al. 2007). Stillman et al. (2003) demonstrated that the number of eggs within the top 5 cm of sediment consumed per second ranged from near zero at densities of 300 eggs m^{-2} to two eggs per second at 25,000 eggs m^{-2}. Similar models predict that surface eggs can sustain current Red Knot populations at densities of 360 m^{-2}, as compared to 19,000 m^{-2} densities of buried eggs (Gillings et al. 2007). Haramis et al. (2007) further concluded that it would take an estimated 16 billion buried crab eggs to provide adequate resources for 40,000 Red Knots alone refueling in Delaware Bay. None of the 16 beaches surveyed in New Jersey and Delaware in 1999 during peak shorebird migration stopovers (23–24 May) contained densities of buried eggs in 0–5 cm cores above 30,000 eggs m^{-2} (i.e., densities deemed necessary to support all sandpipers using the bay), whereas 11 of them contained that density in deeper cores (0–20 cm, Pooler et al. 2003; USFWS 2003). According to recent comparable egg surveys conducted during May 21–28, only 4 of 11 beaches surveyed in Delaware and 1 of 12 beaches in New Jersey averaged this threshold egg density in the 0–5 cm stratum between 2005 and 2007 (Kalasz et al. 2008). In fact, only one new New Jersey beach surveyed in 2005–2007 ever exceeded densities of 30,000 eggs m^{-2} during the entire shorebird migratory period (i.e., May 1–June 6) in any year (Kalasz et al. 2008). While 64% of the Delaware beaches surveyed had egg densities above the threshold at least once during the migratory period, this usually occurred during the final week of the stopover period, when many individuals had already departed for the breeding grounds (NJDEP unpublished data).

Documented changes in sandpiper foraging ecology within Delaware Bay (Baker et al. 2004; Atkinson et al. 2007) and widespread declines in sandpipers along the Atlantic flyway (Morrison et al. 2006; Bart et al. 2007) indicate that current resource levels in the bay may be below the threshold needed to sustain populations that depend on Delaware Bay as a critical migratory stopover site. According to empirical models that account for body mass and morphometry (Kvist et al. 2001), Red Knots passing through Delaware Bay must reach target weights between 180 and 220 g to reach breeding grounds with sufficient energy stores (Piersma [2000] Energetics of body mass changes in red knots staging in Delaware Bay in May 1998. Unpublished report, Nether Inst Sea Res, Trexel, The Netherlands; Baker et al. 2004; Morrison et al. 2005). Red Knots must reach this target before a certain "departure" date that will allow them to complete their flight to the breeding sites at the appropriate time, as evidenced by mass gain rates that are two to three times higher toward the end of their migration stopover period than at the beginning (Atkinson et al. 2007). This pattern is especially pronounced in birds that arrive later in the season and in those at lower weights (Atkinson et al. 2007). However, the proportion of Red Knots reaching the target departure mass decreased significantly between 1997 and 2003 (Robinson et al. 2003; Baker et al. 2004; Niles et al. 2007). In 2003 and 2005, birds also did not increase mass gain rates toward the end of the stopover period, indicating that they were unable to attain rates of ingestion or assimilation to reach target weights within the limited departure window (Atkinson et al. 2007). This was likely a result of the lower egg densities available to sandpipers in recent years (Botton et al. 1994; Niles et al. 2007).

Similar patterns in weight gain have been observed in other sandpiper species using Delaware Bay during spring migration. For example, the proportion of Ruddy Turnstones captured in Delaware Bay that were at threshold departure mass (150 g) declined significantly between 1997 and 2002, although daily rates of mass gain during this period only declined slightly (Niles et al. 2001; USFWS 2003). Analysis of individually marked turnstones captured between 1997 and 2000 similarly revealed significant differences in mass gain among years, although there was no consistent negative trend in gain rates (Robinson et al. 2003). Mizrahi (1999) observed that based on energetic consumption models (Castro and Myers 1988) some Semipalmated Sandpipers captured in Delaware Bay from 1996 to 1998 likely did not have sufficient fat reserves to make it to their breeding grounds in Canada and that many of those that did reach the breeding grounds would have done so with few remaining energy stores. More recent data (2000–2007) show that Semipalmated Sandpipers captured in the bay were of significantly lower mass and demonstrated lower mass gain rates than were observed in the mid-1990s (Mizrahi, unpublished data). Based on these data, Semipalmated Sandpipers appear to be departing Delaware Bay at lower weights than in previous years (Mizrahi, unpublished data).

6.2 Population Responses

The changes in fattening rates observed in Delaware Bay during spring migration appear coincidental with population changes in several species of North American sandpipers (Morrison et al. 2001, 2006; Bart et al. 2007). For instance, the *rufa* subspecies of Red Knot, one of the smallest sub-specific populations of Red Knot known worldwide (Piersma and Davidson 1992), has declined from an estimation of 170,000 individuals in the late 1980s to approximately 20,000 currently (Gonzalez et al. 2001; Morrison et al. 2004). This is noteworthy because most *rufa* Red Knots stage in Delaware Bay during spring migration and evidence suggests that numbers in the bay are also declining precipitously. It was estimated that approximately 100,000 Red Knots passed through the bay annually during spring migration in the 1980s, with peak counts over 95,000 (Clark et al. 1993). In contrast, recent estimates are under 25,000 with peak counts under 15,000 (Niles et al. 2007, Morrison et al. 2007). Decreases in the *rufa* subspecies has also been especially pronounced in the Patagonia and Tierra del Fuego wintering areas (Morrison et al. 2004). A large proportion of Red Knots that migrate through Delaware Bay are known to winter in this region (Atkinson et al. 2007).

The Semipalmated Sandpiper population that breeds in eastern Canada and passes through Delaware Bay in spring (Morrison 1984) has decreased by approximately 43% since 1982 (Morrison et al. 2006), with significant changes in numbers observed along the Atlantic coast (Bart et al. 2007). A decline of 68% between 1982 and 2004 was estimated for the Bay of Fundy, through which approximately 75% of the global population passes during fall migration (Morrison et al. 2006). Declines have also been documented in eastern populations of several other sandpiper species that migrate through Delaware Bay in the spring and likely depend on horseshoe crab eggs, including Least Sandpiper (Morrison et al. 2006; Bart et al. 2007), Sanderling (Howe et al. 1989), Dunlin (Morrison et al. 2006), and Short-billed Dowitcher (Bart et al. 2007).

The dramatic declines in sandpiper numbers in recent years have caused alarm about the future sustainability of some populations. Some survival models indicate that the *rufa* subspecies of Red Knot that passes through Delaware Bay could be extinct by 2010 (Baker et al. 2004), based on the mean survival estimate from 1998 to 2001 (i.e., 53.9%). Annual survival rates from this period were highly variable (i.e., 45–63%, 95% CI), but were still well below those reported for *islandica* Red Knot, which ranged from 76% when the population was declining to 80% when the population was stable (Boyd and Piersma 2001). Modeling efforts by Hitchcock and Gratto-Trevor (1997) determined that among five sandpiper population parameters they considered (i.e., fecundity, adult mortality, juvenile survival, delayed recruitment, and immigration), adult mortality would have the strongest effect on population change. Adult sandpiper survival rates appear closely associated with body stores acquired during spring migration (Morrison et al. 2006). Baker et al.

(2004) reported that Red Knots departing Delaware Bay at low weights are less likely to be seen later in the flyway, implying lower survival. Data collected at Plymouth Beach, Massachusetts, during fall migration also indicated that fat levels in individual Semipalmated Sandpipers were positively associated with annual return rates (Pfister et al. 1998). Fecundity rates are also likely affected by the conditions encountered during stopover, as reproduction is predicted to decrease logarithmically as energy stores at departure decrease (Piersma and Baker 2000).

Although conditions at the breeding and wintering grounds also may impact sandpiper population health, several lines of evidence indicate that the loss of horseshoe crabs in Delaware Bay may be driving the negative trends observed in recent years for some species. Populations of some species that winter in Tierra del Fuego, but do not migrate through Delaware Bay (e.g., Hudsonian Godwit, *Limosa haemastica*), are stable or increasing (Morrison et al. 2004; Jehl 2007). Recent data also indicate that declines in the number of Semipalmated Sandpipers breeding in eastern Canada (e.g., Churchill, Manitoba) are attributable to mortality outside of the breeding season, as breeding success and habitat suitability have not appeared to change within the region (Jehl 2007). Several species that depend on Delaware Bay as their last stopover, but do not winter or breed in the same regions, are experiencing declines, such as Sanderling, Semipalmated Sandpiper, Least Sandpiper, Ruddy Turnstone, and Dunlin (Morrison et al. 2006, Jehl 2007). Some of the sharpest declines have been documented in species that rely heavily on surface horseshoe crab egg availability, such as Red Knot and Semipalmated Sandpiper, and which have also shown marked reductions in mass gain potential during stopover in Delaware Bay (Robinson et al. 2003; Baker et al. 2004; Niles et al. 2007; Mizrahi, unpublished data).

7 Conclusions

Several sandpiper species migrating in the Western Atlantic track the availability of horseshoe crab eggs in space and time during northbound passage to the breeding grounds. Historically, this meant that each spring hundreds of thousands of individuals converged on Delaware Bay, the epicenter of horseshoe crab spawning in North America, to exploit what was once a readily available and profitable food resource. Rapid energy accumulation, combined with efficient energy delivery that occurs during periods of intense exercise, such as long-distance flights, appear to underlie the strong relationship between migrating sandpipers and horseshoe crab eggs in Delaware Bay. That other prey items could provide the same synchrony in availability, economy of acquisition and processing, and nutritional effectiveness as horseshoe crab eggs seems unlikely.

The consequences of reduced crab egg availability are clear. Declines in weight gain potential of sandpipers staging in Delaware Bay can compromise their ability to complete migration successfully, survive harsh conditions they encounter frequently on the breeding grounds, or make the behavioral and physiological transitions necessary to initiate breeding. These outcomes, alone or in concert, can affect population viability adversely especially if diminished egg availability persists, as it has for nearly a decade. Dramatic population declines over the last decade in some species migrating through Delaware Bay are well documented and one, the Red Knot, is perilously close to extinction.

Although we provide clear evidence linking the viability of some sandpiper species with declines in Delaware Bay horseshoe crabs, acknowledging other threats that potentially contribute to declines in sandpiper populations is important. In some years, sandpipers arrive late to Delaware Bay or in poor physiological condition (USFWS 2003; Niles et al. 2007; Mizrahi, unpublished data), which could be related to conditions birds experience on their wintering grounds or at stopover locations in South America. Threats such as industrial development, oil exploration, and aquaculture, to name a few, could affect known sandpiper wintering and stopover areas (USFWS 2003). Although the core *rufa* Red Knot wintering areas along the Patagonia and Tierra del Fuego coastlines appear to be in good ecological condition and have not changed significantly in the last 15 years (USFWS 2003), much less is known about other wintering or stopover locations in South America. For example, in 2006 between 400 and 1,000 Red Knots died at a stopover site in Uruguay, apparently from harmful algal bloom contamination (Baker personal communication). Surveys conducted in the late 1980s along the northern coast of South America (Morrison and Ross 1989) have not been repeated, although this region supports nearly 85% of the world's Semipalmated Sandpipers and significant numbers of other sandpiper species (Morrison personal communication).

Human disturbance also can adversely affect sandpipers during the non-breeding season (i.e., migration, wintering, Pfister et al. 1992; McGowan et al. 2002; Thomas et al. 2003). Observations during spring migration in Delaware Bay suggest that human disturbance can directly affect the use of certain beaches by sandpipers (USFWS 2003; Burger et al. 2007; Niles et al. 2007) and can exacerbate interspecific competition from gulls (Burger et al. 2007). However, actions to abate this problem (e.g., beach closures) have been implemented in Delaware Bay over the last few years (Niles et al. 2007).

We believe that although other perturbations may contribute to overall declines in Delaware Bay sandpipers, their affect is minor compared to the loss of a viable horseshoe crab population that consistently produces a superabundance of eggs. Since 1998, the ASMFC and state environmental protection agencies have taken measures to reduce the horseshoe crab harvest quota. Given that horseshoe crabs must be 9–11 years old before recruiting into the spawning population (Shuster and Sekiguchi 2003), these efforts must persist into the foreseeable future to insure the recovery of horseshoe crab populations and the long-term health of migratory sandpipers in Delaware Bay.

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Horseshoe Crabs, Their Eco-biological Status Along the Northeast Coast of India and the Necessity for Ecological Conservation

J.K. Mishra

Abstract Horseshoe crabs *Tachypleus gigas* and *Carcinoscorpius rotundicauda* are distributed along the northeast coast of India extending from the extreme north of West Bengal through Orissa to the northern coast of Andhra Pradesh. High congregations of these animals are found along the breeding beaches of Orissa and West Bengal during full moon and new moon high tides. Both of these Indian species have significantly different breeding patterns particularly in relation to their habitat and laying of eggs. Although at times both the species inhabit the same body of water, *C. rotundicauda* selects mudflats of mangroves and *T. gigas* have the priority for sandy estuarine and creek zones for spawning.

Environmental conditions of horseshoe crabs along the Indian coast still remain free from any kind of pollution. Also there is no known commercial exploitation for the production of amebocyte lysate or bait. But the degradation and destruction of breeding beaches by excess human activities have been posing a serious threat. This is in turn affecting the breeding migration of adult pairs (in amplexus) to come ashore for breeding. Over time, it has been observed that the number of nests (identified by their concave shapes) following the high tide has been decreasing, indicating a decline in the number of breeding pairs on the beaches. By taking the example of the status of *T. tridentatus* in Japan, a concerted effort should be made both at the national and international levels to protect the breeding beaches of horseshoe crabs of the world and allow these precious guests to use the space as an incubator for their fertilized eggs as ever before to grow up to trilobites and swim freely to their nourishing grounds.

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1 Introduction

Horseshoe crabs in the world are now represented by four extant species (Sekiguchi and Nakamura 1979). Two species namely *Tachypleus gigas* and *Carcinoscorpius rotundicauda* inhabit coastal waters of northeast coast of India along the Bay of Bengal (Fig. 1). Sekiguchi et al. (1976, 1988) had earlier reported the availability of the species along the coast of West Bengal. The very presence of these animals in a coastal zone indicates the health of the environment (Chen et al. 2004), as it indicates that these conditions are suitable for their survival, reproduction and development. But it is now a major concern that the population of *Limulus polyphemus* along the east coast of United States is in a declining trend (Rudloe 1982, Widener and Barlow 1999, Carmichael et al. 2003) as is also the case for *Tachypleus tridentatus* along the coast of Japan (Botton 2001). But the reasons for the population depletion for these two species and the degree of their exploitation are different. In the case of *Limulus*, exploitation is economically driven by fisheries for bait and LAL (Berkson and Shuster 1999, Carmichael et al. 2003), whereas the Japanese species lost its

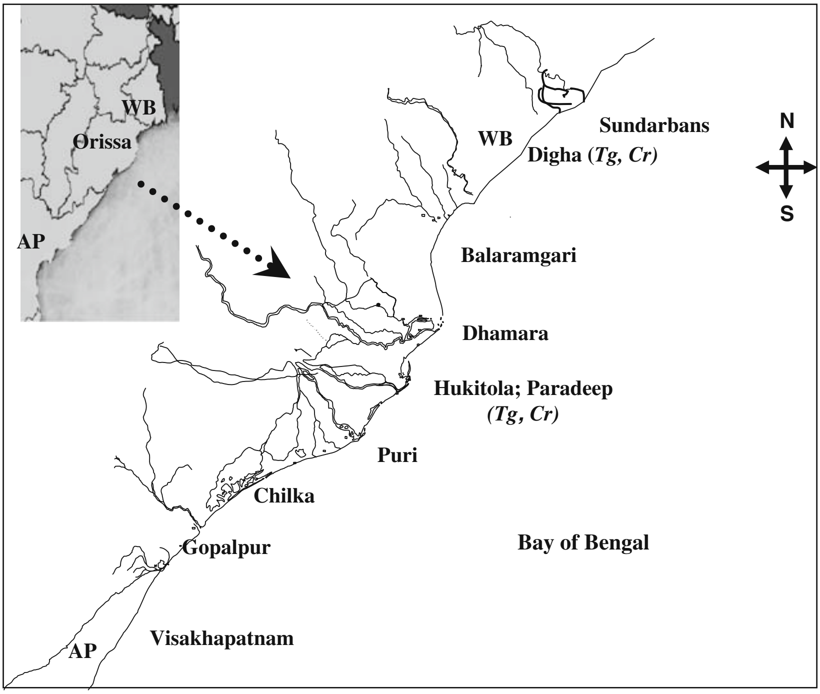

Fig. 1 Location of study sites and distribution of *T. gigas* (*Tg*) and *C. rotundicauda* (*Cr*) along the northeast coast of India. Horseshoe crabs were observed at the three southernmost sites, and nesting was observed at the five northernmost sites. AP = Andhra Pradesh; WB = West Bengal

ground primarily because of the loss of spawning habitat (Botton 2001). But, in both the cases, the threat to the horseshoe crab population is man made and needs attention.

In the case of two Indian species, *T. gigas* and *C. rotundicauda*, there is no known source of commercial exploitation for the production of amebocyte lysate or for bait as in the case of *Limulus* (Levin et al. 2003). But the animals are being used by traditional healers in the form of a liquified substance for curing joint pains (Fig. 2) and at times females are preyed upon by crows for their eggs but not to the extent that *Limulus* eggs are preyed upon by shorebirds along the east coast of United States (Botton and Shuster 2003). Though the above factors may not be the sole cause of population declines in Indian species, it has been observed during this study period that horseshoe crab populations along the Indian coast, particularly *T. gigas,* are declining very fast. Several studies have been carried out on Indian horseshoe crabs (Roonwall 1944, Sekiguchi et al. 1976, Chatterji et al. 1988, Debnath 1991, Mishra 1994) but they lack information on the population density. However, other studies suggest that there is a significant decline in the *L. polyphemus* population along east coast of United States (Widener and Barlow 1999, Berkson and Shuster 1999, Carmichael et al. 2003, 2004, Botton et al. 2006) and in *T. tridentatus* populations along the coast of Japan, Hong Kong, and Taiwan (Morton 1999, Botton 2001, Chen et al. 2004).

Fig. 2 A Traditional healer selling medicine prepared out of *T. gigas* and *C. rotundicauda* on the streets of Kolkata (West Bengal), India

The objective of this chapter is to discuss the eco-biological status of *T. gigas* and *C. rotundicauda* including their distribution pattern, nature of coexistence, nesting behavior, and the necessity for their ecological restoration along the northeast coast of India.

2 Materials and Methods

This chapter is based on the studies carried out by the author extending over a period of 19 years from 1988 to 2007. During this period horseshoe crab habitats along the northeast coast of India including West

Bengal (WB), Orissa, and Andhra Pradesh (AP) were investigated (Fig. 1). An extensive survey was carried out in studying the natural habitat of *T. gigas* and *C. rotundicauda.* Simultaneously nesting behavior and ecological conditions of the breeding zone of both the species were also investigated. However, most of the studies were carried out with special reference to *T. gigas* due to easy accessibility to its natural habitat and the breeding beaches.

Spawning activity of the species was studied by egg laying behavior of the animal and the number of nests they make in each high tide to lay eggs. Egg densities were quantified by counting the number of eggs in each nest at Balaramgari (Lat. 21° 17’ N; Long. 87° 00’ E) in the vicinity of Budhabalanga estuary and at Kasafal in the vicinity of Haskura estuary (Lat. 21° 31’ N; Long. 87° 09’ E), where the congregation of breeding pairs of *T. gigas* were found to be highest (Mishra, 1991, 1994, Mishra et al. 1992).

3 Results

It was found that the horseshoe crabs are distributed along the northeast coast of India, extending from the Sunderbans (estuarine mangroves of Ganges) in the extreme north of West Bengal (WB) coast through the coasts of Orissa and northern Andhra Pradesh (AP) in the south of the Bay of Bengal (Table 1). Sekiguchi et al. (1976) reported that *T. gigas* are distributed along the coast of WB. During this study, it was observed that *C. rotundicauda* are available particularly along the coast of WB including the Sunderban mangroves of River Ganges up to Digha in concurrence with other reports (Chatterji et al. 1988, Itow et al. 2004). *T. gigas* are distributed along the coast of Orissa up to the coast of Visakhapatnam along northern AP in the south at a depth of up to 20–30 m. Sekiguchi (1988) reported that at Digha and Junput (coast of WB), both *T. gigas* and *C. rotundicauda* are available. Similarly along the coast of Orissa, coexistence of these two species were observed at Hukitola Bay (Lat. 20° 24’ N; Long. 86° 48’ E), which was found to be a unique habitat of sandy estuarine beaches and mudflats associated with mangrove forests suitable for nesting by both species. Although the distribution of horseshoe crabs stretches along a vast coast line of northeast Bay of Bengal with varying coastal geomorphology, the breeding migration and nesting behavior of the species were unique to the individual species and they navigate to particular breeding zones of their preference for egg laying.

It was found that *T. gigas* prefers the sandy estuarine zones for laying eggs, whereas *C. rotundicauda* lays eggs in the mangrove mud flats (Sekiguchi et al. 1976, 1978, Chatterji and Parulekar 1992, Mishra 1994, Itow et al. 2004). Also the nesting pattern and number of eggs in each nest varies in these two species

Table 1 Distribution and nesting sites of *T. gigas* and *C. rotundicauda* along the northeast coast of India. '**O**' stands for availability and '–' for non-availability of eggs and juveniles

Name of the place surveyed	Species	Location of availability	Nest site with fertilized eggs	Juveniles
Canning (WB)	*C. rotundicauda*	Mangrove mud flats	O	–
Bakhali (WB)	*C. rotundicauda*	Mangrove mud flats	O	O
Digha (WB)	*C. rotundicauda*	Coastal waters	–	
Talachua (Orissa)	*T. gigas*	Estuarine sandy beaches	O	–
Haskura Estuary (Orissa)	*T. gigas*	Estuarine sandy beaches	O	–
Panchkura Estuary (Orissa)	*T. gigas*	Estuarine sandy beaches	O	–
Balaramgari (Orissa)	*T. gigas*	Estuarine beaches	O	Trilobites of *T. gigas* with ebbing tide
Dhamara (Orissa)	*T. gigas*	Creeks	–	–
Puri (Orissa)	*T. gigas*	Devi estuarine beaches	O	O
Hukitola (Paradeep, Orissa)	*T. gigas*, *C. rotundicauda*	Sandy beaches and mangrove mud flats	O O	Juveniles of *C. rotundicauda* of different sizes
Chilika (Orissa)	*T. gigas*	20–30 m	–	–
Gopalpur (Orissa)	*T. gigas*	20–30 m	–	–
Visakhapatnam (AP)	*T. gigas*	20–30 m	–	–

(Table 2). In case of *T. gigas*, breeding is found to be perennial with maximum during the months of July to February. The intensity of nesting was closely related with the textural properties (mean grain size) of the sediments of the estuarine breeding beach at Balaramgari. The species preferred a range of 63–125 μm mean grain size for nesting and any deviation from this affected their nesting behavior (Mishra, 1991). This phenomenon was in confirmation with the shifting of the nesting site from one place to another accompanied by the changes in sand grain size driven by the monsoon pattern at this breeding beach. Similarly eggs and juveniles were found in the mangrove mud flats at Hukitola. Though the breeding time of *C. rotundicauda* in Indian waters is not

Table 2 Nesting behavior of *T. gigas* and *C. rotundicauda*

Species	Natural habitat	Nest size (cm)		Number of eggs	Egg size (mm)	Larva
		Diameter (cm)	Depth of egg laying (cm)			
T. gigas	Off shore water 20–30 m depth	12–30	10–13	60–720	3.7	Trilobites found swimming to the sea with the ebbing tide
C. rotundicauda	Mangrove mud flats	–	3–7	80–200	2.3	Juveniles found in the mangrove mud flats

yet studied, in the waters of Thailand they breed throughout the year (Sekiguchi and Nakamura 1979).

The most important observation was on the destruction of breeding beaches, which is very important for the egg-laying activity of both the species. Along the coast of Orissa at Balaramgari, there is a tremendous change in the intertidal sands due to the shrinkage of nesting area caused by human activities such as maintenance work by fishing trawlers on the beach itself (Fig. 3) and shifting of beach sands for construction purposes at times. These may be the major cause of damage to the main nesting sites (about 200 m^2) of *T. gigas* at Balaramgari and there is a sharp decline in the number of nests (identified by their concave shape) (Fig. 4).

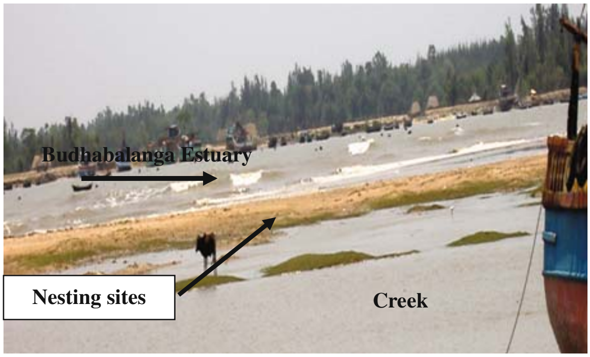

Fig. 3 Breeding beaches at Balaramgari in the vicinity of Budhabalanga Estuary, showing the sand bar adjacent to creek where nesting by *Tachypleus gigas* occurs

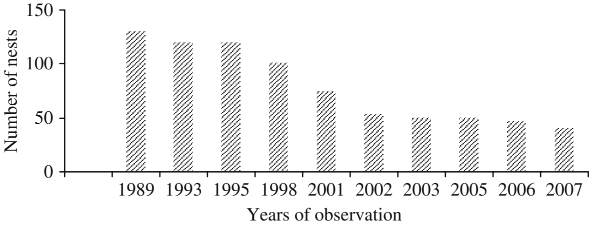

Fig. 4 Number of fresh horseshoe crab nests found at Balaramgari, northeast coast of India

4 Discussion and Conclusions

Breeding migrations of horseshoe crabs are found throughout the year, which is dependent upon the preparedness of the breeding beaches with a maximum percentage of sand with grain size between 63 and 125 μm as in case of *T. gigas*. This phenomena was observed in one of the much studied site at Balaramgari, where breeding pairs navigate to either site of the estuary sensing the availability of the preferable sands in that area (Mishra 1991) at different times of the year. However, the mechanism behind these environmental phenomena needs a detailed study.

Several anthropogenic activities, mainly related to fisheries and construction works along the coastline, are posing serious threats by destroying the natural breeding beaches. This restricts the migrating pairs from finding a suitable nesting site for spawning. Unlike their American counterpart (Rudloe 1982, Berkson and Shuster 1999, Widener and Barlow 1999, Carmichael et al. 2003, Levin et al. 2003) horseshoe crabs in India are not subjected to commercial exploitation for biomedical, fertilizer, and bait purposes. The real threat comes from the human population, which plays an important role in the coastal ecology (Botton et al. 2006). In addition, natural calamities such as super cyclones and tsunamis cause serious damage by shifting the sands from the beaches.

A constructive approach is needed to protect the intact, undisturbed breeding zones in India to help honeymooning horseshoe crab couples to come ashore and lay eggs. As it is evident from the present status of breeding beaches at Balarmgari, it is a matter of concern that the day is not far away when we will not be able to encounter breeding pairs. Efforts should be made by creating public awareness, educating fisher folks, providing alternative sites for boat building and repairing, and if required, enacting laws to protect these precious guests from the brink of extinction from our coast.

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American Horseshoe Crabs, *Limulus polyphemus*, in Mexico: Open Possibilities

Jaime Zaldívar-Rae, René Elías Sapién-Silva, Martha Rosales-Raya, and H. Jane Brockmann

Abstract Little is known about Mexican *Limulus polyphemus*, the southernmost population of the species. We present an overview of work on Mexican horseshoe crabs, their situation, and perceived threats and opportunities regarding the conservation of the species. Horseshoe crabs occur along the western, northern, and eastern coasts of the Yucatán peninsula, and are genetically distinct from populations in the United States. Spawning aggregations and nests are found continuously throughout the year, commonly in protected lagoons where mangrove (*Rhizophora mangle, Laguncularia racemosa*, *Avicennia germinans,* and *Conocarpus erectus*) and sea grass *(Thalassia testudinum*) communities proliferate. Populations are thought to be dwindling since the 1960s and *Limulus* is listed as "in danger of extinction" in Mexican legislation since 1994. The most important localities are within protected areas. Direct exploitation is not an important threat, but coastline modification (especially of mangrove areas and coastal lagoons) for housing and tourism is a major concern. Additional potential threats are the oil industry and shrimp fishery in the southern Gulf of Mexico, but their effects on horseshoe crab populations have not been assessed.

1 Introduction

Knowledge about the biology and ecology of Mexican *Limulus polyphemus*, the southernmost population of the species, is scant (Escalante et al. 1980; Gómez-Aguirre 1980; Bonilla-González et al. 1986; Anderson and Shuster 2003). This chapter aims at providing an overview of the available information on the biology of horseshoe crabs in Mexico and our perspective on potential threats and opportunities for the study and conservation of these animals in Mexico.

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1.1 *The Study of* **Limulus polyphemus** *in Mexico: A Historical Account*

The first description of horseshoe crabs in the Western world was provided by Friar Diego De Landa (1566; translated by Tozzer 1941) in his “Account of the Things of Yucatán.” Although he classified it as a fish, he clearly described the round shell, slender tail, and many legs of the horseshoe crab and used its Mayan name “Mex.” He also stated that the only edible part of the animal was its eggs, which were eaten by the Mayans. The next written accounts of Mexican horseshoe crabs date from the late 19th century. In them, Milne-Edwards (1879) and Ives (1891) mentioned that horseshoe crabs were abundant along the southern west coast of the Yucatán Peninsula in the Laguna de Términos, Campeche, and along the northern coast of Yucatán at Dzilam. Their presence along the coasts of the Yucatán Peninsula was later confirmed by Zarur-Menez (1961), Ramírez et al. (1963), Gómez-Aguirre (1979), and Bonilla-González et al. (1986). Occasional sightings in Veracruz, on the western portion of the Gulf of Mexico, were reported by Chávez and Muñoz-Padilla (1975). González-Guzmán et al. (1967), at the National Autonomous University of Mexico (UNAM), described the cytology of hemolymph collected from horseshoe crabs of Laguna de Términos, Campeche, and correctly inferred a role for amebocyte granules in the coagulation of hemolymph.

González-Guzmán et al. (1967) got their specimens from Dr. Samuel Gómez-Aguirre of UNAM’s Institute of Biology, who had begun his pioneering work with populations in the southern Gulf of Mexico in 1964. Dr. Gómez’s interest in *L. polyphemus* spanned 40 years until his passing in 2006, and his work included the first calls for attention to a decline in Mexican horseshoe crab populations (Gómez-Aguirre 1979, 1980, 1983, 1985, 1993, 2002). Dr. Gómez also led several groups of Mexican biology students in the first morphometric studies of the species and quantified exuviae, dead, and live individuals along the coasts of the Yucatán Peninsula (Bonilla-González et al., 1986; Barba-Macías et al., 1988; Álvarez-Añorve et al., 1989; Gómez-Aguirre 1993; Gómez-Aguirre and Yáñez-Martínez 1995). He also provided the first descriptions on particular aspects of the biology of Mexican horseshoe crabs (e.g. Gómez-Aguirre and Flores-Morán 1987; Gómez-Aguirre et al., 1992).

2 Distribution and Habitat

Mexican populations of *L. polyphemus* are restricted to the coasts of the Yucatán Peninsula, between about 18°N and 21°N (Chávez and Muñoz-Padilla 1975; Gómez-Aguirre 1979; Vázquez-García and Villalobos-Figueroa 1980; Bonilla-González et al., 1986), with rare sightings in Veracruz (Chávez and Muñoz-Padilla 1975; Fig. 1). In 1985, Dr. Gómez-Aguirre and his students found a population on the northern shore of Bahía de la Ascensión in Quintana

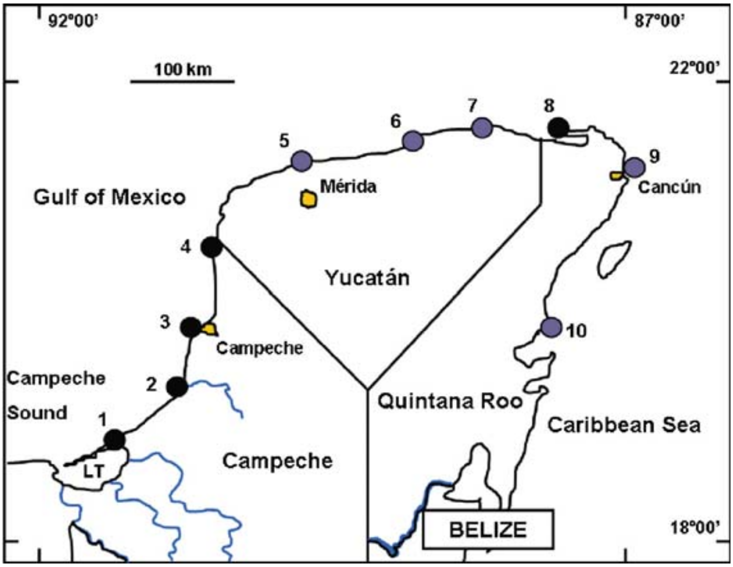

Fig. 1 Distribution of American horseshoe crabs, *Limulus polyphemus*, in Mexico. *Black* and *gray circles* represent localities where mating aggregations have been recorded and localities where live or dead individuals or exuviae have been observed, respectively. Localities: LT = Laguna de Términos; 1 = Isla Aguada, Cabrera and Isla Pájaros; 2 = Icahao and Champotón; 3 = City of Campeche; 4 = Isla Arena and Celestún; 5 = Progreso and Yucalpetén; 6 = Dzilam de Bravo; 7 = San Felipe and Río Lagartos; 8 = Holbox; 9 = Cancún; 10 = Bahía de la Ascensión

Roo, on the Caribbean coast of the Peninsula (Bonilla-González et al., 1986), confirming an anecdotal account by Shuster (1979). This observation contradicts the speculation that the strong northward flowing Yucatán Current and the very narrow continental shelf on the Caribbean coast might prevent southward migrations from northern localities (Anderson and Shuster 2003).

Most Mexican localities for horseshoe crabs are within coastal lagoons. Although rivers are scarce in the Yucatán Peninsula (Fig. 1), large volumes of underground fresh water flow into these coastal lagoons and create estuarine systems. Red, black, and white mangrove and buttonwood (*Rhizophora mangle*, *Laguncularia racemosa*, *Avicennia germinans*, and *Conocarpus erectus*, respectively) and vast seagrass beds (predominantly *Thalassia testudinum*) are common in these coastal lagoons and estuaries, and *L. polyphemus* are commonly associated with these communities (Gómez-Aguirre 1979; Bonilla-González et al., 1986; Barba-Macías et al., 1988). Indeed, the presence of horseshoe crabs on the Caribbean coast of the Peninsula may be explained by coastal lagoons providing suitable habitats without exposure to strong currents and with the sheltering effect of the Mesoamerican Barrier Reef. These shallow water areas also provide valuable nursery grounds for juveniles, which require many years to mature.

3 Morphometry

Following the pattern for the species, in Mexico male horseshoe crabs are significantly smaller than females, with inter-sexual size ratios of 0.73–0.80 (based on intergenal distances; RE Sapién-Silva, unpublished data), similar to those observed in the United States (0.75–0.79 based on prosoma widths; Shuster 1979). There are significant differences in size between populations along the coasts of the peninsula: animals from Champotón are significantly larger than those from Laguna de Términos, while the latter are significantly larger than those from the area near Progreso, Río Lagartos, and Holbox (see Table 1).

Differences between populations do not follow a latitudinal pattern (RE Sapién-Silva, unpublished data) as has been described for U.S. populations (Shuster 1979; Riska 1981). Horseshoe crabs from Champotón are similar in size to those from Chesapeake Bay measured from Shuster (1979), while those from Río Lagartos are smaller than those from the Gulf coast of Florida (RE Sapién-Silva, unpublished data). A similar pattern was detected by one of us (HJ Brockmann, unpublished data) upon comparing horseshoe crabs from San Felipe-Río Lagartos, western Florida, and Delaware Bay (Table 1). If size has a genetic basis, the size differences between Mexican populations may indicate limited gene flow among them.

4 Population Genetics

There seems to be no gene flow between the Yucatán populations and those in Florida (Anderson and Shuster 2003). This is supported by the findings from King et al. (2005) that the population at San Felipe-Río Lagartos (Fig. 1) on the northeastern coast of Yucatán is highly genetically differentiated from populations in the United States. The San Felipe-Río Lagartos population also showed low genetic diversity and heterozygosity, suggesting that geographic isolation, small population size, and inbreeding may have spurred stochastic processes leading to a loss of diversity. King et al. (2005) suggested that the marked genetic differences between the Mexican and United States populations may warrant a taxonomic revision of the genus. An assessment of the genetic structure of Mexican populations has not yet been conducted.

5 Reproduction

In Mexico horseshoe crabs are known to breed from Campeche to northern Quintana Roo, particularly near the mouths of the Laguna de Términos (Punta Cabrera, Isla Aguada, and Isla Pájaros; Gómez-Aguirre 1979; Bonilla-González, et al. 1986; Rosales-Raya 1999), in the estuarine systems of Celestún, Isla Arena

Table 1 Sizes of horseshoe crabs from populations in México, Florida, and Delaware

Measure (cm)	Laguna de Términos[a]	Champotón[a]	Progreso[a]	San Felipe-Río Lagartos[a]	Holbox[a]	San Felipe-Río Lagartos [b]	Florida[b]	Delaware Bay[b]
Females								
Prosoma width								
Mean	–	–	–	–	–	18.8	21.6	25.5
SE						0.32	0.32	0.27
(N)						(39)	(30)	(50)
Interocular distance								
Mean	11.05	14.0	10.2	10.5	10.4	10.8	13.8	16.1
SE	0.05	0.15	0.75	0.20	0.21	0.20	0.17	0.19
(N)	(395)	(37)	(7)	(40)	(26)	(39)	(50)	(50)
Males								
Prosoma width								
Mean	–	–	–	–	–	14.0	16.1	20.0
SE						0.14	0.17	0.16
(N)						(55)	(40)	(75)
Interocular distance								
Mean	7.9	9.7	8.3	7.7	7.5	7.7	9.7	12.0
SE	0.04	0.13	0.22	0.10	0.10	0.10	0.11	0.11
(N)	(333)	(27)	(8)	(44)	(44)	(55)	(60)	(75)

[a] Measured by R.E. Sapién-Elías from specimens collected in Laguna de Términos (southern Campeche coast), Champotón (central Campeche coast), San Felipe-Río Lagartos (on the northern Yucatán coast), and Holbox Island (northern Quintana Roo coast).

[b] Measured by H.J. Brockmann from specimens collected in San Felipe-Río Lagartos, Florida (Seahorse Key, on the west coast of Florida near Cedar Key, Levy County), and Delaware Bay (Bowers Beach, DE).

(M Rosales-Raya, personal observations), in the estuarine areas at the mouth of Río Lagartos (Brockmann, personal observations), on Holbox (R. Salazar-González, personal communication), and at the mouth of the Champotón River (Rosales-Raya 1999). Juveniles were very abundant in the lagoon at Yucalpetén (Brockmann, personal observations) (see Fig. 1 for locations of these sites). Mating aggregations were also common in the City of Campeche in the late 1970s and early 1980s, but do not seem to occur nowadays (M Rosales-Raya, personal observations; Fig. 1). In the Yucatán Peninsula, horseshoe crabs spawn on small beaches limited by mangroves or on the edges of small mangrove islands. These are generally low-energy beaches with little slope, most of whose area is flooded during high tides. Organic matter abounds in the substrate and microbial decomposition is high in these beaches, as indicated by the common characteristic smell of hydrogen sulfide. These generally poor conditions for egg development may partly explain why densities of nesting aggregations in Mexico (from tens to a couple hundred pairs at a time in a particular site) and overall abundances are very low in comparison with those in the United States. Also unlike the U.S. populations, Mexican horseshoe crabs seem to spawn throughout the year, at least in the Campeche populations (Álvarez-Añorve et al. 1989; Barba-Macías et al. 1988; Bonilla-González et al. 1986; Rosales-Raya 1999).

Little is known about the reproductive behavior of Mexican horseshoe crabs. Gómez-Aguirre et al. (1992) documented the sexual differentiation of a captive male collected in Laguna de Términos, which emerged from a molt with the first pair of pedipalps modified into claspers with the attenuated fixed claws characteristic of virgin males (Gómez-Aguirre et al. 1992; Brockmann 2003b). There is no size-based assortative mating in Mexican populations (RE Sapién-Silva, unpublished data) and mating aggregations of many males and one female are uncommon in Mexico. In Laguna de Términos mating pairs are the rule and two males mating with the same female are rarely seen (RE Sapién-Silva, M Rosales-Raya, personal observations).

Horseshoe crabs require 8–10 years to develop from newly metamorphosed juveniles to adults (Shuster and Sekiguchi 2003). Juveniles spend their first 2–3 years in shallow inshore areas just off the breeding beaches, feeding on worms and small mollusks. Juvenile horseshoe crabs were observed in large numbers feeding in a shallow lagoon near Yucalpetén, swimming into and out of the lagoon with each high tide cycle (HJ Brockmann, unpublished data).

Martha Rosales-Raya (1999) studied the geochemical characteristics of nests in three nesting beaches within the Laguna de Términos (Isla Aguada, Cabrera, and Isla Pájaros) and one at the mouth of a small stream north of the Champotón River (Icahao). The percentage of silt-clay in substrates from these four sites did not exceed 40%, a composition considered suitable for nesting (Sekiguchi et al. 1977; Botton et al. 1988). Water salinity in nests increased with the proportion of silt-clay and very fine sand and consolidation of the substrate, and was as high as 59% in the most consolidated substrates (Isla Pájaros). In addition, anaerobic conditions were common and nests were shallow (exposed to 12.4 cm deep) in these substrates. It was nevertheless puzzling that nest density on Isla Pájaros was higher than in other locations with more benign conditions.

6 Ecological Relationships

There are no studies on the diet of Mexican horseshoe crabs, but several bivalve genera in the Yucatán Peninsula (see García-Cubas 1981; González et al. 1991; and references therein) are likely constituents, based on what is known about horseshoe crab feeding preferences in Delaware and New Jersey (Botton 1984; Botton and Haskin 1984; Botton and Ropes 1989). Bonilla-González et al. (1986) reported the presence of seagrass, *Thalassia* sp., leaves in the gut of a female specimen.

Although there are no studies on the predators of Mexican horseshoe crabs and their eggs, many species of shorebirds that are known to feed upon horseshoe crab eggs in spawning beaches of the United States (Wander and Dunne 1981; Botton and Loveland 1993; Clark et al. 1993; Botton et al. 1994, 2003) also occur on the coasts of the Yucatán Peninsula (see lists in MacKinnon 1992, 2005). We have observed laughing and ring-billed gulls (*Larus atricilla*, *Larus delawarensis*) and black vultures (*Coragyps atratus*) feeding on overturned horseshoe crabs and ants harvesting eggs from nests exposed after low tide (J Zaldívar-Rae and RE Elías-Sapién, personal observations). Nothing is known about the predators of adult horseshoe crabs in the Yucatán, but common predators elsewhere are also found here, including sea turtles (Seney and Musick 2007).

7 Status of Horseshoe Crabs in Mexico and Threats

7.1 Legal Status

Mexican law regards *L. polyphemus* as “in danger of extinction” since 1994 (SEMARNAP 1994; SEMARNAT 2002). This category overlaps IUCN’s “endangered” and “critically endangered” categories and includes species whose distribution or population size is drastically diminished or whose biological viability is at risk due to drastic modification or destruction of their habitat, over-exploitation, disease, or predation. Since 2005, the Mexican federal agency in charge of programs to conserve species at risk is the Comisión Nacional de Áreas Naturales Protegidas (CONANP, National Commission for Natural Protected Areas), but so far no specific program for the protection and conservation of horseshoe crabs has been implemented.

7.2 Human Use

Elderly locals report that when they were young they occasionally used female horseshoe crabs as food (M Rosales-Raya; unpublished data) cutting them open for their eggs (G Veronica Rios, unpublished data), and there are reports of the use of horseshoe crabs to feed pigs (Chávez and Muñoz-Padilla 1975). However, these uses have been rare to non-existent for several decades. The traditional octopus fishery (*Octopus maya* and *Octopus vulgaris*) of Campeche and Yucatán once relied on horseshoe crabs as bait (M Rosales-Raya; unpublished data). However, due to a growing international demand for octopus over the past 30 years (Pérez-Pérez et al. 2007, Hernández-Flores et al. 2001) and to the legal protected status of horseshoe crabs, fishermen now favor more abundant and commercially available bait such as spider crabs (*Libinia dubia*).

7.3 Shrimp Fishery

Given the income it generates, shrimp is the most important fishery in the Gulf of Mexico. Over 600 trawlers from ports in the five Mexican states of the Gulf of Mexico sail the continental shelf off the coasts of Campeche in Mexican waters to catch four species of peneid shrimp (*Farfantepenaeus duorarum*, *Farfantepenaeus aztecus*, *Litopenaeus setiferus*, and *Xiphopenaeus kroyeri*), concentrating on the waters just outside the Laguna de Términos (Fernández-Méndez et al. 2001). Trawling nets are designed to be dragged over the sea bottom and most of the trawling activity is carried out in shallow waters. This means that horseshoe crabs and other benthic organisms have likely been negatively affected for decades. However, no quantification of horseshoe crab by-catch or mortality due to trawling has been conducted.

7.4 Offshore Oil Industry

The area off the coast of Campeche, known as the Campeche Sound (Fig. 1), contains the largest and most important oil fields in México. Since the discovery of these oil fields in the 1970s, production has steadily increased and now constitutes more than 80% of national crude oil (García-Cuéllar et al. 2004). The environmental impact of oil industry activities in the Campeche Sound includes the dispersal of heavy metals, polycyclic aromatic hydrocarbons, sulfur oxides, nitrogen oxides, sulfuric acid, volatile organic compounds, and total petroleum compounds, with varying detrimental effects on marine ecosystems (García-Cuéllar et al. 2004). The benthic communities suffer the most severe effects (Botello et al. 1996). Major oil spills during the 1980s had severe consequences in estuaries and coastal lagoons, damaging the reproduction and growth of populations of several important fisheries (Soto et al. 1982;

Botello 1987; Botello et al. 1996). Fortunately, spills and their extent have been reduced since the 1990s and frequent environmental auditing of Petróleos Mexicanos (PEMEX), the national oil company, is contributing to the development of safer practices (Guédez-Mozur et al. 2003; PEMEX 2000, 2002).

The potential role of pollution from the oil industry on the decline of horseshoe crab populations in Laguna de Términos has not been studied. However, this appears to be a valid hypothesis given that (a) the prevailing currents in the eastern Gulf of Mexico head south (Leipper 1954), possibly carrying and concentrating pollutants in the southern part of the gulf and Laguna de Términos; (b) horseshoe crabs use benthic habitats in coastal lagoons and estuaries, where dense crude precipitates and lingers; (c) exposure to oil affects juvenile development (Strobel and Brenowitz 1981); and (d) their diet is based on bivalves, which are known to bioaccumulate pollutants (e.g. Bryan 1979; Porte and Albaigés 1994; Bolning 1999; Gold-Bouchot et al. 2007).

7.5 Coastline Modification

The most widespread threat to horseshoe crabs in Mexico is shoreline modification. Coastal cities in the Yucatán Peninsula are experiencing rapid human population increases and, as a result, these cities are spreading into adjacent coastal areas to develop tourism, services, and housing infrastructure. Further coastline modification results from the construction of summer houses around coastal villages, to supply a growing market for second homes for those living in inland cities. In general, the increase in human population in coastal areas has caused the disappearance of nesting and nursery habitats for horseshoe crabs and the degradation of adjacent water bodies due to pollution from garbage and sewage, limited water fluxes, or the filling of coastal lagoons to expand city areas.

Another example of shoreline modification is the use of artificial barriers to reduce the effects of sea erosion on certain beaches. Owners of beachfront property in these areas face an increased risk of their houses being destroyed by hurricanes and storm surge or due to the repeated action of high tides. To prevent this, artificial reefs made of rocks or plastic mesh tubing are being placed parallel to the wave line, in order to favor the deposition of sand on the beach-facing side of the barrier. This strategy has indeed reduced the energy of these beaches by reducing their slope and creating a smoother beach profile (SEMARNAT 2007; Álvarez-Del Río 2003). Horseshoe crabs use beach slope as a cue to get back to the sea after spawning, and a smooth beach profile may increase their probability of getting stranded (Brockmann 2003a). Additionally, the sand in most of Yucatán's beaches is very fine, which provides a large surface area for microbial growth. Such growth creates anoxic conditions which impede egg development (Brockmann 2003a). Therefore, a gentle beach slope may exacerbate anoxia and unsuitability for egg development, because the substrate in the intertidal zone becomes water-saturated.

7.6 Rising Sea Levels

Beaches where horseshoe crabs used to spawn are eroding due to sea-level rise (M Rosales-Raya, personal observations). This is particularly evident on the west side of the Peninsula where erosion has resulted in the moving of the intertidal zone to unsuitable substrates. For instance, the former nesting site of Isla Pájaros in Laguna de Términos used to be a thick mangrove islet that remained dry even at high tide for at least the past 30 years, but has now turned into a sparse group of trees, whose substrate is completely submerged even at low tide.

7.7 Tourism

Owing to its great appeal as a tourism destination and the resulting economic development, the Caribbean coast of the Yucatán Peninsula (state of Quintana Roo) is experiencing one of the highest rates of population increase in the world, and horseshoe crab populations in this area have already been affected. Large coastal lagoon systems where horseshoe crabs were usually found have already been severely damaged by sewage discharges, pollution by landfills, and the cutting of mangrove forests to build the hotel infrastructure. Pristine coastal lagoons and associated fresh water "cenotes" (sinkholes) are becoming coveted amenities for tourism projects in the area, and the potential damage to the small horseshoe crab populations they may harbor is yet to be established.

8 Opportunities

In spite of all the threats faced by horseshoe crab populations in Mexico, there are grounds for hope and opportunities to preserve populations that are still healthy, and restore those that have been damaged.

8.1 Education of the Local Public

Many people in the Yucatán Peninsula recognize horseshoe crabs, including thousands of families from inland cities and towns who spend their summer vacations in coastal communities. This creates the opportunity to educate a mixed "captive audience" of local and urban people (many of them children and youngsters) on why horseshoe crabs and their habitats are important and unique. These people are likely to transmit their knowledge to other citizens, increasing the base of moral, political, and financial support for further conservation efforts.

8.2 Citizen Initiatives

There are a growing number of non-governmental organizations dealing with the conservation of coastal systems in the Yucatán Peninsula. Particularly, two of us (RE Sapién-Silva, J Zaldívar-Rae) are now working with a group of young Mexican biologists to create a new non-governmental organization, among whose aims will be the conservation and restoration of horseshoe crab populations and their habitats. We intend to achieve this through several strategic lines: (a) educating both urban and coastal local communities, (b) promoting sustainable development in coastal communities, (c) involving local communities in conservation programs, (d) contributing to the scientific study of *L. polyphemus* in Mexico, (e) informing and counseling decision-makers on the status of horseshoe crabs and their habitats in Mexico, and (f) building alliances with governmental agencies, other national and international initiatives, environmentally responsible companies, and other stakeholders.

8.3 Protected Areas

Many of the most important horseshoe crab localities are now within federal or state protected areas; particularly, parts of offshore horseshoe crab habitat and the nesting and nursery areas of Laguna de Términos, Celestún, Río Lagartos, Isla Arena, and Holbox are within federal Areas for the Protection of Flora and Fauna or Biosphere Reserves managed by CONANP since the year 2000 (see map in www.conanp.gob.mx/sig/). CONANP has been developing a model of conservation based on the sustainable development of local communities within protected areas and their area of influence, and strongly encourages the involvement of local communities, academic institutions, non-governmental organizations, and the general public (SEMARNAT-CONANP 2007). As a result of increased awareness of environmental issues among Mexican society, federal and state budgets for conservation are steadily increasing, even in spite of budget cuts in other governmental areas. A large portion of these resources is going into hiring and training personnel, reinforcing the infrastructure, and building up the capacity for the operation of protected areas. In sum, the emerging governmental conservation policies, programs, and increasing governmental budgets should be incorporated in a strategy to protect and preserve horseshoe crab populations.

8.4 Research and Higher Education Institutions

There are good conditions for carrying out research and developing collaborations with academic institutions and researchers in the Yucatán Peninsula and the rest of Mexico. Particularly, the Yucatán Peninsula hosts several national and state research institutions and universities, many with respectable infrastructures, including state-of-the-art communication technologies and equipment. Hundreds of local professionals in biology, natural resource management, fisheries, environmental science, chemistry, and chemical and industrial engineering graduate from these institutions every year. With regard to horseshoe crab populations and their habitats, this wealth of human resources is as yet untapped to carry out research and conservation and monitoring programs, as well as to devise management schemes for healthy populations and habitats and to conduct mitigation and restoration actions in disturbed areas.

9 Gaps in Knowledge and Priority Actions

Finally, we would like to point out some priority work that needs to be done in the near future. In order to develop a baseline body of information to design a conservation strategy, carry out key actions, and evaluate their impact, we need to determine the population sizes offshore and how these relate to the numbers of live and dead animals and exuviae that can more easily be monitored along the shores. In addition, it is important to establish where the key recruitment, feeding, and juvenile development areas are. Devising protocols for particular populations and habitats will enable us to reinforce the capacity of officials in charge of protected areas by training them and establishing monitoring procedures that can be applied on a constant basis to evaluate the status of horseshoe crab populations.

In order to determine management units for conservation and remediation of the damage that the populations may have suffered, information from population surveys will have to be coupled with that from studies of the genetic structure of Mexican populations. The molecular technologies and know-how to obtain these data are already available in Mexico.

Obtaining and maintaining the support of local communities, particularly those groups making direct use of coastal areas and resources in the Yucatán Peninsula, is as important as obtaining scientific data and managing populations. Therefore, it is paramount to start educating children and adults, especially while they are experiencing horseshoe crab habitats and coastal ecosystems first-hand. This includes educating children from coastal communities to understand that they will be the future stewards and main beneficiaries of such resources. At the same time, it is necessary to train current natural resource owners and users in sustainable practices and to promote the diversification of their sources of income, so that they can improve their living conditions while reducing exploitation pressures on particular elements of ecosystems.

A risk map for *L. polyphemus* in Mexico is needed, in order to identify the main threats and causes of disturbance to particular populations or management units and to achieve a more effective conservation and management strategy. Such an exercise will allow us to identify specific problems in particular locations, opportunities and strengths to address them, the stakeholders involved, the key actions to be taken, the timescales for these actions, and the resources that will be required.

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Basic Habitat Requirements of the Extant Species of Horseshoe Crabs (Limulacea)

Carl N. Shuster Jr. and Koichi Sekiguchi

Abstract Because horseshoe crabs (Limulacea) are ecological generalists that exist in more-or-less discrete populations within a variety of habitats throughout their ranges, we cannot explore all the possibilities within this short chapter. We have concentrated, therefore, on the two major habitats that have supported large population of horseshoe crabs (the Seto Inland Sea, Japan, and Delaware Bay, USA) and the species with which we are most familiar (the Japanese horseshoe crab *Tachypleus tridentatus* and the American species *Limulus polyphemus*), including differences between the populations, their habitats, and their distribution, including how the last Great Ice Age probably affected that distribution.

In general, the attributes of prime habitats include quality and quantity of those habitats beneficial to the species throughout their life cycle: tidal streams and marshes; lengthy sandy beaches with low wave force; inshore, often intertidal shallow-water areas; a large food resource; a favorable hydroclimate; the deeper water region of the bay; and the immediate continental shelf. Because horseshoe crab behavior helps to identify the range of environmental parameters within which the crabs are more successful, we also considered the major life stages in their life cycle.

1 Introduction

Understanding the natural history and ecology of horseshoe crabs is paramount to conservation of the species because the crabs provide the clues or background essential to their conservation. In our earlier discussion (Sekiguchi and Shuster 2009) we reviewed the large-scale, global constraints on the abundance and distribution of horseshoe crabs. Here the focus is limited mainly to two habitats, the Seto Inland Sea of Japan and Delaware Bay, USA, and its

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species, *Tachypleus tridentatus* and *Limulus polyphemus*, respectively. The extent and quality of these habitats are of interest to this symposium because where these species propagate is most critical to the survival of the species; thus, available habitat is one of several critical conservation issues.

Horseshoe crabs (Limulidae) are ecological generalists (Eldredge 1991; Loveland et al. 1997), adapting to manifold environments throughout their extensive geographic distributions in the shallow waters bordering the eastern coastlines of North America and Asia. Because each species appears to have a slightly different spawning behavior and spawning area, several questions arise, for example:

Is this of any consequence in considering the conservation of the species?
Are there many or a few characteristics that define a high-quality spawning locale?
Are the rates and amounts of natural and geological processes (i.e., hydroclimatic factors and sedimentation: erosion–transport–deposition) sufficient to produce suitable spawning habitats?
Will conservation of shorelines (i.e., protection against development) be enough or will it be necessary to manipulate and manage habitats?

2 Environment of the Major Life Stages

Many environmental parameters are common to each major life cycle stage. The difference is mainly in site (Sekiguchi 1988). For example, *Tachypleus tridentatus* occupies three zones. Spawning occurs during July and August in sandy beaches, near the high tide line. Embryological development occurs intertidally; the early instars are also in the intertidal areas and shallow water. Later stages and adults are usually in deeper water. But, according to Kawahara (1982), small instars (1.4–7.0 cm prosomal width, PW) bury themselves in the mud during high tide and emerge and feed in pools remaining at low tide. As they grow they extend their range and by November move away from the beach area and go into hibernation. By the following March to April they began to feed. Dredging from only a limited area, some 20 m deep during winter months, confirms that *T. tridentatus* passes the cold-water months in offshore waters. When the water temperature rises above 18°C, they migrate from the deeper waters to shallow water (Nishii 1975). Not much is known about the habitat in the more southern part of the range of the species except it is not the same as in China and Japan with part of the contrast being muddy environments in the north and sandy in the south.

In *L. polyphemus*, the embryos develop in an intertidal area, usually a sandy beach. The trilobite larvae and the first-year stages (instars 1–6; Shuster 1979, Botton et al. 2003) as well as those of the second (instars 7–9) and third year (instars 10 and 11) are usually spent in the near shore area. Studies in the vicinity of the Rutgers Laboratory on the Cape May, New Jersey shore of Delaware

Bay, have demonstrated the distribution of the first-year instars. On September 11, 1951, Shuster (1979) found instars 1–6 unevenly distributed on the first five intertidal bars (flats) from shore (Fig. 1). In 1998 and 1999, from to 17 August, Botton et al. (2003) followed the juveniles and found instars 1–4, on bars 1, 3, and 5. Second-year instars (usually 7–9) and third-year instars (10 and 11) are usually spent in the same area, just off shore in shallow-water area (Shuster, personal observation). That each succeeding stage migrates further and further from the shoreline toward deeper water is indicated by trawl surveys; instar 10 (PW = 38 mm) was trawled at depths from about 1 to 7 m, instar 12 (PW = 50 mm) from about 4 to 8 m, and instar 14 (PW = 81 mm) was from about 6 to 8.5 m (S. Michels, personal communication). These mid-sized juveniles (instars 12–15), in those estuaries where the populations also wander out onto the continental shelf, move seaward where the later stages and adults are common (Anderson and Shuster 2003). Adults usually migrate throughout the ranges occupied by their specific population (Swan 2005, King et al. 2005). This pattern of behavior and movement from one habitat to another is mirrored by *Tachypleus tridentatus* in Japan, but much less is known about the habitats and migrations of the other Asiatic species, *T. gigas* and *Carcinoscorpius rotundicauda* (Sekiguchi 1988, Chatterji 1994).

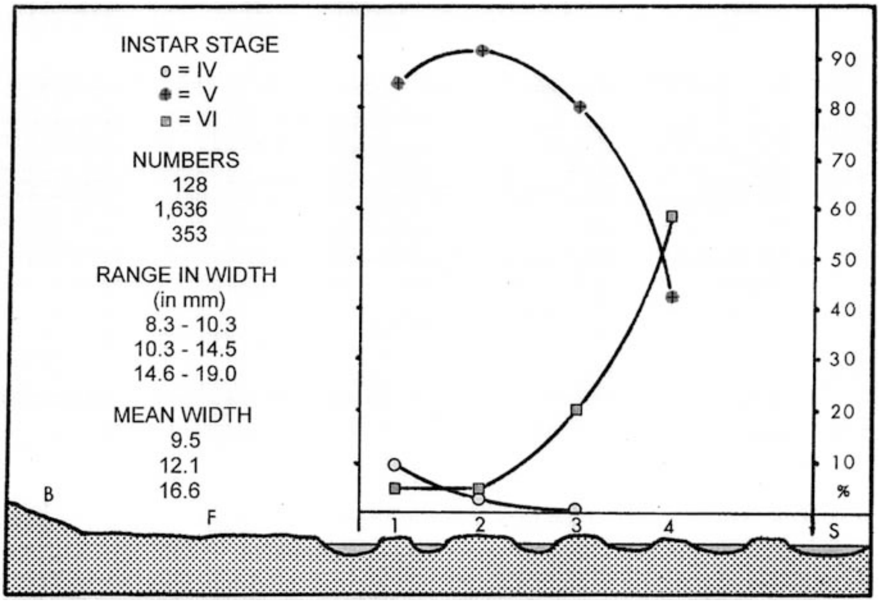

Fig. 1 Distribution of three *Limulus polyphemus* instars (IV–VI) on September 11, 1951, on four intertidal bars in the vicinity of the Rutgers Cape Shore Laboratory in lower Delaware Bay, New Jersey (based on Shuster 1979). Legend: B = beach; F = intertidal flat

3 Comparison of Two Major Habitats

Historically, the Seto Inland Sea of Japan and the Delaware Bay, USA, have been preeminent as habitats with the largest populations anywhere of their respective horseshoe crab species. Yet, despite their general characteristics as inland, shallow-water coastal seas, they are not very similar (Ichiro et al. 2008, Bryant and Pennock 1988). Dissimilarities began in ancient times. In Japan, descendants of the ancient natives still populate the area and that is reflected in the rich cultural history of the area. At Delaware Bay, the natives were replaced by a foreign culture only a few hundred years ago that brought about an abrupt change not only in the coastal environment but also in its civilization (Shuster 1960a, b).

3.1 The Seto Inland Sea, Japan

The Inland Sea is the largest enclosed coastal sea of Japan. It is a large body of water, some 440 km long (275 miles) with its axis oriented in a north-northeast direction, centered on 133° 32’ E and 34° 40’ N. Due to the lack of major rivers, the elongated configuration of the sea and its restricted connections to the open ocean, circulation between the inland sea and the ocean is poor. Pollutants tend to concentrate and are slowly eliminated from the sea (Ichiro et al. 2008).

The sea was once the site of large spawning groups of *T. tridentatus*, the only species of horseshoe crab indigenous to the waters of Japan (Fig. 2). But, the reclamation of land by dikes and polders eliminated most spawning areas (Fig. 3) and worsening water quality probably added to the loss of quality habitat (Itow et al. 1991, Itow 1993). Numerous rice paddies were created just behind some of the dikes, in areas of former tidal marsh not converted to other uses. Status reports on the horseshoe crabs in Japanese waters are published by the Japanese Society for the Preservation of the Horseshoe Crab (Sekiguchi 1989, 1993).

The distribution of *T. tridentatus* in Japanese waters has been summarized by Sekiguchi (1988). The most famous habitat was at Kasaoka City in Okayama Prefecture. There, at Oe-hama Beach on the western shore of Kanaura Cove, egg clusters were once common and the site attracted the attention of many scientists including Ôwatari (1913, 1922), Goto and Hattori (1929), and Oka (1937). Nishii (1975) and Tsuchiya (1980, 1982) have continued studies in the area. The area, designated as a natural preserve to protect horseshoe crabs in 1928 through the efforts of Ôwatari, is now part of a large-scale reclamation project begun in 1969. A large polder enclosed almost all of the former Kasaoka Bay. During our research in 1994 (Botton et al. 1996), Dr. Sekiguchi escorted Dr. Mark Botton and the first author to the former beach. Standing there, trying to envision the past, we had mixed feelings about the new land resource versus the demise of a historically significant horseshoe crab spawning habitat.

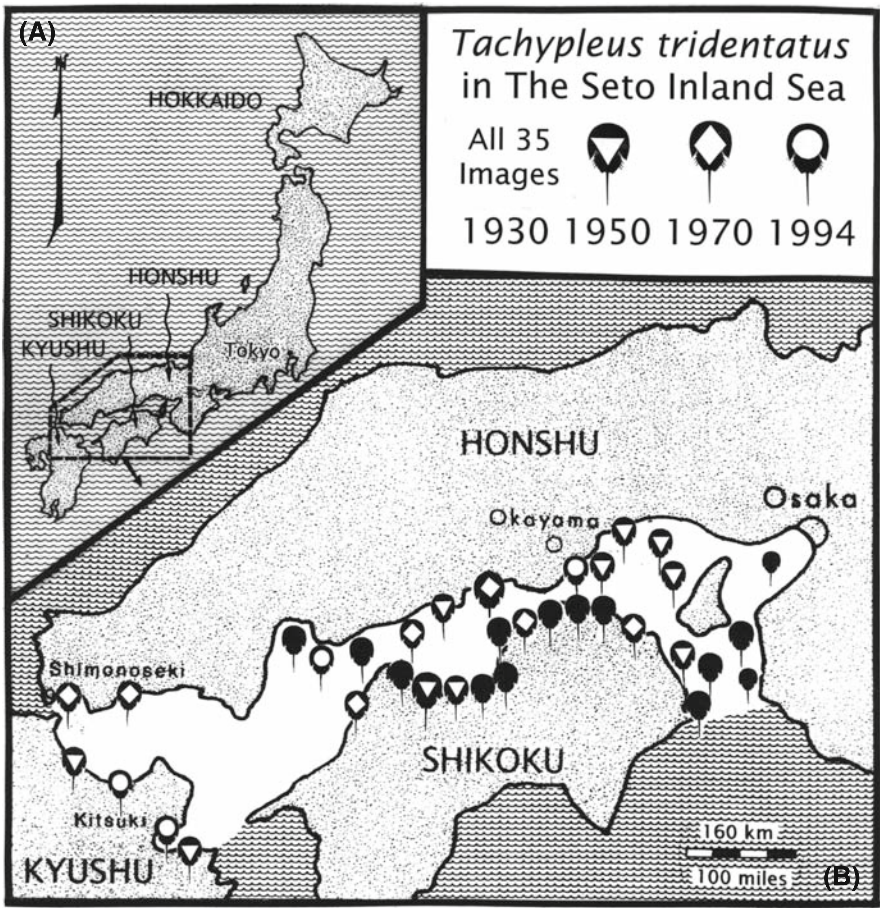

Fig. 2 *Tachypleus tridentatus* once occurred throughout the Inland Sea and south of Osaka Bay and on the northwest coast of Kyushu. (**A**) The main Japanese Islands. (**B**) Outline drawing of the Seto Inland Sea showing recorded sites for *T. tridentatus*, 1930–1994 (combined from four drawings by T. Itow, personal communication). These sites declined from 35 in 1930 (all images) to 21 in 1950 (*triangles*), 11 in 1970 (*diamonds*), and 4 in 1994 (*circles*); the decline first occurred in the eastern portion of its range

3.2 Delaware Bay, USA

Delaware Bay has the largest spawning population of horseshoe crabs anywhere. It is probably axiomatic, therefore, that it has one of the best habitats anywhere. Overall, in examining the big picture, the different habitats occupied by different stages in the life cycle of *L. polyphemus* are abundant and in juxtaposition or near to each other. As a horseshoe crab habitat it is only a few thousand years old, however. Basically, as the last Great Ice Age ended, the

Fig. 3 Part of extensive tide flats at Kitsuki Bay, on the eastern shore of Kyushu, Japan. *Top*: two photographs, combined, give this panoramic view of tidal flats taken from Kitsuki Castle in 1994. *Bottom*: under a bridge, showing details of the dike and a small patch of sand in which horseshoe crab eggs were located; Dr. Sekiguchi is on the *left*

ice melted and sea level rose, the low-lying continental margin was increasingly flooded, creating the continental shelf. When the rivers moved ever nearer to their present positions, they left behind their old valleys and sediments as sea level rose at an average rate of about 3 m each 1,000 years from 8,000 years to 3,000 years ago along the Delaware coast (Belnap and Kraft 1977). Afterward sea level rise slowed down gradually, rising only 5 m in 3,000 years. The finest-grained sediments, mostly mud, were deposited in the shallowest areas of Delaware Bay – the nursery areas of the youngest life stages of *L. polyphemus*. Coarser-grained sediments were deposited in the central part of the bay due to the tidal stirring and tidal activity in the bay (Biggs 1986).

3.2.1 A Moderately Large Estuary

The drainage area of the estuary of the Delaware River is more than 34,600 km^2 and has a greatly varying rate of discharge. During the 20th century the water flow measured at the Trenton, New Jersey gauge ranged from 11 to 17.8 trillion l (1.1–4.7 trillion gallons annually: Sharp 1988). The bay contains about 488 billion m^3 (635 billion cubic feet of water = 4,740 trillion gallons). The bay has an area of some 1,846 km^2 (720 square miles: Shuster 1959).

Fingerlike extensions of the shoal water areas point down-bay, the result of interactions of the tides and river flow transporting and depositing sediments. The expansive shoal at the mouth of the Maurice River probably is former land that was drowned by rising sea level in recent geologic past (Fig. 4). Slightly over 80% of the bay is shallower than 10 m deep, for an accumulative area of 1,490 km^2 (580 miles^2) of a total 1,845 km^2 (720 miles^2).

3.2.2 Extensive Tidal Marshes

Tide marshes contribute to the biological productivity of the Delaware River estuary (Kalber 1959, Daiber and Roman 1988). Both sides of the bay have extensive tide marshes: 13% of Delaware and a larger area in New Jersey. Horseshoe crab activity occurs in marshy areas, as at a juvenile nursery area (Point Judith, Rhode Island) and at some spawning sites at Delaware Bay where they are hidden from sight within an extensive maze of narrow, interconnecting tidal streams in back-bay marshes (Shuster, personal observation).

3.2.3 Long and Sandy Shoreline

The shoreline along its western shore, the State of Delaware, is 89 km (55 miles); that along New Jersey is 29 km (18 miles) longer, largely due to the northeast embayment formed by Maurice Rive Cove (Egg Island flats) (Fig. 4). The total of 206 km (128 miles) is characterized by long stretches of sandy beaches.

3.2.4 Shoreline Habitats as Incubators

The role of a sandy beach or the edges of mangroves as incubators of horseshoe crab eggs is undeniable. Other spawning sites such as intertidal flats, marshy areas, gravel, and shell piles occur less frequently. Because the environment at such sites varies in time and from place to place, it is necessary to study spawning areas, the shore zone, and the hydroclimate, to understand the initial habitat of *L. polyphemus* and the other species, wherever they may be. Sandy beaches can also be refrigerators. Botton et al. (1992) reported that a small component of the trilobite larvae produced in one year may overwinter to the next. This occurs within a 3 m band in the mid-region of a sandy Delaware Bay beach (New Jersey), at sediment depth > 15 cm. Up to 104 live trilobites per m^2 were found throughout the winter and into the spring.

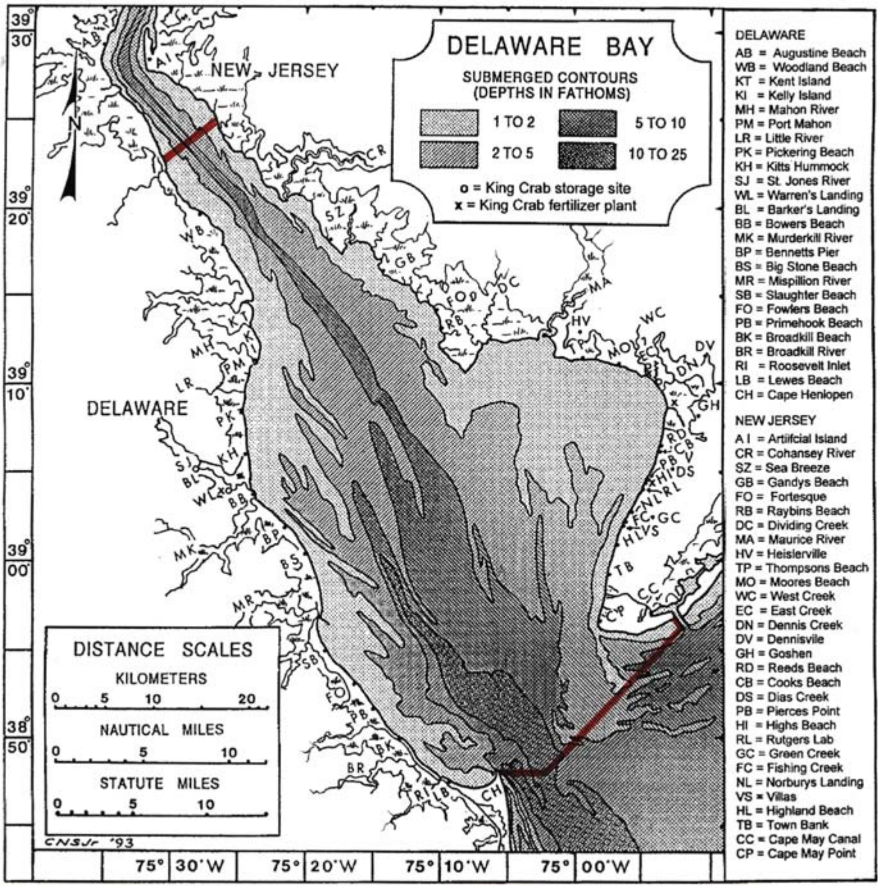

Fig. 4 Tidal marshes and submerged contours of Delaware Bay. Landmarks and sites associated with *Limulus polyphemus* are indicated by *letters* (identified in the column on the *right side* of the chart). Maurice River Cove is the large shoal area (1–2 fathoms) in the northeast corner of the bay along the shore of New Jersey. The area of the bay was calculated as between the mouth of the Smyrna River and the region of Lower Deep Creek in the upper estuary, to the tip of Cape Henlopen to Overfalls lightship to the ocean entrance to the Cape May Canal, the mouth of the bay; marked by *heavy lines* (based on Shuster 1959 and Kraft 1988)

Botton et al. (1988) demonstrated that horseshoe crabs do a certain amount of discrimination in selecting spawning sites. But this is not a fixed axiom; *L. polyphemus* spawns in many unlikely sites despite the apparent ability to discriminate. In Georgia (B Winn, personal communication), crabs spawn in the grass that has grown up in oyster shell piles in the marsh as well as in the soft sand of the bars. Although it is not common, spawning in shoreline shell piles, muddy margins, peat banks, etc., have all been observed in Delaware Bay. In a sense, it seems that when the crabs are ready to spawn they try to do so, adjusting to the local conditions, regardless of the nature of the habitat.

Brady and Schrading (1997) defined four beach characteristics best suited to horseshoe crab spawning as one measure of the feasibility of using channel dredge spoil to replenish beaches of Delaware Bay. They rated the suitability of the habitat as indices in 0.1 increments, from 1 (highest) to 0 (lowest). The most suitable ranges (1 down to 0.8) of the four criteria, measured at the mean high water line, were (1) sand at least 14.5 in. deep over peat; (2) moisture content of sediments 3.7 in. (92 mm) below the surface, from 1.5 to 7.5%; (3) slope of the beach from 4.5 to 9.5%; and (4) sediment grain size at 3.7 in. below the surface from 0.4 to 1.1 mm. The chemistry of beaches may also be involved. A seemingly erratic behavior of spawners on certain beaches led Botton et al. (1988) to the discovery that sensitivity to sulfate emanating from peat beneath a shallow-sand layer on those beaches deterred crabs.

3.2.5 Middle-Atlantic Continental Shelf

This shelf is one of the world's most studied (Stearns 1969). It encompasses a surface area of some 60,000 square nautical miles (1 nautical mile = 1.85 km) of drowned coastal lowland. The shelf varies in width from 24 km (14 miles) off Cape Hatteras, North Carolina, to 190 km (118 miles) of New York. The volume of sediments covering the continental shelf and slope is astronomical (Kraft 1988). All the sediments – sand and mud and even gravel – were eroded from rocky uplands during some 100,000 millions of years and transported by the rivers and deposited at the margin of the ocean. As the weight increased, the edge of the coastal plain and the continental shelf began sinking, filling a basin some 200 miles wide with 45,000 ft of sediments.

After the last ice age that ended some 13,000–11,000 years ago, the former edge of the continent began to be flooded by the rising sea. During the low stand of sea level, ice-fed streams had transported glacial outwash of gravels and sands onto the drowned shelf. Major rivers crossed the shelf in submarine canyons further transporting sediments, mainly modified glacial outwash and moraines. Other features included old shorelines and terraces, submarine river channels and floodplain deposits, deltaic structures, sand ridges, and mobile sand waves (Emery and Schupi 1972). Niches in the resulting habitat are filled with many benthic species, several of which are prey of the horseshoe crab, notably surf clams. The area is also the summertime habitat for juvenile loggerhead turtles, predators of horseshoe crabs and probably the most numerous of the sea turtles in the mid-Atlantic area. Whether *L. polyphemus* is attracted to the shelf by the food resource, a place to spread out, or some other factor(s), it is where a large portion of the Delaware Bay population grows up and migrates to each year. Several age classes of the female crabs are represented on the shelf: large juveniles, those that have recently molted into adults (during the late fall), and the first and second-year spawners. All are protected by the National

Marine Fisheries Service reserve established in 2001 (Fig. 5). This large horseshoe crab sanctuary and the bay-wide moratorium on the harvest of female crabs, parts of the Atlantic States Marine Fisheries Commission Horseshoe Crab Management Plan, are sufficient to insure that there will be sufficient spawning females to provide eggs for the migrating shorebirds on the shores of Delaware Bay.

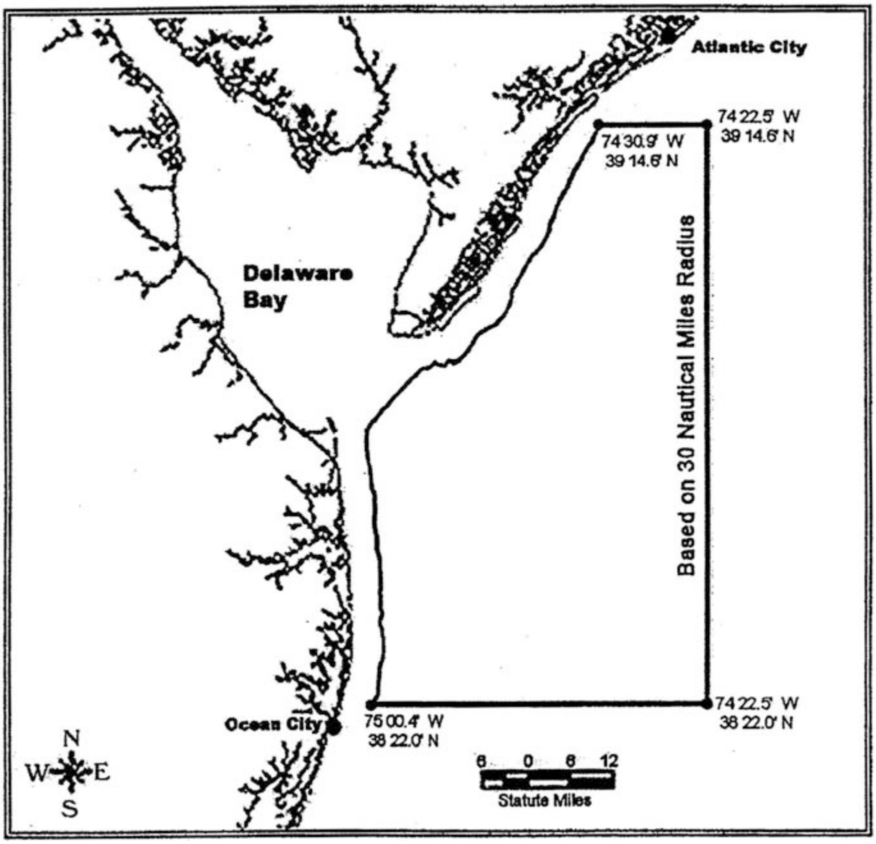

Fig. 5 The Carl N. Shuster Jr. Horseshoe Crab Reserve is a sanctuary about 2.5 times that of Delaware Bay. It was established by the National Marine Fisheries Service in 2001 to protect the reproductive stages of *Limulus polyphemus*, particularly the large females

Changing coastlines and decreasing shallow-water habitats as the glaciers formed would have affected the three Indo-Pacific species, particularly *T. tridentatus* that has a latitudinal range today similar to that of *L. polyphemus*. Considering this geologic history, we assume that the present distribution of the four extant species of Limulacea, particularly the northward extensions of the ranges of *L. polyphemus* and probably also *T. tridentatus*, began after last Great Ice Age. What happened to the genetic composition of the four species during these drastic geologic changes that undoubtedly impacted the habitat and distribution of the four species? And what may be happening? How much will the melt-down of the polar ice cap and attendant changes in the hydroclimate influence the future survival and distribution of the Limulidae? If what we believe happened in the distribution of *L. polyphemus* after the last Great Ice Age, that it expanded its distribution northward from Florida or Georgia to New England as the northern waters warmed, then the species should in time establish breeding populations in more northern climes; *L. polyphemus* in Canadian waters and *T. tridentatus* in more northern waters of Japan and on the coast of Asia. This poses an interesting situation. If migratory shorebirds were feeding on horseshoe crab eggs at that time of the last ice age that probably would have been in the region of Florida–Georgia, and their tundra breeding areas may have been much farther south than they are at present. Would the wintering grounds of the birds been in South America as today? Botton et al. (2003) have also pondered similar questions.

3.2.6 Tides and the Hydroclimate

The maximum ranges and types of tides on shores can be used to characterize spawning areas. For example, populations of *L. polyphemus* exist on the northern shores of Yucatan and along the Florida coast to at least Dauphin Island to the west of the entrance to Mobile Bay. However, there are no spawning populations in the western portion of the Gulf of Mexico. Because all other environmental parameters, food, hydroclimate, sandy beaches, etc., are suitable but the tides are not, it has been deduced that the tides are the limiting factor (Shuster 1979). This may be true for the Indo-Pacific species.

The zonation of water and temperature in a sandy beach, as described by Pollack and Hummon (1971), is an appropriate guide to the distribution of horseshoe crabs nests and survival of the early developmental stages. The zonation of water, as measured by % saturation in beaches, is modified by tidal amplitude (Shuster 1982). Within the Gulf of Mexico the beaches are wetter and present a narrower spawning area due to a 1 m or so tidal amplitude (Brockmann 2003) compared to beaches with greater tidal amplitudes and consequent broader spawning areas, as at Delaware Bay with 2 m amplitudes (Shuster and Botton 1985) and 3 m on Cape Cod (Shuster 1950).

Hedgpeth (1957) described hydrographic climate polygons portraying annual temperature/salinity relationships (plotted as monthly averages). Changes in temperature and salinity regimes from year to year in Delaware Bay have been described by such polygons (Shuster 1960a). Such changes (Fig. 6) probably would have occurred in the water content of the beaches around Maurice River Cove. *L. polyphemus* is active in the bay area during the months from April to November when the water is at least 15°C (59°F) (F. Eicherly, pers. comm.).

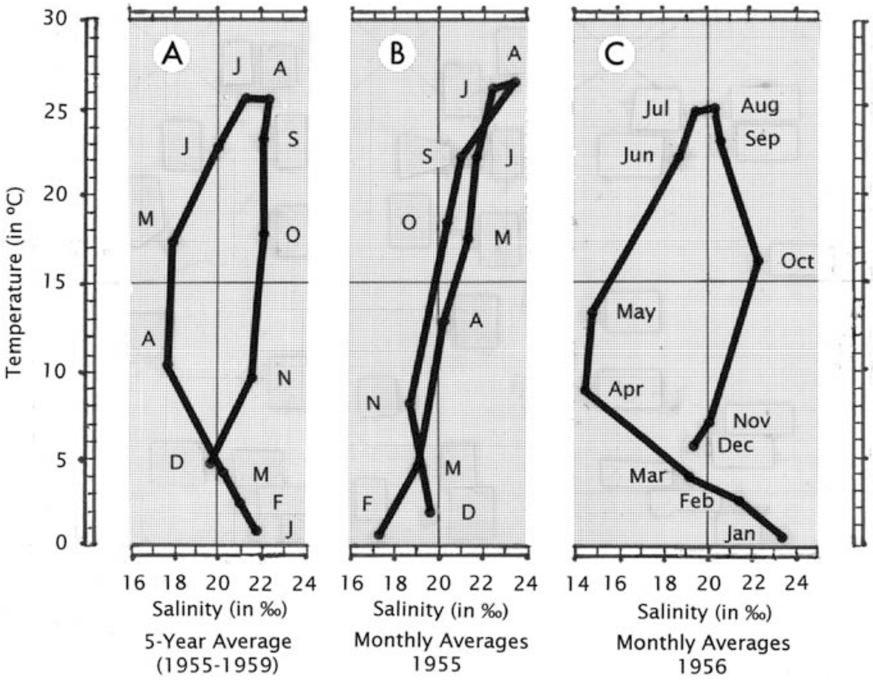

Fig. 6 Temperature–salinity polygons for oyster beds (Bar Grounds) in Delaware Bay: showing 2 years during the period 1955–1959 (**A**) that had markedly different polygons (*B*=1955) and (*C*=1956) (modified from Shuster 1960a). The monthly averages for 1955 (**B**) were almost exactly reversed compared to the 5-year average (**A**). May through August was marked by low river flow and extremely dry weather. Then hurricanes "Connie" and "Diane" passed over the Delaware River basin, in August 12–13 and 19–20, respectively. Higher-than-average river flows during April and May of 1956 (**C**) resulted in a marked decrease in the salinity. Note: the cross-hairs on the graphs designate the 15°C temperature, a threshold at which adult horseshoe crabs are moving about in Delaware Bay (according to bayman Frank Eicherly), and an average salinity of 20%

3.3 Spatial Requirements

The numbers of males accompanying nesting females differs markedly in the four extant species. *L. polyphemus* males typically outnumber the females at least 3:1, whereas the three Indo-Pacific species tend toward single pairs (Brockmann and Smith 2009). Does this mean that greater shoreline strands are required by *L. polyphemus* for the same amount of nesting potential of the other three species because the satellite males take up a lot of space? Or, is the greater egg production by each female *L. polyphemus* compensatory?

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The Relationship Between Small- and Large-Scale Movements of Horseshoe Crabs in the Great Bay Estuary and *Limulus* Behavior in the Laboratory

Winsor H. Watson III, Susanne Y. Schaller, and Christopher C. Chabot

Abstract The overall goal of our research program is to determine the short- and long-term patterns of horseshoe crab (*Limulus polyphemus*) movements in the Great Bay estuary and then seek an understanding of the endogenous and exogenous processes that give rise to these patterns. Small- and large-scale movement data were obtained from 27 horseshoe crabs tracked using ultrasonic telemetry for at least a year. During mating season animals were most active during high tides, but they did not increase their activity or approach mating beaches during every high tide. During the remainder of the year tidal or daily patterns of activity were less evident, and the extent of their movements gradually decreased as water temperatures dropped in the late fall and winter. During the spring, when water temperatures exceeded 10°C, tagged animals moved several km up into the estuary into shallow water ($<$ 4 m) 1 month prior to spawning. A similar temperature threshold was also evident in laboratory experiments, with little rhythmic behavior expressed at temperatures below 11°C. Mating activity lasted approximately 1 month and was followed by a period of high activity. In the fall, most animals moved downriver into deeper water, where they remained during the colder months. Thus, the majority of *Limulus* exhibited a seasonal pattern of movement, remaining within a 3 km stretch of the estuary. In the laboratory, animals expressed both daily and tidal rhythms of locomotion. Those with daily rhythms were more likely to be diurnal than nocturnal, but both tendencies were evident. The clock involved in modulating their locomotory activity appears to be separate from the clock controlling their circadian rhythm of visual sensitivity. When animals were exposed to "artificial tides," created by changing water depth every 12.4 hours, they expressed clear tidal rhythms of activity that were synchronized to the imposed tides. Similar data were obtained from horseshoe crabs in running wheels placed in the estuary. However, if the running wheels were attached to a floating dock, so water depth did not change with the tides, the horseshoe crabs were primarily diurnal. Thus,

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while endogenous biological clocks are capable of controlling many aspects of horseshoe crab locomotion, the actual patterns manifested in the field are strongly influenced by the water depth changes associated with the tides, as well as light levels and seasonal changes in water temperature.

1 Introduction

American horseshoe crabs, *Limulus polyphemus*, offer an interesting and accessible model system for investigating different types of biological rhythms and the environmental factors that influence their expression. Their most well-documented behavior, mating, occurs every year, in the early spring and summer, and appears to be very closely associated with natural tidal rhythms (Rudloe 1980; Cohen and Brockmann 1983; Barlow et al. 1986; Brockmann 2003). They are also known to possess a circadian clock that influences the lateral eyes, making them much more sensitive to light at night (Barlow et al. 1980; Kaplan and Barlow 1980; Barlow 1983). Thus, these animals appear to have one or more biological clocks that are capable of keeping track of the circa 12.4 hour tidal cycle as well as the more typical 24 hour daily cycle (and possibly seasons). Our overall objectives during the past several years have been to determine the following: (1) How far do they move within the estuary and do their movement patterns vary with the seasons in a systematic manner? (2) How much of *Limulus* behavior is under the influence of a tidal clock vs. a circadian clock? (3) What environmental factors determine which biological rhythm they express during different times of the year?

Throughout our investigations we have used a combination of field and laboratory studies to address the aforementioned questions. Field studies are necessary to establish the “normal” behaviors of horseshoe crabs and develop hypotheses about the factors that influence the expression of different types of behaviors. Laboratory investigations allow us to control some of these factors and thus dissect out the specific mechanisms that give rise to various types of biological rhythms and the environmental stimuli that modulate their expression.

In this chapter we will first discuss how ultrasonic telemetry has allowed us to identify the long-term seasonal movement patterns of horseshoe crabs within the Great Bay estuary, NH. We will then provide data from laboratory experiments that document how, and under what conditions, horseshoe crabs will express both daily and tidal patterns of locomotion. Finally, with the foundation provided by our laboratory data, we will examine the fine-scale movements of horseshoe crabs in the field and discuss how and why they express particular types of activity at different times of the year.

2 Seasonal Movements of Horseshoe Crabs in the Great Bay Estuary

The Great Bay estuary in NH, like most estuaries, is characterized by large seasonal fluctuations in temperature and salinity (Fig. 1). It has a large population of horseshoe crabs, but very little is known about their movements and habitat preferences within the estuary. It is likely that, like other mobile estuarine species, such as striped bass and lobsters, horseshoe crabs undertake seasonal movements to optimize their survival under these complex conditions. They may also make use of the salinity and temperature gradients within the estuary to guide their movements, in a manner similar to lobsters (Crossin et al. 1998; Jury et al. 1994, 1995, 2000; Watson et al. 1999; Dufort et al. 2001).

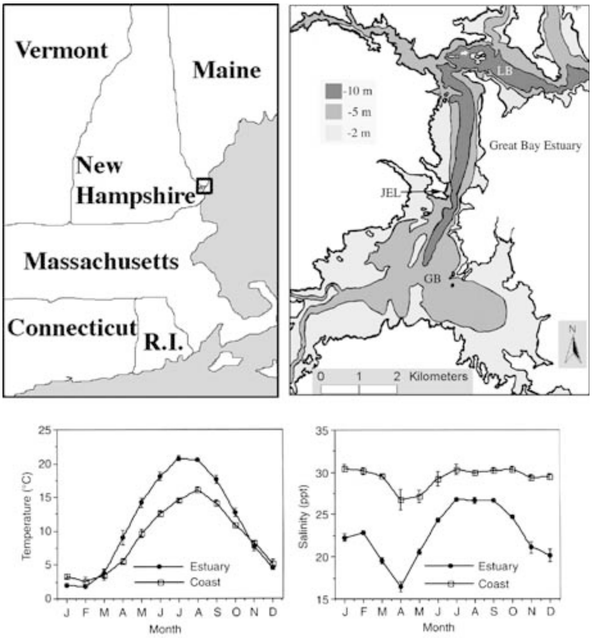

Fig. 1 The Great Bay estuary. *Top left*: Location of the estuary (*box*), which empties into the Gulf of Maine. *Top right*: Enlarged view of the estuary showing depth contours. Depth values are for low tide. JEL=Jackson Estuarine Laboratory. GB=Great Bay, LB=Little Bay. *Bottom panel* shows average monthly temperatures and salinities in the estuary, in the vicinity of JEL, in comparison with values obtained along the NH coast

2.1 *Movements of* Limulus *in the Great Bay Estuary*

Between the fall of 2005 and the spring of 2007 we tracked 27 horseshoe crabs (6 males, 21 females) as they moved around within the estuary. Each animal was fitted with an acoustic transmitter (VEMCO V13L coded tags, 13 mm diameter, 33 mm long, 6 g in water, battery life > 1 year), either internally (*n*=15), in the frontal area, or externally (*n*=12), glued on the dorsal carapace. Animals tagged in the fall of 2005 (*n*=8) were captured in lobster traps in deeper portions of the estuary, brought to the surface, fitted with transmitters, and then released back in the channel (Fig. 2). In 2006 all animals ($n=19$) were fitted with transmitters while spawning at a beach adjacent to the UNH Jackson Estuarine Laboratory.

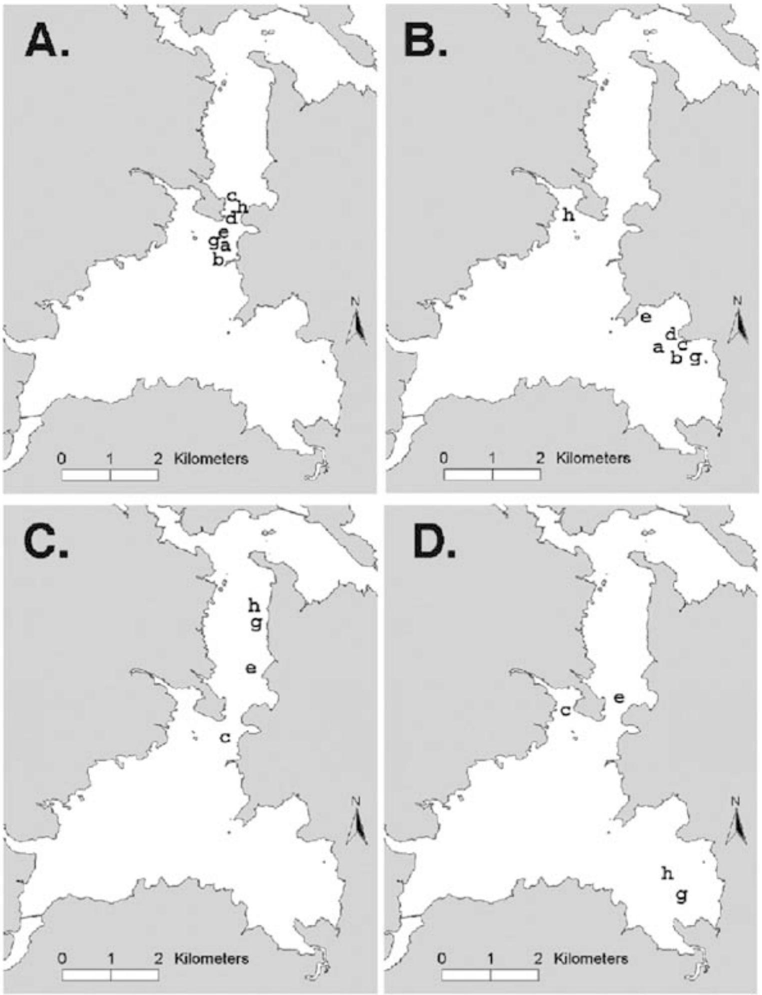

Fig. 2 Seasonal movements of horseshoe crabs in the Great Bay estuary. (**A**) Positions of seven of the eight animals (labeled a–g) tagged in the fall of 2005, during the winter of 2005–2006. They are located primarily in the deep channel (see Fig. 1). (**B**) Positions of those same animals in the spring of 2006. Note how they have moved up the estuary into shallow water. (**C**). Locations of four of the same animals in winter of 2006–2007. (**D**) Positions of those same animals in spring of 2007

Horseshoe crabs were tracked using both VR100 receivers with hydrophones and VR2 listening stations (VEMCO Ltd., Halifax, NS, Canada) deployed throughout the estuary. Each time a horseshoe crab fitted with a coded transmitter moved within the range (approximately 300 m) of a VR2, the ID of the animal, time, and date were logged. If animals were fitted with depth/temperature transmitters, those data were also logged. In contrast, the VR100 was used to search the estuary for animals on a weekly basis, from May to October and monthly basis during the colder months. When animals were located, all positions were recorded in GPS coordinates and data were analyzed and mapped using ArcView.

After being tagged in the fall of 2005, most of the horseshoe crabs moved very little during the late fall and winter and were primarily located in the deepest parts of the estuary (10–15 m; Fig. 2). Then, between April 18 and the middle of May, seven of the eight horseshoe crabs moved upriver into much shallower water (2–4 m) (Fig. 2). The following year (2006–2007) a similar pattern was observed, both with 7 of 8 of the original animals from 2005 and the additional 18 animals tagged in the spring of 2006. They spent the cold months in deeper water, downriver from spawning locations, and then moved up into the estuary 2.3 ± 0.5 km (mean ± SE) in April and May when water temperatures exceeded 10.5°C (Fig. 2). In May of 2007 it was remarkable to observe how, during a short 2-week period, all nine of the animals that over-wintered in the lower portion of Little Bay moved at least 1 km upriver. While all animals may have been responding to seasonal changes in ambient light levels, or an internal circannual clock of some type, we favor the hypothesis that increases in activity were triggered by the rapid warming of the water that typically occurs each spring at this time.

Movements up into the estuary in the spring typically resulted in most animals moving into much shallower water about a month prior to spawning (compare positions of animals in Fig. 2, with the depth profile in Fig. 1). Of the 19 animals tagged while spawning in the spring of 2006, 7 returned to the Jackson Estuarine Laboratory (JEL) and likely spawned there, 5 moved past JEL, and the remaining animals were not relocated in the spring. Following spawning in May and June, animals became quite active in the summer (July–August), moving a mean distance of 7.3 ± 1.5 km and residing in a range of depths. It is likely, but not confirmed, that animals are searching for and consuming a great deal of prey during this time of year.

Animals began to decrease their activity in September and this trend continued throughout the fall (Fig. 3). The average distance traveled declined to 3.0 ± 0.6 km in September and October as animals moved into wintering sites, and there was also a tendency to reside in the deeper channels and along the banks of the channels. From December 2006 through March of 2007 animals traveled very little (0.6 ± 0.1 km) and remained in deeper water (range 2.2–22.2 m, median 16.3 m). This tendency to be very inactive in the winter was confirmed using the fixed array telemetry system described in a subsequent portion of this review. We were able to continuously track the same three animals for more than 3 weeks and there was no apparent movement by any of them during this time.

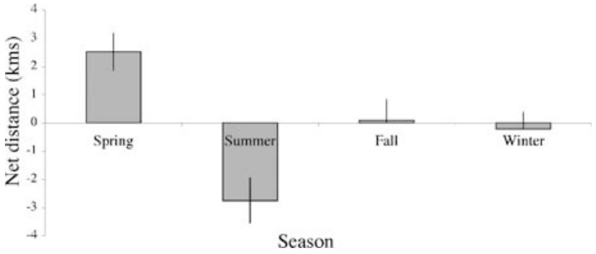

Fig. 3 Net distance moved by horseshoe crabs tagged in 2005 in Great Bay during time periods indicated in 2005–2007. These calculations were made by designating the position of each animal at the beginning of a given time period, such as the spring, as zero. Movements of the animal up the estuary, relative to this position were given positive values. Movements down the estuary, toward the coast, were given negative values. All movements by each animal were totaled to give a net movement per animal and then these values were averaged to obtain a mean for the population

3 Expression of Tidal and Circadian Rhythms in the Laboratory

Limulus reliably express both daily and tidal rhythms of locomotion in the laboratory (Fig. 4, Chabot et al. 2004, 2007). While most animals express either tidal (approximately 12.4 hour) or diurnal (24 hour cycle, active in the day) pattern of activity, some animals were nocturnal. Interestingly, when we monitored the sensitivity of one of the lateral eyes to light (electroretinograms, ERGs), while simultaneously recording locomotor activity, we found that patterns of locomotion were independent of visual sensitivity (Fig. 5; Watson et al. 2008). Thus, the circadian clock that enhances the sensitivity of the eyes at night (Barlow 1983) does not appear to play a role in modulating locomotion.

Locomotion is influenced by a number of factors, including light, changes in water depth, and water temperature. Of these, water depth appears to have the greatest impact. When animals in the laboratory are exposed to artificial "tides," with 12.4 hour changes in water depth of as little as 25 cm, the majority synchronize their activity to the imposed cycles (Fig. 6; Chabot et al. 2008, Watson et al. 2008). Moreover, once the artificial tides are stopped, animals will continue to express a tidal rhythm in phase with the imposed tides, indicating that the artificial tides are entraining an endogenous tidal clock.

The activity cycles of some horseshoe crabs also correlate quite well with changes in light levels (Fig. 4; Chabot et al. 2008; Watson et al. 2008). When exposed to 14:10 LD cycles some animals have a tendency to be most active in the day and some at night (Fig. 4). However, many horseshoe crabs will also spontaneously express tidal rhythms under LD cycles, indicating that under LD conditions tidal rhythms are not completely suppressed. Moreover, on occasion, an animal expressing a tidal cycle will briefly become synchronized to dawn and dusk periods (Chabot et al. 2007; Watson et al. 2008), suggesting

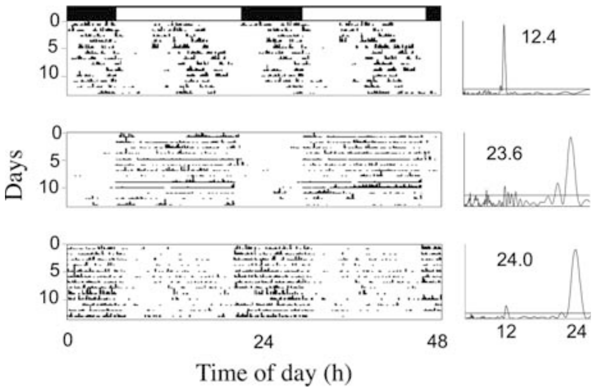

Fig. 4 Three types of activity patterns expressed by horseshoe crabs in the laboratory. All recordings were obtained from male horseshoe crabs walking inside a *Limulus* "running wheel" (Chabot et al. 2004, 2007). Data are double-plotted to facilitate visual recognition of patterns. The light dark paradigm (14:10, LD) is presented on the *top* of the figure. *Right panels* – Lomb–Scargle periodogram analyses of respective actogram sections, and the *numbers* indicate the dominant period. The *top panel* illustrates a tidal rhythm, the *middle panel* a diurnal rhythm, and the *bottom panel* is data taken from an animal that was nocturnal

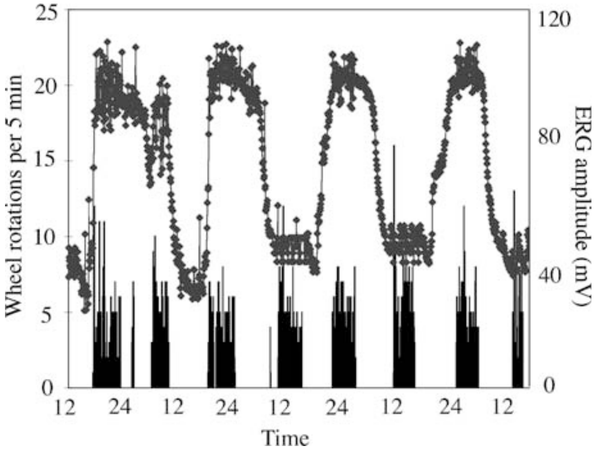

Fig. 5 Circadian rhythm of eye sensitivity and tidal rhythm of locomotion recorded simultaneously from the same horseshoe crab in constant dim illumination. Electroretinograms (ERGs) were obtained from one of the two lateral eyes using a clear plastic cup filled with seawater as the active electrode. Light pulses were delivered to the eye every 30 seconds, using a green LED glued inside a black plastic cup that was secured over the eye with electrode. This arrangement made it possible to simultaneously record ERGs while animals walked inside a plastic running wheel (details of running wheel in Chabot et al. 2007). Sensors on the wheel enabled us to record wheel rotations. It appears that locomotion is being driven by an endogenous tidal clock while visual sensitivity is under the control of a circadian clock

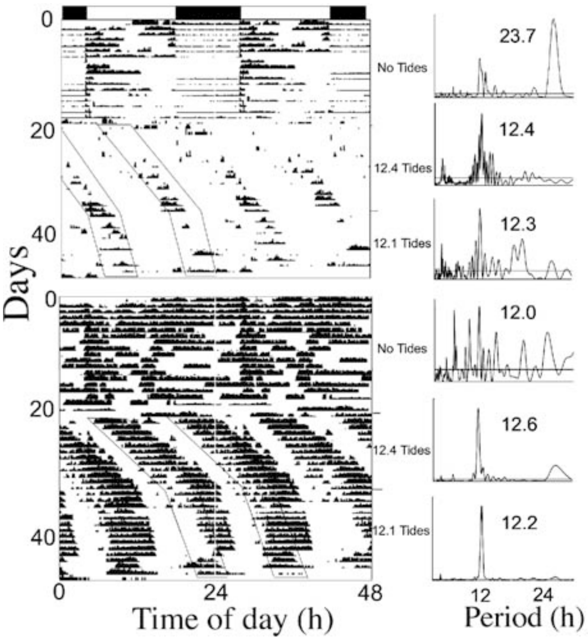

Fig. 6 The effects of "artificial tides" (water depth cycles) on the locomotor activity of two horseshoe crabs. LD cycle indicated by *black/white bars* at *top*. Periods of time when the water depth changed with a tidal cycle, delivered at periods of 12.4 and 12.1 hour, are indicated by *open boxes* on the actograms. Water pressure began to increase at the time indicated by the *left side* of the *boxes*. Maximum pressure occurred by the time indicated by the *right side* of the *boxes*. *Right panels*: Lomb–Scargle periodogram analyses of respective actogram sections. Largest peak value above horizontal line of significance ($P>0.01$) indicated by *numerical value*

that light can even have some influence over the expression of tidal rhythms. The complex ways that light and tides work together to modulate the activity of horseshoe crabs is certainly an area that is worth pursuing in the future.

Horseshoe crabs seem to exhibit different types of activity during different times of the year. They are typically quite inactive in the winter, they mate and express tidal rhythms in the spring and early summer, and then they move into deeper water and are fairly active during the remainder of the year (we do not know what types of activity cycles they express during these times of year). We have been trying to determine what environmental cues might cause them to switch from one pattern of activity to another, and the most logical choices are light and water temperature. When horseshoe crabs were exposed to warm water the majority of them expressed tidal rhythms of activity, even if the L:D cycle was 9:15, typical of winter (Chabot et al. 2008). In contrast, if the water temperature was reduced below 12°C, they did not express a tidal rhythm of locomotion, even if they were exposed to a L:D cycle of 14:10, typical of the spring and summer (see additional data on this subject in Section 4.2) This supports the hypothesis, expressed earlier, that in NH when water temperature rises rapidly in the spring and exceeds 10–12°C, horseshoe crabs become quite active and start moving toward areas where they typically breed.

4 Expression of Tidal and Daily Rhythms of Activity in the Field

While it is generally assumed that horseshoe crabs tend to be more active at night, especially during nighttime high tides, our laboratory data indicated that animals with a daily pattern of activity were just as likely to be diurnal as nocturnal (Chabot et al. 2004, 2007). In order to test this theory in the field one of our first studies involved conducting *Limulus* surveys at two different mating beaches in the Great Bay estuary, in the spring of 2006. We found no statistically significant difference in the number of horseshoe crabs observed mating during the day vs. the night (Fig. 7). In Adam's Point Cove, we observed 24.7 ± 6.1 (mean ± SEM, n=24 days) animals along a 50 m transect during the day and 32.4 ± 6.8 animals during the night (P=0.3119; paired t-test, df=23). The results from a nearby beach (0.5 km away) were similar (27.5 ± 5.7 *Limulus* during the day and 35.4 ± 9.8 during the night; P=0.4381; df=23). The small tendency for more animals at night than during the day might be due to either the influence of light or the fact that in this location nighttime high tides tend to be higher than daytime high tides.

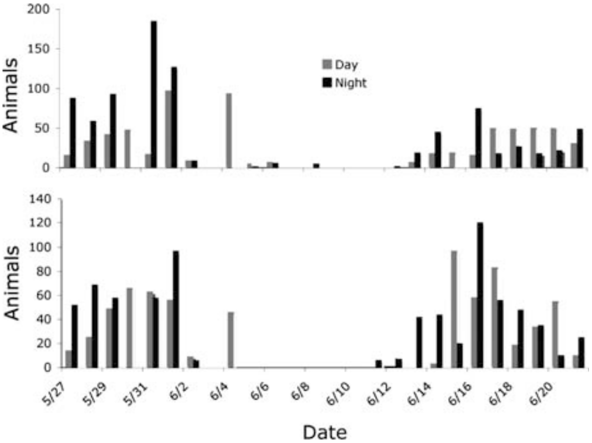

Fig. 7 Day and night counts of spawning horseshoe crabs at two different locations. Note that on any given day, the numbers of animals mating at night and during the daytime are either similar or vary in an unpredictable manner. The drop in counts from 6/2/06 to 6/13/06 was due, in part, to a drop in salinity as the result of heavy rains

4.1 High-Resolution Tracking Using Ultrasonic Telemetry

The primary study site for investigations of small-scale horseshoe crab movements in the field was a cove near the Jackson Estuarine Laboratory (Fig. 1). We fitted 18 horseshoe crabs with ultrasonic transmitters while they were mating and then tracked them with a fixed array telemetry system (VRAP, VEMCO Ltd) for as long as they remained in the vicinity of the array. The VRAP system is capable of continuously monitoring the position of horseshoe crabs with an accuracy of approximately1–2 m, depending on the position of the animal relative to the buoy array, noise levels in the area, and habitat (Golet et al. 2006). In this particular situation, the system worked very well, except for the difficulty of accurately obtaining positional fixes when horseshoe crabs moved into very shallow (0–1 m) water while mating.

Of the 18 animals tracked in the spring of 2006, we obtained good data from 17 for an average of 2.5 days each (range of 1–6). The majority (14) returned to the same cove to spawn during the high tides subsequent to being fitted with transmitters. However, they did not approach the beach at every high tide. Between tides they returned to deeper water (approximately 5–8 m), but rarely sought the deepest water available (within 100 m of the mating beach the water depth was > 20 m; Fig. 8). Overall, during the mating season, lasting from late May until the end of June, the six animals for which data were available for at least four high and four low tides were significantly more active during high tides than low tides (P=0.0079, df=5; Fig. 9A). We also determined, for those same six animals, the percentage of each high or low tide when they were completely inactive. While the trend was to be more inactive during low tides, it was not statistically significant (P=0.1089, df=5; Fig. 9B). Interestingly, while there was a trend to be more active in the day than night, it was not statistically significant (unpaired *t*-test, P=0.536, df=32; Fig. 9C). Thus, at least during the mating season, our field data confirm our findings in the laboratory, indicating (1) a strong tendency to be active during high tides and (2) only a weak, if any, influence of LD or a circadian clock on the control of locomotion. Rather, it is possible that the circadian rhythm of eye sensitivity evolved to allow horseshoe crabs to see equally well whether they are active during the day or night. This is particularly adaptive during the mating season, when they must be active during both the day and night if they are driven to breed during each high tide.

4.2 The Influence of Depth and Temperature on Activity Rhythms in the Field

During the late fall, winter, and early spring when water temperatures were below about 10°C, horseshoe crabs in the field were very inactive (Fig. 3). Then, as the estuarine waters warmed up rapidly in the spring they become much more active and began migrating up into the estuary and into shallower areas. We

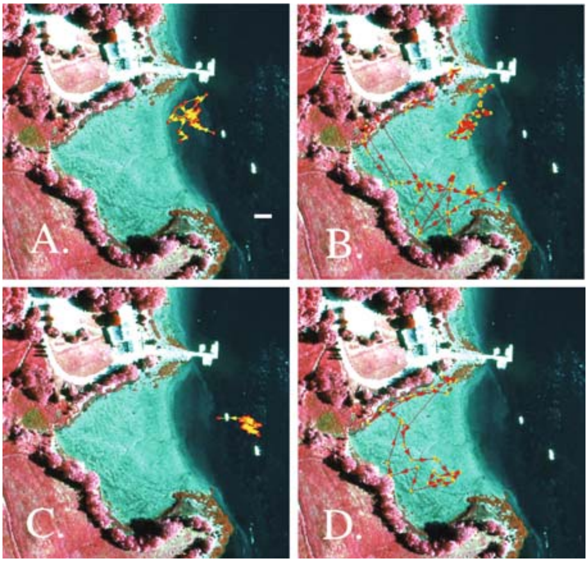

Fig. 8 Activity of a female horseshoe crab during high and low tides. Points were obtained with a fixed array telemetry system approximately every 5 minutes and 2 hours of data are presented in each panel. Points are displayed on an infrared image of the area around the Jackson Estuarine Laboratory, NH. Land and foliage are red, shallow water is light blue/green and deeper water is dark blue/green. This animal was fitted with an ultrasonic transmitter on 6/16/06, while spawning at high tide on the beach along the northern shore of the cove (near where the dock meets the shore). During low tide, around midnight, it was relatively inactive, in about 5 m of water (**A**). The following high tide (6:40 AM), the next day, it returned to spawn and was much more active (**B**). Then it moved into deeper water (approximately 7–8 m) and was inactive during the next low tide (**C**), followed by high activity and spawning during the following high tide (7:30 PM); (**D**). *White scale bar* in panel A is 20 m

propose that the combination of increases in water temperature and moving into shallower water, where they can better sense the changes in depth associated with tidal cycles, trigger the expression of a tidal pattern of activity. Our preliminary data (Fig. 10) suggest that tidal rhythms are not expressed in the laboratory below about 11°C (which is consistent with the threshold for increased activity in the spring). When they move into shallow water it leads to both increases in water temperature and larger relative changes in water depth. To test the theory that animals readily express tidal rhythms in shallow water in the field, we recently recorded the activity of eight horseshoe crabs in running wheels similar to those used in the laboratory, in approximately 3 m of water near the Jackson Estuarine Laboratory (Fig. 11). There was a clear tendency for these animals to be active during high tide. To determine if the primary tidal zietgeber entraining animals to the natural tides cycles was changing water depth, or other factors associated with the changing tides, such as

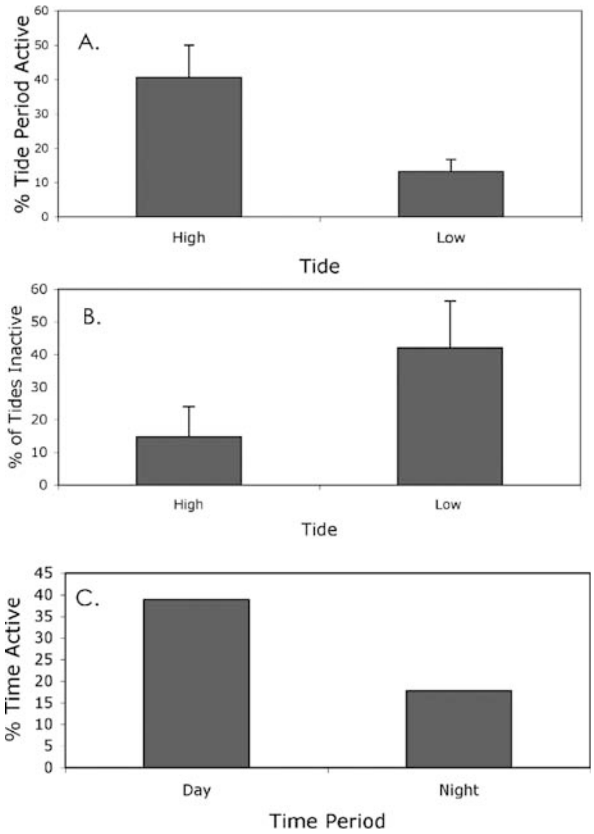

Fig. 9 The activity of *Limulus* in the field during different phases of a tide cycle (**A** and **B**) and during the day vs. night (**C**). These data were obtained from analysis of the movements of 17 animals tracked for periods of time ranging from 1 to 6 days. Positional fixes were obtained about every 5 minutes for each animal. Tracks for individual animals were played back and observers determined the time periods when animals were clearly moving significant distances in a given direction (small movements were not easily distinguishable from background noise due to positioning errors) or clearly inactive. These data were used to calculate the percentage of each 6 hour high or low tide when animals were either active (**A**) or inactive (not moving at all, **B**). Values do not necessarily add up to 100% because there were time periods when movements were ambiguous (approximately 50% of the time in (**A**) and (**B**) and 35% of the time in (**C**)). For the tide analyses (**A**, **B**), only animals that were active for at least four high and four low tides were used (n=6). The day vs. night analyses were carried out with all 17 animals. Differences were statistically significantly different in (**A**), but not in (**B**) and (**C**)

fluctuations in salinity or temperature, we also placed animals in running wheels under a floating raft. In that situation the raft went up and down with the tides so there was no change in depth, even though animals were exposed to fluctuations in temperature, salinity, and other variables. Under these circumstances, most of the animals ignored the tidal cues and were most active during

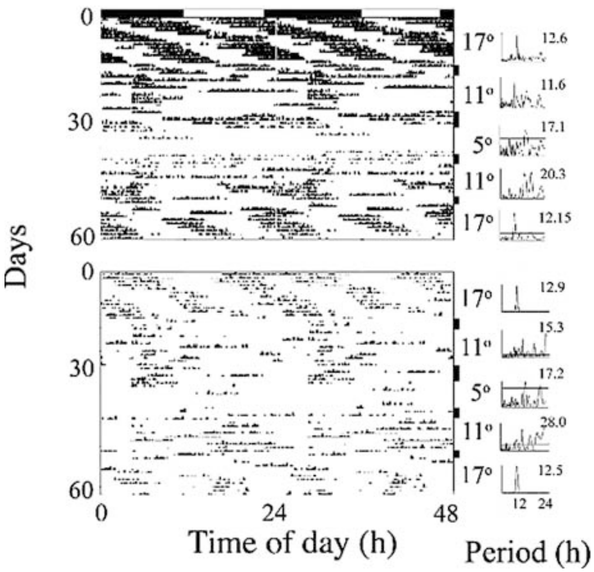

Fig. 10 Circatidal rhythms in two different male *Limulus* exposed to three temperatures and a 15:9 (summer) LD cycle. LD cycle indicated by black and white bars at top. Periodograms and plots are similar to Figs. 4 and 6. Notice how robust tidal rhythms of activity are only expressed when the temperature is 17°C

the day (Fig. 11). Thus, in the field, as well as the laboratory, light, temperature, and the changes in water depth typically associated with the tides all influence both the extent of horseshoe crab movements and their tendency to express a given pattern of movement. Temperature appears to be permissive, allowing rhythms to be expressed once a certain temperature threshold is exceeded. However, while the temperature in Great Bay changes by as much as 5°C during each tide cycle, these temperature fluctuations do not appear to be sufficient to entrain the tidal rhythm of horseshoe crabs (Fig. 11). In contrast, the depth changes associated with each tide have a profound impact on expression of tidal rhythms. Horseshoe crabs will also synchronize their activity to LD cycles, if tidal cues are weak. Thus, *Limulus* provides a very interesting model system for investigating the way that different environmental signals influence the expression of endogenous rhythms.

Temperature and other variables that change with the tides have also been shown to impact circatidal rhythms in other intertidal species. Temperature changes associated with the tidal cycle are sufficient to entrain several crab species (Williams and Naylor 1969), as are pressure fluctuations for both crabs and fish (Naylor and Atkinson 1972; Abelló et al. 1991; Northcutt et al. 1991). Salinity changes appear to be effective for estuarine crabs (*Callinectes sapidus*; Forward et al. 1986), but not for green crabs (Palmer 1995). Finally, periodic agitation is sufficient to entrain tidal rhythms in two species of isopods (Klapow 1972; Hastings 1981). It is interesting to note that a recent paper indicates that agitation cycles are also effective entraining agents for juvenile horseshoe crabs (Ehlinger and Tankersley 2006). In adult *Limulus,* however, water depth changes appear to be more effective than turbulence, temperature, or salinity

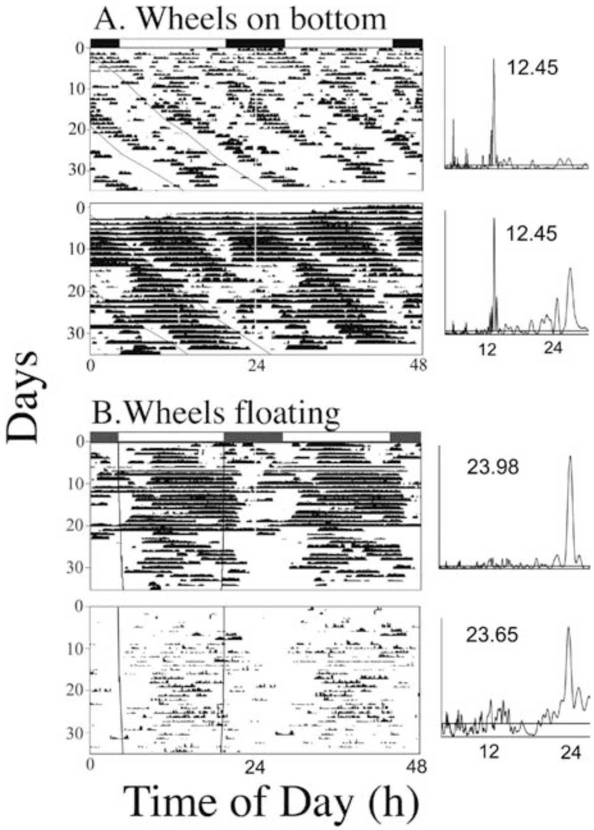

Fig. 11 Double-plotted actograms (*left panels*) of locomotor activity of male horseshoe crabs in running wheels in the estuary. (**A**) One set of animals was anchored on the *bottom* of the estuary, in 4 m of water, and they were subjected to natural tidal pressure changes (approximately 3 m, 300% from low to high). The majority of these animals synchronized their activity to the natural tide cycles. (**B**) The other set of animals were in running wheels secured underneath a floating dock, so they experienced natural tidal cycles, but no change in depth. *Black* and *white bars* at the top signify the approximate photoperiod; *angled lines* in the actogram signify mean high tide. Lomb–Scargle periodograms of the actograms are presented in the *right panels*. Values indicate highest significant peaks of activity above *horizontal line* of significance ($P < 0.01$)

cycles (Chabot et al., 2008). Barlow et al. (1986) proposed that the number of animals mating during any given high tide is proportional to the relative height of that tide. Taken to the extreme, in areas where there is little change in water depth with the tides, such as in the Indian River estuary in Florida, mating is not very well synchronized to the tides. Furthermore, evidence supporting the view that most mating activity occurs during the new and full moons comes primarily from certain areas in Florida where significant changes in water depth may only occur during this period of the month (Rudloe 1980). Yet, despite all these data, we still know little about how horseshoe crabs, or any marine invertebrates for that matter, detect changes in water depth (Morgan 1984; Macdonald and Fraser 1999; Fraser 2006). This is certainly an area worthy of future investigation.

5 Summary and Conclusions

Horseshoe crabs express a number of biological rhythms in the field and the laboratory. They possess at least two endogenous biological clocks, one tidal and one circadian. The tidal clock influences locomotion and is readily entrained by the depth changes associated with tidal cycles, but not the associated fluctuations in temperature and salinity. The circadian clock controls eye sensitivity, but it does not appear to have an impact on locomotion. Nevertheless, patterns of locomotion are influenced by LD cycles, with some horseshoe crabs expressing nocturnal patterns of behavior, while others are diurnal, both in the laboratory and in the field.

The combination of endogenous clocks, and sensitivity to natural stimuli, results in the patterns of activity observed in the field. In the winter, when temperatures are low, animals move very little and they tend to inhabit deeper regions of the estuary. When water temperatures rise above about 10°C in the spring, animals become more active, they tend to move further up into the estuary, and often they migrate into shallower areas adjacent to breeding beaches. In shallow water the natural fluctuations in water depth, associated with the tides, synchronize and entrain the endogenous tidal clock so that animals are not most active at high tide. Following cues that are poorly understood at the present time, they find and approach breeding beaches at high tide. At low tide they move into moderately deep water and are primarily inactive at this time. When mating season is over, activity remains high in the summer and it is not clear what rhythms they express the most. As the water temperature drops in the fall, they move into deeper water to spend the winter. While our combination of laboratory and field studies has provided some insight into the behavior of horseshoe crabs in their natural habitat, and the internal and external forces that guide this behavior, many questions remain to be resolved.

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Ecology of Horseshoe Crabs in Microtidal Lagoons

G.S. Ehlinger and R.A. Tankersley

Abstract The American horseshoe crab, *Limulus polyphemus,* typically inhabits estuaries and coastal areas with pronounced tides that have a significant influence on their physiology, behavior, and ecology. Most notably, changes in water level associated with the tides have been shown to underlie temporal and spatial patterns in spawning. However, horseshoe crabs also inhabit areas that lack significant tides and associated cues that are known to mediate reproduction. Our investigations of the potential impact of conditions in microtidal lagoon habitats on the timing and frequency of spawning of *L. polyphemus* adults and the development and survival of embryos and larvae have focused on populations inhabiting the Indian River Lagoon (IRL), a large, shallow estuary along the east coast of Florida, USA. Extensive surveys of adult activity along beaches indicated that spawning occurs year-round but is aperiodic. Larval abundances in the plankton are low and more closely associated with changes in water level than adult spawning activity. Laboratory experiments indicated that embryos and larvae are able to tolerate extreme salinities and temperatures that occur in temperate and tropical microtidal lagoons in the summer. Embryos complete development and molt at salinities below 60, yet fail to develop at temperatures $\geq$ 35°C. Larvae tolerate salinities of 10–70, but survivorship declines significantly at more extreme salinities (i.e., 5, 80, and 90). Results indicate that both temperature and salinity influence the rate of development, but only the extremes of these environmental factors have an effect on survival. In general, the reproductive ecology and early life history of *L. polyphemus* in microtidal lagoons differ markedly from those documented for populations inhabiting tidal areas. This difference is likely due to extreme salinity and temperature conditions during summer reproductive periods and the lack of tidally related synchronization cues for spawning and regular, periodic beach inundation.

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1 Introduction

The American horseshoe crab *Limulus polyphemus* (Linnaeus) is a common inhabitant of the sublittoral zone of estuaries throughout the Atlantic and Gulf coasts of North America. Throughout much of its geographic range, spawning and larval hatching occur synchronously with tidal and lunar cycles (Rudloe 1979, 1980, 1985, Botton et al. 1988). Details of their reproductive biology are best known for populations inhabiting Delaware Bay and the Gulf Coast of Florida, USA (Cohen and Brockmann 1983, Rudloe 1985, Shuster and Botton 1985, Botton et al. 1988, Penn and Brockmann 1994). In these areas, mating occurs in the spring and early summer when females migrate to shallow areas during spring tides to spawn on sandy beaches (Cohen and Brockmann 1983, Rudloe 1985, Shuster and Botton 1985, Botton et al. 1988, Penn and Brockmann 1994). Peak spawning generally occurs near the time of high tide during new and full moons and appears to be under endogenous control (Rudloe 1980, Cohen and Brockmann 1983, Barlow et al. 1986). Eggs develop in the sand, hatching into trilobite larvae approximately 24–28 days after fertilization (Botton and Loveland 2003). Most trilobites emerge and enter the water column when inundated during spring tides (Rudloe 1979, 1980, Jegla and Costlow 1982, Laughlin 1983, Sugita 1988, Penn and Brockmann 1994). Newly hatched trilobite larvae exhibit a diurnal vertical migration pattern, with larvae remaining buried during the day and entering the water column at night (Rudloe 1979, 1980).

Timing of spawning and larval hatching of horseshoe crabs varies among areas with different tidal regimes (Cohen and Brockmann 1983, Rudloe 1985, Shuster and Botton 1985, Botton et al. 1988, Penn and Brockmann 1994). While seasonal and tidal patterns in spawning have been well documented for *L. polyphemus* inhabiting areas with semi-diurnal tides (Shuster 1982, Rudloe 1985, Barlow et al. 1986, Shuster et al. 2004), horseshoe crabs along the Gulf Coast of Florida that experience diurnal tides exhibit a spawning pattern that is consistent with local tides (Rudloe 1979, 1980, 1985, Cohen and Brockmann 1983). However, horseshoe crabs also occur in microtidal lagoons that lack regular tidal changes and periodic shoreline inundation.

Limulus polyphemus generally occurs in estuaries where the salinity ranges from 5 to 34. While *L. polyphemus* densities are highest in euhaline areas near the mouths of estuaries, they also inhabit oligohaline regions (Shuster 1982). Although adult and juvenile *L. polyphemus* live in subtidal benthic habitats, embryonic and early larval development occurs in intertidal areas. Adult *L. polyphemus* nest near the waterline in the mid- to upper intertidal zone and deposit up to 20,000 eggs 10–25 cm below the sediment surface (Shuster and Botton 1985, Brockmann 1990, Penn and Brockmann 1994). Horseshoe crabs deposit their eggs in the mid to upper beach where conditions are optimal for egg development since the sediments at higher elevations are warmer and drier and those at lower elevations are often anoxic (Penn and Brockmann 1994). Nests are laid in sandy areas that are regularly inundated in tidal systems, but have variable frequencies and periods of inundation in microtidal areas (Rudloe 1985, Penn and Brockmann 1994). Nevertheless, because of the location of the nests on the beach, *L. polyphemus* embryos and larvae are potentially exposed to larger fluctuations in temperature and salinity than those experienced by subtidal adults. During low tide, nests may be exposed to freshwater during periods of rain and experience rapid changes in temperature as a result of heating of the beach by sunlight. Thus, developing embryos are expected to be capable of tolerating rapid and wide fluctuations in environmental conditions.

Adult horseshoe crabs also inhabit microtidal lagoons that are characterized by a much broader salinity range (5–55) due to shallow conditions and high rates of evaporation and freshwater input (Pritchard 1967, Robertson 1970, Shuster 1982, Botton et al. 1988, Ehlinger et al. 2003). In estuarine habitats of New England and the mid-Atlantic region, low salinities caused by snow melt and freshwater run-off are more common than high salinity conditions. Therefore, most studies conducted on the effects of salinity on embryonic development have focused on tolerance of hypoosmotic stress. Optimal salinity range for larval development is between 20 and 30 (Jegla and Costlow 1982, Laughlin 1983, Sugita 1988). Development time of embryos to the larval stage (trilobite larva) increases at salinities above and below this range (Jegla and Costlow 1982, Laughlin 1983, Sugita 1988). Temperature has also been found to affect the rate of embryonic development and the duration of posthatch intermolt stages, with the optimal temperature for development ranging between 25 and 30°C (Jegla and Costlow 1982).

Placement of *L. polyphemus* nests in the upper littoral zone of sandy beaches affords developing embryos protection from aquatic predators, with most surviving to hatching (Rudloe 1979). However, this nesting pattern creates problems for the emergence and dispersal of larvae into the aquatic environment. Embryos hatch into trilobite larvae in approximately 28 days and emerge into the water when inundated during nocturnal high tides near the time of full moon (Rudloe 1979, Penn and Brockmann 1994). However, because of the wide geographic distribution of *L. polyphemus*, tidal conditions experienced by developing embryos vary significantly. In microtidal lagoons, the frequency of nest inundation varies throughout the species' range and is not always predictable. Given the diverse range of shoreline inundation patterns, hatching of *L. polyphemus* eggs may be facilitated by environmental triggers that help synchronize larval hatching with water levels on the beach, especially in nontidal areas. If an environmental cue associated with inundation triggers hatching of *L. polyphemus* larvae, then larvae will hatch and emerge when conditions are favorable for entry into the plankton.

In tidally dominated estuaries and coastal habitats, periodic (tidal) changes in water level help maintain optimal conditions for horseshoe crab embryonic development (Penn and Brockmann 1994). However, in shallow microtidal estuaries, submergence of nesting areas on the beach depends on freshwater input and wind forcing. Therefore, spawning and nesting activities may be restricted to periods when wind events result in significant increases in water level. Moreover, the lack of regular beach inundation may cause conditions within the sediments to be unsuitable for embryonic development, hatching, or larval emergence. Thus, the paucity of tidally related synchronization cues and regular beach inundation is hypothesized to have a significant impact on the mating activities of adult crabs and the development and hatching patterns of larvae.

Our investigations of the potential impact of conditions in microtidal lagoons on the timing and frequency of spawning of *L. polyphemus* adults and the development and survival of embryos and larvae have focused on populations inhabiting the Indian River Lagoon complex (IRL). The IRL consists of three shallow (1–3 m deep) sub-basins, the Indian River, Banana River, and Mosquito Lagoon, that extend approximately 250 km parallel to the Atlantic coast of Florida (Smith 1987, De Freese 1991). Although significant tidal changes occur in the immediate vicinity of the five inlets that link the IRL to the ocean, most of the system is virtually tideless (tidal amplitudes < 5 cm), with wind forcing and freshwater input responsible for changes in water level (Smith 1993).

Like many microtidal lagoons, the IRL experiences temperature and salinity conditions that are more extreme than those found in most estuaries (Pritchard 1967, Robertson 1970). Temperature and salinity values as high as 45°C and 55, respectively, have been reported in the IRL during summer spawning periods (IRLNEP 1994). These extreme environmental conditions may influence embryonic development, hatching success, and larval survival. This chapter summarizes the results of a series of studies investigating the reproductive ecology and early life history of *L. polyphemus* in the microtidal IRL. The studies focus on the potential impact of microtidal conditions on the timing and frequency of spawning and embryonic and larval development in extreme temperature and salinity conditions.

2 Methods and Results

2.1 Spawning and Larval Abundance

Adult and larval surveys conducted at 21 sites in the IRL (Fig. 1) from February 1999 to August 2000 (see Ehlinger et al. 2003 for detailed methodology) indicated that adult spawning activity varies seasonally with peaks in the abundance of spawning pairs occurring in the late winter/early spring (Ehlinger et al. 2003, Figs. 2 and 3). During the study period, short episodic peaks in spawning were often separated by long intervals (3–4 weeks) in which no mating occurred (Figs. 2 and 3). Spawning is therefore aperiodic and lacks any apparent lunar or semi-lunar periodicity.

Larvae were collected in plankton samples from late spring to early summer, but were only found at 12 of the 21 sites (Fig. 3). In general, larval abundance was unrelated to spawning activity since the time interval (lag) between peaks in

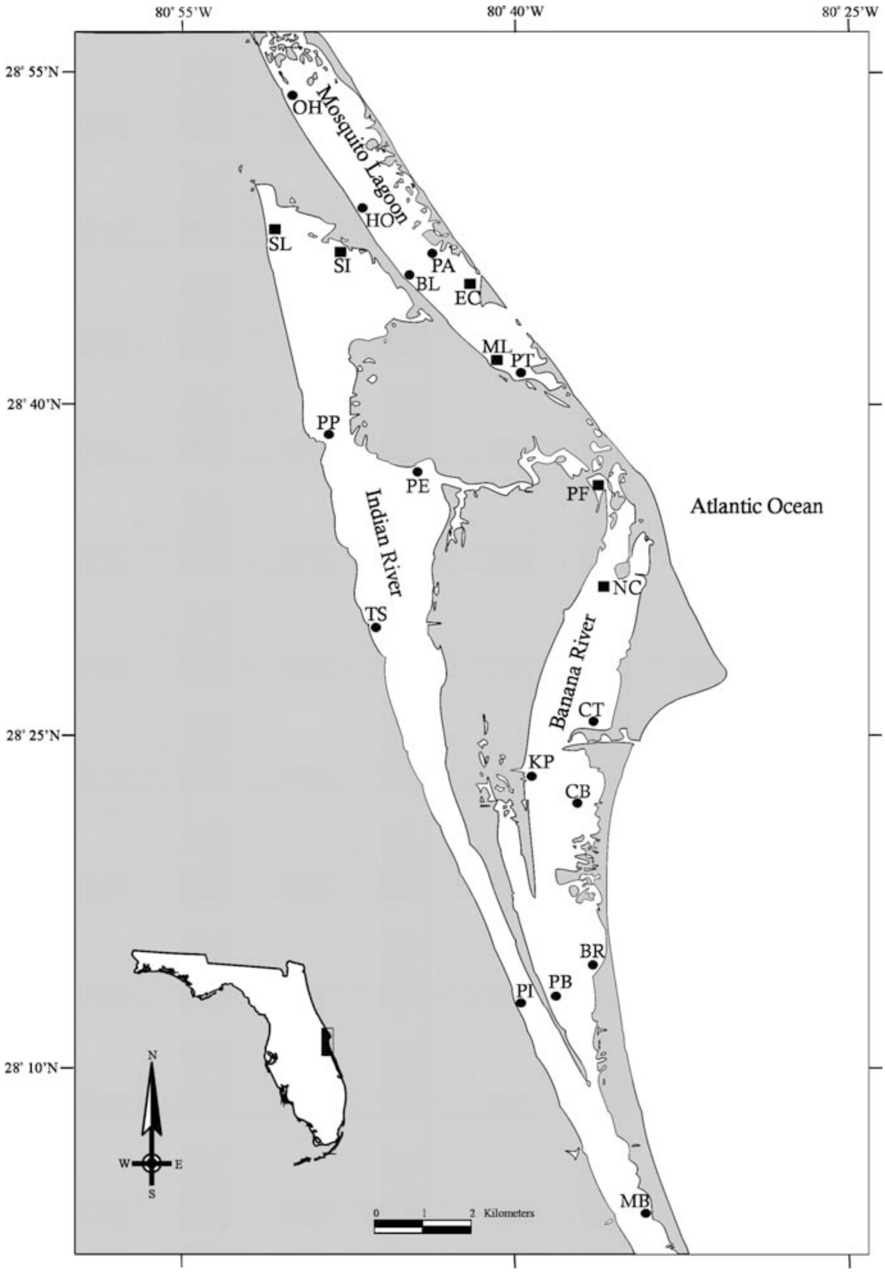

Fig. 1 Study sites in the Indian River Lagoon system. Six study sites (■) were sampled during Phase I of the study from February 1998 to December 1999 for adult *Limulus polyphemus*. Twenty-one study sites (■ and ●) were sampled during phase II of the study from February to August 2000 for adult and larval *Limulus polyphemus*. Redrawn from Ehlinger et al. (2003)

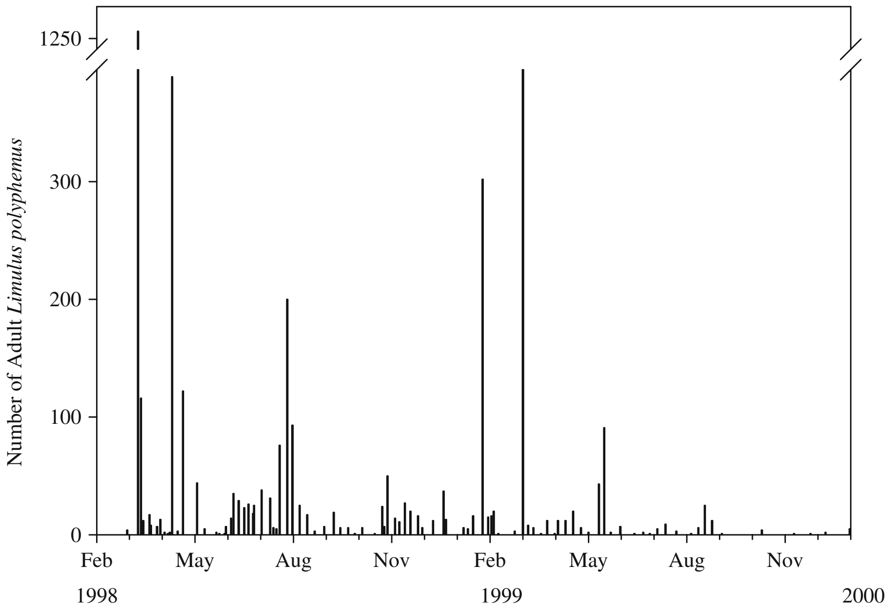

Fig. 2 Temporal pattern of spawning by *Limulus polyphemus* during Phase I (February 1998–December 1999) of this study. Redrawn from Ehlinger et al. (2003)

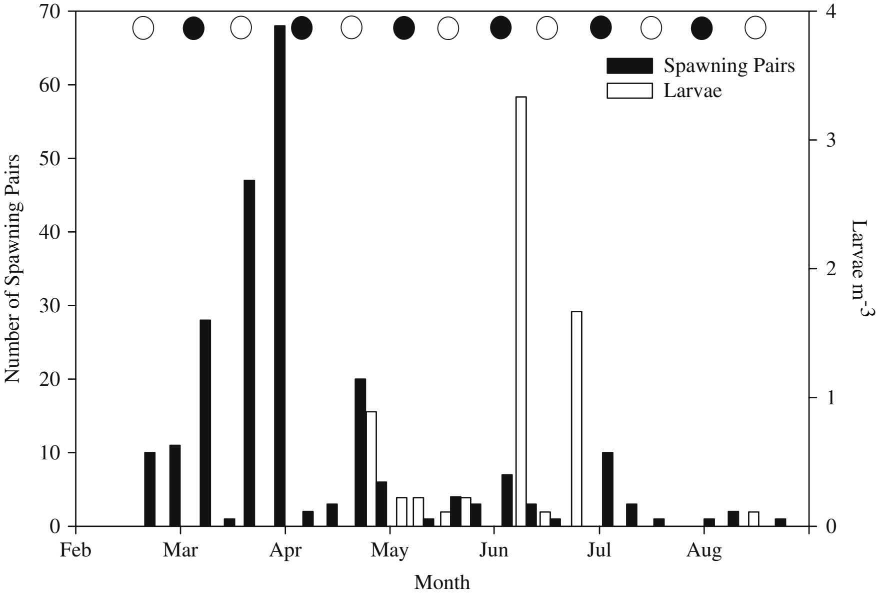

Fig. 3 Temporal pattern of spawning and larval hatching of *Limulus polyphemus* during Phase II (February–August 2000) of this study. *Open circles* represent full moons and *closed circles* represent new moons. Redrawn from Ehlinger et al. (2003)

adult and larval densities (≈ 8 weeks, Fig. 3; Ehlinger et al. 2003) exceeded the duration of embryonic development (≈ 4 weeks, Rudloe 1979, Sekiguchi 1988, Penn and Brockmann, 1994). Moreover, spawning activity throughout the IRL is not synchronized since peaks occur at different sites at different times. The distribution and abundance of larvae is not always linked to the spatial distribution of spawning adults since larvae were frequently found at sites where spawning was rarely observed.

Attempts to correlate the abundance of adults and larvae with environmental and hydrologic variables indicated that spawning and larval hatching appear to be triggered or associated with different cues (Ehlinger et al. 2003). For adults, seasonal abundances were associated with a decrease in temperature/salinity. However, the presence of spawning adults is not associated with any other environmental variable that might serve as a synchronization cue. Conversely, the presence of larvae is associated with periods of high water when beach width and salinity levels are low.

2.2 Tolerance of Embryos and Larvae to Temperature and Salinity

The ability of horseshoe crab embryos to tolerate temperature and salinity conditions in the IRL was tested by rearing artificially fertilized eggs under different temperature and salinity combinations (see Ehlinger and Tankersley 2004 for detailed methodology). Results indicated that both high temperatures and salinities have a significant effect on the rate and success of embryonic development (Ehlinger and Tankersley 2004). At 35 and 40°C, eggs fail to hatch after 75 days at all salinities tested (salinities of 30–60; Ehlinger and Tankersley 2004). Normal development and hatching occur at all other temperatures (25–40°C). Temperature and salinity also had a significant effect on time to hatching (Fig. 4). Embryonic development takes significantly longer at 25°C

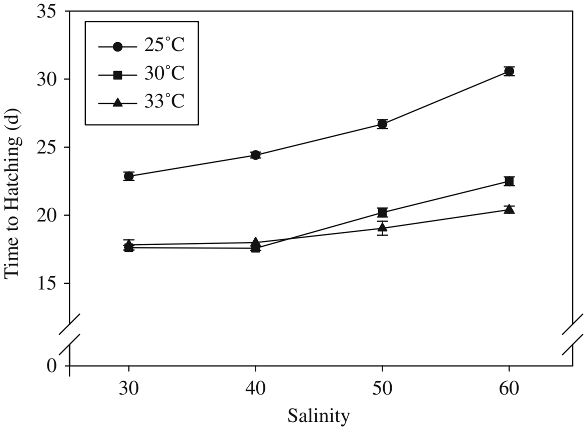

Fig. 4 Mean (± SE) number of days from fertilization to hatching of *Limulus polyphemus* embryos at 25, 30, and 33°C and at salinities of 30, 40, 50, and 60. No hatching occurred in any of the test salinities at 35 and 40°C. $N = 30$ for each trial. Redrawn from Ehlinger and Tankersley (2004)

compared to 30 and 33°C at all test salinities (Fig. 4). At 25°C, time to hatching increases significantly as the salinity increases (Fig. 4). Embryos maintained at 30 and 33°C and salinities of 30 and 40 have similar hatching rates, but hatching is delayed significantly in more hypersaline conditions (salinities of 50 and 60, Fig. 4). The optimal temperature and salinity conditions for development is 30–33°C and 30–40, respectively (Ehlinger and Tankersley 2004).

Experiments designed to test *Limulus polyphemus* larval tolerance of different salinities found that trilobite larvae are able to survive for at least 30 days at salinities ranging from 10 to 70 (see Ehlinger and Tankersley 2004 for detailed methodology). Mortality only occurs in the very extreme salinities of 5, 80, and 90 (Fig. 5). Comparisons of the survivorship curves among salinity treatments indicate that survivorship is only significantly reduced when larvae are maintained at a salinity of 90 (Fig. 5). Time to 50% mortality (TM_{50}) is ≈16.0 days in a salinity of 90 (Ehlinger and Tankersley 2004).

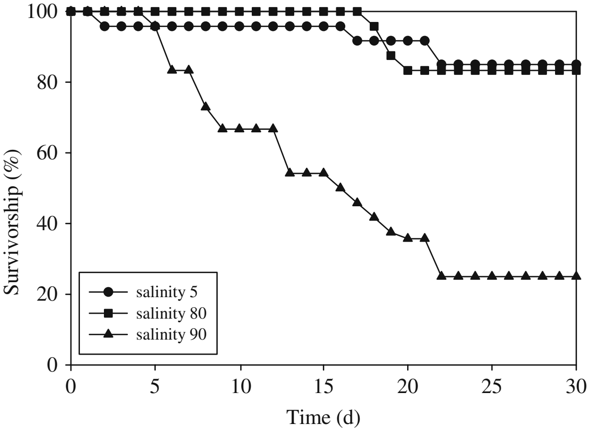

Fig. 5 Kaplan–Meier survivorship curves for *Limulus polyphemus* larvae subjected to salinities ranging from 5 to 90 for 30 days. All larvae survived in salinities from 10 to 70. $N = 24$ for each trial. Redrawn from Ehlinger and Tankersley (2004)

2.3 Hatching Triggers

Embryos hatch into trilobite larvae in approximately 28 days and move toward the surface of the substrate to enter the water when inundated during nocturnal high tides near the time of full moon (Rudloe 1979, Penn and Brockmann 1994). Given the diverse range of tidal patterns that occur throughout its range, we hypothesized that the release of *L. polyphemus* larvae from nests in the sand may be facilitated by one or more environmental triggers that serve to synchronize hatching with periods of high water on the beach. If an environmental cue associated with inundation triggers hatching, then larvae will hatch and emerge when conditions are favorable for entry into the water column, thereby increasing their chance of survival. This is especially important in microtidal systems since inundation of the beach is aperiodic. To test this hypothesis, developing embryos were exposed to different treatments that mimicked conditions experienced during periods of high water (hydration, agitation, and osmotic shock; Ehlinger and Tankersley 2003). When exposed to conditions representative of high tide in the field, hatching levels of late-stage (Stage 21) embryos increase significantly relative to levels in control treatments (Fig. 6; Ehlinger and Tankersley 2003). For all treatments tested (hydration, agitation, hydration and agitation, and hydration with agitation and sand), most hatching occurred within the first 2 h following exposure (Fig. 6). Most embryos (>65%) in the treatments involving agitation hatched during the exposure period (Fig. 6). Hatching levels in the hydration-only treatment increased rapidly following exposure, but were significantly lower than the other treatments (agitation, hydration and agitation, and hydration with agitation and sand). At the end of the monitoring period, hatching levels were significantly higher for embryos exposed to hydration and agitation with sand than for all other treatments. These results support the field findings of Rudloe (1979) who found that peaks in larval abundance in the plankton occur when localized storms with strong onshore winds coincide with high tide, producing unusually heavy surf. Botton and Loveland (2003) also found that peaks in larval abundance occur during periods of rough surf.

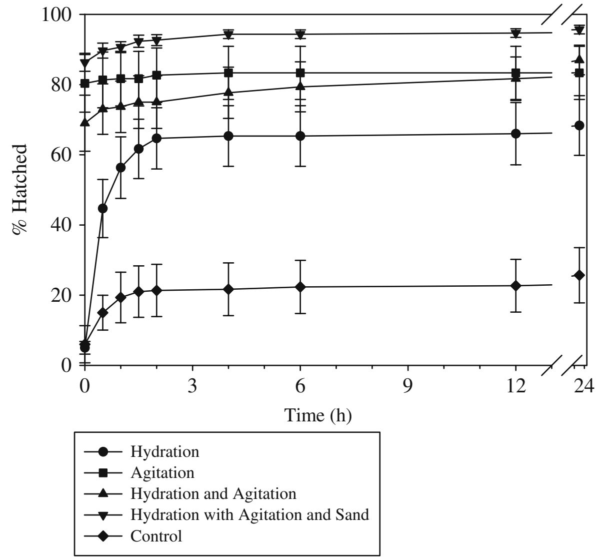

Fig. 6 Percentage ($\bar{X} \pm$ SE) of hatched larvae over 24 h following exposure to one of the four hydration and agitation treatments for 30 embryos ($N = 10$). Embryos in control conditions were maintained in rearing conditions (moist paper towels) and were not inundated with seawater or agitated by shaking. Redrawn from Ehlinger and Tankersley (2003)

When embryos are placed in isoosmotic seawater (salinity of 60) relative to their perivitelline fluid, hatching increases sharply and levels off at 40% after 2 h (Fig. 7, Ehlinger and Tankersley 2003). Hatching levels are significantly higher than in the treatment in which embryos received no inundation. Hatching is facilitated when embryos are exposed to seawater that is hypoosmotic to their perivitelline fluid. Ehlinger and Tankersley (2003) found that the perivitelline fluid of embryos collected from the Banana River, FL, and Delaware Bay, NJ,

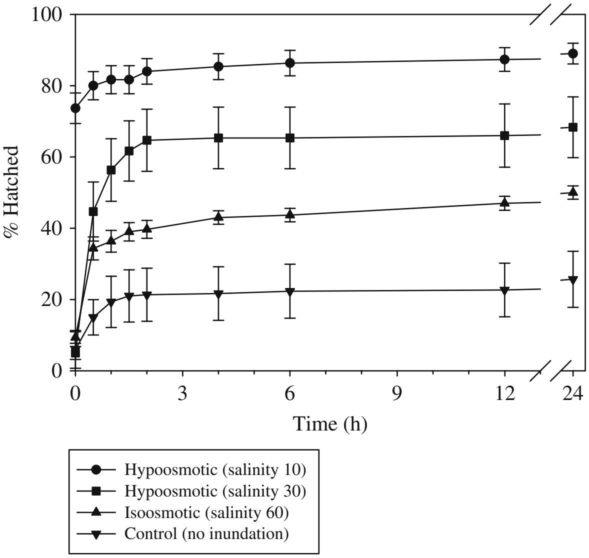

Fig. 7 Percentage ($\bar{X} \pm$ SE) of hatched larvae over 24 h after exposure to osmotic shock for 30 embryos ($N = 10$). Two hypoosmotic treatments were tested (salinities of 10 and 30) and an isoosmotic treatment (salinity of 60). Embryos in control conditions were maintained on moist paper towels and were not placed in seawater. ($N = 300$). Redrawn from Ehlinger and Tankersley (2003)

was hyperosmotic to the surrounding seawater and porewater collected at the same time, thus, when inundated, embryos experience a hypoosmotic shock. Hatching significantly increased when embryos were placed in hypoosmotic seawater with a salinity of 10 and 30 (Fig. 7, Ehlinger and Tankersley 2003). In all treatments, most hatching occurred within the first 2 h following exposure (Fig. 7) which would time hatching with inundation of the nest and allow the larvae to enter the water column.

2.4 Discussion

In areas with regular tides, spawning by *L. polyphemus* is controlled by an endogenous oscillator that is entrained by external cues associated with the tides (Rudloe 1979, 1980, Barlow et al. 1986). However, in microtidal lagoons where tidal changes are negligible and high water occurs due to wind forcing and freshwater input, *L. polyphemus* does not appear to respond to localized external cues. Instead, spawning in the IRL is aperiodic and unpredictable (Figs. 2 and 3). In another microtidal system, St. Joseph Bay, FL, horseshoe crabs spawn when triggered by changes in water level (Rudloe 1985). However, spawning in the IRL is not triggered by environmental cues.

Larvae generally appear in the plankton 3–4 weeks after peaks in spawning (Rudloe 1979, 1980, Penn and Brockmann 1994). During our sampling period in the IRL, larvae were present in the plankton in May and June, approximately 8 weeks after peak periods of spawning (Fig. 3). This substantial lag between spawning and the appearance of larvae in the water column indicates that hatching may be decoupled from spawning. This decoupling is most likely due to the lack of regular inundation of nests by tidal changes in water level.

In tidal areas, larvae hatch and emerge from the nest when it is inundated during nocturnal high tides associated with full moons (Rudloe 1979) or when there is a strong onshore wind that creates wave action (Botton and Loveland 2003). Hatching and emergence from the nest appears to be triggered by environmental stimuli in microtidal systems since the appearance of larvae in the plankton was associated with periods of high water. This pattern is consistent with the results of Rudloe (1979) who found that larvae exhibit strong lunar and diel patterns in hatching and emergence in areas where nests are inundated by high tides associated with full moons. This pattern is also consistent with Botton and Loveland (2003) who found that the abundance of planktonic horseshoe crab larvae in lower Delaware Bay was most closely linked with strong onshore winds that generated wave heights above 30 cm. This is similar to other marine arthropods, such as *Carcinus maenas* and *Rhithropanopeus harrisii*, which exhibit tidal and lunar rhythms in tidal areas but lack the similar rhythms in areas without significant tidal changes (Naylor 1960, Cronin and Forward 1979, 1983). The paucity and irregularity of shoreline inundation in the IRL may contribute to the low densities of larvae found in the plankton. In our sampling, the maximum density was 4 larvae m^{-3} (Fig. 3) compared to over 700 larvae m^{-3} reported in plankton tows on the Gulf Coast of Florida (Rudloe 1979). Without regular inundation, larvae may be stranded in the upper beach and unable to leave the nest and enter into the water column.

Horseshoe crab nests are typically located 10–20 cm below the sediment surface at the high water line. Thus, developing embryos and larvae are often exposed to wide and rapid fluctuations in temperature and salinity (Penn and Brockmann 1994). In microtidal areas, shoreline inundation is irregular and *L. polyphemus* nests may not be submerged for months. In the IRL, conditions in the shallow waters where horseshoe crabs nest have been documented to reach temperatures and salinities as high as 45°C and 55, respectively (Ehlinger and Tankersley 2004). *Limulus polyphemus* embryos and larvae develop and hatch in constant temperatures up to 35°C and in salinities as high as 60 (Figs. 4 and 5). *Limulus polyphemus* populations inhabiting the IRL experience higher temperatures and salinities than those in the more northern portion of its range. Therefore, populations inhabiting the IRL may be able to withstand higher temperatures and salinities, as a result of acclimatization, resulting in slightly higher optimal ranges for development and growth (30–33°C and salinities of 30–40). Botton et al. (2006) found that horseshoe crabs have adapted to living in a thermally stressful environment by maintaining a high baseline level of cellular stress proteins such as heat shock protein 70 (Hsp70). They found that there was a slight elevation of Hsp70 only among heat-shocked trilobite larvae in a 6 h recovery treatment and that Hsp70 levels did not differ significantly between control and heat-shocked embryos, thus intertidal embryos and larvae do not have to synthesize inducible Hsps when stressful temperatures are encountered (Botton et al. 2006). This is advantageous because embryos and larvae encounter regular heat shocks due to diurnal and tidal temperatures changes. Thus, the high salinities experienced by developing embryos and larvae in the IRL do not account for the low larval densities found, but the high temperatures may account for the low larval densities.

The ability of *L. polyphemus* embryos and larvae to tolerate a wide range of salinities is an important adaptation to the extreme conditions in microtidal lagoons. Tolerating such a wide range of conditions may be an advantage in species that live in a highly variable, unpredictable environment (Anger 1991). Physiologically tolerant embryos and larvae, which are capable of surviving extreme temperature and salinity regimes, may be a factor that has contributed to the evolutionary success of *L. polyphemus*.

In the IRL, larvae occur in the plankton during periods of high water. Laboratory studies indicate that larvae are triggered to hatch when they are exposed to environmental cues that are associated with inundation (Ehlinger and Tankersley 2003). Although larval hatching in *L. polyphemus* occurs in the absence of external stimuli (Jegla 1979), hatching is facilitated when embryos are subjected to environmental conditions associated with periods of high water, including hydration, agitation, and osmotic shock (Figs. 6 and 7). Hatching in response to these environmental cues is likely adaptive since it would increase the likelihood that they would hatch and emerge from the nest during periods of inundation, thereby preventing them from being stranded. In tidal systems, larvae hatch and leave nests when inundated at high tide. Thus, the environmental cues that facilitate hatching occur with a regular periodicity, which increases the chances that larvae will be released into the water column (Rudloe 1979). In microtidal lagoons, hatching is triggered by environmental cues that are associated with inundation. During embryonic development in the nests, the perivitelline fluid within the eggs becomes hyperosmotic to the surrounding water, most likely due to desiccation (Sekiguchi 1988). Thus, when the nest is inundated, the eggs experience an osmotic shock, which triggers hatching (Fig. 7). Increased hatching levels in *L. polyphemus* exposed to only hydration supports the findings of Rudloe (1979) that *L. polyphemus* embryos enter the water column immediately when the nest is inundated. During periods of high water, the nests would be inundated which would facilitate hatching and larval release from the nest, leading to a greater presence of larvae in the plankton. Increased hatching when hydrated is particularly important in microtidal and nontidal systems where shoreline inundation is unpredictable and controlled by wind forcing, not changes in water level associated with tides (Ehlinger et al. 2003). If the nest is briefly inundated, *L. polyphemus* larvae would hatch and emerge into the water column during periods of high water, thus preventing stranding in the nest and problems of nest release in a nontidal system. Similar responses to mechanical stimulation have been reported for other species which undergo development in intertidal areas. Waves and mechanical agitation that simulate the action of the rising tide trigger larval hatching in the capelin, *Mallotus villosus* (Frank and Leggett 1981) and in the isopod *Excirolana chiltoni* (Enright 1965). The inconsistency of planktonic larvae in the IRL may be due to the fact that the nests are inundated aperiodically, resulting in the observed decoupling between peaks in larval abundance and spawning (Ehlinger et al. 2003).

Patterns of spawning and larval hatching in *Limulus polyphemus* populations in microtidal lagoons differ from those in tidal areas due to the absence of tidally associated zeitgebers. Adult spawning becomes asynchronous and larvae hatch when they are inundated, resulting in peaks in larval abundance during periods of high water. Consequently, methodologies used to assess population size and reproductive periodicity of *L. polyphemus* in tidal systems are ineffective in microtidal lagoons. Because of the asynchronous spawning and lack of tidally related synchronization cues, the spatial and temporal distribution of adult and larval horseshoe crabs in microtidal lagoons may be patchy, making it difficult to accurately assess the density and structure of the population.

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Phylogeography, Demographic History, and Reserves Network of Horseshoe Crab, *Tachypleus tridentatus*, in the South and East China Seaboards

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Abstract Molecular genetic data can be used to diagnose the status of populations and provide the management information. The loss of habitats and population degradation of the tri-spine horseshoe crab, *Tachypleus tridentatus*, in Asia urged the need to study patterns of phylogeography and population demography for its conservation. In this study, we first reviewed the marine reserves for *T. tridentatus* in the South and East China Seaboards, and genetic variation of mitochondrial (mt) AT-rich region was then examined for 114 *T. tridentatus* individuals. They were collected from eight populations in the northern South China Sea and East China Sea, including Zhoushan (ZS), Yangjing (YH), Beihai (BB), and Kinmen (TK) along the southeast coast of Mainland China, Penghu Island, Tiexiawei (TT), and Dongwei (TD) and in the Taiwan Strait, and Budai (TB) and northern coast in Taiwan (TN). Pairwise tests of genetic differentiation (F_{ST}) indicated two populations, Budai and Tiexianwei, were significantly different from others. The genetic connectivity of the other six populations showed a pattern consistent with an isolation-by-distance model of gene flow. Mismatch distribution analyses indicated that three populations, Beihai, Yangjing, and Zhoushan, had a pattern consistent with range expansion and three populations appeared to be in equilibrium (northern Taiwan, Kinmen, and Dongwei). Budai and Tiexianwei, located in semi-enclosed embayments had patterns of nucleotide substitution consistent with recent population bottlenecks. Those populations that appear to have undergone recent range expansion are all along the Mainland China coast and could have been affected by glacial sea-level fluctuations along the northern South and East China Seaboard. Populations estimated to have undergone a bottleneck may have had reduced gene flow due to geographic barrier that contributed to inbreeding depression based on evidence of lower genetic diversity. Finally, a greater understanding of genetic connectivity could be used to realize the dispersal pattern and apply for reserve network design of horseshoe crab.

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1 Introduction

1.1 Intraspecific Variation and Phylogeography of Horseshoe Crabs

Population genetic studies of marine species have shown that, especially along continental margins, high dispersal potential is often associated with only mild genetic differentiation over large scales (Palumbi 1992). These results suggest high levels of gene flow between populations. There may often be some mechanisms limiting the actual dispersal of marine species with high dispersal potential (Scheltema 1986), such as isolation by distance, behavioural limits to dispersal, climate change, tectonism, sea-level fluctuations, coastal hydrography and geography, and natural and anthropogenic extirpations, and most of the time these complex mechanisms operated at once (review in Palumbi 1994; Charlesworth 2003; Edwards and Beerli 2003).

Horseshoe crabs, the well-known living fossil, with the reputation for extreme conservatism in morphotypic evolution have been considered the 'archetype of bradytely' and 'a classic example of arrested evolution' (Fisher 1984). However, intraspecific geographic variation of morphology among the four extant horseshoe crab species, *Tachypleus tridentatus, T. gigas, Limulus polyphemus,* and *Carcinoscorpius rotundicauda*, have been described (Sekiguchi et al. 1976; Sokoloff 1978; Rudloe and Rudloe 1981; Shuster 1982; Yamasaki et al. 1988; Chiu and Morton 1999, 2003). At the molecular level, extant horseshoe crabs appear to have intraspecific genetic variation and patterns of population differentiation in allozymes, mitochondrial DNA, and microsatellites (Selander et al. 1970; Saunders et al. 1986; Orti et al. 1997). In *L. polyphemus*, the level of allozyme heterozygosity ($H = 0.057$) proved similar to mean estimates for many animals (Selander et al. 1970); and levels and pattern of intraspecific differentiation were similar to those of several other invertebrates and vertebrate species inhabiting the same coastal range in the Southeastern United States. A consensus genetic break distinguishes that populations were found in Georgia northwards from those in Florida's Atlantic and Gulf coasts (Saunders et al. 1986; Avise 1992).

Horseshoe crabs appeared to exhibit marked population subdivisions with mitochondrial COI marker (Pierce et al. 2000). Geographic subdivision of *L. polyphemus* populations along the northwestern Atlantic coast were defined as four to five clades of populations using microsatellite loci in a regional level (King et al. 2005). More recently, population subdivisions were found in mitochondrial AT-rich region of *T. tridentatus* in relatively much smaller geographic scale (about 60 km in distance) and showed the sensitivity and potential of AT-rich region for phylogeographic study (Yang et al. 2007). The possible cause of subdivision of horseshoe crabs in above studies could be due to geographic restriction or isolation by distance.

1.2 Status of **Tachypleus tridentatus**

The life cycle of the horseshoe crab is highly dependent on environmental conditions of the coastal zone; thus, they have served as a flagship species for monitoring the health of coastal zones because it is large enough to observe (Sekiguchi and Sugita 1980; Rudloe and Rudloe 1981; Chen et al. 2004). The American horseshoe crab, *Limulus polyphemus*, is ecologically and economically important in the east coast of United States. Nine species of migratory shorebirds rely on the eggs of American horseshoe crab for food during their spring migration northwards to Canada. Recent studies have shown a decline in both the American horseshoe crab populations and in the shorebird populations they sustain (Clark 1996). Furthermore, the biomedical industry utilizes horseshoe crab blood, which has an extensive infection fighting system, thereby improving the ability of pharmaceutical and medical device manufacturers to assure that their products are free of contaminating endotoxins (reviewed in Walls 2002). The population density of tri-spine horseshoe crab, *T. tridentatus* was once high along the coast of Mainland China, especially in the northern South China Sea coast of Fujian, and in the region of Hainan Island and Beibu Bay (Sekiguchi 1988). During April to October in the 1980s, the most numerous horseshoe crab adults came ashore as mated pairs to lay eggs in Beibu Bay. However, the observations of *T. tridentatus* populations in Taiwan, Japan, Hong Kong, Singapore, Malaysia, Borneo, and Thailand recently indicated that they are in a dwindling condition (Itow 1993; Chatterji 1994; Chiu and Morton 1999; Botton 2001; Chen et al. 2004; Yang et al. 2007). The productivity of the horseshoe crab was highest in Pingtan, Fujian in 1980s (Mikkelsen 1988). However, the 1970s productivity of the horseshoe crab industry in Pingtan was reduced by approximately 80–90 % relative to the 1950s, and there were not enough individuals to support such an industry until the 1990s. Based on the estimation by the Pingtan Ocean and Aquatic Bureau, the productivity of horseshoe crabs was about 15,000 pairs in 1984, but reduced subsequently to 9,500 pairs in 1995, 3,700 pairs in 1998, and 1,000 pairs in 2002 (Huang et al. 2002). Although this was local data, it is rare that survey data are available from other cities. It could also reflect the serious decline of *T. tridentatus* in South and East China seas. The serious decline resulted from various factors, including water pollution, loss of suitable spawning and nursing grounds, and overharvesting for food, biomedical, and chitin industry in China due to trade with Japan or the United States.

1.3 Review in Nature Reserves and Genetic Connectivity for Horseshoe Crabs

Nature reserves can offer no-take habitats surrounded by areas unsuitable for supporting populations of target species or maintaining community diversity due to habitat fragmentation, degradation, or loss (Terborgh and van Schaik 1997). An important factor in the establishment of marine reserves is the process by which they are nominated and designated (Brody 1998). Design of marine reserve systems requires an understanding of larval transport in and out of reserves, whether reserves will be self-seeding, whether they will accumulate recruits from surrounding exploited areas, whether small reserves can protect assemblages of taxa with limited dispersal (Roberts and Hawkins 1997; Shanks et al. 2003), and whether reserve networks can exchange recruits with long dispersal (reviewed in Palumbi et al. 2003 and Palumbi 2004). The outcomes of ecological and genetic studies are providing information about species-level connections, gene flow, dispersal capabilities, and metapopulation dynamics (reviewed in Palumbi et al. 2003 and Palumbi 2004). By evaluating species with high dispersal capacities, patterns of genetic homogeneity could be used to establish upper limits of propagule exchange and connectivity on shorter, ecological times scales (i.e., seasonal, annual) since genetic homogeneity can be sustained by exchanging a limited number of individuals, orders of magnitude lower than what is required to sustain a metapopulation over ecological timescales (Cowen 2002). Limited dispersal capacities might reduce gene flow among populations, making them prone to subdivision, even when the distance among populations is small. Single-species models have been important in demonstrating how dispersal distance and the shape of the dispersal curve (Botsford et al. 2001), as well as adult mobility, influence reserve success (Polacheck 1990; DeMartini 1993; Guénette and Pitcher 1999). Direct measurements of mean larval dispersal are needed to understand connectivity in a reserve system, but such measurements are extremely difficult. Genetic patterns of isolation by distance have the potential to add to direct measurement of larval dispersal distance and can help set the appropriate geographic scales on which marine reserve systems will function well (Palumbi 2004). Analytical and simulation studies of isolation-by-distance models have shown that genetic distances between populations increase with increased geographic distance. Pairwise gene flow estimate are higher for more adjacent populations and lower for populations that are more distant. The relationship between population genetic distance and geographic distance depends on the configuration of the stepping-stone lattice, the mutation rate, and the migration rate among adjacent populations (Slatkin 1993).

The American horseshoe crab landings were reduced in 2002 by approximately 45% below the coastwide quota of 2.3 million crabs. The Addendum II approved by Horseshoe Crab Management Board was also recommended that the National Marine Fisheries Service (NMFS) prohibit the harvest of horseshoe crabs in federal waters off Delaware. The Carl N. Shuster Jr. Horseshoe Crab Reserve, encompassing nearly 1,500 square miles of federal waters off the mouth of the Delaware Bay, was established by the NMFS on March 7, 2001 (ASMFC 2002; ASMFC 2008 Shuster 2003). At Kinmen Island, Taiwan, a total of 800 ha of coastal areas have been designated as protected area for the tri-spine horseshoe crab, based on the Fishery Law, in December 1999, and fishing has been abandoned for the 5 years from 2000 to 2004 (Chen et al. 2004). The deadline was recently extended to 2008. In China, horseshoe crabs are not only ‘Grade II Protected Animal of China’ in the ‘List of State Key Protected Wildlife,’ but also province-level ‘key protected aquatic wildlife’ in Zhejiang, Fujian, Guangdong Province, and Guangxi Zhuang Autonomous Region where there are abundant populations. However, unsustainable harvests still happened even in these provinces and the juvenile habitat and spawning ground were lost due to urban development or coastal aquaculture. Scientists started to call on protection for horseshoe crabs since 1980s in China, and proposed the planning of Pingtan Horseshoe Crab Nature Reserve in 1998, but no substantial progress has been achieved ever since (Huang et al. 2002). Now, there are seven city-, county-, or province-level of horseshoe crab nature reserves that have been established since 2001 in Gaungdong province concentrically in northern South China Sea (see Fig. 1), but not in other places along the coast of East Asia (except in Japan) (GEPA 2004). These marine reserve networks serve as the hope for protecting the habitat and recovering depleted populations of horseshoe crabs in the future, if this management plan is effective.

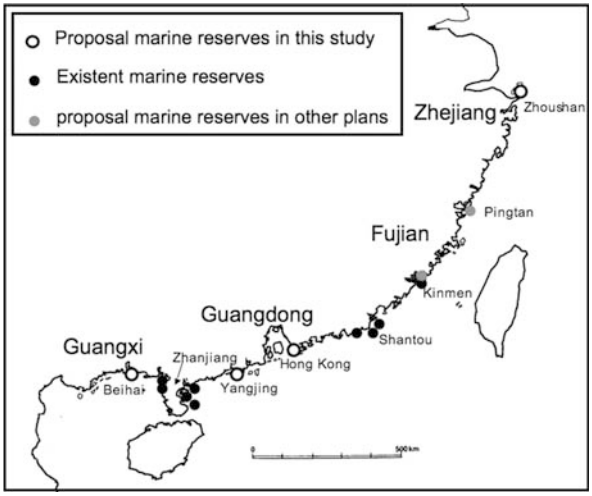

Fig. 1 Existent and proposed marine reserve networks for horseshoe crabs in the South and East China Seaboards. *Dark circles* represent existent reserves (Chen et al. 2004; GEPA 2004). *Gray circles* represent the proposed reserves from other projects (Hung 2002). *Empty circles* represent the proposed reserves based upon the present study

In the present study, a nested hierarchical analysis was used to investigate the genetic structure of tri-spine horseshoe crab, *Tachypleus tridentatus*, along the full geographic range along the coast of southeast China and Taiwan Strait at different scales. A maternally inherited molecular marker, the mitochondrial AT-rich region, was used. Historical and recent perspectives on biogeography and population demography would be focused on. Genetic structure, gene flow, and the potential mechanisms (sea surface currents, demography, and others) creating any population division will be elucidated, allowing for recommendations of effective marine reserves establishment in Taiwan as well as coastal regions of the South and East China seas.

2 Material and Methods

2.1 Sampling

A nested hierarchical sampling strategy (1,000, 200, and 50 km) was designed to collect *Tachypleus tridentatus*. Two regions, Taiwan Strait and Southeastern China (Zhoushan (SZ), Yangjing (YH), and the other in Beihai (BB)), were targeted at a scale of 1,000 km. Across Taiwan Strait, four replicate sites were designed to cover the range of 200–300 km (Kinmen, Penghu Islands, and Northern Taiwan). Intensive sampling was conducted at three localities at the Taiwan Strait (two are in Penghu Archipelago and one is at the west coast of Taiwan, Budai) to cover the range of few kilometres to 10–50 km (Fig. 2). This sampling design covered the fine scale to the regional scale of populations. In the Taiwan Strait, three populations, Kinmen (TK), Dongwei (TD), and Northern Taiwan (TN), were in open coast; two populations, Tiexianwei (TT) and Budai (TB), were in semi-enclosed bays. The book gill or leg muscle tissues were preserved in 70% ethanol, and then horseshoe crabs were released subsequently.

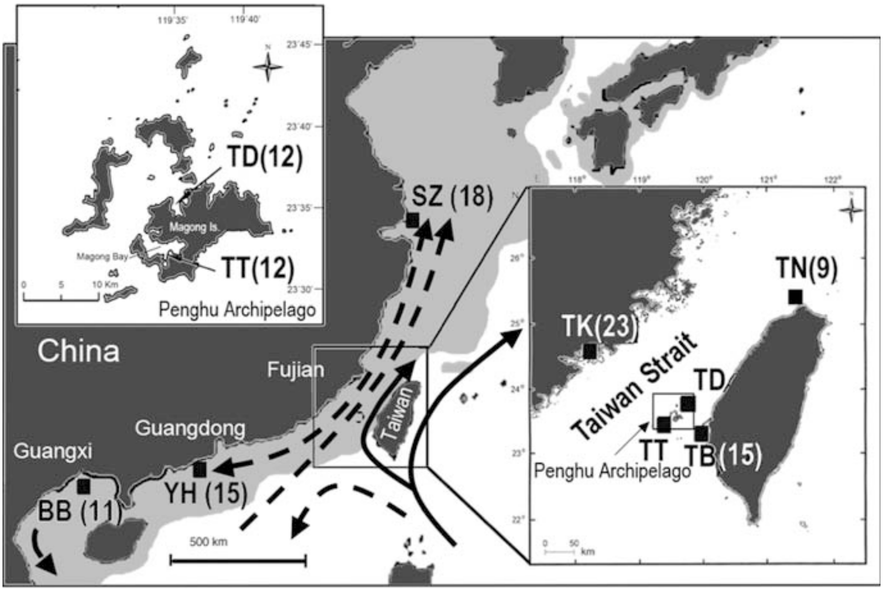

Fig. 2 Map of collection sites, geographic, and hydrographical patterns. The *dark square* represents the sampled sites and the sample size is shown beside the site name. Two sampled sites Donwei (TD) and Tiexianwei (TT) are located in Penghu Archipelago. It is notable that TT is in the inner bay, Magong Bay. The *slide arrow* represents the annual sea current and the *dashed arrow* represents the seasonal sea current (Hu et al. 2000; Xu and Oda 1999)

2.2 DNA Extraction, PCR Amplification, and Automated Sequencing

Techniques for DNA extraction, PCR, and sequencing of the mt AT-rich region in tri-spine horseshoe crab have been established (Yang et al. 2007). In general, total genomic DNA was extracted from either branchial appendage or leg muscle tissue with the Blood & Tissue Genomic DNA Extraction Miniprep System Kit (VIOGENE). The AT-rich region spanning between the 12S ribosomal RNA gene and tRNA- methionine were amplified by the primers Hb-12S (5'-GTCTAACCGCGGTAGCTGGCAC-3') and Hb-trna (5'-GAGCCCAATAGCT TAAATTAGCTTA-3'). PCR amplification reactions were performed as described in Yang et al. (2007). The amplified DNA was extracted using a DNA extraction column (Viogene, Taipei) under conditions recommended by the manufacturer. Nucleotide sequences were determined from both ends by direct sequencing of the PCR products with an ABI 377 Genetic Analyzer.

2.3 Population Structure and Demographic Analyses

DNA sequences were edited using DNASTAR software (DNASTAR, Madison, WI) and aligned using Clustal X (Thompson et al. 1997). Summary statistics, such as number of haplotype, haplotype diversity, nucleotide diversity (Nei 1987), and fixation indices (F_{ST}) were performed using Arlequin 3.11 (Excoffier et al. 2005). Fixation indices (F-statistics) (Wright 1951) are applicable to mtDNA under the assumption of uniparental, non-recombining inheritance to represent population difference. Hudson et al. (1992) gave an expression to estimate F_{ST} values based on sequence data: $F_{\mathrm{ST}} = 1 - \mathrm{Hw}/\mathrm{Hb}$, where Hw is the average nucleotide variation among individuals within a subpopulation and Hb is the average variation between subpopulations. This allows for an estimation of gene flow, $N_{\mathrm{e}}m = 1/2\,[1/F_{\mathrm{ST}} - 1]$, where N_{e} is the effective number of females and m is the migration rate. F_{ST} values and $N_{\mathrm{e}}m$ were calculated with Arlequin 3.11 (Excoffier et al. 2005).

Statistical parsimony (Templeton et al. 1992) was used to construct a statistically supportable haplotype network using the program TCS 1.21 (Clement et al. 2000). The historical demography of *T. tridentatus* was examined using mismatch distribution analysis (MDA) based on the observed number of differences among all pairs of haplotypes (Rogers and Harpending 1992) by Arlequin 3.11. Simulation studies have shown that the distribution is multimodal in samples drawn from populations at demographic equilibrium, a pattern reflecting stochastic lineage loss; a unimodal distribution suggests a stationary population growth (range expansion) and a Poisson distribution indicates a recent bottleneck.

To examine the population genetic structure of *T. tridentatus* for the presence of a pattern of isolation by distance among populations, F_{ST} estimates were plotted against geographic distance between samples by using the program IBD (Bohonak 2002). Reduced major axis (RMA) regression was used to calculate the slope and intercept of this relationship. A Mantel test was used (10,000 permutations) to assess whether the association between logarithm of genetic distance (F_{ST}) and logarithm of geographic distance is statistically significant. Zero or negative values of genetic distance was set to 0.0001 for they could not be log-transformed. Geographic distances between samples were calculated according to the minimum coastline distance.

3 Results

The total length of the AT-rich region was 369 bp in *Tachypleus tridentatus*. In total, 22 unique haplotypes of the AT-rich region were identified from 114 *T. tridentatus* individuals collected from eight sites (sequence data of 47 individuals were from Yang et al. 2007). Population-specific nucleotide composition, number of haplotypes, haplotype diversity, and nucleotide diversity are presented in Table 1. These data indicated that all populations had an average of haplotype diversity ($h \pm$ SD) as 0.65 ± 0.31 (ranged from 0.46 to 0.94) and nucleotide diversity ($\pi \pm$ SD %) as 0.42 ± 0.21 % (ranged from 0.26 to 0.70) excepting for the extreme value as 0 representing no genetic diversity from TB population. Population from Tiexianwei (TT) had lower haplotype diversity (0.46 ± 0.17) and

Table 1 Localities and molecular characters in the AT-rich region

Region	Populations	ID	Sites location	N	N_h	N_eh	$h \pm$ SD	$\pi \pm$ SD (%)
TS	Kinmen	TK	23°25'N, 118°18'E	23	6	1	0.70 ± 0.07	0.40 ± 0.06
TS	Tiexiawei	TT	23°31'N, 119°34'E	12	4	2	0.46 ± 0.17	0.26 ± 0.12
TS	Dongwei	TD	23°34'N, 119°35'E	12	4	1	0.56 ± 0.15	0.44 ± 0.11
TS	Budai	TB	23°22'N, 120°08'E	14	1	0	0	0
ECS	Northern Taiwan	TN	25°10'N, 121°43'E	9	5	1	0.81 ± 0.12	0.54 ± 0.2
ECS	Zhoushan	SZ	30°02'N, 121°58'E	18	12	3	0.94 ± 0.04	0.70 ± 0.1
SCS	Beihai	BB	21°30'N, 109°05'E	11	8	5	0.93 ± 0.07	0.55 ± 0.07
SCS	Yangjing	YH	21°37'N, 111°50'E	15	7	2	0.80 ± 0.08	0.50 ± 0.13

The region of each population was indicated. TS: Taiwan Strait, including Kinmen (TK), Tiexiawei (TT), Dongwei (TD), and Budai (TB). ECS: East China Sea, including northern Taiwan (TN) and Zhoushan (ZS). SCS: South China Sea, including Yangjing (YH), Beihai (BB). Sample size (N), number of haplotypes (N_h), haplotype diversity (h), nucleotide diversity (π) (± standard deviation; SD) (%) and, number of endemic haplotype (N_eh).

nucleotide diversity (0.263 ± 0.122%), and population from the Budai (TB) population contain a single haplotype without diversity.

Pairwise *F*-statistics (F_{ST}) revealed significant isolation of two populations, TT ($F_{ST} = 0.063$–0.792, $p < 0.05$) and Budai ($F_{ST} = 0.293$–0.792, $p < 0.001$) (Table 2). P value of pairwise F_{ST} from populations along the continental coast (TK, YH, and BB) were all not significant ($F_{ST} = -0.023$ to 0.032, $p > 0.05$).

Patterns of genetic connectivity of *T. tridentatus* populations are shown in Fig. 3. The width of arrows represents the level of genetic connectivity among

Table 2 Pairwise *F*-statistic (F_{ST}) values of genetic differentiation and female migrants per generation (N_em) values of gene flow among horseshoe crab populations. F_{ST} values are above the diagonal and N_em values are below the diagonal

	TK	TD	TT	TB	TN	SZ	BB	YH
TK	–	–0.036	0.153*	0.482**	–0.049	0.099	0.023	–0.025
TD	∞	–	0.063	0.518**	–0.036	0.143*	0.061	0.003
TT	2.774	7.386	–	0.776**	0.078	0.348**	0.260**	0.138*
TB	0.537	0.465	0.144	–	0.471**	0.199*	0.253**	0.451**
TN	∞	∞	5.878	0.562	–	0.093	0.021	–0.059
SZ	4.530	2.997	0.937	2.013	4.876	–	0.017	0.071
BB	21.430	7.710	1.425	1.474	23.772	29.262	–	0.004
YH	∞	178.071	3.136	0.608	∞	6.542	138.389	–

*$p < 0.05$, **$p < 0.001$, ∞: unrestricted gene flow.

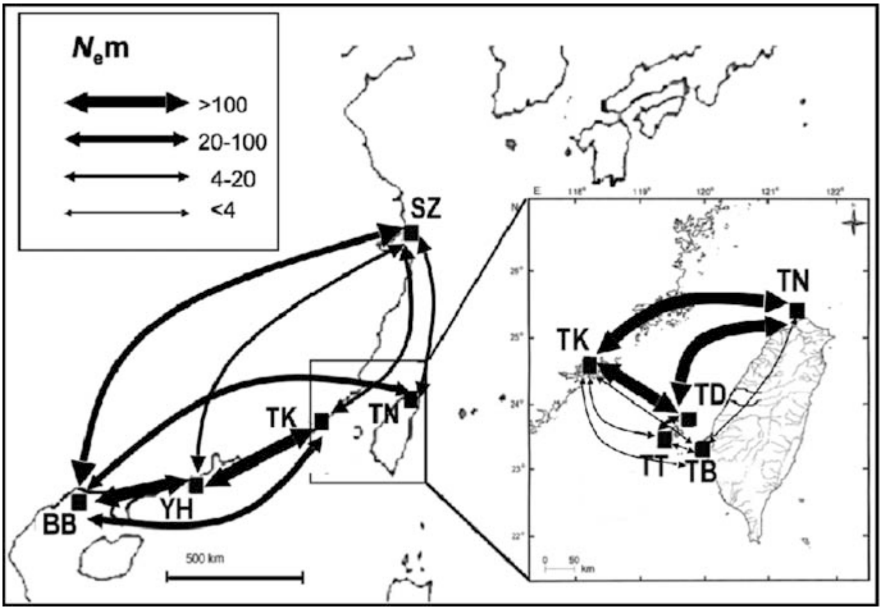

Fig. 3 Patterns of genetic connectivity of *T. tridentatus* populations. The width of *arrows* represents the level of genetic connectivity among populations that was revealed by effective female migrants per generation (N_em) values

populations that was revealed by effective female migrants per generation (N_em) values. It showed the pattern of higher connectivity between adjacent populations along the continental coast but the pattern did not appear within the Taiwan Strait. TT and TB populations showed the weaker connectivity with other populations.

The haplotype network was constructed by using statistical parsimony and contained two dominant haplotypes (Fig. 4). The two major haplotypes have been shown that demographic expansions lead to star-shaped genealogies (Slatkin and Hudson 1991). The most frequent haplotype was shared with almost all populations except for Budai (TB), and the other common haplotype was shared by four populations from distant sites. There was only single haplotype shared with one of the common haplotype in TB for 14 samples (see also in Table 1).

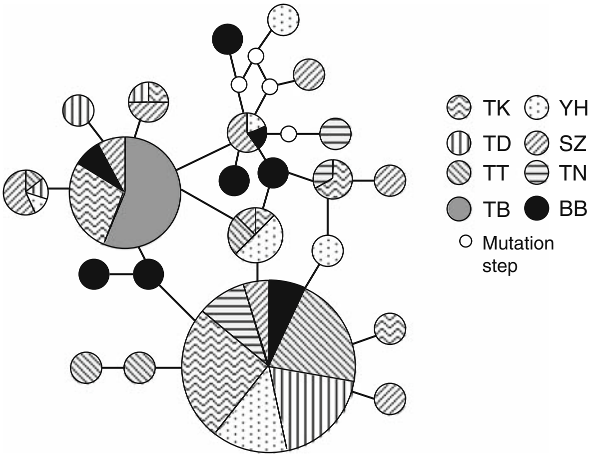

Fig. 4 A statistical parsimony network of *T. tridentatus* haplotypes. Each *circle* represents a unique haplotype. The size of circle represents the frequency of haplotype. *Solid lines* connect haplotypes by a single mutational change with a probability higher than 95%

Based on mismatch distribution analysis (MDA), a slope distribution was observed only for the *T. tridentatus* population at TT indicating a bottleneck. The consequence of bottleneck at TB might be more serious resulting in the only one haplotype. A unimodal distribution suggested that populations at SZ, YH, and BB were under range expansion. TK, TN, and TD were at demographic equilibrium (Table 1 and Fig. 5).

A pairwise analysis of logarithm of F_{ST} values among locations showed a negative trend without indicating isolation by distance (IBD) ($r = -0.08$, Fig. 6a). The Mantel test that compared logarithm of geographic distance and logarithm of F_{ST} matrices did not indicate a significant difference between matched and randomized values ($p = 0.536$). Figure 6b shows log linearized F_{ST} values plotted against corresponding log pairwise

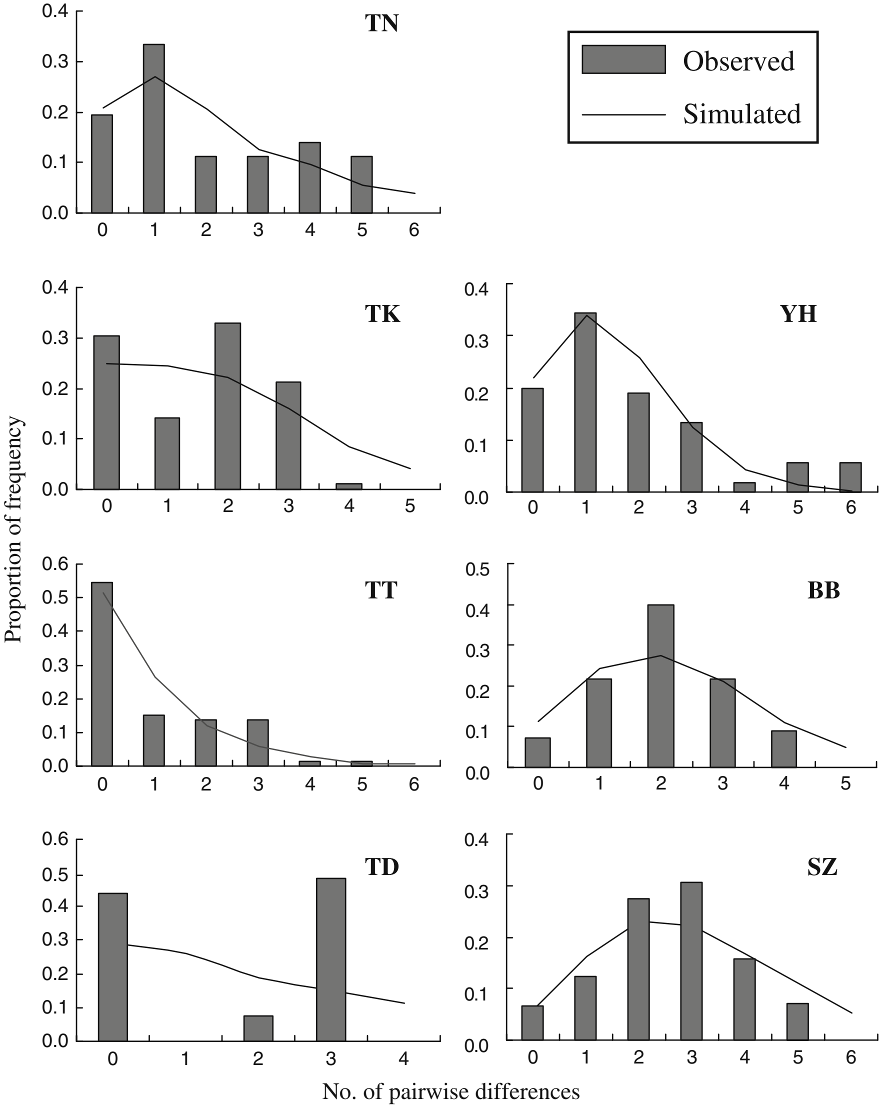

Fig. 5 Mismatch distribution analyses of the *Tachypleus tridentatus* AT-rich region pairwise sequence differences as computed by Arlequin 3.11 The observed distributions (*bars*) are compared to model distribution illustrated by the curve (*line*). A mismatch distribution for the Budai population could not be illustrated due to the presence of a single haplotype

geographic distance between six populations excluding the distinct populations, TT and TB (r = 0.58). The Mantel test indicates a significant difference between matched and randomized values (p = 0.018) suggesting IBD.

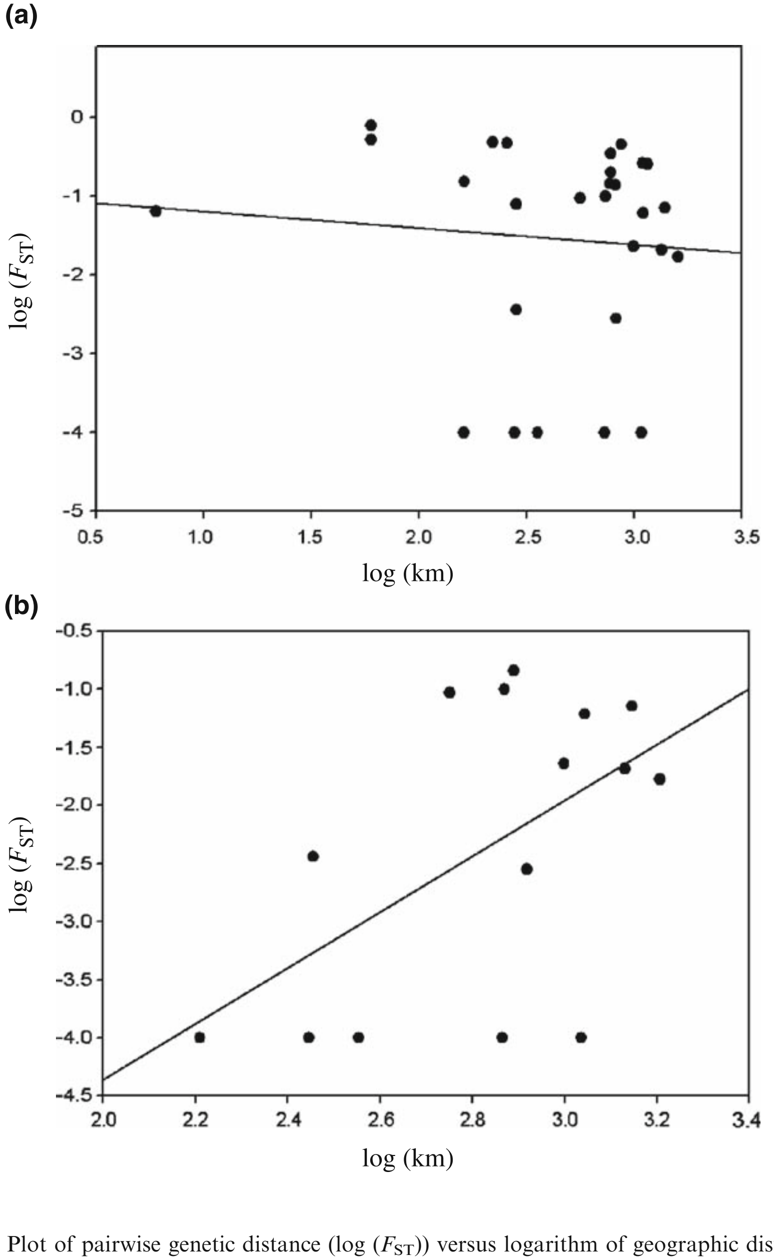

Fig. 6 Plot of pairwise genetic distance (log (F_{ST})) versus logarithm of geographic distance (log (km)) among populations for (**a**) all *Tachypleus tridentaus* samples in this study and (**b**) samples without considering Budai (TB) and Tiexiawei (TT) populations (see detail explanations in text). The RMA regression line overlays the scatter plots. The regression $y = -2.70x + 5.49$, $r = -0.08$, $p > 0.05$ for all samples. The regression was $y = 4.81x - 16.19$, $r = 0.58$, $p < 0.05$ for samples without considering TB and TT populations

4 Discussion

4.1 Phylogeographic and Dispersal Patterns

Genetic differentiation of *Tachypleus tridentatus* detected in this study based on mtDNA AT-rich region revealed no differentiation among most populations expect for two distinct populations. One of them was detected in Tiexiawei (TT) from Yang et al. (2007), the other was also found in Budai (TB) within Taiwan Strait (Table 2) in this study. DNA sequence analysis of the mitochondrial cytochrome oxidase I gene (COI) indicates minimal contemporary gene flow between Delaware Bay and Chesapeake Bay crab populations (Pierce et al. 2000). The collection site in Budai was also located in a lagoon similar to the semi-enclosed bay in TT. Our results also supported that a geographic barrier was a more important factor in subdivision among *T. tridentatus* populations than geographic distance, even at a distance of more than 1,000 km (for example between Beihai (BB) and Zhoushan (SZ)) (Table 2 and Fig. 3). For species that have limited adult migratory abilities, dispersal of planktonic larvae may maintain interconnections between geographically disjunct populations (Grimm et al. 2003). According to ecological observations, the hatched trilobite larvae of *Limulus polyphemus* swim freely for a short period (about 6 days) and settle to the bottom in shallow waters of the intertidal zone near their natal beaches (Shuster 1982). However, the strong tendency of *Limulus* larvae to remain close to the beach suggests that their capability for long-range dispersal between estuaries is extremely limited. The limited larval dispersal potential might also be consistent with the genetic subdivision among mid-Atlantic horseshoe crab populations (Pierce et al. 2000; Botton and Loveland 2003; King et al. 2005). Moreover, *T. tridentatus* larvae tend to stay in their nests immediately after hatching, where they usually reside over winter, then leave the nests and reside at or near their natal beaches in the following spring or summer (Kawahara 1982). Juveniles of *T. tridentatus* bury themselves in the sandy-mud bottom during high tide and feed during low tide (Sekiguchi 1988; Chen et al. 2004; Chiu and Morton 2003). This suggests that larvae of *T. tridentatus* might have less ecological dispersal potential than *L. polyphemus*. Coastal marine organisms utilize nearshore estuarine habitat as nursery grounds for larvae or juveniles. In contrast to pelagic or reef-based species, where reproduction often involves long-distance transport of larvae, such coastal species have developed strategies to minimize offshore transplant of larvae (Checkly et al. 1990; Sponaugle et al. 2002). The genetic connectivity of populations across Taiwan Strait was not always consistent with the direction of sea currents (Figs. 2 and 3), i.e., populations between TK and TN or TD or even between TN and YH had stronger connectivity with more than 100 effective female migrants per generation ($N_e m$) but did not follow the direction of sea currents. Instead of transportation of juveniles by sea current, another possible explanation might be migration by adults contributing to such a pattern. Based on a release-and-recaptures experiment of 12,500 *L. polyphemus* adults in mid-Atlantic (Chincoteague, Virginia and Ocean City, Maryland), the mean distance between site of release and site of recapture for all resighted *L. polyphemus* (3.45%) was 68.3 km; maximum distance moved was 493.7 km (Grogan 2004). If the adult movement pattern of *T. tridentatus* was similar to *L. polyphemus,* it might be possible for adult *T. tridentatus* to continuously migrate across Taiwan Strait (approximately 130–250 km in width and 60 m in maximum depth) therefore maintaining genetic homogeneity since the last glacial maximum. Based on the patterns of larval behaviour and conflict between genetic connectivity and sea currents, we suggest that adults' dispersal could be more important than larval dispersal for maintaining high gene flow in *T. tridentatus*. These results were also suggested by King et al. (2005) and Pierce et al. (2000) in regard to regional dispersal capacity especially in male-mediated dispersal.

4.2 Glaciation Events and Small-Population Effect on Demographic History

There was no differentiation of *Tachypleus tridentatus* along the open coast even at a distance of almost 2,000 km between the BB and ZS populations ($F_{ST} = 0.017$, $p > 0.05$). However, considerable differentiation was observed using microsatellite loci among populations along the Atlantic coast as genetic distances (chord) ranged between 0.25 (approximately 400 km) and 0.45 (approximately 2,200 km), while all pairwise F_{ST} values (0.014–0.092) were statistically significant ($p < 0.001$) (King et al. 2005).

Based on the analysis of genetic data combined with known geographic events, the picture of demographic history of *T. tridentatus* in East Asia can be elucidated. Both star-like haplotype network pattern (Fig. 4) and unimodal mismatch distribution analysis indicated range expansion of three populations, BB, YH, and ZS along Mainland China (Fig. 5). There are several marine species in this area which also showed patterns of range expansion, such as redlip mullet, *Chelon haematocheilus* (Liu et al. 2007), and crimson snapper, *Lutjanus erythropterus* (Zhang et al. 2006), along the coast of South and East China seas that could be due to the glacial sea-level fluctuation. There were approximately 12 major and many smaller scale glaciations in the Pleistocene (Crowley and North 1991), yet it is difficult to point to a particular sea-level fluctuation as the cause of each cladogenic event. Glacial maxima were associated with declines in sea level of 120–140 m in north South and East China seas board (Lambeck et al. 2002). The population of *T. tridentatus* in Hakata Bay, Kasaoka Bay (Japan), and Amoy (close to Kinmen) was isolated quite recently. Sekiguchi (1988) proposed that the ancestral populations distributed along the coast connecting Japan and China were scattered into the present isolated populations by the large-scale sea elevation that occurred towards the end of the Pleistocene period (during last glacial maximum). It is one part of the process of the distribution of horseshoe crab that raises questions, such as the unknown colonization routes of southern population of horseshoe crabs and how glacial events affect the population demographic history. Lack of phylogeographic structure along the Mainland China could reflect the recency of the range expansion and insufficient time to attain migration–drift equilibrium (Slatkin 1993) on *T. tridentatus*.

Differences in demographic history also explain the patterns of mtDNA sequence variations and genetic subdivisions among *T. tridentatus* populations. Low haplotype diversity, low nucleotide diversity, and an approximate Poisson mismatch distribution imply the genetic consequences of a population bottleneck (e.g., population in Tiexianwei) (Nei et al. 1975; reviewed in Avise 2000, 2004; see also in Table 1). It could be that dispersal is prevented by geographic barriers or by a life history pattern that act to prevent transport of larvae between populations. Alternatively, transport may occur, but migrating individuals may not have a high chance of recruitment into new habitats. Third, recruitment may occur, but selection may prevent migrants from growing and entering the breeding population (Koehn et al. 1980). No matter which the explanation, the genetic results show the existence of a heterogeneous spatial mosaic of marine dispersal. At the fine scale within Taiwan Strait, the geographic barriers hypothesis might apply to *T. tridentatus* of TT and TB because they occur in semi-enclosed areas, and as a consequence the population there might have limited genetic exchange with populations outside the bay (Fig. 3). A similar demographic differentiation was also observed in *L. polyphemus* in Delaware (DB) and Chesapeake bays (CB) of the East Coast of the United States. The population in DB was suggested to have undergone a recent population bottleneck, whereas the CB population was suggested to be a stable population at equilibrium (Pierce et al. 2000). The recent population bottleneck in the Delaware Bay can be attributed to population reduction by over-harvesting for commercial bait early in the last century (Pierce et al. 2000). Budai was one of few sites where populations of horseshoe crabs survived in Taiwan after long-term marine pollution along the west coast of Taiwan. Besides the geographical isolation, over-harvesting and loss of habitat for natal grounds in Magong Harbour and Budai Harbour might also be causing decline of population and consequent loss of genetic diversity in the TT and TB populations of *T. tridentatus*, respectively.

5 Conclusion and Conservation Implications

The present study of *Tachypleus tridentatus* consists of eight locations at various geographic types of coast: open coast of mainland, islands, and semi-closed bays. Those populations along open mainland coast that were under range expansion could be due to the effect of glacial sea-level fluctuation, and those populations at open island coast were under migration–drift equilibrium. Only two distinct populations were found and both of them were in a semi-enclosed bays in the Taiwan Strait (data from Yang et al. 2007 and the present study), and such pattern were also found in *Limulus polyphemus* populations in Delaware Bay and Chesapeake Bay (Pierce et al. 2000). The demographic histories showed these two distinct populations could be under recent bottleneck and have lost genetic diversity. A population with lower genetic diversity might result from inbreeding depression due to decreasing population size in a short timeframe, resulting in reduced evolutionary potential in a long-term time scale. For conservation purposes, an effective management strategy in the recovery of small inbred populations with low genetic diversity is to introduce individuals from other populations to improve their fitness and restore genetic diversity (Frankham et al. 2005). Moreover, horseshoe crabs are still not in wildlife conservation species list in Taiwan and we hope our results along with continuous surveys (such as in Chen et al. 2004) will promote horseshoe crabs to wildlife conservation species list.

Single, small reserves are unlikely to provide the best balance of conservation and fisheries' benefits and network of marine reserves are likely to be necessary (Botsford et al. 2001). The isolation-by-distance (IBD) model was the best description for gene flow between populations of *T. tridentatus* except for those distinct Budai (TB) and Tiexiawei (TT) populations and was almost unrestricted ($N_e m > 100$ to ∞) at the geographic distance of about 500 km. According to the pattern of IBD detected among mainland populations, spacing reserves less than 500 km apart could function well to sustain the gene flow between adjacent *T. tridentatus* populations along the open coast, creating an efficient design for a marine reserve network in northern South and East China seas. According to the implication for the genetic connectivity, proposed marine reserves of *T. tridentatus* following the rule could be set up in Beihai, Yangjing, Hong Kong, Kinmen (and Xiamen), and Zhoushan (from south to north in order) for the continuous reserves network (Fig. 1).

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Genetic Structure of Japanese Populations of *Tachypleus tridentatus* by mtDNA AT-Rich Region Sequence Analysis

Shin Nishida and Hiroko Koike

Abstract *Tachypleus tridentatus* is distributed from coastal Southeast Asia to western Japan. The northernmost population of this species, the Japanese population, is rapidly decreasing due to loss of tidal flats and spawning beaches, and the deterioration of coastal waters. To examine the genetic structure and the genetic diversity of the Japanese population, over 290 samples from nine localities were analyzed using hemolymph, egg, and muscle. The AT-rich region (control region, 369 base pairs) of mitochondrial DNA (mtDNA) was analyzed. Sequences of nuclear integrations of mtDNA (numt) were found in this species, with high sequence similarity to mtDNA. Therefore, to identify "true" mtDNA sequences, long PCR was conducted to amplify the majority of the circular mtDNA molecule. Specific primers were then designed for amplification of the mtDNA AT-rich region. Seven haplotypes were identified based on the sequence of the AT-rich regions from the Japanese populations. All haplotypes were related and closely connected by a single substitution. Haplotype AT1 was dominant and was observed in all regions examined. Two genetic groups were detected based on distribution of haplotypes and significant F_{ST} or Φ_{ST}. Sampling localities in the eastern group were almost monomorphic for AT1, with a few rare haplotypes, resulting in a low haplotype diversity, while the western group was comprised of haplotypes AT1, AT2, and AT3, and consequently higher haplotype diversity than in the eastern groups. These results suggested that the northernmost population of this species might have been formed recently and that the dispersal rate has been relatively low, leading to the formation of genetically distinct populations.

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1 Introduction

Tachypleus tridentatus is distributed from coastal Southeast Asia to western Japan (Sekiguchi 1988; Fig. 1). Members of this species generally inhabit a cove or bay that is protected from surf and becomes a tideland during low tide (Sekiguchi 1988). They molt at least 13 times (for male) and 14 times (for female) as they grow from the larval stage to sexual maturity (Harada 2003). This period exceeds 10 years for captive breeding from an egg (Harada 2003). They lay their eggs in sandy beaches near the high tide line. Hatched larvae of the American horseshoe crab, *Limulus polyphemus*, swim freely for approximately 6 days (Shuster 1982) and then settle to the bottom of water at or near their natal beach. On the other hand, larvae of *T. tridentatus* swim very little compared with *L. polyphemus* and usually stay in their nest after hatching and pass the winter there (Sekiguchi 1988).

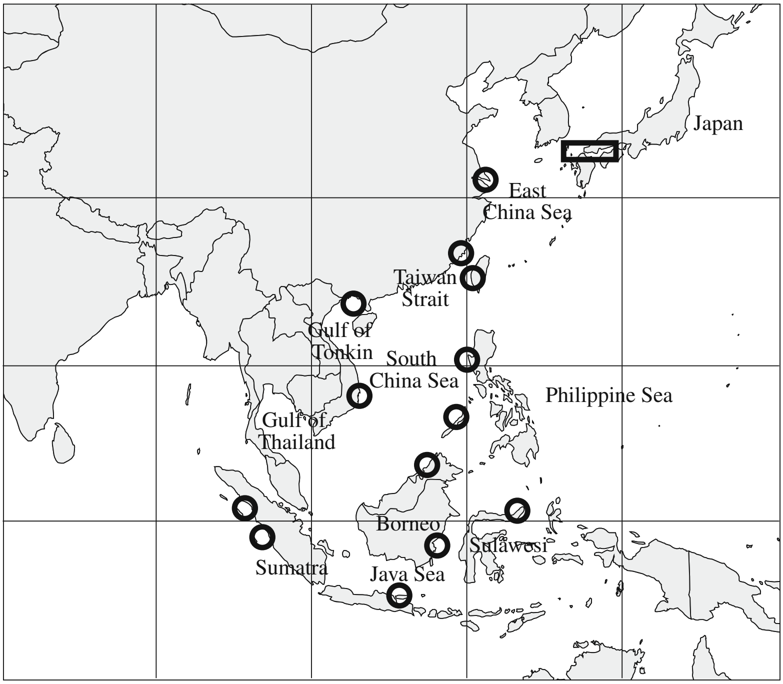


Fig. 1 Worldwide distribution of *Tachypleus tridentatus* (from Sekiguchi 1999) and the area analyzed (*square*) in this study

Observations of the behavior of larvae at Imari Bay (Sakemi 1997) suggested that at the spring tide event most of the larvae moved from the hatching sandy beach during the night at high tide and planktonically moved near the tideland via the ebbing tide. It has been suggested that larvae of this species exhibit phototaxis (Sakemi 1997). The juvenile stage (up to 7 cm in prosomal width) is spent in the tideland near the hatching beach, and locomotive activity is relatively low (50 m/month) (Kawahara 1982). Larger juvenile and sub-adult individuals may gradually move to deeper water depending on their growth stage. Adult individuals winter in deep water (about 20 m deep) offshore and move to shallow water near sandy beaches for spawning when the seawater temperature increases in the late spring (Nishii 1975; Sekiguchi 1988). These observations indicate that horseshoe crabs stay in a relatively limited area near their natal beach and should lead to genetic differences among spawning localities.

The northernmost population of *T. tridentatus*, the Japanese population, is rapidly decreasing due to loss of tidal flats and spawning beaches and the deterioration of coastal waters (reviewed in Sekiguchi 1988; Itow 1993). In Japan, the species is included in the Red List and the Red Data Book of Japan as critically endangered (CR + EN) (Ministry of the Environment 2000, 2006). The Japanese population and also probably the Asian populations of this species are facing extinction and are in urgent need of conservation measures and protection.

Molecular genetic analysis is a powerful tool for investigating genetic differentiation within a population, genetic structure, and diversity throughout the history of a population. Analysis of populations of the American horseshoe crab (*L. polyphemus*) along the eastern coast of North America using several genetic markers detected a major genetic “break” between the northern and southern populations in a region in northeastern Florida by allozyme (Selander et al. 1970), mitochondrial DNA restriction fragment length polymorphism analysis (Saunders et al. 1986), and microsatellite (King and Eackles, 2004). Sequence analyses of mitochondrial cytochrome *c* oxidase subunit I (*COI*) (Pierce et al. 2000) suggested that there has been limited gene flow between the Delaware Bay and upper Chesapeake Bay populations, which occupy a small geographic range on the eastern coast of North America.

There is little genetic information about *T. tridentatus*. Analysis of mitochondrial (mt) DNA by restriction fragment length polymorphism (RFLP) (Sugawara et al. 1988) detected no genetic differences among the Hakata Bay, Kasaoka Bay (located in Seto Inland Sea at Okayama prefecture), and Amoy, China, populations. Although the sample size was small, this result indicated that there is a close relationship between the Japanese and Chinese populations. Recently, Yang et al. (2007) investigated three local populations from the Taiwan Strait (Kinmen Island beside mainland China, and Tiexianwei and Dongwei of Magong Island) using sequence analysis of the mtDNA AT-rich region (control region). A significant subdivision was detected between populations on Kinmen Island and Tiexianwei on Magong Island, which represent a relatively small geographic range. In contrast, there were no significant genetic differences between the two populations on Magong Island: Tiexianwei and Dongwei, or between Kinmen Island and Dongwei populations.

The mtDNA AT-rich region is a highly variable, noncoding region that is useful for phylogeographic studies and population genetic surveys, although the high AT content poses technical and analytical problems (Vila and Björklund 2004). In this study, we carried out a high-resolution analysis of the mtDNA AT-rich region to examine the genetic structure and the genetic diversity of the Japanese population of *T. tridentatus*. Our results provide useful information for the conservation of this species.

2 Materials and Methods

We analyzed the mtDNA of 294 samples (Table 1) derived from hemolymph, egg, and muscle, from nine localities in Japan: Kujukushima-Sasebo Bay ($n=69$) at Nagasaki prefecture; Imari Bay ($n=16$) at Saga prefecture; Kafuri Bay ($n=48$), Hakata Bay ($n=56$), Tsuyazaki tidal flat ($n=18$), and Sone tidal flat ($n=43$) at Fukuoka prefecture; Wama tidal flat ($n=7$) and Morie Bay ($n=29$) at Oita prefecture; and Yamaguchi Bay ($n=8$) at Yamaguchi prefecture. All sampling localities have a spawning beach except for Wama tidal flat. These samples consist of individuals of various ages, collected in 2005 and/or 2006 from each locality, except for few samples from captive individuals and specimens in other years, and egg samples from Sone tidal flat in 2000.

Table 1 List of the nine local populations and the samples from each that were analyzed in this study

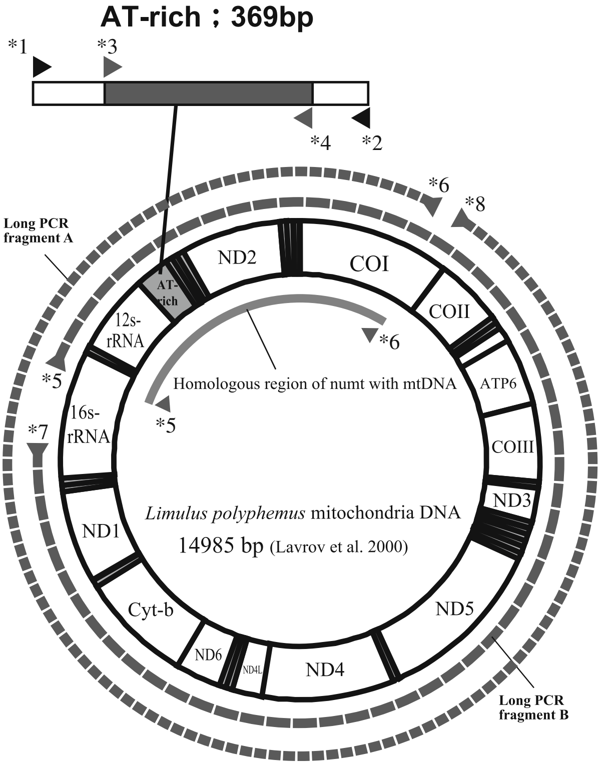
Local population/Samples	Year of sampling	Hemolymph	Egg	Muscle	Total
Kujukushima-Sasebo Bay	2005	39			39
	2006	30			30
	Total	69			69
Imari Bay	2005	8	4	4	16
Kafuri Bay	2002			1	1
	2006	24	22	1	47
	Total	24	22	1	48
Hakata Bay	2004			1	1
	2005	33	4		37
	2006	17		1	18
	Total	50	4	2	56
Tsuyazaki tidal flat	2006	11		3	14
	Others			4	14
		11	0	7	18
Sone tidal flat	2000		16		16
	2005			3	3
	2006	24			24
	Total	24	16	3	43
Wama Bay (Usa)	2005	7			7
Morie Bay (Kitsuki)	2005	6	20		26
	Others	3			3
	Total	9	20		29
Yamaguchi Bay	2005			8	8
Total		202	66	26	294

Approximately 100 μl of hemolymph were collected by direct cardiac puncture using a 0.45 (26G) × 13 mm SB needle and a 1 ml tuberculin syringe (Terumo Corp.) after cleaning the dorsal cardiac membrane with 70% ethanol. The hemolymph was placed into 100 μl of RSB buffer (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 25 mM EDTA-2Na, pH 7.4) in a 1.5 ml microcentrifuge tube. As soon as possible after sampling, individuals were released at the same place they were caught. Pierce et al. (2000) reported a procedure for DNA isolation from hemolymph that involves nuclease-like digestion of high-molecular weight DNA using proteinase K. Therefore lysis buffer containing guanidine thiocyanate (Reagent 1, IsoQuick Nucleic Acid Extraction Kit, ORCA Research Inc., USA) was used as a protein denaturant for the hemolymph samples. Muscle from dead individuals and eggs were stored in 99% ethanol. Approximately 5–10 mg of sliced muscle tissue or egg were placed in a solution of 310 μl of RSB buffer, 15 μl of 10% SDS, and 15 μl of 20 mg/ml proteinase K, and then incubated for 2 hours at 55°C on a rotator for protein digestion. Nucleic acids were extracted using the IsoQuick Nucleic Acid Extraction Kit.

At the time of this study, there had been no reports of AT-rich sequences in this species. Initially, we amplified a mid-length fragment (ca. 4 kb), which included the AT-rich region, extended through end of the 16s rRNA, and included the entire coding region of the *COI* gene, using the following primers: HC-16sH.hc (5’-ATTATGCTACCTTCGCACGGTCAATATAC-3’) and HC-COIH.hc (5’-GGCTAATAATATGAGAAATTATTCCAAATCC-3’), which were designed based on known sequences of this species (GenBank accession Nos. U09387 and U09393, Avise et al. 1994). However, the amplified 4 kb fragment was identified by sequence analysis as nuclear integrations of mtDNA (numt, a partial sequence has been registered in GenBank under AB353282), with high sequence similarity to mtDNA. To our knowledge, this is the only report of this sequence in horseshoe crabs. To examine the “true” mtDNA sequence, we used long PCR to amplify most of the sequence of the circular mtDNA (Fig. 2). Long PCR fragment A (from/to 16s rRNA) was amplified using the primers HC-16sH.hc and 16sL-edge.hc (5’-GGCGGAGAAGTTCTAAATGAGGGTCTGTTC-3’); fragment B (from/to *COI*) was amplified using the primers HC-COIH.hc and COIL-edge.hc (5’-TACCACTTTTTTCCCTCAACACTTTTTAGG-3’). Long PCR was carried out in a reaction volume of 25 μl, which contained approximately 50 ng of total DNA, 1 × GC buffer I, 0.4 mM of each dNTP, 0.2 μM of each primer, and 1.25 units TaKaRa LA *Taq* DNA polymerase (TaKaRa Bio Inc.). Shuttle PCR (two temperature PCR) was performed as follows: preheating at 94°C for 1 min, 30 cycles of denaturation at 98°C for 10 s, annealing and extension at 60°C for 15 min, and post-extension at 72°C for 10 min.

The following specific primers for the amplification of the mtDNA AT-rich region were designed to compare numt and mtDNA sequences: HC-mt12s1.hc (5’-GTCTAACCGCGGTAGCTGGCACAATTTTG-3’) and HC-mtRGln1.hc (5’-TTTTAGTGTAAAGGCACATTGAATTTTG-3’) for PCR, and HC-12sB.hc (5’-CTAAGTATAATTATATTATTCATATAGCTAAG-3’) and tRgl-seq.hc

Fig. 2 Schematic representation of the circular mtDNA molecule, with regions analyzed and primer positions used in this study. *Triangles* indicate positions of the primers. *1, HC-mt12s1.hc; *2, HC-mtRGln1.hc; *3, HC-12sB.hc; *4, tRgl-seq.hc; *5, HC-16sH.hc; *6, HC-COIH.hc; *7, 16sL-edge.hc; *8, COIL-edge.hc



(5'-ATGGATCAGACCATTAAAATTAGGTAAAGG-3') as internal primers for sequencing. PCR was carried out in a reaction volume of 25 μl, which contained approximately 50 ng of total DNA, 1 × PCR buffer, 0.2 mM of each dNTP, 0.2 mg/ml BSA, 0.2 μM of each primer, and 0.625 units TaKaRa Ex *Taq* Hot Start Version DNA polymerase (TaKaRa Bio Inc.). The PCR reaction parameters consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 45 s.

The products of long PCR and some of shorter PCR products were subcloned using the TOPO TA Cloning Kit (Invitrogen Corp.) into the PCR 2.1 TOPO-vector and then transformed into the TOP 10F' strain of *E. coli*. Positive colonies were selected and screened for the correct product insert. After the initial screen, the PCR products from each clone were sequenced.

PCR products were enzymatically purified (PCR Product Pre-Sequencing kit, USB Corporation), and direct sequencing was carried out using a Dye Terminator Cycle Sequencing Kit (Beckman Coulter Inc.), according to the manufacturer's protocol. Cycle sequencing consisted of 30 cycles of 96°C for 20 s, 50°C for 20 s, 60°C for 150 s. Capillary sequencing was conducted using the CEQ2000XL DNA sequencer (Beckman Coulter Inc.) and data were analyzed using CEQ Software, Sequence Analysis ver. 2 (Beckman Coulter Inc.). Alignment of the sequence data was performed manually using MEGA 3.1 (Kumar et al. 2004).

Geographic heterogeneity in the frequency distribution of haplotypes was tested using an exact test. Genetic variation in populations was expressed as gene diversity (*h*, Nei 1987), which was estimated from the frequency distribution of haplotypes. Nucleotide diversity (π; Nei 1987) was estimated from the average number of nucleotide differences per site between two sequences. The population genetic structure was quantified by conventional *F*-statistics (F_{ST}; Wright 1951; Weir and Cockerham 1984) and the analysis of molecular variance (AMOVA, Φ_{ST}; Excoffier et al. 1992) was done using Arlequin (ver. 3.11; Excoffier et al. 2005). A network tree was constructed by the median-joining (MJ) method (Bandelt et al. 1999) using Network (ver. 4.201; http://www.fluxus-engineering.com).

3 Results

A total of six transitions (Table 2) were detected in the AT-rich region of the Japanese populations defining seven haplotypes (AT1–AT7; GenBank accession Nos. AB353283–AB353289; Table 3 and Fig. 3). We found no significant difference in years on the haplotype frequencies for each sampling locality. AT1 was the most dominant haplotype, observed in all regions. The frequency of AT1 in each locality was greater than 0.5. AT2 and AT3 were detected in the western part of the area analyzed and defined the western group (Kujukushima-Sasebo Bay, Imari Bay, and Kafuri Bay). Kujukushima-Sasebo Bay and Imari Bay were comprised of AT1, AT2, and AT3, and the frequency of three haplotypes was similar in both localities. Four individuals with the AT4 haplotype were identified from both Kafuri Bay and Morie Bay, which are geographically separate. AT6 was found only in Kafuri Bay from hemolymph samples and its frequency was 0.125 (six individuals). The eastern group (Hakata Bay, Tsuyazaki tidal flat, Sone tidal flat, Wama Bay, Morie Bay, and Yamaguchi Bay) was defined as almost monomorphic for AT1 with the occurrence of some rare haplotypes. AT5 and AT7 were detected only in the Sone tidal flat and Tsuyazaki tidal flat, respectively.

Table 2 Variable nucleotide sites of mtDNA AT-rich region in seven haplotypes from the Japanese populations

	Nucleotide position					
Haplotype	1 4 9	1 9 5	2 4 4	2 6 9	2 9 2	3 3 5
AT1	C	T	A	C	C	G
AT2	.	.	.	T	.	.
AT3	.	.	.	T	T	.
AT4	.	.	.	.	T	.
AT5	.	C	.	.	.	.
AT6	.	.	G	.	.	.
AT7	T	.	.	T	T	A

Table 3 Distribution of the mtDNA AT-rich region haplotypes, haplotype diversity (*h*), and nucleotide diversity (π) for the nine localities in Japan

Sampling Localities/ haplotypes	AT1	AT2	AT3	AT4	AT5	AT6	AT7	Total	*H*	π(%)
Kujukushima-Sasebo Bay	35	21	13					69	0.62 ± 0.03	0.22 ± 0.18
Imari Bay	9	4	3					16	0.63 ± 0.09	0.23 ± 0.19
Kafuri Bay	37	1	2	2		6		48	0.39 ± 0.08	0.14 ± 0.13
Hakata Bay	56							56	0.00 ± 0.00	0.00 ± 0.00
Tsuyazaki tidal flat	17						1	18	0.11 ± 0.10	0.12 ± 0.13
Sone tidal flat	42				1			43	0.05 ± 0.04	0.01 ± 0.03
Wama Bay (Usa)	7							7	0.00 ± 0.00	0.00 ± 0.00
Morie Bay (Kitsuki)	27			2				29	0.13 ± 0.08	0.04 ± 0.06
Yamaguchi Bay	8							8	0.00 ± 0.00	0.00 ± 0.00
Total	238	26	18	4	1	6	1	294	0.33 ± 0.03	0.13 ± 0.12

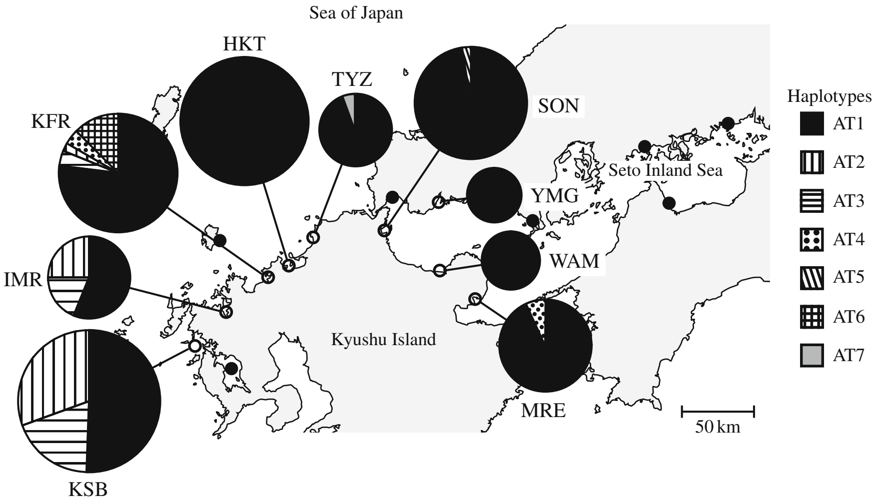


Fig. 3 Geographic distribution of the mtDNA AT-rich region haplotypes of Japanese *Tachypleus tridentatus*. *Open circle* show other habitats of this species. KSB, Kujukushima-Sasebo Bay; IMR, Imari Bay; KFR, Kafuri Bay; HKT, Hakata Bay; TYZ, Tsuyazaki tidal flat; SON, Sone tidal flat; YMG, Yamaguchi Bay; WAM, Wama tidal flat; MRE, Morie Bay

The haplotype diversities (h) and the nucleotide diversities (π) for each sampling locality are shown in Table 3. The haplotype diversity was 0.33 for the overall Japanese population. Those of sampling locality in the western group (h = 0.39–0.63, overall; h = 0.57) were higher than in the eastern group (h = 0–0.13, overall; h = 0.05). Nucleotide diversity was 0.0013 for the overall Japanese population, and those of sampling localities in the western group (π = 0.0014–0.0023, overall; π = 0.0021) were higher than in the eastern group (π = 0–0.0012, overall; π = 0.0002).

The results of the exact test for geographic heterogeneity in haplotype frequency distribution are shown in Table 4. Significant differences were detected between Kujukushima-Sasebo Bay and Imari Bay compared to all other localities with the exception of some pairs. There were no significant differences among localities in the eastern group and between Kujukushima-Sasebo Bay and Imari Bay in the western group. Kafuri Bay differed from others in western group and Hakata Bay. As a result, significant difference between western and eastern group was detected with high probability value ($P < 0.01$).

The degree of genetic differentiation was measured using F_{ST} (Table 4) and Φ_{ST} (Table 5). F_{ST} values ranged from zero, wherein all subpopulations have equal allele frequencies, to one, wherein all the subpopulations are fixed for different alleles. The parameter Φ_{ST} is analogous to F_{ST}, but incorporates genetic distances among alleles (for a review, see Allendorf and Luikart 2007). Typically, an F_{ST} value above approximately 0.15 is considered an indication of significant differentiation among fragments (Frankham et al. 2002). Pairwise comparisons showed that there was significant differentiation between the neighboring localities of Imari Bay and Kafuri Bay (F_{ST} = 0.10; Φ_{ST}= 0.20), and Kafuri Bay and Hakata Bay (F_{ST} = 0.15, Φ_{ST}= 0.09). Kujukushima-Sasebo Bay and Imari Bay were genetically different from the other localities (F_{ST} = 0.10–0.51; Φ_{ST}= 0.17–0.55). No significant differences were detected among the eastern localities. F_{ST} and Φ_{ST} values between western and eastern groups were 0.25 and 0.23, respectively, and were significant ($P < 0.01$).

Table 4 Pairwise F_{ST} values (lower matrix) and probability values of exact test (upper matrix) between the nine localities in Japan

	1	2	3	4	5	6	7	8	9
1 Kujukushima-Sasebo		0.93	***0.00***	***0.00***	***0.00***	***0.00***	0.06	***0.00***	***0.03***
2 Imari Bay	-0.03		***0.01***	***0.00***	***0.00***	***0.00***	0.17	***0.00***	0.08
3 Kafuri Bay	***0.14***	***0.10***		***0.00***	0.27	***0.01***	0.80	0.15	0.80
4 Hakata Bay	***0.34***	***0.51***	***0.15***		0.24	0.44	-1.00	0.12	-1.00
5 Tsuyazaki tidal	***0.22***	***0.23***	0.04	0.07		0.51	1.00	0.29	1.00
6 Sone tidal	***0.29***	***0.39***	***0.10***	0.01	0.00		1.00	0.16	1.00
7 Wama Bay (Usa)	***0.20***	0.18	0.02	0.00	-0.07	-0.08		1.00	-1.00
8 Morie Bay	***0.23***	***0.26***	***0.04***	0.07	-0.01	0.02	-0.05		1.00
9 Yamaguchi Bay	***0.21***	0.20	0.03	0.00	-0.05	-0.06	0.00	-0.04	

Note: In bold and italic, P <0.05.

Table 5 Pairwise Φ_{ST} (analysis of variance by AMOVA) values between the nine localities in Japan

	1	2	3	4	5	6	7	8	9
1 Kujukushima-Sasebo Bay									
2 Imari Bay	-0.04								
3 Kafuri Bay	***0.23***	***0.20***							
4 Hakata Bay	***0.37***	***0.55***	***0.09***						
5 Tsuyazaki tidal flat	***0.20***	***0.17***	0.01	0.07					
6 Sone tidal flat	***0.33***	***0.45***	***0.06***	0.01	0.02				
7 Wama Bay (Usa)	***0.23***	***0.21***	-0.02	0.00	-0.07	-0.08			
8 Morie Bay (Kitsuki)	***0.28***	***0.33***	0.03	0.07	-0.01	0.00	-0.05		
9 Yamaguchi Bay	***0.23***	***0.23***	-0.01	0.00	-0.05	-0.06	0.00	-0.04	

Note: In bold and italic, $P < 0.05$.

Figure 4 shows the MJ network for the haplotypes of the AT-rich regions of species from Japan and the Taiwan Strait (GenBank accession Nos. DQ516529–DQ516538; Yang et al. 2007). All haplotypes were closely related and connected by a single substitution. Although the Japanese populations were genetically distinct from the Taiwan Strait populations, they were relatively close, as AT2 and AT7 were shared by both populations.

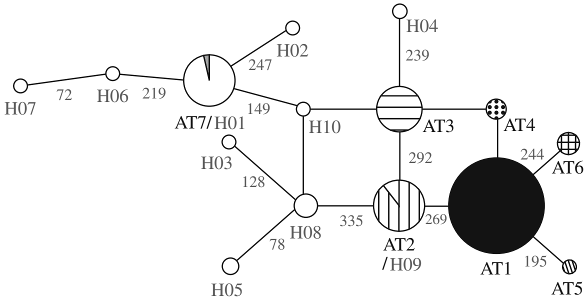


Fig. 4 The MJ network for mtDNA AT-rich region haplotypes from Japan (AT1–AT7) and the Taiwan Strait (in white, H01–H10, Yang et al. 2007). The area of the circles is proportional to the frequency of the haplotype. Each *line* represents a mutational step with the indicated numbers.

4 Discussion

Genetic subdivisions were identified in Japanese populations of *T. tridentatus* that occupy a small geographic area using analysis of the mtDNA AT-rich region. Previously, RFLP analysis of mtDNA (Sugawara et al. 1988) failed to detect any genetic variation among populations of Hakata Bay, Kasaoka Bay (not analyzed in this study), and Amoy, China, although the sample size was small.

Sequence analysis of the AT-rich region of mtDNA provided high-resolution genetic information, and we identified seven haplotypes with six transitions in Japan. The western group was defined by haplotypes AT2 and AT3. In contrast, the eastern group was almost monomorphic for AT1, and lacked AT2 and AT3. The geographic boundary between the two groups is the Itoshima Peninsula located between Kafuri Bay and Hakata Bay. The degree of genetic differentiation between the two groups was relatively high ($F_{\mathrm{ST}} = 0.25$; $\Phi_{\mathrm{ST}} = 0.23$). In an exact test of haplotype frequency distributions, F_{ST} and Φ_{ST} values indicated that the Kafuri Bay was genetically significantly different from the neighboring localities in Imari Bay and Hakata Bay. Thus, the Japanese population can be divided into three genetic units: western, Kafuri Bay, and eastern. The geographic distances between them are relatively small. Imari Bay and Kafuri Bay are approximately 70 km apart, and Kafuri Bay and Hakata Bay are only about 40 km apart, and separated by relatively shallow water (< 20–30 m deep). There seems to be no barriers between them because adult individuals live on bottom under around 20 m deep.

In the Taiwan Strait, a significant population subdivision ($F_{\mathrm{ST}} = 0.14$) was detected between Kinmen and Tiexianwei at Magong Island which represent a relatively small geographic range (< 150 km) (Yang et al. 2007). On the other hand, two populations of Mogong Island, Tiexianwei and Dongwei, are separated by 15 km and showed moderate gene flow, while there appeared to be unlimited gene flow between Kinmen and Dongwei. Tiexianwei is located at the head of Mogong Bay and is semi-closed. Such geographical features and consequent loss of habitat might have limited the migration of individuals to or from the bay (Yang et al. 2007). A population subdivision was also reported for the American horseshoe crab, *L. polyphemus* (Pierce et al. 2000). The haplotype frequency distribution between Delaware Bay and upper Chesapeake Bay populations on the eastern coast of North America was significantly different based on *COI* sequence analysis, indicating limited gene flow on small geographic scale (Pierce et al. 2000). These results, together with life history information, suggest that the dispersal rates of this species are relatively low and that natal homing may be relatively strong, at least for females, leading to the formation of genetically distinct populations. Geographic features and the structure and condition of habitats should also strongly affect the migration of this species.

The network tree showed that the whole Japanese population is clearly genetically distinct from the whole Taiwan Strait population. However, they are relatively genetically close, since all haplotypes had a continuous connection, and AT2 and AT7 were shared by both populations. Population differentiation and the maintenance of a close relationship could be explained by "isolation by distance" over a short time period. Haplotype H04 from the Taiwan Strait could have been derived from AT3, which indicates that AT3 may also be found in China. AT2, AT3, and possibly AT7 would be the ancestral haplotypes for the Japanese population.

These results clearly show the population history of the Japanese population. The distribution of *T. tridentatus* appears to have continuously expanded from south to north. The ancestral populations, including AT2, AT3, and AT7, migrated from Southeast Asia to the western part of Japan through the Taiwan Strait. AT1 was the endemic haplotype for the Japanese population and therefore would emerge in the ancestral Japanese population. Later, a small number of individuals composed of mainly AT1 moved into Hakata Bay and rapidly expanded in the eastern part of Japan, forming the eastern group. The haplotype diversities and nucleotide diversities for each locality reflect each population history. The western group maintained ancestral polymorphisms leading to relatively high genetic diversity, while the eastern group may be strongly influenced by the founder effect, and thereby exhibit less genetic diversity.

Although it is difficult to estimate the time for this divergence/dispersal event because the evolutionary rate of the AT-rich region in this species is unknown, glacial and warmer interglacial events during the middle to late Quaternary period would strongly affect migration of this species. In the first phase, during the glacial events when the sea level was lower than 100 m (Ohshima 1990), the East China Sea was dry, and the western Kyushu was connected to the southern Asian continent. In the subsequent warmer period, new habitat areas, such as tideland and sandy beaches, were continuously created by marine transgression. Species could migrate into Japan around the coastal line through these new habitats. In the second phase, the Holocene climate optimum period, part of the eastern group would be formed in the Seto Inland Sea, as this marine area emerged during this period.

The identification of a population subdivision in the Japanese population of *T. tridentatus* is useful information for defining the management units for the conservation of this species. Two genetic groups, the western group including at least two genetic units (for western and Kafuri) and the eastern group, were detected based on distribution of haplotypes and significant F_{ST}. Moreover, this genetic data with the network tree and values of genetic diversities revealed unique population history for each group. Genetic differentiation among local populations must be considered when introducing individuals from other habitats and breeding programs to restore the local population. Almost all of the populations in Japan, and most likely other Asian populations of this species as well, are decreasing due to human activities and fragmentation of their habitat. The genetic diversity of this northernmost population is conventionally low. Therefore, fragmentation of their habitat would lead to a rapid loss of genetic diversity and promptly increase their risk of extinction. Further study using microsatellite DNA markers should identify genetic differentiation in the Japanese population at a higher level of sensitivity, and provide information about male activity, and be useful for the genetic monitoring of stocks for the conservation of this species.

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School, Kawakami, Y., Iwaoka, C., Hayashi, O., Takahashi, S., Hayakawa, O., Sakemi, R., Sugimoto, S., Nishihara, S., Shiokawa, N., Harada, N., Ono, G., Teshima, T., Maeda, K., Wada, T., Okamura, T., Kotoh, S., Ohira, Y., Michiyama, A., Kido, Y., Shibata, K., Mansyo, M., and Hamada, M.

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Part IB
Reproduction, Physiology, and Development

Fig. 1b A group of horseshoe crabs spawning on an intertidal beach (photograph by M. Botton)

Reproductive Competition and Sexual Selection in Horseshoe Crabs

H. Jane Brockmann and Matthew Denman Smith

Abstract The four species of horseshoe crabs share many similarities but differ in the intensity of reproductive competition. Although all horseshoe crabs nest synchronously, only *Limulus polyphemus*, the American horseshoe crab, has intense male–male competition (high operational sex ratios and multi-male groups around nesting pairs) and very high female nesting densities. These differences in reproductive competition are reflected in differences between American and Asian species (*Tachypleus gigas*, *T. tridentatus*, or *Carcinoscorpius rotundicauda*) in their reproductive structures and in egg and sperm size. However, the American and Asian species do not differ in the degree of sexual dimorphism in body size. The intensity of reproductive competition is correlated with nesting density in *L. polyphemus*, but this is not the case in the Asian species, which rarely have multi-male groups or highly male-biased operational sex ratios.

1 Introduction

Horseshoe crabs have an unusual reproductive system unlike that of any other arthropod (Sekiguchi 1988b, Giese and Kanatani 1987): all four species have external fertilization and broadcast spawning with close pairing but no brooding. The unusual reproductive behavior of the four extant species is in many ways very similar (Table 1). In all four species males attach to females (in amplexus) with modified prosomal appendages; the male–female pairs migrate into shallow water where they spawn; females oviposit in clusters by injecting their eggs into the substrate; and the eggs are fertilized as they are laid with aquatic free-swimming sperm (Brockmann 2003a; although females occasionally arrive in the nesting area without males, they do not lay eggs). These clusters of eggs are left buried 7–20 cm below the surface where they develop and the pair returns to the sea. Despite similarities, differences exist in the

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Table 1 Comparison of reproductive patterns in horseshoe crabs

Characteristic	*Limulus polyphemus*	*Tachypleus tridentatus*	*Tachypleus gigas*	*Carcinoscorpius rotundicauda*
Nesting habitat	Bays and estuaries on undisturbed sandy beaches with low organic content in the upper intertidal; nest in 20 cm water or less$^{2,3,17, 20,22, 27,29}$	Sheltered, low-profile sandy beaches with low organic content between the upper and lower tide lines in 30 cm water^{3,10,12,16,22,23}	Brackish estuaries and rivers and sandy estuarine beaches near the high-tide mark^{6,8,22,23,24}	Brackish estuaries and rivers in muddy sand and mangrove swamps^{6,10,14,23,24}
Length of breeding season	MA: May–June^{13,15,28} NY: late May–July^{4} DE Bay: May–June^{26} FL: March–Nov^{20}	Japan: mid-July to mid-August^{6,12,16,22,23} S. China: May–August^{11}	Gulf of Thailand (lat. 10°N): April–August^{22,23} Bay of Bengal, India (lat. 21°N): throughout the year^{6,8}	Gulf of Thailand: throughout the year^{22,23} Bay of Bengal, India: early pre-monsoon^{14}
Timing of nesting	Nest on high tides of new and full moon^{1,20,30}	Nest on high tides of new and full moon^{5,12,22}	Nest on high tides of new and full moon^{6,8,24}	Nest on high tides of new and full moon^{6,14}
Density: pairs/100 m	Variable, 1–1863 pairs, median = 40 (FL; see Table 2)	1–48, normally 30 pairs^{22}	1–20 pairs^{6,8}	50–100 pairs^{14}
OSR (M:F)	1.5–6.9^{1,20,30}	Normally 1.0^{3,22}	1.09^{6,8,31}	1.0–1.4^{14}
Egg numbers/ female/ year	14,500–63,500^{15} 88,000^{28}	20,000^{16,23,28}	1242–6565^{6,7} 8000^{23,28}	3540–13,490^{7,14,23,28} 4217–10,982^{6,5}
Eggs/cluster	3650^{26,28}; 640–1280^{15}	561–888^{22}; 200–300^{5,23,28}; 100–631^{11}; up to 1000^{7,12}	40–213^{24}; 200–300^{6}; 400^{16,23,28}	20–72^{14}; 43–139^{6}; 80–150^{23,28}

Table 1 (continued)

Characteristic	*Limulus polyphemus*	*Tachypleus tridentatus*	*Tachypleus gigas*	*Carcinoscorpius rotundicauda*
Egg size	1.6–1.8 mm[15,23,25,28]	3 mm[5,12,23,25] 3–3.3 mm[16,28]	3.25–4 mm[19,24,25,28] 1.54–2.09 mm[7,16] 4.0 mm[23]	2–2.2 mm[7,23,24,28] 1.5–2.3 mm[5,25] 1.25–1.5 mm[14]
Female:male size ratio;	1.28 (see Table 2)	1.14–1.20[3,32]	1.29[32]	1.04[32]
Prosomal width*	Male: 200.2 mm Female: 256.9 mm[32]	Male: 244.2 mm Female: 278.4 mm[32]	Male: 163 mm Female: 210 mm[32]	Male: 128.7 mm Female: 133.8 mm[32]
Claspers on male	First pair thickened; hemichelate[32]	First and second thickened; hemichelate[32]	First and second thickened; hemichelate[32]	First and second slightly swollen; chelate[32]

* Size varies considerably between sites in all four species (see Table 2 for variation in *L. polyphemus*).

[1] Barlow et al. (1986); [2] Botton et al. (1988); [3] Botton et al. (1996); [4] Botton et al. (2006); [5] Chatterji and Parulekar (1992); [6] Chatterji (1994); [7] Chatterji (1995); [8] Chatterji et al. (1992); [9] Chen et al. (2004); [10] Chiu and Morton (2003); [11] Chou and Cheng (1950); [12] Goto and Hattori (1929); [13] James-Pirri et al. (2005); [14] Khan (2003); [15] Leschen et al. (2006); [16] Mikkelsen (1988); [17] Penn and Brockmann (1994); [18] Rama and Rao (1972); [19] Roonwal (1944); [20] Rudloe (1980); [21] Sekiguchi (1988a); [22] Sekiguchi (1988b); [23] Sekiguchi and Nakamura (1979); [24] Sekiguchi et al. (1977); [25] Sekiguchi et al. (1988); [26] Shuster and Botton (1985); [27] Shuster (1950); [28] Shuster (1982); [29] Smith et al. (2002); [30] Smith et al. (2002b); [31] van der Meer Mohr (1941); [32] Yamasaki et al. (1988).

reproductive biology of the four species, which may be associated with ecological and environmental differences.

All four species of horseshoe crabs migrate for spawning into shallow water. *Limulus polyphemus*, *Tachypleus gigas*, and *T. tridentatus* nest on sheltered, intertidal beaches (Chiu and Morton 2003, Sekiguchi 1988b) where the sand is well aerated, low in organic matter, and low in mud and silt (Botton et al. 1988, 2006, Penn and Brockmann 1994, Smith et al. 2002a). This substrate provides a suitable environment for development until the larvae emerge and metamorphose into juveniles (the timing of which depends on temperature and population; Laughlin 1983). These nesting areas are usually in estuaries and bays that are protected from high waves and that have shallow, nearby flats that provide feeding grounds for the young juveniles (Brady and Schrading 1997, Chiu and Morton 1999, 2003, Sekiguchi 1988b). As they grow larger, juveniles move into progressively deeper water (Shuster 1982). Unlike the other three species, *Carcinoscorpius rotundicauda* nests well up into the mouths of rivers, on mudflats, and in mangroves (Khan 2003, Mikkelsen 1988). Horseshoe crabs are capable of picking out favorable habitat within much larger, unsuitable areas (Botton et al. 2006), so their nesting is often concentrated in a few high-quality patches.

The concentrated nesting of horseshoe crabs may also be due to two additional factors. First, it is possible that horseshoe crab nesting may be social, i.e., that horseshoe crabs are attracted by the presence of conspecifics. Social nesting is often thought to evolve in response to predation, which may be important for *L. polyphemus*, at least at some points in their long life cycle (Botton 2009). Second, there is good evidence that horseshoe crabs are philopatric. Larvae metamorphose shortly after emerging from the nest and often settle near their natal beaches (Botton et al. 2003a, Rudloe 1981). Both tagging (Baptist et al. 1957, Leschen et al. 2006, Rudloe 1980, Sokoloff 1978, Swan 2005) and tracking studies of animals equipped with sonar (Moore 2004, Brousseau et al. 2004) demonstrate that most individuals remain within a limited area throughout the year. Phenotypic studies (Botton and Loveland 1992, Burton 1983, Itow 2004, Kato et al. 2005, Miyazaki et al. 1987, Palumbi and Johnson 1982, Riska 1981, Rudloe 1985, Sekiguchi et al. 1976, Sekiguchi et al. 1978, Shuster 1955, 1982, Sokoloff 1978) and genetic analyses (Itow 2004, King et al. 2005, Pierce et al. 2000, Saunders et al. 1986) demonstrate substantial differences among populations that could only be maintained with little gene flow. Such high levels of philopatry mean that horseshoe crabs will concentrate in areas where eggs and juveniles develop successfully.

In addition to being concentrated in certain areas, nesting is also highly synchronized. In all four species, migration to the nesting area for spawning is associated with high water levels (Sekiguchi 1988b). Pairs of horseshoe crabs leave the tidal flats with the incoming tide, apparently in response to increased water flow rates (Brockmann in prep) that might be detected by water flushing through the book gills and pressure-sensitive areas on the carapace (Chen et al. 2004, Kaplan et al. 1976, Sekiguchi 1988b, Shuster and Botton 1985, Waterman and Travis 1953). Specifically, migration to nesting sites is timed to the spring tides, i.e., to the several days of extra high tides just before, during, and after the new or full moons (each such period is called a tidal cycle) (Barlow et al. 1986, Cohen and Brockmann 1983, Sekiguchi 1988b, Smith et al. 2002b). In general, the number of horseshoe crabs migrating to shore is higher when tides are higher (Barlow et al. 1986, 1987, Barlow et al. 1986, Brockmann in prep, Chatterji et al. 1992, Sekiguchi 1988b, Schaller 2002; although this is apparently not true of all populations, Smith et al. 2002a). Also, they prefer the higher of two unequal tides in a day (Barlow et al. 1986, 1987, Cohen and Brockmann 1983, Rudloe 1980). In areas with little or no tide, migration occurs at times of high water, such as when wind-blown surge increases water levels (Brockmann unpublished ms, Ehlinger et al. 2003, Rudloe 1985). The strong synchronization with high tides or high water levels means that large numbers of horseshoe crabs may nest at the same time.

Further, horseshoe crab spawning is seasonal. In areas where the temperature falls below 16–20°C, horseshoe crabs are inactive (Chiu and Morton 2003, Sekiguchi 1988b), apparently moving to deeper water and burying in the substrate (Sekiguchi 1988b, Wenner and Thompson 2000; but the most northern population of *L. polyphemus* in Maine remains active down to 12–14°C; Schaller 2002). This limited temperature range for activity means that horseshoe crabs in many parts of their range have a limited breeding season. But temperature is not the only factor limiting reproduction because all four species of horseshoe crabs seem to have a well-defined breeding season of 1–3 months (Barlow et al. 1986, Chatterji 1994, Rudloe 1980, Sekiguchi and Nakamura 1979), which further contributes to their synchrony of spawning (Sekiguchi 1988b).

In all four species, the synchronous and seasonal spawning of horseshoe crabs that is associated with high tides (or high water), and that is focused on a few suitable sites, can result in high nesting densities. The extreme occurs in Delaware Bay, where beaches may be literally paved with nesting *L. polyphemus*. In fact, there are so many nesting horseshoe crabs that females dig into nests laid earlier in the season (Smith 2007). Also in this species, large numbers of unpaired males converge on the nesting beaches and crowd around the nesting pairs as satellites to form spawning groups. Paternity analyses demonstrate that in these mating groups, satellite males (in addition to attached males) fertilize many of the eggs that females lay (Brockmann et al. 1994). High nesting densities and high operational sex ratios (OSR, males:females) with strong sperm competition mean that both males and females can face high levels of reproductive competition. But in other areas or at other times, when population sizes are smaller or spawning is less synchronous or less focused on a few favored beaches, females rarely exhume the nests of others (female competition) and few satellites gather around the nesting pairs (male competition) (Brockmann 1990, Carmichael et al. 2003, Leschen et al. 2006, Penn and Brockmann 1994, Shuster and Botton 1985). This means that in *Limulus* the level of reproductive competition is highly variable. *Tachypleus* and *Carcinoscorpius* are consistently described as showing little or no reproductive competition (Sekiguchi 1988b). Botton et al. (1996) suggest that the lack of satellite males and group spawning in the Asian horseshoe crabs may be explained by their much smaller population sizes.

In this chapter we first detail the pattern of reproductive competition in *L. polyphemus* and evaluate whether differences in the presence of satellites and satellite groups within and among populations can be explained as flexible responses to different female nesting densities. We then return to the Asian species of horseshoe crabs to evaluate whether their lower levels of reproductive competition can be explained by their lower densities.

2 Reproductive Competition in Male *L. polyphemus*

2.1 Reproductive Competition in Limulus

Reproduction in *L. polyphemus* is often highly competitive. Female nesting densities may be remarkably high (e.g., 10–80 females m^{-2}; Smith et al. 2002b), in fact, so high that females frequently dig over previous nesting sites. This causes eggs to be exhumed by the later arriving individuals and results in considerable egg mortality (Botton et al. 2003b, Kraeuter and Fegley 1994, Smith 2007). Even at high female densities, male-biased OSRs (operational sex ratio, males:females) are the rule. Male biases result from the fact that only a small proportion of females in the population nest at any one time (Brockmann 2003b, Cohen and Brockmann 1983) and females normally complete their nesting with only a few visits to the beach, whereas males return repeatedly throughout the breeding season (Leschen et al. 2006, Rudloe 1980, Sekiguchi 1988b). Females are determinate layers, i.e., all eggs to be laid in a given breeding season are mature at the onset of spawning, so once all eggs are laid the female leaves the breeding area (Leschen et al. 2006). Attached males normally remain with the same female throughout her nesting but then detach when the female's breeding is complete and she returns to offshore feeding areas (Brockmann and Penn 1992). There may be differences among populations in the length of attachment: attached pairs have been observed overwintering on Cape Cod (Barlow et al. 1987, Moore 2004) whereas in Florida (Seahorse Key; SHK) animals usually remain together for only one tidal cycle (mean length of attachment is 3.7 ± 6.1 s.d. days; Brockmann and Penn 1992) but occasionally may stay together longer (up to 51 days; Brockmann 2003b).

Spawning groups form when unattached (i.e., unpaired) males crowd around a nesting pair as satellites (Barlow et al. 1987, Shuster 1953, Shuster and Botton 1985, Sydlik and Turner 1990). They are attracted to a pair either before nesting has begun or after nesting is underway (Schwab and Brockmann 2007); they use visual cues and are equally sensitive during the day and at night (Barlow et al. 1987, Barlow and Powers 2003). Males also approach cement models of horseshoe crabs that have been placed in shallow water or along the shoreline. When encountering two models, males prefer to approach the one that contrasts more strongly with the background, the one that is shaped like a horseshoe crab (Barlow et al. 1982, 1987, Barlow and Powers 2003) and the larger of the two models (Schwab and Brockmann 2007). Males also prefer to approach a model placed over a site where females have been nesting previously; they are particularly attracted to a site where a female with many satellites has nested before (Hassler and Brockmann 2001); and when satellite males are removed from a female, that female is more likely to regain satellites than a female that had not previously attracted satellites (Brockmann 1996), which suggests that chemical cues are involved.

Whether approaching a model or a nesting female, the satellite males take up characteristic positions around the pair (Brockmann 1990). The favored position over the female's incurrent canal (Fig. 1) results in particularly high levels of paternity, but satellites in all positions may achieve at least some paternity (Brockmann 2003b, Brockmann et al. 1994, 2000). Males jockey for position around the female, sometimes working their way under the front margin of the attached male's prosoma. This position results in somewhat higher paternity for the satellite (Brockmann et al. 1994, 2000). Other factors also affect paternity including tidal current speed (faster current favors satellites) and female size (attached males have higher paternity with larger females) (Brockmann et al. 2000). In many externally fertilizing invertebrates, both sperm limitation and

Fig. 1 Photograph of a spawning group of *L. polyphemus* at Seahorse Key, FL (Levy County). The attached male (*center*) is lighter and has fewer encrusting epibionts than the five satellites that surround him. Two of the satellites have taken up positions over the female's incurrent canal (indicated with *arrows*). Paternity analyses reveal that these two satellites will fertilize most of the eggs that the female lays (photograph by HJB)

sperm competition are involved (Levitan 1998, Yund and McCartney 1994), but in *Limulus* nearly all eggs (96–100%) are fertilized whether satellites are present or not (Brockmann 1990).

2.2 Assortative Mating and Alternative Tactics

High levels of reproductive competition are associated in many species with size-assortative mating (Andersson 1994, Harari et al. 1999, McLain 1987). In *L. polyphemus* unattached and paired males do not differ in overall size (Botton and Loveland 1992, Brockmann 1990, 1996, Brockmann and Penn 1992, Cohen and Brockmann 1983, Penn and Brockmann 1995) or in clasper size (Botton et al. 1996, Brockmann 1990, Cohen and Brockmann 1983, Duffy et al. 2006). Replacement males (i.e., when a female returns to the beach with a different attached male than on the previous tide) are not different in size from the original mates (Brockmann 1990, Brockmann and Penn 1992). This means that there is no assortative mating based on size (Brockmann 1990, Cohen and Brockmann 1983, Pomerat 1933) and no size advantage for large males. Botton and Loveland (1992), Loveland and Botton (1992) and Suggs et al. (2002) argue that this is because there are no size-related constraints on amplexus.

Nonetheless, differences in physical condition exist between males that are found paired and unpaired males in both Florida and Delaware Bay populations (Fig. 1; Brockmann 2003b). Attached males are less likely to be covered with epibionts; their eyes are less likely to show deterioration or fouling; their prosoma is lighter and less pitted; and they are less likely to show damage or to be missing the claws used to clasp females (Brockmann 2002, Brockmann and Penn 1992, Duffy et al. 2006, Penn and Brockmann 1995, 1996). These lighter males have less damage from the flatworms (*Bdelloura candida*) that lay eggs on their gills (Groff and Leibovitz 1982, Watson 1980a,b) and they have a heavier mucous secretion on their prosoma (Harrington and Armstrong 2003), which is thought to protect them from fouling, infections, and possibly UV light (Brockmann 2003b). Taken together, these data suggest that attached males are younger (their terminal molt occurred more recently) than unattached males (Brockmann 1996, 2003b).

Younger males are also behaviorally different from older individuals. They are more active and more likely to pair; they pair more quickly; they are more likely to remain paired; and they are more likely to find a female if they are detached (Brockmann and Penn1992). Younger males are also less likely to return to the nesting area and return less frequently than males in poorer condition (Brockmann 2002, Brockmann and Penn 1992). In addition, males in good condition are more likely to right themselves if overturned and less likely to become stranded after the tide (Penn and Brockmann 1995), a major cause of mortality among spawning males (Botton and Loveland 1989).

The physical and behavioral differences between attached and unattached males mean that there are two condition-dependent mating tactics in *L. polyphemus* (Brockmann and Penn 1992). Although some males switch between tactics, most of the time they are confined to one tactic or the other (Brockmann 2002). Brockmann (2002) has also shown that being unattached is not just a result of males not finding females: males in good condition that are prevented from attaching do not just come ashore and take up satellite positions around the nesting pairs. Rather, males in good condition are less likely to become satellites even when they cannot attach, which further supports the view that younger and older males use different mating tactics. There are no effects of satellite male condition on paternity but when the attached male is in worse condition, the satellite male's paternity improves (Brockmann et al. 2000).

2.3 Sexual Size Dimorphism and Reproductive Competition

In many species of animals, the degree of sexual size dimorphism is positively correlated with reproductive competition (Clutton-Brock et al. 1977, Jehl and Murray 1986, Mitani et al. 1996). In turn, reproductive competition is positively correlated with density (Conner 1989) and with a male-biased OSR (Kvarnemo and Ahnesjö 1996). *L. polyphemus* is sexually dimorphic with females larger than males. Populations differ in the degree of sexual dimorphism (F:M ratio; $\overline{x} = 1.29 \pm 0.04$ SD; range = 1.18–1.35; $n = 15$; Table 2) and in the level of reproductive competition: nesting density averages 0.74 ± 1.27 s.d. crabs/m^2 (range = 3.54–0.01; $n = 10$; Table 2) and OSR averages 2.9 ± 1.28 s.d. males per female (range = 1.1–5.8; $n = 15$; Table 2). Despite large differences in reproductive competition, the degree of sexual dimorphism does not correlate with the level of reproductive competition in females (nesting density) or males (OSR) (least-squares multiple regression, $F_{3,8} = 0.31$, $p = 0.813$). Instead, variation in size dimorphism may be due to other ecological factors such as resource availability or to environmental factors such as temperature.

2.4 Group Size, OSR, and Spawning Density

The intensity of sperm competition within a population varies markedly because mating group sizes and OSR are highly variable. Even when many pairs are nesting, at least some are without satellites; and even when few pairs are nesting some usually have satellites (Fig. 2; Brockmann 1996, Cohen and Brockmann 1983, Hassler and Brockmann 2001). In Delaware Bay (DE) the mean number of satellites per pair ranges from 0.2 to 5.2 and increases with the number of pairs on the beach (Fig. 2B), and 5–87% of the nesting pairs have no satellites (this is more likely at lower nesting densities; Fig. 2A; Brockmann 1996). Similarly, in a population from the west coast of Florida, Seahorse Key

Table 2 Comparisons of factors associated with reproductive competition among populations of *Limulus polyphemus*

State	Location	Study year	Sex	Prosomal width (cm) ± SD (*n*)	Dimorphism (F:M)	OSR (M:F) (n = # of tides)	Density	Source
ME	Hog Bay	2001	Male	14.7 (951)	1.183	2.8 (17)	0.16 crabs/m^2	Schaller (2002)
			Female	17.4 (337)				
	Thomas Point Beach[a]	2001	Male	14.8 (26)	1.297	1.7 (17)	0.30 crabs/m^2	Schaller (2002)
			Female	19.2 (14)				
	Bagaduce River[a]	2001	Male	13.9 (9)	1.259	1.07 (8)	0.06 crabs/m^2	Schaller (2002)
			Female	17.5 (12)				
MA[b,c]	Monomoy NWR	2000–2002	Male	18.8 ± 1.5 (909)	1.298	1.9 (14)	0.06 crabs/m^2	James-Pirri et al. (2005)
			Female	24.2 ± 2.2 (447)				
	Pleasant Bay[d]	2000–2002	Male	17.9 ± 1.3 (1775)	1.279	5.8 (12)	0.04 crabs/m^2	James-Pirri et al. (2005)
			Female	22.9 ± 2.4 (298)				
	Nauset Estuary	2000–2002	Male	17.5 ± 1.7 (433)	1.337	1.6 (6)	0.01 crabs/m^2	James-Pirri et al. (2005)
			Female	23.4 ± 2.1 (256)				
	Cape Cod Bay	2000–2002	Male	17.4 ± 1.6 (2942)	1.304	2.9 (27)	0.007 crabs/m^2	James-Pirri et al. (2005)
			Female	22.7 ± 1.9 (759)				
NJ[e]	Cape May	1977	Male	20 ± 1.4 (1058)	1.283	5		Shuster and Botton (1985)
			Female	25.7 ± 2.0 (653)				
	Cape May	1979	Male	20 ± 1.4 (1058)	1.283	3		Shuster and Botton (1985)
			Female	25.7± 2.0 (653)				
	Cape May	1999	Male	20 ± 1.4 (1058)	1.283	3.5[f] (86)	0.75 females/m^2 ≈2.63 crabs/m^2	Smith et al. (2002b)
			Female	25.7 ± 2.0 (653)				
DE[f]	DE Bay	1977 1979	Male	20 ± 1.4 (75)	1.275	3		Shuster and Botton (1985)
			Female	25.5 ± 1.9 (50)				

Table 2 (continued)

State	Location	Study year	Sex	Prosomal width (cm) ± SD (*n*)	Dimorphism (F:M)	OSR (M:F) (n = # of tides)	Density	Source
	DE Bay[c]	1999	Male	20 ± 1.4 (75)	1.275	3.5 [f] (86)	1.01 females/ m^2 ≈3.54 crabs/ m^2	Smith et al. (2002b)
			Female	25.5 ± 1.9 (50)				
SC[d]	Otter Island	1999	Male	23.7	1.299	1.96		Wenner and Thompson (2000)
			Female	30.8				
FL	Apalachee Bay	1976–1978	Male	16.9 ± 1.3 (1552)	1.324	3.6 (66)		Rudloe (1980)
			Female	22.3 ± 2.2 (742)				
	SHK	2004–2005	Male	16.6 ± 1.2 (586)	1.35	2.1 (115)	0.55 crabs/m^2	Brockmann, unpublished data
			Female	22.5 ± 1.5 (322)				

[a] Several survey dates were clearly past the breeding season and were not included in calculating average density.
[b] Density was averaged from all years and day and night surveys.
[c] Numbers were estimated from graph.
[d] Populations at these sites have been heavily harvested, thus reported OSR may be an overestimate due to a harvesting preference for large females.
[e] Data on body size from Loveland and Botton (1992).
[f] Unpublished data on body size from H.J. Brockmann.
[g] At beaches with a spawning density >0.35 females/m^2.

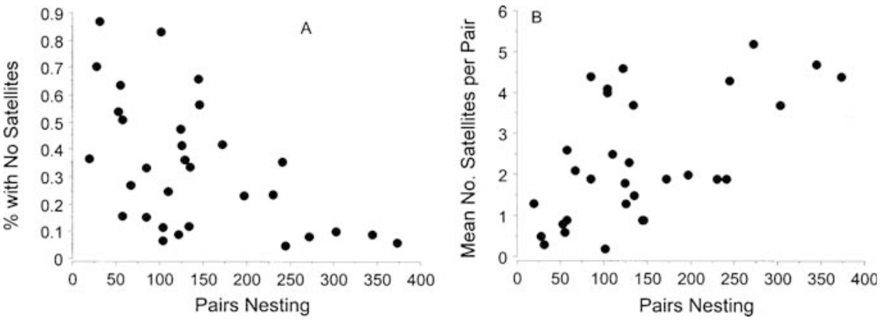

Fig. 2 Correlation between nesting density and spawning groups at Cape Henlopen, Delaware Bay (May and June 1991–1993; data from Brockmann 1996). (**A**) The percent of the population without satellites is negatively correlated with nesting density ($r^2 = 0.28$, $p = 0.002$, df = 30); (**B**) The mean number of satellites per pair is positively correlated with density ($r^2 = 0.33$, $p = 0.0007$, df = 30)

(SHK, 2004–2005), mean group size varies from 0 to 2.5 and is correlated with nesting density ($r^2 = 0.13$, $p < 0.0001$, df = 114); 23–100% of the pairs are without satellites, and this is negatively correlated with nesting density ($r^2 = 0.3$, $p < 0.0001$, df = 114). In these studies the number of lone males (unattached males that are not in groups) varies greatly (38–91% of all males present in DE; 0–100 % at SHK) and correlates with density (DE: $r^2 = 0.28$, $p = 0.002$, df = 30; SHK: $r^2 = 0.1$, $p = 0.004$, df = 114). However, OSR as a measure of reproductive competition does not always correlate with nesting density (SHK: $r^2 = 0.1$, $p = 0.004$, df = 114) because there are both high and low OSRs at low densities.

The OSR varies widely within and among populations (Table 2; Carmichael et al. 2003). For example, in DE the median OSR is 3.5 (males:females) at times when many crabs are nesting (>0.35 females/m^2) and 1.5 when fewer crabs are present (Smith et al. 2002b). On Cape Cod, MA, some populations show low mean OSR (Nauset Estuary: 1.6), whereas other nearby locales show much higher OSR (Pleasant Bay: 5.8), but overall there is no correlation between OSR and spawning density ($r^2 = 0.03$, $p = 0.47$, $n = 10$; James-Pirri et al. 2005). A number of factors may account for variation in OSR. Unattached males are more vulnerable to being overturned in the waves than attached males (Penn and Brockmann 1995) and when overturned many of these unattached, poor-condition males are unable to right themselves. Perhaps for this reason, OSR is lower with higher waves (Smith et al. 2002b). At SHK the OSR is higher toward the end of a tidal cycle and toward the end of the season (Brockmann, unpublished ms) but in Maine the numbers of unattached males decline later in the season (Schaller 2002). Perhaps because of these many interacting factors, in general, some populations show a higher OSR when more nesting pairs are present (Rudloe 1980, Smith et al. 2002b) and several show no such relationship including populations from Maine (Schaller 2002) and New York (Hanna 2001). Taken together, these within-population and among-population comparisons mean that differences in male competition (i.e., differences in OSR and frequency of group spawning) are not simply a reflection of changes in the number of nesting females between tides.

3 Differences in Reproductive Competition Among Horseshoe Crab Species

3.1 Reproductive Competition in Asian Horseshoe Crabs

The evidence for reproductive competition in *Tachypleus* and *Carcinoscorpius* is quite limited. Population sizes and nesting densities of *T. tridentatus* and *C. rotundicauda* are thought to be low throughout most of their range and most studies describe population OSR as 1:1 (Chen et al. 2004, Chiu and Morton 1999, Khan 2003). *T. tridentatus* normally nest as pairs where the maximum number of pairs present is 48 and normally fewer than 30 (Sekiguchi 1988b). Satellite male behavior appears to be almost non-existent. Sekiguchi (1988b) observed several males in a tank clasping onto paired couples, but states that males do not normally associate in this way under natural conditions where a male–female pair is the normal condition. Botton et al. (1996) observed no unpaired males during their study of *T. tridentatus*. Although he does not comment on seeing satellites or groups, a picture in Mikkelsen (1988, p. 82) clearly shows two male *T. tridentatus* traveling in tandem with the accompanying legend “a cluster of males surrounding a female of the same species” from a site near Beihai, China, on the Beibu Gulf (where the numbers of nesting pairs are higher than elsewhere in the range). Botton et al. (1996) observed a satellite male with a mated pair of *T. gigas* in an aquarium and the behavior of this animal was identical to that of satellite *L. polyphemus*. Chatterji (1994) describes *T. gigas* populations in the Bay of Bengal as low with 1–17 females nesting on a beach each day with a total of 235 females and 255 males over the entire nesting season and an OSR of 1.09, but he has no descriptions of groups. The picture of reproductive competition in the Asian horseshoe crabs, then, is very different from that observed in *L. polyphemus*.

3.2 Is Low Reproductive Competition the Result of Low Density?

The available data suggest that *T. gigas* may have the highest levels of reproductive competition of the Asian species. Chatterji et al. (1992) provide sufficient data for *T. gigas* to test the hypothesis that unattached males are more common when more nesting pairs are present. They observed 1–16 pairs on 36 high tides nesting along the Balramgari coast of Orissa. Although the pairs often nested close together (based on figures in Chatterji 1994), there was never more than one unattached male present on any tide and no mention is made of satellite groups. Unattached males were no more likely to be present on a high tide when there were many nesting pairs than when there were few pairs (Mann–Whitney U Test, $N_1 = 17, N_2 = 19, p = 0.36$). For comparison, we examined a similar data set from SHK using Florida *L. polyphemus*, i.e., presence or absence of unattached males on all tides where there were 1–16 pairs nesting (0–49 unattached males were present on those tides; 2004–2005 data). Unattached males were significantly more likely to be present when there were more nesting pairs even within this limited range of nesting densities (Mann–Whitney U Test, $N_1 = 16$, $N_2 = 28$, $p = 0.01$). We conclude then (assuming overall equality of population sex ratios) that the pattern of male mating tactics in *T. gigas* is different from that found in *L. polyphemus* and that differences in the presence of satellites is not just a reflection of low nesting densities.

3.3 Sexual Size Dimorphism

The Asian horseshoe crabs are sexually dimorphic with males smaller than females (Table 1; Shuster 1982, Yamasaki et al. 1988). As in *L. polyphemus*, considerable variation in body size can be found throughout the range of the Asian species, but the relationship between male and female sizes remains about the same (Chiu and Morton 2001, Itow et al. 2004, Kato et al. 2005, Sekiguchi et al. 1978). The mechanism underlying size dimorphism is a difference in the growth pattern between males and females (Botton and Loveland 1992). Sekiguchi et al. (1988) showed that male *L. polyphemus* and *T. tridentatus* take 16 molts to reach maturity while females mature in 17. Thus, the consistency of dimorphism between populations is a result of a difference in the number of molts, regardless of adult body size. This is further demonstrated by Wenner and Thompson (2000) who found that the adult female to male size ratio (1.29) was the same as the mean juvenile pre-molt to post-molt ratio (size increase per molt) calculated over the first 11 instars. Despite the fact that the American horseshoe crabs face far more intense reproductive competition than any of the Asian species, the level of sexual dimorphism in *L. polyphemus* does not differ from the Asian species. This is consistent with the view that sexual selection in externally fertilizing animals is similar for males and females (Levitan 1998). For horseshoe crabs it might also be that factors other than sexual selection are affecting sexual dimorphism. For example, because larger females lay more eggs (Chatterji 1995, Chatterji et al. 1992, Khan, 2003, Leschen et al. 2006, Schwab and Brockmann 2007), fecundity selection may favor delayed sexual maturation of females, whereas selection may favor earlier maturation for males since there appears to be no size advantage in male–male competition. Another hypothesis suggests that physical constraints related to amplexus may favor males that are smaller than females (Botton and Loveland 1992).

3.4 Correlates of Reproductive Competition

Differences in reproductive competition between Asian and American horseshoe crabs are mirrored by differences in their internal and external anatomy. In all female horseshoe crabs, eggs are produced by the lining of the oviduct; mature eggs evaginate through the ovarian walls, break off, and accumulate in the lumen of the oviduct (Dumont and Anderson 1967) leading to the paired gonopores, located on the operculum. The posterior oviducts of female *L. polyphemus* are more developed than in the other three species and divided into several tubes (Yamasaki et al. 1988). During the breeding season these posterior branches are filled with the ovulated eggs that will be laid during that season. The more highly developed and numerous oviducts allow female *L. polyphemus* to store larger numbers of eggs prior to spawning and to lay far more eggs compared to the Asian species (Mikkelsen 1988, Sekiguchi and Nakamura 1979, Shuster 1982). In all four species larger females lay more eggs (Chatterji 1994, 1995, Chatterji and Parulekar 1992, Khan 2003, Leschen et al. 2006, Schwab and Brockmann 2007), presumably because of their greater storage abilities.

L. polyphemus eggs are substantially smaller and females carry many more eggs and deposit many more at one time than in any of the three Asian species (Table 1). Smaller eggs mean smaller newly hatched larvae, and *Limulus* has the smallest larvae of the four species (Shuster 1982). This variation represents different strategies in the life history trade-off between egg size and egg number. The smaller, more numerous eggs laid by *L. polyphemus* may be an adaptation to their higher OSR, higher densities, and much greater female competition or to higher predation on their eggs and larvae compared with the Asian species. We are currently investigating variation in egg size and number and the potential causes of differences among populations of *L. polyphemus*.

The reproductive system of male *L. polyphemus* also differs from that of the other horseshoe crabs in several important ways (Yamasaki et al. 1988). Sperm are produced in the walls of a reticulated network of fine tubules that make up the testes with a complex network of sperm ducts leading to the paired gonopores, which are located on the operculum. Unlike the Asian species, *L. polyphemus* has more complexly branching and proliferated distal sperm ducts and "sperm sacs" or organs that store mature sperm in the testes (Yamasaki et al. 1988). This means that male *L. polyphemus* are capable of storing mature sperm, perhaps as an adaptation for sperm competition or as an adaptation for fertilizing the eggs of the highly fecund females. It may even be possible for males to adjust their ejaculate to the intensity of sperm competition as has been demonstrated in some insects (Schaus and Sakaluk 2001, Wedell 1999) and other species. Selection may have favored the loss of these structures in the Asian horseshoe crabs because of the lower risk of sperm competition and the fact that fewer eggs are laid at one time.

Limulus sperm are quite different from the sperm of the Asian species. The internal microstructure of the flagellum is different and because of this *Limulus* sperm swim much faster than the sperm of the Asian species (Ishijima et al. 1988). After reaching the egg, fertilization begins when the sperm's acrosomal filament is explosively released through the sperm head and literally screws through the jelly of the egg (Fahrenbach 1973, Sekiguchi et al. 1988, Tilney 1975). Presumably because of the reduced size of the egg and surrounding jelly, the acrosomal filament of American horseshoe crab sperm is shorter than that found in the sperm of the Asian species although the overall sperm size is the same (Hong et al. 1995). *Limulus* eggs are fertilized after multiple sperm attachment (Brown and Knouse 1973) and multiple acrosomal reactions (Fahrenbach 1973, Yamasaki et al. 1988). All horseshoe crabs must have mechanisms to prevent polyspermy, but *Limulus* must have particularly strong blocks since there are often many spawning males (and hence more sperm) with one female. The differences in sperm structure and behavior among horseshoe crab species may be adaptations to differences in the level of sperm competition.

The external anatomy of the four species also differs in ways that may parallel differences in reproductive competition (Yamasaki et al. 1988). *L. polyphemus* has one pair of claspers that hold the terminal spines (posterior processes) of the female. In contrast *T. tridentatus*, *T. gigas*, and *C. rotundicauda* have two pairs of claspers that are used in amplexus (Botton et al. 1996, Shuster 1982, Yamasaki et al. 1988). The anterior pair attaches along the opisthosomal margin or to the moveable spines and the posterior pair attaches to the terminal spines. The result is that attached male *Tachypleus* are more firmly attached and ride farther forward on the female's opisthosoma than male *Limulus*. Additionally, the anterior portion of the male's prosoma is more arched than in the female, and this is particularly noticeable in *T. tridentatus*. The presumed function of this prosomal arch is to allow the male to fit over the female's opisthosoma (Shuster 1982). While differences in reproductive competition may be responsible, there are many alternative hypotheses to explain variation in patterns of amplexus and body shape between American and Asian species. Unfortunately, there have been no studies on the costs and benefits associated with differences in either (1) the number of claspers (or the resulting attachment strength and ride height) or (2) prosomal arch size. Despite the lack of data, some potential trade-offs have been discussed. First, Botton et al. (1996) suggest that the different patterns of amplexus in the Asian species have been selected to assure fertilization by the attached male since, unlike *Limulus*, satellites are rarely if ever present during spawning. Alternatively, past competition could have selected for morphology that reduced vulnerability to sperm competition (i.e., dual claspers that increase attachment strength), thus making satellite behavior less successful than in *Limulus*. Second, the increased attachment strength of the Asian species may make them less likely to become detached from females in rough waves (especially beneficial because unattached males are more likely to become stranded). In contrast, Botton et al. (1996) suggest that the higher ride height (because of the two pairs of claspers) of the Asian species may actually make them more vulnerable to wave action. Lastly, variation in prosomal arch size may affect how males are attached to females. The resulting fit between a male and a female may influence susceptibility to both wave action and sperm competition. Variation in arch size between species has not been quantified. However, in *Limulus*, arch height varies significantly within populations and potentially among populations (M. D. Smith unpublished data). We are currently investigating the fitness costs and benefits of arch variation and how factors such as density, OSR, and individual condition influence fitness trade-offs. These studies will help us to understand if and how reproductive competition influences external anatomy and patterns of amplexus in the four species.

4 Conclusions

In addition to their unusual external fertilization, horseshoe crabs differ from many other species in having a limited amount of time for breeding. In most populations pairs are limited to only a few hours a day over 5 days every 2 weeks for 1 or 2 months if the seas are relatively calm and if they can find a suitable nesting site. Males engage in scramble competition, clasping females and remaining with them through much of their breeding period. This means that horseshoe crabs, and particularly *L. polyphemus*, have a mating system that is closer to the explosive breeding system found in some anurans and fishes than it is to the mating patterns of most other arthropods (Brockmann 1990). Like other explosive breeders with scramble competition, males seek out females rather than females approaching males; there is no assortative mating based on size; there are no size differences between paired and unpaired males; males often clasp inappropriate objects; and they mate guard by remaining in amplexus for days. As in other explosive breeders (Myers and Zamudio 2004, Tennessen and Zamudio 2003, Zamudio and Chan 2008), *L. polyphemus* males show condition-dependent alternative mating tactics and relatively high levels of multiple mating and multiple paternity. In several groups of anurans and *L. polyphemus*, high sperm production is correlated with a high probability of group spawning (Byrne et al. 2002), which suggests that satellite behavior is not merely the result of unusually high population densities, but rather is an evolved trait that increases individual reproductive success under particular ecological conditions. Short-term changes in densities or operational sex ratios (OSR) select for the ability to change tactics quickly (Arak 1983, Wells 1977). Populations of *Limulus* in different regions experience much greater variation in local densities, OSR, and lengths to their breeding seasons than do the Asian species, and this selects for individuals that can fine-tune their behavioral responses and maximize fitness by varying their mating tactics depending on the immediate social context (Semlitsch 1985, Sullivan 1989). Under these conditions selection favors males that can switch to satellite behavior when the payoff to searching is low, thus favoring the evolution of satellite behavior and spawning groups as we see in the American horseshoe crab. Alternatively, when there is little variation in population density, OSR, or breeding season length, and especially if densities are so low that females are hard to find, selection favors males that search out females and stay with them, as in the Asian horseshoe crabs. Although low population densities may be a recent phenomenon due to habitat destruction and exploitation, the morphological differences between the four species in reproductive structures, eggs, and sperm suggest long-standing patterns of far greater reproductive competition in American than in Asian horseshoe crabs.

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Vision in Horseshoe Crabs

Robert B. Barlow

Abstract *Limulus* has been a superb animal model for understanding vision in higher animals, including man. Nobel-prize winning research on the lateral eye of *Limulus* by H. K. Hartline revealed fundamental principles of retinal function applicable to all eyes. The function of the *Limulus* lateral eye is now well understood as is its essential role in the animal's mating behavior.

1 Introduction

"For such a large number of problems there will be some animal of choice or a few such animals on which they can be most conveniently studied." August Krogh's "Principle" made in 1929 captured well H. Keffer Hartline's decision several years earlier, to study vision in horseshoe crabs. The same is true of A. V. Hill's comment also made in 1929: "we may often throw light upon function or process in the higher animals…by the choice of a suitable organ…in some animal far removed in evolution." Hartline devoted nearly his entire life studying the remarkable eyes of horseshoe crabs, animals that are truly "far removed in evolution." Close inspection of the animal reveals relatively large eyes that look nothing like our own (Fig. 1). What could they possibly teach us about vision in other animals? Hartline provided the answer: they reveal how eyes provide vision – not just in horseshoe crab but in all animals. His groundbreaking discoveries of the physiological properties of *Limulus* eyes laid the foundation for our current understanding of eye function. For his pioneering work, Hartline shared the Nobel Prize in Medicine or Physiology (Hartline 1969).

Hartline began his wonderful journey in research on the *Limulus* eye in 1926 at the Marine Biological Laboratory (MBL) in Woods Hole, MA (Fig. 2). He had gone there hoping to find an animal with relatively simple eyes. His interest in vision began earlier at Lafayette College, where as an

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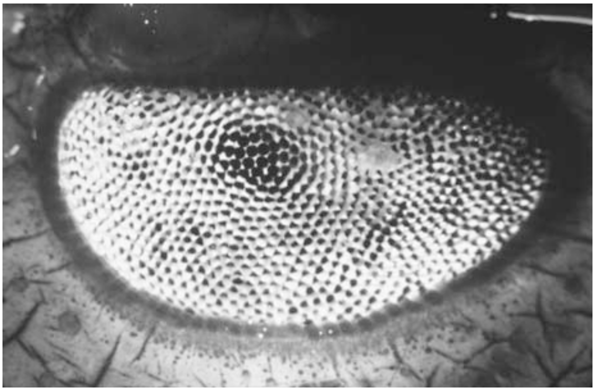

Fig. 1 The compound eye of *Limulus*. A pair of these large eyes, each containing about 1,000 ommatidia (*black disks*), is perched high on each side of the prosoma and provide *Limulus* with wide fields of vision (Herzog and Barlow 1992). The crab can see to not only each side but also ahead, behind, and above. The individual ommatidia are the largest known retinal receptors in the animal kingdom. They are roughly 100 times the size of rods and cones in the human eye

undergraduate he carried out a meticulous study of how pill bugs respond to light. This strictly behavioral study triggered in him a keen interest to understand how light causes changes in an animal's behavior, beginning with action of light on an eye. At the time, he was convinced that the eyes of frogs would be ideal for understanding how light-sensitive cells in the retina begin the process of vision. In earnest, he tried to record nerve signals from frog eyes while a medical student at Johns Hopkins University. But he quickly became frustrated first because the frog's eye proved to be more complex than he imaged and second because he could only study them at night. Daytime was packed with classes and labs – that were of little interest to him and kept him from his frogs. Hartline often joked that Johns Hopkins awarded him a medical degree only if he promised never to touch a patient. After graduating, he questioned whether frog eyes could provide the insight about sight he had hoped for and decided to go to Woods Hole to search for a marine organism that possessed a simpler and more accessible eye.

Hartline always enjoyed telling the story of how he stumbled across a large female horseshoe crab as he strolled along the water's edge on a beach in Woods Hole. He knelt down to look at her eyes and marveled at their size – especially the individual light receptors, the ommatidia (Fig. 1). The receptors were so large that he could see them without a magnifying glass. Numbering about 1,000 in each eye, the individual ommatidia are roughly 100 times the size of rods and cones in the human eye. They are, in fact, among the largest light receptors in the animal kingdom. In awe of their size, Hartline reasoned that if he could see them so easily, he might be able to record their electrical responses to light and understand how they provide vision to the animal.

Hartline's first experiment with the *Limulus* eye was to record a light-evoked electrical signal, termed the electroretinogram (ERG), that he had successfully

Fig. 2 H. Keffer Hartline (1903–1983) sitting on his "think bench" in the woods behind his home near Baltimore

recorded from the human eye. The amplitude of the ERG provides a good measure of retinal sensitivity, and he set about to measure it with a simple cotton wick electrode placed in contact with the cornea of the lateral eye. Because highly sensitive physiological amplifiers did not exist in the late 1920s, he connected the wick electrode to a mechanoelectrical device known as a string galvanometer that proved sensitive enough to record light-induced changes in ERG amplitude (Hartline 1928). While exploring retinal tissue with a wick electrode, Hartline detected brief, tiny electrical events in the optic nerve trunk that he thought might be individual nerve impulses. This possibility excited him, and he managed to locate a vacuum tube – a recent innovation – and built an amplifier that had sufficient sensitivity to detect small nerve signals (Barlow 1986).

In the summer of 1931, Hartline returned to MBL with his colleague, Clarence Graham, and his new highly sensitive vacuum tube amplifier. They found it relatively easy to record masses of electrical impulses even from the tiniest nerve bundles dissected from the optic nerve trunk of *Limulus*, but impossible to record nerve impulses from a single nerve fiber – which was their goal. On their final day in the laboratory that summer, they succeeded in recording from a single optic nerve fiber when they tested the last *Limulus* in the tank – a large, crusty-shelled adult having dull, scarred eyes – "a miserable specimen" according to Hartline. Stunned at their unexpected, last-minute good fortune, he and Graham furiously set about to learn what they could before packing up the lab to leave the following day.

The outpouring of results from these and subsequent Woods Hole single nerve fiber recordings was enormous, touching on most every aspect of retinal physiology and laying the foundation for all future research. A thorough description of these fundamental contributions together with Hartline's studies using intracellular recording techniques would fill several volumes (Ratliff 1974). There are also numerous studies by others including me who were inspired by Hartline's pioneering work. Reviewing the entire body of work, I complied a "Top 10" list of discoveries and present them here.

1.1 Neural Coding of Light Intensity

In their first recordings from the *Limulus* lateral eye, Hartline and Graham (1932) discovered that the discharge of impulses from single optic nerve fibers increased with light intensity. They found a near linear relationship between the rate of discharge and the logarithm of light intensity similar to that between intensity and brightness in human vision known as the Weber–Fechner Law. They also found that a single nerve fiber with its attached photoreceptor responded over a wide range of intensities that may be as great as 1–1,000,000. Kaplan and I repeated their experiment without excising the eye as they did but leaving it with its blood supply intact in the animal. We were indeed surprised to discover that the eye in vivo has a much greater sensitivity, responding over an intensity range of $1–10^{10}$ (Barlow and Kaplan 1971). Several years later, my laboratory was doubly surprised to find that a circadian clock in the animal's brain further increases lateral eye sensitivity at night (Barlow et al. 1977). Remarkably, a single *Limulus* photoreceptor can signal the brain about individual photons at night and operate under bright sunlight during the day, a range of $1–10^{14}$. Human vision operates over a similar wide range – from the dimmest visible star to the noonday sun – but does so with two types of photoreceptors, rods and cones, and a far more complex retina.

1.2 Spectral Sensitivity

Graham and Hartline (1935) also studied the spectral sensitivity of single photoreceptors and found that their optic nerve response varies with the wavelength of light, peaking in the blue-green region of the spectrum. Years later

Hubbard and Wald (1960) found that the visual pigment extracted from *Limulus* photoreceptors absorbed light in the same region of the spectrum. This spectral match laid the foundation for understanding the cellular mechanisms of color vision of all animals.

1.3 Temporal Summation of Light

Hartline (1934) discovered that the *Limulus* eye functions as an adding machine, summing the influences of individual photons in brief flashes to produce a visual response. The ability of a single photoreceptor to sum the influences of single photons delivered within a short period of time, termed the critical duration, indicates that the photochemical reactions exerted in a photoreceptor by a light flash depend only on energy (intensity × duration). The reciprocal relationship between intensity and duration of brief flashes is known as the Bunsen–Roscoe Law (Hartline 1934). Adherence to it by both humans and horseshoe crabs points to similar photochemical reactions in both human and horseshoe crab eyes.

1.4 Light and Dark Adaptation

Hartline and Graham (1932) found that after the onset of light, the discharge of impulses from a single optic nerve fiber was initially high and then decreased to a lower rate indicating rapid sensory adaptation. After a hiatus of research caused by World War II, Hartline revisited the property of adaptation and with MacDonald (1947) examined both the decrease in sensitivity of the eye caused by light (light adaptation) and the subsequent increase in sensitivity after light offset (dark adaptation). The phenomena of dark and light adaptation are familiar to all who have experienced changes in their vision upon entering a dark movie theater on a sunny day and then after leaving the theater. Before the *Limulus* studies, the ability to adapt visual sensitivity to ambient lighting conditions was well known, but the origin of adaptation, eye vs brain, was not. Hartline and MacDonald (1947) found that light and dark adaptation begin in single photoreceptors of the *Limulus* eye and stated that this was “strong presumptive evidence” for a photoreceptor origin of adaptation in the visual systems of many higher animals, including humans.

The above four “Top 10” discoveries were made from recordings of single optic nerve fibers. These findings convinced Hartline that visual phenomena common to many higher species – as visual adaptation and spectral sensitivity – originated in the retina of this primitive animal. These discoveries were made in the first half of the last century. Studies carried out after 1950 probed the cellular mechanisms underlying optic nerve responses. These later studies were enabled by the advent of glass microelectrodes that have tips tiny enough to penetrate the cell membrane of *Limulus* photoreceptors and record their initial electrical responses to light.

1.5 Photoreceptor Potential

Masters at delicate surgical manipulations, Hartline and Graham succeeded in removing a single photoreceptor unit, an ommatidium, from the eye during their early studies. Using a small electrode, they recorded a minute electrical current, they termed an "action current", that coincided with the generation of impulses in the optic nerve. They suggested that the action current of an ommatidium initiated nerve impulses, but their electrode was too large to probe the inner workings of cells within an ommatidium. Using the new-developed glass microelectrode, Hartline, Wagner, and MacNichol (1952) later succeeded in impaling a single photoreceptor cell, called a retinular cell, and recorded its response to light. They found that a light flash depolarized the transmembrane potential and believed that this photoreceptor potential was "intimately related to the initiation of nerve impulses." Tomita (1956) and MacNichol (1956) then showed that this photoreceptor potential results from an increase in cell membrane conductance and is indeed related to the generation of nerve impulses. These germinal studies in Hartline's laboratory and many others throughout the world led to a detailed understanding of how both invertebrate and vertebrate photoreceptors respond to light.

1.6 Single Photon Detection

Single photoreceptors can respond to the smallest amount of energy: a single photon of light. Hecht et al. (1942) came to this conclusion indirectly from their behavioral study of human visual sensitivity. Yeandle (1958) provided direct physiological evidence for this remarkable result 16 years later when he recorded elemental voltage events from *Limulus* photoreceptors (retinular cells) exposed to very dim light. The discrete voltage events, he called "quantum bumps," increase in frequency as photon flux increases and sum to form the photoreceptor potential that leads to the generation of optic nerve responses as discussed above. A graduate student of Hartline, Alan Adolph (1964), extended this work and reported marked fluctuations in the amplitudes of the discrete quantum bumps. Fred Dodge et al. (1968), also in Hartline's lab, examined the quantum bump amplitudes using techniques of linear systems analysis and found that bump amplitudes decrease as their frequency increases with increasing light intensity, adapting the eye to brighter light. Their "adapting bump" model provided the first comprehensive explanation for light adaptation in the retina, any retina.

1.7 Lateral Inhibition

Hartline's discovery of lateral inhibition in the *Limulus* eye marked a milestone in vision research and is largely the reason he was awarded the Nobel Prize. Interestingly, his discovery was accidental as are many groundbreaking discoveries. “I turned on the room lights and the optic nerve response decreased” said Hartline, recounting an experiment he had performed on the *Limulus* eye in 1949. “Why should the response decrease when I increase the light intensity?” He had experienced this phenomenon countless times, but had not appreciated its significance. Why he was suddenly alerted to the effect of room light is not clear, but he finally grasped its meaning: illuminating one region of the *Limulus* eye can inhibit the responses of ommatidia in a neighboring region. The concept of lateral inhibition was born (Hartline et al. 1956). It has proven to be a fundamental principle of all visual systems, including that of humans. By enhancing the contrast between light and dark areas in the visual field – a phenomenon known as simultaneous contrast, lateral inhibition influences most everything we see. In 1865, Ernst Mach hypothesized that this ability of human vision could be explained by mutually inhibitory interactions in the retina. Physiological support of Mach’s idea waited many years: it was found in a visual system far simpler than our own.

Hartline’s discovery of lateral inhibition initiated a remarkable line of research extending to the present day. He and his coworker Ratliff (1957, 1958) found that the optic nerve responses from individual ommatidia could be quantitatively expressed in terms of the algebraic sum of inhibitory influences of neighboring ommatidia. Studying with Hartline as his last graduate student, I extended his work with Ratliff by measuring the receptive fields of lateral inhibition in the eye (Barlow 1969). A fellow graduate student, David Lange, and I detected an essential nonlinearity in the inhibitory interactions (Barlow and Lange 1974) that led to an accurate description of the eye’s response to stationary patterns of illumination (Barlow and Quarles, 1975). Ratliff et al. (1967, 1974) analyzed the responses to dynamic pattern of illumination using linear systems analysis. Finally graduate students in my lab, Erik Herzog, Scott Jackson, and Christopher Passaglia, together with Frederick Dodge and I developed a comprehensive cell-based model of the *Limulus* eye that incorporates all known physiological properties of the eye and predicts its response with better than 95% accuracy (Passaglia et al. 1997, 1998). These achievements stand today as the only complete quantitative analysis of neural integration among an ensemble of sensory receptors. The well-known Hartline–Ratliff formulation and its later extension have been the starting point for many treatments of information processing in more complex neural systems. It led to a comprehensive description of the neural code the eye sends to the brain as discussed later in this chapter.

1.8 Circadian Rhythms in Visual Sensitivity

The *Limulus* lateral eye exhibits extraordinary day–night changes in sensitivity. A circadian clock located in the brain transmits efferent optic nerve activity to the eyes at night increasing their sensitivity to light by about 1,000,000 times over daytime levels (Barlow et al. 1977). This discovery was also accidental. It was made by Stanley Bolanowski, Michael Brachman, and me while recording the lateral-eye ERG during a graduate laboratory exercise at Syracuse University in 1976. As described above, Hartline had recorded the ERG years before, but not at night. We were astonished to see the amplitude of the ERG increase at night and then decrease the following day while the crab was kept in constant darkness. We were doubly astonished to see the day–night rhythm disappear when we severed the optic nerve trunk. Suspicious that the optic nerve trunk may be transmitting efferent activity from the brain, we teased apart the individual fibers of the nerve trunk searching for brain-generated activity. We detected efferent activity in a few fibers but only at night. We recorded the activity and were delighted to find that stimulating the optic nerve with the recorded activity the following day transformed the eye to its highly sensitivity nighttime state: we could play the role of the circadian clock!

The so-called simple eye of this living fossil has evolved remarkably complex, sophisticated mechanisms to increase retinal sensitivity. Table 1 lists the multiple changes in anatomy, physiology, and metabolism that combine to produce the nearly 1,000,000-fold increase in nighttime sensitivity. Circadian rhythms in vision are not unique to *Limulus*; they are widespread among both invertebrates and vertebrates (Barlow et al. 1989, 2001). In most cases, visual sensitivity appears to be under the joint control of a circadian oscillator and light. Why visual systems of some animals need to anticipate changes in light intensity rather than respond directly to them is not known. Interestingly, the large nighttime increase in *Limulus* eye sensitivity nearly compensates for the nighttime decrease in ambient light intensity.

Table 1 Circadian rhythms in the *Limulus* lateral eye

Retinal property	Day	Night
Efferent input	Absent	Present
Gain	Low	High
Noise	High	Low
Quantum bumps	Short	Long
Frequency response	Fast	Slow
Dark adaptation	Fast	Slow
Lateral inhibition	Strong	Weak
Cell position	Proximal	Distal
Screening pigment	Clustered	Dispersed
Aperture	Constructed	Dilated
Acceptance angle	6°	13°
Photomechanical movement	Trigger	Prime
Photon catch	Low	High
Membrane shedding	Trigger	Prime
Intense light effects	Protected	Labile
Visual sensitivity	Low	High

1.9 Horseshoe Crabs Use Vision to Find Mates

Discovery of robust rhythms in *Limulus* eye sensitivity intensified a long-standing question: What does the horseshoe crab use its eyes for? Hartline often joked that he had spent many decades "studying vision in a blind animal." After learning about our discovery of circadian rhythms in *Limulus*, Hartline reminded me that no one had succeeded in uncovering a role for vision in the animal's behavior. Intent on finding one, I spent many dark cold nights diving with *Limulus* at the bottom of Buzzard's Bay near Woods Hole... and learning very little. I did learn, however, that crabs turned sharply away from shadows of downwelling moonlight that I cast on their eyes with my underwater clipboard. These shadow responses could be interpreted as predator avoidance behaviors, but it seemed unlikely to me that the retinal circadian rhythms evolved for this purpose. A more plausible explanation was suggested by my MBL colleague Colleen Cavanaugh who noted that *Limulus* often mate at night and they may need sensitive vision to find mates. Their predominant nocturnal migration to shallow waters during the flood tides of full and new moons is well known (Barlow et al. 1986). Upon reaching the water's edge, males seek and clasp onto females who then build nests and deposit eggs. We tested the possible role of vision by offering males cement castings of female shells and other shapes placed in the shallow mating areas. Needless to say, we were delighted to see males swarming around the castings, especially those painted black (Barlow et al. 1982). We were also amazed that the black castings evoked the entire male mating sequence: approach, mounting, and sperm release. In fact the males were so attracted to the castings that they would not leave them as the tide receded, risking dehydration and sea gull attack. We rescued these tenacious, love-struck males detaching them from the castings and returning them to the sea. The great attraction of males to the cement castings of females eliminated a role for chemical cues. These experiments provided the first clear evidence for a role of vision in the animal's behavior – males use vision to find mates!

How well can *Limulus* see? We tested their vision by observing the behavior of males in the vicinity of submerged cement castings using a suspended overhead video camera fitted with an image intensifier for nighttime observations. We found that males detected the castings nearly as well day and night (Powers et al. 1991) with greater sensitivity for higher contrast castings (Herzog et al. 1996). Females avoided the castings as did juveniles (Ridings et al. 2002). We concluded that the large 1,000,000-fold circadian increase in sensitivity of the lateral eyes at night enables the animals to detect potential mates.

How many ommatidia does a male use to see a female? The coarsely faceted lateral eyes provide wide-field vision but with very low resolution. Males may be operating near the optical limit of their lateral eyes by using as few as four ommatidia, about 1% of the eye's receptors, to detect a female at a distance of about 1 m. Sacrificing what little acuity they have to increase their visual sensitivity at night, it is indeed surprising that the animals can see so well in their underwater habitat.

1.10 Neural Code for Vision

What does the eye tell the brain? Forming images of mates with less than 1% of the eye's receptors raises the question of what information the eye sends to the brain when a crab sees a mate underwater. Recording the information transmitted to the brain by the many optic nerve fibers is not feasible. Fortunately, as described above the neural network of the *Limulus* eye has been characterized so thoroughly that its properties can be modeled precisely with a realistic, cell-based model of the eye, one that is capable of computing the entire ensemble of optic nerve responses the eye sends to the brain (Passaglia et al. 1998). With it we analyzed neural coding underlying mate detection by recording the lateral eye's view of its underwater world with a shell-mounted camera (CrabCam, Fig. 3) while simultaneously recording the response of an optic nerve fiber from an ommatidium looking in the same direction as the CrabCam. Upon returning to the laboratory, we played back the videotaped scene to the computational model and calculated the ensemble of optic nerve responses, or "neural images," to the scene. Finding that the response they recorded from the single nerve fiber matched well that computed for the equivalent receptor of the model, we examined the computed neural images for putative neural codes of potential mates. Incredibly, we found that the eye responded vigorously to mate-size objects moving across its visual field, that is, the eye appears "tuned" for detecting horseshoe crabs. We concluded that its spatial and temporal properties are optimized for detecting moving, crab-like objects (Passaglia et al. 1997).

Fig. 3 A mini-video camera, CrabCam, mounted on an adult horseshoe crab records what the right lateral eye sees. A recording chamber mounted anterior to the eye contains a microsuction electrode (black cylinder on right) that records the response of a single optic nerve fiber through a whole drilled in the carapace. A white cap seals the recording chamber. The waterproof electrode and camera simultaneously record a nerve fiber's activity and the eye's underwater view as the crab searches for mates

The lateral eyes of this so-called living fossil are not as primitive as one might expect. They are elegant in design, incorporating many of the integrative mechanisms found in more complex vertebrate eyes. They possess universal excitatory and inhibitory mechanisms that "tune" the eye to transmit robust signals to the brain about mate-like objects. Circadian mechanisms of adaptation enable the eyes to operate over wide ranges of light intensity. Even on the darkest, overcast moonless nights, they can tell the brain about potential mates (Atherton et al. 2000). Under such conditions, *Limulus* can see what we cannot.

2 What Is the Neural Basis of the Crab's Visual Behavior?

The answer to this question would surely rank as the "11th" in our list of Top 10 discoveries from *Limulus* vision research. It remains unanswered but certainly not uninvestigated. Wilska and Hartline (1941) were the first to probe the *Limulus* brain and succeeded in recording responses from neurons in the optic ganglia. They detected cells that responded only to the cessation of illumination, "OFF responses" similar to those recorded from ganglion cells of vertebrate retina and completely unlike the "ON responses" that characterize responses from the *Limulus* eye. Thirty years later Max Snodderly (1971), a student of Hartline, extended their work and found greater complexity with different types of light responses in the two optic ganglia of the brain. He noticed that neurons in the first optic ganglion, the lamina, only responded to light offset, i.e., they were exclusively OFF cells, a finding later confirmed by my student, Christopher Passaglia (1997). In the second optic ganglion, the medulla, Snodderly reported all three types of responses, ON, ON-OFF, and OFF; some having large receptive fields ranging from 25 to 100% of the eye, as confirmed by Passaglia. Using fluorescent dyes that he could inject from an intracellular recording electrode, Passaglia tracked neuronal processes across the medulla and branching into the lamina terminating as far as 2 mm away from the cell body in the medulla. He also found that neurons in the brain integrate the eye's output over periods of 250–500 ms. This temporal integration together with spatial integration from the convergence of optic signals from small regions of the eye onto brain neurons improves the signal-to-noise properties of neural images computed with our cell-based model (see above) for the nighttime state of the eye (Hitt et al. 2000).

The circadian increases in the eye's sensitivity combined with spatial and temporal filtering in the brain can yield detectable visual signals even under very low nighttime levels of illumination. The circadian and neural integrative mechanisms may help explain how *Limulus* can see so well at night, but they do not reveal how the brain processes the information it receives and generates responses to behaviorally relevant visual stimuli. With the same fluorescent dye cell-marking technique used by Passaglia, Kazuo Mori in my lab detected nerve cells that respond well to the contrast, both positive and negative, of mate-size objects that move across the visual field with the approximate speed of a horseshoe crab (Mori et al. 2007). Moreover Mori found that processes of these cells appear to be anatomically associated with neurons that mediate the motor output of the brain. Although preliminary, these findings lay the foundation for completing the pathway from eye input to brain output.

As noted at the beginning of this chapter, Hartline chose to study “a suitable organ” (A.V. Hill) in “some animal of choice” (A. Krogh) and moved forward the entire field of vision research. He chose the lateral eye of *Limulus* with the hope of understanding how light causes changes in an animal’s behavior, an interest inspired by his undergraduate research on pill bugs. How sensory information is coded and decoded to produce a behavioral output is a fundamental question in neuroscience. The relative simplicity of the *Limulus* eye has provided a clear window into the peripheral coding of visual information, but the brain’s decoding is not clear. The *Limulus* brain is not simple – no brain is. How it processes the eye’s neural code is not completely understood. We must probe deeper into the brain to find how visual inputs control motor outputs. Perhaps then we will know how the animal sees.

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Sperm Attachment on the Egg of Malaysian King Crab, *Carcinoscorpius rotundicauda*

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Abstract The structures of mature gametes of *Carcinoscorpius rotundicauda* were investigated using scanning electron microscopy (SEM). In general, morphology of the sperm was found to be similar to the other three horseshoe crab species, *Limulus polyphemus, Tachypleus tridentatus*, and *Tachypleus gigas*. The sperm consists of a head, 4.6 μm, containing the nucleus capped with an acrosomal vesicle, and a long flagellum, 33 μm. The spherical egg (2.25–2.58 mm in diameter) consists of two main parts: yolk and chorion. The chorion is composed of two layers: the thin and hard outer layer, or basement lamina (3.03 μm), which has irregular-sized pores on its outer surface, and the thick inner layer, vitelline envelope (43.6 μm). The egg membrane uniformly surrounds the yolk and shows no evidence of a micropyle. Sperm attachment to egg envelope includes three successive steps: initial attachment, acrosomal reaction, and sperm penetration. The initial attachment is observed to occur while spermatozoa are motile. In this step, large numbers of sperm attach to the egg surface. This is followed by the acrosome adhesion, whereby the acrosomal vesicle opens and releases its contents to adhere to the egg material. This is indicated by the flattened appearance of the sperm acrosome. Most of the attached undergo this step and pass through the inner layer of egg. In the last step, the sperm head penetrates through the egg envelope.

1 Introduction

The horseshoe crab fertilization system is considered as primitive, since fertilization occurs externally. A considerable quantity of gametes can be obtained year around from each sexes and used in fertilization experiments (Brown 1976). Horseshoe crabs come ashore in pairs, with a male attached to the opisthosoma of the female body using its chelate claspers. The female is ready

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to lay eggs, and she may spawn all of her eggs within just a few days (Shuster and Botton 1985). She buries herself in the sand, bending down the front part of her body (prosoma), pressing forward and pushing the sand from underneath with her walking legs and out behind with the last pair of legs specially modified for digging (Vosatka 1970). As digging, the female makes the water-soaked, fine sediments more fluid by paddling with her legs (Brockmann 2003). The eggs are released in a continuous stream into the fluid sand 5–20 cm below the surface (Rudloe 1980, Brockmann 1990). As the eggs are laid, they are fertilized by the male, externally with aquatic, free-swimming sperm. Eggs are laid in discrete clusters; in *Limulus polyphemus*, there are some 2,000–4,000 eggs per cluster, but the number is highly variable (Shuster and Botton 1985, Brockmann 1990). Newly laid eggs are extremely sticky (Brown and Clapper 1981) and adhere tightly to one another and to sand grains (Rudloe 1980). After laying one cluster (which takes 3–15 min), the female pushes forward 10–20 cm before laying the next batch of eggs.

The eggs the female has left behind develop in the sand for 2–4 weeks through four embryonic molts, hatching into "trilobite" larvae (Sekiguchi et al. 1988). The non-feeding larvae remain in their clusters in the sand for several additional weeks until the next tidal inundation when they swim into the sea (Rudloe 1979). Within a few weeks depending on temperature (Laughlin 1983), the free-swimming trilobite larvae molt into tiny, spiny juvenile horseshoe crabs which live on the near-shore sand flats (Sekiguchi et al. 1988).

Fertilization in *Carcinoscorpius rotundicauda* has not previously been described, although extensive investigations have been performed on the American horseshoe crab, *L. polyphemus* (Brown 1970, Brown and Humphreys 1970, Brown and Knouse 1973, Clapper and Brown 1980a, b, Cooper and Brown 1972, Mowbray et al. 1970, Brown and Clapper 1980, Bannon and Brown 1980). Here, we describe the structure of mature gametes and the sperm–egg reaction in *C. rotundicauda* using scanning electron microscopy (SEM). The changes occurring in gametes following contact to each other were observed.

2 Materials and Methods

2.1 Materials

Mature individuals of male and female *C. rotundicauda* were collected using fish traps from Pulau Lumut, Port Klang, Malaysia during full and new moon high tides. Gametes were collected from healthy matured animals. Eggs were collected by gentle pressure proximal to the female genital pores. Horseshoe crab sperm were collected using a syringe from male genital pores. Sperm was diluted to a 10% sperm suspension with seawater with 33 ± 2 ppt. Sperm concentration and motility were examined under a light microscope. The sperm concentration was measured using a haemocytometer. The sperm suspension (1 ml) was mixed with mature eggs (30–40 eggs in each container) immediately after collection. Fertilized eggs were washed with seawater after 5 and 60 min in order to observe different stages of sperm attachment and fertilization, and then processed for SEM viewing.

2.2 Scanning Electron Microscopy (SEM)

Sperm, unfertilized eggs, and fertilized eggs were prepared for SEM viewing. Samples were prefixed with osmium tetroxide and refrigerated at 4°C overnight, washed with 0.1 sodium cacodylate buffer for 3 times (10 min each), and dehydrated in an acetone series. Samples were then transferred into the specimen basket and dried in a critical point dryer for 30 min. Samples were placed onto the stub using double-sided tape and coated with gold in sputter coater and finally viewed under SEM.

3 Results

3.1 Morphology of Mature Gametes

The *C. rotundicauda* spermatozoan consists of three main parts: head, midpiece, and a long flagellum (Fig. 1A). A cap-like structure (acrosomal vesicle) was observed on the head (Fig. 1B). The head has an uneven surface. This species has a single long flagellum.

The mature egg of *C. rotundicauda* is round and yolky with an elastic membrane. The eggs measured between 2.25 and 2.58 mm in diameter and were mostly greenish yellow in color. It has a sticky membrane which adheres to the wall of the glass vessel or the tip of the forceps.

The egg consists of two main parts: the yolk and the outer membrane or chorion (Fig. 2). The chorion is a thick membrane with a rough surface with irregularly sized pits Fig. 3) . As noted by Sekiguchi (1988), the chorion itself consists of two layers: a thin outer layer or basement lamina, which originates from the ovarian epithelium, and an inner layer called vitelline envelope, formed by the egg itself. The thickness of the basement lamina was 3.03 μm, while the vitelline envelope was 43.6 μm. The thickness of outer layer is approximately 1/14 the inner layer (Fig. 4).

3.2 SEM Observation on the Attachment of Sperm to the Egg

The egg and sperm of *C. rotundicauda* were observed under light microscope prior to fertilization. As in most animals, sperm motility was initiated at the time of insemination and dilution with seawater. Sperm count was between

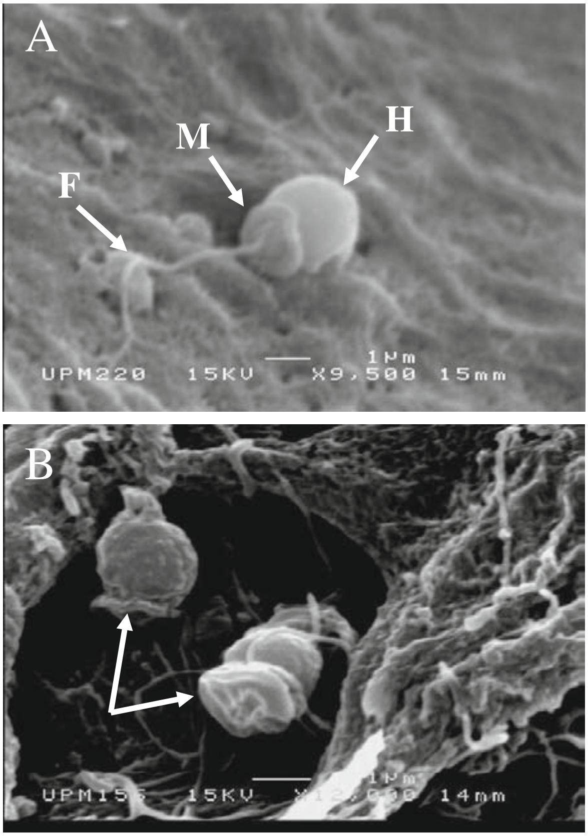


Fig. 1 (**A**) SEM observation on the matured spermatozoa. (**B**) SEM observation on mature spermatozoa showing cap-like acrosomal vesicle on the head. F, flagellum; H, head; M, midpiece

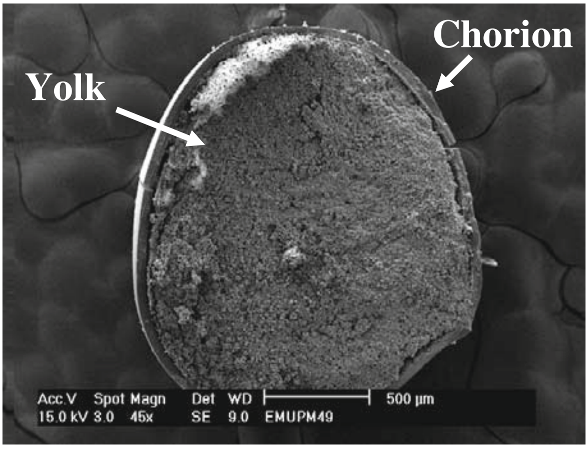


Fig. 2 Cross section of matured *C. rotundicauda* egg under SEM

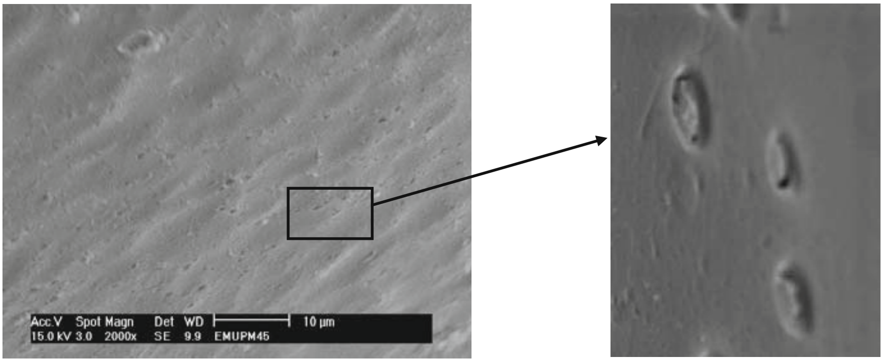
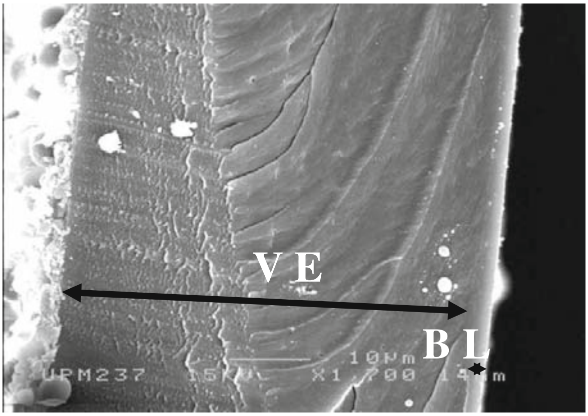


Fig. 3 Irregular-sized pits on the chorion surface as observed under SEM

Fig. 4 Cross section of the chorion observed under SEM. VE, vitelline envelope; BL, basement lamina



0.003 × 10^9 and 0.023 × 10^9 sperm/ml in matured *C. rotundicauda*. The sperm mixed with mature eggs swam toward the egg surface and surrounded it like a halo. At higher magnifications, almost all of spermatozoa in the region were motile and swam directly to the egg surface. Once the sperm attachment occurred, motility subsided and the halo disappeared.

The egg membrane consisted of the basement lamina and vitelline envelope, which uniformly surround the yolk. No micropyle was observed. Samples of eggs and sperm mixture were observed under SEM. Large numbers of spermatozoa were attached to the egg surface (Fig. 5). Each sperm adheres perpendicularly to the surface by means of the acrosomal vesicle (Fig. 6). The apical tip was observed to attach to the egg and no apparently morphological change in the sperm was evident at this stage (Fig. 7). Here the basement lamina of the egg participates in the initial attachment, followed by the acrosomal reaction (Fig. 8). At this stage, the acrosomal vesicle opens to release its contents into

Fig. 5 The egg surface covered with numerous spermatozoa at different magnifications (5 min after insemination). A, acrosome; F, flagellum; N, nucleus

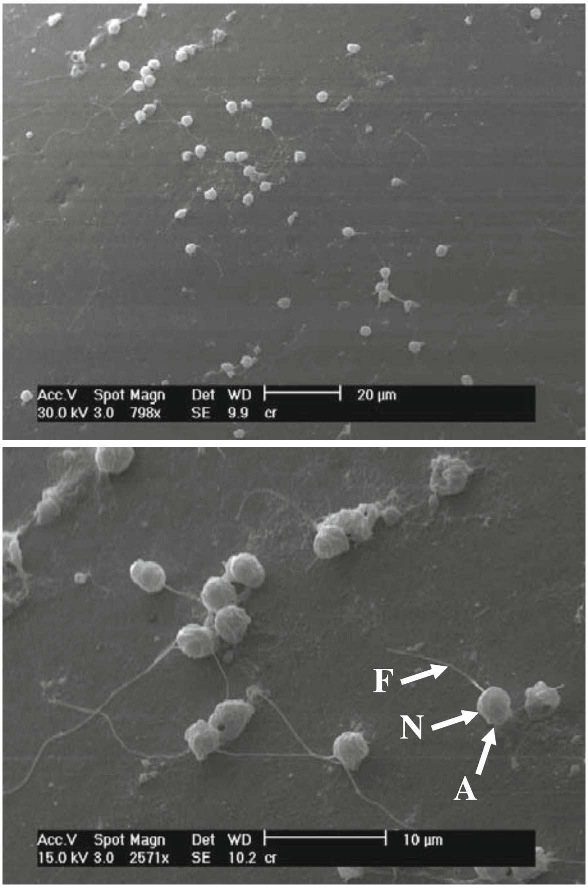


Fig. 6 Primary attachment of the spermatozoa to the egg surface (5 min after insemination)

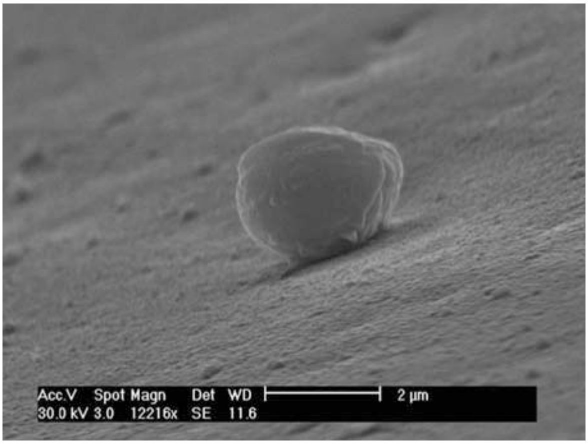


Fig. 7 Spermatozoa attached to the basement lamina of the egg. BL, basement lamina; F, flagellum; H, sperm head

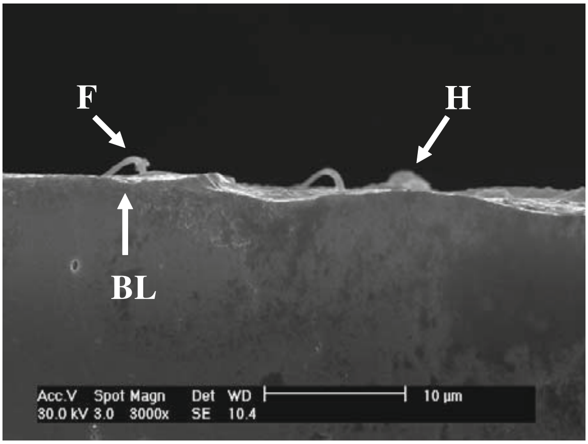
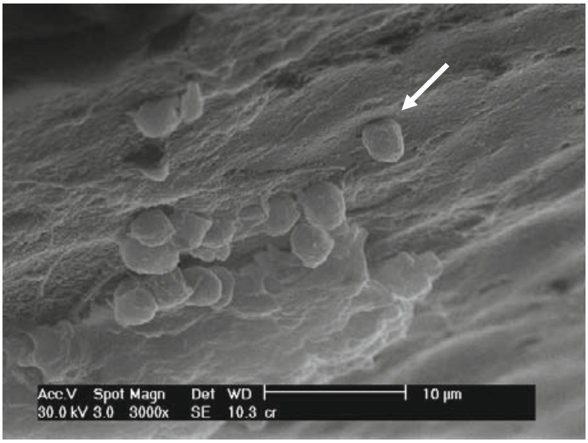


Fig. 8 Attachment of the acrosomal vesicle (*Arrow*) (5 min after insemination)



the egg materials. At this stage, observation was difficult particularly when the acrosomal filaments were projected into the egg chorion. Sperm at this stage is indicated by the flattened appearance of the acrosome (Fig. 9). Each acrosomal filament passes through the basement lamina. These filaments may be digesting a pathway or mechanically forcing their way through the vitelline envelope, as suggested by Brown (1976) for *L. polyphemus*. After the acrosome reaction, the secondary reaction occurs whereby the fibrous components of the flagellum are incorporated into the sperm body and flagellum coils. However, it was difficult to find the one sperm in this step. The projection of the acrosomal filament is a part of the acrosome reaction, while the penetration of the acrosomal filament through the egg envelope is a direct result of this reaction. Most of the attached undergo this step and pass through the inner layer of egg (Figs.10 and 11).

Fig. 9 Sperm penetrating the egg envelope (15 min after insemination). H, sperm head; F, flagellum

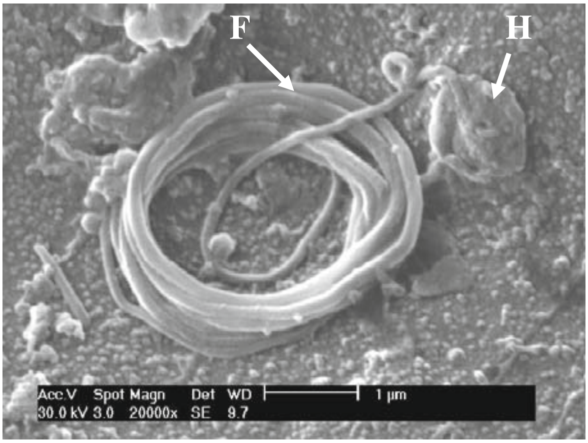


Fig. 10 Numerous sperm penetrated the egg chorion (60 min after insemination)

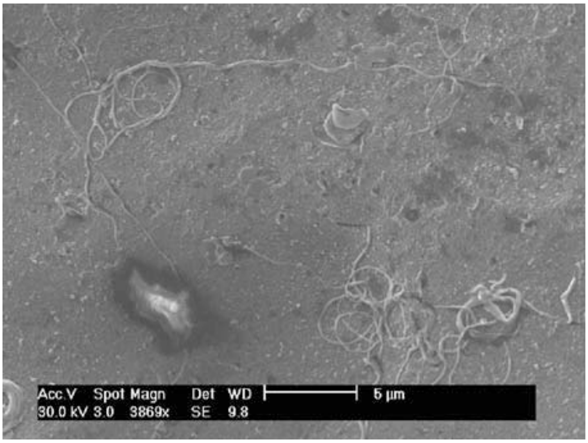
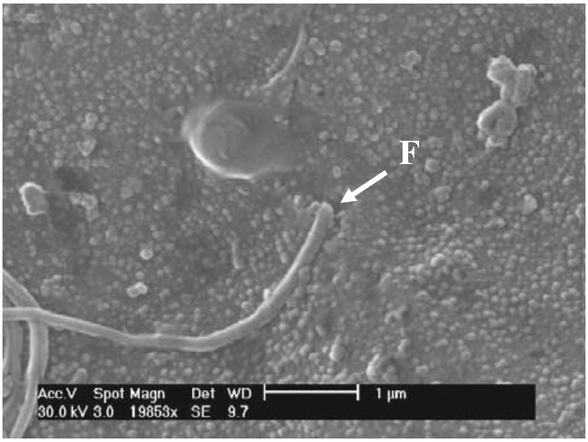


Fig. 11 Spermatozoon passes through the basement lamina



4 Discussion

In general, morphology of the sperm was found to be similar to the other three horseshoe crab species, *L. polyphemus, Tachypleus tridentatus*, and *Tachypleus gigas* (Sekiguchi 1988). It consists of three main parts: head, midpiece, and flagellum. Sekiguchi's (1988) measurement of the flagellum of *C. rotundicauda* was 37.7 µm, which is larger than what he reported for the other three species. He also reported that the sperm head size was 5 µm, the same as for *L. polyphemus* and *T. gigas* and larger than what he reported for *T. tridentatus*. The cap-like structure on the head is the acrosomal vesicle and is different from *L. polyphemus*. It is a thick hemisphere in *L. polyphemus*, while it look like a flat disc in *C. rotundicauda* (Sekiguchi, 1988).

Among the three Asian species of horseshoe crab, *C. rotundicauda* has the smallest-sized egg, measuring 2.25–2.58 mm in diameter, compared to *T. tridentatus* and *T. gigas*, which average 3.0 and 3.7 mm diameter, respectively (Sekiguchi 1988). The eggs are yellowish green, while *T. gigas* and *T. tridentatus* eggs are yellow and those of *L. polyphemus* are bluish grey to pinkish grey (Sekiguchi, 1988).

As in the other species of horseshoe crabs, *C. rotundicauda* has an egg consisting of yolk surrounded by a chorion with an inner basement lamina and outer vitelline envelope (Brown and Humphreys 1971). There is no space between the two layers in *C. rotundicauda* and the other Asian species, whereas an open space exists in *L. polyphemus* (Sekiguchi, 1988). The thickness of the outer layer was measured to be 1/14 the inner layer, while in *L. polyphemus* it is 1/10 of the inner layer. SEM observation showed some irregular-sized pores on the chorion surface.

Observations of the spermatozoa under light microscopy showed high concentration of spermatozoa in this species. Sperm of *C. rotundicauda* were observed to be motile before mixing with the egg, but in *L. polyphemus* the spermatozoa are non-motile even when diluted with seawater and motile only when in close proximity with the egg (Clapper and Brown 1980a). The sperm was observed to swim toward the egg surface forming a halo-like region around the egg similar to those seen in *L. polyphemus* (Clapper and Brown 1980b).

Although in some species (e.g., *Arbacia*) a single sperm can approach, attach, penetrate, and fertilize an egg (Halliday and Verrell 1984), in most species the normal number of spermatozoa attaching to the egg is usually more than one (Austin 1969a). High sperm concentration (0.003–0.023 $\times$ 10^9 sperm/ml) resulted in numerous sperm attachments to the egg surface. In *Arbacia*, too many spermatozoa attaching to the egg can cause pathological polyspermy resulting in abnormal development (Austin 1969b). On the other hand, in *C. rotundicauda,* as with *L. polyphemus* (Brown 1976) and several species of crustacean decapods (Binford 1913), it is normal to have large numbers of spermatozoa attached to the egg surface.

Like *L. polyphemus* (Brown 1976), *C. rotundicauda* spermatozoa undergo two stages of egg attachment: initial and secondary attachment. The initial attachment occurs as the sperm apical tip come into contacts with the basement lamina. The secondary reaction involves binding of the acrosomal materials to the egg surface. At this stage, each sperm is attached perpendicularly to the egg surface. The acrosomal reaction, the release of acrosomal contents, occurs after sperm attachment to the egg. The nuclear content of the sperm is then transferred through the egg envelope.

Even though many sperm initially attach to the egg surface and pass through the egg envelope, only one sperm will be able to transfer its nucleus into the egg and fertilize the egg. Therefore, the egg controlled the penetration of sperm through the vitelline plasma membrane.

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Distribution and Development of *Limulus* Egg Clusters on Intertidal Beaches in Delaware Bay

Richard G. Weber and David B. Carter

Abstract Accurate knowledge of where spawning *Limulus* females place their egg clusters in beaches is important for sampling egg cluster density, which in turn is important in studies of habitat use, monitoring *Limulus* egg production, determining potential shorebird forage, and for investigating the effects of beach erosion or replenishment. We examined *Limulus* egg cluster placement on seven western shore Delaware Bay beaches. Depth to center of recently laid clusters ranged from 3.5–25.5 cm, with an average depth to center of 15.5 ± 3.5 cm (n=533). Centers of 88.0% (469) of all clusters were within 20 cm of the undisturbed beach surface, and 98.1% of all clusters (523) extended 1 cm or more into the 20 cm horizon. Clusters were found only in the upper 85% of the foreshore, beginning at the spring tide high-water mark, even though beach widths varied (n = 6,132 clusters in 80 transects). Intact, recently laid clusters contained from 2,524 to 16,835 eggs, with an average size of 5,786 ± 2,834 eggs (n =26). Beach sediment temperatures in early May ranged from 13.7°C to 24.2°C (mean = 17.0° ± 3.0°C SD); in early June from 16.4 to 29.7°C (mean = 21.6° ± 3.3°C SD); and in early July from 22.4 to 30.4°C (mean = 26.8° ± 1.9°C SD). Under these conditions, the first trilobite larvae (<25 larvae per beach sampled) normally began to appear in our sediment samples during the first week in June, suggesting that about 35 days are required for development of the earliest eggs in a normal spring. Because egg clusters on all beaches were confined to 85% of the upper foreshore, and most clusters were within reach of a 20 cm deep sample, future studies to assess cluster densities should be designed to sample within that portion of a beach.

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1 Introduction

An accurate knowledge of where spawning *Limulus* females place their egg clusters in beaches is important for sampling egg cluster density, which in turn is important for studying habitat use, monitoring horseshoe crab egg production, determining potential shorebird forage, and for investigating the effects of beach erosion or replenishment. Delaware has periodically replenished selected bayfront beaches since 1961. Concerns about possible effects of beach replenishment on *Limulus* spawning resulted in a research program to find how *Limulus* eggs are distributed during spawning. During 1998–2005, we conducted a series of studies which examined *Limulus* egg cluster placement on western shore Delaware Bay beaches in May, June, and early July. We were particularly interested in the depth at which clusters were placed and in the distribution of clusters across beaches.

Literature concerning depth at which *Limulus* egg clusters are placed in Delaware Bay beaches is sparse. Hummon et al. (1976) found egg clusters "from 2–6 cm depth at neap low to 12–24 cm depth at mean high water level" in Roosevelt Inlet, Delaware. Penn and Brockmann (1994) reported a depth of 9.3 ±3.9 cm for 112 clusters examined at Cape Henlopen State Park, Delaware. In New Jersey, Botton et al. (1994) mention 15–20 cm as the depth where "most egg clusters are initially deposited."

Information about egg cluster distribution across Delaware Bay beaches is also sparse. Shuster (1982) discovered that "On Delaware Bay beaches, egg nests [clusters] are found in a broad band starting about 3 meters from the low-water line to the spring high-tide water mark." Shuster and Botton (1985) reported that in New Jersey, clusters were absent from the lowest 3 m of the beach. Williams (1986), also working in New Jersey, found "the middle section of the intertidal zone contained significantly more eggs than either end ..." Brockmann (2003b) reported that in Delaware, nesting occurred over 61% of the beach, and spawning was not concentrated near the high tide line. Bayfront beach widths are variable and relate to the extent of a beach's tidal flat. Larger tidal flats give narrower foreshores, which seem to be preferred for spawning by *Limulus* females (Smith et al. 2002). Because beach widths vary, the area selected for egg cluster sampling must be proportional to foreshore width, rather than a fixed span (Jackson et al. 2002).

During our studies of cluster depth and distribution, we became curious about the average number of eggs per cluster in Delaware Bay beaches. Not every aggregation of *Limulus* eggs exhumed from a beach is an intact cluster, as laid by a female. Spawning females frequently damage previously laid, intact egg clusters and disturb the surrounding sediments, a process called bioturbation (Kraeuter and Fegley 1994). Even the simple act of digging up clusters with a shovel can cause parts to break off, especially if egg development has made the cluster friable. Hummon et al. (1976), examining a beach in Roosevelt Inlet, Delaware, reported finding clusters that were "comprised [of] 75–300 eggs." In

New Jersey, Shuster and Botton (1985) examined four egg clusters and found them to average 3,650 (±232.5) eggs. Shuster and Botton (1985) reported great variability in the amount of sediment present in each cluster, with gravel from one cluster being approximately three times the weight of the eggs.

Finally, our regular collection dates during these studies gave us an opportunity to examine beach sediment temperatures. The time required for *Limulus* eggs to develop and hatch is controlled by temperature (French 1979), although salinity also affects development rate (Jegla and Costlow 1982). Warmer temperatures give shorter times to hatching (French 1979; Jegla and Costlow 1982). French (1979) found that eggs maintained at approximately 15°C took 45 days to hatch, but eggs maintained at approximately 23°C required only 28 days.

2 Background

The American horseshoe crab, *Limulus polyphemus* L., occurs in coastal and estuarine environments from northern Maine to the Yucatán peninsula (Shuster 1982). The species is especially abundant along the mid-Atlantic coast and reaches its peak abundance in the Delaware Bay (Shuster 1982). *Limulus* spawning activity is most intense during the nocturnal full and new moon tides of May and June (Rudloe 1985; Barlow et al. 1986). However, these spawning peaks may occur later if onshore winds prevent spawning on the nights of full and new moon high tides (Shuster 1982). In Delaware Bay, considerable spawning also takes place at other times during the lunar cycle, on both day and night high tides (Shuster and Botton 1985; Penn and Brockmann 1994). As a result, within Delaware Bay, egg clusters are not concentrated along the spring high tide line as they are in other parts of their range (Brockmann 2003b). Female *Limulus* spawn near the time of high tide, on the beach foreshore, where they burrow into the beach sediments to approximately the level of their compound eyes (Cohen and Brockmann 1983). A spawning female is accompanied by at least one male, which has grasped her opisthosoma with his claspers and follows behind her. She may also be attended by one or more unattached “satellite” males (Brockmann 1990; Loveland and Botton 1992). A female burrowed into the beach, ready to spawn, moves ventral appendages (the flabellae; Sekiguchi 1988), creating a current of water beneath her body. When spawning, she pulls water under herself, from behind. It is expelled, from beneath her body, through the lateral gaps at the hinge line between her prosoma and opisthosoma (Barthel 1974; Brockmann 1994). The attached male may also assist in creating this forward flow of water (Brockmann et al. 2000; Brockmann 2003a). The moving water carries smaller sediment particles from beneath the female’s body, leaving behind particles too large to be transported by the strength of the flow she and the male generate. When a cavity has been cleared beneath her operculum, the female releases several thousand eggs into it (Penn and Brockmann 1994), which the male fertilizes. Shortly afterward, she moves forward through the sand, away from the eggs she has just deposited. She may pause again to lay another cluster (Kingsley 1892; Brockmann 1990), or may push up and out of the beach to return to the water. As the female moves away from eggs she has just laid, the moist sand slumps down, confining the fresh eggs among whatever larger sediment particles remained in the cavity when they were laid (Shuster and Botton 1985; Shuster and Sekiguchi 2003).

As first laid, a *Limulus* egg is encased in a soft, flexible chorion and is not tightly filled or turgid. It is also covered by a sticky film (Rudloe 1979; Brown and Clapper 1981; Shuster and Sekiguchi 2003). As the female moves away, the soft, newly laid eggs are pressed together by the weight of the sand above them. They are not tightly filled spheres, so they deform slightly as they are compacted, forming numerous flattened and concave areas where they touch, thereby increasing the areas of contact between eggs (Fig. 1). During the next hour or so, the chorions become somewhat tougher and less flexible and the sticky film on the eggs cures, cementing the group of eggs and sediment particles together with a surprisingly sturdy, slightly flexible, bond. The resulting "rubbery" cluster of eggs contains variable amounts of coarse sediment particles (Fig. 2) (Shuster and Botton 1985; Shuster and Sekiguchi 2003). Clusters are not uniform in size or shape, but do tend to be ovoid, slightly flattened, and thinner

Fig. 1 Photograph of eggs inside a recently laid *Limulus* egg cluster, which has been broken apart to show the flattened and concave surfaces caused when the soft, newly laid eggs were pressed together by the moist sand around them. These broad areas of contact, coupled with the sticky material surrounding each egg when it is laid, combine to make new clusters resilient, with a "rubbery" feel when handled. As development proceeds, eggs become turgid and rounded which decreases areas of contact, the sticky bond between them breaks down, and the cluster becomes friable and is easily broken apart

Fig. 2 *Limulus* egg clusters, showing the variable sizes and amounts of beach sediment particles they contain. The *bottom* two clusters are intact; the *top* two have lost eggs along their *lower left* edges

(from top to bottom) than wide. For the next week or two, depending on temperature, the cluster will persist as a resilient, rubbery aggregation of eggs and sediment. As embryonic development progresses, the individual eggs become more rounded and turgid, which decreases the former broad contact areas, while the adhesiveness of the initial bond also begins to break down. When these changes have progressed sufficiently, a cluster becomes friable and is easily crumbled apart with a fingertip, even when still mostly supported by surrounding sand. Thus, the resilience of a cluster when probed with a fingertip can provide a useful index to how long it has been since the female deposited it. A cluster found in the beach has been laid within the previous one or two weeks, if it does not crumble when probed with a fingertip. If no storms have disturbed the beach sediments during that time, then the distance from the beach surface to the cluster is the depth at which the female originally placed it. The cluster will remain at the depth it was placed unless another spawning female disturbs the area where it rests, or the depth of sediment covering it is affected by storm waves. Depending on the angle at which storm waves strike a beach face, sediment may be removed from the beach, decreasing sediment depth above a cluster, or moved from higher to lower levels of the beach, thereby deepening sediment above a cluster (Jackson et al. 2002).

The depth at which *Limulus* egg clusters are placed is a function of the female's body size and of the depth to which she burrows into the beach (Shuster and Sekiguchi 2003). Spawning females commonly burrow into a beach until their compound eyes are near the beach surface (Cohen and Brockmann 1983), so at each subsequent spawning tide, additional females will burrow into the same section of beach, to spawn at the same depth as any previously laid clusters. Inevitably, some previously laid clusters are disturbed each time spawning takes place. This process, and the associated disturbance of beach sediments, has been called bioturbation (Kraeuter and Fegley 1994). Bioturbation from heavy spawning, combined with moderate wave action, can alter sediment depth (Jackson et al. 2005). Collisions with previously laid clusters are density dependent, and heaviest near the center of the beach foreshore; however, even at low spawning densities, as many as 20% of the existing clusters may be disturbed (Smith 2007). The effect of such a collision on the disturbed cluster is partly determined by its age. That is, whether it is new and tough, or whether it has been in place long enough for embryonic development and cement deterioration to make it friable. A collision between a spawning female and a previously laid egg cluster breaks the cluster into several smaller aggregations of eggs, or "clusterlets." Succeeding cohorts of spawning females can further break up clusterlets into individual eggs. When sediments containing clusterlets and dissociated eggs are activated by waves, eggs are moved upward toward the beach surface, where they eventually come within the reach of foraging shorebirds (Pooler et al. 2003).

In this chapter, we report studies done during 1998–2005 to refine our understanding of where female *Limulus* deposit their eggs in western shore Delaware Bay beaches and of the clusters themselves.

3 Study Beaches

Locations of our study beaches are shown in Fig. 3. All are in the mid-bay region, where western shore spawning activity is most intense (Smith and Michels 2006), from Port Mahon, south to Slaughter Beach. The straight-line distance between Port Mahon and Slaughter Beach is 28 km. Tides on these beaches are semi-diurnal, with a mean range of 1.4 m and a spring range of 1.7 m (Nordstrom et al. 2006). Through the lunar cycle, nocturnal high tides range from 0.3 to 0.5 m higher than diurnal tides, which spreads *Limulus* spawning across the upper foreshore, the span between the high tide line and the beach break at the beginning of the low tide terrace (=tidal flat). Most of these sites are low-energy, bayfront beaches, comprised of mixed sand and gravel, and having a tidal flat exposed at low tide. Exceptions are the area of Slaughter Beach we sampled, which did not have a tidal flat exposed at low tide, and Mispillion Inlet, at the mouth of the Mispillion River, which is protected from bay surf action by riprap jetties, and which also does not have an exposed tidal flat. Beaches with tidal flats exposed at low tide have shorter foreshores and, on bayfront beaches, females prefer to spawn on such narrow beaches (Smith et al. 2002).

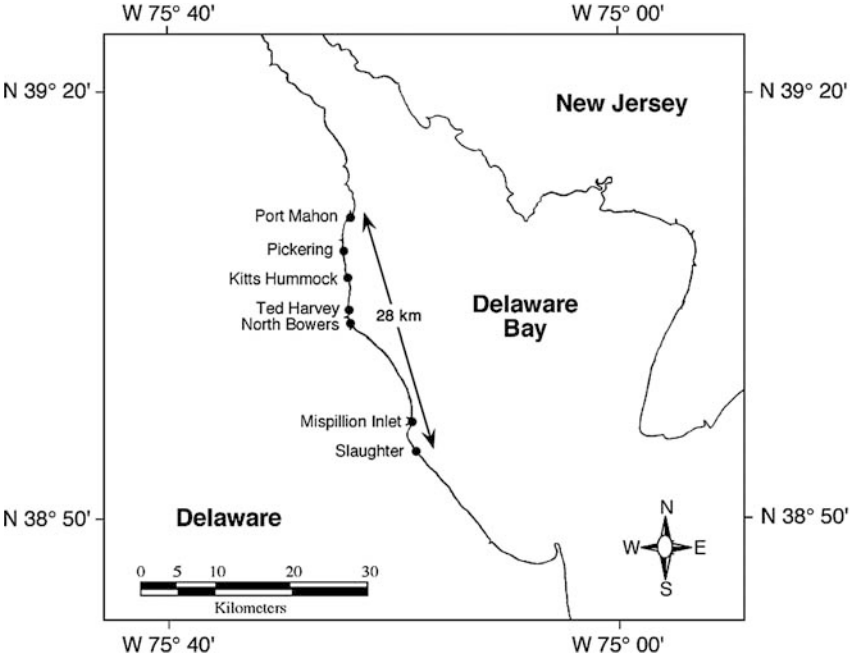

Fig. 3 Map of Delaware Bay showing locations of beaches where *Limulus* egg cluster distribution was studied. The *straight-line* distance between Port Mahon and Slaughter Beach is 28 km

4 Methods

For convenience, we have arranged this section, and the following Results section, by topic.

4.1 Egg Cluster Depth

In 1998, 1999, and 2000, we examined the depth at which female *Limulus* place their egg clusters. Port Mahon, Ted Harvey, and North Bowers beaches were sampled each year. Slaughter Beach was sampled only in 1998, and Pickering Beach was sampled only in 2000. There had not been unusually strong onshore winds for at least 10 days before any sample date, so beach surfaces had not been altered recently by wave action. Sampling was done in June, when the heaviest spawning was past and sample dates were selected to be two or more days away from new and full moon tides. This was done to minimize chances of bioturbation having altered sediment depth. When sampling, we examined 1–3 sites on each beach, measuring the depths of 15–30 clusters at each site, on a total of 25 sites. We sampled by making trenches approximately 1 m long by

0.3 m deep, at right angles to the water line, midway between the nocturnal high tide wrack line and the beach break where the tidal flat begins. A small garden trowel, oriented vertically, was used to shave away the trench face at approximately 1 cm per pass.

When a cluster was exposed, it was first probed with a fingertip to determine whether it was friable, or firm. If it seemed firm, a fingertip was used to expose its outline in the sand wall. A cluster which survived both these tests without breaking was considered to be fresh enough that its depth would be close to the depth at which the female had originally placed it. Measurements from beach surface to a cluster's top and bottom were taken at the cluster's maximum thickness and were referenced to the bottom edge of a 1 m long board laid on the beach surface along the edge of the trench. Using the board as a reference minimized the effect of any small, local surface depression or elevation on the measurements. We did not attempt to measure maximum cluster width. To do so would have required removing the cluster, which frequently caused the local sand wall to collapse, requiring removal of considerable sand to again reach unperturbed beach.

4.2 Cluster Distribution Across the Foreshore

We examined the distribution of egg clusters across beach foreshores in 2000 and 2001. We sampled Pickering, Kitts Hummock, Ted Harvey, and North Bowers beaches each year, using from 6 to 15 transects per beach. To do this, we used a single-bottom plow, pulled by a tractor, to make a series of 0.3 m deep furrows (transects) from the nocturnal high tide wrack line (approximately the spring tide high water level) down to the beginning of the tidal flat. Transects were at right angles to the water line, and spaced 3–10 m apart. We marked off each transect in 30 cm intervals, then used a garden rake and our fingers to locate all clusters present in each 30 cm span of material turned out of the furrow. Clusters were replaced into furrows as counted, and when counting in each transect was completed, it was refilled and the surface smoothed to the original level. After the next tide cycle, no evidence of the operation remained.

4.3 Cluster Distribution Along Sample Transects

We tested the foreshore cluster distribution we had observed in the 2000–2001 study, described above, by using core-sampled transects. Based on the 2000–2001 results, the transects used in this study spanned only 85% of the distance from the nocturnal high tide wrack line down toward the foot of the beach, where the tidal flat began. On beaches with no tidal flat exposed at low tide (Mispillion Inlet and Slaughter Beach), we used 85% of the distance from the high tide wrack line to the low water level. We used a tape measure to determine the 85% point for each transect we sampled.

In this study, we sampled during 2002–2005 at Port Mahon, Pickering, Kitts Hummock, North Bowers, Mispillion Inlet, and Slaughter beaches. Half of these beaches, Port Mahon, Mispillion Inlet, and Slaughter Beach, were new beaches, which were not sampled during the 2000–2001 study of cluster distribution across the foreshore. There were five sample dates per season, each selected to fall between new and full moon tides, and more than 3 days before or after new or full moon, so that any spawning activity associated with those lunar tides would be represented in the samples. On each sample date, we used a pair of core-sampled transects per beach, separated by at least 100 m. Transects were oriented at right angles to the water line, and consisted of 20 evenly spaced core samples. Sample sediment cores were 5.7 cm in diameter × 20 cm deep. The six beaches sampled during this study had different distances across their intertidal zones; thus, different transect lengths were required. However, it was possible to space the 20 core samples evenly, and thus proportionally, along each transect's length by using marked bungee cords. Stretching a marked bungee cord over a span to be sampled indicated the exact location where each sample core should be taken. Equal spacing of marks on the cords made the 20 cores taken across each beach fall into proportional locations, from the nocturnal high tide wrack line where the first core was taken, down to the 20th core, at the point 85% of the distance from wrack line to the tidal flat. Thus, any ranked sample core would be located at the same percentage distance down across the beach. For example, the twelfth sample core would be located at a distance 60% from the wrack line (60% = 12/20*100).

Upon collection, each core sample was passed through a 1.3 cm mesh screen into a bucket, to reveal any clumps of eggs. Because clusters in cores sometimes break apart as the core is taken, one, or more, firm aggregation of eggs that did not pass through the 1.3 cm mesh was recorded as a single cluster for that core. We also recorded the core position (number) where each cluster was found. Thus, by summing all clusters found in all twelfth cores (and in the other core positions as well) during this study, it was possible to obtain a composite view of cluster distribution downward across the intertidal zones of the beaches we sampled.

4.4 Egg Cluster Size

We examined cluster size by digging a 0.3 m deep trench in the mid-beach area. Each cluster that we uncovered in the trench was examined closely by two people familiar with egg clusters, to determine whether it was friable, and intact (without obvious breaks along edges). All friable clusters were discarded, and every intact cluster was collected. We counted all eggs by hand, rather than using volumetric estimates of total numbers. We collected 26 egg clusters; 14 from Port Mahon in 2003, and 12 from Pickering Beach in 2005.

4.5 Beach Temperature and Egg Development

During the spawning seasons of 2002–2005, we measured beach temperatures on Port Mahon, Pickering, Kitts Hummock, North Bowers, and Slaughter beaches. We took two measurements at each site, separated by at least 100 m. Temperatures were always measured near low tide, and beaches had been under the influence of air temperature and insolation for varying amounts of time prior to measurement. Readings were taken at a depth of 20 cm, with digital probe thermometers placed near the center of the intertidal span.

5 Results and Discussion

5.1 Egg Cluster Depth

We measured the depth and thickness of 533 clusters. Collection data and summaries of cluster depth and thickness data appear in Table 1. Cluster thickness (top to bottom) ranged 0.6–7.6 cm, with an average thickness of 3.5 ± 1.1 cm

Table 1 Summary of sampling to examine depths at which *Limulus* egg clusters were placed on Delaware Bay beaches, 1998–2000. Sampling was done approximately midway between the nocturnal high tide wrack line and beginning of the tidal flat. A total of 25 sites were examined, which yielded 533 clusters. Thickness is the distance from top to bottom of a cluster. Beaches are listed north to south

Beach	Year	Sample date	Sites examined	Total clusters	Mean depth to center (cm)± SD	Mean thickness (cm)± SD
Port Mahon	1998	8, 17, 29 June	3	48	13.2 ± 2.9	3.4 ± 1.4
	1999	8, 9, 23 June	3	75	15.0 ± 2.9	3.6 ± 1.2
	2000	13 June	2	54	16.6 ± 3.9	3.9 ± 1.1
Pickering Beach	2000	13 June	2	50	18.3 ± 3.4	4.0 ± 1.1
Ted Harvey	1998	8, 17, 29 June	3	45	15.3 ± 2.8	3.5 ± 2.8
	1999	15 June	2	50	14.1 ± 3.2	3.6 ± 0.9
	2000	14 June	1	25	14.5 ± 1.6	3.3 ± 0.7
North Bowers	1998	8, 17, 29 June	3	45	16.5 ± 2.4	3.2 ± 1.0
	1999	9 June	2	59	14.6 ± 3.1	2.9 ± 0.8
	2000	14 June	2	51	18.3 ± 3.9	3.3 ± 0.8
Slaughter Beach	1998	17, 29 June	2	31	13.2 ± 2.7	3.8 ± 0.9

SD. Depths of clusters ranged 3.5–25.5 cm, with an average depth to center of 15.5 ± 3.5 cm SD. For all clusters, centers of 4.9% (26) were within 10 cm of the beach surface (only 1 cluster was in the 0–5 cm horizon), 39.5% (210) were in the 10–15 cm horizon, 43.6% (232) were in the 15–20 cm horizon, 12.0% (64) were in the 20–25 cm horizon, and 1 cluster was below 25 cm. Figure 4 shows the depth-to-center distribution of clusters in the entire sample set.

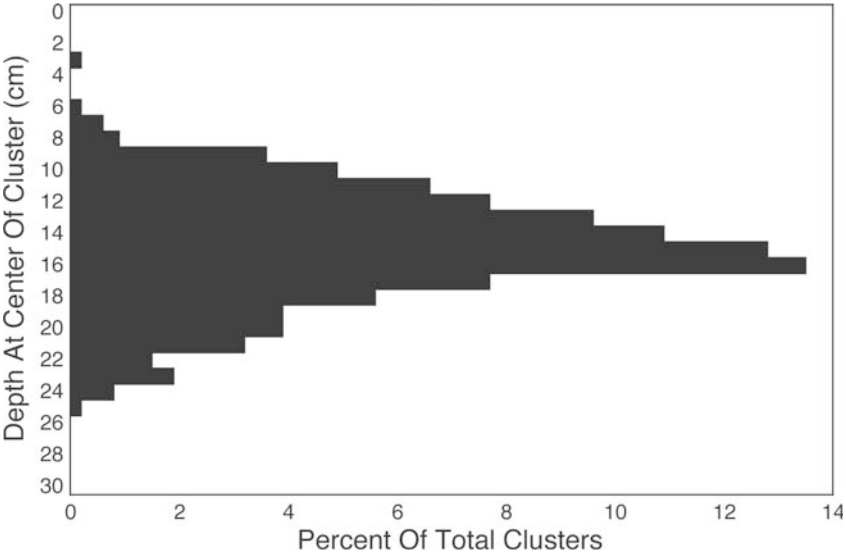

Fig. 4 Depth-to-center distribution of *Limulus* egg clusters observed at mid-beach on Port Mahon, Pickering, Ted Harvey, North Bowers, and Slaughter beaches in June, 1998–2000. A total of 533 clusters were found at the 25 locations examined. Of these, 83.1% (443) were in the 10–20 cm horizon

5.2 Cluster Distribution Across the Foreshore

We examined a total of 80 transects and found 6,132 clusters. On all beaches, all clusters were found in the upper 85% of the span from the nocturnal high tide wrack line to the beginning of the low tide terrace (tidal flat) (Fig. 5). Of these, 90.9% (5,588 clusters) were found between 25 and 75% of the distance between the nocturnal high water wrack line and the beginning of the tidal flat. These results suggest that female *Limulus* place their egg clusters only in the upper 85% of western Delaware Bay beach foreshores at the current population abundance. The average number of clusters per transect varied from beach to beach in both years, and the average number of clusters per transect was highest in 2001 (Table 2). Difference between years is most easily explained by the date of sampling. Sample dates in 2000 were in mid-May (Ted Harvey, North Bowers) and late June (Pickering, Kitts Hummock). These are slightly before,

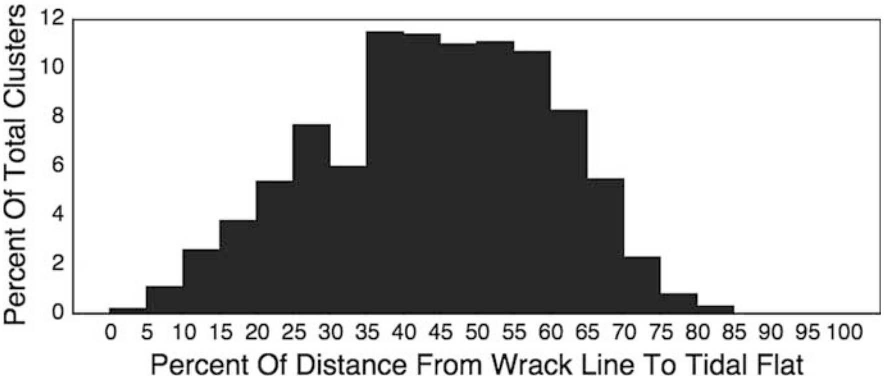

Fig. 5 Distribution of *Limulus* egg clusters observed across the foreshores of Pickering, Kitts Hummock, Ted Harvey, and North Bowers beaches in May and June, 2000 and 2001. All of the 6,132 clusters found in the 80 transects examined were in the upper 85% of beach foreshores. Of those, 90.9% (5,588 clusters) were found between 25 and 75% of the distance between the nocturnal high water wrack line (approximately the spring high tide water level) and the beginning of the tidal flat. The tidal flat would be off the chart, to the right

Table 2 Summary of sampling to examine *Limulus* egg cluster distribution across Delaware Bay beach foreshores, 2000–2001. Transects consisted of 0.3 m deep furrows, spaced 3–10 m apart, running from the nocturnal high tide wrack line (approximately the spring tide high water level), down to the beginning of the low tide terrace (tidal flat). A total of 80 transects were examined, which yielded 6,132 clusters. Beaches are listed north to south

Beach	Year	Sample date	Transects	Transect length (m)	Mean clusters per transect ± SD
Pickering Beach	2000	30 June	6	12.5	38.7 ± 11.7
	2001	31 May	15	14.3	213.9 ± 74.0
Kitts Hummock	2000	27 June	10	8.5	25.8 ± 7.6
	2001	30 May	10	11.3	60.6 ± 20.0
Ted Harvey	2000	16 May	10	8.5	31.9 ± 8.4
	2001	5 June	10	7.0	66.6 ± 42.0
North Bowers	2000	15 May	9	13.7	28.1 ± 8.6
	2001	29 May	10	14.3	64.9 ± 24.4

and somewhat after the normal seasonal spawning peak. Spawning is just beginning in early May, and by the end of June, both the water temperature and beach sediments have warmed so that many clusters have developed to the late embryo and larval stages, which we did not count. The 2001 sample dates were in late May (Pickering, Kitts Hummock, North Bowers) and early June (Ted Harvey), very close to the normal seasonal spawning peak.

5.3 Cluster Distribution Along Sample Transects

Figure 6 shows the distribution of the 260 clusters collected along core-sampled transects during the 2002–2005 seasons. Distribution of the egg clusters found in these sample cores was similar to that observed in the 2000–2001 study of cluster distribution across the foreshore, even though half of the sampled beaches had not been part of the 2000–2001 study. Of clusters in cores, 76.9% (200) were found in the 5th through 15th cores (25–75% of the transect length). These results add additional support to evidence from the 2000–2001 test, described above, that even though beach foreshore widths may vary, sampling only the upper 85% of a west Delaware Bay beach foreshore will adequately sample the span in which female *Limulus* place their egg clusters. Thus it would seem best for studies measuring egg cluster densities to index the sampling protocol to either the spring high water level or the nocturnal high tide wrack line.

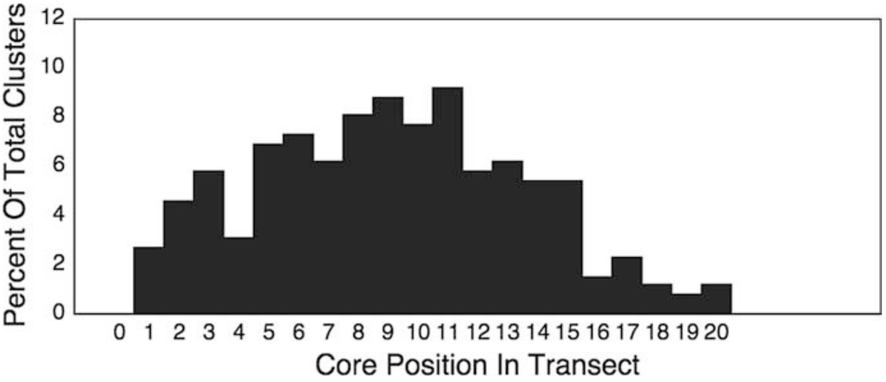

Fig. 6 Distribution of 260 *Limulus* egg clusters found in individual cores along core-sampled transects on Port Mahon, Pickering, Kitts Hummock, North Bowers, Mispillion Inlet, and Slaughter beaches in May, June and July, 2002 through 2005. Transects spanned 85% of the distance from nocturnal high tide wrack line to the tidal shelf, and were sampled with 20 cores. Cores were kept evenly, and proportionally, spaced across the varying beach widths by use of marked bungee cord lines which could be stretched to fit all beaches. Of those clusters, 76.9% (200) were found in the 5th through 15th cores (25–75% of the transect length). The zero value on the Core Position axis approximates the 0–5% position on Fig. 5. This offset is necessary because core sample dates were approximately midway between dates of the somewhat higher lunar tides when spawning is most intense. The tidal flat would be off the chart, to the *right*

5.4 Egg Cluster Size

Cluster size data appear in Table 3. After separating eggs from sediment, we found there was little uniformity in the amounts of sediment present in clusters, which agrees with the observations of Shuster and Botton (1985). For the total

Table 3 Summary of *Limulus* egg cluster size and sediment data. All eggs were counted by hand. Sediment was air dried, then weighed on a scale with 0.1 gm resolution

			Eggs per cluster			Sediment per cluster (gm)		
Beach	Year	Clusters	Minimum	Maximum	Mean ± SD	Minimum	Maximum	Mean ± SD
Port Mahon	2003	14	2,524	16,835	5,744 ± 3,599	7.5	68.7	31 ± 15.4
Pickering Beach	2005	12	2,587	9,158	5,836 ± 1,714	7.1	95.0	40 ± 24.8
For total sample		26	2,524	16,835	5,786 ± 2,834	7.1	95.0	34.8 ± 20.4

sample, air-dried sediment weights per cluster ranged from 7.1 g to 95 g (mean 34.8 ± 20.4 g SD). These sediment weights ranged from 33–352% (mean 157% ±73.7% SD) of damp egg weight. Sediment particle sizes present in the clusters were representative of larger particles in the surrounding sediment. Minimum numbers of eggs per cluster (2,524 eggs in 2003; 2,587 eggs in 2005) and average number of eggs per cluster (5,744 eggs in 2003; 5,836 eggs in 2005) were similar in both samples, but maximum numbers were quite different (16,835 eggs in 2003; 9,158 eggs in 2005) (Table 2). The average number of eggs we found per cluster (5,786 ± 2,834 SD) for the entire sample is higher than has been previously reported for Delaware Bay beaches by Hummon et al. (1976) and Shuster and Botton (1985).

5.5 Beach Temperature and Egg Development

Mid-beach sediment temperatures in early May ranged from 13.7 to 24.2°C (mean = 17.0° ± 3.0°C SD); in early June from 16.4 to 29.7°C (mean = 21.6° ± 3.3°C SD); and in early July from 22.4 to 30.4°C (mean = 26.8° ± 1.9°C SD). Temperatures did not differ appreciably between beaches on any given day. These values represent the highest temperatures to which developing eggs are exposed and exist for only a few hours during the diurnal low tide. The average temperatures at which eggs develop are somewhat lower than these values, due to the twice-daily flooding with much cooler bay water.

The first few trilobite larvae (<25 larvae per beach sampled) normally began to appear in our sediment samples during the first week in June, suggesting that about 35 days are required for development of the earliest eggs in a normal spring. These were larvae of the current season, not larvae that had overwintered (Botton et al. 1992), because larvae did not show up in our earlier spring samples.

Although we normally began to find the first larvae in samples taken during the first week of June, the date of their appearance in some years was retarded or advanced by prevailing weather. For example, May 2003 was unusually cool and overcast. As a result, average daily bay water temperatures stayed below 15°C (Smith and Michels 2006), spawning was delayed, and the first larvae did not appear in our samples until 7 July – a month later than normal. By contrast, May 2004 was unusually warm, water temperatures rose above 15°C early in May, spawning began earlier than normal (Smith and Michels 2006), and the first larvae appeared in our samples on 26 May, about 2 weeks earlier than normal. Thus, the date the first larvae of the season appear can vary widely between years.

6 Summary and Conclusions

On the western shore Delaware Bay beaches, we sampled for *Limulus* egg clusters during studies reported here, we found the average depth to center of recently laid egg clusters was 15 ± 3.5 cm SD ($n = 533$ clusters). Cluster thickness (top to bottom) ranged from 0.6 to 7.6 cm, with an average thickness of 3.5 ± 1.1 cm SD. Most clusters (98.1%; 523 clusters) were $\geq$2 cm in thickness. Centers of 88.0% (469) of all clusters were within 20 cm of undisturbed beach surface, and centers of all egg clusters were within 25.5 cm of undisturbed beach surface. It is not necessary for a core sampler to reach to the center of a cluster for the cluster to be represented in a core. In our core sampling study, firm aggregations of eggs which did not pass through a 1.3 cm mesh were used as indication that the core included part of a cluster. Using that same criterion for the 533 clusters found in this study would mean that a 20 cm deep core could be expected to reach and sample 98% of the clusters present in an undisturbed beach, i.e., all clusters $\geq$2 cm in thickness.

All egg clusters on all beaches ($n = 6{,}132$ clusters in 80 transects) were found in the upper 85% of beach foreshores (the span between the high tide line and the beach break at the beginning of the tidal flat), with 90.9% (5,588) of the clusters within the 25–75% part of that span, even though foreshore widths varied. We then tested this 85% coverage of the upper foreshore using core-sampled transects, adding three beaches which had not been included in the original sample. In transects spanning only the top 85% of the foreshore, sampling with 20 evenly spaced core samples per transect produced a cluster distribution similar to that observed when using continuous trenches to sample the entire foreshore width. We found 76.9% (200) of all core-sampled clusters between 25 and 75% of the transect length ($n = 260$ clusters). These results suggest that taking evenly spaced core samples across the upper foreshore can adequately sample egg cluster density. The results also suggest that female *Limulus* place their egg clusters only in the upper 85% of western Delaware Bay beach foreshores at current population abundance, even though beach widths may vary. Thus it would seem reasonable for future studies investigating egg cluster densities to index the sampling protocol to either the spring high water level, or the nocturnal high tide wrack line, and sample downward across the foreshore, 85% of the distance to the tidal flat, or the low water level if there is no tidal flat exposed at low tide.

Intact egg clusters ranged in size from 2,524 to 16,835 eggs with an average of 5,786 ± 2,834 SD eggs per cluster ($n = 26$). Minimum numbers of eggs per cluster (2,524 in 2003; 2,587 in 2005) and average number of eggs per cluster (5,744 in 2003; 5,836 in 2005) were similar in both years. The average number of eggs per intact cluster (5,786 ± 2,834 SD) is higher than previously reported for Delaware Bay by Shuster and Botton (1985). Extrapolations to obtain estimates of total egg numbers from counted egg clusters should take this into account.

Beach sediment temperature measured at mid-beach, and at 20 cm depth, averaged 17.0° ± 3.0°C SD in early May, 21.6° ± 3.3°C SD in early June, and 26.8° ± 1.9°C SD in early July. The earliest trilobite larvae usually appeared in samples taken during the first week of June; however, prolonged cool weather caused larvae to appear a month later in 2003, while unseasonably warm weather caused them to appear 2 weeks earlier than normal in 2004. These differences appeared to be due to both to the date at which the earliest eggs were laid and to temperatures within beach sediments where the eggs were located. Early warming of the bay causes spawning to begin earlier than normal (Smith and Michels 2006), and the warmer beach sediments accelerate larval development; delayed warming produces the opposite effects.

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Comparisons in Prosomal Width and Body Weight Among Early Instar Stages of Malaysian Horseshoe Crabs, *Carcinoscorpius rotundicauda* and *Tachypleus gigas* in the Laboratory

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Abstract The three Southeast Asian horseshoe crab species are diminishing not only locally but also regionally and protection of them is now an urgent matter. Two species of horseshoe crab, *Tachypleus gigas* and *Carcinoscorpius rotundicauda*, were artificially inseminated, and the eggs were incubated at 28±1°C and in the salinity of 33±2 ppt. The fertilized eggs hatched after 42 days and 41 days with hatching rates of 98.1 and 98.9% for *T. gigas* and *C. rotundicauda,* respectively. This study reveals that in the identical laboratory condition, *C. rotudicauda* underwent more frequent molting than *T. gigas*. After 328 days of rearing, 63.8 and 22.9% of *C. rotundicauda* larvae had molted to the 6th and 7th instars, respectively, while 56.6 and 20.1% of *T. gigas* at the end of 355 days of rearing had molted to the 5th and 6th instars, respectively, but only 0.6% had molted to the 7th instar. There is a wide variation in the molting rate among larvae obtained from synchronized inseminated eggs and reared under uniform laboratory conditions.

1 Introduction

In contrast to American horseshoe crab, *Limulus polyphemus*, which spawns in the spring (Brockmann 2003), the species found in peninsular Malaysia, *Carcinoscorpius rotundicauda* and *Tachypleus gigas*, migrate to the shore to lay eggs each full moon through out the year (Hajeb et al. 2005). Pairs of horseshoe crabs in amplexus (male clasping the opisthosoma of the female) typically come ashore with the high tides, and females deposit eggs in multiple small clutches in nests 10–20 cm deep in the sand. As the eggs are laid, they are fertilized externally by the male in amplexus (Leschen et al. 2006). The eggs develop in the sand for 2–4 weeks through four embryonic molts, hatching into “trilobite” larvae. The non-feeding larvae remain in their clusters in the sand for several

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additional weeks until the next tidal inundation when they swim into the sea. Within a few weeks depending on temperature, the free-swimming trilobite larvae molt into tiny, spiny juvenile horseshoe crabs which live on the near-shore sand flats (Brockmann 2003).

The mating behavior of *T. tridentatus* (except for the absence of satellite male) was reported to be fundamentally similar to *L. polyphemus*, despite more than 100 million years of isolation between two groups (Botton et al. 1996). Within the first year, *L. polyphemus* molted to the 6th instar larvae, while *T. tridentatus* molted to the 2nd instar stage (Sekiguchi 1988). The relationship between the sizes of different body parts of the first four instar stages of *C. rotundicuda* and *T. gigas* was reported by Sekiguchi (1988), but further information about later instars of these two species is incomplete. This study was conducted to measure the relationship between body size (width of prosoma) and body weight of later instar stages of Malaysian native species, *C. rotundicuda* and *T. gigas*, in the laboratory.

2 Materials and Methods

Adult males and females of *T. gigas* and *C. rotundicauda* were caught from Pulau Lumut, Selangor, Malaysia, and were kept in 34–35 ppt aerated seawater before artificially inseminating their eggs. Five females were dissected and their eggs were pooled after being removed from their ovary. The eggs were washed with filtered seawater several times to clear their surface from internal fluid, since it has been suggested to have inhibitory effect on fertilization (Sekiguchi 1988).

The eggs were fertilized by sperm collected from the male genital pore using a 1-ml syringe, which was diluted with seawater to make a 10% (v/v) sperm solution, following methods of Ehlinger and Tankersley (2004). A sample of 250 and 400 eggs from the pooled fertilized eggs of *T. gigas* and *C. rotundicauda*, respectively, were used for study. The fertilized eggs were incubated at 28°C in complete darkness to simulate their nests in nature. The water was changed every 12 h similar to the tidal conditions. The instar larvae were reared in plastic trays (17 × 35 cm) and fed with *Artemia* nauplii. Immediately after hatching/ molting, the instar larvae were weighed and their prosomal widths measured with digital calipers. The rearing period under similar conditions for *C. rotundicauda* and *T. gigas* was 328 and 355 days, respectively.

3 Results

Fertilization rates were 19.2 and 14.8% for *C. rotundicauda* and *T. gigas*, respectively. The first trilobite larvae of *C. rotundicauda* hatched after 41 days of incubation (Table 1); within 9 days after first hatching, 98% of the eggs were hatched (i.e., from 41 to 50 days post-fertilization). The first *T. gigas* eggs hatched one day later on the 42nd day of incubation, and 98% of the eggs hatched within 20 days after the first egg hatched (i.e., <62 days post-fertilization). After rearing

Table 1 Comparison of hatching and molting periods, earliest and latest days of hatching and molting, and final distribution of instar larvae of *Tachypleus gigas* and *Carcinoscorpius rotundicauda*

	T. gigas instars							*C. rotundicauda* instars						
	1st	2nd	3rd	4th	5th	6th	7th	1st	2nd	3rd	4th	5th	6th	7th
Days of larval rearing/incubation in which 50% of eggs/larvae hatched or molted	47	84	127	193	254	–	–	45	87	122	160	209	269	–
Days of larval rearing/incubation in which 75% of eggs/larvae hatched or molted	50	87	142	208	297	–	–	48	92	128	174	220	279	–
First day of hatching/molting	42	73	104	146	195	239	332	41	67	99	130	161	191	326
Last day of hatching/molting	64	182	238	337	339	344		55	147	180	260	282	316	–
Existing instar larvae after rearing period (%)	0	0	1.89	20.75	56.6	20.13	0.63	0	0	0	2.76	10.55	63.76	22.93

for 355 days, the dominant instar stage for *T. gigas* was the 5th instar, which included 56.6% of the total larvae. The slowest growing larvae were 3rd instars (1.9% of total larvae), and the fastest growing larvae were 7th instars (0.6% of total larvae). After rearing for 328 days, most (63.7%) of the *C. rotundicauda* were 6th instars. The slowest growing larvae were 4th instars (2.8% of total larvae), and the fastest growing larvae were 7th instars (22.9% of total larvae).

Total mortality during the rearing period (from the date of 1st instar hatching to the end of rearing period) was 34.5 and 39.9% for *T. gigas* and *C. rotundicauda*, respectively. About 22% of the 1st instar larvae of *T. gigas*, which were not able to molt to the 2nd instar, remained in the same instar stage for about 170 days of rearing period and then died. By comparison, only 0.8% of the 1st instars of *C. rotundicauda* failed to molt. The frequency of developmental abnormalities in *T. gigas* was noticeably less than *C. rotundicauda*. Sources of mortality included overfeeding, which accounted for 8.8% of mortality in the *C. rotundicauda*, and inability to molt due to their abnormality, which accounted for 6.6% occurred among 1st instar larvae.

After each molt, body size and weight increased in *C. rotundicauda* and *T. gigas*, but the growth increment was not constant across stages. The incremental growth of body weight in *T. gigas* was 131% from the 1st instar to the 2nd, smaller (109%) between the 2nd and 3rd instar, and relatively constant (132–135%) over the next instars (Fig. 1, Table 2). The incremental growth in body weight in *C. rotundicauda* was 71% from the 1st to the 2nd instar, but it was much higher thereafter (Fig. 1, Table 2). The incremental growth in prosomal width in the *T. gigas* was greatest between the 1st and 2nd instar and then decreased slightly between the next three instars. In *C. rotundicauda,* the incremental growth in prosomal was similar (34–40%) across all stages (Fig. 2 and Table 2). The body weights in both species were highly correlated with prosoma widths (Fig. 3).

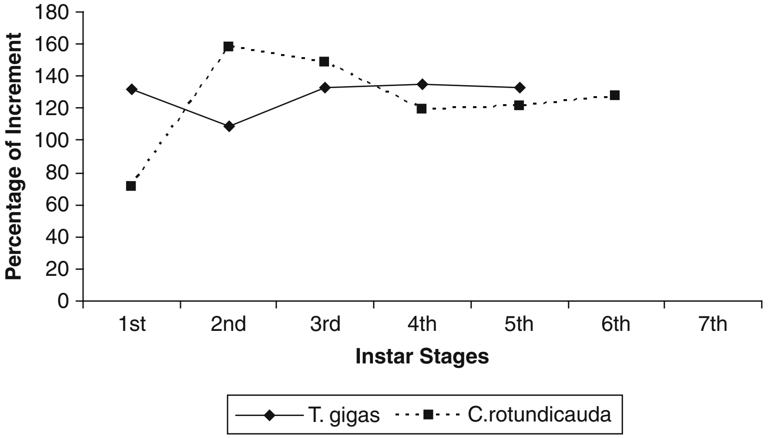

Fig. 1 Comparison between increment of body weight of early instar larvae in *T. gigas* and *C. rotundicauda*

Table 2 Prosomal width (PW), incremental growth in PW (IPW), weight, and incremental growth in weight (IW) for early instars of *Tachypleus gigas and Carcinosorpius rotundicauda*. Means and standard deviations are shown for PW and weight

Instar	*T. gigas*				*C. rotundicauda*			
	PW (mm)	IPW (%)	Weight (g)	IW (%)	PW (mm)	IPW (%)	Weight (g)	IW (%)
1st	6.98 ± 0.26	55.7	0.035 ± 0.00	131.4	3.67 ± 0.13	36.0	0.007 ± 0.00	71.4
2nd	10.87 ± 0.58	41.6	0.081 ± 0.08	108.6	5.00 ± 0.23	39.5	0.012 ± 0.00	158.3
3rd	15.39 ± 0.60	36.0	0.169 ± 0.02	133.1	6.98 ± 0.34	35.0	0.031 ± 0.00	148.4
4th	20.93 ± 0.77	34.0	0.394 ± 0.05	134.8	9.42 ± 0.80	33.6	0.077 ± 0.02	119.5
5th	28.04 ± 1.04	31.4	0.925 ± 0.11	132.8	12.59 ± 0.84	35.6	0.169± 0.04	121.3
6th	36.85 ± 1.51	–	2.153 ± 0.25	–	17.08 ± 1.42	37.0	0.374 ± 0.09	127.8
7th	–	–	–	–	23.39 ± 2.51	–	0.852 ± 0.22	–

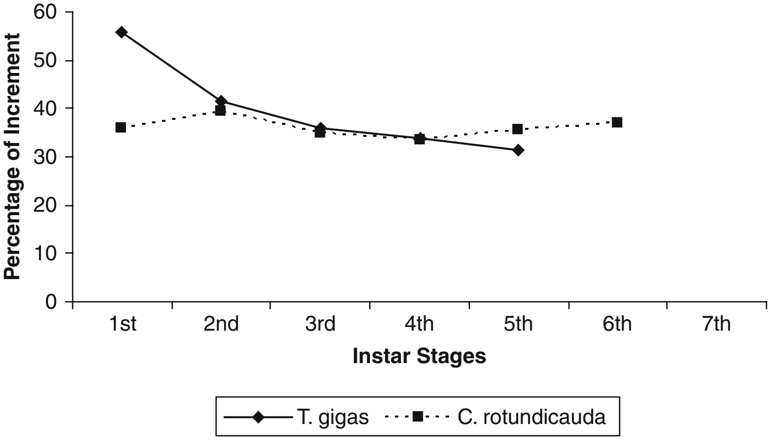

Fig. 2 Comparison between increment of prosomal width of early instar larvae in *T. gigas* and *C. rotundicauda*

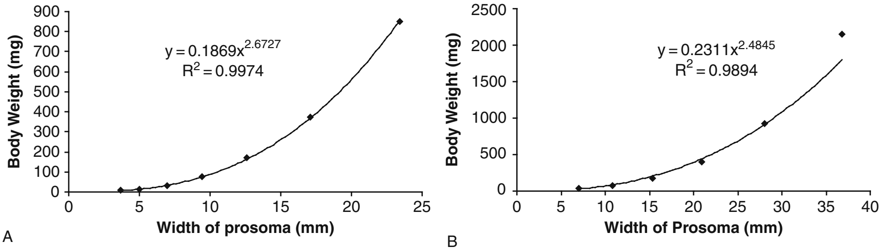

Fig. 3 (**A**) Relationship between body weight and prosomal width of *C. rotundicauda* in early instar larvae. (**B**) Relationship between body weight and prosomal width of *T. gigas* in early instar larvae

4 Discussion

During a rearing period of 328 days, *C. rotundicauda* larvae molted six times to reach the 7th instar (approximately 1 molt/1.5 month). This is in contrast with Chiu and Morton (2001), which assumed one growth stage in first year and three growth stages in the second year for this species. During a rearing period of 355 days, *T. gigas* larvae molted five times to reach the 6th instar (Table 1). According to Sekiguchi (1988), *L. polyphemus* and *T. tridentatus* molted to the 6th and 2nd instars within a year, respectively. The prosomal width of *C. rotundicauda* for the first six instars in this study (Table 2) was noticeably smaller than those reported by Sekiguchi (1988), or by applying the calculated growth rate factor (1.26) suggested by Chiu and Morton (2001). These differences may be due to the intraspecific and population differences, but it is interesting that the difference in the prosomal width is compensated in the 7th instar. The prosomal widths of first two instars of *T. gigas* in this study were smaller than those reported by Sekiguchi (1988), but it was compensated in the next stages.

The average prosomal width increments between the first six instars of *T. gigas* and seven instars of *C. rotundicauda* were 39.7 and 36.1%, respectively. This is greater than 24.20 and 24.00% reported for *C. rotundicauda* and *T. tridentatus* by Lee and Morton (2005) in Hong Kong but closer to the 38.5% reported by Yeh (1999) for *T. tridentatus* in Taiwan. Although Lee and Morton (2005) suggested that the differences in the prosomal width increment may be attributed to the latitudinal differences in the habitat, there is a possibility that it may be due to the age of pre-molt individual, which was mentioned earlier by Shuster (1954) and Carmichael et al. (2003) for *L. polyphemus* and Kawahara (1982) for *T. tridentatus*.

The relationship between prosomal width and wet weight of *C. rotundicauda* and *T. gigas* indicated that the weight gain in both experimental species is faster than the increment in carapace width (Fig. 3). This agrees with reports for different locations and species (Lee and Morton 2005; Chiu and Morton 2001; Chatterji et al. 1988; Chatterji 1994).

About 22 and 0.8% of *T. gigas* and *C. routundcauda* larvae, respectively, remained alive in the 1st instar stage and did not molt for 170 days before being dead in the same instar stage. Botton et al. (1992) reported delayed molting in *L. polyphemus* larvae that did not emerge from the sandy nests overwintered without any molting. Although the occurrence of morphologically abnormal larvae was higher in *C. rotundicauda*, the inability of 1st instar *T. gigas* to molt to the next stage might be a type of hidden abnormality. Because we used contaminant-free seawater in the laboratory, the occurrence of abnormal embryos was unexpected. However, the region where our adults were collected is not a conserved area, and it is possible that there was some transfer of contaminations during gametogenesis.

Although the eggs of *T. gigas* and *C. rotundicauda* were fertilized in the laboratory and incubated and reared in identical conditions, at the conclusion of the experiment, there were *T. gigas* larvae ranging from 3rd to 7th instar and *C. rotundicauda* ranging from 4th to 7th instar. These larvae were all the same age, and presumably there was little genetic variation among them. The causes of this variability in developmental rate have important implications for aquaculture efforts aimed at restoring depleted populations of horseshoe crabs (e.g., Mishra 2009; Schreibman and Zarnoch 2009) and merit further study. In addition, the information about body weight and prosomal width in the laboratory obtained from two native species of Malaysia might be the initial critical step for their further ecological studies.

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Emergence Behavior of Juvenile *Tachypleus tridentatus* Under Simulated Tidal Conditions in the Laboratory and at Two Different Sediment Temperatures

Christine N. Lee and Brian Morton

Abstract Previous local studies estimated that the densities of emerged, feeding, juvenile *Tachypleus tridentatus* (prosomal width = 17.1–36.9 mm), obtained from surface counts at Ha Pak Nai, Hong Kong, varied from 4 individuals · 1,000 m^{-2} in summer (July–September) to 0 individuals · 1,000 m^{-2} in winter (December) 2002. To determine if such figures reflected true densities, juveniles were kept in tanks with sediment from the nursery ground at temperatures of between 15–20°C (winter) and 25–30°C (summer) under simulated tidal cycles. After a week's acclimation, their emergences were recorded, as was the depth of sediment to which they burrowed. No individuals emerged under imitated conditions of low tide at winter temperatures whereas 23% emerged at summer ones, indicating that sediment temperatures override circatidal activities when they fall below 20°C. The estimated abundance of juveniles on a nursery beach in the summer of 2002 should therefore be $4.16/0.23 = 18$ individuals · 1,000 m^{-2}. During the imitated low tide, nearly all juveniles, which did not emerge at the substratum surface, buried themselves to a depth of <3 cm, irrespective of sediment temperature. Our results also showed that only 5% of the tested juveniles, regardless of temperature, were ever identified above the substratum during high tides. Overall, the present study confirms that field estimations of juvenile *T. tridentatus* abundance should include temporal patterns because emergence varies with temperature and tidal state.

1 Introduction

Horseshoe crabs have been identified as indicator species of healthy and clean intertidal sandy beaches (Morton and Morton, 1983). Three species have been recorded from Hong Kong, that is, *Carcinoscorpius rotundicauda* (Latreille, 1802), *Tachypleus gigas* (Müller, 1785), and *Tachypleus tridentatus* (Leach, 1819) (Hill et al., 1978; Hill and Phillipps, 1981; Morton and Morton, 1983;

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Mikkelsen, 1988). In the wake of habitat destruction and commercial fishing, *T. gigas* is now believed to be locally extinct, whereas the former two species are now similarly at risk from various impacts upon their shrinking populations (Chiu and Morton, 1999a, b, 2003a; Morton and Lee, 2003).

Population studies are indispensable to determining the current plight of horseshoe crab species in Hong Kong. Contrary to anecdotal documentation of adults coming ashore to breed, systematic samplings of juveniles during receding tides at their nursery grounds have been conducted by Chiu and Morton (1999a) and Morton and Lee (2003). These two studies suggested that in Hong Kong, situated in the subtropical transition zone between the temperate Northern Pacific and tropical Indo-Pacific and, therefore, subject to the alternation of hot, humid summers and cold, dry winters, sediment temperature might serve as one of the critical local factors determining the emergence of juveniles from the sediment to feed (Zhou and Morton, 2004). Hence, this might also affect estimates of population numbers.

At Shui Hau, Chiu and Morton (1999a) recorded only six juvenile *T. tridentatus* per sampling trip in winter, as compared with 47 juvenile *T. tridentatus* per sampling trip at the same place in summer. Similarly, in Ha Pak Nai, Morton and Lee (2003) recorded no juvenile *T. tridentatus* at winter sediment temperatures of <20°C as compared with an average summer density of four juvenile *T. tridentatus*·1,000 m^{-2}. Although such temporal variations in abundance could be thought of as an indication of the summer spawning season, or of an offshore migration (e.g., Botton et al., 2003), these results also raise questions about whether different temperatures solicit various behaviors. The aims of the present study were thus to investigate and compare the emergence behavior of juvenile *T. tridentatus* at the times of low tides at temperatures of 25–30 and 15–20°C, reflecting summer and winter sediment temperatures in Hong Kong, respectively.

2 Methods

2.1 Experimental Aquaria

Sets of aquaria comprised a small tank (40 × 40 × 40 cm^3) contained within a bigger one (55 × 55 × 55 cm^3) (Fig. 1). The bigger tank essentially acted as a seawater storage for the smaller one. The smaller, inner, tank had holes drilled along the upper portion of its walls so as to share its water volume with the bigger, outer, one. The bottom also had a perforated platform to allow aeration of the contained sediment from beneath. A 10-cm layer of sand, excavated from a horseshoe crab nursery ground, was put onto the platform and changed every week. Two horizontal slits, each ~3-mm high and ~30-mm apart, were made on one wall of the inner tank. Each set of tanks also included a closed seawater circulation system with its own reservoir. Water temperatures of some sets of

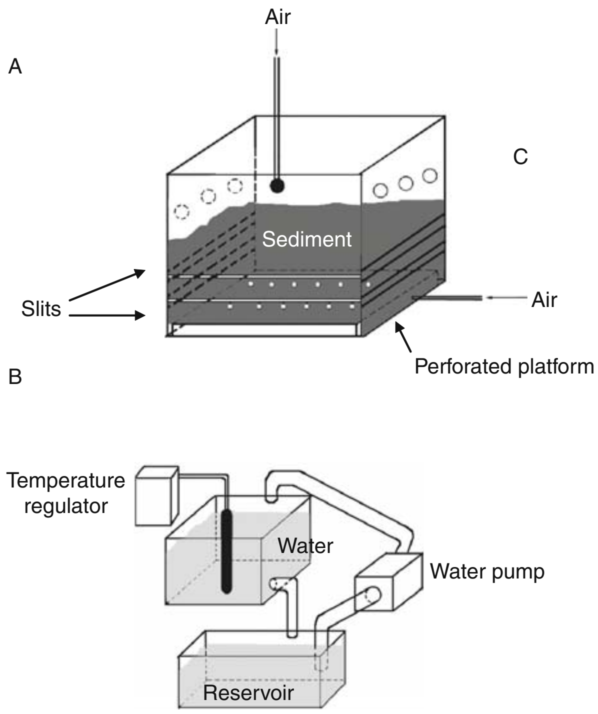

Fig. 1 The experimental setup for the study. (**A**) The inner aquarium; (**B**), the outer bigger one with a reservoir

tanks were maintained at 25–30°C to mimic summer conditions in Hong Kong, while remaining, winter, ones experienced temperatures between 15 and 20°C. The temperatures of the tanks were allowed to stabilize for 24 h before experimentation under the dark:light cycle of 12:12.

2.2 Acclimation Procedures

As it is generally believed that juvenile horseshoe crabs emerge from the sediment primarily to forage, 22 captive individuals used in this study, with prosomal widths ranging from 17.1 to 36.9 mm, were all fed with a regular supply of minced squid and prawn for 2 weeks at 25–30°C. They were then admitted to aquaria at either 15–20 or 25–30°C for a week's starvation, depending on the temperature category they were going to be experimented upon, that is, 15–20°C for the 11 individuals in winter tanks and 25–30°C for another 11 in the summer ones.

2.3 Experimental Procedures

Juveniles were placed in their own smaller, inner, tank with a plastic plate covering both the inner and outer tanks. After living in the experimental tank for 48 h, the emergence (or burrowing) behavior of each juvenile was recorded. Subsequently, water inside each smaller tank was drained off to expose the sediment surface. Further, the water level of the outer tank was lowered to slightly below the sediment surface of the inner one. This allowed the temperature regulator to continue to operate in the residual water of the outer tank, under the cover of the plastic plate over both the inner and outer tanks, so as to maintain the constant temperature of the sediment in the inner one. Ninety minutes later, the location (emerged or buried) of each juvenile was again recorded. If the juveniles were buried, the smaller tank was removed from the outer one and two plastic plates were inserted into the slits on its side to separate the sediment layers, as designed, into ~30-mm thick sections thereby halting the vertical movement of the horseshoe crabs inside the sediment. Each sediment layer was then removed to record how deep the horseshoe crabs had burrowed into it.

At the end of the experiment, the tested juveniles went through the same acclimation procedures again, that is, a week of being regularly fed before another week of starvation at the temperature at which they were experimented. The two groups of animals were then interchanged, that is, those experimented upon in the winter aquaria were put into the summer ones and vice versa. To investigate if the order of temperature treatments affected their burrowing behavior, the chi-square test with Yates' correction was employed.

To study whether or not juveniles would emerge from the sediment during every simulated low tide in their favored conditions, those which did so were, following re-acclimation, put into the same temperature aquaria once again in a repeat of the initial experiment, as described above.

3 Results

At simulated low tides, individuals 3, 8, 16, 18, and 20 (Table 1) emerged in the summer aquaria (representing 23% of the tested juveniles), whereas not one did in the winter ones. There was no statistically significant association between their emergence with the type of temperature tanks they first experienced ($\chi^2 = 0.020$; $P > 0.05$). All juveniles which buried themselves in the substratum in both summer and winter aquaria never did so below a depth of 30 mm, except for individual 5, which buried to a depth of between 30 and 60 mm in the latter one.

Under conditions of simulated high tides, only one of the 22 tested individuals was identified above the substrata of the aquaria under both summer and winter conditions ($\chi^2 = 0.095$; $P > 0.05$). Trails made by the juveniles under high-tide conditions, including the emerged ones, were identified more often in summer aquaria (6) than in winter ones (2), irrespective of the type of temperature tanks they first experienced ($\chi^2 = 0.069$; $P > 0.05$). This suggests a relatively higher level of activity in the summer aquaria.

Table 1 *Tachypleus tridentatus*. The emergence of tested juveniles from the substratum in the various experimental setups

Type of tank first experimented		Winter tank		Summer tank	
		Emergence at high tide	Emergence at low tide (depth to which it buried)	Emergence at high tide	Emergence at low tide (depth to which it buried)
Individuals first experimented upon in winter tank	1	No, but there was a trail	No (0–3 cm)	No	No (0–3 cm)
	2	No	No (0–3 cm)	No	No (0–3 cm)
	3	No	No (0–3 cm)	Yes	Yes
	4	No	No (0–3 cm)	No	No (0–3 cm)
	5	No	No (3–6 cm)	No, but there was a trail	No (0–3 cm)
	6	No	No (0–3 cm)	No	No (0–3 cm)
	7	No	No (0–3 cm)	No	No (0–3 cm)
	8	No	No (0–3 cm)	No, but there was a trail	Yes
	9	No	No (0–3 cm)	No	No (0–3 cm)
	10	No	No (0–3 cm)	No, but there was a trail	No (0–3 cm)
	11	Yes	No (0–3 cm)	No	No (0–3 cm)
Individuals first experimented upon in summer tank	12	No	No (0–3 cm)	No	No (0–3 cm)
	13	No	No (0–3 cm)	No	No (0–3 cm)
	14	No	No (0–3 cm)	No	No (0–3 cm)
	15	No	No (0–3 cm)	No	No (0–3 cm)
	16	No	No (0–3 cm)	No, but there was a trail	Yes
	17	No	No (0–3 cm)	No	No (0–3 cm)
	18	No	No (0–3 cm)	No, but there was a trail	Yes
	19	No	No (0–3 cm)	No	No (0–3 cm)
	20	No	No (0–3 cm)	No	Yes
	21	No	No (0–3 cm)	No	No (0–3 cm)
	22	No	No (0–3 cm)	No	No (0–3 cm)
Total number of emerged juveniles:		1	0	1	5

The five juveniles that emerged from the substratum after drainage in the summer aquaria (individuals 3, 8, 16, 18, and 20 in Table 1) were, following re-acclimation, put back separately into the same condition to repeat the experiment. None of these individuals, however, re-emerged either prior to or after drainage.

4 Discussion

Both Chiu and Morton (1999a; 2004) and Morton and Lee (2003) conducted samplings of juvenile horseshoe crabs at their nursery grounds in Hong Kong at approximately 3 h prior to the predicted time of spring low tides; juveniles are reported to be most active (Rudloe, 1979; Kawahara, 1982). The nursery grounds are so flat that, by this time, about half of the intertidal mud was already exposed. Within the exposed area, a majority of emerged, feeding, *T. tridentatus* were identified at 60 m down from the shoreline (Morton and Lee, 2003). The low estimated abundance of juvenile *T. tridentatus* in summer and virtual absence of emerged juveniles in winter in previous Hong Kong studies lead to a question regarding their behavior in response to sediment temperature.

In a behavioral study of *T. tridentatus* in Hong Kong, Chiu and Morton (2004) questioned whether the seasonal abundance pattern of emerged juveniles at their nursery grounds is a result of differential metabolic activities at various temperatures or a seasonal migration into subtidal areas. The virtual disappearance of *T. tridentatus* juveniles from other Asian nursery beaches in winter has been suggested to result from a declining aerobic metabolism under the influence of low temperatures, leading to a period of dormancy (Kawahara, 1982; Chiu and Morton, 1999a, 2004), as has also been asserted by Rudloe (1981) for juveniles of the American horseshoe crab, *Limulus polyphemus*. It is also known that juveniles can endure periods of starvation for as long as 1 year (Sekiguchi, 1988, Weng and Hong, 2001). Juvenile *L. polyphemus* have also been hypothesized to migrate to subtidal areas during the winter in Delaware Bay (Botton et al., 2003). The present study confirms that in Hong Kong *T. tridentatus* juveniles become inactive and remain buried in the substratum of their nursery ground under, simulated, winter temperatures. This is because more trails or emergence should have been observed if juveniles had retreated to deeper waters. The possible difference between the suggested behaviors of *L. polyphemus* and those of *T. tridentatus* in this study might be attributable to the much colder temperatures in Delaware Bay than in Hong Kong in winter.

Activities of *L. polyphemus* trilobite larvae have been shown to have an endogenous circatidal rhythm (Rudloe, 1981; Ehlinger and Tankersley, 2006). In such experiments, however, individuals were held concurrently under both winter and summer temperature conditions, with all other conditions comparable to each other, and still different behaviors were recorded. Our results thus demonstrate that sediment temperature might override such a rhythm when it falls below 20°C.

Even at summer temperatures, however, only 23% of juveniles of *T. tridentatus* became active and emerged from the substratum under simulated low tides. Similarly, of these, none re-emerged from the substratum at the next low tide. This is also known from our fieldwork in 2002. That is, juveniles do not emerge from the substratum on every receding tide, suggesting that such a behavior occurs only at the population level. A similar behavior is seen in adults, which do not come ashore to spawn on consecutive high tides (Sekiguchi, 1988). Such a lack of behavioral pattern might be key to the continued survival of horseshoe crabs over geological time. If all adults and juveniles emerged from the water or sediments, respectively, at a predictable time, the chances of mortality from predation, for example, would be higher. Extrapolating the 23% rate of juvenile *T. tridentatus* emergence from this study, the average density of juvenile *T. tridentatus* at Ha Pak Nai during the summer of 2002 by Morton and Lee (2003) is readjusted from 4.2 to 18 individuals$\cdot$1,000 m^{-2}.

Juvenile *T. tridentatus* burrowed to a depth of ~3 mm, irrespective of sediment temperature. This might be due to the possible occurrence of anoxic sediments at deeper levels, although the depth of the redox layer was not determined. They might also need to maintain a constant flow of seawater over their book gills that could be difficult to achieve if they have buried too deep into the substratum. Possibly, also, the juveniles might burrow for protection from predation. Identification of natural predators might provide more insights into such juvenile behavior. The actual reason behind this behavior requires further investigation.

Because of the high sediment load in the water column of the horseshoe crab nursery grounds at Ha Pak Nai (Kot and Hu, 1996; Chiu and Morton, 2003b), attempts to sample here during high-tide periods have never been successful. This leads to questions of whether individuals also emerge from the substratum when submerged. In this study, under simulated high-tide conditions at winter temperatures, however, most individuals not only buried themselves immediately but remained so; only two of the 22 experimental animals were being either seen on the surface of the substratum or had a trail identified. Conversely, 6 of 22 individuals (27%) were either recorded on the surface of the substratum or left a trail at simulated high tides at summer temperatures. Of the six occasions when juveniles were either emerged or produced trails before aquarium drainage (simulated high tide), two re-buried themselves until the end of the experiment. Such a disappearance at trail ends has also been sporadically encountered in the field (Morton and Lee, 2003; Chiu and Morton, 2004). It is unknown if the trails were made soon after the juveniles were put onto the substratum at the beginning of the experiment. Nevertheless, the number of trails seen before drainage approximated the number of emergences identified after drainage at the end of the experiment in summer aquaria. This suggests that juveniles could also occur above the substratum when they are covered by water at the time of high tide. Such an observation contrasts with the generalization that juveniles are active only during low-tide periods (Kawahara, 1982; Sekiguchi et al., 1988; Chatterji, 1994). Overall, the present study identifies the need to take the temperature-linked emergence behavior of juvenile horseshoe crabs into account when estimating their abundances at nursery beaches via surface counts.

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Distribution of Juvenile Horseshoe Crabs in Subtidal Habitats of Delaware Bay Using a Suction-Dredge Sampling Device

William H. Burton, Frederick S. Kelley, and Elizabeth A. Franks

Abstract Surveys of horseshoe crab juvenile distribution and abundance typically do not include shallow subtidal areas primarily because of the logistical difficulties with sampling in this habitat. A suction-dredge was constructed consisting of a "T"-shaped sampling head; an 8-hp trash pump, a 15.2-m-long non-collapsible hose-pipe; and a gunwale-mounted catch-basin to survey newly hatched juvenile horseshoe crabs in shallow subtidal habitats of lower Delaware Bay. Suction-dredge data were compared to catches taken in 4.9-m-small trawl collections at a series of 84 shallow water stations in 4 separate sampling events conducted in the summer and fall of 2004. Suction-dredge sampling for standard tow lengths of 15.2 m produced orders of magnitude higher catches compared to the trawls which were towed for an average of 109 m. The dredge was capable of sampling many stations in a single day and did not damage juveniles excessively. There was evidence for gradual bayward migrations of juvenile horseshoe crabs, as higher densities were found close to the spawning beaches in July, but progressively higher densities were found in offshore transects in August. Few juveniles were collected in September and October. This study demonstrated that juvenile horseshoe crabs were abundant in shallow subtidal areas of Delaware Bay, and that suction-dredge sampling could provide a valuable tool to monitor horseshoe crab populations.

1 Introduction

There is limited information on population dynamics and abundance of horseshoe crabs (*Limulus polyphemus*), as traditional population and harvest data have historically not been collected for this species (Walls et al. 2002). Some offshore trawling studies have been conducted (Botton and Ropes 1987, Hata and Berkson 2003), as well as spawning surveys and egg-count surveys (Smith et al. 2002, ASMFC 1998). However, juvenile horseshoe crabs may be missed in

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these studies, due to the unique habitats they occupy. After emerging from egg clusters buried in the sand, juvenile horseshoe crabs occupy the intertidal zone and shallow subtidal areas where they undergo several molts before migrating to deeper waters (Rudloe 1981, Shuster 1982). Surveying the abundance of newly hatched, epibenthic horseshoe crabs in shallow water habitats of estuaries presents a unique challenge because traditional sampling devices such as small trawls are not very effective at collecting 5–40 mm prosoma width juvenile horseshoe crabs. Quadrat sampling in mud flats at low tide can only be conducted in very shallow water and only a small area can be effectively sampled. The distribution of juvenile horseshoe crabs in shallow water habitat and their seasonal use of these areas are not well known due to the difficulty with current sampling methods. In this chapter, we describe a suction-dredge sampler to survey for juvenile horseshoe crabs in shallow water habitats of Delaware Bay, and report on their distribution and abundance at selected beaches during summer and early fall of 2004.

2 Methods

A suction-dredge sampling device was constructed and tested in shallow nearshore habitats in lower Delaware Bay (Fig. 1). The dredge itself consisted of a “T”-shaped intake pipe assembly powered by an 8 hp centrifugal trash pump. The head-pipe was constructed from a 1 m length, 8 cm diameter PVC pipe, perforated with 20 intake holes of 2.5 cm diameter. The intake holes were arranged symmetrically in two rows on the wall of the pipe that contacted bottom substrate. The head-pipe was closed at each end by threaded caps, which could be removed in the field to facilitate cleaning. The head-pipe was joined at the center with a PVC pipe of the same dimension to form the “T”. To provide rigidity and strength, the entire assembly was attached to a delta-wing-shaped piece of 2-cm-thick plywood. The plywood base also allowed us to add weight (approximately 22 kg) to the head-pipe end of the dredge to keep it in close contact with the bottom. We used a non-collapsible hose-pipe, 15.2 m in length and 7.62 cm in diameter to connect the suction head to the centrifugal pump. A similar length of hose-pipe ran from the outlet of the pump around the bow of the sampling vessel and back to a catch-basin attached to the port gunwale of the research vessel. The catch-basin was constructed of plywood and measured 1.8 m in length, 0.6 m in height, and 0.5 m in depth. The bottom of the basin was fitted with hardware cloth of 0.4 cm mesh.

At higher boat speeds (4–5 knots) the delta wing kept the suction head on the surface, and with the pump running, allowed for flushing between fixed sampling stations. In operation, a target station was approached using the vessel’s global positioning system (GPS) while moving with the tide. The sampling vessel was then slowed to about 2 knots which allowed the dredge to sink to the bottom. When the suction head contacted the bottom, the outlet hose was

Fig. 1 Detail of the underside of the suction-dredge head and plywood delta wing

monitored for sediment discharge. As soon as the discharge turned sufficiently muddy, the outlet hose was directed into the catch-basin while a 15.2 m tag-line was deployed over the starboard side to standardize distance towed. At the end of the tow, the outlet hose was removed from the catch-basin; boat speed was increased to raise the dredge from the bottom. When the discharge became clear the dredge was ready for the next sampling station. All sampling was conducted during daylight hours.

Juvenile horseshoe crabs collected by the suction-dredge were sorted from material within the catch-basin and each crab was inspected for viability (many shell casts closely resembling live crabs were also collected). Up to 30 crabs were measured using calipers for prosoma width and all crabs were counted.

Within 2 or 3 days after finishing the suction-dredge survey, trawl sampling was conducted using a 5.2-m semi-balloon otter trawl with 3.2-cm stretch mesh in the wings and body, and 1.3-cm stretch mesh liner in the cod end. Trawl sampling was done primarily to characterize fish, blue crab, and adult horseshoe crab populations at our study sites but also collected 5–40 mm prosoma width juveniles. This is the same equipment used by the Delaware Division of

Fish and Wildlife in their annual Delaware Bay trawl surveys. Two-minute tows were conducted at each station. Beginning and ending coordinates were recorded to measure distance towed; tow length averaged 109 m among 336 trawls conducted during the study. Counts and prosomal widths of all 5–40-mm horseshoe crabs taken by the small trawl were recorded.

We conducted juvenile horseshoe crab surveys in Delaware Bay in nearshore habitats ranging in depths from 1 to 4 m adjacent to seven spawning beaches with the suction-dredge and the small trawl. Beaches surveyed in New Jersey included West Egg Island, East Egg Island, and East Point while the Delaware beaches included Kelly Island, Port Mahon, Kitts Hummock, and North Bowers Beach (Fig. 2). The surveys were conducted monthly from July to October 2004. Each survey area was divided into three transect corridors, positioned parallel to shore and approximately 0.2 nautical miles (0.4 km) in breadth. In order of proximity to shore, the corridors were defined as nearshore, mid-shore, and offshore habitats. Along each transect corridor, four station targets were positioned at equal distances of approximately 0.2 nautical miles (0.4 km) apart. In total, 12 stations were positioned at each beach. In each sampling event, 84 stations were surveyed using both suction-dredge and trawl methods (7 beaches × 3 transects × 4 stations).

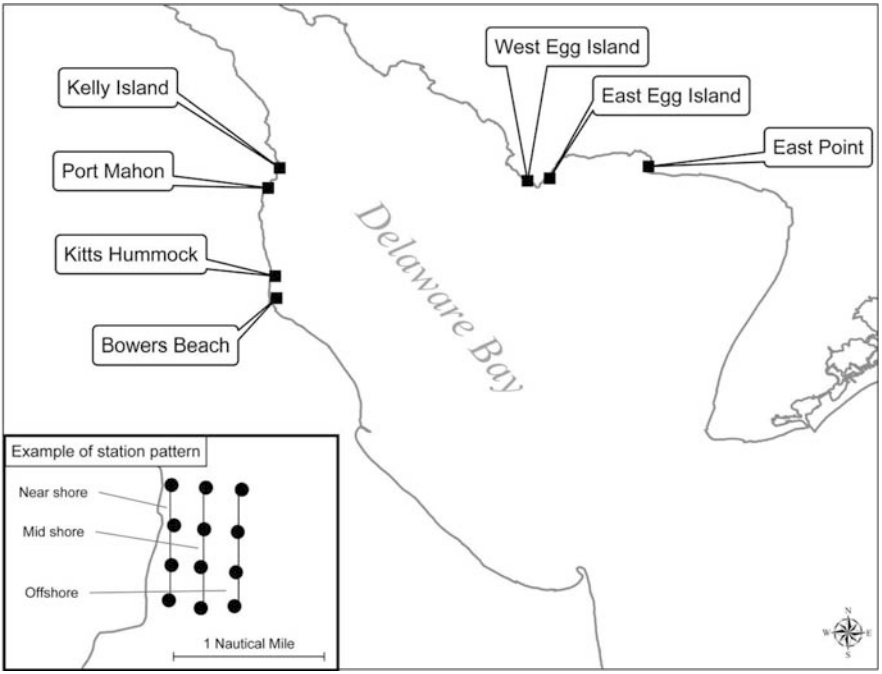


Fig. 2 Locations of the seven horseshoe crab spawning beaches surveyed for juveniles in the summer and fall of 2004 in Delaware Bay

Suction-dredge and trawl catches of juvenile horseshoe crabs were expressed in numbers per 100 m^2 of bottom sampled and compared to evaluate each gear's relative efficiency. Spatial and temporal movement patterns of juvenile horseshoe crabs were evaluated by combining the data from all seven beaches and calculating the average numbers of juvenile crabs taken by standard suction-dredge tow by transect and sampling month.

3 Results

The operation of the suction-dredged proved to be very effective. Close inspection of the suction-dredge catches of juvenile horseshoe crab revealed that very few specimens were damaged by the pump. Minimal clogging of the T sampler occurred except in bottom habitats where excessive filamentous bryozoans existed (generally in the higher salinity New Jersey beaches sampled).

On all four sampling events, the suction-dredge collected an order of magnitude more 5–40 mm juvenile horseshoe crabs relative to the small trawl (Table 1). Markedly higher densities were estimated from the suction-dredge which was towed for only 15.2 m relative to the small trawls that were towed an average of 109 m.

Densities of juvenile horseshoe crabs were highest in the nearshore transect in July relative to the mid-shore and offshore transects (Table 2). During August, fewer crabs were taken nearshore relative to the mid-shore and offshore transects. Suction-dredge sampling during September and October found few juveniles in any of the three transects, suggesting that the majority of juveniles had migrated out to deeper waters by September.

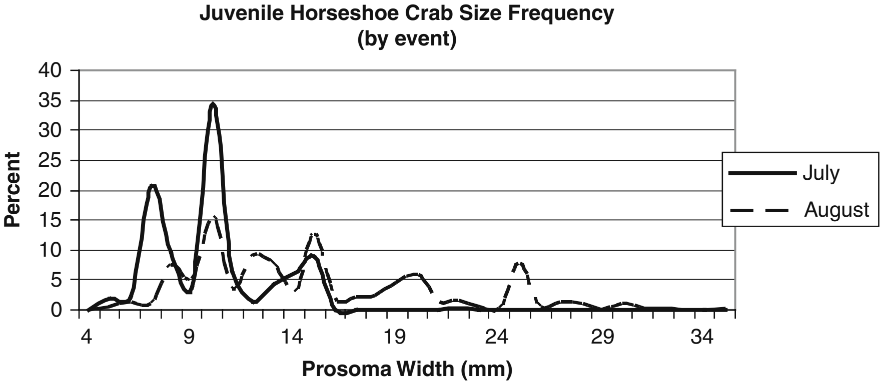
The juvenile horseshoe crab data from the suction-dredge collection were examined for prosoma width frequencies by sampling event for the combined catches taken in New Jersey and Delaware. Three potential cohorts were observed in the data, particularly during the months of July, September, and October (Fig. 3). In July, three peaks of 7-, 10-, and 15-mm-wide horseshoe crabs existed. This pattern was not observed in the August survey, but in the September and October survey three distinct prosomal widths existed in the size data at 13.5, 17, and 24 mm. The peak spawning migration in the Delaware Bay area generally occurs during the evening new and full moon tides in May and June (Shuster 1982, Shuster and Botton 1985). During May and June 2004, two new (May 19 and June 17) and two full moons (May 4 and June 3) occurred. These data suggest that three major spawning events occurred during these four moon phases. Separate size analyses for the New Jersey and Delaware data indicated that the tri-modal size frequencies were mostly a function of the Delaware collections.

Table 1 Average densities (SD) of horseshoe crab juveniles per 100 m^2 of bottom sampled among 12 stations ($n = 12$) sampled in the shallow nearshore habitats adjacent to seven spawning beaches sampled in Delaware Bay in 2004

	July		August		September		October	
	Dredge	Trawl	Dredge	Trawl	Dredge	Trawl	Dredge	Trawl
New Jersey Beaches								
West Egg Island	303.5 (795.7)	3.7 (4.1)	54.2 (47.0)	0.6 (1.0)	7.0 (13.8)	0.1 (0.1)	7.7 (13.6)	0.1 (0.3)
East Egg Island	73.6 (71.4)	6.4 (7.9)	58.8 (41.8)	0.9 (1.6)	7.7 (13.0)	0.02 (0.05)	5.4 (8.4)	0.1 (0.1)
East Point	10.1 (19.6)	0.3 (0.7)	4.6 (7.4)	0.2 (0.4)	0.8 (2.7)	0.02 (0.07)	2.3 (8.1)	0 .0 (0.0)
Delaware Beaches								
Kelly Island	240.0 (141.8)	6.4 (5.4)	68.9 (49.7)	0.2 (0.4)	12.4 (31.8)	0.0 (0.0)	1.6 (3.6)	0.0 (0.0)
Port Mahon	514.1 (273.7)	20.8 (12.6)	133.2 (44.5)	0.6 (0.5)	2.3 (8.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Kitts Hummock	493.2 (612.4)	5.4 (4.0)	538.1 (499)	2.4 (3.3)	90.6 (71.4)	0.1 (0.1)	65.0 (55.8)	0.5 (0.5)
Bowers Beach	329.0 (434.9)	5.5 (3.8)	581.4 (577)	9.7 (15.3)	125.4 (97.9)	0.0 (0.0)	154.1 (130.3)	0.2 (0.3)

Table 2 Mean numbers (SD) of horseshoe crab juveniles taken in 15.2 m tows with the suction-dredge sampler among all seven study beaches by transect and sampling event

Event	Nearshore (*n* = 28)	Mid-shore (*n* = 28)	Offshore (*n* = 28)
July	45.5 (69.4)	15.9 (19.7)	29.1 (40.1)
August	7.6 (12.8)	24.8 (35.1)	34.0 (53.6)
September	3.0 (5.4)	4.0 (6.9)	4.4 (8.9)
October	2.9 (4.8)	3.5 (6.3)	4.6 (11.7)



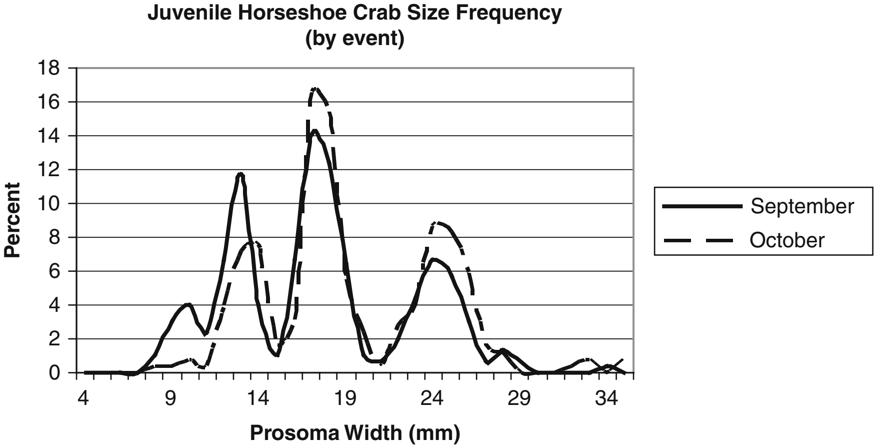


Fig. 3 Size frequency analysis of horseshoe crab prosomal widths for all suction-dredge collections (New Jersey and Delaware data combined) by sampling event

4 Discussion

The suction-dredge was much more effective at collecting juvenile horseshoe crabs relative to the small trawl. Although the total catches were much smaller in the trawl collections, the spatial trends among sampling beaches mirrored the dredge data suggesting that the small trawl was sensitive enough to characterize the relative abundances of juveniles among beach habitat. While the suction-dredge was clearly a more effective sampler, the gear efficiency is unknown but could be quantified by conducting a depletion experiment. By conducting multiple passes over the same sampling lane and monitoring the reduction in catch rate between passes, the dredge's catch efficiency could be calculated. The sampling efficiency could then be applied to survey data to estimate the total population size in a given area.

Subtidal densities of juvenile horseshoe crabs (per 100 m^2 of bottom) obtained through suction-dredge sampling were greater than those obtained in the small trawl and were also much greater than densities estimated in other studies for juvenile Asian horseshoe crabs – *Tachypleus tridentatus* in the Philippines and *Carcinoscorpius rotundicauda* in Singapore (Almendral and Schoppe 2005, Hong 2004). Almendral and Schoppe (2005) estimated 1.47 juvenile horseshoe crabs *(T. tridentatus)* per 100 m^2 in suitable substrata by walking the intertidal zone at Aventura Beach, Palawan, Philippines. Based on data for juvenile horseshoe crabs (*C. rotundicauda*) in Singapore generated by Hong (2004), densities of about 2.05 individuals per 100 m^2 were estimated. Juvenile densities found in our study often exceeded the 158.1/100 m^2 juvenile *L. polyphemus* found through quadrat sampling in intertidal areas by Carmichael et al. (2003) in Pleasant Bay, Cape Cod, Massachusetts.

Suction-dredge sampling of newly hatched horseshoe crabs may be a better indicator of annual spawning success than adult counts conducted during the spring spawning events and post-spawning surveys of egg masses deposited on the beach. Although the data are not presented in this chapter, our survey included quantitative sampling of egg masses on the subject beaches and there was a positive correlation between egg densities and densities of horseshoe crab juveniles sampled by the suction-dredge. Many external factors can alter the numbers of horseshoe crab juveniles that successfully hatch in a given year including storm events that can erode buried egg masses, desiccation, annual changes in the intensity of predation by shorebirds and gulls, and fertilization success. By the time the newly hatched horseshoe crabs reach 5–40 mm prosomal widths their chances of reaching adult stages is greatly increased and hence may be a better life stage to annually monitor for fisheries management needs. Monitoring juvenile abundance in shallow water habitats could also provide an index to future recruitment into the adult population.

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Part II
Conservation

Part IIA
Commercial Use and Management of Populations and Habitat

Fig. 2a Stacks of horseshoe crabs awaiting processing into fertilizer in the 1920's (courtesy Delaware State Archives)

History of Horseshoe Crab Harvest on Delaware Bay

Gary Kreamer and Stewart Michels

Abstract Horseshoe crab (*Limulus polyphemus*) harvest on Delaware Bay is not a new event. Early settlers to the New World reported use of horseshoe crabs by Native Americans for food, tools, and to enrich soils for growing crops. Literature from the mid- to late 1800s documents the use of horseshoe crabs for fertilizer and to supplement livestock feed. By the 1870s and for almost a century thereafter, well over a million crabs were harvested annually from Delaware Bay, in support of a regionally significant "cancerine" (fertilizer) industry. Subsequent to the cessation of the cancerine industry in the mid-twentieth century, relatively low-scale use of horseshoe crabs as bait for American eel and other fisheries existed. This use exploded in the 1990s, as eel markets expanded and use of horseshoe crabs for bait in a rapidly emerging whelk (*Busycon* spp.) pot fishery intensified along the East Coast of the United States. With horseshoe crabs spawning in mass along the shores of Delaware Bay, and little or no regulations in place, harvest pressure once again approached levels of the fertilizer use days. Simultaneously, an ongoing need for bleeding of horseshoe crabs to provide *Limulus* amebocyte lysate (LAL) for biomedical use, and growing recognition of the importance of horseshoe crab eggs on Delaware Bay as a key stopover food source for migratory shorebirds prompted concerns about observed declines in the population, resulting in implementation of significant management measures to ensure the sustainability of the species.

1 Introduction

The harvest and use of the American horseshoe crab, *Limulus polyphemus*, on Delaware Bay, is not a recent phenomenon. In this chapter, we will summarize aspects of that harvest relative to three phases, each corresponding roughly to a

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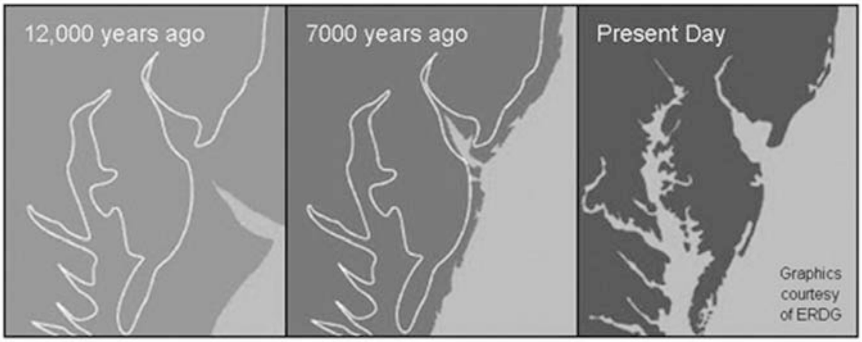
particular time period and focus of usage: (1) use by indigenous inhabitants of the bay area for food and tools in the centuries prior to European settlement; (2) agricultural (fertilizer and livestock feed) use by European settlers in the 1800s and 1900s; and (3) concentrated harvest of crabs for bait and biomedical applications during the last several decades.

Before exploring those aspects, it is important to put into context the relative timeframe for horseshoe crab/human interactions on Delaware Bay. Although Delaware Bay is clearly the current global hotspot for horseshoe crab spawning and fossil evidence shows that horseshoe crabs have been inhabiting the planet for more than 350 million years, these two facts are sometimes erroneously put together to imply that horseshoe crabs have been coming in mass into Delaware Bay to spawn for hundreds of millions of years. In fact, the Delaware Bay was formed less than 12,000 years ago after the last ice age (Kraft 1988), and development of the estuarine tidal nature of the Bay is thought to be less than 5,000 years old (Custer 1984; Fig. 1). Given these timeframes, it seems likely that the use of Delaware Bay for spawning by horseshoe crabs probably increased gradually over the last 3,000 or 4,000 years.



As the last ice age ends, Delaware Bay begins to form.

The Bay develops further as rising seas spread inland.

Sea level rise slows. Delaware Bay fills to present levels.

Fig. 1 The formation of Delaware Bay (graphics courtesy ERDG, www.horseshoecrab.org)

2 Native American Use of Horseshoe Crabs

We do not know precisely when humans began to use of horseshoe crabs in the Delaware Bay. Given the probable abundance and ease of harvest of spawning horseshoe crabs and the resourcefulness of native peoples in using what nature provided, it seems likely that the nomadic hunter-gatherers who inhabited the bay area in the millennia before European settlement (Custer 1984) found ways to use *Limulus* for food and tools. One can find numerous references in the literature to the use of horseshoe crabs by native peoples, including the use of carapaces to bail out canoes and the telsons for spear tips (Grant 2001; Shuster 2003; Hall 2007). However, because no such artifacts have been discovered and documented from archaeological digs in this region, it has not been proven that such uses existed or were prominent. Because these peoples produced no written history and horseshoe crab chitin would tend not to be preserved over such extended time periods, it is unlikely that direct evidence would be uncovered to support such uses.

Most of what we know about native use of *Limulus* has been extrapolated from sketchy accounts of early European chroniclers of the New World. One of the earliest references was in 1590 by English biologist Thomas Harriot who alluded to horseshoe crabs or "Seekanauk" (native American name for horseshoe crab) as *a kind of crusty shell fish which is good meat, about a foot in breadth, having a crusty tail, many legs like a crab, and her eyes in her back.* (Harriot 1972)

The artist John White, who accompanied Harriot as recorder, produced the scene shown in Fig. 2 illustrating native fishing methods. This piece includes the first known depiction of *Limulus* (see Fig. 2, bottom right). It also shows in the far background, natives fishing with spears, which Harriot (1972) describes in caption as follows: *They have a striking method of fishing in the rivers. For, since they lack iron and steel, they put the hollow tail of a certain fish like a sea crab onto their reeds or long rods for a point, with which at night or by day they spear fish and heap them up in their small boats.* According to certain interpretations of this passage (Feest 1978; Hulton 1984; Chartier 2006; www.virtualjamestown.org/images/white_debry_html/white.html), the "hollow tail" is the telson of horseshoe crab. However, since earlier in Harriot's writings he mentions, "sea crabs, such as we have in England," separately from his description of horseshoe crabs, it may well be that the spear tips he describes are not derived from horseshoe crabs at all, but from some form of crustacean (C. Blume, Delaware Division of Parks and Recreation, personal communication.).

Recently, we received a request from a Native American, Buddy "White Cloud" (Jett, of the Patawomeck Indian Tribe of Virginia, personal communication) for several horseshoe crab telsons, which he intends to mount on hardwood shafts "to recreate the type of fishing spears and gigs that our ancestors may have used." Mr. Jett made his request after reading Harriot's description of horseshoe crabs. We sent Mr. Jett several *Limulus* telsons for use in crafting spears, and we anxiously await the results of his efforts. Regardless of how that endeavor pans out, many questions remain to be resolved regarding traditional use of *Limulus* by native peoples.

Fig. 2 An illustration entitled "The Method of Fishing of the Inhabitants of Virginia" by John White for Harriot's 1590s writing (Hulton 1984). By Permission of The Manners' Museum, Newport, News, VA

3 Use of Horseshoe Crabs in Agriculture

By the time Europeans settled the Delaware Bay area, native people were supplementing what they fished, hunted, and gathered by growing their own food (Custer 1984). Samuel de Champlain, in his early 1600s explorations of the New World noted use of horseshoe crabs by native people living along the Maine coast to manure their corn crops (Morison 1972). According to Fowler (1946), Champlain also observed these natives using a hoe that was made from the shell of a horseshoe crab. Although not documented for Delaware Bay, it is speculated that natives there similarly learned to work dead horseshoe crabs into the soil to improve their crop yields and passed this knowledge on to the colonists (Hall 2007).

There is ample evidence that by the 1800s, farmers in the Delaware Bay area had adopted this practice (Shuster 2003). The early literature is replete with testimonials to the efficacy of this approach: "The dead bodies of the (crabs) themselves are hauled up in wagons for manure, and when placed at the hills of corn, in planting time, are said to enrich the soil, and add greatly to the increase of the crop." (Brewer 1840); "Mr. Springer of Dyer's Creek, with a compost of 7,000 crabs, 20 loads of muck, 2 coal-pit bottoms, 7 or 8 loads of hay, and manure applied on 6 acres of sandy loam, raised 151½ bushels of wheat." (Goode 1887); and "On land which would not grow wheat at all up to that time, crops of 20, 25 and even 30 bushels to the acre have been raised by the use of these crabs composted with earth" (Fowler 1908). Similar accounts of the use

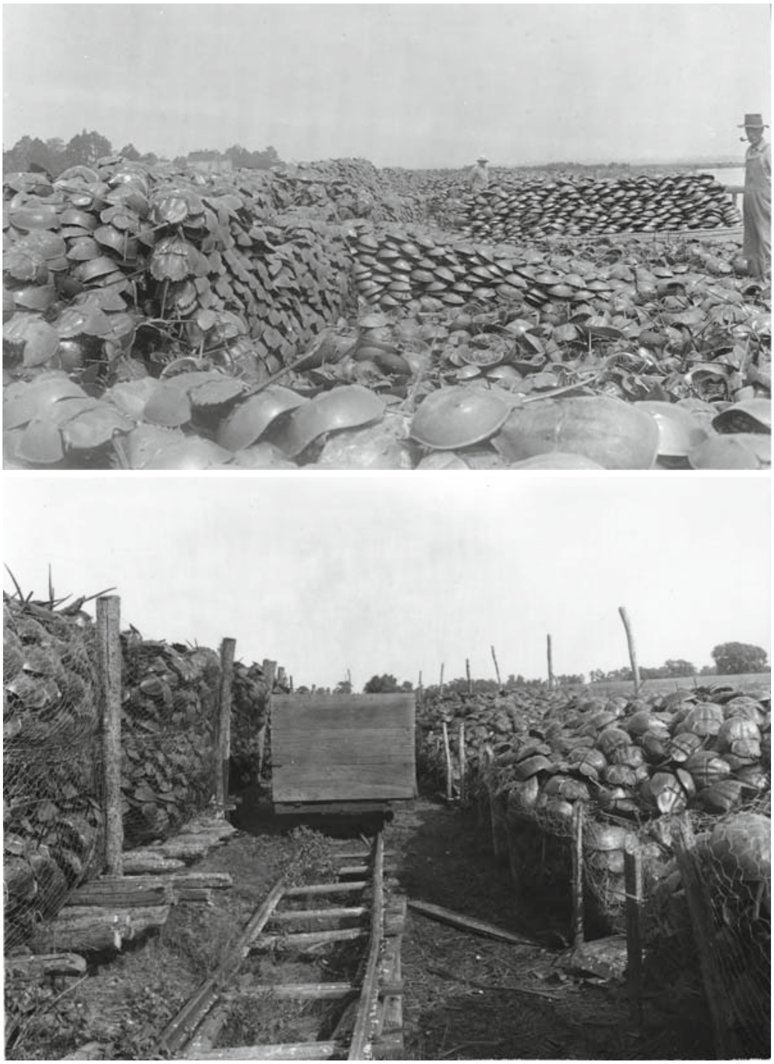


Fig. 3 "King Crabs" stockpiled to dry for fertilizer use near Bowers Beach, DE in the 1920s (photos courtesy of Delaware Public Archives)

of composted horseshoe crabs to fertilize other crops exist, including reference to their use in Delaware peach orchards during the mid-1800s (Shuster 2003).

It is also documented that farmers used a portion of the crabs gathered as food for livestock feed, either fed whole to hogs or made into a mash for chickens (Shuster 2003). Writings from that time also describe deposits of eggs so thick on bay beaches that farmers shoveled them up by the wagonload to use as chicken feed (Goode 1887).

Through the late 1800s and early 1900s, as more and more lands were cleared for farming, the need for more horseshoe crabs to fertilize the fields grew. Factories sprung up on both sides of the bay to process the masses of crabs harvested into a fertilizer meal. Because horseshoe crabs were thought to be crustaceans, the product was called “cancerine,” meaning “derived from crabs” (Shuster 2003).

The magnitude of horseshoe crabs harvested for agricultural use during that period of time is astonishing. In 1856, more than a million crabs were taken from a 1-mile stretch of New Jersey beach (Cook 1857), and in 1 year (1880), over 4 million crabs were harvested from the Bay (Smith 1891). As late as the 1920s, archival photos attest to the masses of crabs harvested and stockpiled for this purpose (Fig. 3).

Shuster (2003) describes the methods used to collect and process horseshoe crabs to make cancerine. Masses of crabs were collected effectively on the New

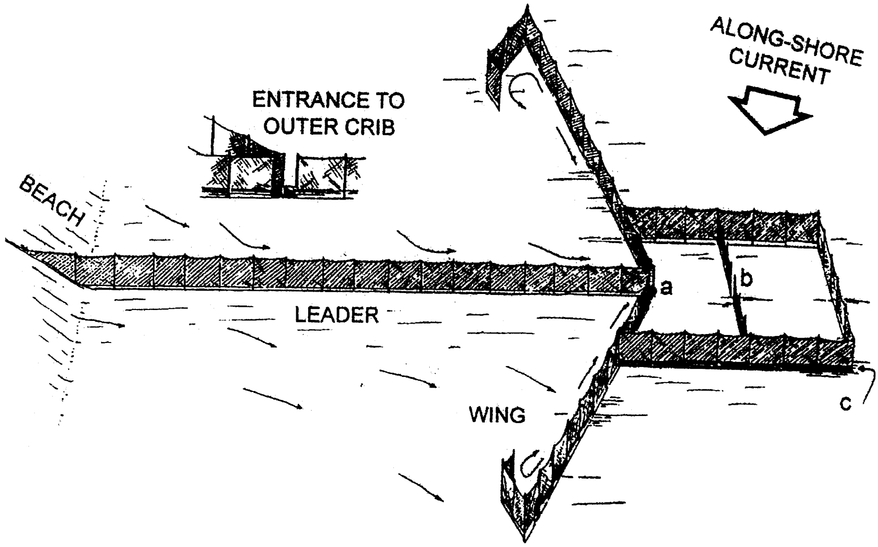


Fig. 4 The “pound” method of harvesting horseshoe crabs for fertilizer use in New Jersey. As the tide receded, crabs leaving the beach after spawning were guided by the leader and wings and trapped in the outer crib (illustration used with permission of Carl N. Shuster Jr.)

Jersey side of the bay by use of special arrays of wooden stakes called “pounds,” which were strategically placed in nearshore bay waters to direct and trap large numbers of crabs as they moved to and from the spawning beaches (Fig. 4; Smith 1891; Shuster 2001).

At low tide, crabs were removed by horse-drawn wagon or scow and transported to drying areas or directly to fertilizer plants (Figs. 5, 6). Inside these

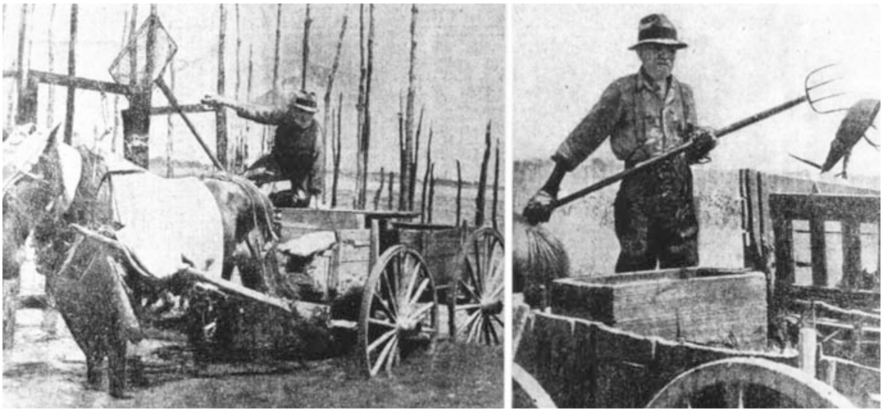


Fig. 5 In the 1800s, horse-drawn wagons were commonly used to remove horseshoe crabs from the spawning beaches (photo courtesy of Delaware Public Archives)

Fig. 6 “Cancerine” plant in Milford, DE (circa 1920s). Note the wagonload of crabs with telsons sticking up pulled up in front of the barn (photo courtesy of Delaware Public Archives)

plants were massive grinders for crushing the crabs and sometimes furnaces to steam them prior to turning them into the ground meal product that was ultimately bagged and sold to farmers for fertilizer (Shuster 2003). Over a million horseshoe crabs per year for over a half century were harvested from Delaware Bay for use as fertilizer (Fig. 7).

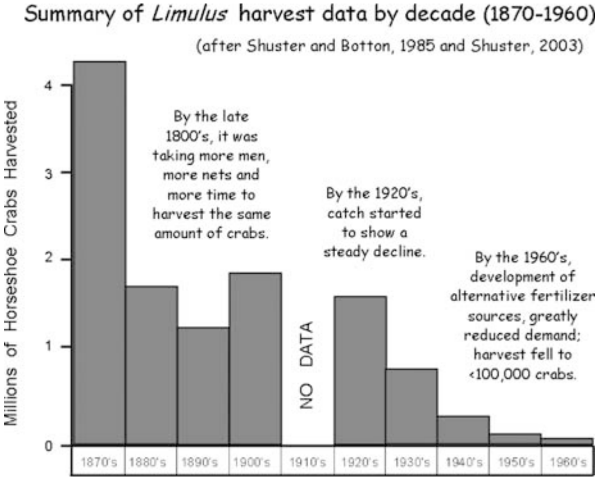


Fig. 7 A century of horseshoe crab harvest on Delaware Bay (1870s–1960s). Adapted from Shuster and Botton (1985)

By the late 1800s, there was evidence of declining populations, as it was taking more men, gear and time to harvest the levels of previous decades (Smith 1891). By the 1930s, harvest levels had declined dramatically (Shuster 2003). Fortunately by the 1960s, development of alternative fertilizer sources had offset the demand for using horseshoe crabs. By the 1970s, all cancerine plants in Delaware and New Jersey had either shut down or had been converted to other purposes (Shuster 2003). This provided the *Limulus* population on Delaware Bay with a brief chance to rebuild before the next wave of harvest pressure arrived.

Figure 8 provides a comparison of the economic value of past and present horseshoe crab harvest. Although not adjusted for inflation and other economic indicators, the value of horseshoe crabs has increased from the 1800s when a single crab was worth just a fraction of a penny to recent times when a crab is worth a dollar or more.

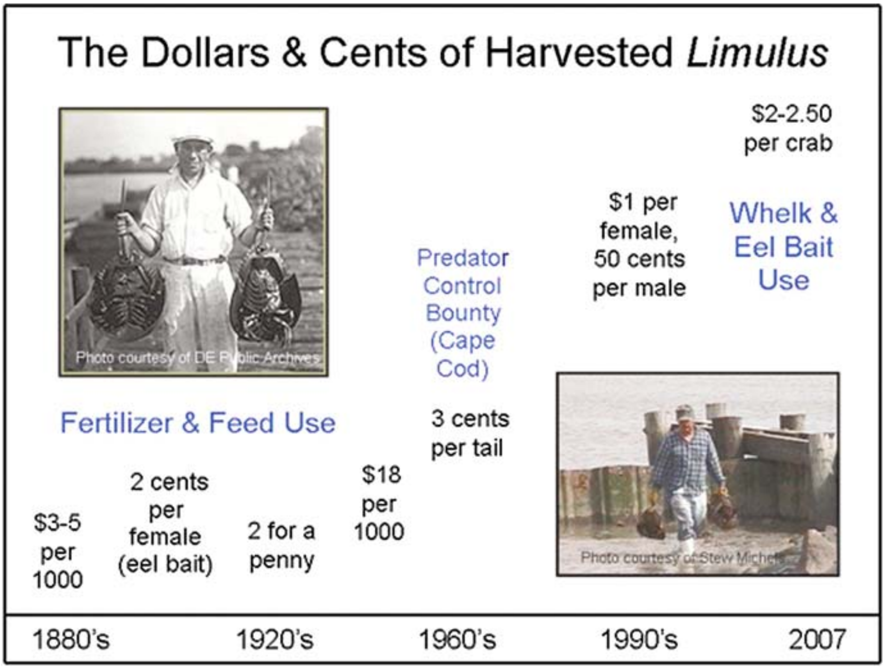


Fig. 8 A summary of data on the economic value of harvested horseshoe crabs from 1870s to the present (compiled from information in: Smith 1891 and Shuster 2003)

4 Recent Use of Horseshoe Crabs on Delaware Bay

Similar to horseshoe crab harvest in previous centuries, horseshoe crabs have continued to be used and valued in recent decades. In the 1990s, there was an explosion of use of horseshoe crabs as bait in coastwide whelk and eel fisheries and for biomedical applications. Horseshoe crabs have become important to a substantial and growing eco-tourism industry.

4.1 American Eel Pot Fishery

Horseshoe crabs serve as a primary bait source in the American eel (*Anguilla rostrata*) pot fishery in the Delaware Bay region. The use of horseshoe crabs for this purpose dates back to at least the 1800s when eels were landed to supply domestic food markets (Goode 1887). Eels landed in recent years have been used to supply domestic and international (Europe and Asia) food markets (Manion et al. 2000). Annual reported eel pot landings in the mid-Atlantic declined from the late 1980s to the present. However, this decline may not reflect a decreasing demand for horseshoe crabs as bait, as eel prices remained high (Fig. 9) (http://www.st.nmfs.noaa.gov/st1/commercial/). In the 1990s, eel

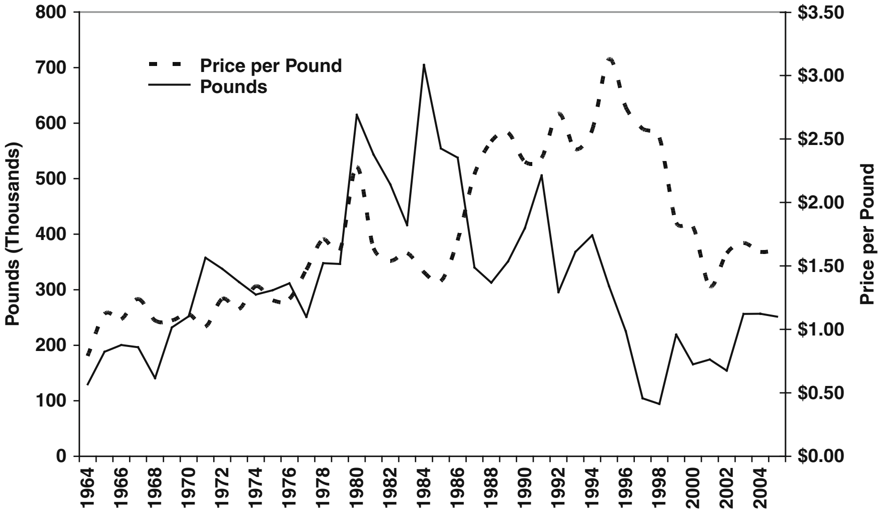


Fig. 9 Annual reported US mid-Atlantic American eel pot landings (thousand of pounds) and price per pound (exvessel value updated to 2007 dollars). Source: http://www.st.nmfs.noaa.gov/st1/commercial/

landings shifted to smaller eels to use as bait for recreational fishing of striped bass (*Morone saxatilis*).

Through the 1990s Delaware Bay eel potters typically used a half to a whole horseshoe crab to bait each pot (Munson 1998). The eel pot fishery in the Delaware Bay area almost exclusively uses female horseshoe crabs. Fishermen

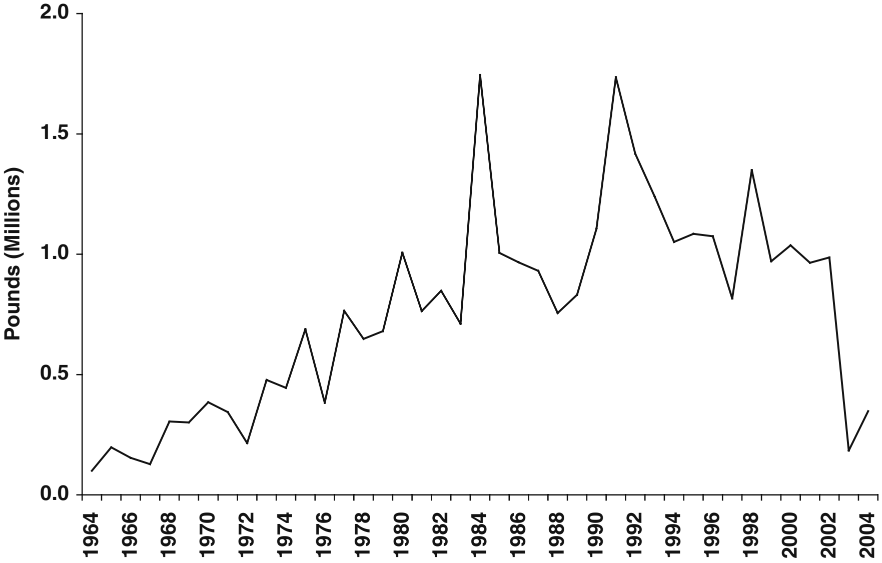


Fig. 10 Annual reported whelk (conch) pot landings (millions of pounds) for the US Atlantic Coast. Source: National Marine Fisheries Service

routinely report that horseshoe crabs are by far the best bait that can be used to pot eel, though many have tried alternatives, such as clam, shrimp heads, and cat food.

4.2 Whelk Pot Fishery

Horseshoe crabs are also used as bait in the Delaware Bay whelk (*Busycon* spp.) pot fishery. Whelk landings support domestic and international food markets. Domestically, whelk meat is used principally in ethnic markets in the Northeast United States, whereas international use is concentrated in Asia (Manion et al. 2000). Coastwide-reported whelk pot landings increased substantially in the mid-1980s and remained high through at least 2002 (Fig. 10) (http://www.st.nmfs.noaa.gov/st1/commercial/). Growth of this fishery was attributable to restrictions and declines in other fisheries, which forced fishers to find alternative sources of income.

The whelk pot fishery uses male and female horseshoe crabs, though female crabs are preferred. Through most of the 1990s, whelk pot fishermen typically used a whole female or two male horseshoe crabs to bait each pot.

4.3 Other Fisheries

Horseshoe crabs in the Delaware Bay were also used as bait in the catfish (Ictaluridae), minnow (Cyprinidae), and killifish (Cyprinodontidae) trap fisheries. There is no documented information on the extent to which horseshoe crabs are used in the catfish fishery, but use for this purpose is generally considered minimal relative to the eel and whelk pot fisheries. Munson (1998) reported that four minnow/killifish harvesters operating in NJ used an average of 4,125 horseshoe crabs annually. There were no similar records of horseshoe crab use in this fishery from Delaware, though there are anecdotal reports of individuals using horseshoe crabs for this purpose.

4.4 Biomedical Horseshoe Crab Fishery

Horseshoe crabs have been harvested from the Delaware Bay for use in the manufacture of *Limulus* amebocyte lysate (LAL) since 1982 (B. L. Swan, Limuli Laboratories, personal communication). LAL is used as the worldwide standard for detecting bacterial endotoxins in intravenous drug and medical devices. Horseshoe crabs are bled and returned to the water alive. There is some mortality associated with the harvesting, transport, and bleeding process. Reported mortality associated with bleeding has ranged from 2.1 to 15% (Swan 2001; Thompson 1999). There is only one biomedical bleeding facility located on the Delaware Bay and it is the smallest of the facilities coastwide. Biomedical landings specific to the Delaware Bay are not available due to confidentiality concerns; however, the use of horseshoe crabs for this purpose is annually monitored by the Atlantic States Marine Fisheries Commission (ASMFC). Coastwide mortality associated with bleeding horseshoe crabs has remained below 57,500 crabs since 1998 (ASMFC 2007).

4.5 Horseshoe Crab Bait Landings

Reported horseshoe crab landings increased in response to demand of the crabs for bait through the late 1990s (Fig. 11) (http://www.st.nmfs.noaa.gov/st1/commercial/). Though true increases are difficult to assess due to incomplete reporting, corroborating evidence suggests that a precipitous increase in landings occurred. For example, the number of horseshoe crab hand harvest permits issued in Delaware increased from 10 in 1991 to 132 by 1997 (Whitmore and Greco 2005). Based on estimates from the ASMFC, average Delaware Bay area (DE, NJ, MD, PA and VA) landings were nearly 2 million crabs annually for the years 1995–1997 (ASMFC 2007) (Fig. 12). Virginia landings were included in the estimates, as a large proportion of the Virginia's harvest occurred in federal waters just off the mouth of Delaware Bay until establishment of the Carl N. Shuster Sanctuary in 2001. Development of the Interstate Fishery Management Plan for Horseshoe Crab (FMP) and its subsequent addenda, the use of bait savings devices by American eel and whelk fishermen, and the establishment of the Horseshoe Crab Sanctuary reduced the horseshoe crab harvest in the Delaware Bay area to less than 500,000 crabs by 2004.

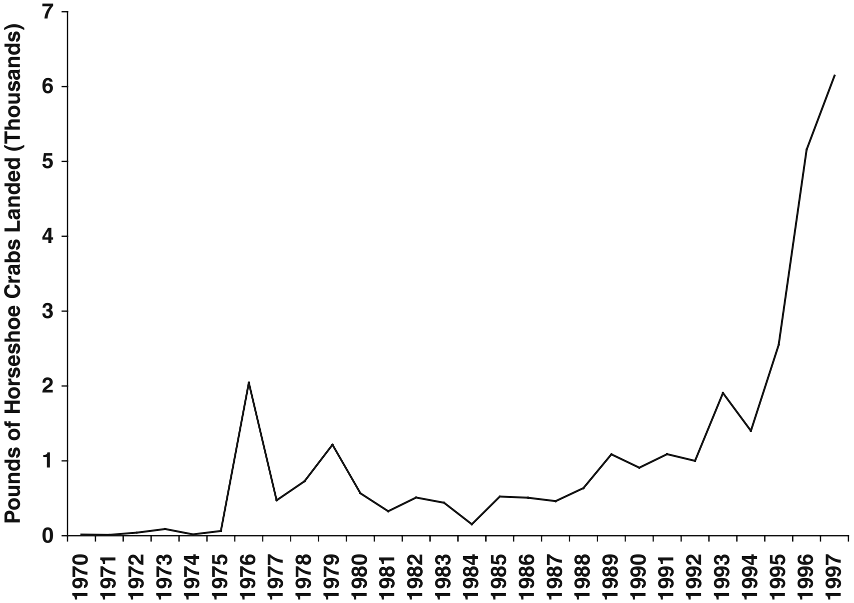


Fig. 11 Annual reported horseshoe crab landings (thousands of pounds) for the US Atlantic Coast. Source: National Marine Fisheries Service

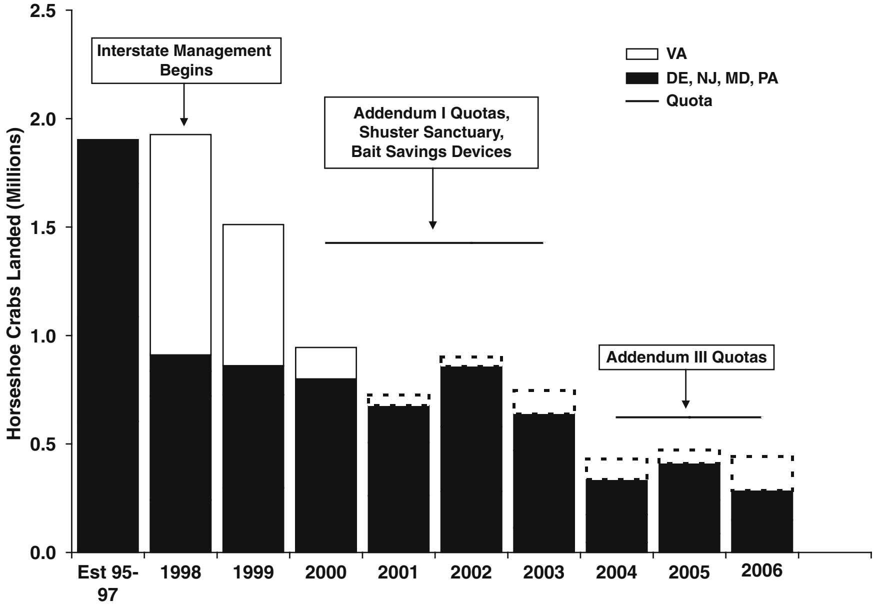


Fig. 12 Annual reported horseshoe crab landings (millions of crabs) from the states of DE, NJ, PA, and MD (*black bar*) and VA (*white bar*). Solid outline around white bar indicates a large proportion of these landings were Delaware Bay origin crabs; dashed outline around white bar indicates large proportion of landings from crabs not of Delaware Bay origin. Atlantic States Marine Fisheries Commission's mandated quota represented by solid horizontal line. Source: Atlantic States Marine Fisheries Commission

4.6 Ecotourism

An important non-consumptive use of the horseshoe crab comes in the form of ecotourism. The relationship between migratory shorebirds and horseshoe crab eggs in the Delaware Bay became well established by the early to mid-1980s. The crabs spawning and migratory shorebirds feeding upon their eggs attract birders, naturalists, researchers, and film crews to the Delaware Bay from around the world to view and document the spectacle. These visitors significantly contribute to local economies. Manion et al. (2000) estimated that expenditures related to horseshoe crab/migratory shorebird viewing contributed $6.8–$10.3 million per year to the economy of greater Cape May, NJ.

The birding and conservation communities have been the driving force behind horseshoe crab conservation efforts. Birding and conservation groups were largely responsible for the first horseshoe crab legislation aimed at protecting Delaware Bay horseshoe crabs in 1990 and they have played a prominent role in local and coastwide management since. Though there are indications that the Delaware Bay segment of the horseshoe population is stable or improving (Michels et al. 2007; Hata 2007), there continues to be a significant concern that current population levels are not sufficient to support migratory bird populations. Thus, precautionary horseshoe crab conservation efforts continue, primarily in the form of fishery regulations, but also including habitat protection and beach augmentation. It remains to be seen how the horseshoe crab population on Delaware Bay will respond in the long term to these conservation measures, and how this translates to future developments in the rich and growing history of human connection to and reliance on these animals.

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Biomedical Applications of Limulus Amebocyte Lysate

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Abstract This year celebrates the 30th anniversary of the licensing of *Limulus* amebocyte lysate (LAL) by the US Food and Drug Administration (FDA) as a test for the presence of endotoxin in biologicals, pharmaceutical drugs, and medical devices. LAL is currently recognized by several major pharmacopoeias and is used worldwide. That a suitable alternative for the detection of endotoxin has not supplanted LAL is indicative of its superior reliability. Since its discovery, LAL has proven its usefulness not only to detect harmful levels of endotoxin (as pyrogens) in pharmaceutical products, but has become an indispensable tool in controlling endotoxin in processes and equipment used to produce pharmaceuticals. Indeed, the exquisite sensitivity of LAL compared to other assays for endotoxin/pyrogen has proven extremely useful in monitoring high-purity water used as a prime ingredient or processing agent for all biologicals, drugs, and devices. LAL has also become the assay of choice for researchers studying both the clinical and the environmental effects of endotoxin. To highlight its usefulness, various specific applications of LAL including modifications of the assay to allow testing of complex substances will be described. Finally, although horseshoe crab mortality associated with LAL production is low, the LAL industry has taken steps to find a synthetic substitute and to produce reagents and methods that use much less LAL than traditional assays. That the horseshoe crab has uniquely contributed a test that profoundly affects the safety of pharmaceuticals should be celebrated and rewarded by continuing to protect this valuable resource.

1 Introduction

This year, 2007, marks the 30th anniversary of the licensing of *Limulus* amebocyte lysate (LAL) by the US Food and Drug Administration (FDA) as an alternative to the United States Pharmacopoeia's (USP) Pyrogen Test (PT)

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for endotoxin in human and veterinary drugs (US Department of Health and Human Services 1987). Since this approval, LAL has become accepted worldwide. Besides inclusion in the USP as the Bacterial Endotoxins Test (BET) (US Pharmacopoeia 1995), LAL test methods are also included in the European Pharmacopoeia (European Pharmacopoeia Commission 1987) and the Japanese Pharmacopoeia (The Pharmacopoeia of Japan 1992). Although the PT remains as an official test, in most cases the BET has completely replaced the PT, i.e., is the required test for certain drugs or is an acceptable alternative.

While LAL has been criticized as not being a true pyrogen test, i.e., there are other pyrogens (fever-causing agents), endotoxin has been shown to be the most common and most potent pyrogen and the one that is most likely (if not the only) pyrogen to contaminate injectable drugs and devices (Twohy et al. 1984). Thus, LAL and the horseshoe crab that is its source of manufacture have become invaluable to insure the safety of the world's pharmaceutical drug supply.

Currently LAL is commercially manufactured from amebocytes carefully harvested from the blood of the North American horseshoe crab, *Limulus polyphemus*, and the Asian horseshoe crab, *Tachypleus tridentatus* (Levin et al. 2003). LAL can also be obtained from the two other species, *Tachypleus gigas* and *Carcinoscorpius rotundicauda.* A synthetic version of LAL based on a recombinant form of the major endotoxin-reactive component of *C. rotundicauda* has been developed (Ding et al. 1999) and is commercially available (Lonza 2007).

This paper focuses mainly on the non-pharmaceutical applications of LAL. With the exception of a few specific examples to illustrate pharmaceutical use, general pharmaceutical applications and related regulations, LAL manufacture and commercialization, and clinical applications have been thoroughly reviewed elsewhere (Levin et al. 2003, Novitsky 1982a, Hochstein and Novitsky 2003, Novitsky 1994, 1996, 1999, Jorgensen 1986)

2 LAL Biochemistry

The biochemical basis of LAL is thought to play a key role in the horseshoe crab's (HSC) ability to ward off infection (Armstrong 2003). As the HSC does not have an immune system, as do the vertebrates, microbial defense is left to a humoral system. The mechanisms of action of the biochemical components that make up LAL originate in the HSC's amebocytes. These components not only recognize bacteria (those of the gram-negative type) but also fungi (those containing β-D-1,3-glucan) (Morita et al. 1981). One can only speculate that early HSCs, known to us through the fossil record, had a similar system and that the protection from infection it provided contributed to the HSC's survival through the millennia. Figure 1 shows *Mesolimulus walchi* (most likely a juvenile) and its tracks at the end of a journey some 150 million years ago when met its end, not to infection, but to stranding and burial in a tropical lagoon (Barthel 1990).

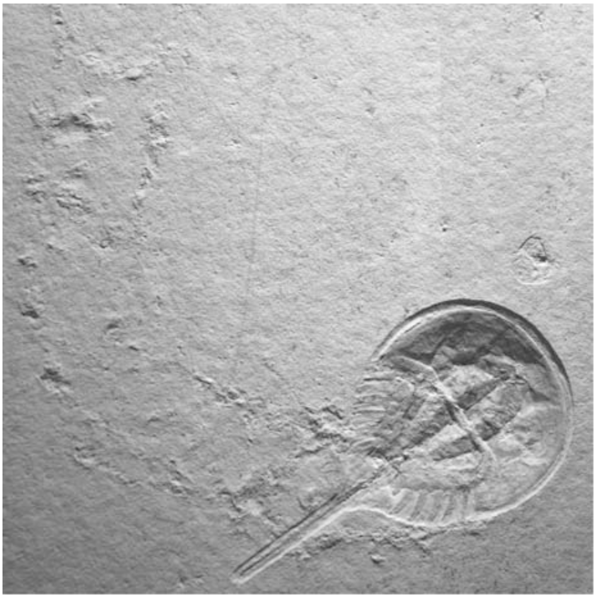

Fig. 1 Fossil of *Mesolimulus walchi* with tracks from Solnhofen Plattenkalk, Eichstätt, Bavaria, approximately 150 mya (prosomal width = 5.8 cm) *Photo by author*

To adequately understand the advantages and limitations of LAL, it is important to have at least a fundamental understanding of the biochemistry underlying the LAL test and to a certain extent, the chemistry of the substance detected by LAL – endotoxin. It should be noted that commercial LAL differs according to brand/manufacturer. Basically, all LAL is made from blood collected by "bleeding" adult HSCs of both sexes and separating the amebocytes from the plasma or hemolymph. The amebocytes are then broken or lysed to release the biochemical components that form the active ingredients of LAL. Differences in manufacturing occur in all steps. For example, some manufacturers use glass and stainless steel implements for blood collection, while others use plastic. Amebocytes can be lysed by suspending in distilled water, by alternately freezing and thawing, or by mechanical rupture. Various chemicals can also used to prevent the amebocytes from coagulating or from premature rupture during blood collection. Variation in manufacturing results in qualitative and quantitative differences between brands of LAL. This is especially evident when certain samples, usually of complex chemical composition, are tested with multiple brands. The consensus biochemistry of the LAL reaction is shown in Fig. 2 as are the various modifications to allow the assay to be read. The assay can be read in a variety of manners and once again the method of manufacture will differ for the type of assay chosen. The originally described LAL assay utilized the physiological "clotting" reaction. This assay is commonly referred to as the gel-clot test (Novitsky 1988). It basically is an end point test based on the highest dilution of sample that causes the LAL reagent to form a solid clot in a small test tube in a certain period of time at a fixed incubation temperature. A variation of this assay utilizes the turbidity or cloudiness that is formed leading up to clot formation as an end point. This type of assay is generally more sensitive and/or more rapid to perform than the gel-clot assay. In order to accurately read this test, however, a spectrophotometer is required. Finally, the component of the LAL that causes turbidity and subsequently a gel-clot to form, coagulogen, can be replaced by a synthetic peptide that contains the amino acid sequence that is cleaved by the clotting enzyme. This peptide also contains a chromogen (or fluorogen), i.e., a chemical that changes color (or fluorescence) when an adjacent chemical is modified. The LAL reagent that utilizes this synthetic peptide is referred to as the chromogenic assay. The chromogen most commonly used is para-nitroaniline which is colorless until the peptide is acted on and then turns yellow. A spectrophotometer using a wavelength appropriate to the chromogen's spectra is required. In one variation of this assay, the yellow dye is converted to its diazo derivative that is a deep purple. This later variation is useful for samples that have an inherent yellow color (Novitsky 1999).

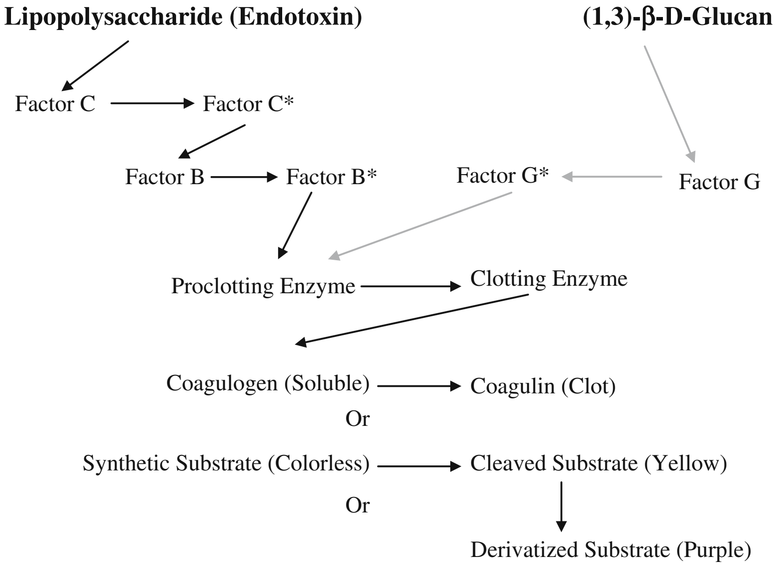

Fig. 2 Consensus biochemistry of the LAL reaction. Gray arrows denote alternate glucan-mediated pathway

3 Source and Chemistry of Endotoxin

Endotoxin is a component of the outer cell envelope of gram-negative bacteria. A more descriptive term for endotoxin is lipopolysaccharide (LPS). A generalized structure of LPS is shown in Fig. 3. Endotoxin or LPS occurs wherever bacterial contamination has occurred. Since LPS is refractory to degradation by most physical and chemical methods that would kill or remove bacteria, it can and does remain in solutions that are sterile. As water is an excellent growth environment for bacteria, especially gram-negative bacteria, water is typically the major source of endotoxin (Dawson et al. 1988). Since water serves as the major ingredient or processing component for injectable drugs, vaccines, and medical devices, it is not surprising that insuring water is as free of endotoxin as possible is a major concern of pharmaceutical manufacturers and testing of purified water with LAL constitutes its single largest use (Novitsky 1984, 1987).

O-antigen---Outer Core---Inner Core---Lipid A

O-antigen = Polysaccharide composed of repeating sugar sequences

Outer Core = Oligosaccharide

Inner Core = Heptoses + 2–keto-3-deoxy-octonic acid (KDO)

Lipid A = Phosphate + fatty acids

Fig. 3 Schematic of the general structure of lipopolysaccharide (endotoxin)

The toxic component of LPS and the component that causes LAL to react is the lipid A portion of the molecule (Fig. 3). Lipid A is highly conserved between different species of LPS, i.e., LPS from different species of bacteria, and because of this, equal weights of LPS from widely diverse species of bacteria react similarly. While this makes LAL a perfect general test for endotoxin, LAL cannot be used to differentiate between species.

LPS manifests its toxicity by causing a febrile (pyrogenic) response in animals when a sufficient amount is injected into the blood stream or cerebrospinal fluid. Hence LPS/endotoxin is also referred to as a pyrogen. At a high enough dose, LPS can be fatal. Fortunately, LPS can be tolerated in massive doses if ingested by healthy individuals. In fact, the normal bacterial flora of mammalian digestive systems is mainly gram-negative and is present in extremely great quantity. There is ample evidence, however, that inhaled endotoxin can cause pulmonary problems in susceptible individuals (Milton et al. 1987).

4 Pharmaceutical Applications

Soon after the discovery of LAL, the utility of this test to pharmaceuticals became apparent. LAL was demonstrated to be both a rapid and an accurate test for short-lived pharmaceuticals, i.e., radio drugs (Cooper et al. 1970). One of the authors, J. Cooper, then joined the Bureau of Biologics at the FDA and together with HD Hochstein helped start the FDA's program to investigate the feasibility of using LAL as an alternative to the PT for all pharmaceuticals and medical devices (Levin et al. 2003). The LAL test also quickly earned the distinction as a referee for disputed PT results. In one notable instance, the LAL test was used to prove that high levels of endotoxin in the swine flu vaccine introduced in 1976 was responsible for the adverse effects reported to the Centers for Disease Control (CDC) attributed to the vaccine (HD Hochstein, personal communication).

In the years following developments at the FDA and while clinical LAL studies continued to dominate most scientific interest LAL, a wide variety of LAL applications began to appear in the literature. The most notable of these are summarized in the following sections.

4.1 Purified Water

Since water is common to all drugs and devices, either as an integral component or as a processing agent (wash water), and since water is easily contaminated by gram-negative bacteria and hence endotoxin, it is not surprising that water is the substance that accounts for the largest number of LAL tests in the pharmaceutical industry. It is reasoned that if water contains endotoxin below a certain level, it will be unlikely to increase endotoxin in the final product that would render that product pyrogenic. The level as accepted by the USP and FDA is 0.25 Endotoxin Units (EU) ml^{-1} (a EU is equivalent to about 1 ng of purified endotoxin obtained from a strain of *Escherichia coli*). For comparison, ordinary bottled drinking water can easily contain several EU ml^{-1}. A special subset of water testing relates to renal dialysis. Although artificial kidneys (actual reverse osmosis filters) are tested with LAL as medical devices, they become contaminated during use and especially with reuse. In addition, the water used to prime the dialysis machine can add endotoxin to the unit if not closely monitored with LAL. Adverse patient reactions due to endotoxin have been widely documented and although monitoring with endotoxin is required, the dialysis industry has been slow to adopt this critical test (Novitsky 1982b).

4.2 Intravenous Drugs

Since endotoxin toxicity is manifested most commonly when injected into the blood stream, it is recognized that intravenous (IV) solutions should contain levels of endotoxin well below the pyrogenic dose. Although the water used to formulate IV drugs is required to already have sub-pyrogenic levels of endotoxin as determined by LAL testing, the final formulation and sometimes even the individual components are tested with LAL to insure the final IV solution (and its container) is within the allowed endotoxin limit. The endotoxin limit for IV drugs is slightly higher than water and based on the anticipated dose that will be used for the particular drug.

4.3 Biologicals

Biologicals include pharmaceuticals made from substances obtained from humans and animals, e.g., clotting factors and insulin. Biologicals also include vaccines which can contain bacterial, or animal components, e.g., from chicken eggs. It is recognized that gram-negative bacteria can easily contaminate biologicals and batches can contain large amounts of endotoxin. Fortunately, biologicals are usually given in relatively low doses and often administered by intramuscular injection. Even so, in 1976 following adverse reactions to a new batch of swine flu vaccine, in one of the earliest uses of the LAL test, it was determined that the batch of vaccine had a particularly high level of endotoxin that was responsible for the adverse effects. Antibiotics, while not strictly characterized as biologicals, also contain large and variable amounts of endotoxin since they have a microbial source (Case et al. 1983)

4.4 Medical Devices

Medical devices such as syringes, catheters, and needles are usually quite clean as a result of their method of manufacture. However, implanted devices, e.g., porcine heart valves, or orthopedic devices of rather complicated manufacture, can contain levels of endotoxin that cause localized inflammation and ultimate rejection of the device. In these cases, it is particularly important that the devices be checked by LAL. Generally the devices are rinsed with LAL-negative water and then the rinse tested. In some cases, the device itself can be immersed in the LAL reagent and the reaction of endotoxin that tightly adheres to the device can be measured. In this later case, the chromogenic version of the LAL test is used so that the reaction solution can be transferred to a reading device to score the test.

4.5 Recombinant Drugs

Relatively recently, i.e., sometime after the acceptance of LAL as an alternative to the PT, recombinant drugs were introduced. These are drugs produced through genetic engineering and are produced by bacteria, fungi, or mammalian cell culture. One can appreciate that recombinant drugs produced by the gram-negative bacterium *E. coli* would have a high potential of being contaminated with endotoxin from its producer organism. LAL is especially critical in drugs produced in this manner as it is used as a tool to follow the purification of the drug. Although the fungi (mainly yeast) and cultured mammalian cells used to produce certain drugs do not contain endotoxin per se, the media used for their growth can contain significant amounts of endotoxin that can subsequently contaminate the final product.

4.6 Stored Blood

Between 1987 and 1991, nine cases of red cell-associated *Yersinia enterocolitica* sepsis were reported to the CDC (Arduino et al. 1989). In some of these cases, it was determined through LAL analysis that most of the severe adverse effects (including seven deaths) noticed with patients who received transfusions of the contaminated red cells were not related to the infection per se but rather to the large amount of endotoxin associated with the contaminating bacteria. In one case, over 20,000 ng ml^{-1} of endotoxin was detected. Although it was hoped that LAL could be used routinely to screen stored red cells immediately prior to transfusion, sampling (i.e. removing a sample from the blood bag immediately prior to transfusion) without compromising the sterility of the unit(s), and sending a sample to the laboratory (versus bedside testing), made the LAL test impractical for this application. In addition, the incidence of infection was extremely low (43 million units collected between April 1987 and October of 1990 administered to 13 million patients resulted in nine reported cases and seven deaths). The problem was eventually solved by shortening the storage time for collected red blood cells. The shorter storage time did not allow potential contaminating bacteria to grow to levels (and produce sufficient endotoxin) to cause the problem.

5 Environmental Applications

5.1 Seawater and Marine Sediments

The oceans are teeming with bacteria. It was originally thought that in the marine environment, bacteria could only grow near the surface where there was abundant food, reasonable temperature, and low pressure. With the advent of the LAL test, it was shown that bacterial-associated endotoxin was present throughout the water column down to the deepest parts of the ocean, i.e., >4,000 m (Watson et al. 1977). Furthermore, endotoxin as determined by LAL was shown to highly correlate with bacterial number and even more so with bacterial biomass (carbon content). Thus, LAL provided a rapid, indirect measure of bacteria. The test was extended to study bacteria in sediments as well as in shore waters and was included in a benchmark survey of the Georges Bank to assess the potential impact of offshore drilling (Hobbie et al. 1987). Due to relatively high concentrations of nutrients in coastal waters where horseshoe crabs live and breed, large numbers, e.g., $>10^8$ bacteria ml^{-1} can occur. The HSC therefore lives in a veritable bacterial soup (Watson and Novitsky 1991). The same biochemistry that enabled scientists to measure the number of bacteria and amount of endotoxin in the water, also acts to protect the HSC in this environment.

5.2 Fresh Water

Gram-negative bacteria also occur in abundance in fresh water. Several studies have examined the utility of the LAL test to predict the portability of water assuming the total number of bacteria, indirectly determined from endotoxin content, would correlate with the presence of harmful bacteria or fecal contamination (Jorgensen et al. 1979). In one study, a high degree of correlation between the LAL test and the fecal coliform counts was demonstrated (Evans et al. 1978). This study, however, was a serendipitous occurrence and the LAL test has never gained acceptance as a test for potable water.

5.3 Air Quality

Several studies have shown that inhaled endotoxin can cause respiratory problems (Hasday et al. 1999, Milton et al. 1987, Sloyer et al. 2002). Environments most likely to manifest this type of problem are those where large amounts of aerosols containing gram-negative bacteria and endotoxin occur, e.g., metal working shops and sewage treatment plants (Sloyer et al. 2002). Another type of respiratory problem can be encountered in dusty environments. In cotton milling plants it has been shown that endotoxin associated with cotton dust is responsible for much of the respiratory problems encountered (Milton et al. 1987). The cotton dust/endotoxin association is interesting from an ecological standpoint. Prior to the cotton bole rupturing during the ripening process, the bole is essentially sterile. Once open, however, the raw cotton fibers are quickly colonized by gram-negative bacteria. Additional colonization occurs during processing and depending on the amount of moisture, nutrients, and temperature, large numbers of bacteria can be present along with their accompanying endotoxin. Even if the bacteria are destroyed during processing, the endotoxin usually remains tightly associated with the cotton fiber and can be released along with fine particles of cotton fiber during processing. Tobacco can also contain large amounts of adsorbed endotoxin, and one study employing LAL has shown that endotoxin released during cigarette smoking can cause respiratory problems (Hasday et al. 1999).

5.4 Endotoxin in Space

Recently a specially modified version of the LAL test was tested in space (Charles River Laboratories 2007). While providing on-the-spot results for the astronauts for their air and water systems, this was also good news for those of us back on earth in that the test design uses much less LAL reagent than other commercially available assays (Charles River Laboratories 2007).

6 Food Quality

It is well known that many types of food can be and are contaminated by gram-negative bacteria. As with water, studies have shown that presence of large amounts of endotoxin can be indicative bacterial contamination, either current or historical. This is especially true of meat. Thus, the presence of endotoxin was shown to correlate well with meat quality (Jay 1997). Likewise, the endotoxin content of fish has been shown to correlate with quality (Sullivan et al. 1983). While gram-negative bacteria are not the major source of milk spoilage, they are good indicators proper sanitization of the processing plant and of keeping quality of milk, especially for milk intended for ultrapasteurization (Mikolajcik and Brucker 1983). A modification of the LAL assay that uses a blue dye to more easily observe a gel-clot endpoint in microtiter plates was developed especially for analyzing milk (Mottar 1987).

7 Medical Research

7.1 Biological Effect(s) of Endotoxin

While one of the most obvious manifestations of endotoxin is a febrile response, reactions at the humoral and cellular level are manifold and complex. LAL has therefore become an indispensable tool for researchers studying the effects of endotoxin in mammalian models and to investigate the role of endotoxin in existing diseases (Romero et al. 1988, Warren et al. 1985, Novitsky et al. 1985).

7.2 Search for an Antiendotoxin Drug Therapy

In the study of gram-negative infection and sepsis, endotoxin plays a key role in the pathophysiological response of the host (Riveau et al. 1987). It has been postulated that if the adverse effect(s) of endotoxin could be abrogated, sepsis survival could be improved. Since the toxic portion of endotoxin (LPS), i.e., lipid A, is the same part of the LPS molecule that causes the LAL reaction, anti-endotoxin compounds could be screened with LAL prior to testing in animals (Novitsky et al. 1985, Warren et al. 1987a, b, c). Several studies have shown this to be an effective strategy and in fact an endotoxin-neutralizing compound with therapeutic potential was isolated from *Limulus* hemolymph and was studied extensively (Alpert et al. 1992, Garcia et al. 1994, Novitsky et al. 1996, Stack et al. 1997, Warren et al. 1992). A recombinant form of this protein was also constructed and found to be active (Andrä et al. 2004, Kuppermann et al. 1994, Nelson et al. 1995, Saladino et al. 1994, 1996a, b, Siber et al. 1993, Wainwright et al. 1990, Weiner et al. 1996).

8 LAL Reactivity to Fungal Glucan

8.1 Results of the Academic Medical Center Consortium (AMCC) Sepsis Study

While LAL is used in Japan as a clinical test for the presence of endotoxin in blood as one tool to diagnose sepsis, this is not allowed in the United States unless approved by the FDA. Part of the approval requires a clinical study to demonstrate utility. In 1988, LAL was included as part of the AMCC Sepsis study to see if there would be sufficient utility to license LAL as an aid in diagnosing sepsis. Prior to this trial numerous independent studies indicated the likelihood that LAL was a predictor of gram-negative sepsis. Unfortunately, under the conditions of this trial, no clinical utility could be demonstrated (Bates et al. 1998, Ketchum et al. 1997). An interesting result of the trial, however, was that a strong correlation was found when a patient was shown to have both a bacterial and a fungal infection (Ketchum et al. 1997). This finding, along with a growing literature indicating that the LAL reagent under certain conditions could also react with a fungal cell wall component, β-D-glucan, lead to more research into the feasibility of making an LAL test that was sensitive specifically to glucan.

8.2 Development of a Test for Fungal Infection

In 1988, Japanese investigators described an alternate pathway that resulted in the LAL reaction (Morita et al. 1981). This is shown in Fig. 2 (gray arrows). Numerous studies in Japan using the two different LAL formulations: one sensitive only to endotoxin and the other sensitive to both endotoxin and glucan proved the value of LAL in detecting fungal infections and eventually resulted in a fungal diagnostic (Miyazaki et al. 1992, Obayashi et al. 1995). Subsequent research resulted in an LAL reagent sensitive to only glucan, simplifying the assay. Although the glucan assay was already widely used in Japan, further development in the United States was necessary before the approval of Fungitell$^{\mathrm{TM}}$ by the FDA as an aid in the diagnosis of invasive fungal infection (Associates of Cape Cod, Inc. 2007). While not a universal test for fungal infection, the glucan assay has been reported to be especially useful for the early detection of *Aspergillus* and *Candida* infections (Associates of Cape Cod, Inc. 2007).

9 Conclusions

From the numerous examples presented, it is clear that LAL has a proven track record as an endotoxin test. As such, it is now considered the gold standard. Although the importance of LAL should justify its continued use for biomedical applications, especially as the major pyrogen test for pharmaceutical products, all of us concerned with the preservation of the HSC need to be vigilant and encourage the LAL industry to be a leader in conservation efforts (Novitsky 2001). To this end, the LAL industry has already made great strides in commercializing a recombinant-based LAL reagent and has introduced tests that use much less LAL than the original assay. While it is exciting to see a new health-related assay from the HSC – the fungal diagnostic based on the glucan pathway – this assay will undoubtedly put new pressures on the harvesting of HSCs. With increased awareness of its biomedical importance and attention to conservation to help ensure its survival, the HSC will continue to disclose life-saving secrets and help our understanding of healthy as well as disease-causing biochemical mechanisms.

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The Effect of Hemolymph Extraction Volume and Handling Stress on Horseshoe Crab Mortality

Lenka Hurton, Jim Berkson, and Stephen Smith

Abstract We evaluated mortality associated with hemolymph extraction of 0, 10, 20, 30, and 40% of estimated hemolymph volume among low-stress and high-stress groups of horseshoe crabs. In addition to bleeding, the high-stress group underwent simulated transport and handling procedures associated with the biomedical industry's bleeding process. Mortality rates of the unbled animals were not significantly different between the stressed and unstressed groups. Of the bled animals, there was a significantly higher mortality rate (8.3%) in the stressed group when compared to the unstressed group (0%), suggesting a possible synergistic effect between hemolymph extraction and external stressors. Within the stressed group, mortality was significantly associated with bleeding ($P = 0.009$). Within the stressed group, mortality was significantly associated with the amount of blood withdrawn ($P = 0.009$). Mortality rate ranged from 0 to 29.4% over the range of 10–40% hemolymph volume withdrawn. Insight was provided into the possible synergistic effect of blood extraction and external stressors associated with biomedical transport and holding methods on horseshoe crab mortality. Future strategies to reduce horseshoe crab mortality associated with biomedical bleeding must not only take into consideration the effects of bleeding but also the physiological stressors attributable to harvest, transport, and holding conditions.

1 Introduction

The Fishery Management Plan (FMP) for horseshoe crabs has set forth regulations impacting biomedical companies (ASMFC 2007), which catch and bleed horseshoe crabs to extract a compound from this invertebrate's hemolymph, or blood, for the production of *Limulus* amebocyte lysate (LAL). LAL is a clotting agent derived solely from horseshoe crab blood cells (Mikkelsen 1988) and is a

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product used for protecting public health (Berkson and Shuster 1999). It is used by the pharmaceutical and medical industries to ensure that their products, such as intravenous drugs, vaccines, and implantable medical devices, are not contaminated with endotoxins from pathogenic Gram-negative bacteria (Mikkelsen 1988, Novitsky 1991). If a human is exposed to endotoxins a pyrogenic response (i.e., fever) ensues (Novitsky 1991), and in larger amounts endotoxic shock and even death can occur (Miller and Hjelle 1990). With LAL's integration into the pharmaceutical industry as a gold standard for product quality control many other widespread applications have been developed, including clinical diagnostic tests for such illnesses as Gram-negative bacterial meningitis and typhoid fever (Ding and Ho 2001). The LAL test is capable of detecting femtogram levels of endotoxin (Ding and Ho 2001) making it the most effective and economical test for detecting endotoxin contamination. Its increasing medical and pharmaceutical applicability makes it a highly valued product in a global market. Ensuring continuous production of LAL to meet increasing domestic and global needs requires a stable population of horseshoe crabs and is part of the larger management issue.

Considering the extensive use of horseshoe crabs in the biomedical industry since the 1950s, relatively few studies have been conducted concerning the effects of bleeding on the animals. Biomedical companies do not know how much blood adult horseshoe crabs of a given size possess. Hence, it is important to fully understand how much blood extraction and under what physiological, or stress, conditions will result in an acceptable level of mortality. The ASMFC FMP regulations require biomedical companies to return bled horseshoe crabs to the ocean within 72 h of capture, to report harvest data, and characterize mortality (HCTC 1998). Companies must also remain beneath a mortality threshold of 57,500 crabs, where if exceeded the board is required to consider action (HCTC 1998). Mortality rates resulting from the bleeding process are reported to be as high as 20% (Rudloe 1983, Thompson 1998, Kurz and James-Pirri 2002, Walls and Berkson 2003). Bleeding methods vary between biomedical companies. However, it is standard to withdraw blood gravitationally via heart puncture using a large gauge needle until blood flow slows to an intermittent drip (B. Walls, Versar Inc., pers. comm.). Blood extracted from individual animals was reported by Rudloe (1983) to range from 100 to 300 ml. Walls (2001) reported from 8.4 to 218.7 ml of blood extracted from male horseshoe crabs and up to 267.8 ml in females (E. Walls, Versar Inc., Columbia, MD, unpub. data) with the upper extraction volumes potentially impacting horseshoe crab morbidity.

A variety of methods can be used to estimate blood volume, ranging from traditional methods such as exsanguination to more popular dilution methods using dyes and radioactive isotope tracers (St. Aubin et al. 1978). Because horseshoe crabs have an open circulatory system, a method that can quantify blood in the major vessels, muscle, tissue spaces, and sinuses would provide the most accurate estimate. A dye dilution method using inulin is capable of penetrating into these spaces and is a non-lethal and minimally invasive colorimetric assay (Steinitz 1938). Inulin has been used in numerous blood volume studies on other species, such as rock lobster (*Panulirus longpipes*) (Dall 1974), crab (*Carcinus maenas*) (Harris and Andrews 1982), and snail (*Littorina littorea*) (Jones and Kamel 1984). There are two existing estimates of blood volume for juvenile horseshoe crabs (Robertson 1970, Shuster 1978). Robertson (1970) used inulin to estimate extracellular volume and total hemolymph volume. Shuster (1982) used exsanguination to estimate that the total blood volume of one 3 kg female was 300 ml. A blood volume estimate could be used to assess the percentage of blood loss resulting from biomedical bleeding, which is but one of several stages of the biomedical bleeding process.

Throughout the typical biomedical bleeding process, horseshoe crabs are subjected to a variety of potential stressors (i.e., air exposure, increased temperature, handling, blood loss, and trauma). Each LAL producer has its own bleeding process involving different methods of capture, distance and method of travel to the bleeding facility, different holding times and conditions, different bleeding methods, and methods of returning bled crabs that is most appropriate to that company's setting and situation (Walls and Berkson 2003). Typically, horseshoe crabs are collected for biomedical bleeding using trawls, dredges, or hand-harvest (HCTC 1998). Animals may be held on the deck of a boat or in containers for several hours during collection, transported to the bleeding facility in trucks (that may or may not be air-conditioned), held in the laboratory's cold room for several hours at an air temperature of 16–18°C, bled for a period of time, and then held in the cold room or in the truck until transport back to the dock (E. Walls, Versar Inc., Columbia, MD, pers. comm.). Bled horseshoe crabs are required to be returned to their approximate capture point within 72 h of capture or enter the bait harvest (HCTC 1998). The numerous stressors to which the horseshoe crabs are exposed to throughout the biomedical bleeding process are likely to affect mortality rate. The effects of environmental stressors (i.e., harvest method, handling) may even have impacts on mortalities incurred in horseshoe crabs that had been harvested for biomedical bleeding but were ultimately not bled. In the 2006 season, 309,289 crabs were harvested for biomedical purposes alone, with 1.5% (4,639 crabs) rejected prior to bleeding due to mortality, injury, or slow movement (ASMFC 2007).

To formulate effective resource management strategies, impacts on a resource from all stakeholders must be characterized. With biomedical companies subject to regulation by the ASMFC, not only does horseshoe crab mortality have to be assessed from this stakeholder but also future management regulations could potentially progress toward more restrictive harvest regulations or a decrease in mortality threshold of bled animals. In this type of modulating management framework, reducing the biomedical industry's impact on horseshoe crab mortality may be needed, in which case the effects of bleeding as well as the bleeding process on the animals are critical data that are lacking. As there is relatively little information on blood volume of horseshoe crabs, the first study objective was to estimate hemolymph volume across a representative size range of adult *Limulus*. The second objective was to quantify mortality associated with blood extraction at fixed levels and stress from simulated transport and handling procedures.

2 Methods

2.1 Specimen Collection

Specimens were obtained from Cambrex BioScience Walkersville, Inc. (Cambrex), a LAL producer, during early fall of 2002 (for the blood volume study) and summer of 2003 (for the bleeding mortality studies). Horseshoe crabs were captured using a standard trawling procedure (Hata and Berkson 2003) off the coast of Ocean City, Maryland. After capture, horseshoe crabs were brought to Cambrex's bleeding facility in Chincoteague, Virginia. These specimens were then transported in an air-conditioned van to the Horseshoe Crab Research Center (HCRC) at Virginia Polytechnic Institute and State University in Blacksburg, Virginia. The animals were maintained in a recirculating aquaculture system in appropriate environmental conditions (Brown and Clapper 1981, Smith and Berkson 2005). We chose salinities and temperatures that are within the normal range of tolerance of the horseshoe crab and are acceptable for maintaining test animals in good condition (Brown and Clapper 1981, Bullis 1981, Smith and Berkson 2005), which for the blood volume study was salinity of 27–30% and water temperature of 21–23°C.

The animals were allowed to acclimate up to 2 weeks, during which they were tagged, weighed, sexed, and measured. Tagging involved drilling two 3/32″ holes into the prosoma at its thinnest point and attaching a laminated oval fish tag (Floy Tag, Seattle, WA) with two 3/32″ wide cable ties. Measurements were inter-ocular (IO) width and prosomal width (PW). In the blood volume study, the average IO of male horseshoe crabs was 12.0 cm (ranging from 10.5 to 14.0 cm) and PW of 20.6 cm (ranging from 17.5 to 23.0 cm). The average female IO was 16.5 cm (ranging from 14.0 to 20.0 cm) and PW was 27.0 cm (ranging from 22.0 to 33.0 cm). In both bleeding mortality studies, the average IO of male horseshoe crabs was 12.3 cm (ranging from 10.0 to 14.5 cm), and PW of 21.2 cm (ranging from 17.5 to 25.0 cm). The average female IO was 16.1 cm (ranging from 12.5 to 18.5 cm) and PW was 26.4 cm (ranging from 20.5 to 30.0 cm). Only animals that were uninjured (i.e., no cracked carapaces or missing legs) and free of epibionts were used in the two bleeding mortality studies, as it is standard practice in the biomedical bleeding industry.

2.2 Blood Volume Study

A total of 60 specimens, 30 males and 30 females, ranging from 0.90 to 4.40 kg were selected to provide a representative size range of adult horseshoe crabs. Hemolymph volume was estimated by a dye dilution method using stable inulin

(Roe et al. 1949; J. Shields, Virginia Institute of Marine Science, pers. comm., 2002). A working solution of 30 mg/ml of inulin (Sigma Chemical Co.) was prepared in sterile filtered seawater (Sigma Chemical Co.) and then sterile filtered using 20 μm filters. Initial experiments indicated a dosage of 200 mg of inulin per 1 kg of body weight to be appropriate (Hurton 2003). Based on this dosage, a portion of the working solution was injected through the arthroidial membrane into the cardiac sinus. Preliminary experiments also showed that full mixing was achieved within 6 h at a 21.5°C holding temperature (Hurton 2003). After 7 h, 1 ml of hemolymph was withdrawn from the cardiac sinus to provide enough hemolymph for one sample per test animal ($n = 60$). The samples were centrifuged at 10,000*g* to isolate the cell-free hemolymph. Samples then underwent a colorimetric assay (Roe et al. 1949; J. Shields, Virginia Institute of Marine Science, pers. comm. 2002) and the absorbance, at 520 nm wavelength, was measured. The spectrophotometer was calibrated before analysis using a blank and checked thereafter with the blank every 15 samples to ensure that the machine's absorbance readings did not drift.

Once inulin concentration in the hemolymph samples was determined relative to the standard curve, blood volume was estimated as follows (Martin et al. 1958, Jones and Kamel 1984):

$$V = [d(c_1 - c_2)/c_2] - d \tag{1}$$

where V is the blood volume, d the injected volume, c_1 the injected concentration of inulin, and c_2 the final concentration of inulin. This equation is based on the standard equation for dilution with one additional term ($-d$), which corrects for the additional fluid volume added to the animals hemolymph when injecting the inulin solution.

Data were analyzed with the aid of SAS (Statistical Analysis System, Version 8, 1999) using a significance level of $\alpha = 0.05$. Preliminary analysis indicated that a linear model with weight and nonlinear model with IO were the best-fits to characterize their correlation with blood volume. The relationships between weight and blood volume for males and females were evaluated by analysis of covariance. The IO width was regressed with the blood volume data by a nonlinear function and fitted with an exponential curve.

2.3 Bleeding Mortality Study

The first part of the bleeding mortality study used 100 male and 100 female horseshoe crabs, which were collected in July 2003, to test the effect of blood extraction volume on mortality. From this sample, 100 males and 100 females were selected to test only the effects of various levels of blood extraction on mortality. This sample of horseshoe crabs was termed the "low-stressed" group because they were not exposed to external stressors associated with simulated holding and transport. The selected horseshoe crabs ranged in size in an effort to provide a representative sample of animals bled by some biomedical companies (E. Walls, Versar Inc., Columbia, MD, pers. comm.). Horseshoe crabs were randomly assigned to one of five bleeding treatments. Each bleeding group was comprised of equal numbers of males and females. Horseshoe crabs were bled 0 (control), 10, 20, 30, or 40% of their predicted blood volume. Predicted blood volume was calculated using the relationship between blood volume and IO width as follows (see Sect. 1.4.1, Hurton et al. 2005):

$$H = 25.7e^{0.1928(IO)} \tag{2}$$

where H is the hemolymph volume in ml and IO the inter-ocular width in cm.

The bleeding process of this component involved removing horseshoe crabs from their holding tank, positioning them in a bleeding rack, and disinfecting the surface of their arthroidial membrane with a 70% ethanol-soaked cotton swab. An 18-gauge needle was inserted through the membrane into the cardiac sinus to extract the predetermined amount of blood. The animals were then placed in a holding container without water for 15–20 h. Bled horseshoe crabs were not immediately put back in water to prevent them from absorbing water and regaining their blood volume. This time out of water approximated the duration that horseshoe crabs may be out of water after bleeding at some biomedical bleeding facilities (E. Walls, Versar Inc., Columbia, MD, pers. comm.). The 5-h difference in holding time was deemed not to confound treatment effects. Bleeding of individual crabs from the five treatment groups occurred by random selection, hence there were at random animals from all of the bleeding treatments out of the water from 15 to 20 h. Temperatures were recorded using a temperature logger and maintained at 21°C. The animals were returned to their holding tanks and mortality was monitored for 2 weeks.

The second part of the bleeding mortality study used 110 male and 85 female horseshoe crabs, which were collected in August 2003, to test the interactive effects of blood extraction volume and external stressors on mortality. Due to difficulty in obtaining the desired 200 study animals, 195 horseshoe crabs were selected for this study. These animals comprised the “high-stressed” group. The external stressors (i.e., air exposure, increased temperature) originated from simulated transport and holding procedures of the biomedical bleeding process.

During the first week after transport, the horseshoe crabs were held in the same conditions, handled in the same manner, and assigned to the same bleeding treatments as the low-stressed group. The bleeding process of this study involved removing horseshoe crabs from their holding tank and placing them in holding containers located outside where they were exposed to air, sun, and increased temperatures. During this 6-h period, the air temperature rose from 21 to 29°C to simulate the horseshoe crabs’ time on the deck of a trawler. Next, the animals were moved into a small, moving truck, which was not air-conditioned. This phase simulated transportation to the bleeding facility and holding time until placement into the cold room of the laboratory. During this 4-h period, the outdoor temperature increased to 31°C and the temperature inside the closed truck peaked at 36°C. After this, the animals were transferred to the HCRC's tank room where they remained for 16 h at 21°C. This phase mimicked the holding time in a facility's cold room prior to bleeding. The animals were bled according to their assigned treatment group and in the same manner as in the first mortality study. During this 8-h period, the room temperature was 22°C. Once the bleeding was completed, the horseshoe crabs were moved back into the truck for 13 h to simulate holding time and transport to the boat. The overnight temperature inside the truck dropped from 24 to 20°C. In the morning, the horseshoe crabs were returned to the water in their recirculating aquaculture tanks and mortality was monitored for 2 weeks.

Fisher's exact test was used to evaluate: (1) differences in mortality of unbled horseshoe crabs between the high-stressed and low-stressed groups, and (2) differences in mortality of bled crabs between the low-stressed and high-stressed groups. Logistic regression was used to examine the association between mortality and bleeding in the high-stressed group with horseshoe crab sex as an additional parameter in the analysis. Data were also assessed to see if the increase in bleeding amount was correlated with an increase in mortality by pooling the frequency of mortalities of males and females in each bleeding treatment of the high-stressed group. These pooled mortality frequencies were tested against bleeding treatment to determine if there existed a correlation within the high-stressed group between mortality and percent of blood volume extracted by employing a regression with a fitted quadratic curve.

3 Results

3.1 Blood Volume Study

The linear relationship of blood volume to weight for male horseshoe crabs (linear regression, $n = 30$, $P < 0.001$, $r^2 = 0.8567$) was characterized by the equation:

$$H_m = 257.2w - 5.693 \tag{3}$$

where H_m is the male hemolymph volume in ml and w the wet body weight in kg. The relationship between weight and blood volume is presented in Fig. 1. The female correlation of blood volume to weight is described by the equation:

$$H_f = 215.0w - 107.0 \tag{4}$$

where H_f is the female hemolymph volume in ml and w the wet body weight in kg. The relationship between weight and blood volume is presented in Fig. 2.

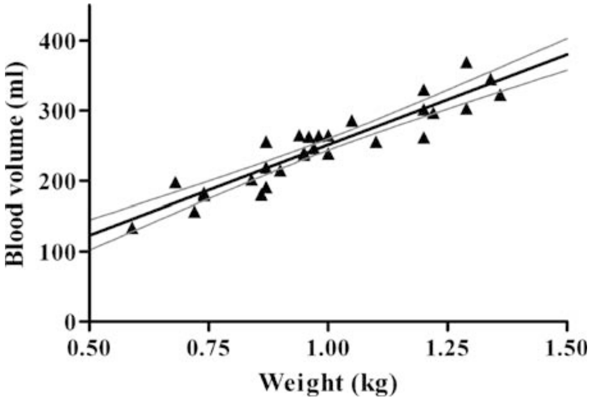

Fig. 1 The linear regression of wet body weight against estimated hemolymph volume for male horseshoe crabs ($n = 30$, $r^2 = 0.8567$, $P < 0.001$) is characterized by the equation $H_m = 257.2\ w - 5.693$, where H_m is the male hemolymph volume (ml) and w the wet body weight (kg). The population mean is bounded by the 95% confidence interval of the mean (*grey bounding lines*)

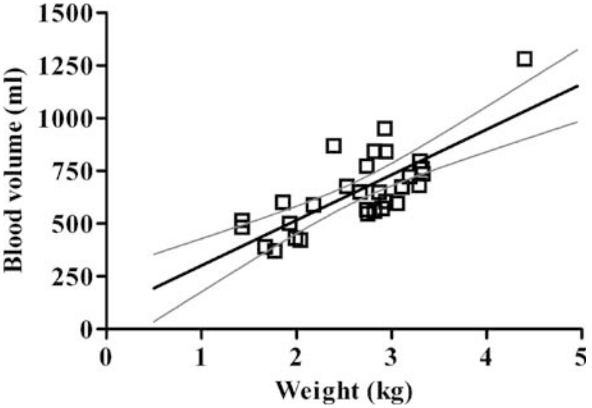

Fig. 2 The linear regression of wet body weight against estimated hemolymph volume for female horseshoe crabs ($n = 30$, $r^2 = 0.5772$, $P < 0.001$) is characterized by the equation $H_f = 215.0\ w - 107.0$, where H_f is the female hemolymph volume (ml) and w the wet body weight (kg). The population mean is bounded by the 95% confidence interval of the mean (*grey bounding lines*)

No significant difference was found between males and females in either slope or intercept of their respective regression lines. However, females did demonstrate greater variability in blood volume than males (Fig. 2). Blood volume of the horseshoe crabs represented as a percentage of wet body weight was 25 ± 2.2% (mean ± S.D.) for males and 25 ± 5.1% (mean ± S.D.) for females.

In the field, it was often more straightforward to measure IO width rather than weight. The exponential relationship between blood volume and IO width (non-linear regression, $n = 60$, $P < 0.0001$) is represented by the equation:

$$H = 25.7e^{0.1928(IO)} \tag{2}$$

where H is the hemolymph volume in ml and IO the inter-ocular width in cm. This relationship is presented in Fig. 3.

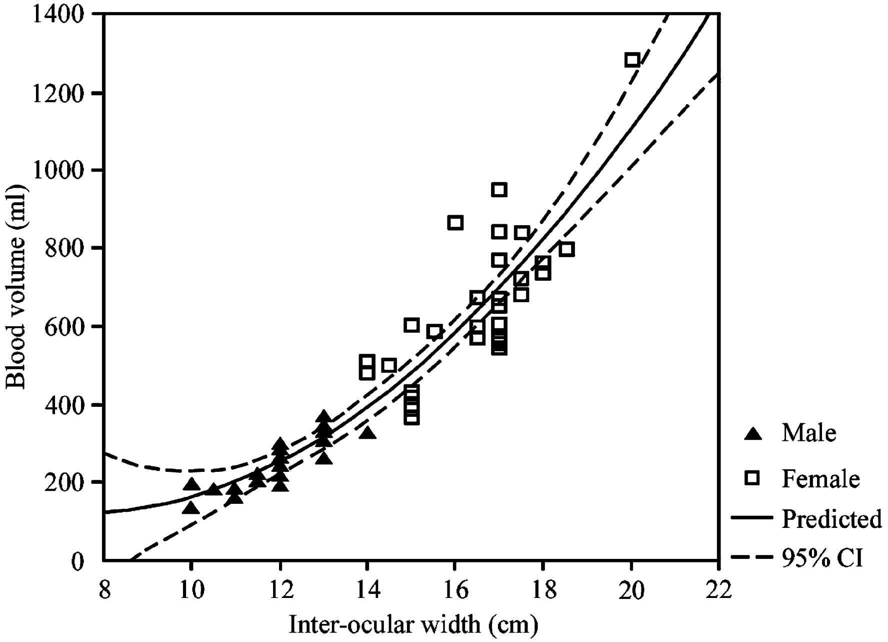

Fig. 3 Non-linear regression of IO width against estimated hemolymph volume with an exponential fit ($n = 60$, $P < 0.0001$) is expressed by the equation $H = 25.7\ e^{0.1928\ (IO)}$, where H is the hemolymph volume (ml) and IO the inter-ocular width (cm). The population mean is enclosed by a 95% confidence interval of the mean (*dashed lines*)

3.2 Bleeding Mortality Study

No horseshoe crab mortality occurred in any of the five treatments in the low-stressed group, suggesting that post-bleeding mortality in *Limulus* did not arise solely from the effects of blood loss. There were a total of 14 mortalities distributed throughout the bleeding treatments among horseshoe crabs under higher stress conditions. All mortalities occurred within the first 7 days of the study.

In the high-stressed group the bled horseshoe crabs had an overall mortality rate of 8.3% compared to the 2.6% mortality rate of unbled crabs, suggesting a relationship between mortality and bleeding under high-stressed conditions ($n = 195$, $P = 0.0088$; Fig. 4). The bleeding variable was significant (logistic regression, $n = 156$, $P = 0.0160$); yet, sex was not a significant variable (logistic regression, $n = 156$, $P = 0.6100$), with seven deaths in total occurring in each group. Mortality rates reached 13.6% for males in the 30% bled treatment and female mortality was as high as 29.4% in the 40% bled group (Table 1). With male and female mortalities pooled, the frequency of mortality increased as the bleeding percentage increased (non-linear regression, $n = 5$, $P = 0.006$, $r^2 = 0.994$; Table 1, Fig. 4).

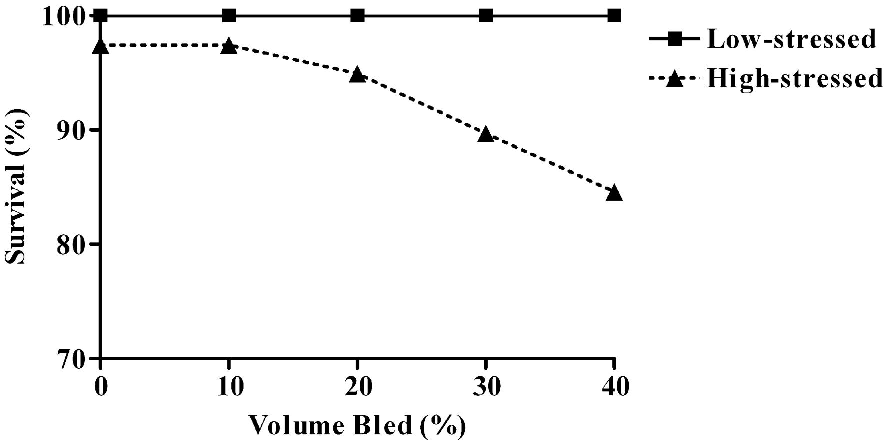

Fig. 4 Comparison of horseshoe crab survival between the low- and high-stressed groups ($n = 200$ and 195, respectively) across the five bleeding treatments, where bleeding treatments were a percentage of the total estimated blood volume for each individual horseshoe crab

Table 1 Comparison of mortalities observed in the high-stressed group between unbled horseshoe crabs and horseshoe crabs bled at different levels ($n = 195$)

	Mortality (no.)			Mortality (%)		
% Bled	Male	Female	Total	Male	Female	Total
0	1	0	1	4.5	0	2.6
10	0	1	1	0	5.9	2.6
20	2	0	2	9.1	0	5.1
30	3	1	4	13.6	5.9	10.3
40	1	5	6	4.5	29.4	15.4
Total	7	7	14	6.4	8.2	7.2

Mortality rates were not significantly different between low- and high-stressed groups for unbled crabs (Fisher's exact test, $n = 79$, $P = 0.4937$). Of the bled crabs, there was a mortality rate of 0% in the low-stressed group and an average rate of 8.3% in the high-stressed group, suggesting a significant difference in mortality rates between the two groups (Fisher's exact test, $n = 316$, $P < 0.0001$; Fig. 4).

Though this study's data suggested a significantly increased mortality rate in the high-stressed group as opposed to the low-stressed group, it is important to note the variability present in mortality throughout treatments. It was observed that within the high-stressed group the male and female horseshoe crabs had different and variable responses in the different bleeding treatments (Table 1). For example, males had two and three mortalities in the 20 and 30% bleeding treatments, respectively; yet only one mortality in the 40% category. Females only appeared to have a spike in mortality in the 40% category. More than likely, much of this variability is an artifact of a sample size that is not large enough. With male and female data pooled, survival appears to proceed in a decreasing trend as bleeding percentage increases (Fig. 4). Further investigation with a larger sample size or a different experimental design could elucidate a more definitive trend in horseshoe mortality rates in horseshoe crabs exposed to external stressors and different bleeding treatments, as well as whether one sex may be more susceptible to bleeding mortality than the other.

4 Discussion

Hemolymph volume was linearly related to horseshoe crab weight. Horseshoe crab hemolymph volume as a percentage of body weight falls within the range of those in other arthropods, which share the feature of an open circulatory system. The blue crab (*Callinectes sapidus*) has a blood volume of 25.5% of wet body weight (Gleeson and Zubkoff 1977) when compared to the crab *C. maenas* with a blood volume of 33.0% of wet body weight (Harris and Andrews 1982). Other arthropod blood volume estimates are as follows: rock lobster (*Panulirus longipes*) with 17.8% (Dall 1974), freshwater crayfish (*Cambarus virilis*) with 25.6% (Prosser and Weinstein 1950), and scorpion (*Heterometrus fulvipes*) with 33.4% (Kumari and Naidu 1987).

Shuster's (1982) single blood volume estimate from a 3,000 g adult female horseshoe crab was 300 ml or 10% of body weight. This study presents blood volume estimates of both adult male and female horseshoe crabs spanning a representative size range of the species ($n = 60$). The results indicate that the mean blood volume is 25% of wet body weight or 2.5 times that of Shuster's (1982) estimate. One explanation for the discrepancy between Shuster's (1982) results and those within this study is that Shuster (1982) estimated blood volume by exsanguination. As horseshoe crabs have an open circulatory system, a significant amount of blood can remain in the body within the muscle, tissue spaces, and sinuses. Clotting can also occur, triggered by trauma to the arthroidial membrane during exsanguination, and would further hinder measurement of total hemolymph volume.

Even though the inulin dye dilution method is more accurate in estimating hemolymph volume than exsanguination, various factors can influence blood volume estimates in general. For instance, water volume of the blood varies with the salinity of the horseshoe crab's environment and with the length of time the animal is out of the water (Shuster 1978). Lower salinity would increase blood volume and exposure to air would decrease blood volume. Data from this study did not address the issue of changing blood volume in horseshoe crabs. Information pertaining to horseshoe crab blood volume change is currently unknown or anecdotal. However, some of these factors have been documented to influence blood volume in other species. Blood volume was influenced by seasonal changes in shore crabs (*Carcinus mediterraneus*) (Devescovi and

Luču 1995), salinity in the gastropod *L. littorea* (Taylor and Andrews 1988), nutritional state in various decapod crustaceans (Dall 1974, Depledge and Bjerregaard 1989), and parasitism in *L. littorea* (Jones and Kamel 1984). Therefore, our results may not be reflective of horseshoe crab blood volumes at other time periods, environmental parameters, or in other health conditions. For the purposes of this study, environmental parameters were kept as constant as possible between the various batches of horseshoe crabs used in both the blood volume and bleeding mortality experiments.

It is also important to note that females had greater variability in estimated hemolymph volume for the same size classes (Figs. 2 and 3). For example, of the 30 females injected with inulin, 10 females had an inter-ocular width of 17 cm. These females were estimated to have hemolymph volumes from 548 to 951 ml. Yet, the prediction for this size class is 681 ml (Eq. (2)). Hence, the estimated hemolymph volumes were up to 270 ml greater and 133 ml less than the predicted value. Variability of blood volume among females may be attributable to whether the female is carrying eggs and in the maturation stage of the eggs. Eggs in a gravid female would displace space in the body that would otherwise be filled with hemolymph in the absence of eggs. Therefore, it might be postulated that blood volume could vary seasonally in females corresponding to the spawning season from May to July. Determining if this conjecture is correct and incorporating this parameter into derived blood volume relationships would possibly improve blood volume predictions for females.

This study examined blood volume of horseshoe crabs in a controlled environment, which may not be completely reflective of blood volumes in a natural population. Even though these values should be considered approximations, they are the first blood volume estimates for a range of sizes of adult horseshoe crabs. These results decrease the uncertainty regarding the total blood volume of adult horseshoe crabs and, as in this study, can be integrated into a bleeding mortality assessment of horseshoe crabs that provides an additional dimension in interpreting an experimental outcome.

Based on this study's use of 200 horseshoe crabs, no impact on mortality was observed with blood extraction up to 40% of the horseshoe crab's blood volume under conditions of low stressors. This result suggests that despite hypovolemia and altered blood chemistry (Hurton 2003) horseshoe crabs may be relatively tolerant to the removal of a large amount of blood when in the absence of other stressors and allowed to regain their blood volume by immediate return to water.

Horseshoe crab mortality from blood loss, however, was significant in the presence of higher levels of stress, including external stressors (i.e., lengthy air exposure, elevated temperatures) as applied in our study. Novitsky's (1991) aquarium studies reported that up to 30% of blood volume can be safely extracted, suggesting that the animals in that study may have been similar to our low-stress group (Novitsky 1991). It is likely that, under different conditions, horseshoe crab response may be altered. Findings from our study indicate that a combination of external effects and sub-lethal hemorrhage result in significantly increased mortalities, possibly from a synergistic interaction between the two types of stressors. Studies in other species exposed to various stressors also indicated that mortality might be affected by a synergistic combination of effects from multiple stressors (Schisler et al. 2000, Schulz and Dabrowski 2001, Hatch and Blaustein 2003). Such results emphasize the importance of considering the cumulative impact of multiple stressors.

Biomedical companies can now estimate blood volume extracted using the identified blood volume relationships (Sect. 1.4.1 and Hurton et al. 2005). Calculated upper-estimated blood volumes for male crabs (Eq. (3)) weighing 1.4 kg and females (Eq. (4)) weighing 3.3 kg are approximately 354 and 602.5 ml, respectively. Extrapolating from the reported maximum extracted blood volumes for male and female horseshoe crabs (218.7 and 267.8 ml, respectively) (Walls 2001, B. Walls, Versar Inc., unpub. data 2001) and the estimated blood volumes, males could be having up to 62% and females up to 44% of their blood volume extracted. A previous estimate of maximum blood volume extraction from horseshoe crabs was 50 ± 13% (mean ± SD) of predicted blood volume (Hurton 2003), indicating that it is possible for blood extraction to exceed our tested 40% bleeding treatment. In the context of this study, these bleeding proportions would have mortality implications when the animals are in a physiologically taxed state. Yet, animals under low-stressed states with these values would likely have negligible effects on mortality. Hurton (2003) found that no mortality resulted when attempting to extract the maximum volume of blood from animals which, in that study, could be considered to be under low-stress conditions as the animals were immediately returned to the water after bleeding. In the current study, the high-stressed condition mortality rates were greater than the low-stressed state throughout all bleeding treatments tested (Fig. 3), suggesting that environmental stress could be a key contributor to the synergistic effects occurring in the bleeding process. Therefore, it may be a more effective approach to reduce external environmental stressors on horseshoe crabs in the bleeding process rather than solely focusing on reducing bleeding amounts.

In the typical biomedical bleeding process, horseshoe crabs undergo time on a boat deck or in collection bins, transport, storage in a cold room, bleeding, holding time, and transport back to the ocean. At each of these stages of processing, the animals are exposed to a number of stressors of varying magnitudes, including exposure to air for extended time periods, elevated temperatures, dehydration, hypovolemia, and likely other unknown stressors. Each biomedical company has their own bleeding process that contributes a variable nature to horseshoe crab bleeding mortality in the biomedical industry. More importantly, the process components can be manipulated to achieve particular objectives such as attempting to decrease horseshoe crab mortality. For example, it is possible that horseshoe crabs exposed to greater lengths of time to air and higher temperature would lose moisture from their exposed gills and likely become dehydrated. Dehydration results in decreased blood volume, increased blood osmotic concentration, and possible viscosity (Wilkens and Young 2006), all of which can contribute to physiological stress. Dehydration rates in horseshoe crabs are unknown, but have been examined in *Austropotamobius pallipes*, a freshwater crayfish, which is a facultative air breather that can survive about 3 days out of water (Taylor et al. 1987). The study reported that when exposed to air (70–80% relative humidity) for 27 h, crayfish dehydrated and had a 25% decreased blood volume. Yet, when exposed to water-saturated air (100% relative humidity), crayfish did not have a decrease in blood volume. Similarly, horseshoe crabs exposed to water-saturated air may be less likely to become dehydrated, thereby potentially decreasing the effects of one type of stress acting on the animal's physical condition.

Possible stress-reducing actions that could be considered include holding horseshoe crabs in an air-conditioned environment (i.e., during time in the truck and in the cold room), decreasing their time out of the water, covering them with wet burlap while out of water to keep them moist and shaded from the sun, drenching the animals with water periodically, and/or increasing the relative humidity of the air in the holding room. These suggested actions could play a role in helping to decrease the physiological stress that horseshoe crabs experience throughout the bleeding process. Implementing these actions could decrease the biomedical industry's impact on the strained horseshoe crab population, help keep the industry within the FMP's established mortality threshold, and reduce some tensions within this horseshoe crab controversy.

Acknowledgments Sources for portions of this chapter originate from articles formerly published in Marine and Freshwater Behaviour and Physiology (see Hurton 2005 in References and http://www.informaworld.com) as well as Fishery Bulletin (Hurton, L. and J. Berkson. 2006. Fishery Bulletin 104:293–298). This work is a result of research sponsored in part by the NOAA Office of Sea Grant, US Dept of Commerce, under Grant No. NA96RG0025 to the Virginia Graduate Marine Science Consortium and Virginia Sea Grant College Program. Cambrex Bio Science Walkersville, Inc. generously supplied us with horseshoe crabs for the study. We wish to thank Dr. Eric Hallerman of the Department of Fisheries and Wildlife Sciences at Virginia Tech for his helpful advice throughout this project. In addition, we thank Penelope Pooler and Keun Pyo Kim of the Department of Statistics at Virginia Polytechnic Institute and State University for their statistical counseling. A special thanks is extended to numerous colleagues for their assistance in transporting, tagging, and/or bleeding horseshoe crabs for this study: Vincent Caruso, Michelle Davis, Elisabeth Franks, Whitney Grogan, Dr. Dave Hata, Dr. Jay McGhee, René Olsen, Alison Sasnett, Mary Tilton, and Dr. Alison Williams. We greatly appreciate the time and effort of all involved.

Abbreviations

ASMFC	Atlantic States Marine Fisheries Commission
Cambrex	Cambrex BioScience Walkersville, Inc.
FMP	Fishery management plan
HCRC	Horseshoe Crab Research Center
IO	inter-ocular
LAL	*Limulus* amebocyte lysate

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Horseshoe Crabs in Hong Kong: Current Population Status and Human Exploitation

Paul K.S. Shin, HiuYan Li, and Siu Gin Cheung

Abstract An updated survey, using both random transect and walk-through search methods, at 17 shores in Hong Kong in summer and winter showed that juvenile horseshoe crabs (*Tachypleus tridentatus* and *Carcinoscorpius rotundicauda*) were significantly reduced by over 90% in density since 2002. Of the shores where juveniles were found, the highest density of *T. tridenatus* was 0.31 and lowest 0.08 ind 100 m^{-2}. Juvenile *C. rotundicauda* was only found using the walk-through search method, with the highest record of 1.17 and lowest 0.17 ind hr^{-1} $person^{-1}$. The mean prosomal width of juvenile *T. tridentatus* obtained from the walk-through survey varied from 2.6 to 5.5 cm, which corresponded to an age of 4–8 years old. A larger size range for *C. rotundicauda* was, however, noted, from 2.5 to 9.0 cm.

The degree of human exploitation of adult horseshoe crabs (*T. tridentatus*) in Hong Kong was estimated through interviewing 34 seafood restaurants, 150 fish sellers and fish handlers, and fishermen in two local fish wholesale markets over a 13-month study period. A total of 1,023 horseshoe crabs were caught in 2004–2005, with 72% from mainland Chinese waters. Apart from releasing back to sea, an average sale of 17 horseshoe crabs per month was estimated. While the sale of horseshoe crabs was low by comparison with other marine species of economic importance, human exploitation still contributes a potential threat and puts further pressure on the mature population of horseshoe crabs in Hong Kong due to their long maturity period and declining densities of the juveniles.

1 Introduction

Once reportedly abundant on shores in Hong Kong by local villagers in the 1950s, adult horseshoe crabs are rarely found on shores these days. From Mikkelsen (1988), there were records of three Asia-Pacific horseshoe crab

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species, *Tachypleus tridentatus*, *T. gigas* and *Carcinoscorpius rotundicauda* in Hong Kong. More recently, only *T. tridentatus* and *C. rotundicauda* were reported (Chiu and Morton 1999a) and a decline of their presence in the northeastern waters of Hong Kong was also noted (Chiu and Morton 1999b, 2003a). The decline in horseshoe crab populations is not confined to Hong Kong, but has been observed in Japan, Taiwan, and Thailand as well (Sekiguchi 1988; Itow 1998; Chen et al. 2004). It is apparent that the threat for further population decline and possible extinction might be imminent if there are no effective conservation measures to save horseshoe crabs in the Asia-Pacific (Earle 1991).

In Hong Kong, information on species identification (Chiu and Morton 2003b), characteristics of nursery beaches (Chiu and Morton 1999b; Morton and Lee 2003), ecology and biology (Chiu and Morton 1999a), growth and allometry (Chiu and Morton 1999a, 2003a), and behavior of juveniles in the field (Chiu and Morton 2004) and laboratory (Morton and Lee 2003) is available. With continuing urban developments and disturbances to coastal habitats, the threat to the existence of horseshoe crab populations remains apparent. The objectives of this study are to update the current population status on the distribution of juvenile horseshoe crabs at local shores and assess the extent of human exploitation of adult horseshoe crabs in Hong Kong.

2 Materials and Methods

2.1 Population Distribution Study

A total of 17 Hong Kong soft shores were surveyed in this study (Fig. 1), including Tsim Bei Tsui, Sheung Pak Nai, Pak Nai, and two locations at Ha Pak Nai in northwestern New Territories; and San Tau, Shui Hau Wan, Pui O Wan, Tai Ho Wan, Sham Wat, Yi O, Tung Chung, and Hau Hok Wan on Lantau Island, where horseshoe crabs once were reported in abundance (Chiu and Morton 1999b). In eastern waters and on Lamma Island, surveys were also conducted at Luk Keng, Lai Chi Wo, and Pak Kok Wan in northeastern New Territories, and Sok Kwu Wan on Lamma Island (Fig. 1). The 13 shores in northwestern New Territories and on Lantau Island were surveyed in summer from September to November 2004 and in winter from December 2004 to February 2005 while the three shores in northeastern New Territories and one shore on Lamma Island were surveyed from 2005 to 2006. Summer distribution studies in Luk Keng, Pak Kok Wan, and Sok Kwu Wan were carried out from August to October 2005. For Lai Chi Wo in northeastern New Territories, the summer survey was undertaken in June 2006. The winter distribution studies of these four sites were conducted in January 2006.

A transect method as adopted by Chiu and Morton (1999b) was used in this study. At each shore, four horizontal transects were set equally apart from the

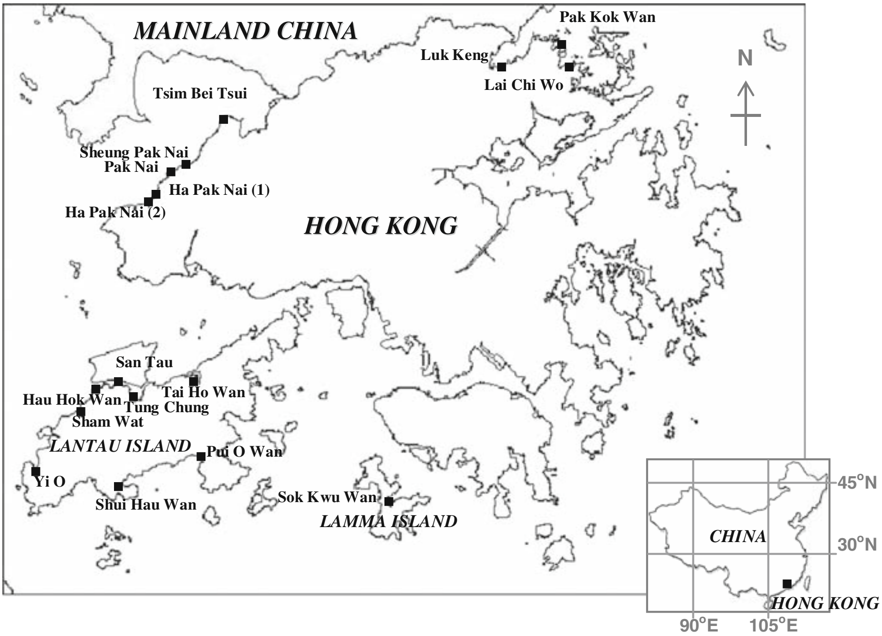

Fig. 1 The 17 survey shores for juvenile horseshoe crabs in New Territories, on Lantau Island and Lamma Island of Hong Kong

lower tidal level at 0.7–1.6 m above chart datum (CD). The length of each transect was similar to the width of the shore, and along each transect five quadrats (8 m × 8 m) were randomly selected for counting of juvenile horseshoe crabs (Fig. 2). The total survey area on a shore was 1,280 m^2. The number of each horseshoe crab species found on the sediment surface within the quadrat was counted and their prosomal width measured using a Vernier caliper. For survey locations near freshwater streams, the sampling was further stratified by studying additional 20 random quadrats (0.5 m × 0.5 m) within the stream area. As horseshoe crabs may bury in the sediment and could not be observed by the present sampling method, such bias was assessed by using 20 random quadrats (area of 2 m^2) and each sample was examined by digging sediment up to 5 cm depth in the distribution study in northeastern New Territories and on Lamma Island in 2005–2006. Within each quadrat, sediment temperature, salinity, and dissolved oxygen were also monitored using a glass thermometer, a hand-held refractometer (Model ATAGO S/Mill-E), and an oxygen electrode (YSI Model 58), respectively.

Apart from the transect method, the population density of horseshoe crabs was also obtained by a walk-through survey. At each shore, two persons were involved in search of juvenile horseshoe crabs by walking along the four horizontal transects in a fixed time duration of 3 hours during low tide. All juvenile horseshoe crabs found were counted and the prosomal width of each

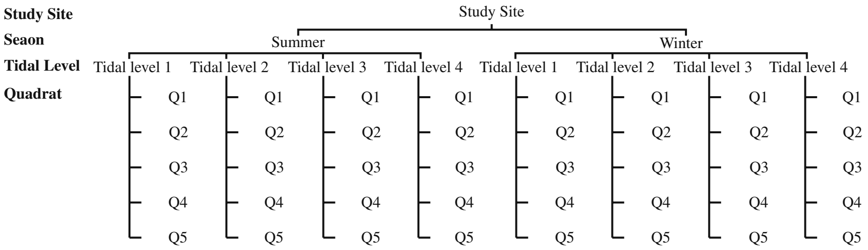

Fig. 2 The transect sampling design for survey of juvenile horseshoe crabs on Hong Kong shores

individual was measured. The data were normalized by calculating the population density per unit search effort, i.e., number of horseshoe crabs/hour/person.

2.2 Human Exploitation Study

A monthly market survey from September 2004 to September 2005 was conducted at 11 popular sites for the sale of seafood in Hong Kong. In total, 34 seafood restaurants and 150 fish sellers were interviewed monthly for information on the sale of adult horseshoe crabs. Monthly interviews of fishermen from two local fish wholesale markets were also carried out, to record if horseshoe crabs were caught in local waters.

2.3 Data Analysis

As the population density data obtained from the transect method did not follow the normal distribution, differences in population density between the survey sites were compared using non-parametric Kruskal–Wallis (KW) test to assess the effects of site, tidal level, and season on the population density of juvenile horseshoe crabs. A Bonferroni adjustment was used to correct for Type I error, and significance for each KW test was evaluated against $\alpha = 0.05$ divided by the number of comparisons being made. Thus, the significance level for each KW test was $P = 0.017$. For data on the hydrological parameters and size of juvenile horseshoe crabs obtained from the walk-through method, data were normally distributed. Hence, a three-way repeated-measures ANOVA was used to address the interacting effects among the survey shores, tidal levels, and seasons on the hydrological parameters and size of horseshoe crabs. No data transformation was used prior to these analyses. When significant differences among treatments were observed, Tukey multiple comparison tests were performed to determine the differences between sites and tidal levels with a significance level of $P = 0.05$. All the statistical analyses were undertaken using software SPSS 11.0.

3 Results

3.1 Population of Juvenile Horseshoe Crabs

Over the survey period, sediment temperature in summer averaged at 29.4 ± 2.2°C, interstitial salinity 25.1 ± 7.2‰, and dissolved oxygen 6.0 ± 1.7 mg l^{-1}, whereas in winter, sediment temperature averaged at 17.0 ± 3.6°C, interstitial salinity 32.5 ± 6.6‰, and dissolved oxygen 7.7 ± 1.4 mg l^{-1}. Temperature varied significantly among the survey shores ($F_{16,\ 156} = 83.392$, $P < 0.001$), tidal levels ($F_{3,\ 156} = 8.558$, $P < 0.001$), and seasons ($F_{1,\ 156} = 5187.483$, $P < 0.001$). Salinity also varied significantly with the sampling shores ($F_{16,\ 203} = 18.854$, $P < 0.001$), tidal levels ($F_{3,\ 203} = 5.728$, df = 3, $P = 0.001$), and seasons ($F_{1,\ 203} = 117.47$, $P < 0.001$). However, dissolved oxygen level in the sediment varied significantly with the survey shores ($F_{15,\ 170} = 9.241$, $P < 0.001$) and seasons ($F_{1,\ 170} = 69.180$, $P < 0.001$) but not tidal levels ($F_{3,\ 170} = 0.471$, $P = 0.703$).

Among the 17 shores, juvenile *T. tridentatus* were only found at 6 shores and no *C. rotundicauda* were noted using the transect method. Figure 3 shows the mean density of juvenile *T. tridentatus* recorded in both summer and winter surveys. In summer, Tsim Bei Tsui and one location at Ha Pak Nai had the highest mean density of 0.31 ± 0.96 and 0.23 ± 0.76 ind 100 m^{-2}, respectively, followed by another location at Ha Pak Nai (0.16 ± 0.48 ind 100 m^{-2}) and San

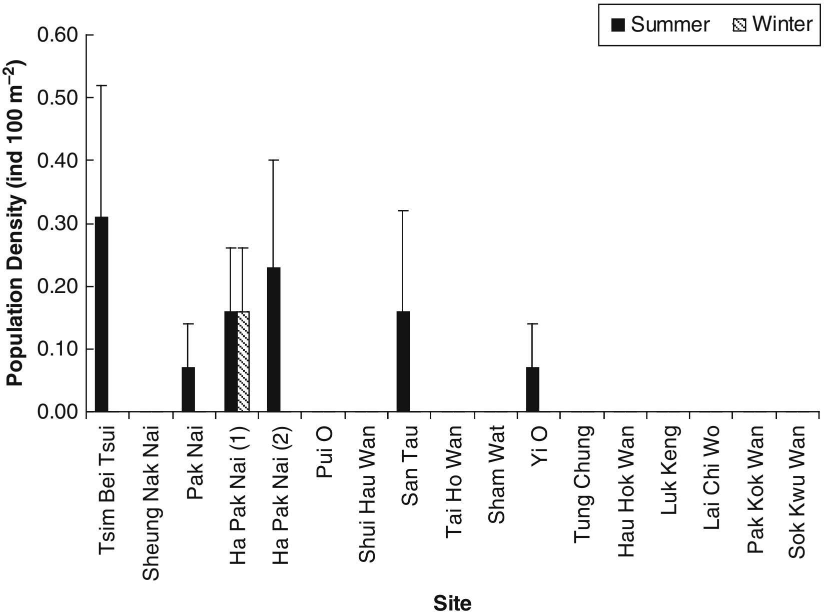

Fig. 3 Mean density (+SE) of juvenile horseshoe crabs (*Tachypleus tridentatus*) recorded by the transect sampling method in the present surveys

Tau (0.16 ± 0.70 ind 100 m^{-2}). Pak Nai and Yi O had the lowest mean density of 0.08 ± 0.35 ind 100 m^{-2} among the six shores. In winter, horseshoe crabs were only found at one site located at Ha Pak Nai with the average density of 0.16 ± 0.48 ind 100 m^{-2}. However, no significant difference in densities was noted among shores (KW test, $H = 29.811$, $P = 0.019$). For spatial variations within shores, the density on the upper shore (tidal level 1) was higher than that on the lower shore (tidal levels 2–4) in both summer and winter (Fig. 4); the differences, however, were not statistically significant (KW test, $H = 1.625$, $P = 0.654$). There was also no significant difference (KW test, $H = 4.554$, $P = 0.033$) in the distribution of horseshoe crabs in the two sampling seasons. The mean density was 0.06 ± 0.39 and 0.01 ± 0.12 ind 100 m^{-2} in summer and winter, respectively. No horseshoe crabs were recorded in stream areas, and no horseshoe crabs were found by the digging method in the distribution study in northeastern New Territories and on Lamma Island.

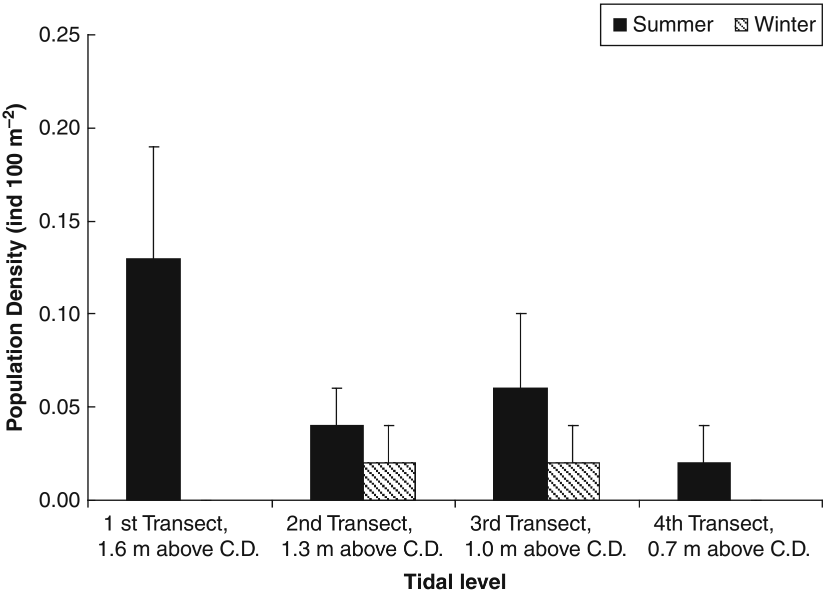

Fig. 4 Mean density (+SE) of juvenile horseshoe crabs (*Tachypleus tridentatus*) found at the four tidal levels (1 = the uppermost transect; 2 = the second transect; 3 = the third transect; 4 = the fourth transect, 0.7 m above chart datum (C.D.)) recorded by the transect sampling method in the present surveys

More individuals of *T. tridentatus* were collected by the walk-through survey than the transect sampling in both summer and winter (Fig. 5). While direct comparison of data was not feasible owing to different sampling efforts, the walk-through survey might be a better strategy to collect sufficient data for

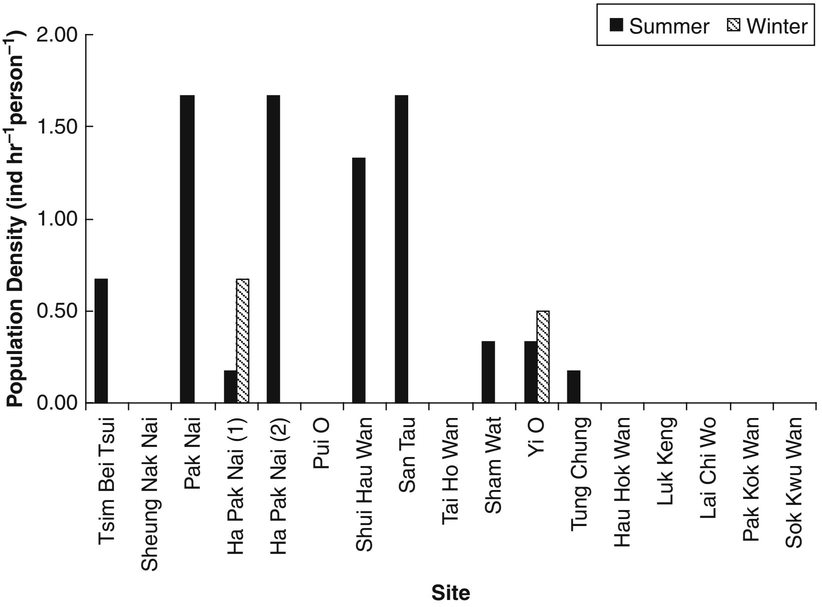

Fig. 5 Population density of juvenile horseshoe crabs (*Tachypleus tridentatus*) found by the walk-through survey

juvenile horseshoe crabs occurring at low densities on local shores. Similar to the transect sampling method, higher densities of horseshoe crabs were found at Tsim Bei Tsui, Ha Pak Nai (2), San Tau, and Pak Nai. No individual was found at Shui Hau Wan by transect sampling but eight individuals were found by the walk-through survey in the summer study. No individual of *C. rotundicauda* was found using transect sampling but 30 and 4 individuals were found in summer and winter, respectively, using the walk-through survey (Fig. 6) with highest densities being found at Tsim Bei Tsui and Pak Nai. Only *C. rotundicauda* was found in northeastern New Territories including Luk Keng and Lai Chi Wo.

Figure 7 shows the mean prosomal width of juvenile *T. tridentatus* obtained from the walk-through survey in both summer and winter. Their mean carapace width varied from 2.6 to 5.5 cm. According to the size–age relationship established by Sekiguchi (1988), these corresponded to an age of 4–8 years old. Horseshoe crabs in Shui Hau Wan had the highest average prosomal width of 5.6 ± 1.25 cm, while those in Tung Chung had the lowest average prosomal width of 2.7 ± 0.27 cm (Fig. 7). The size of juvenile *T. tridentatus* did not vary significantly with the survey shores ($F_{8,\ 35} = 1.721$, $P = 0.128$), seasons ($F_{1,\ 35} = 0.265$, $P = 0.61$), tidal levels ($F_{3,\ 35} = 1.048$, $P = 0.383$), or their interaction ($P > 0.05$) as tested by three-way repeated-measures ANOVA. From the present data, the largest average size of horseshoe crabs was collected at 1.3 m above CD and smallest at 0.7 m above CD; the difference, however, was not statistically significant.

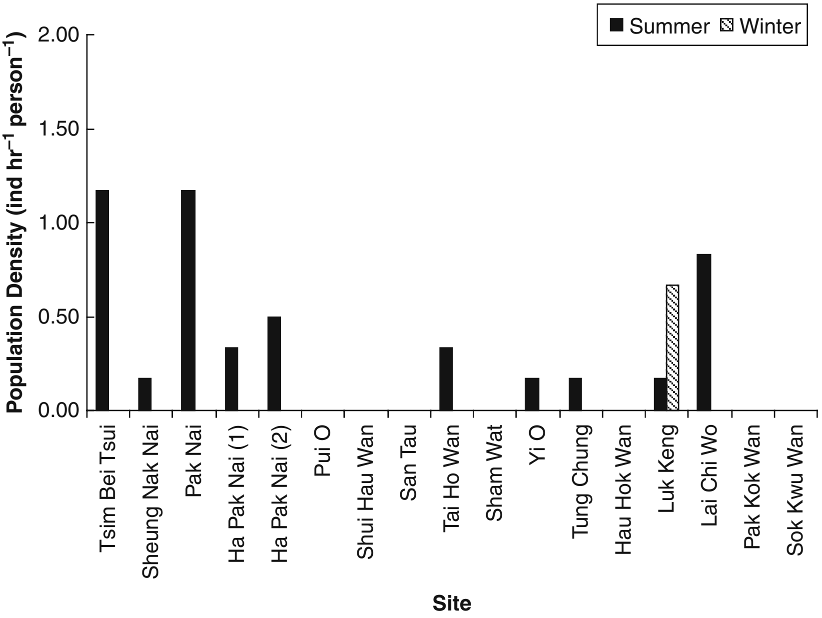

Fig. 6 Population density of juvenile horseshoe crabs (*Carcinoscorpius rotundicauda*) found by the walk-through survey

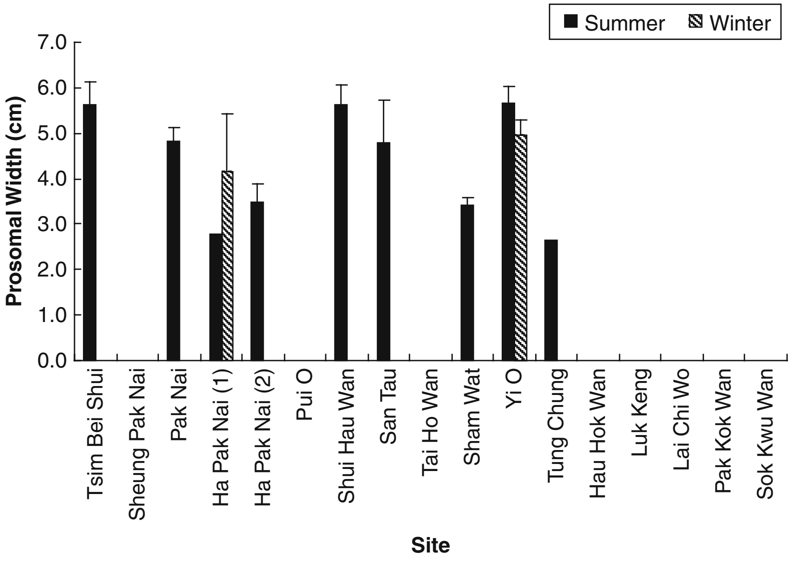

Fig. 7 Mean (+SE) size (prosomal width) of juvenile horseshoe crabs (*Tachypleus tridentatus*) recorded by the walk-through survey at the 17 shores in summer and winter

Figure 8 shows the prosomal width of *C. rotundicauda* obtained at different survey shores and ranged from 2.5 cm (Ha Pak Nai (1)) to 9.0 cm (Lai Chi Wo). The size of horseshoe crabs did not vary significantly with tidal levels ($F_{3,\ 18} = 2.92$, $P = 0.062$), seasons ($F_{1,\ 18} = 14.307$, $P = 0.932$), and their interaction ($F_{1,\ 18} = 0.119$, $P = 0.734$) but varied significantly with the survey shores ($F_{9,\ 18} = 14.307$, $P < 0.001$). From the present data, the largest horseshoe crabs were collected from 1.0 m above CD and smallest at 0.7 m above CD; the difference, however, was not statistically significant.

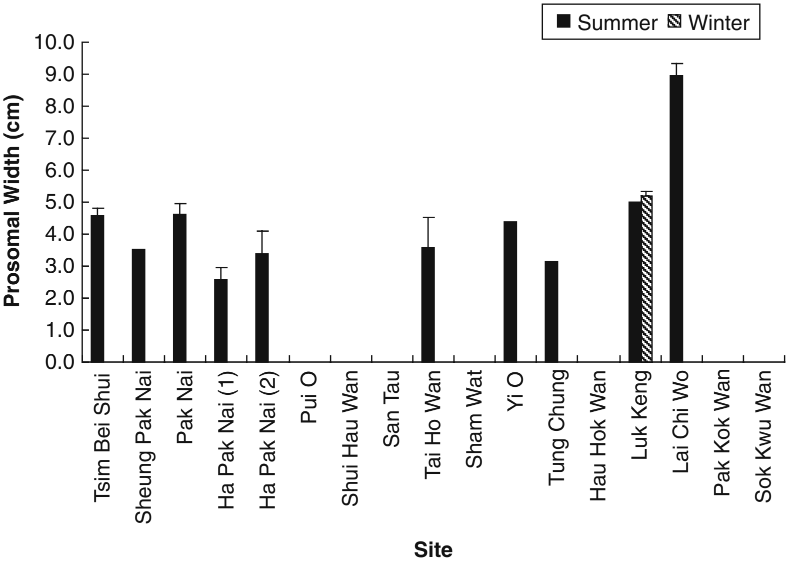

Fig. 8 Mean (+SE) size (prosomal width) of juvenile horseshoe crabs (*Carcinoscorpius rotundicauda*) recorded by the walk-through survey at the 17 shores in summer and winter

3.2 Human Exploitation of Adult Horseshoe Crabs

By interviewing the fish sellers in fish stalls, seafood restaurants, and wholesale markets, we determined that a total of 1,023 adult horseshoe crabs (mostly *T. tridentatus*) were caught throughout the year, from September 2004 to September 2005 (13-month period) (Fig. 9). Most of them were caught by shrimp trawlers, occasionally by netting and cage fishing methods. In terms of seasonal variations, more catch (about 50 ind month^{-1}) was recorded from September to December 2004 than that of the remaining months in 2005 (about 9–10 ind month^{-1}). This result was also confirmed by the interviews with fishermen that more horseshoe crabs were found in the winter than summer time. Of these adult horseshoe crabs, only 32% (332 individuals) were obtained from Hong Kong and 68% (691 individuals) from mainland Chinese waters (Fig. 9). The size ranged from 3 to 60 cm, with an average prosomal width of 30–40 cm and weight of 3 kg.

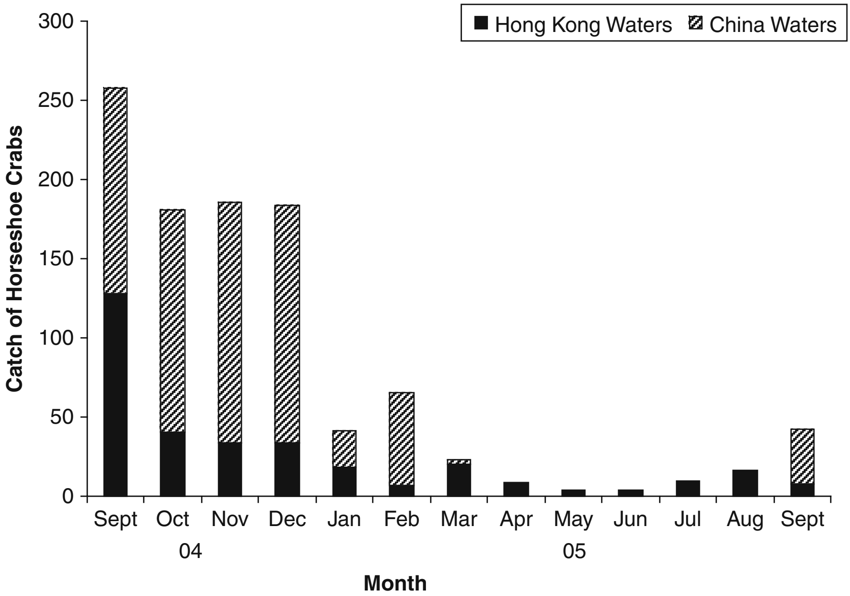

Fig. 9 Number of catch of adult horseshoe crabs (*Tachypleus tridentatus*) from both Hong Kong and mainland Chinese waters, from September 2004 to September 2005

Owing to the low market value and rarity in comparison to other marine economic species such as fish, shrimps, and crabs, about one-third of the horseshoe crabs caught in the trawl net were released immediately back to sea by the fishermen and the remaining two-thirds (about 690 individuals) were retained on board and sold to fish wholesale markets and seafood restaurants in Hong Kong. The present interview surveys noted that the majority (62%) of the adult horseshoe crabs were sold and used for the Chinese traditional ‘set-free’ rituals at sea, while the remaining (28%) were kept and served as delicacy dishes in local seafood restaurants. ‘Set-free’ rituals are practices by Chinese Buddhist followers who believe that if they release live animals back to the wild, they will have relinquished their sins of killing animals in their daily life. An average sale of 17 adult (mostly female) horseshoe crabs per month was estimated from the present surveys, with 45% of them being obtained from Hong Kong waters (Fig. 10).

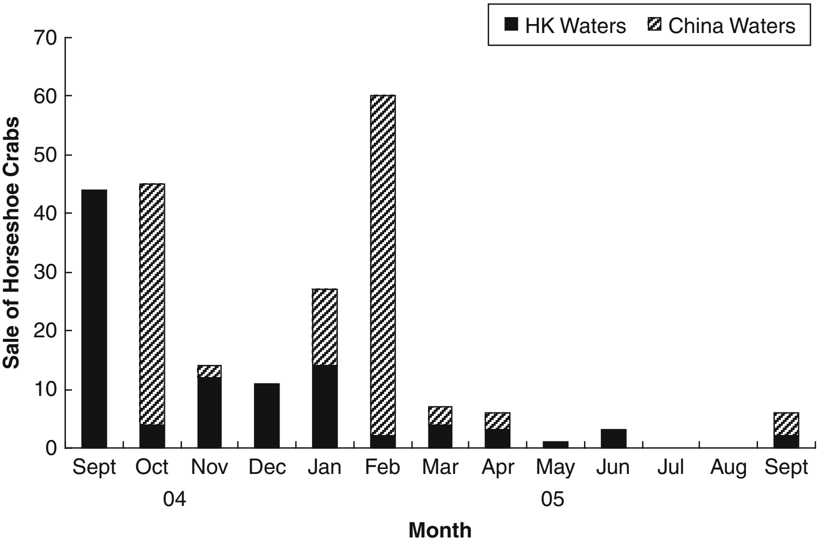

Fig. 10 Estimated sale of adult horseshoe crabs (*Tachypleus tridentatus*) caught from both Hong Kong and mainland Chinese waters for delicacy dishes in Hong Kong seafood restaurants, from September 2004 to September 2005

4 Discussion

4.1 Spatial Variation of Juvenile Horseshoe Crab Population Density

In this chapter, both western and eastern shores of Hong Kong were investigated. Most of the shores where juvenile horseshoe crabs were found are protected from wave action. In particular, those shores on the west of Hong Kong experience lower salinity in the summer owing to increased freshwater discharge from a large river system during the summer rains in mainland China. More juveniles and co-occurrence of *T. tridentatus* and *C. rotundicauda* were noted at some of these shores. The salinity at the eastern shores is relatively oceanic and stable in both summer and winter. In this chapter, only juveniles of *C. rotundicauda* were found at two of the eastern shores.

Local surveys of horseshoe crabs were conducted twice in the past, once in the period of 1995–1998 and once in 2002. In the survey from 1995 to 1998 (Chiu and Morton 1999a), horseshoe crabs were recorded from more mudflat locations than the 17 shores in this chapter (Morton and Lee 2003). Based on the present findings, juvenile horseshoe crabs were still recorded at Tsim Bei Shiu, Pak Nai, and two locations at Ha Pak Nai by the transect sampling method, while few horseshoe crabs were located in Sheung Pak Nai. One shore, Nim Wan, where juvenile horseshoe crabs were found in the previous survey has been developed as a landfill site since 1993.

This study also confirmed the presence of horseshoe crabs on Lantau Island. Based on the present results, horseshoe crabs were found at Shui Hau Wan and San Tau. However, no horseshoe crabs were found on Lamma Island and northeastern New Territories by the transect sampling method, where juvenile horseshoe crabs have been reported using the similar survey method during the study period from 1995 to 1998 by Chiu and Morton (1999a).

4.2 Decline in Juvenile Horseshoe Crab Populations

As compared with previous data, the population density of horseshoe crabs is shown to decline in the past few years. An intensive population distribution study of horseshoe crab was conducted at eight stations along the coast of northwestern New Territories from May to December in 2002 (Morton and Lee 2003), with similar sampling strategies to that of the present survey, i.e., six horizontal transects, from the shoreline down to the lower shore, with a total covering area of 1,200 m^2 (Morton and Lee 2003). In the 2002 study, horseshoe crabs were found at Sheung Pak Nai, Pak Nai, and two locations in Ha Pak Nai, with the densities of 0.10, 1.97, 1.55, and 1.14 ind 100 m^{-2}, respectively. Based on the present data in summer 2004, horseshoe crabs were recorded at all these four stations; however, no horseshoe crabs were found at Sheung Pak Nai by transect sampling, while only the density of 0.08, 0.16, and 0.23 ind 100 m^{-2} were found at Pak Nai and the two locations at Ha Pak Nai. Comparing these data, a sharp decline in horseshoe crab populations by over 90% in the past 2 years is apparent.

Another significant finding in the present survey was that no *C. rotundicauda* was found at all the survey shores in the two seasonal samplings, while this species was found at Ha Pak Nai during the survey from 1995 to 1998 (Chiu and Morton 1999a) and observed along the coast from Sheung Pak Nai to Pak Nai in 2002 (Morton and Lee 2003). In the 2002 intensive survey, only four *C. rotundicauda* were recorded in the 8-month sampling period, from May to December 2002. In this study, no *C. rotundicauda* was found at Sheung Pak Nai, Pak Nai, and Ha Pak Nai. This may imply a significant decline or even disappearance of *C. rotundicauda* within this area. Possible reasons for such declines may be continuing urban developments in the hinterland of the shores and coastal infrastructural projects, leading to habitat destruction and/or degradation and water pollution (Morton et al. 1996). Recently completed and proposed developments on the west of Hong Kong include a bridge link corridor between Hong Kong and mainland China, reclamation for a theme park on Lantau Island, establishment of container port terminals, and construction of a bridge linking west of Hong Kong with other places in mainland China.

4.3 Impact of Human Exploitation of Adult Horseshoe Crabs

There are no data on the population size and habitat range of both adult horseshoe crab species in Hong Kong waters. Potential breeding sites where juvenile horseshoe crabs are found are mostly flat, muddy shores with minimal human disturbances. Anecdotal records on adult populations were from fishermens' accounts based on their by-catch of horseshoe crabs during their trawling activities. Judging from the sharp decline of juvenile horseshoe crab populations on local shores, the sale of adult horseshoe crabs in local seafood restaurants, which seems relatively low as compared to other marine economic species, may be one of the factors for such a population decline in recent years. Based on the results of the market surveys, the sale of horseshoe crabs, especially the gravid females, would reduce production of young, significantly affecting the population due to the long maturity period and low breeding success of the adults in the wild. Human exploitation, therefore, contributes a potential impact and even puts further pressure on the scattered populations of juvenile horseshoe crabs in Hong Kong. While some of the adult horseshoe crabs are caught and kept for display in seafood restaurants, without proper care and food supply, they may eventually die in captivity. This also reduces the number of mature potential breeding horseshoe crabs in the wild and further decreases the chance of successful pair matching and breeding. The traditional 'set-free' ritual also creates a potential threat to the survival of released horseshoe crabs, as they may not be able to adapt to different habitats.

5 Conclusion and Recommendations

This chapter confirmed the sharp decline of juvenile horseshoe crabs (*T. tridentatus* and *C. rotundicauda*) on shores in Hong Kong. Habitat destruction and/or degradation and water pollution caused by continuing urban developments in the hinterland of the shores and coastal infrastructural projects are the possible major factors leading to such declines. While human exploitation of adult horseshoe crabs seemed to be low in comparison to other marine economic species, the sale of adult gravid females as delicacy dishes in seafood restaurants could result in reduction of reproductive pairs in the wild and subsequent decline in mating pairs and success of maintaining viable juvenile populations on the shores. To protect juvenile horseshoe crabs and their nursery grounds, it is proposed that these shores should be designated as Sites for Special Scientific Interest by the Hong Kong government, so that urban developments at these shores can be kept minimal. In the longer term, a comprehensive conservation strategy including public education and awareness, designation of horseshoe crabs as endangered species, ban for human consumption, and introduction of artificial breeding programs should be developed to prevent eventual loss of both *T. tridentatus* and *C. rotundicauda* in Hong Kong waters.

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Comparative Status and Assessment of *Limulus polyphemus* with Emphasis on the New England and Delaware Bay Populations

David R. Smith, Michael J. Millard, and Ruth H. Carmichael

Abstract Increases in harvest of the American horseshoe crab (*Limulus polyphemus*) during the 1990s, particularly for whelk bait, coupled with decreases in species that depend on their eggs has reduced horseshoe crab abundance, threatened their ecological relationships, and dictated precautionary management of the horseshoe crab resource. Accordingly, population assessments and monitoring programs have been developed throughout much of the horseshoe crab's range. We review and discuss implications for several recent assessments of Delaware Bay and New England populations and a meta-analysis of region-specific trends. These assessments show that the western Atlantic distribution of the horseshoe crab is comprised of regional or estuarine-specific meta-populations, which exhibit distinct population dynamics and require management as separate units. Modeling of Delaware Bay and Cape Cod populations confirmed that overharvest caused declines, but indicated that some harvest levels are sustainable and consistent with population growth. Coast-wide harvest was reduced by 70% from 1998 to 2006, with the greatest reductions within Delaware Bay states. Harvest regulations in Delaware Bay starting in the late 1990s, such as harvest quotas, seasonal closures, male-only harvest, voluntary use of bait-saving devices, and establishment of the Carl N. Shuster Jr. Horseshoe Crab Reserve, were followed by stabilization and recent evidence of increase in abundance of horseshoe crabs in the region. However, decreased harvest of the Delaware Bay population has redirected harvest to outlying populations, particularly in New York and New England. While the recent Delaware Bay assessments indicate positive population growth, increased harvest elsewhere is believed to be unsustainable. Two important considerations for future assessments include (1) managing Delaware Bay horseshoe crab populations within a multi-species context, for example, to help support migratory shorebirds and (2) anticipating the potential for harvest restrictions within Delaware Bay to redirect harvest onto outlying populations that cannot sustain the increased harvest.

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1 Introduction

Concern about the status of the American horseshoe crab (*Limulus polyphemus*) began in the 1980s (Shuster and Botton 1985) and has increased greatly since the late 1990s because of increased harvest of horseshoe crabs and awareness of the critical ecological relationship between horseshoe crabs and migratory shorebirds in Delaware Bay (Berkson and Shuster 1999; Walls et al. 2002; Odell et al. 2005). Harvest of horseshoe crabs for bait in the eel (*Anguilla rostrata*) and whelk (*Busycon carica* and *Busycotypus canaliculatus*) fisheries was effectively unregulated until the late 1990s, but harvest has decreased since then due to a series of mandatory regulations (AMFSC 2004) and voluntary use of bait-saving devices (bait bags) that reduce the amount of crab needed to bait a pot. Horseshoe crabs also are harvested to extract blood for production of *Limulus* amebocyte lysate (LAL), a highly sensitive assay for bacterial endotoxin (Novitsky 1984; Novitsky 2009). LAL-related mortality, estimated at <57,000 per year, is small compared to harvest for bait, which was nearly 840,000 in 2006 (ASMFC 2007).

Although early efforts at horseshoe crab population assessment trace back to the late 1950s (Shuster 1950, 1979; Baptist et al. 1957) and 1980s (Botton and Ropes 1987, 1988a), comprehensive, statistically robust assessments within and among populations coastwide largely began in the past decade. Comprehensive population assessments were prompted in the 1990s when the Atlantic States Marine Fisheries Commission (ASMFC) established a formal fisheries management plan for horseshoe crabs (ASMFC 1998). This plan required periodic and more consistent assessments of horseshoe crab populations coastwide (ASMFC 2004). Increased interest in the value of horseshoe crab eggs as a food source for migratory shorebirds has caused a demand for data on the status of the horseshoe crab populations and development of a multi-species approach to managing horseshoe crabs, particularly in Delaware Bay (Davis et al. 2006; Smith et al. 2006; Sweka et al. 2007). Assessments of smaller horseshoe crab populations in New England estuaries, particularly along Cape Cod, have provided a useful contrast to those of Delaware Bay. These studies point out the potential for management actions to redirect harvest onto outlying populations, which may be more vulnerable to harvest pressure (Gibson and Olszewski 2001; Carmichael et al. 2003; Rutecki et al. 2004; James-Pirri et al. 2005; Grady and Valiela 2006).

Here we synthesize recent population assessments in the context of (1) life history characteristics that are pertinent to monitoring and (2) current management principles and systems. From this information we draw conclusions about current population status and discuss implications for management actions and future assessments. Although important considerations for management of horseshoe crab populations, for simplicity, we exclude food resource and habitat assessments from this synthesis, but refer the reader to recent literature on trophic dynamics (Gaines et al. 2002; O'Connell et al. 2003; Carmichael et al. 2004) and habitat studies (Smith et al. 2002b; Jackson et al. 2008).

2 Distribution and Life History Traits Pertinent to Assessment

Breeding populations of the American horseshoe crab are unevenly distributed along the western Atlantic coast from Maine to Florida and in the Gulf of Mexico to the Yucatán Peninsula (Anderson and Shuster 2003). A large geographic discontinuity exists in the Gulf of Mexico west of Mobile Bay, which isolates the Yucatán population. A range-wide survey of genetic structure based on microsatellite DNA suggests that the distribution of American horseshoe crabs is comprised of multiple population units divided among large geographic regions: Gulf of Maine, mid-Atlantic, Atlantic Florida, Gulf Florida, and Mexico (King et al. 2005).

The life history of the American horseshoe crab is characterized by high fecundity, high egg and larval mortality, and low adult mortality (Botton and Loveland 1989; Loveland et al. 1996; ASMFC 2004). Spawning occurs on low-energy estuarine beaches. Spawning activity tends to be heaviest during spring tides associated with full and new moons (Cohen and Brockmann 1983; Barlow et al. 1986; Smith et al. 2002a). In the northern and middle Atlantic, the spawning season extends from late spring to mid-summer, i.e., late April through July (Shuster and Botton 1985; Widener and Barlow 1999; Schaller et al. 2005; James-Pirri et al. 2005). In the southern Atlantic, the spawning season extends from spring to summer, i.e., March through July (Thompson 1998). In the Gulf of Mexico, the spawning season extends from spring through fall, i.e., March through November (Rudloe 1980; Cohen and Brockmann 1983).

A female horseshoe crab will spawn on multiple tides and deposit eggs in multiple locations within the same tide (Brockmann 1990; Leschen et al. 2006). The female burrows into the sand and deposits eggs in multiple nests while moving forward (Shuster and Botton 1985; Brockmann 2003). Males remain above the sand while externally fertilizing the eggs (Sekiguchi 1988; Brockmann and Penn 1992).

The operational sex ratio of horseshoe crabs on the spawning beaches is skewed toward males because of behavior and population demographics. One male attaches to the female in amplexus, while unattached males surround the female during fertilization such that males spend more time on spawning beaches than females (Brockmann and Penn 1992). Hence, the operational sex ratio on spawning beaches is expected to be male biased compared to the sex ratio of the whole population. While juvenile populations typically show a balanced sex ratio, the sex ratio among all adults in the whole population, not just while spawning, has been observed to be somewhat skewed toward males in Delaware Bay (2.2:1 M:F; Smith et al. 2006) and Pleasant Bay, MA (2.3:1 M:F; Carmichael et al. 2003). This difference has been attributed to higher fishing or natural mortality among adult females compared to males, but also might be due to males maturing earlier than females.

In Pleasant Bay, MA, fecundity is size specific (Leschen et al. 2006). In Delaware Bay where the median female prosomal width is 265 mm (Smith

2007), fecundity was reported to be roughly 80,000 eggs per female (Shuster and Botton 1985). In Pleasant Bay where the mean female prosomal width is 230 mm (James-Pirri et al. 2005), fecundity is <60,000 eggs per female (Leschen et al. 2006). Botton and Loveland (1992) reported that in 1987 mean female prosomal width was smaller in Great Bay, NH (161 mm), than in Delaware Bay (258 mm).

Females bury eggs 15–20 cm below the beach surface (Shuster and Sekiguchi 2003; Weber and Carter 2009). Nests are disturbed when subsequent spawning causes bioturbation (Jackson et al. 2005). In areas of high spawning density, eggs are exhumed to the beach surface where they become temporarily available for consumption by other species (Jackson et al. 2005; Nordstrom et al. 2006). Nest disturbance increases with spawning density, but weather-mediated wave energy introduces a great deal of uncertainty into the rate that disturbed eggs are exhumed to the surface (Smith 2007). In Delaware Bay where horseshoe crab abundance is at its highest, exhumed eggs are critical for migratory shorebirds during their stopover each spring (Botton and Harrington 2003).

Rate of egg development in situ depends on temperature, oxygen, moisture, and disturbance from waves and bioturbation (French 1979; Jegla and Costlow 1982; Penn and Brockmann 1994; Jackson et al. 2008). Under ideal environmental conditions larvae can emerge within 2–4 weeks, although some larvae have been observed to overwinter and hatch the following spring (Botton et al. 1992). Larvae and juveniles remain nearshore in the intertidal flats and subtidal areas, usually close to breeding beaches (Botton and Loveland 2003). After the first couple of years, juveniles move out to deeper waters (Botton and Ropes 1988a).

It is generally thought that horseshoe crabs molt 16–17 times over the 9–12 years it takes to reach sexual maturity (Shuster 1979; Shuster and Sekiguchi 2003). Maximum age is thought to be 17–19 years of age (Ropes 1961; Botton and Ropes 1988b; Swan 2005). After reaching sexual maturity, it is believed that molting ceases; however, that is the subject of some debate (Carmichael et al. 2003; Shuster and Sekiguchi 2003; Smith et al. 2009). Carmichael et al. (2003) concluded in a population study in Pleasant Bay that it was plausible that adults molt throughout their life. In contrast, an analysis of over 36,000 adult horseshoe crabs from Delaware Bay concluded that adults experience a terminal molt, with sexual size dimorphism due to shorter development times for males than females (Smith et al. 2009). Whether or not adult horseshoe crabs continue to molt is an important assumption in population modeling (Grady and Valiela 2006; Sweka et al. 2007).

The general migratory pattern of horseshoe crabs is believed to be that (1) juveniles move to deeper bay water as they mature, (2) juveniles either reach sexual maturity in the estuary or migrate to and mature in the ocean, and (3) adults migrate annually from the ocean or deep bay waters to spawn on estuarine beaches (Baptist et al. 1957; Shuster 1979; Shuster and Botton 1985; Botton and Ropes 1988a; Botton and Loveland 2003; Smith et al. 2009). There is considerable evidence, however, that migratory patterns may be more complex. Smith et al. (2009) suggested horseshoe crabs in Delaware Bay exhibit sex-specific migratory patterns. Until about age 8 years, juveniles of both sexes remain within the bay. After age 8 years, females begin to migrate to the continental shelf as older juveniles and mature in the ocean. In contrast, males tend to remain within the bay to mature. After reaching maturity, both sexes migrate from the ocean or deep bay waters to spawn on the estuarine beaches. Evidence from Delaware Bay and New England waters suggest some adults overwinter in local embayments (Widener and Barlow 1999; Smith et al. 2006; Moore and Perrin 2007). For example, Botton and Ropes (1988a) concluded that horseshoe crabs in estuaries north of Long Island, NY, remained within the estuaries or close to shore in the ocean. In fact, Moore and Perrin (2007) and Watson et al. (2009) found that horseshoe crabs remained within local embayments year-round in Maine and New Hampshire, respectively. In contrast, the greatest proportion of the Delaware Bay horseshoe crabs appears to migrate to the continental shelf (Botton and Ropes 1988a, Hata 2008).

Although there are few specific estimates of mortality, horseshoe crabs are thought to have high mortality during early life stages and relatively low natural mortality among adults (Botton and Loveland 1989; Loveland et al. 1996; Botton et al. 2003; Carmichael et al. 2003). Juveniles experience particularly high mortality during the first year (Botton et al. 2003; Carmichael et al. 2003). Botton et al. (2003) estimated that 1.5 larvae survive to the end of the first summer out of 100,000 eggs, and Carmichael et al. (2003) estimated that 1 larva survived to the end of the first year out of 100,000 eggs. Survival of juveniles beyond the first year to sexual maturity is thought to be relatively high. Carmichael et al. (2003) estimated that 78% of year 1 juveniles survive to sexual maturity.

Eggs typically are deposited in beach sediments typically 5–25 cm deep and away from potential predators (Weber and Carter 2009). During mass spawning, however, eggs may be exhumed onto the beach surface or into the swash and are readily consumed by foraging fish, shorebirds, and other predators or scavengers (Botton and Shuster 2003, Nordstrom et al. 2006). These naturally exhumed eggs are unlikely to develop due to desiccation in the absence of predation, so their consumption is thought to be inconsequential to horseshoe crab recruitment. Because the quantity of exhumed eggs is related to spawning density, the importance of inter-specific relationships between horseshoe crabs and egg consumers is greatest in estuaries with high horseshoe crab abundance, such as Delaware Bay.

The most important source of natural mortality among adults is thought to be stranding associated with spawning (Botton and Loveland 1989). Botton and Loveland (1989) concluded that stranding mortality, which they estimated to be about 10% of the total population in Delaware Bay in the mid 1980s, is likely to vary among estuaries because it is affected by population density, weather and tidal conditions, and beach geomorphology. The condition of the individual, which is probably age related, is also a factor in stranding-related mortality (Penn and Brockmann 1995). Quantitative information on adult survival is very limited, but Carmichael et al. (2003) used ratios of size frequencies from 1 year of sampling in Pleasant Bay, MA, to estimate adult survival assuming that adult females continue to molt. Other sources of natural mortality include predation by sharks and loggerhead sea turtles (*Caretta caretta*) (ASMFC 1998; Keinath 2003; Seney and Musick 2007). The overall effect of predation on horseshoe crab populations, however, is not well understood.

3 Fisheries and Harvest Regulations

Historical and recent episodes of overharvest have caused declines in horseshoe crab population abundance (Shuster 2003). Recent examples include Delaware Bay (ASMFC 2004) and Mashnee Dike on Cape Cod, MA (Widener and Barlow 1999). Historically, during the late 19th century and early 20th century horseshoe crabs were harvested in the millions for use as fertilizer and livestock feed (Shuster and Botton 1985; Shuster 2003; Kraemer and Michels 2009). Harvest during the late 20th century to the present, which has been either for bait, scientific collection, or biomedical products, has also numbered in the millions (Shuster 2003; Rutecki et al. 2004; Kraemer and Michels 2009). The primary methods used to harvest horseshoe crabs include hand, trawl, and dredge.

Horseshoe crabs are harvested for use as bait in the whelk pot fishery and American eel pot fishery. Females are preferred in the eel and whelk fisheries and are harvested selectively. Males are used in the whelk fishery, but not in the eel fishery. Since horseshoe crabs are sacrificed for bait, this use results in 100% mortality of harvested crabs (Rutecki et al. 2004).

Horseshoe crabs are also harvested for their blood by the biomedical industry for the production of *Limulus* amebocyte lysate (LAL). Horseshoe crabs are bled and released alive for LAL production and most animals survive the bleeding process (Hurton et al. 2009). Hurton et al. (2009) found that the effect of bleeding on mortality depends on the amount of stress that the horseshoe crab experiences before and after the bleeding process. Hence, estimates of mortality associated with LAL production have ranged from 7.5 to 15% (Rudloe 1983; Walls and Berkson 2003; Hurton et al. 2009). Although the magnitude of coast-wide harvest for bait far exceeds harvest for LAL production, in specific estuaries biomedical harvest can exceed bait harvest (Rutecki et al. 2004). Recent collection for biomedical purposes was approximately 350,000 (ASMFC 2007), but total coast-wide mortality due to bleeding for LAL is estimated to be <57,000 coastwide, which was only 6% of horseshoe crab bait harvest in 2006 (ASMFC 2007).

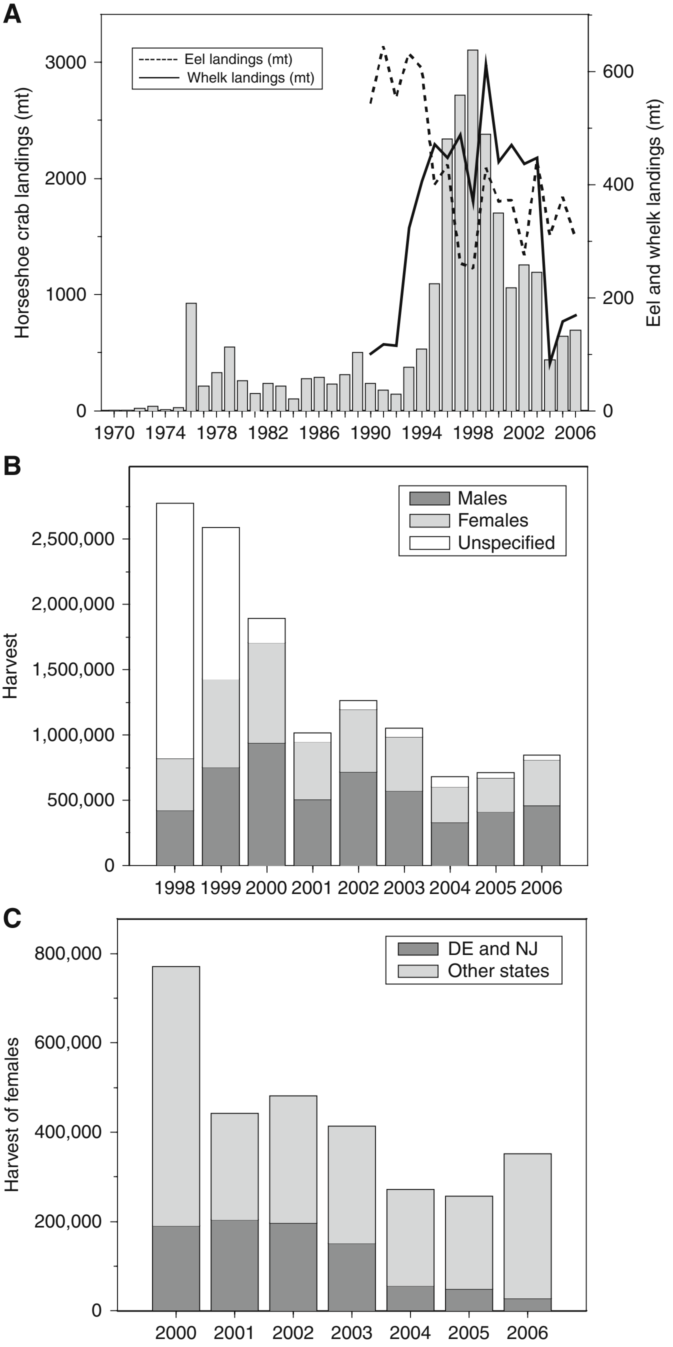
Scientific harvest typically supplies research animals for neuroscience, physiology, behavior and vision studies, among others. Although some research animals are released after use, most are sacrificed, and this use typically is associated with 100% mortality (Rutecki et al. 2004). Research use is responsible for the smallest portion of horseshoe crabs harvested annually (ASMFC 1998; Rutecki et al. 2004).

During the 1990s, the increase in harvest of horseshoe crabs was largely due to increased use as whelk bait (Fig. 1A). According to National Marine Fisheries Service (NMFS) records, landings by the whelk pot fishery increased during the 1990s and was positively correlated with horseshoe crab landings (Spearman $r = 0.76$, $P = 0.01$; Fig. 1A). In contrast, commercial landings by the American eel pot fishery in Atlantic states declined from 544 mt in 1990 to 252 mt in 1998 during the period when horseshoe crab landings increased substantially (Fig. 1A). Eel and horseshoe crab landings were negatively correlated during that time (Spearman $r = -0.83$, $P = 0.002$).

Prior to 1998, harvest of horseshoe crabs for bait was largely unregulated and underreported (ASMFC 1998). The decline in horseshoe crab abundance during the 1990s prompted the Atlantic coastal states, ASMFC, and NMFS to implement a series of harvest controls. Declines in the Delaware Bay region were particularly influential because of the ecological relationship between migratory shorebirds and horseshoe crabs in that region (Odell et al. 2005). These harvest controls along with voluntary use of bait-saving devices have been largely successful at reducing harvest; harvest declined by 70% between 1998 and 2006 (Fig. 1B). Harvest in Delaware Bay is within the range of reported landings before the early 1990s. According to NMFS records (http://www.st.nmfs.noaa.gov/st1/commercial/index.html), reported landings of horseshoe crabs from New Jersey, Delaware, Maryland, or Virginia in 2006 (352 mt) were between landings in 1989 (365 mt) and 1990 (232 mt) despite nonmandatory reporting prior to 1998.

Harvest reductions have been greatest in the Delaware Bay states where female landings declined by 85% from 190,000 in 2000 to 28,000 in 2006 (Fig. 1C). In 2005, ASMFC implemented a 2-year moratorium on the harvest of females from the Delaware Bay states; the ban began in 2006 (ASMFC 2007). Another important regulation was implemented in 2001 when NMFS established a 3,885 km^2 no-take sanctuary at the mouth of Delaware Bay, which was named the Carl N. Shuster, Jr. Horseshoe Crab Reserve after the renowned horseshoe crab scientist. The Shuster Horseshoe Crab Reserve reduces harvest on the continental shelf and in particular protects older juvenile and newly mature females, which could otherwise be harvested prior to spawning. According to population models, survival of juveniles exerts the greatest influence on population growth (Grady and Valiela 2006; Sweka et al. 2007).

The recent moratoriums in Delaware Bay states were accompanied by surges in landings elsewhere as harvest was redirected from Delaware Bay to other regions. This pattern was widespread in New England. Between 2005 and 2006, landings went from 73,740 to 171,900 in MA, from 8,260 to 15,270 in RI, and from 15,240 to 25,280 in CT. Also, landings increased in NY from 155,100 in 2005 to 172,380 in 2006 to 284,120 in 2007. Landings in VA increased from 97,960 in 2005 to 155,700 in 2006, but declined to approximately 90,000 in 2007.


A
Eel landings (mt)
Whelk landings (mt)
Horseshoe crab landings (mt)
Eel and whelk landings (mt)
0
1000
2000
3000
0
200
400
600
1970
1974
1978
1982
1986
1990
1994
1998
2002
2006
B
Males
Females
Unspecified
Harvest
0
500,000
1,000,000
1,500,000
2,000,000
2,500,000
1998
1999
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2003
2004
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2006
C
DE and NJ
Other states
Harvest of females
0
200,000
400,000
600,000
800,000
2000
2001
2002
2003
2004
2005
2006

This pattern of redirected harvest raises questions of sustainability and has triggered additional regulations. For example, harvest for bait was prohibited in Pleasant Bay, MA, in response to rapid increase in landings as fishing pressure was redirected from Delaware Bay to other populations (AS Leschen, Massachusetts Division of Marine Fisheries, personal communication).

It is not uncommon for particular states and jurisdictions to impose regulations more stringent than the ASMFC mandates. For example, New Jersey implemented a total moratorium on horseshoe crab harvesting within New Jersey waters Additional restrictions have been implemented on Federal lands in Cape Cod where harvest is banned within the Monomoy National Wildlife Refuge and Cape Cod National Seashore (MJ James-Pirri, University of Rhode Island, personal communication). In Rhode Island, harvest is prohibited during the period 48 hours preceding and 48 hours following the new and full moons during the months of May, June, and July (S Olszewski, Rhode Island Marine Fisheries, personal communication).

4 Coastal Assessments

We review three recent assessments. The first is a review of regional meta-analyses of trends in fishery-independent surveys, which were conducted as part of a coast-wide assessment (ASMFC 2004). The second is a review of assessments specific to the Delaware Bay population. The third is a review of assessments specific to New England populations. The assessments in Delaware Bay and New England provide a contrast between methods applied in estuaries that are large (Delaware Bay) and small (particularly Cape Cod embayments).

4.1 Regional Meta-analyses

There are a number of fishery-independent surveys that catch horseshoe crabs along the Atlantic coast. Although most of the surveys are inefficient at catching horseshoe crabs because of gear or survey design, the surveys can yield important information on trends when combined for analysis. Meta-analysis

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Fig. 1 Reported harvest of horseshoe crabs. (**A**) Coast-wide reported landings (mt) of horseshoe crabs, American eels, and whelk reported by Atlantic coast states. Only pot and trap harvest from 1990 on was included for the eel and whelk fisheries (source NMFS records at http://www.st.nmfs.noaa.gov/st1/commercial/index.html). (**B**) Harvest by sex (numbers of individuals) during the period of active management 1998–2006 (source ASMFC). (**C**) Harvest of females landed in Delaware Bay states vs. other states during 2000–2006, which is a period when sex was determined for most of the harvest (source ASMFC) methods are a set of techniques that are useful for combining independent data to gauge whether the datasets are showing the same patterns or effects.

Meta-analyses were structured by region. The regions were the Southeast Region, the Mid-Atlantic/Delaware Bay Region, the New York Region, and the New England Region. There were three surveys for the Southeast Region, eight surveys for the Mid-Atlantic Region, four surveys for the New York Region, and four surveys for the New England Region. The ASMFC stock assessment report includes detailed descriptions of each survey (ASMFC 2004). The latest year included at the time of the assessment was 2003, but for some of the surveys the available data ended in 2002. The catch indices from the fishery-independent surveys varied in scale. Thus, the indices were standardized by subtracting the mean and dividing by the standard deviation to make the results comparable.

Within some regions the trends in the surveys were heterogeneous, which influenced the groupings for meta-analysis. The Southeast and Mid-Atlantic Region surveys showed similar patterns and were analyzed together. The New York Region surveys showed varied trends and were divided into two groups: western Long Island/Long Island Sound and eastern Long Island. The New England Region surveys showed varied trends and were divided into two groups: Cape Cod and Narragansett Bay.

The meta-analyses involved fitting separate linear regressions to the standardized indices and then combining the regression results to see if a consensus conclusion could be reached. The following meta-analysis techniques were used (Manly 2001): (1) the Fisher's method tests the hypothesis that at least one of the surveys shows a significant decline; (2) the Stouffer's method tests the hypothesis that there is a consensus among survey-specific analyses for a decline; (3) a weighted standardized slope along with confidence intervals tests the hypothesis that the surveys show a significant decline on average.

The summary of the meta-analyses results is presented in Table 1. Detailed results can be found in ASMFC (2004: Appendix C). In the Southeast Region, the time series were comparably short (maximum duration was 1995–2003), and there was no evidence for decline. In the Mid-Atlantic/Delaware Bay Region, there was a clear preponderance of evidence that populations declined from the late 1980s to the present. In the New York Region, there was no evidence for a linear decline; however, patterns differed among the two groupings. In western Long Island/Long Island Sound, there was evidence of population increases; three of the five surveys showed a significantly positive trend and a fourth was a marginally significant positive trend. In eastern Long Island/Peconic Bay, the current levels were consistent with levels during the mid-1980s; however, there has been a decline from peak levels during the early to mid-1990s. In the New England Region, the Narragansett Bay surveys indicated population decline from the mid-1970s to the present. However, around Cape Cod the trends were less clear. Although there was some evidence for declines, the data were also consistent with a hypothesis of population stability.

Table 1 Summary of horseshoe crab population meta-analyses from fishery-independent data collected up to 2003 (ASMFC 2004), grouped by region because patterns in relative abundance were region specific. Three meta-analytic techniques were employed to test for (1) at least one survey showing a decline (Fisher's method), (2) consensus support for declines among surveys (Stouffer's method), and (3) a weighted average slope showing a decline (confidence interval of weighted slope <0; Manly 2001). WLI = western Long Island, ELI = eastern Long Island, NB = Narragansett Bay, CC = Cape Cod

			Support for decline				
Region	Number of surveys	Duration	% showing decline*	At least one survey supports decline?	Consensus support?	Weighted slope supports decline?	Population status
Mid-Atlantic	8	1988–2003	75	Yes	Yes	Yes	Decline
New England	(NB) 4	1975–2002	100	Yes	Yes	Yes	Decline
	(CC) 4	1978–2002	25	Yes	Yes	No	Decline
New York	(WLI) 5	1987–2003	0	No	No	No	Stability or increase
	(ELI) 2	1980–2003	0	No	No	No	Consistent with mid-1980s, declined from peak (1990s)
Southeast	3	1995–2003	0	No	No	No	Stability

*(P-value ≤ 0.10).

It is important to note that the meta-analysis was completed prior to the most recent harvest regulations enacted in 2004 through 2006, which greatly reduced harvest pressure in Delaware Bay, but redirected harvest pressure to outlying populations, particularly in New York and Massachusetts. The redirected harvest caused concern among managers that increased harvest outside of Delaware Bay is not sustainable, prompting localized harvest closures (e.g., Pleasant Bay, Massachusetts) and proposals for other harvest restrictions (AS Leschen, Massachusetts Division of Marine Fisheries, personal communication).

4.2 Delaware Bay Assessments

Recently, there have been several assessments specific to the Delaware Bay population based on population estimates, trend analyses, and population models (Smith et al. 2002a; Hata and Berkson 2003 and 2004; Davis et al. 2006; Smith et al. 2006; Sweka et al. 2007; Smith and Michels 2006). Population estimates have been based on benthic trawl surveys (Hata and Berkson 2004) and mark–recapture studies (Smith et al. 2006). Trend analyses have been based on ongoing surveys that were designed specifically for monitoring horseshoe crab populations, including a trawl survey (Hata and Berkson 2003) and a bay-wide spawning survey (Smith et al. 2002a; Smith and Michels 2006). Population modeling included application of surplus production models (Davis et al. 2006) and age-based projection models (Sweka et al. 2007).

Methods for estimating absolute and relative abundance have included spawning surveys (Smith et al. 2002a), offshore trawl surveys (Hata and Berkson 2004), and tagging studies (Smith et al. 2006). Because Delaware Bay is a large estuary and horseshoe crabs migrate seasonally, great care must be taken to sample adequately in space and time to get a representative sample of the population. Smith and Michels (2006) evaluated the effect of spatial and temporal coverage on trends based on the spawning survey and concluded that misleading inference results from inadequate sample coverage. For example, the timing of spawning in Delaware Bay is determined, in part, by water temperature. Heavy spawning does not occur until after water temperatures are consistently above 15°C (Smith and Michels 2006). As a result, during years when water temperatures are warm, spawning starts early and the season lacks a strong single peak in spawning. During years when water temperatures are cool, spawning starts late and the season has a strong single peak that coincides with warming temperatures. Thus, if the survey were conducted only during peak spawning, then trends in peak spawning do not accurately reflect trends in spawning activity throughout the spawning season. Trawl surveys must consider issues of gear inefficiency as well as spatial and temporal coverage of tows (Hata and Berkson 2003). Tagging studies have to consider issues of tag loss, tag-induced mortality, and heterogeneity of capture (e.g., sex-specific recapture probability) (Smith et al. 2006).

Estimates for absolute abundance have ranged widely. Trawl-based estimates were 7.1 million adults offshore of Delaware Bay (Hata and Berkson 2003). In contrast, tagging-based estimates were 20 million spawning adults (male and female combined) in Delaware Bay during 2003 (Smith et al. 2006). Smith et al. (2006) discussed reasons for the disparate estimates including gear inefficiencies, unknown proportion of the population that remained in the estuary, and bias due to assumption violation. ASMFC (2006) evaluated the effect of assumption violations on the mark–recapture estimates, in particular the sensitivity of the model to overlooked tags during the spawning survey. Overlooking tags during recapture would result in overestimation of abundance and underestimation of harvest rate. The authors investigated a range of possible rates of overlooking tags and found that harvest remained below 10% of estimated population abundance (ASMFC 2006: Appendix B).

Although estimates of absolute abundance have varied, the recent trends in relative abundance have been consistent regardless of method (Table 2). Estimates of relative abundance of adults from the offshore trawl survey (Hata 2008) and the spawning survey (Michels et al. 2008) have been highly correlated ($r = 0.77$ for females, $r = 0.94$ for males) for the years 2002 through 2007 when both surveys have been implemented (Fig. 2). The spawning survey has been implemented following a consistent statistical design since 1999 (Smith and Michels 2006). Density of spawning males has increased significantly (slope $= 0.196$; $\mathrm{SE} = 0.0438$; $P = 0.003$). Density of spawning females has not changed significantly during that period (slope $= 0.011$; $\mathrm{SE} = 0.0103$; $P = 0.31$). Because males mature earlier than females (Shuster and Sekiguchi 2003; Smith et al. 2009), the increasing density of spawning males could be a leading indicator of significant increases in female spawning density. Based on the offshore benthic trawl survey, Hata (2008) concluded that all demographic groups (i.e., juveniles, adult males, and adult females) of the Delaware Bay horseshoe crab population have been increasing since 2003 (Table 2). For example, catch per tow from the offshore benthic trawl survey has increased by 1.4 on average ($\mathrm{SD} = 0.16$) each year since 2003. Hata (2008) also observed an increase in the smallest size classes caught by the trawl gear (i.e., >100 mm or >7 years old), indicating increased recruitment to the population. Smith et al. (2009)

Table 2 Summary of recent trend assessment by demographic group for horseshoe crabs in the Delaware Bay region

Demographic group	Assessment
Adult females	Stable[1] or Increase[2]
Adult males	Increase[1,2]
Juveniles	Increase[2,3]

Data come from surveys designed specifically to monitor horseshoe crabs:

[1] Delaware Bay spawning survey from 1999 to 2007 (Michels et al. 2008).

[2] Offshore benthic trawl survey from 2002 to 2007 (Hata 2008).

[3] Bay-wide tagging study from 2003 to 2005 (Smith et al. 2009).

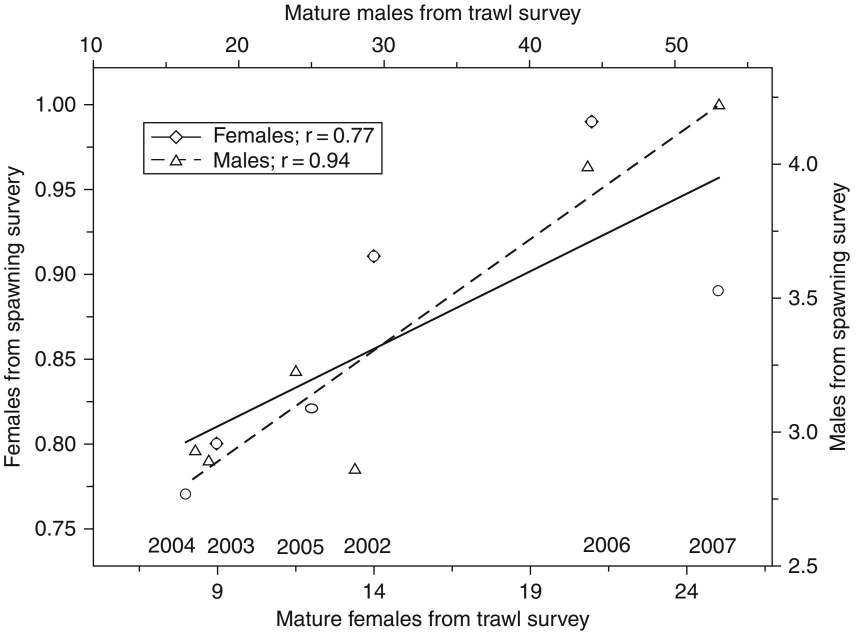

Fig. 2 Comparison between the offshore benthic trawl survey (Hata 2008) and Delaware Bay spawning survey (Michels et al. 2008) during the years when both were conducted. Correlation between the results were $r = 0.77$ for females and $r = 0.94$ for males

observed expanding size distributions among juveniles (n = 9,075) during 2003–2005 (Kolmogorov–Smirnov Test, $P < 0.001$).

Observations of recent increases in horseshoe crab abundance were widespread within the Delaware Bay region. The long-term trends from the late 1980s to the present in fishery-independent catches of horseshoe crabs indicate a population that declined during the 1990s and began a recovery in the early 2000s (Fig. 3). We updated the meta-analysis of ASMFC (2004), which was summarized in Section 4.1, using data from surveys conducted within the Delaware Bay region from 1999 to 2007. These years correspond to the time since harvest regulation began. Surveys were NMFS spring and fall trawl survey, which combines the catches of adults and juveniles (B Kramer, NMFS, unpubl. data), state-specific Delaware Bay trawl surveys in Delaware and New Jersey, which record separately the catches of adult females, adult males, and juveniles (SF Michels, Delaware Division of Fish and Wildlife, J Brust, New Jersey Division of Fish and Wildlife unpubl. data), Delaware Bay spawning survey, which counts separately adult females and adult males (Michels et al. 2008), and offshore benthic trawl survey, which records separately the catches of adult females, adult males, and juveniles (Hata 2008). These surveys are described in Appendix C of ASMFC (2004). Among the 13 surveys, 7 (54%) had a significantly positive slope ($P \leq 0.10$). Three surveys

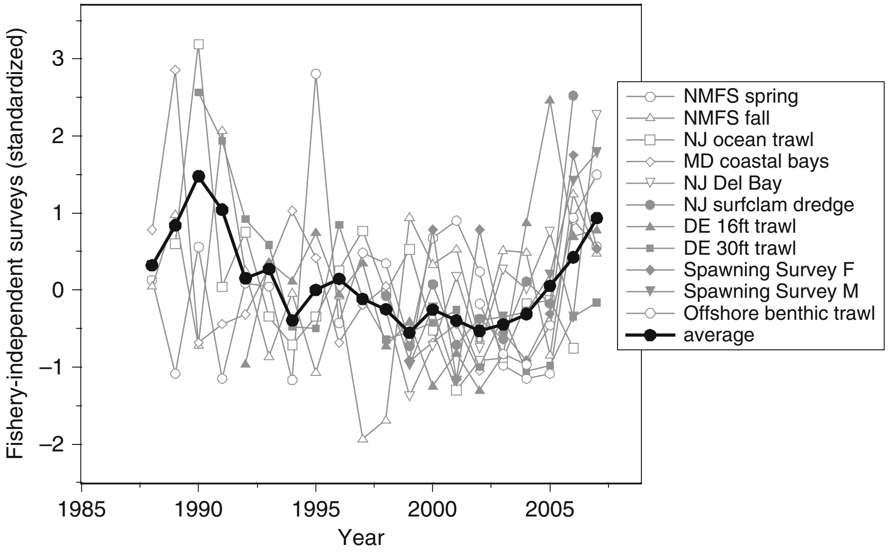

Fig. 3 Standardized catches from fishery-independent surveys, which consistently catch horseshoe crabs in the Delaware Bay region. Surveys were listed and described in ASMFC (2004). Standardization was by subtracting the mean and dividing by the SD. The dark line is the average of the standardized catches

caught juveniles (New Jersey and Delaware trawls and offshore benthic trawl), and all showed significantly increasing catches. Four surveys recorded sex-specific catches of adults (New Jersey and Delaware trawls, offshore benthic trawl, and spawning survey), and three out of four showed significant increases in catches of males and one out of four showed significant increases in catches of females. There was no evidence of declines (Fisher's method: S = 6.61, df = 26, *P* = 0.99). Also, the weighted slope among all surveys was significantly positive (RMSE-weighted slope = 0.22, SE = 0.045, 90% CL = 0.14–0.30).

Consistent with an expanding population, evidence for increases was strongest among juvenile catches, evident among adult males, and tentative among adult females. This pattern reflects the horseshoe crab maturity schedule, which determines that an expanding population would appear first among juveniles, then among adult males, and finally among adult females. Males mature 1–2 years earlier than females (Smith et al. 2009).

Various aspects of the population dynamics of the Delaware Bay population have recently been assessed by modeling (Davis et al. 2006; Sweka et al. 2007). Davis et al. (2006) applied an age-aggregated production model to a set of standardized fishery-independent and fishery-dependent datasets. They concluded that the Delaware Bay population was depleted and was subject to high fishing mortality. Sweka et al. (2007) created an age-structured population model to evaluate effect of harvest (magnitude and timing) and density-dependent egg mortality on population dynamics. The age-structured model was parameterized with survival and fecundity from the literature. They found that the probability of the population increasing was high (>80%), with low and medium egg mortality and harvest less than 200,000 females per year. They concluded that population growth was most sensitive to early life stage survival.

In general, these assessments of the Delaware Bay population of horseshoe crabs indicate that (1) relative abundance declined during the 1990s, but has stabilized since 2000 and is showing signs of increase; (2) fishing mortality rate in the 1990s exceeded fishing mortality associated with maximum sustainable yield (F_{MSY}), peaked around 1998, and has been declining since then; and (3) current harvest in Delaware Bay is at or below harvest in the early 1990s (ASMFC 2006). Harvest regulations and controls implemented since the late 1990s, such as harvest level reductions, seasonal closures, and the establishment of the Carl N. Shuster Jr. Reserve, have coincided with an abatement of the declines in abundance that were observed in the 1990s. Recent monitoring data indicate an increasing horseshoe crab population in Delaware Bay (Hata 2008; Michels et al. 2008).

Because the goals of horseshoe crab management in Delaware Bay are linked to shorebird populations, it is important to recover the horseshoe crab population to a level that will provide eggs for sufficient shorebird weight gain during the migratory stopover (Berkson and Shuster 1999; Sweka et al. 2007). In Section 6, we discuss current assessments and management frameworks that are taking into account the multi-species objectives in horseshoe crab management.

4.3 New England Assessments

Horseshoe crab populations in New England, in particular those around Cape Cod, have been the subject of groundbreaking studies and assessments. In contrast to the Delaware Bay region where there is one large and intermixing horseshoe crab population (King et al. 2005; Swan 2005), the New England region is composed of a series of coastal embayments, which support populations with relatively limited movement between them (James-Pirri et al. 2005; Moore and Perrin 2007). A consistent conclusion arising from these assessments has been that the population characteristics differ among the embayments and management must be local (Rutecki et al. 2004; James-Pirri et al. 2005).

Gibson and Olszewski (2001) used a production model to assess horseshoe crab populations in Rhode Island. Because harvest statistics were inaccurate for some of the time series, the production model was applied in a novel way, treating the relative abundance indices as accurate and using the model to reconstruct the unknown harvest statistics. The resulting model was used to project population growth under various harvest regimes. The resulting assessment indicated that stock recovery (to maximum sustainable yield biomass) would take 10 years under no harvest and 20 years under limited harvest at 26 mt or fishing mortality (F) below 0.25. Harvest was reduced based on modeling results, and recent monitoring suggests the restrictions have contributed to population growth (S. Olszewski, Rhode Island Div of Fish and Wildl., personal communication).

Widener and Barlow (1999) observed a dramatic decline of the horseshoe crab population that spawned in Mashnee Dike, a small embayment in western Cape Cod. The decline was attributed to high harvest of the Mashnee Dike population during the 1990s. The observations at Mashnee Dike provide an important, but limited, example of how a local population can be overharvested and why management needs to be local. The dramatic decline and near-extirpation observed at Mashnee Dike (95% reduction between 1984 and 1999) apparently was not a widespread phenomenon throughout Cape Cod (Carmichael et al. 2003; James-Pirri et al. 2005) and certainly does not represent all coast-wide patterns (ASMFC 2004; Table 1).

Carmichael et al. (2003) assessed abundance and size structure of horseshoe crabs during late spring and summer 2001 in Pleasant Bay, MA, which is a shallow estuary on Cape Cod. The shallow depths and clear water made the bay conducive to the use of quadrat and transect sampling throughout most of the bay to count individual horseshoe crabs of all sizes and extrapolate to estimate abundance. A complete size distribution was described, assuming that catchabilities of all sizes were equal, and a life table approach (i.e., ratios of size-specific abundances) was used to estimate size-specific mortality. This was the first comprehensive effort to estimate abundance and population structure in Pleasant Bay and has provided the basis for assessments of harvest rate (Rutecki et al. 2004) and population modeling (Grady and Valiela 2006) for the Pleasant Bay population.

James-Pirri et al. (2005) measured interannual variation in spawning activity, size structure, and movement in four Cape Cod embayments, including Pleasant Bay. Spawning was patchily distributed throughout Cape Cod with high spawning densities isolated to a few hot spots compared to most spawning areas in Cape Cod. They suggested these few spawning hot spots were responsible for the majority of spawning activity on Cape Cod. Spawning densities on Cape Cod was much lower (e.g., two orders of magnitude lower) than on Delaware Bay beaches, which explains, in part, why horseshoe crab eggs are not a primary food source for shorebirds on Cape Cod. James-Pirri et al. (2005) observed differences in adult sizes among several Cape Cod embayments with embayment-specific growth rates offered as a possible hypothesis.

4.4 Comparison of Delaware Bay and New England Assessments

The Delaware Bay region is dominated by one large population, and the management objectives are driven by the inter-specific relationship with migrant shorebirds. As a result, current assessments have focused exclusively on the Delaware Bay population, and methods for multi-species management are evolving (Hata and Berkson 2003; Davis et al. 2006; Smith et al. 2006; Sweka et al. 2007; Hata 2008; Michels et al. 2008). In contrast, the New England region is comprised of many embayment-specific populations with unique characteristics, such as spawning densities, size structure, and harvest pressure. Consequently, New England assessments have been a mix between single (Gibson and Olszewski 2001; Carmichael et al. 2003) and multiple (Rutecki et al. 2004; James-Pirri et al. 2005; Grady and Valiela 2006) population focus.

4.4.1 Assessment Methods

Differences in water depth, water clarity, and bottom type have resulted in different sampling methods among regions. In the shallow and clear waters of Pleasant Bay, Carmichael et al. (2003) used quadrat and transect sampling to sample all sizes of horseshoe crab. These methods have been used in Delaware Bay, but only in nearshore habitats to sample young-of-the-year juveniles (Botton et al. 2003). Because of the large size and deep waters in the Delaware Bay, trawl and dredge surveys are required to monitor horseshoe crabs in the Delaware Bay and in the Atlantic Ocean where older juveniles and adults migrate (Hata and Berkson 2003; Smith et al. 2006; Hata 2008).

Despite differences in sampling methods, similar assessment and population estimation methods have been applied in Delaware Bay and New England. Surveys of spawning activity and egg density have been applied in both regions (Widener and Barlow 1999; Smith et al. 2002a; Pooler et al. 2003; James-Pirri et al. 2005). Tagging has been used in both regions to assess movement (Shuster 1950; Baptist et al. 1957; Swan 2005; James-Pirri et al. 2005; Moore and Perrin 2007). Methods employed for analysis of size structure have been similar (Carmichael et al. 2003; Smith et al. 2009). Surplus production models have been applied to the Delaware Bay population (Davis et al. 2006) and to the Narragansett Bay population (Gibson and Olszewski 2001). Matrix projection models have been applied to the Delaware Bay population as an age-structured model (Sweka et al. 2007) and to three Cape Cod estuaries as a stage-structured model (Grady and Valiela 2006).

4.4.2 Assessment Results

The derivation of intrinsic growth rate of horseshoe crab populations presents an interesting and potentially important contrast that has emerged among the assessments. The intrinsic growth rate is the maximum per capita rate that a population can grow. Interestingly, the greatest contrast is not between Delaware Bay and New England assessments, but between theoretical, empirical, and modeling derivations of intrinsic growth.

Theoretical and Empirical Estimates of Population Growth

Gibson and Olszewski (2001) used theoretical models (Robertson 1979; Boudreau and Dickie 1989) to predict that intrinsic rate of growth for horseshoe crabs is in the neighborhood of 0.5 (finite growth rate = 1.6). Empirical data from Narragansett Bay based on the University of Rhode Island Graduate School of Oceanography trawl survey indicated that during 1968–1975 the horseshoe crab population underwent a period of increase typified by density-independent growth. The rate of increase for that period was 0.52, which agrees closely with Gibson and Olszewski's theoretically based estimates. There is also agreement with the recent trajectory of population growth apparent in the offshore benthic trawl survey of the Delaware Bay population (Hata 2008). The instantaneous growth rate calculated from the offshore trawl survey averages 0.35, which is below the intrinsic growth rate of 0.5. Growth from the trawl survey was measured during a time when the population was being harvested and was large enough for density-dependent bioturbation to affect egg development (Smith 2007). Hence, observed growth rate is expected to be lower than the intrinsic growth rate.

Modeled Estimates of Population Growth

In contrast to theoretical and empirical estimates, the intrinsic growth rates derived from recent age- and stage-based models are an order of magnitude lower than predicted by Gibson and Olszewski (2001) or observed in data from Rhode Island or Delaware Bay. Sweka et al. (2007) computed intrinsic growth rate based on an age-structured model parameterized with survival and fecundity from the literature. Their model resulted in a growth rate of approximately 0.05 for low-spawning biomass, which corresponds to intrinsic growth rate. Grady and Valiela (2006) computed an intrinsic growth rate of 0.05–0.1 based on their stage-structured model. Given the agreement between the theoretical and empirical growth rates (i.e., intrinsic growth rate appears to be in the order of 0.5), the intrinsic growth rates from Sweka et al. (2007) and Grady and Valiela (2006) might be low and their population projections might be conservative. One possible explanation for the discrepancy is that the estimates of early life and juvenile stage survival could be underestimated because both Sweka et al. (2007) and Grady and Valiela (2006) found that population growth was most sensitive to early life and juvenile stage survival. Thus, small negative bias in early life and juvenile stage survival would result in large negative bias in population growth.

5 Evidence for Local or Regional Management Initiatives

Review of the available life history and population assessment data reveals several lines of evidence, which suggest that management of horseshoe crab populations should be specific to either embayments or regions (some of this evidence has been discussed in detail above). Here we summarize the most compelling points. First, movement of crabs may be regionally or locally limited. Botton and Loveland (2003) found that larvae exhibit limited capability to disperse from natal beaches. Genetic structure indicates that males disperse at higher rates than females, and female-mediated gene flow among embayments is limited (Pierce et al. 2000, King et al. 2005). Accordingly, tagging data indicate that although capable of migrating great distances, a majority of adult crabs remain within local regions and some overwinter in local embayments (ASMFC 2004; James-Pirri et al. 2005; Swan 2005; Smith et al. 2006; Moore and Perrin 2007). These data are further supported by stable isotope analyses, which indicate adult crabs are loyal to local feeding grounds (Carmichael et al. 2004, O'Connell et al. 2003).

Second, trends in horseshoe crab abundance and population dynamics differ among regions (ASMFC 2004). In particular, smaller sized populations such as those in Cape Cod waters may be localized based on spawning densities, size structure, and movement patterns (Carmichael et al. 2003; James-Pirri et al. 2005). Since different types of harvest (bait, biomedical, or scientific) select for different size and sex segments of the population, different populations may experience different harvest pressures due to their location-specific population dynamics (Rutecki et al. 2004).

Third, different embayments and regions are subject to different types of harvest for different purposes. In Delaware Bay waters, commercial harvest is conducted by hand and dredge (Kraemer and Michels 2009), while in areas such as Cape Cod most harvest is conducted by hand from local beaches (Rutecki et al. 2004). In Delaware Bay, the majority of harvested crabs are collected for bait. In contrast, among Cape Cod populations, the primary purpose for which crabs are harvested (bait, biomedical, or scientific) varies by embayment (Rutecki et al. 2004) with bait harvest predominating except in Pleasant Bay where only biomedical harvest is permitted (AS Leschen, Massachusetts Division of Marine Fisheries, personal communication). Since mortality associated with each harvest type varies, the extent of harvest pressure and depletion by overharvest also necessarily varies among embayments (Widener and Barlow 1999; Rutecki et al. 2004). Hence, there is strong support for local management based on regional or sub-regional population structure and harvest pressures.

6 Future Assessments: Control of Redirected Harvest and Multi-species Adaptive Resource Management

For the past decade and for the foreseeable future, management of the American horseshoe crab has been and will be driven by concern for the migratory shorebirds that depend on horseshoe crab eggs in Delaware Bay (Odell et al. 2005). Focus on Delaware Bay causes two central challenges for coast-wide management. First, standard fishery management methods for determining sustainable harvest do not meet the main objective of managing the Delaware Bay horseshoe crab population to support shorebird needs. Second, as harvest of the Delaware Bay population is reduced to meet shorebird needs, harvest pressure is redirected to outlying populations, which might not be able to sustain increased harvest.

Standard fishery management methods often aim to find the fishing rate that leads to a maximum sustainable yield. While this is traditionally an important fishery management goal, it misses the mark when managing simultaneously for multiple related species. In the case of the Delaware Bay horseshoe crab population, the fishery management goal is to manage harvest consistent with continued use by current and future generations of the fishing and non-fishing public, migrating shorebirds, and other dependent wildlife (ASMFC 1998). There is general agreement among managers and researchers that within Delaware Bay a sustainable red knot (*Calidris canutus rufa*) population should be the driving force of horseshoe crab management actions. Red knots have declined dramatically in recent years and decreased availability of horseshoe crab eggs in Delaware is a likely factor (Baker et al. 2004).

The ASMFC is currently developing a structured decision or adaptive resource management process (Clemen and Reilly 2001; Williams et al. 2002) for multi-species management. The elements of the decision include objective(s), management alternatives, models to predict consequences of management, and monitoring programs to track results of management. The multi-species objectives are to regulate a sustainable harvest of horseshoe crab populations and provide sufficient horseshoe crab eggs to support red knot recovery goals. Management alternatives include a range of male and female harvest levels. The predictive model has four components: (1) a horseshoe crab dynamics model, (2) a model that links horseshoe crab abundance to egg availability, (3) a model that links egg availability to red knot weight gain, and (4) a red knot dynamics model that relates weight gain to red knot adult survival and reproduction. The monitoring programs that have been developed over recent years can satisfy the needs of the adaptive resource management process. A working group comprised of shorebird and horseshoe crab researchers is actively developing the adaptive resource management process.

Apparent progress toward recovery of the Delaware Bay horseshoe crab population (Michels et al. 2008; Hata 2008) could be a Pyrrhic victory for populations along the Atlantic coast if harvest is redirected to smaller embayments that cannot sustain the increased harvest pressure. In the implementation of an adaptive resource management process, allowable harvest should be determined for Delaware Bay that is consistent with multi-species objectives, and harvest in embayments outside the Delaware Bay region should not be permitted to expand beyond what is sustainable. Thus, effective coast-wide management will require identifying local populations, determining sustainable level of harvest for each population, and re-evaluating existing quotas to match sustainable levels. A focused and integrated effort among horseshoe crab biologists, researchers, and managers throughout the Atlantic coast is required to achieve this goal.

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An Integrative Approach to Horseshoe Crab Multiple Use and Sustainability

Jim Berkson

Abstract This chapter is designed as a perspective piece, using personal opinions to further the discussion about horseshoe crab management, a relatively new topic. I argue that horseshoe crab management and the science that management supports has been one-dimensional, focused almost exclusively on the commercial harvest. Fortunately, we can use lessons learned from fisheries management over time to guide us and adapt these lessons specifically to horseshoe crab management. Progress will require the incorporation of concepts including precautionary management, integrated assessments, cumulative impacts, and ecosystem-based management as we move beyond resource conservation to sustainable resource utilization.

1 Introduction

This chapter will address the topic of horseshoe crab management, unlike most of the chapters in this book, which address horseshoe crab research. It will focus on the future instead of the past. Ultimately, with this chapter, I'll try to expand on the current debate over the goals for management, the methods used to create and evaluate management measures, and, ultimately, the current direction we're heading in terms of management. This is meant to be a perspective piece, using my own opinions, opinions that I believe are drawn from sound fact. I will focus on the horseshoe crab (*Limulus polyphemus*) population with which I am most familiar, the Delaware Bay population. My bottom line argument is that we are thinking far too narrowly in terms of management

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Opinions expressed in this chapter are those of the author and not necessarily those of the National Marine Fisheries Service, NOAA, or any agency.

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for this ecologically, commercially, and medically essential species. Fortunately, we can apply many of the lessons that have been learned with regard to fisheries management over the years in setting our course forward.

The best place to begin this chapter is with an examination of its title: "An integrative approach to horseshoe crab multiple use and sustainability."

- "Multiple use" is a term used to refer to a natural resource that has more than one application valued by humans. It is commonly used to describe natural resources such as forests (Schuler and Meadows 1975), water (Boelee et al. 2007), and even marine-protected areas (Brown et al. 2001). The majority of harvested marine species aren't typically considered to have multiple uses to humans, but horseshoe crabs are an exception (Berkson and Shuster 1999). They are caught in a commercial fishery, are used as bait in whelk and eel fisheries, provide food in the form of eggs to migratory shorebirds, and provide a chemical used in the testing of vaccines and implantable devices (Berkson and Shuster 1999). The importance of the horseshoe crab population goes beyond its value to any one individual user or interest group.
- "Sustainability" means the property of being maintained without giving way or yielding, i.e., the property of continuing existence without loss. It's hard to find anyone not in favor of managing a natural resource sustainably. The problems arise when those involved try to agree as to what should be sustained. With regard to natural resources, goals have included sustainable development (Kaasa 2007), sustainable communities (King and Hood 1999), sustainable fisheries (Ault et al. 2006), sustainable landscape (Pavlovic and Benjamin 1999), and sustainable ecosystem-based management (Pavlovic and Benjamin 1999). Environmentalists, resource users, and policymakers alike favor the term sustainability, but in the case of the horseshoe crab, to what are they referring? Environmentalists refer to the migratory shorebird population. Commercial fishermen refer to the commercial fishery. Pharmaceutical companies refer to *Limulus* amebocyte lysate (LAL) production. The goal of the current management plan is to manage the population for its continued use by current and future generations of the fishing and non-fishing public, migrating shorebirds, and other dependent wildlife including federally listed sea turtles. In summary, the management plan is trying to keep all aspects sustainable.
- An "integrative approach" is a method which brings together pieces of a system. Integrative approaches to natural resource management examine ways to combine the multiple dimensions of a system when assessing management options. The world of the horseshoe crab is obviously complex, with many factors acting as stressors on the population, including the commercial fishery, the biomedical industry's bleeding programs, pollution, habitat destruction, and climate change. But, horseshoe crab management is currently one-dimensional. Management is focused on the commercial fishery. In fact, it is assumed that the persistence and sustainability of the horseshoe crab population, its multiple uses, and the migratory shorebird

> populations are primarily, if not solely, dependent on the management of the commercial fishery. This one-dimensional view moves forward despite our knowledge that the system is more complex than this and there are many additional stressors that we must monitor and manage. Is this one-dimensional approach serving us well?

If we combine these technical explanations and their context, we end up with the following:

Effective management requires (1) an acknowledgment by all involved that the importance of the species transcends its value to any one user or interest group; (2) methods, which will allow true sustainability to be achieved for all users; and (3) an integrative approach to management, which incorporates all substantive population stressors.

Before we can move toward improved horseshoe crab management, we need to make a very basic change with respect to the way we think about the organism. It's time to move beyond fascination and admiration of this species to concentrate on its conservation and utilization. We are clearly concerned with its conservation. The symposium that was the basis for this book was entitled the "International Symposium on the Science and Conservation of Horseshoe Crabs." Internationally, horseshoe crab persistence is threatened, and there is a need to raise the awareness of this issue and to do the research and monitoring necessary to develop sound management practices to conserve these species. But it's important to point out that our end point need not be for conservation alone. Horseshoe crabs are a utilized species (Berkson and Shuster 1999). Whether for fertilizer, bait, blood for the biomedical industry, or human consumption, the species is utilized. We must acknowledge that this is not by necessity a bad thing, should there be a sufficient population of horseshoe crabs to allow for utilization. Like so many other renewable natural resources, humans are fortunate to share the planet with yet another species that helps us survive and progress. Our goal should be to move beyond conservation to the point of sound and sustainable utilization. We need to think of horseshoe crabs as a renewable natural resource requiring management and, from there, to implement the research, monitoring, and management practices that will support effective conservation and utilization. This may seem obvious to many, but providing the scientific information needed to effectively manage the population has only recently become a priority. A quick review of the table of contents of this book on horseshoe crab conservation demonstrates how little of the overall science conducted has been designed to address management issues.

Over the past 100 years, we have learned a great deal about fisheries management, so fortunately we don't have to reinvent the wheel. My approach is to start by reviewing a number of the lessons previously learned from fisheries management and adapt them to horseshoe crab management. I provide a few lessons that I suggest might be particularly applicable to horseshoe crab management. Lesson 1 addresses one of the most fundamental, yet often unrecognized, points, i.e., just what are we managing?

2 Lessons

2.1 Lesson 1: Natural Resources Management Is About Managing Humans

A common mistake made by people new to the world of resource management is that we control the resources. In fact, we can't manage the resource (horseshoe crabs), but we can manage the humans affecting the resource (Decker and Enck 1996). Whether it's commercial fishermen, industrial polluters, or shoreline developers, the people are the actual targets of management actions. This is an important lesson because it forces us to realize that economics, sociology, and politics are all part of the management process. The need for a useful and logical guiding principle in this complicated process leads us to Lesson 2.

2.2 Lesson 2: It Is Essential to Incorporate the Precautionary Principle into Management

The more uncertain the status of the resource in question, the more conservative must be the management actions. That is the basis of the precautionary principle. While the concept of the precautionary principle was created to deal with public health, over time it has been extended for use in environmental concerns (Garcia 1994). It began to be applied to international fisheries in the late 1980s (Garcia 1994). In the past, actions that could potentially harm the environment were allowed to proceed unless there was strong evidence that the actions would do significant damage to the environment. Under the application of the precautionary principle, the burden of proof changes in a monumental fashion. Actions can only move forward if there is convincing scientific evidence that they won't do harm.

If there is reason to assume that certain damage or harmful effects on the resources of concern will be caused by specific activities, management action may be required to control the activities before a causal link has been established by absolutely clear scientific evidence (Garcia 1994). The precautionary principle changes how risk is assessed and applied in making management decisions. This provides an incentive to collect the information needed to apply proper scientifically based management, because in its absence, uncertainty is high and management must be conservative. The precautionary principle has been applied to fisheries in the United States (Darcy and Matlock 1999), South Africa (Cockcroft and Payne 1999), and Australia (Smith and Pollard 1996).

A 2004 analysis by the Atlantic States Marine Fisheries Commission showed that the Delaware Bay horseshoe crab population had experienced declines in recent years (ASMFC Stock Assessment Subcommittee 2004). Results from a stock assessment published in 2006 (Davis et al. 2006) indicated that the 2003 biomass of the Delaware Bay population of horseshoe crabs was 56% of the 1995 biomass, equating to an annual decline of greater than 7% during the period. In addition, many Delaware Bay horseshoe crab surveys have high levels of variability and uncertainty (Davis et al. 2006). Many are negatively correlated with each other (Davis et al. 2006). All of these facts lead to the implementation of conservative management actions based on the application of the precautionary principle.

Applying the precautionary principle to real-world situations can be extremely complex (VanderZwaag 2002). These challenges do not negate the need to place the burden of proof on those who have the potential to further impact an already-degraded resource. Discussion of the precautionary principle is a good lead-in to Lesson 3, managing the commercial fishery.

2.3 Lesson 3: The Horseshoe Crab Fishery Will Be a Primary Target of Management

There are numerous human-caused stressors affecting the horseshoe crab population other than the commercial fishery. The impacts of pollution have been studied (Botton and Itow 2009) as have possible impacts of habitat loss (Avissar 2006; Smith 2007). Other possible stressors exist as well, such as temperature rise due to global warming. It's at least possible that one or more of these stressors may be having a significant impact on the horseshoe crab population. In fact one or more of these factors may now or at some point in the near future be having a greater impact on the population than the commercial fishery. If so, why should the fishery be managed at this point in time? Management has been focused nearly exclusively on the commercial fishery until now. It certainly makes sense that commercial fishers would ask, "Why pick on us?"

The ultimate reason is because there are laws involved. Both the Atlantic Coastal Fisheries Cooperative Management Act and the Interjurisdictional Fisheries Act were established to regulate coastal fisheries.

But let's look at this in a more practical sense. Given all of the human-caused stressors, which is most likely to be managed first or most heavily? I would argue that the stressor(s) that fit the following criteria most closely will be the one(s) most likely to be regulated:

1. *The stressor that is easiest to observe*. It is far easier to observe harvesters picking up spawning horseshoe crabs on the beach or trawlers coming to port than it is to see the less direct impacts of pollution or habitat degradation.
2. *The stressor that creates the most direct impact on the species*. It becomes a complex task to quantify how stressors, such as pollution, habitat loss, or temperature change, directly affect the vital rates of the horseshoe crab population. In contrast, the impact of the commercial harvest is very direct. Any horseshoe crab harvested this year will not survive and reproduce next year.

3. *The stressor with the history of greatest impact.* The harvest for horseshoe crabs for use as fertilizer and livestock feed was active in the late 19th century with catch records showing a decline from a maximum of over 4 million to less than 100,000 in the 1960s (Shuster and Botton 1985). Very little harvest took place during the 1950s and 1960s, during which the population increased as much as 13-fold (Loveland et al. 1996). This provides strong evidence that the population had been overexploited in the past and that during a time of greatly limited harvest, the population managed to recover. Regardless of what other stressors are impacting the population and how their impacts may have increased in recent years, harvest will be a primary stressor of concern because of its history with the Delaware Bay population.
4. *The stressor that is easiest to manage (Hilborn et al.* 2003). There are many ways to manage a commercial fishery, from seasonal closures to size limits, from quotas to moratoria. Commercial fisheries management has a long, albeit not always, successful history (Hilborn et al. 2003). It's a common argument that it's considerably easier to shut down a commercial fishery 6 months a year than it is to halt habitat degradation caused by its many economic proponents or to stop pollution from entering Delaware Bay.

Whether or not the commercial fishery has the greatest short- or long-term impact on the horseshoe crab population, it will be the primary focus of management. With commercial fisheries regulations having been in place in recent years (Walls et al. 2002), there are early indications that the population may be recovering, based on results of Virginia Tech's horseshoe crab trawl survey, which began in 2001 (Hallerman 2007). This, of course, continues to add weight to the argument that the commercial fishery was responsible for the population decline in the first place and should be the primary target of management.

With our one-dimensional management focus on commercial fishery regulations appearing to allow for population recovery, or at least status improvement, it would be easy to fall into the trap of thinking that our short-term management strategy should be extended indefinitely, leading us to Lesson 4.

2.4 Lesson 4: It Is Wrong to Assume that Severe Harvest Restrictions Must Be Maintained Indefinitely

While severe harvest restrictions are coinciding with current population growth, are they required forever? The horseshoe crab population is a renewable natural resource. Population growth is occurring, demonstrating that more animals are being produced than are needed to replace the previous generation. This surplus production, or a portion of it, should be available to be harvested, allowing the population to grow at a slower rate or maintain its current size. Fishery management plans often allow for a population to be harvested during its rebuilding period and to be harvested once rebuilding has occurred.

Society must determine the target size for the horseshoe crab population. It can be set at carrying capacity, the maximum possible, but that will by definition eliminate the ability to utilize the population. If the target population size is anything less than its maximum possible and the population is growing, it should be possible at some point to relax harvest restrictions, allowing the surplus production to be taken and the remaining population to be sustained. Discussions about the target size for the horseshoe crab population have been driven mainly by the concern about red knots and other migratory shorebirds that rely on the eggs of horseshoe crabs for energy for the final legs of their migration, usually without consideration of the need for or implications of a sustainable horseshoe crab fishery.

Key to all of this is the ability to estimate the number of horseshoe crabs that can be sustainably harvested at any given population size. This requires ongoing monitoring and assessment. Although sensible harvest management must be a long-term focus of management, it alone will likely not be sufficient, leading us to one of the most important lessons, Lesson 5.

2.5 *Lesson 5: It Is Wrong to Assume that Horseshoe Crabs Can Be Managed Through Harvest Restrictions to the Exclusion of Regulating All Other Human Stressors*

Even with the commercial fishery completely eliminated, other human-caused stressors may cause the horseshoe crab population to decline, now or in the future, if left unchecked. If stressors such as habitat degradation and pollution continue at current levels or worsen, horseshoe crab fecundity and survival rates may drop, causing the population to decline, even in the absence of any commercial fishery. Given sufficient habitat loss, pollution spillage, or water temperature increases due to global warming, the population will be in trouble. Even a permanent moratorium on the harvest of all horseshoe crabs may not ensure the long-term persistence of the population or the long-term persistence of species that depend upon them, without effective management of other stressors impacting the population.

Ultimately, we need to be able to evaluate the impacts of all major stressors on the horseshoe crab population. Just as we want to be able to answer questions regarding the impacts of alternative harvest levels, we also need to be able to answer questions such as What are the likely impacts on the horseshoe crab population of removing 20% of the spawning habitat on Delaware Bay? Or, what are the likely impacts on the horseshoe crab population from a specific spill from a chemical plant or a tanker? We are beginning to develop the tools to be able to answer the "what if" questions regarding harvest (Davis et al. 2006), but the data and models to understand the likely impacts from the other significant stressors are not keeping pace. Even more important, we must be able to understand the overall impacts of our combined management decisions, which lead to Lesson 6.

2.6 Lesson 6: Integrated Assessments Are Required to Evaluate the Cumulative Impacts of a Suite of Management Actions

An integrated assessment is one that evaluates the cumulative impacts on a population, populations, species, or ecosystem. Suppose we know the maximum mortality rate acceptable to keep the horseshoe crab population stable on average, over time. Now suppose we assume that mortality comes from four primary stressors: commercial harvest, biomedical harvest, impacts from pollution, and impacts from habitat loss. In order to keep the population stable on average, over time, the combined mortality rate of the four stressors must not be greater than the maximum allowable mortality rate. Is it possible to identify the acceptable level of mortality from any individual stressor, such as the commercial fishery, without knowing the levels of mortality expected from the other three stressors?

Policymakers regularly make decisions impacting each of the stressors. Commercial harvests can increase or decrease. Habitat can be lost or protected. The levels of pollutants released can increase or decrease. Scientists must be able to inform policymakers about the cumulative mortality impacts of these stressors before management decisions are made, to ensure that each decision is not being made in a vacuum, in the absence of all of the information needed to make well-informed management decisions.

Models that lend understanding to the cumulative effects of stressors on natural resource systems have been developed for a wide range of species and systems (Rose et al. 2000; Wu et al. 2000; Schindler and Smol 2006; Oguz and Gilbert 2007).

Assessing the likely effects of a suite of alternative management actions requires the development of predictive, integrated assessments. Integrated assessments are becoming more common as it has become increasingly clear that managing individual stressors independently is insufficient. An assessment of brown trout (*Salmo trutta*) incorporated gravel conditions, water quality, disease rates, water temperature, habitat conditions, stocking practices, angler catch, and flood frequency (Borsuk et al. 2006). Assessments of Columbia River salmon (*Oncorhynchus tshawytscha*) incorporated effects of potential management actions on habitat, hatcheries, hydropower operation, and harvest (Mamorek and Peters 2001).

Currently, we are managing the horseshoe crab population in a vacuum. Commercial harvest is managed in the absence of knowledge of the magnitude of mortality caused by other human-caused stressors. Policymakers do not consider options for commercial harvest management in coordination or consultation with options for the management of other stressors. As a result, we are left with a one-dimensional approach to horseshoe crab management.

Developing an integrated assessment is not an easy task. It requires considerably more monitoring and research than that required to do traditional harvest management. Additional layers of complexity must be added. Rather than trying to include all possible stressors immediately, it is necessary to identify the factors likely to have the greatest impact on the species. Moving to integrated assessments will take time, but the move must begin. Just as it is too limiting to look at one dimension of impacts on the population, it is too limiting to continue to focus on one dimension of the ecosystem, which leads us to Lesson 7.

2.7 Lesson 7: We Must Move Toward Ecosystem-based Management

In recent years, two major reports have been released about the status of the oceans. Both the US Oceans Commission (2004) and the Pew Oceans Commission (2003) call for management to evolve in its focus to the level of the ecosystem, rather than single species. The 2007 re-authorization of the Magnuson-Stevens Fishery Conservation and Management Act (Public Law 109-479) calls for the creation of an advisory panel to develop recommendations to expand the application of ecosystem principles in fishery conservation and management actions.

The science of ecosystem-based management is evolving, as is our ability to actually implement it (e.g., Larkin 1996; Schramm and Hubert 1996; Mooney 1998; Hall and Mainprize 2004; Francis et al. 2007; Marasco et al. 2007; Murawski 2007). Examples of its implementation are now appearing in the literature (Constable et al. 2000; Olsen et al. 2007).

Horseshoe crabs play a vital role in the ecosystem in which they live. They serve as predator, scavenger, and prey (Walls et al. 2002). In the Delaware Bay, they are prey to threatened loggerhead sea turtles and to declining migratory shorebirds, among others (Walls et al. 2002; Hallerman 2007). Human actions which affect horseshoe crabs also affect numerous other species in the ecosystem. Bycatch in Virginia Tech's horseshoe crab trawl survey has included 76 different taxa, including summer flounder (*Paralichthys dentatus*), a harvested species where overfishing is currently occurring, and spiny dogfish (*Squalus acanthias*), a species currently listed on the IUCN Red List as vulnerable (Graham 2007). Pollution, habitat degradation, and climate change affect the ecosystem as a whole. Ecosystem-level stressors such as habitat destruction and degradation are regarded as the leading threat to horseshoe crab populations internationally (Berkson et al. 2009).

Given its important role in the coastal ecosystem and the fact that so many of its predominant stressors are ecosystem level in nature, horseshoe crab management should move from the single-species level to the ecosystem level. Efforts are currently underway to develop a model which will connect the horseshoe crab and migrating shorebird populations (Michelle Davis, Virginia Tech, personal communication). This is an important step in the direction of ecosystem modeling and ecosystem-based management, but it is not ultimately sufficient as other species are involved in the complex, coastal food web.

3 Discussion

Some might argue that in this chapter I argue for ideals: research, monitoring, modeling, and ultimately management beyond what we're capable of achieving. They might say that our resources are insufficient to do more than we are doing, to progress faster than we are progressing. That may very well be true at the present, but before we can move beyond our current approach, we must have a vision of where we want to go. I present one such vision based on the current evolution of fisheries management.

Others may argue that I am not giving the appropriate credit to how far we've come already. They might point out that the research and monitoring now in place didn't exist 10 years ago and is now being utilized to shape management policies which appear to be working. It's true that we have made remarkable strides. The question I raise is not could we have done better, but rather can and will we do better?

As I wrote in the beginning of the chapter, this is an opinion piece, where I try to lay out where I believe horseshoe crab management needs to go in the future. I raise the question as to whether our current data collection programs, research, monitoring, modeling, and management efforts will guarantee the conservation and utilization of the Delaware Bay population of horseshoe crabs, long term, given the full range of ever-changing anthropogenic stresses on the population. Ultimately, management must incorporate the cumulative impacts of all significant stressors at the ecosystem level.

Currently, horseshoe crab management exists within a box. In the box, the commercial fishery impacts the horseshoe crab population, which impacts the migratory shorebird population. This is clearly an oversimplification of the system with which we are working. As scientists and managers, our long-term goal has to be to think outside the box and break through its walls. Remaining within the box long term will not serve well the horseshoe crab population or the other species and resource users which depend upon it.

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Strategies to Conserve and Enhance Sandy Barrier Habitat for Horseshoe Crabs (*Limulus polyphemus*) on Developed Shorelines in Delaware Bay, United States

Nancy L. Jackson and Karl F. Nordstrom

Abstract The ability to successfully manage estuarine shorelines requires balancing ecological function with societal demands. The sandy barriers of Delaware Bay provide important spawning habitat for horseshoe crabs but they are modified for shore protection. This chapter provides a review of the sandy shoreline resources within Delaware Bay; describes the spatial and temporal scales of processes that govern their dimensions, location, morphology, and sedimentary characteristics; compares management programs in the state of Delaware and New Jersey for managing sandy shorelines in the estuary; and provides examples of the status of developed sandy barriers where high levels of horseshoe crab spawning occur.

1 Introduction

Delaware Bay is considered one of the most ecologically important estuaries on the east coast of the United States, providing a rich diversity of physical environments for aquatic and avian species (Sullivan 1994). The bay hosts the largest horseshoe crab population in the mid-Atlantic region and provides foraging areas for the migratory shorebirds that stop over in Delaware Bay during spring (Clark et al. 1993; Botton et al. 1994). The horseshoe crab is considered a keystone species in the estuary and supports the commercial fishing, biopharmaceutical, and ecotourism industries (Eubanks et al. 2000; Manion et al. 2000) and provides an important nutrient source for the migratory shorebirds that feed on horseshoe crab eggs (Baker et al. 2004). Growing concern that the horseshoe crab population in Delaware Bay was unable to withstand continued losses (Berksen and Shuster 1999) prompted federal and state agencies to devise a strategy to protect the species. Traditional management strategies to reverse population declines in aquatic species include harvest restrictions and designation of marine-protected areas. Efforts to sustain horseshoe crab populations have followed

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a similar path with attention to the effectiveness of harvest limits, the uncertainty in population estimates, and the conflicts among the diverse stakeholders over resource allocation (Odell et al. 2005). Federal efforts to manage the horseshoe crab population in Delaware Bay included the creation of the Carl N. Shuster Horseshoe Crab Reserve off the mouth of Delaware Bay. At the state level, Delaware and New Jersey established regulations in the 1990s that imposed a system of permitting, licensing, and area restrictions on the harvest of horseshoe crabs (Walls et al. 2002). The most recent measure imposed a moratorium on the horseshoe crab commercial bait fishery for a 2 year period beginning in 2006 (NJ Federal Register, February 6, 2006), a move that was challenged by the resource users and amended in 2007 to allow harvest of a designated number of male crabs (Bernie's Conchs, LLC & Charles Auman vs State of Delaware, Division of Natural Resources and Environmental Control, 2007).

Management approaches that control harvest can decrease stress on the horseshoe crab population, but a more comprehensive approach to management must incorporate mechanisms for ensuring availability and functionality of habitat. The Atlantic States Marine Fisheries Commission (1998) reports that availability of optimal spawning beaches will be a limiting factor on the reproduction of horseshoe crabs. Horseshoe crabs spawn in the highest densities on the intertidal foreshores of sandy barriers in Delaware Bay (Smith et al. 2002). Horseshoe crabs respond to the wave energy and salinity gradient, avoiding sites where wave energies are too high or salinities are too low. Many of the most heavily used shorelines for spawning are located within the mid-region of the bay (Smith et al. 2002) and many are backed by human settlements that have altered the characteristics of the foreshore fronting them. Botton et al. (1988) found that only 10.6% of the beaches on the east side of Delaware Bay (New Jersey) were optimal for horseshoe crab spawning due to the presence of peat outcrops or hard protection structures on the intertidal foreshore. A similar assessment conducted in 2005 found that the length of shoreline classified as optimal habitat on the east (New Jersey) and west (Delaware) side of the bay totaled 23.9% (Lathrop and Allen 2005). High rates of erosion, combined with local land use decisions (i.e., type, location, and density of development and building guidelines), have resulted in the modification, deterioration or, in some cases, elimination of horseshoe crab spawning habitat (Jackson et al. 2002). Thus, the long-term viability of horseshoe crab spawning habitat in Delaware Bay rests on the ability of responsible agencies, governments, and stakeholders to strike a balance between conservation and development interests. A 2004 survey of state coastal resource managers in the United States identified land use and habitat change as the two most important issues that need to be addressed over the next 5 years (Benoit et al. 2004), reflecting the high priority on balancing ecosystem function with development. This chapter provides a review of the sandy shoreline resources within Delaware Bay, compares management programs in the state of Delaware and New Jersey for managing sandy shorelines in the estuary, and provides examples of the status of developed sandy barriers in New Jersey and Delaware where high levels of horseshoe crab spawning occur.

2 Geomorphic Setting

Delaware Bay is a drowned river valley estuary located on the mid-Atlantic coast of the United States (Fig. 1). Tides are semidiurnal, with a mean tidal range of 1.6 m and a spring tidal range of 1.9 m (NOAA 2006). The shoreline comprises unconsolidated sandy barriers fronting large marsh systems with sediment supplied by eroding low Holocene highlands (Kraft et al. 1979). The barriers initially formed where there was sufficient sediment supply and wave energy capable of reworking the sedimentary deposits (Knebel et al. 1988). The dominant energy reworking the shoreline is from waves generated within the estuary although ocean swell has an influence on the shoreline in the lower reaches near the mouth of the estuary. There are 105 barriers in Delaware Bay, averaging 1 km in length and 50 m in width and separated by rivers or tidal creeks (Lewis et al. 2005). Many of the barriers support human settlements that are located near the shoreline.

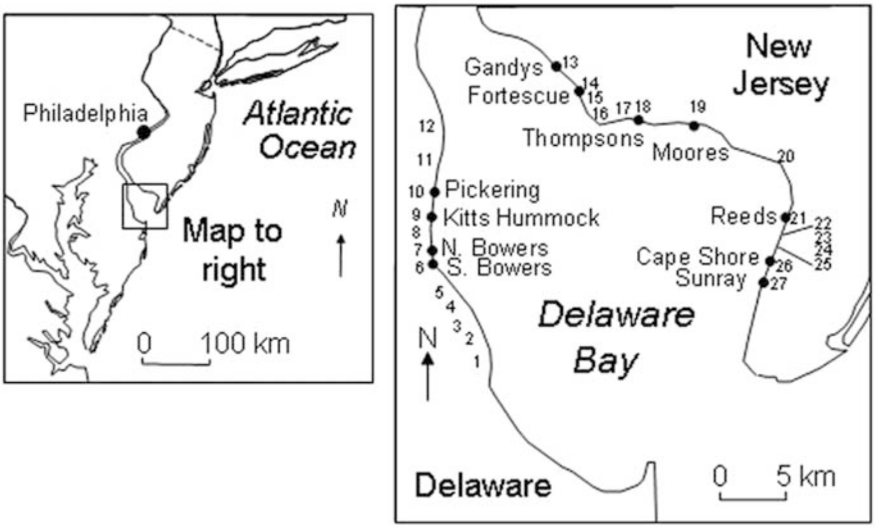

Fig. 1 Locator and site map of the Delaware estuary showing the locations of high densities of horseshoe crab spawning (*solid circles*). Numbers indicate location of communities identified in Table 1

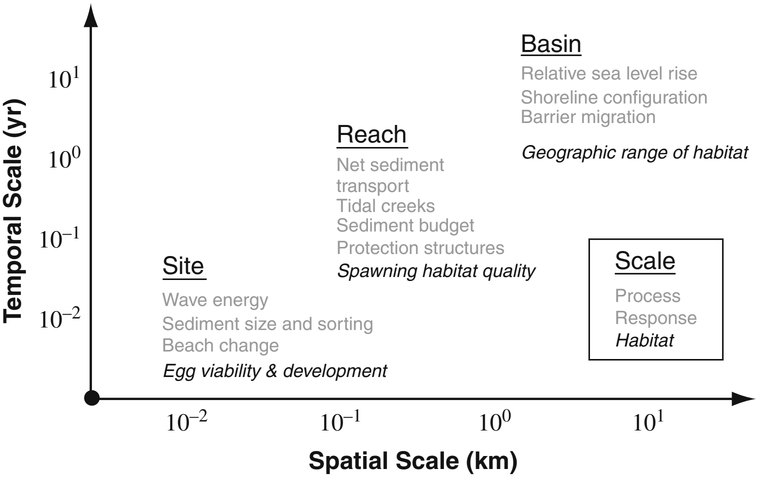

Fig. 2 Spatial and temporal scales of coastal processes and horseshoe crab spawning habitat

The processes that rework the sandy foreshores of Delaware Bay operate over several spatial and temporal scales (Fig. 2). These processes affect the suitability of the foreshore for horseshoe crab spawning by controlling its shape, location, and sedimentary characteristics across and along the shore. Over spatial scales of meters (alongshore) and timescales of hours to days, local tide and wave conditions and associated currents have the greatest influence on changes to beach morphology. The two-dimensional profile of the active beach consists of a relatively steep (4–7°) foreshore comprised of medium sand (Jackson et al. 2005a), where horseshoe crab spawning is generally greatest, and a gently sloping (<0.5°) low-tide terrace. The highest waves and greatest profile change on the west-facing beaches in the estuary occur during high-velocity northwest winds. High-velocity winds from the northeast, generally associated with the passage of low-pressure systems, result in the highest waves and greatest profile change on the east-facing beaches. The elevation of the low-tide terrace controls the magnitude of wave energy reaching the shoreline at lower stages of the tide; the higher the elevation of the low-tide terrace, the greater the dissipation of wave energy.

The suitability of the foreshore as habitat is a function of the ability for eggs to develop in the beach matrix. Development of eggs within the foreshore occurs over a period of weeks, during the spring and early summer, and is a function of oxygen, temperature, and moisture of the interstitial sedimentary environment. The size and sorting of the sediment comprising the beach matrix are controls on rates of infiltration and exfiltration of water which change the oxygen, temperature, and moisture conditions over tidal cycles (Jackson et al. 2008). Bioturbation or wave action during storms can exhume eggs from the beach matrix before they fully develop. The type of beach profile response that occurs in response to storm conditions in the estuary is dependent on the orientation of the shoreline (Nordstrom and Jackson 1992). Most change in profile shape occurs on the upper foreshore when the shoreline is oriented near perpendicular to the direction of the highest waves. Sediment is removed from the upper foreshore, in the zone where horseshoe crab spawning occurs during spring tide, and deposited lower on the foreshore below mean tide. Eggs buried in the upper foreshore are more susceptible to removal by waves during storm events compared to eggs laid lower on the foreshore where deposition occurs. On shorelines oriented at an angle to the highest waves, the foreshore will undergo parallel slope retreat with no change in overall slope. This response is due to the dominance of longshore sediment transport forced by waves at an oblique angle. Horseshoe crab burrowing will contribute to removal of sediment from the upper foreshore and deposition on the lower foreshore and can change the modal beach response during the spawning season from parallel slope retreat to onshore–offshore exchange (Jackson et al. 2005b).

At the reach scale (individual barriers) and over temporal scales of decades, a combination of physical and human actions (most importantly shore protection structures and beach fill) is dominant in shaping the physical characteristics of the shoreline. The dominant physical processes that shape the shoreline are associated with the behavior of the tidal creeks, the sediment budget of the barrier system, and the net transport of sediment. On barriers that are substantially modified by human processes the location of human development and the types and configuration of shore protection will influence the characteristics of the shoreline. Of most importance to horseshoe crabs is the ability to burrow and lay eggs in the foreshore. Smith et al. (2002) found that the lower the wave energy the greater the likelihood for spawning to occur. There exists an alongshore wave energy gradient along the barriers that is a function of breaks in shoreline orientation, the elevation of the low-tide terrace, and the configuration of buildings, roads, and shore protection structures. The result will be alongshore variation in spawning on the barrier over a tidal cycle. Structures on the intertidal foreshore (Fig. 3A) can create isolated shoreline segments that can provide low-energy refuges during high waves but at the expense of eliminating portions of the active foreshore that would be important spawning areas (Botton et al. 1988; Jackson et al. 2002). Beach nourishment (Fig. 3B) is used to offset deficits in the sediment budget of some barriers, particularly those supporting human settlements.

Figure 4 shows the range of types of foreshore environments along a developed barrier on the east side of the estuary (Fig. 1). The northern segment of the island is characterized by high levels of development with bulkheads intersecting the intertidal profile (Fig. 4A). The southern segment of the shoreline consists of a nourished beach backed by a bulkhead and road (Fig. 4B). The source of sediment for the nourishment operation was material dredged from Fortescue Creek. Net transport of sediment to the south has resulted in the creation of sandy depositional areas at the mouth of Oyster Creek (Fig. 4C) and

Fig. 3 Views of Reeds Beach, NJ (**A**) and Slaughter Beach, DE (**B**) showing differences in the characteristics of the shoreline that are backed by human development

within the creek channel (Fig. 4D). These depositional features have become opportunistic sites for both horseshoe crab spawning and shorebird foraging.

The lack of input to the sediment budget on barriers without human infrastructure has led to progressive erosion of the shoreline. Reported rates of erosion are 1.3 m yr^{-1} on the west side of the estuary and 1.9 m yr^{-1} on the east side of the estuary (May et al. 1983). On the west side of the estuary reported rates of barrier transgression are between 2 and 3 m yr^{-1} (Kraft et al. 1979). High rates of erosion can result in the outcropping of peat on the foreshore resulting in a lower quality spawning habitat (Botton et al. 1994).

Estuaries are ephemeral features over geologic timescales and the persistence of these features is a balance between variations in sea level rise and sedimentation (Dalrymple et al. 1992). Vulnerability to sea level rise along the shoreline is highly variable based on type of shoreline and rates of erosion and accretion. The west side of Delaware Bay is classified at low risk to future sea level rise

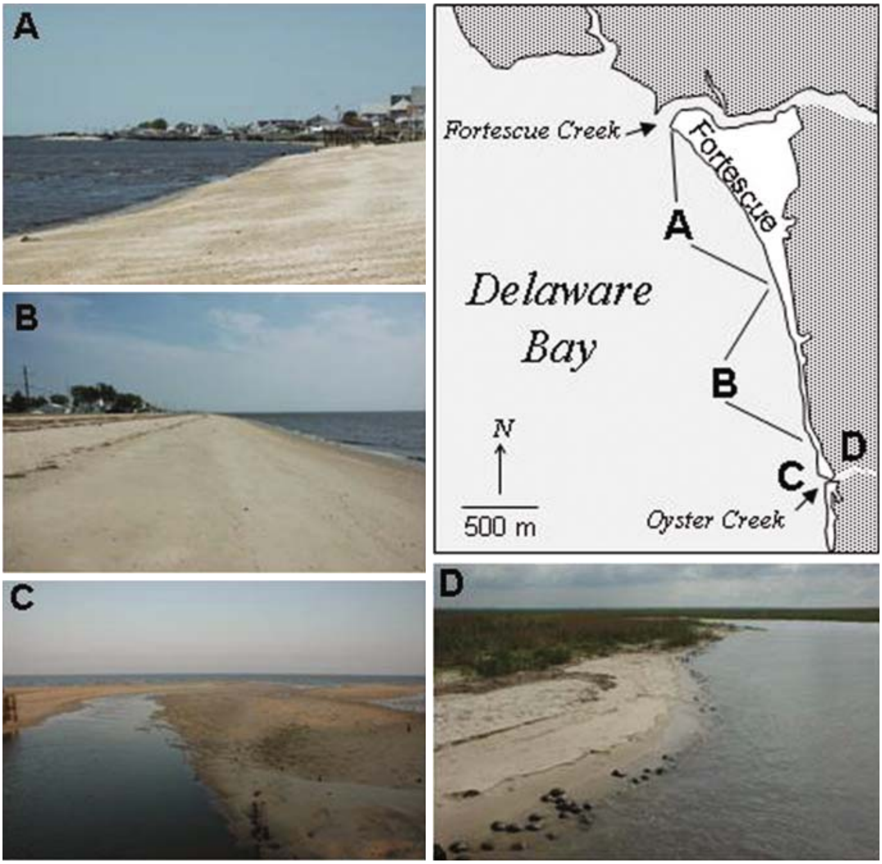

Fig. 4 Map of the barrier of Fortescue, NJ, and photographs showing (**A**) the northern segment of the shoreline backed by human development, (**B**) the southern segment of the shoreline comprising a recently nourished beach backed by a bulkhead and road, (**C**) the inlet of Oyster Creek, and (**D**) the banks of Oyster Creek where sandy deposits have created spawning habitat

whereas the east side is at moderate to high risk (Thieler and Hammar-Klose 1999). Sea level rise can increase water depths within the bay, wave energies reworking the foreshore, and rates of erosion on the shoreline. The response of barriers to a rise in sea level is to migrate inland. Inland migration occurs during storm events when sediment is transported inland via overwash. The net result is the transgression of the barrier across the gently sloping marsh platform. On barriers that are stabilized by shore protection projects, beach nourishment, or bulkheads, inland migration is prevented and the result is persistent erosion of the foreshore. The limited volume of sediment within many of the unnourished barriers suggests that the barriers may not remain intact as they migrate inland.

3 Management Framework

Estuaries are recognized for their ecological value and most policy and management efforts in the United States are directed toward protecting their vital functions and services. An ecosystem-based management approach has been adopted for estuaries that form part of the US National Estuary Program (Imperial and Hennesey 1996). Delaware Bay entered into the National Estuary Program in 1988 and the comprehensive conservation and management plan was produced in 1996 (Delaware Estuary Program 1996). Among the numerous problems articulated in the plan was beach erosion and human development, but like most national estuary programs the attention to shoreline resources has focused on marsh and nearshore bay bottom environments.

At the state level, management of shoreline resources (geomorphological and biological) in Delaware Bay is the responsibility of the states of Delaware, Pennsylvania, and New Jersey under the authority of the Coastal Zone Management Act (CZMA), Endangered Species Act (ESA), Coastal Barriers Resources Act (COBRA), Federal Emergency Management Act (FEMA), Clean Water Act (CWA) (specifically Sections 320 and 404), and the Rivers and Harbors Act. The focus here is action undertaken within Delaware and New Jersey because the beaches of primary importance to horseshoe crab spawning are located in these two states. The Coastal Management Program in Delaware is based on comprehensive coastal legislation, the Delaware Coastal Zone Act of 1971 (7 Del.C., Chapter 70), and the Beach Preservation Act of 1972 (7 Del.C, Chapter 68), whereas the program in New Jersey is based on coordination of several state laws (Knecht et al. 1996) such as the Coastal Area Facilities Review Act (CAFRA) (N.J.S.A. 13:19), the Waterfront

Table 1 Shoreline development and management practices on Delaware Bay

Site[1]	State/ community	Level of development[2]	Active shore protection	Active ecosystem protection
Delaware				
1	Broadkill Beach	High	Groins, Beach Nourishment	Community-based sanctuary
2	Prime Hook			Federal Refuge Area Trust for Public Land COBRA
3	Fowlers Beach	None	None	Federal Refuge Area Community-based sanctuary COBRA
4	Slaughter Beach	High	Groins, Beach Nourishment	Community-based sanctuary
5	Big Stone	None	None	State-imposed seasonal closure State Wildlife Management Area

Table 1 (continued)

Site[1]	State/ community	Level of development[2]	Active shore protection	Active ecosystem protection
				Milford Neck Preserve (Nature Conservancy)
6	South Bowers	Low	Beach Nourishment	
7	North Bowers	High	Beach Nourishment	
8	Ted Harvey	None	Beach Nourishment	State-imposed seasonal closure State Conservation Area
9	Kitts Hummock	High	Beach Nourishment	Community-based sanctuary
		None		COBRA
10	Pickering	High	Beach Nourishment	Community-based sanctuary
11	Little Creek	None	None	State-imposed seasonal closure State Wildlife Management Area
12	Port Mahon	None	None	Port Mahon Preserve (Nature Conservancy)
New Jersey				
Downe Township				
13	Gandys Beach	High	Bulkheads	State-imposed seasonal closure (portion) Gandys Beach Preserve (Nature Conservancy)
14	Fortescue	High	Bulkheads, Beach Nourishment	State-imposed seasonal closure (portion) Glades Preserve (Natural Lands Trust)
15	Raybins Beach	Low	None	State-imposed seasonal closure (portion) State Wildlife Management Area
16	Egg Island	None	None	State Wildlife Management Area
Maurice River Township				
17	Maurice River	None	None	Public utility restoration site
18	Thompsons Beach	Low	None	

Table 1 (continued)

Site[1]	State/ community	Level of development[2]	Active shore protection	Active ecosystem protection
19	Moore's Beach	None	None	State-imposed seasonal closure (portion) State Wildlife Management Area COBRA
Upper Township				
20	Dennis Creek	None	None	State Wildlife Management Area
	Middle Township			
21	Reeds Beach	High	Bulkheads	State-imposed seasonal closure (portion)
22	Cooks Beach	None	None	State-imposed seasonal closure COBRA
23	Kimbles Beach	Moderate	None	State-imposed seasonal closure COBRA
24	Pierces Point	Moderate	Bulkheads	State-imposed seasonal closure (portion)
25	Highs Beach	Low	Bulkheads	State-imposed seasonal closure
26	Cape Shore	None	Groins, Geotextile Tube	State-imposed seasonal closure COBRA
27	Sunray Beach	Moderate	Bulkheads	State-imposed seasonal closure (portion) Sunray Beach Preserve (Nature Conservancy)

[1] Site numbers are identified in Fig. 1.

[2] High (>60% of shoreline reach backed by development); Moderate (30–60% of shoreline reach backed by development); Low (>30% of shoreline reach backed by development).

Source: Orth-Rodgers & Associates, Inc. (2003) Western/Southern Cumberland region municipal profiles. 28 pp.

Development Law (N.J.S.A. 12:5-3), and the Wetlands Act of 1970 (N.J.S.A. 13:9A). State practice in New Jersey relies on more traditional (i.e., construction regulations and setbacks) and non-regulatory (i.e., land acquisition) actions to protect shoreline resources (Bernd-Cohen and Gordon 1999) than on beach nourishment to enhance or restore degraded habitats and enhance ecosystem function (Table 1).

4 Efforts to Conserve or Protect Shore Resources

At the scale of a barrier/marsh system there are generally one or more "special management areas," including sandy barriers and segments of sandy barriers that are subject to COBRA protection, Wildlife Management Areas (WMAs) that are owned and managed by the state division of US Fish and Wildlife, and overlay zones that delineate the geographic reach of state and local construction setbacks and building requirements (Davis 2003). Most of the undeveloped sandy barriers and undeveloped segments on developed sandy barriers have been placed under protection of COBRA that prohibits federal flood insurance and monies for infrastructure rehabilitation.

Controls on federal assistance to build and repair infrastructure are limits to growth on many of the developed barriers. Main access roads to the barriers can flood or be undermined during storms and communities look to state and federal assistance for funds to repair storm damage. Efforts to curb financial assistance for infrastructure along certain reaches of the Delaware Bay shoreline have resulted in the depopulation of two sandy barriers (Moores Beach and Thompsons Beach) (Fig. 1) on the east side of the bay that are now under state jurisdiction and segments of the shore are under COBRA protection.

Beach closures and establishment of community-based sanctuaries are used to prevent access to areas where horseshoe crab spawning occurs. The state of New Jersey has closed all or part of the shoreline during the time of horseshoe crab spawning and shorebird foraging since 2003 (Burger et al. 2004). The state of Delaware also prohibits hand harvesting of horseshoe crabs on state-owned lands during the horseshoe crab spawning season but also provides authority to local landowners to prevent hand harvesting of horseshoe crabs on their private property. Although this type of action does not affect the geomorphology of the shoreline it is a land management strategy that enhances use of foreshore habitat by horseshoe crabs.

Section 3209 of Title 7 outlines provisions for horseshoe crab sanctuaries in Delaware. All lands owned by state and federal entities are designated as horseshoe crab sanctuaries during the spawning season. Private landowners can register their land with the state environmental agency to designate their beach holdings as a horseshoe crab sanctuary. A sanctuary designation makes it unlawful to collect horseshoe crabs at any time. Four communities, by majority vote, have registered their land collectively to create community-based horseshoe crab sanctuaries. This sanctuary strategy is a low-cost approach for shorefront residents to protect horseshoe crab spawning habitat. The weakness is the potential lack of permanence since landowners can agree to deregister their properties by a majority vote. A more permanent, but high-cost, approach is land acquisition by public or private non-profit interests.

Public acquisition of shoreline and adjacent marsh environments in Delaware Bay occurred in response to the need to provide foraging areas for the migratory shorebird population. Most of the land acquisitions have been transfers from private owners to the state governments but there are a few non-governmental organizations that have purchased land on the bayshore (Table 1). Estimates place the amount of land in public ownership on the Delaware Bay shoreline at approximately 40% (Lathrop and Allen 2005). Many of the land holdings include both the sandy barrier and surrounding marsh complex but there are some public lands that comprise the marsh behind the sandy barriers but not the sandy barriers. This situation occurs where private development occupies the sandy barrier. The result is a fragmented arrangement of property rights holdings alongshore and across geomorphic units of the barrier/marsh complexes. Large tracts of marsh are designated wildlife management areas designed to protect key habitat for species such as the migratory shorebird population that arrive during the horseshoe crab spawning season. These birds forage on horseshoe crab eggs found within the swash zone of the foreshore (Nordstrom et al. 2006) and will rest and/or feed within the marsh environment (Burger et al. 1997). Some sandy barriers where a high level of horseshoe crab spawning and foraging takes place are within the holdings but some of the optimal spawning barriers remain outside the special management areas and in private ownership.

5 Efforts to Enhance or Restore Sandy Foreshore Habitat in Developed Areas

The problem of providing horseshoe crab habitat in developed areas is critically linked to the problem of finding suitable means of protecting human infrastructure from beach erosion and flooding. The types of shore protection strategies employed in estuaries are similar to those found on the exposed ocean shoreline (Nordstrom 1989), but the size and extent of the projects reflect the relatively lower wave energies found in estuarine systems. Overall state policy on the west side of the estuary has focused on nourishing the developed sandy barriers and creating new sandy beach environments along undeveloped shoreline reaches to restore or enhance habitat for horseshoe crabs and migratory shorebirds (Fig. 3B). On the east side of the estuary state policy has encouraged beach nourishment but allowed construction of hard protection structures (primarily bulkheads) to reduce erosion and flooding along the developed segments of the shoreline (Fig. 3A).

In Delaware, the Beach Preservation Act (7 Del.C. Chapter 68) enables the Department of Natural Resources and Environmental Control to regulate construction activities and prevent or repair damages from erosion of beaches by shore protection structures. The act establishes a state fund to carry out the purposes of the act. Regulations established under the act discourage the construction of walls (i.e., bulkheads) for shore protection and require a permit for construction of a wall once all other forms of shore protection are deemed inadequate. There are provisions in the regulations for mitigating measures in instances when a structure or activity adversely affects the beach. These measures can include beach nourishment or dune construction and are the responsibility of the owner for the lifetime of the project. The regulations impose a setback on development along the Delaware Bay shoreline that is at least 75 feet landward of the 6 foot contour interval (North Atlantic Vertical Datum). The state has conducted over 50 nourishment operations since the 1960s ranging in size from 1500 to 226,000 cubic meters of sediment.

In New Jersey, the Coastal Zone Management Rules (N.J.A.C. 7:7E-7.11) state that beach nourishment is encouraged for shore protection provided that sediment size and type of fill material are compatible with existing material, the geometry of the project (elevation, width, and slope) is compatible with the existing beach, and that sediment deposition should not result in unacceptable shoaling in downdrift inlets or navigation channels. Structural solutions, such as bulkheads, are permissible if the structure will not cause significant adverse impacts on sediment supply or transport and will cause minimum feasible adverse impact to living marine and estuarine resources. Dredged material may be used, when free of contamination and of appropriate particle size, for beach protection and creation of new wildlife habitats. The state established a shore protection fund (N.J.A.C. 13:19-16.1) that can be used for shore protection projects or for the non-federal share of federal projects. Contributions to the fund are currently set at $25 million annually to support projects on the ocean and bay but most of the projects, to date, have been conducted on the ocean shoreline.

Beach nourishment, when used solely for shore protection, creates a cross-shore profile that is much wider and often higher than pre-nourishment conditions (Jackson et al. 2002) (Fig. 5). From an ecological perspective, the differences in geometry of the cross-shore profile may lead to changes in location of spawning activity particularly when a scarp forms on the intertidal profile due to creation of an overly high backshore (Fig. 5). Sediment for nourishment operations in

Fig. 5 View of Bowers Beach, DE, showing the development of a scarp on the foreshore after beach nourishment

estuaries may be from land-based sources, offshore or from dredging of offshore channels, or the numerous creeks that dissect the shoreline. Of importance to horseshoe crabs are the textural properties of the sediment that can influence development of their eggs in the foreshore. Foreshore sediment may be finer than existing material when the source is derived from adjacent creek channels (Jackson et al. 2007). Comparison of foreshore sediment on unnourished beaches on the east and west side of Delaware Bay reveals similar sediment characteristics. Comparison of unnourished beaches on the east side of the estuary with nourished beaches on the west side reveals coarser grain sizes with a larger percent gravel fraction on the unnourished beaches (Jackson et al. 2005a).

6 Implications

Habitat availability in Delaware Bay is spatially and temporally contingent from year to year due to physical processes in the bay. Changes in salinity, temperature, and wave energy over the spawning season within a given year, and from year to year, can suppress or enhance spawning activity along specific-stretches of shoreline. Storm activity can erode beaches and may reduce their suitability for spawning or egg development. Thus, providing habitat to horseshoe crabs requires an adaptive management strategy that goes beyond protecting properties from erosion or flooding. Management activities should strive toward sustainable outcomes that integrate natural and social processes (Turner et al. 1998) and with opportunities for adaptation as ecosystems respond to management actions (Boesch 2001). At the site scale, beach nourishment provides sandy foreshore habitat in areas where high erosion and flood hazards threaten private property. Questions of whether beach nourishment enhances or impairs beach and nearshore habitat is still a subject of uncertainty (Greene 2002; Jackson et al. 2002; Nordstrom 2005; Peterson and Bishop 2005, Avissar 2006). Several projects initiated by the US Army Corps of Engineers are designed for a combination of shore protection and ecological restoration (i.e., Port Mahon), navigation and ecological restoration (i.e., Mispillion River), or one-time nourishment for horseshoe crab and shorebird habitat (i.e., Reeds Beach to Pierces Point). These projects have not reached construction phase, but planning has involved dialogue among project designers, biologists, and geomorphologists on how to best achieve ecological and coastal management goals.

Current initiatives to manage shore resources on Delaware Bay employ some of the tools common to integrated coastal management, such as designating protective areas and establishing land trusts (Cicin-Sain and Knecht 1998). These regional-scale approaches are gaining popularity as mechanisms for integrating the multiple demands of resource users into the complex regulatory programs that have evolved in the coastal zone (Davis 2003). Successful integrated coastal management is predicated on adequate forms of governance to meet the challenge of balancing ecosystem and development needs (Olsen 2003). The institutional framework for coastal management in Delaware Bay includes several political and administrative jurisdictions at local, state, and federal levels that are responsible for a piece of the total management program, as well as commercial/industrial and environmental/conservation groups that have an interest in resource decision-making. Level of interjurisdictional cooperation is a key factor in moving beyond local-scale concerns to regional-scale actions that improve environmental quality and support the holistic orientation of integrated coastal management. Nelson and Weschler (1998) outline four conditions that suggest the potential for jurisdictions to engage in cooperative actions: (1) level of interest and involvement, (2) knowledge and appreciation for goals of other jurisdictions, (3) availability for regional governance, and (4) previous experience with interjurisdictional cooperation. At the scale of barrier/marsh complexes there is a mosaic of public and private property holdings with different mandates and management objectives. These systems are geomorphically linked but there needs to be better understanding of the physical relationships between the barrier and marsh systems and how these are mediated by management decisions at local and regional scales.

The small volume of sediment in the coastal barriers will result in lower quality habitat for horseshoe crab spawning on both developed and undeveloped reaches, given increases in relative sea level rise. Nourishment may offset these losses at specific sites but there is need to construct a bay-wide geography of future habitat for horseshoe crabs. Achieving broad scale, long-term management outcomes may require the construction of institutional relationships at a “missing” spatial scale where regulatory efforts do not generally take place (Judd 1998). Processes such as sea level rise operate beyond the limits of politically defined local and state spatial scales, an area where few formal institutional arrangements have been established.

The horseshoe crab harvest restrictions promulgated by the Atlantic States Marine Fisheries Commission have brought the issue of potentially declining horseshoe crab populations to the forefront of the public’s attention, but there is significant debate over whether the restrictions will be successful. These restrictions are important for sustaining horseshoe crabs and shorebirds, but this singular focus may be diverting attention from other critical issues, such as habitat loss and change. Without adequate high-quality habitat to support horseshoe crabs, harvest restrictions will be little more than short-term measures that delay inevitable population-level problems.

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Conservation Program for the Asian Horseshoe Crab *Tachypleus tridentatus* in Taiwan: Characterizing the Microhabitat of Nursery Grounds and Restoring Spawning Grounds

Hwey-Lian Hsieh and Chang-Po Chen

Abstract A study of the physical properties of horseshoe crab (*Tachypleus tridentatus*) nursery grounds indicated that juveniles preferred sediments consisting of fine sand with median grain size of 0.14–0.27 mm in diameter, 16.9–23.2% water content when tides receded, 0.23–0.41% TOC content, 0.04–0.07% TN content, 2.3–2.8 $\mu g/cm^2$ chlorophyll *a* content, and poorly sorted substrates. Juvenile horseshoe crab density increased based on the amount of chlorophyll *a* content in the sediment and infaunal polychaete density, suggesting that the juveniles prefer nursery grounds containing abundant prey and its supporting food web. An effort to restore horseshoe crab spawning grounds was conducted by covering 20 cm deep mud substrate (0.23 mm in diameter) with coarse sand (1.10 mm in diameter). Although adults transferred to this site succeeded in laying eggs, the hatching rate was only 33.9%. This low rate may be attributed to small tidal amplitude at this restoration site.

1 Introduction

1.1 Significance of the Present Study

The horseshoe crab is a living fossil and has high economic value in the fishing industry. Some of the harvest is used for human consumption and some products are used in the medical industry (Shuster 2001, Swan 2001). It is also a good indicator species for monitoring the health of coastal zones and has local cultural importance in Taiwan (Chen et al. 2004). The Asian horseshoe crab, *Tachypleus tridentatus*, is one of three horseshoe crabs distributed in the Indo-Pacific region. This species once thrived in the coastal wetlands of Taiwan, but now survives in only a few small areas. A similar decline in abundance and

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endangered status has also been reported by Hong Kong and Japan (Chiu and Morton 1999, Botton 2001). The decline is largely due to loss of suitable habitat caused by anthropogenic impacts such as land reclamation, dike construction, water pollution, and loss due to overfishing for food consumption and biomedical use. This loss is symptomatic of a wider decline in Taiwan's natural resources and biodiversity. This decline is in conflict with efforts to sustain Taiwan's development. In order to replenish what we have lost, the central government administration has recognized restoration of coastal wetlands as an urgent requirement. As a result, implementation of habitat mitigation and restoration has been itemized in the national biodiversity conservation program, which was authorized by Executive Yuan in Taiwan in 2001 (Action Plan in Biodiversity Promotion issued by Executive Yuan August 15, 2001, amended in February 2004).

Since conservation of a range of habitats has been acknowledged to be much more effective than the protection of individual species, we have chosen the horseshoe crab as a "flagship" species (Zacharias and Roff 2001). Preserving or conserving their habitat can secure the whole community, including the target flagship species as well as other species dependent on that habitat. More importantly, the recovery of the flagship species populations will indicate the restoration of intertidal ecosystems and the recovery of coastal natural resources, and therefore is beneficial to humans living on the Taiwan islands. In addition, our experiences in conserving this flagship species can be shared with other countries. Since other researchers are likely to encounter situations similar to that in Taiwan, such as harvest pressure and the loss of suitable habitats, our findings can be especially useful in fostering better connections in the international network of marine-protected areas for horseshoe crabs. To implement the conservation plans for the horseshoe crab, we have set up a three-step strategy:

(1) Develop an understanding of its life history
(2) Characterize its habitat requirements
(3) Using the findings of steps 1 and 2, identify methods for repairing and reconstructing a functional ecosystem

Throughout its life cycle, the horseshoe crab is highly dependent on environmental conditions in all of its coastal habitats. Adults spawn on the coarse sand near the high-tide zone and juveniles inhabit the adjacent intertidal mudflats and then gradually migrate to the deeper subtidal zone for maturation (e.g., Sekiguchi 1988, Chiu and Morton 1999, Anderson and Shuster 2003). *Limulus polyphemus* nest sites are defined by a set of physical properties including a sandy beach and its hydroclimate. Grain size, sediment moisture content, oxygenation, temperature, and depth of sand over peat have been found to be important (e.g., Botton et al. 1988, Brockmann 2003, Shuster and Sekiguchi 2009). At the macrohabitat level, the geomorphology of the spawning beach as mediated by waves, tides, and currents has also drawn great attention (Anderson and Shuster 2003, Jackson et al. 2005). This data review highlights that our knowledge of the horseshoe crab's habitat requirements is primarily based on observations of the American horseshoe crab *L. polyphemus*, especially its spawning ground. In contrast, the habitat requirements of the three Asian horseshoe crab species (*T. tridentatus*, *T. gigas*, and *Carcinoscorpius rotundicauda*) are largely unknown.

1.2 Analyses of Habitat Requirements Through the Life History of T. tridentatus and the Purpose of the Study

According to the three-step strategy outlined above, we have made detailed analyses that allow us to understand the habitat requirements of the horseshoe crab at each important life history stage (Fig. 1). Both the aspect of organism

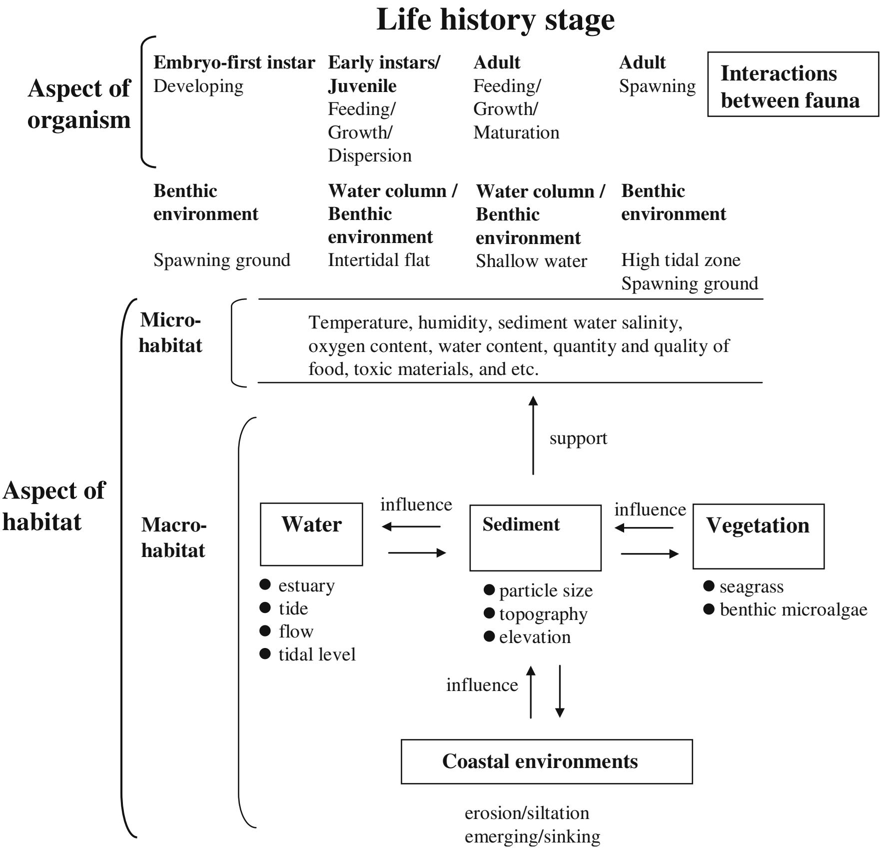

Fig. 1 A scheme showing the analysis of the habitat requirements for a horseshoe crab species throughout its life history

and the aspect of habitat are involved. From the organismal point of view, the horseshoe crab needs different habitats at different life stages. Embryos develop in a benthic environment while embedded in sands on the spawning ground. Newly hatched first instars and early-stage instars are capable of swimming in the water column, and thus are dispersed by currents. Juveniles settle down on tidal flats and commence demersal feeding and growth after a number of molts. As the juveniles grow larger, they gradually leave the tidal flats and migrate to shallow water areas where they become adults. At the start of spawning season, paired mature adults move toward shore and nest on the high tidal zone of sandy beaches. Throughout these stages, they interact with other co-existing fauna. From the habitat point of view, two scales exist for any given habitat: macro and micro. At the macrohabitat scale, the entire coastal environment setting is influenced by the geomorphology and hydrodynamics of that coast, which determines the stability of the coast and whether it is in a state of erosion or deposition. Interactions between geomorphological and hydraulic forces mediate the suitability of beach slope, tidal amplitude, current velocity, and sediment grain size as well as the presence or absence of vegetation for horseshoe crabs to live on. When looking at their microhabitats, components such as sediment grain size, water content, organic content, temperature, salinity, oxygen content, and food availability are critical to the horseshoe crab's distribution.

Due to the lack of records on adults coming to the beach for nesting, mating sites for *T. tridentatus* have not been reported in recent decades in the Taiwan region. However, spontaneous spawning was induced successfully on a natural, sandy beach, thus suggesting that this kind of beach was a potential and suitable nesting ground (Chen et al. 2004). Since we know this potential nesting ground is sandy and have determined its sand grain size range, we may be able to induce adults to spawn in the field where unfavorable substrate is replaced by a favorable one.

In lower mudflats adjacent to a potential spawning ground in higher tidal zones, juveniles from the second instars up to and beyond the sixth instar (carapace length 8–71 mm) are found to be abundant. This kind of mudflat is most likely a nursery ground. Chen et al. (2004) measured several abiotic factors of the potential spawning ground and the nursery mudflat including grain size, water content, total organic carbon, and nitrogen content. Other than this preliminary data, no detailed studies of the microhabitat characteristics of nursery ground have been conducted.

The purpose of the present study is to advance our understanding of the habitat requirements of *T. tridentatus* in the coastal zones of Taiwan so that suitable existing habitat can be preserved and damaged habitats have a chance to be restored. Our approach focuses on the following topics:

(1) Characterization of physical properties of nursery grounds
(2) Evaluation of potential practices for spawning ground restoration
(3) Proposal of future studies regarding habitat restoration and conservation

2 Nursery Grounds: Relationships Between Juvenile Horseshoe Crab Density, Physical Properties of Sediments, and Infaunal Density

2.1 Materials and Methods

2.1.1 Sampling Sites and Time

The physical properties of nursery grounds were examined on Kinmen Island at three sites – Nanshan, Beishan, and Hsiashu – in August of 2005 and July and November of 2006 (Fig. 2). At each sampling site, four 300 m long transect lines, each 50 m apart, were set up parallel to the seashore. On each transect line, ten plots, each 5 m by 5 m, were placed at 30 m intervals and three plots were randomly chosen for the sampling (Fig. 3). A total of 12 plots (3 per transect line) were chosen. Sediment samples were collected, treated, and analyzed as follows.

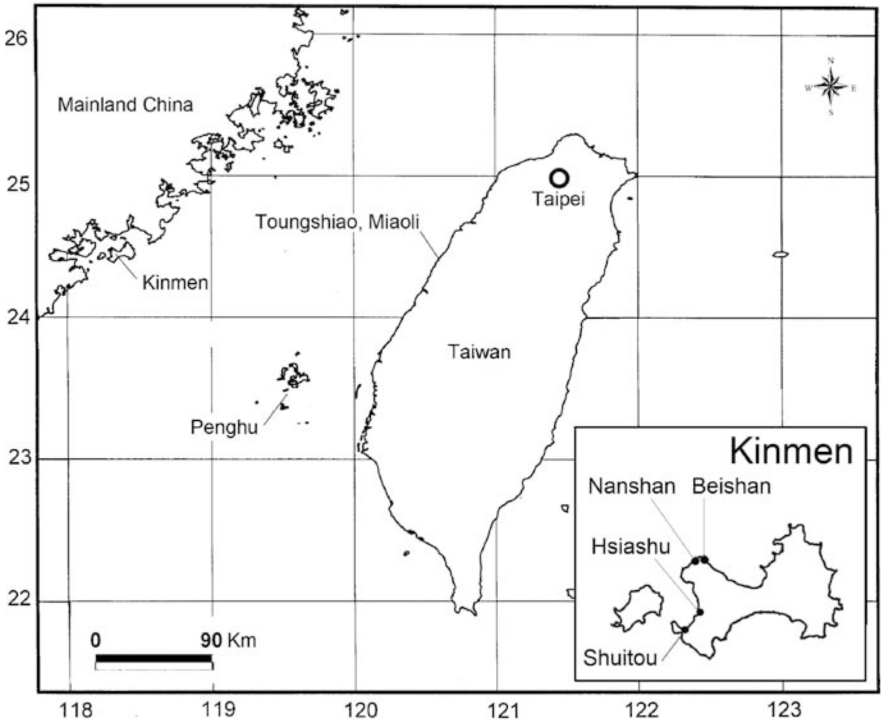

Fig. 2 Map depicting the present study sites of *T. tridentatus* distributed in the Taiwan area. On Kinmen Island, samples from three nursery grounds, Hsiashu, Nanshan, and Beishan, were taken from August 2005 to November 2006. At Toungshiao, Miaoli County, sampling was conducted from a restored spawning ground from August to October 2002

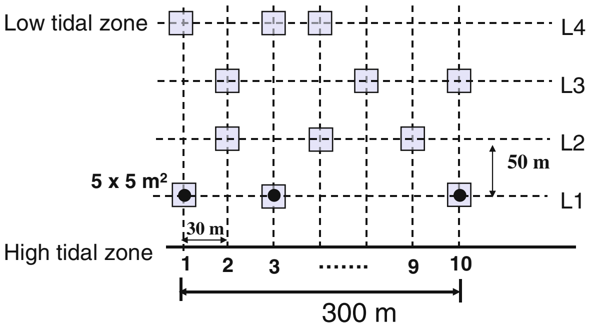

Fig. 3 A scheme showing the sampling plots for each nursery ground on Kinmen Island where sediment samples were collected to study the sedimentary physical properties and densities of juvenile horseshoe crabs *T. tridentatus* and infauna benthos

2.1.2 Measurements of Physical Properties

Granulometry, water content, total organic carbon and nitrogen contents, chlorophyll a content, pH, and salinity: The top 5 cm of sediment on the mudflat was collected separately using a PVC corer of 2.6 cm diameter. For analyzing chlorophyll *a* content, the top 0.5 cm of sediment in a 7.07 cm^2 surface area was collected. During transportation to the laboratory, all samples were kept cool at approximately 4°C while those for chlorophyll *a* content measurements were also kept in the dark. In the laboratory, samples for the measurement of total organic carbon (TOC) and nitrogen contents (TN) were kept at –70°C until cryo-dried.

Granulometry was determined by wet sieving the samples through a Wentworth series of screens with mesh openings from 1.0 mm to 63 μm. Silt and clay contents were measured using pipette methods. Median gain sizes and sorting coefficients were also calculated. Detailed procedures were described in Hsieh (1995).

Water content of the sediments was measured as the percent weight loss after oven-drying at 60°C for 48 h. Water content (% H_2O) = [(wet weight – dry weight)/wet weight] × 100%.

Total organic carbon and nitrogen contents were analyzed using an element analyzer (Perkin-Elmer EA-2400 II). Cryo-dried sediments were sieved through a screen with a mesh opening 0.5 mm in size in order to remove large animal or plant debris. Those that passed through the screen were collected and treated with 1 N HCl to remove all inorganic carbons.

Chlorophyll *a* was extracted by soaking the sample in 90% acetone overnight and analyzed using a fluorometer (Turner Designs, Model: 10-AU). Since no macroalgae and vascular plants were present in the sediment samples, chlorophyll *a* extracted was considered to be from microalgae.

Sediment pH values were measured using a glass electrode pH meter in 1:2 ratio of sediment to deionized water by weight. The sediment was first vigorously mixed with water before performing measurements (Chiu et al. 1999). Sediment salinity was measured using a refractometer.

2.1.3 Estimation of Juvenile Horseshoe Crab Density and Infauna Density

In each aforementioned plot (25 m^2), the living juvenile horseshoe crabs which were found by sight were counted and infaunal benthos were also sampled. Infauna were collected using a PVC corer with a diameter of 10 cm (0.00785 m^2) and treated following the procedures described in Hsieh (1995). The infauna specimens that were retained on a 0.5 mm mesh screen were identified to taxonomic levels higher than species, such as class or order, and the numbers of individuals were counted. The juvenile crab density and infaunal density were expressed as number of individuals m^{-2}.

2.1.4 Statistical Analyses of the Relationships of Juvenile Horseshoe Crab Density with Sedimentary Physical Properties and Infauna Density

Differences in the physical properties among the three sites and the properties that contributed to such differences were determined by factor analysis of ordinations (Press 1972). The six physical properties included in the factor analysis were grain size, silt/clay content, sorting coefficient, TOC content, water content, and chlorophyll *a* content. The relationships between two biotic components, the densities of juvenile horseshoe crab and polychaetes, and five physical properties were determined using Canonical Correlation Analysis (Digby and Kempton 1987). The five physical properties included TOC, grain size, sorting coefficient, water content, and chlorophyll *a* content. The remaining infauna groups were excluded because they were insignificant based on the Canonical Correlation Analyses. All statistical calculations were produced with the SAS PC software application (SAS Institute 2003).

2.2 Results

2.2.1 Physical Properties of Nursery Grounds

Sedimentary physical properties among the three sites exhibited similar pH, sediment salinity, and sediment temperature but dissimilar grain size, silt/clay content, sorting coefficient, total organic content, water content, and chlorophyll *a* content (Fig. 4). The three sites were all sandy; however, they varied in sediment grain size. Nanshan's substrate was the finest while Beishan's was the coarsest and Hsiashu's was intermediate. Median grain sizes at these sites were 0.08 mm (± SE 0.02), 0.41 mm (± SE 0.08), and 0.16 mm (± SE 0.03) in diameter, respectively. Silt/clay content and grain size were closely inversely correlated ($r = -0.79$, $p<0.001$); thus, Nanshan had the greatest silt/clay content with 45.8% whereas Beishan and Hsiashu had 32.0 and 15.1%, respectively. Nanshan and Beishan had similar total organic carbon content (TOC) of 0.39 and 0.43%, respectively. Both values were greater than 0.24% at Hsiashu. The same trend was seen in total nitrogen content as seen in higher values of 0.06 and 0.07% at Nanshan and Beishan than

0.05% at Hsiashu. Nanshan possessed 28.6% water content that was greater than those at Beishan (21.6%) and Hsiashu (19.1%). By contrast, Nanshan and Beishan had chlorophyll *a* concentrations of approximately 1.9–2.0 mg cm^{-2} that were lower than that of 2.6 μg cm^{-2} at Hsiashu. Substrates at all three sites were poorly sorted as seen in sorting coefficients that were all greater than 1.0.

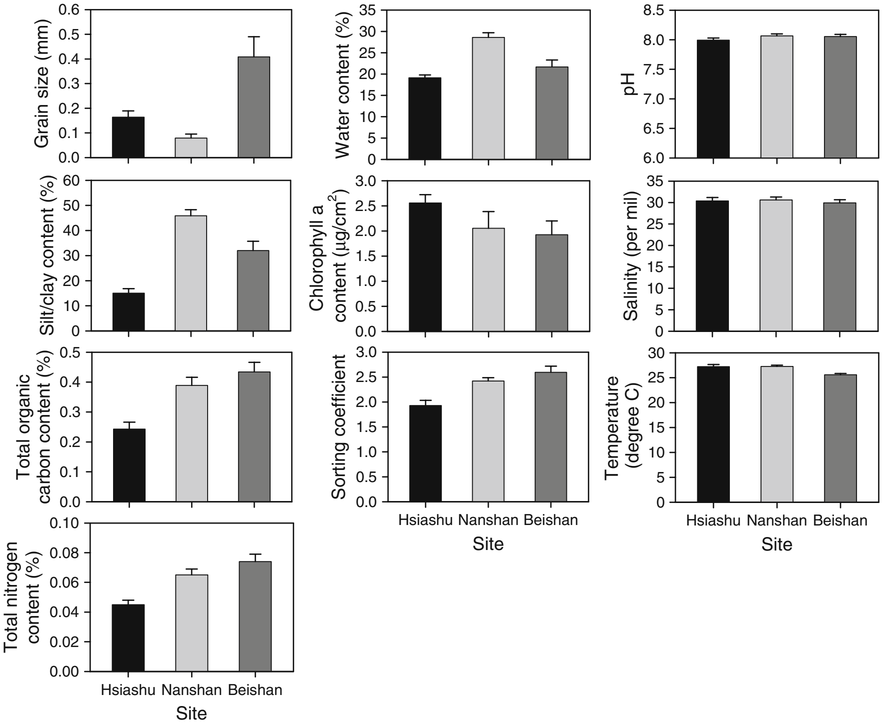

Fig. 4 Measures of benthic physical properties at each nursery ground on Kinmen Island, Taiwan. For each site, data were pooled over all sampling events. Values are means ± SE

2.2.2 Juvenile Horseshoe Crab Density and Infauna Density

Juvenile horseshoe crab density was lowest at the Nanshan site, higher at Beishan, and highest at Hsiashu and averaged 0.006, 0.061, and 0.169 individuals m^{-2}, respectively (Fig. 5).

A total of 13 taxa of infauna were collected from the three sites. They were nemerteans, nematodes, bivalves, gastropods, leeches, polychaetes, oligochaetes, sipunculans, isopods, insects, amphipods, shrimps, and crabs. Compared with the total averaged infauna density of 1783.4 individuals m^{-2} at Hsiashu, those at Beishan and Nanshan were lower having 1026.2 and 1117.4 individuals m^{-2}, respectively. Among these infauna, the polychaetes were the

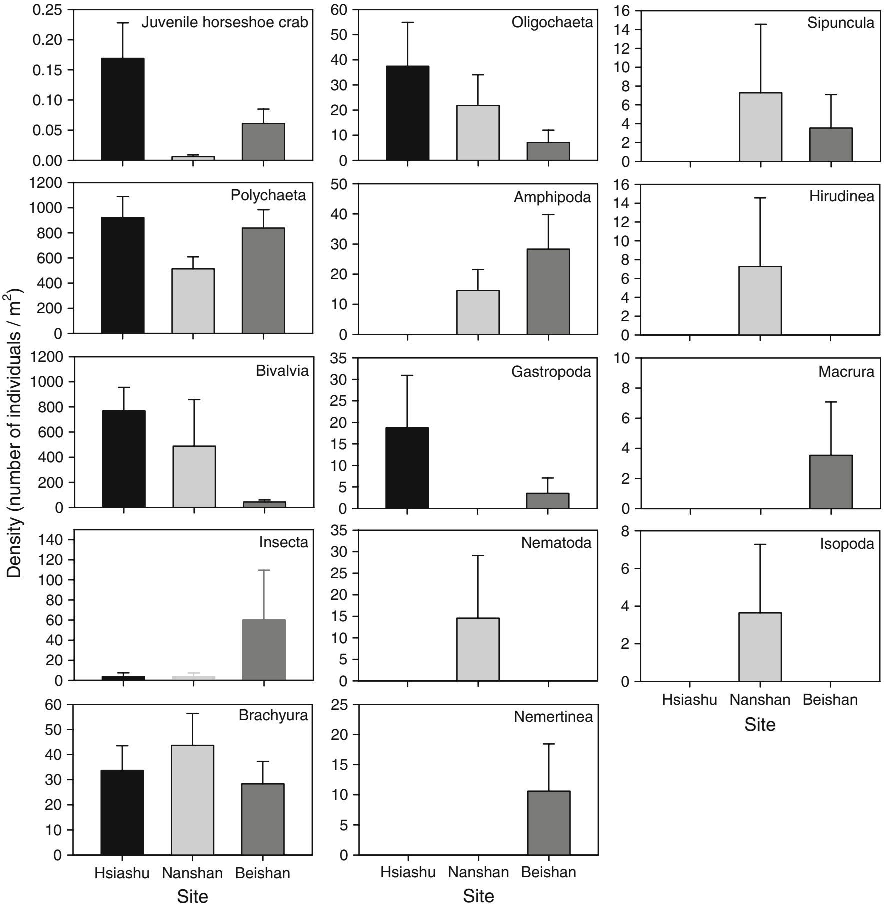

Fig. 5 Density changes of juvenile horseshoe crab *T. tridentatus* and infauna benthos among nursery grounds on Kinmen Island, Taiwan. For each site, data were pooled over all sampling events. Values are means ± SE

most dominant group at each site and bivalves had the next highest densities (Fig. 5). Similar to the distribution of the juvenile horseshoe crabs, polychaete density was lowest at Nanshan site, greater at Beishan site, and greatest at Hsiashu site and was 513.2, 838.6, and 921.7 individuals m^{-2}, respectively (Fig. 5). The remaining taxa had densities fewer than 50 individuals m^{-2}.

2.2.3 Relationships Among Juvenile Horseshoe Crab Density, Infauna Density, and Physical Properties of the Nursery Ground Microhabitat

The first two canonical correlations between two biotic components and five physical components were both significant as seen in Canonical Correlation

Analysis (cumulative proportions = 0.73 and 1.00, respectively; $r_1 = 0.54$, approx. $F_{(10,\ 124)} = 4.32$, $p < 0.0001$; $r_2 = 0.37$, approx. $F_{(4,\ 63)} = 3.01$, $p = 0.02$). In the first canonical variable, densities of juvenile horseshoe crab and polychaete were positively correlated with chlorophyll *a* content, but negatively correlated with water and TOC content. In the second canonical variable, densities of juvenile horseshoe crab were negatively correlated with sorting coefficient (Fig. 6).

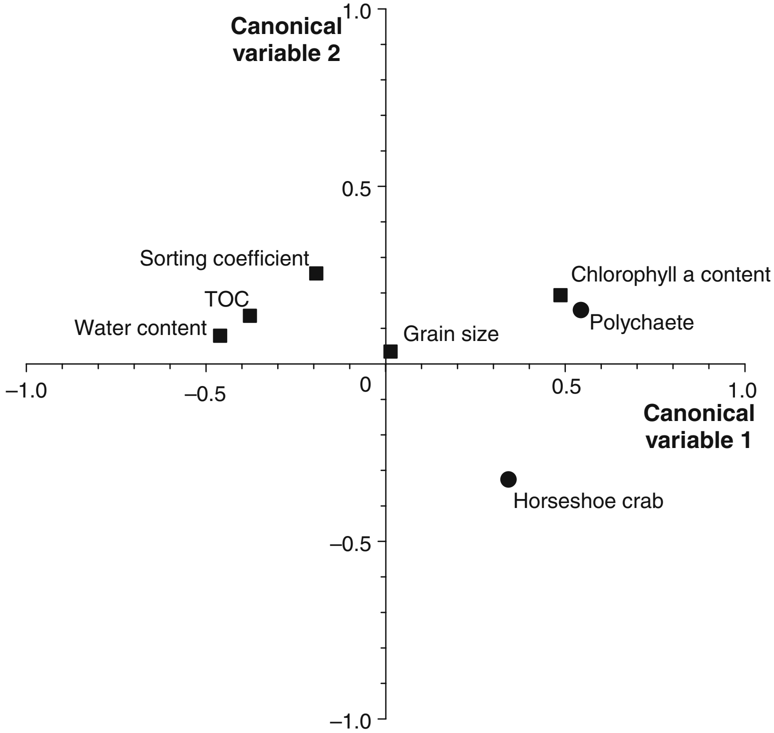

Fig. 6 Ordination and relationships among two biotic (density of horseshoe crab and polychaete) and five sedimentary physical properties by the first two canonical variables using Canonical Correlation Analysis

Among the six physical properties of the sediments, relative importance in differentiating the three sites was demonstrated by Factor Analysis (Figs. 7, 8). The first three principal factors together explained 81% of total variation in the physical properties at the three sites pooled, while the first factor alone explained 48%. On axis factor 1, silt/clay content and chlorophyll *a* content were important elements, having high positive or negative correlation with the axis (factor loading = 0.88 and –0.52, respectively). On axis factor 2, grain size

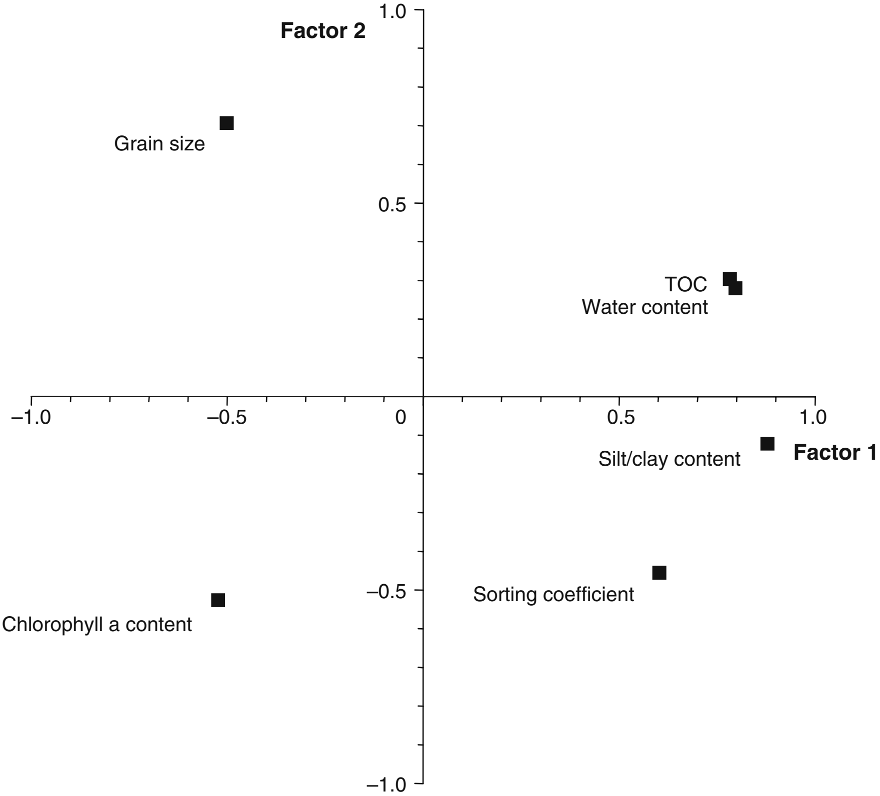

Fig. 7 Ordination of sedimentary physical properties by the first two factor axes, Factors 1 and 2, using Factor Analysis

and chlorophyll *a* content were important also with high positive or negative correlation with the axis (factor loading = 0.71 and −0.53, respectively) (Fig. 7). When all samples were ordinated by axis factors 1 and 2, the three sites overlapped with one another; however, Hsiashu and Nanshan were two distinctive sites within a broadly scattered Beishan site (Fig. 8). Hsiashu differed from Nanshan in having greater chlorophyll *a* content and larger grain size but less silt and clay, water, and TOC content and better-sorted sediments. By contrast, Beishan was characterized by having the largest variation in each of the physical elements that distinguished Hsiashu from Nanshan (Fig. 8).

2.2.4 Microhabitat Requirements of Juvenile Horseshoe Crab

To determine which habitat characteristics are most favored by juvenile horseshoe crabs, we analyzed the data by plotting average juvenile density in each site

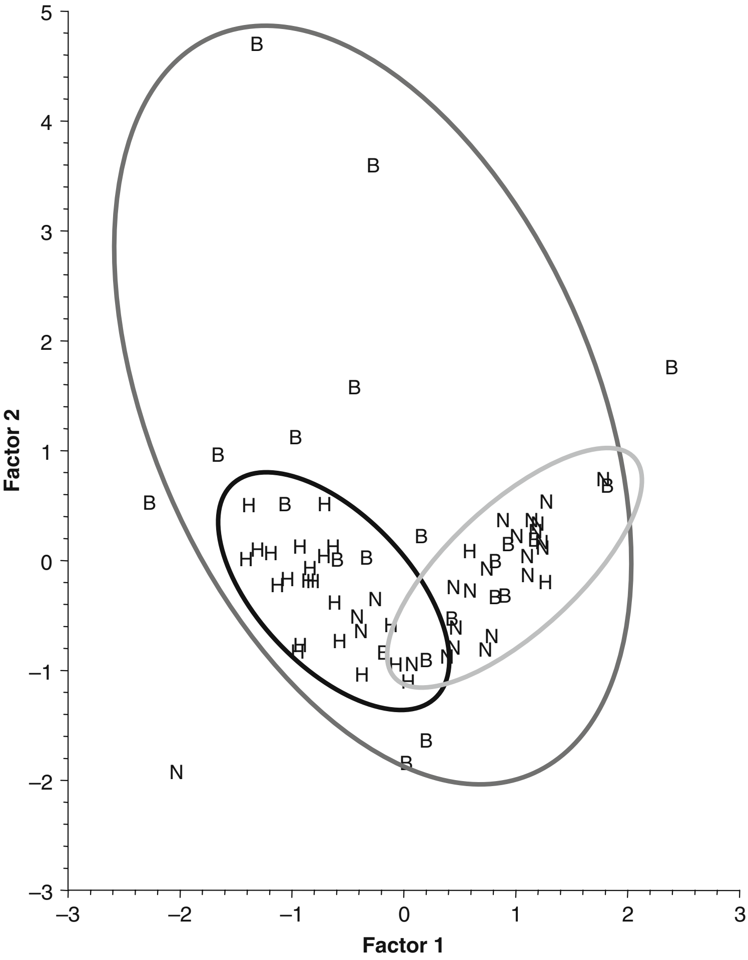

Fig. 8 Ordinations of the three nursery grounds based on their sedimentary physical properties by the first two factor axes, Factor 1 and 2, using Factor Analysis. H: Hsiashu, N: Nanshan, B: Beishan

at Kinmen against averaged value of each of physical properties at that site. The results showed that juveniles preferred sediments consisting of the following physical traits: grain size ranging from 0.14 to 0.27 mm in diameter, silt/clay content ranging from 13.7 to 36.2%, water content ranging from 16.9 to 23.2%, total organic carbon content ranging from 0.23 to 0.41%, total organic nitrogen ranging from 0.04 to 0.07%, chlorophyll *a* content ranging from 2.3 to 2.8 μg cm^{-2}, and in poorly sorted condition with sorting coefficients ranging from 1.87 to 2.76 (Fig. 9).

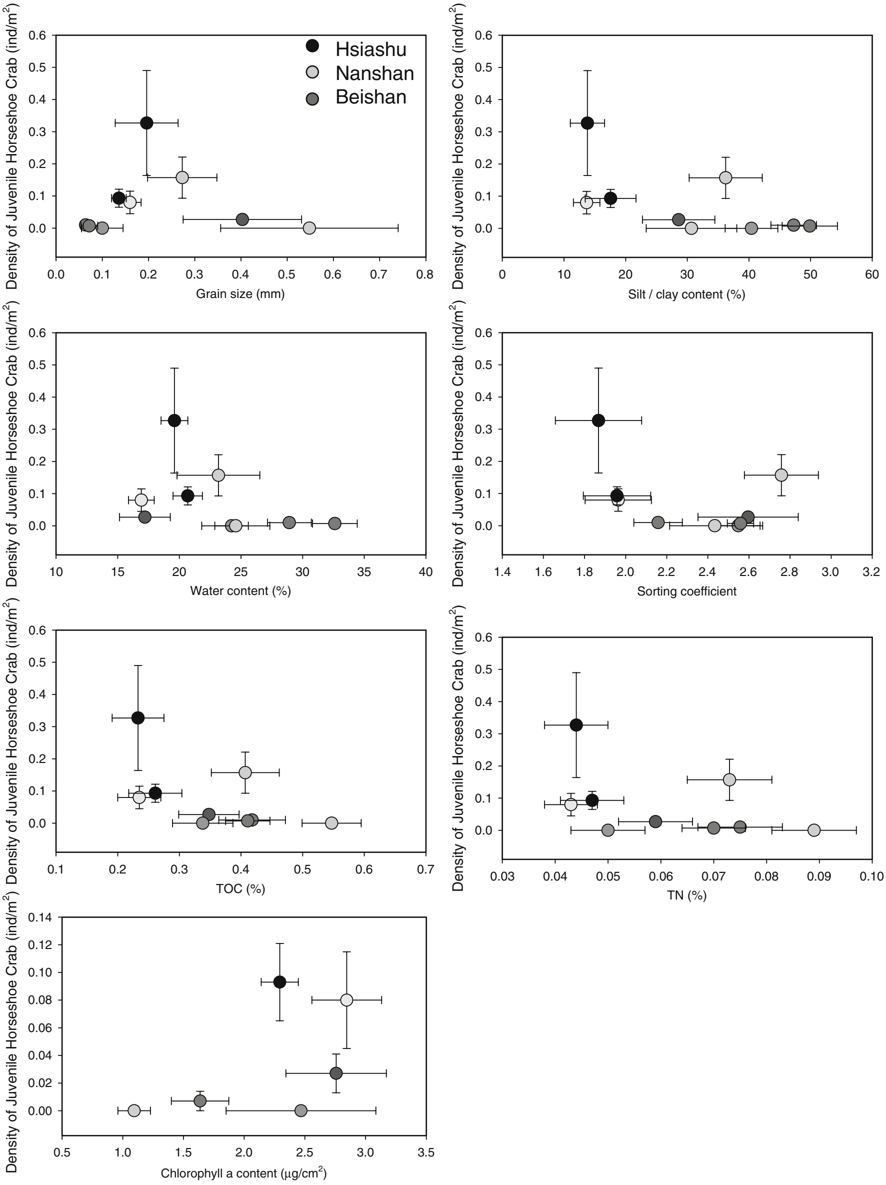

Fig. 9 Relationships between the densities of juvenile horseshoe crab *T. tridentatus* and the sedimentary physical properties showing the preferable conditions of the juveniles

3 Restoration of Spawning Ground

3.1 *Preparation of Nesting Substrate and Induction of Spawning*

Spawning grounds restoration was conducted in a 10 m by 10 m net fenced area on a small mudflat in the summer of 2002 at Toungshiao Town, Miaoli County, in northern Taiwan. The mesh size of the net was approximately 2 mm which was an adequate size for retaining instars in the fenced area. In July 2002, in the high tidal zone of this experimental area, the original mud substrate (averaging 0.13 mm in diameter) was changed by directly placing a layer of coarse sand (averaging 1.1 mm in diameter, a size suggested by the study of Chen et al. 2004) on top of the existing substrate. After flushing by daily tides for approximately 4 weeks, the coarse sand had been spread slightly down the slope and had formed a zone of approximately 5 m in width by 20 m in length and was 20 cm in depth (Fig. 10). In this restored area, sediment water content in the top 10 cm depth ranged from 3.7 to 9.3% as measured at the time when tides receded. Substrates adjacent to the restored zone were kept intact as mud throughout the study period.

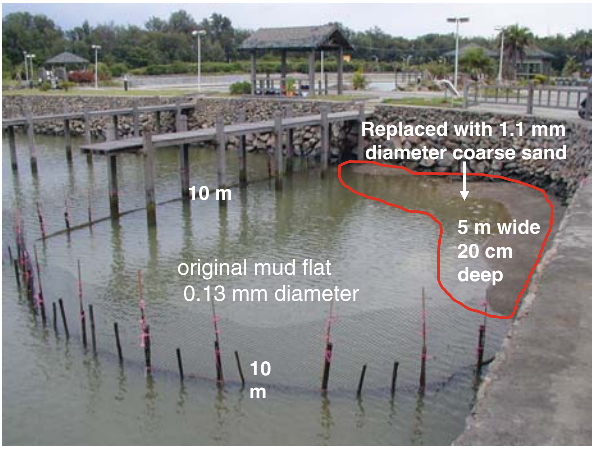

Fig. 10 A small net fenced area showing the restored spawning ground for *T. tridentatus* at Toungshiao, Miaoli County, Taiwan, in July 2002. The original mud substrate in the high tidal zone was replaced by 20 cm depth layer of approximately 1.1 mm diameter coarse sand

3.2 *Hatching Rate and Juvenile Survival Rate*

In August 2002 on the days when the spring tide arrived, five pairs of gravid adults were placed onto the high tidal zone in order to induce spawning. Locations where “spawning foams” emerged from the substrate were marked with wooden rods (Fig. 11). These air bubbles indicate the presence of nests (Chen et al. 2004). Previous studies showed that hatching takes place 40–50 days after fertilization (Chen et al. 2004). Subsequent observations on hatching rate and survival rates of young instars were made 40 days later and were repeated at 7 day intervals for three more observations until early October. Sand covering the nests was gently scraped away to expose the embedded

Fig. 11 Induction of spawning on restored spawning ground during August 12–13, 2002. **a**: gravid adults were put on the beach during tidal floods, **b**: spawning foams, **c**: when wooden sticks were placed beneath the spawning foams, the nests could be relocated after tides receded

offspring (Fig. 12). The number of eggs laid and the numbers of the first-stage instars hatched per nest were recorded (Fig. 13). After each recording, all eggs, offspring, and sand were gently placed back in each nest for continuous incubation until the next observation. During each observation time, a search was made on the restored spawning site for crawling instars. A search was also made on the surrounding mud for the first-stage and older instars.

3.3 Results

In total, four nests were found in Area A, eight nests in Area B, and two nests in Area C. In Area B, one nest had no eggs and another nest lost all of its eggs 1 month later; thus, these two nests were not included in further counting. Fifty days after spawning, no nests in Area B and Area C had hatched first instars. Eggs in the nests in Area C turned green and moldy as early as 12 days after spawning (Fig. 13). Only the nests in Area A produced viable offspring. Hatching rates ranged from 0 to 88.5% and averaged 33.9% over a 50-day period (Fig. 14).

Fig. 12 The nesting localities of the restored spawning ground. Number of nests found in Areas A, B, and C were 4, 6, and 2, respectively

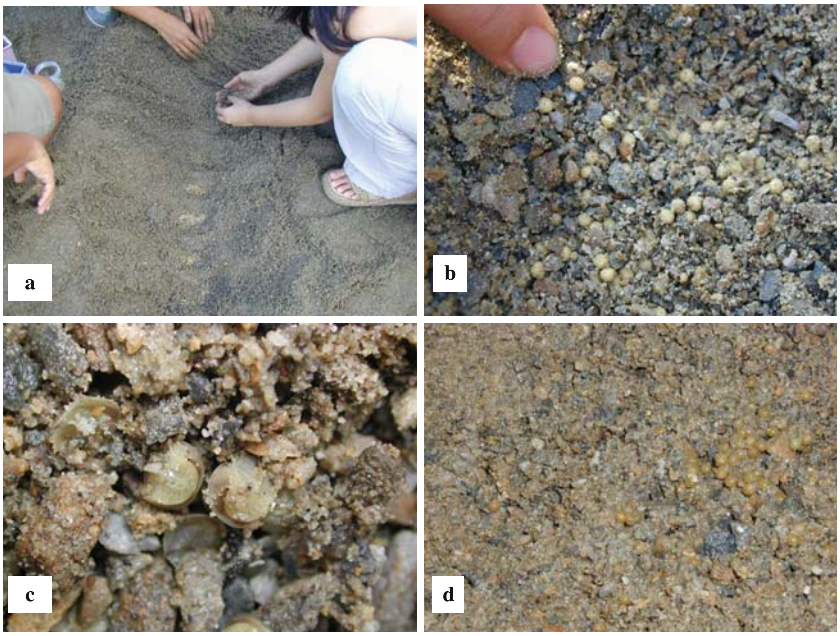

Fig. 13 Developing eggs in the nests. **a**: nests were discovered by removing covering sand, **b**: developing embryos, **c**: the first instars in the nest, **d**: moldy eggs

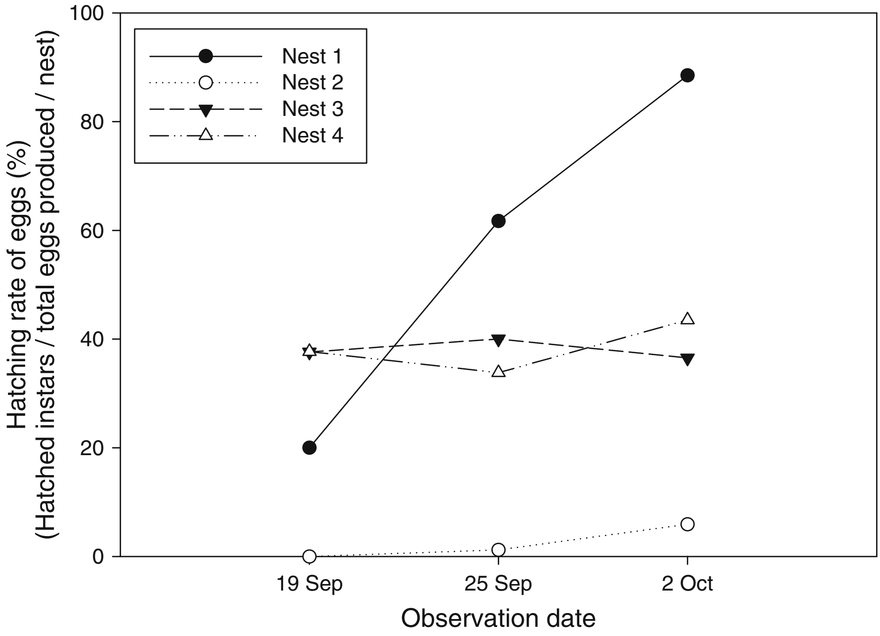

Fig. 14 Hatching rate changes over the study period of approximately 50 days. Fertilization began on August 12–13, 2002

No juveniles were found on the mudflat within the netted area and the surrounding mudflat during the study period, or over the following autumn, winter, and spring, indicating none of the hatched first instars had survived.

4 Discussion

4.1 Macrohabitat Preferences of T. Tridentatus

In Taiwan, Kinmen Island is known as the best place to find juvenile *T. tridentatus*. Although spawning swarms on Kinmen Island have not been observed for decades, we realize from local proverbs that the horseshoe crabs once heavily populated the west coast of Kinmen Island. In one proverb, this thriving phenomenon was described as “horseshoe crabs that died at Shuitou village are smelled at the far distant Gougaun village” (Chen et al. 2002). Shuitou is located in a headland bay that has now been partially used to build Shuitou Harbor. The original bay morphology reveals three areas: sandy beach along the upper tidal zone, mudflat stretching across the lower tidal zone, and an offshore trench zone at a depth of 20–35 m. In addition, the bay is subject to faster near-shore currents relative to the surrounding coasts (3.8 vs. 1.8 knots in

Nanshan and Beishan coast, Taiwan Electronic Navigational Chart Center 2006). Bays with these characteristics have been found to be the most suitable habitat for the horseshoe crabs including *T. tridentatus* since the three habitats – spawning grounds, nursery grounds, and maturation grounds – required by the horseshoe crab to complete its life history are connected to each other as seen in Delaware Bay for *L. polyphemus* (Brockmann 2003, Anderson and Shuster 2003) and in *T. tridentatus* (Sekiguchi 1988, Seino 2009).

Compared to Shuitou, the present study sites at Hsiashu, Nanshan, and Beishan are also headland bays, but are smaller. Also, the latter two sites lack an offshore trench and their subtidal zone is much shallower than 20 m. Therefore, integration of the three types of habitats that horseshoe crabs depend on appears to be not as good at Nanshan and Beishan as at Shuitou. Hsiashu has a greater juvenile density than Nanshan and Beishan. This may be attributed to the closer proximity between Hsiashu and Shuitou, making the Hsiashu macrohabitat similar to that of Shuitou as they are co-located in the same bay. Nanshan and Beishan are located in the range of The Horseshoe Crab Conservation Area, which was designated in order to compensate for the loss of the horseshoe crab's natural grounds due to the construction of Shuitou Harbor. These two sites represent available and suitable habitat, but probably not the best habitat for the horseshoe crabs on Kinmen Island. The same macrohabitat requirements were shown for the Japanese populations of *T. tridentatus* distributed in the Seto Inland Sea where the horseshoe crabs prefer semi-enclosed bays having sand bars present at river mouths with tidal mudflats stretching out of these sand bars (Seino 2009).

4.2 Microhabitat Characteristics of Nursery Ground

The juvenile horseshoe crab density at Hsiashu is approximately 2.5 times and 25 times greater than Beishan and Nanshan, respectively. This difference may be explained by both the abundance of the juveniles' forage base and the food web that supports it as well as by some physical properties. These important biotic determinants include the density of the polychaete prey and the concentration of the polychaetes' food, the microalgae. The abiotic factors include water content and total organic carbon content in the nursery ground sediments.

From the viewpoint of food availability and abundance, Hsiashu may represent a better nursery ground than the Beishan and Nanshan sites. Studies on the horseshoe crab's diets have shown that bivalves, especially thin-shelled and small-sized ones, are the preferable food for the adult *L. polyphemus* whereas early instars can be raised with polychaetes in aquarium conditions (Botton and Shuster 2003). In addition, polychaetes and crustaceans had also been reported as the food of the adult *T. gigas* in India (Botton and Shuster 2003). Diet analysis in juvenile *T. tridentatus* using δ^{13}C and δ^{15}N measures showed that polychaetes were their main prey (Nishida and Koike 2009). According to our study, polychaetes may also be one of the most important food sources for the juvenile *T. tridentatus* as seen from positively correlated relationships between the densities of the juvenile horseshoe crab and the polychaete (see Fig. 6). Moreover, polychaete density at Hsiashu was found to be greater than at Nanshan (921.7 vs. 513.2 individuals m^{-2}). These results imply that Hsiashu produces more of the favorable food, polychaetes, which nourished the juvenile horseshoe crabs, making Hsiashu capable of producing a larger juvenile population.

Regarding the food sources of the polychaetes, deposit-feeding polychaetes assimilate benthic microalgae from sediment surface (Newell et al. 1995, Hentschel 1998, Hsieh et al. 2002). In addition, the chlorophyll *a* content has been regarded as a measure of microalgal biomass present on the sediment surface. In our study, the polychaete abundance is positively correlated with chlorophyll *a* content. Chlorophyll *a* content was greater at Hsiashu than at Nanshan and Beishan, suggesting that Hsiashu possesses higher microalgal biomass (see Figs. 4, 6) and, therefore, more food available for the polychaetes. The positively correlated relationships among the densities of the polychaetes and the juvenile horseshoe crabs and chlorophyll *a* content lead us to the reason why more juveniles inhabit Hsiashu.

From the viewpoint of sedimentary physical properties, Hsiashu may also reflect a better habitat than Beishan and particularly Nanshan. The analyses of relationships between the juvenile horseshoe crab density and the sedimentary physical characteristics show that the juveniles avoid sediments having high water content and high total organic carbon content (see Fig. 6). Waterlogged sediments are known to often lack aeration, resulting in hypoxic or anoxic conditions and even hydrogen sulfide production in the sediments. Oxygen deficiency in sediment has been attributable to organic enrichment (Pearson and Rosenberg 1978). Among the three sites studied, the greater water content and total organic carbon content observed in Nanshan and Beishan sediments may be responsible for the lower densities of the juvenile horseshoe crabs at these two sites as compared to that at the Hsiashu nursery ground.

4.3 The Practice of Spawning Ground Restoration

The present pilot study shows that despite a low average hatching rate, the restoration practice was successful in terms of viable offspring being produced through induction of spawning in a modified substrate. The results from this experiment suggest that restoring spawning ground is feasible and may be a promising approach for enhancing horseshoe crab populations.

The low hatching rate observed may be due to various factors. First, the experiment area is located in the inner part of a bay. This bay is enclosed by dikes and has only one tidal outlet. We suspect that tidal amplitude may be too small to aerate the nests, resulting in embryos dying from mold infection.

Oxygenation of sediment is known to be essential for eggs to develop in the nest. Studies on the characteristics of the nesting site in *L. polyphemus* showed that mating adults avoided anaerobic peat sediments (Botton et al. 1988). Second, repeated exposure and reburying of nests for observations on embryo survivorship may have damaged the fragile embryos. Third, the eggs spawned may be poor in quality because the experiment of spawning induction was conducted at the end of the natural spawning season for the horseshoe crabs.

5 Future Work

5.1 Study on the Geomorphology and Hydrodynamic Regime of the Spawning Ground

Considering the variety of habitats required by the horseshoe crab *T. tridentatus* throughout its life history, the status of its nursery ground in Taiwan is in much better condition than that of its spawning ground. Mudflats are still present on Kinmen Island, Penghu Island, and the main Taiwan island, but sandy beaches suitable for nesting have largely disappeared due to dike construction, reclamation, or erosion. Although we have initiated a small-scale restoration for spawning and have succeeded in breeding some viable first instars, we failed in our overall goal since we lost many embryos and no hatchlings survived to grow. This may be due to the fact that our knowledge of the physical setting of the spawning ground is still rather limited. In the future, before practicing restoration, the geomorphology and hydrodynamic regime of the natural spawning ground, as perceived as a macrohabitat setting by the mating horseshoe crabs, needs to be studied in detail. Important forces shaping the beach morphology and flow regime are bay shape, tidal amplitude, currents, beach slope, sedimentary composition, sediment transportation, and typhoon-induced wave actions. In addition, on spawning beaches the vertical profiles of physical settings such as water content, temperature, oxygenation, and grain sizes also need further study.

5.2 Restoration Practice of Spawning Ground

In order to restore the ecological integrity of the spawning ground of *T. tridentatus*, the practice of restoration needs to integrate ecological, engineering, administrative, and community-based conservational disciplines through the entire process of restoration including planning, design, construction, and management.

Most sections of the coasts in the Taiwan area have been modified, especially on the Taiwan main island. However, a few places in southwestern Taiwan are undergoing or have used beach nourishment to prevent erosion. These places are our target sites for practicing the reformation of spawning grounds. At the present, a small beach at the southern coast of Budai Harbor, Chiayi County, in the southwestern Taiwan seems to be a suitable site. Local NGOs support the recovery of the lost horseshoe crab population that once thrived locally (Chen et al. 2009). Upcoming work for our research team will involve finding and inviting coastal engineers to join the habitat repairs for the horseshoe crabs.

5.3 Evaluation and Conservation of the Existing Nursery Grounds and Potential Spawning Grounds

The status of nursery grounds and potential spawning grounds of *T. tridentatus* on Kinmen Island need to be continuously monitored. Some of the measurements for evaluating the ecological integrity of these habitats include changes in the number of recruits, the size structure of juvenile populations, and the physical properties of nursery grounds and potential spawning grounds. At the macrohabitat level, beach erosion is a warning sign for the loss of potential spawning grounds. To prevent such loss, the geomorphology of the target bays needs to be monitored and evaluated periodically. The monitoring range should extend from the upper sandy beach area through the tidal flat and down to the offshore trench.

The most effective approach to conserve the horseshoe crab *T. tridentatus* is to designate protected areas for it. On Kinmen Island, 800 hectares of tidal flat has been designated as the horseshoe crab protected area (Chen et al. 2004). This action protects only part of its nursery grounds and not its maturation grounds. Adult populations distributed around the Kinmen area are known to live in the trenches of shallow water surrounding Kinmen, Taiwan, and Xiamen, China. As a result, the designation of protected areas where adult horseshoe crabs harvesting is prohibited needs Cross-Strait (Taiwan and China) collaboration.

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The Effects of Water Quality on Horseshoe Crab Embryos and Larvae

Mark L. Botton and Tomio Itow

Abstract It is well established that horseshoe crab eggs can develop successfully across a wide range of temperatures and salinities. However, many estuaries in which horseshoe crabs spawn have been heavily impacted by pollutants, and degraded water quality may be affecting the survival of horseshoe crab eggs laid in such areas. Laboratory bioassays using *Limulus polyphemus* embryos and larvae have shown a very high tolerance to a variety of contaminants, including heavy metals, oil, and organic compounds, in comparison to similar stages in other marine arthropods. Of the metals tested, acute toxicity was highest for mercury and tributyltin (TBT), but much lower for cadmium, zinc, and copper. Possible mechanisms of pollution tolerance, including stress proteins (Hsps), are discussed. Sublethal levels of heavy metals (especially mercury and TBT) induced a variety of embryonic malformations and impaired regeneration of walking legs. The frequency of malformed *L. polyphemus* embryos was low (ca. 1%) both in relatively pristine habitats (lower Delaware Bay, NJ) and in urban estuaries (Sandy Hook Bay, NJ, and Jamaica Bay, NY). In contrast, a much higher percentage of malformed *Tachypleus tridentatus* embryos have been found from several locations in Japan, and severely polluted water may be hampering Japanese efforts to re-establish horseshoe crab populations. Pollutants accumulated by adult females may become incorporated into their eggs and could conceivably be passed up the food chain to egg predators.

1 Introduction

This chapter examines the evidence that pollution has had significant impacts upon horseshoe crabs. In North America, there is evidence of decreasing numbers of *Limulus polyphemus* in the Delaware Bay area (ASMFC 1998) and on Cape Cod, Massachusetts (Widener and Barlow 1999). In southeast Asia, populations of *Tachypleus tridentatus*, *T. gigas*, and *Carcinoscorpius rotundicauda* are

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diminishing in Japan (Botton 2001), China (Chen et al. 2004), Malaysia (A. Christianus, Universiti Putra Malaysia, pers. comm.), and Hong Kong (Morton 1999; Chiu and Morton 2003). No single factor may explain these widespread declines, but it seems likely that pressures on horseshoe crab populations have resulted from some combination of pollution, degradation of the estuarine spawning habitats, and commercial fishing activities (Berkson and Shuster 1999; Botton 2001; Chiu and Morton 2003; Walls et al. 2002; Chen et al. 2004).

Most of the research on the effects of pollution on horseshoe crabs has been conducted on the American (*L. polyphemus*) or Japanese (*T. tridentatus*) species. Both spawn on sandy estuarine beaches (Brockmann and Smith 2009). Embryonic development through the "trilobite" (first instar) stage requires about 21–24 days in *L. polyphemus* and about 62 days in *T. tridentatus* (Sekiguchi 1988). Previous studies have emphasized the effects of pollution on the horseshoe crab eggs, embryos, and larvae because the early developmental stages of marine animals are usually most sensitive to the effects of pollution and because of the potential of contaminant transfer to egg predators such as birds and fishes. Pollutants could be derived from a combination of industrial, municipal, or agricultural wastes that enter the watershed and subsequently enter into the water, sediments, and biota.

At high levels, pollutants may cause mortality. The acute toxicity of a pollutant is typically evaluated in laboratory bioassays in which test organisms are exposed to a series of known concentrations of a pollutant for a time period (commonly 24–96 h), after which mortality is measured. Since pollution in the field may not be pulsed in this fashion, bioassays using continuous exposure may be more environmentally realistic. In the case of either acute or continuous exposure, the toxicity of the pollutant to the test population is frequently expressed as the median lethal concentration (LC_{50}), which is the concentration that results in 50% mortality. Pollutants may also cause sublethal effects at concentrations below those that cause mortality. For example, animals exposed to sublethal levels of contaminants might develop abnormally or take longer to molt than those raised in cleaner water. Lastly, if horseshoe crab eggs are accumulating pollutants, birds or other consumers ingesting contaminated eggs might experience adverse effects. We consider some of the possible modes by which pollutants enter into horseshoe crabs and discuss some of the physiological mechanisms that might help explain the survival of the embryos and larvae in stressful environments.

2 Bioassay Studies Using Horseshoe Crab Embryos, Larvae, and Early Juveniles

2.1 Heavy Metals

Botton et al. (1998a, b) and Botton (2000) studied the toxicity of acute and continuous exposure of cadmium, mercury, copper, zinc, and tributyltin (TBT) to stage 20 embryos (third embryonic molt; refer to Sekiguchi 1988 for descriptions of this and other developmental stages) and trilobite larvae of *L. polyphemus* (Table 1). In general, percent survival decreased as the duration of exposure to the pollutant increased, and the tolerance of larvae was typically greater than embryos. It should be noted that in these studies, LC_{50}s were estimated very conservatively; they were based on the percentage of animals that survived and molted to the next stage, rather than simply on the number of animals alive after 24, 48, or 72 h. The two heavy metals that were most toxic, TBT and mercury, have no known metabolic roles. Conversely, crabs were more tolerant of copper and zinc, which are metabolically useful in hemocyanin synthesis and as enzyme co-factors (White and Rainbow 1985). The concentrations of heavy metals that horseshoe crab embryos and larvae could survive were much greater than other marine arthropods, and the levels of heavy metals in lower Delaware Bay were unlikely to cause significant mortalities (Botton et al. 1998a, b; Botton 2000). The tidal freshwater and oligohaline areas of this estuary, including the Philadelphia metropolitan area, have the highest concentration of pollutants, but the concentrations are very much reduced in the mesohaline portion of the bay where most horseshoe crab spawning takes place (Church et al. 1988; Albert 1988; Riedel and Sanders 1998).

Table 1 Median lethal concentrations (LC_{50}) of some heavy metals to *Limulus polyphemus* embryos and trilobite larvae at various exposure periods. LC_{50} values are in mg l^{-1} except for tributyltin, which is in μg l^{-1}. Modified from Botton et al. (1998a, b) and Botton (2000)

Stage	Metal	24 h	48 h	72 h	Continuous
Stage 20 embryo	Tributyltin	44	20	14	
	Mercury	12.8	5.1	3.1	3.2
	Copper	151	180	171	185
	Cadmium	>1,000	503.3	171.9	39.5
	Zinc	>1,000	>1,000	715	170
Trilobite	Tributyltin	>1,000	742	594	42
	Mercury	56.0	19.6	7.6	0.7
	Cadmium	304.0	139.9	167.7	71.7
	Copper	>1,000	855	637	136
	Zinc	>1,000	>1,000	>1,000	87

2.2 *Oil*

Oil is a pervasive pollutant in the marine environment, and the potential impact of an oil spill is a particular concern to horseshoe crabs in the Delaware Bay system, given that the Port of Philadelphia is the third largest petrochemical port in the United States. Since 1972, there have been nine oil spills of more than 10,000 gallons in the Delaware Estuary, the most recent of which was the tanker Athos I (http://www.ocean.udel.edu/oilspill/). This vessel struck a submerged

18,000 lb anchor near its berth in Paulsboro, NJ, on November 20, 2004, and released an estimated 265,000 gallons of heavy crude oil. None of the major oil spills in the Delaware Estuary had a negative impact on horseshoe crabs. Nearly all took place outside the spawning season, and the majority also occurred in the Port of Philadelphia itself, far upriver from the spawning beaches. The Anitra spill (42,000 gallons) took place at Big Stone Anchorage in the lower bay on May 9, 2006, and certainly had great potential to impact nearby horseshoe crab eggs and shorebirds on nearby spawning beaches. Because of the comparatively shallow depth of the shipping channel in the Port of Philadelphia, it is necessary for larger tankers to transfer their oil to smaller, shallower draft vessels which then convey the oil to the refineries. The Anitra spill took place during this "lightering" procedure. Fortunately, the effects of the Anitra spill were minimized because of opportune wind patterns following the spillage.

The effect of oil pollution on horseshoe crab eggs and early instars has been studied in laboratory bioassays. Laughlin and Neff (1977) showed that *L. polyphemus* eggs exposed to water-soluble fractions (WSF) of No. 2 fuel oil generally had high hatching success, except at the highest oil concentration (50% WSF). Rearing temperature and WSF concentration had little effect on the resulting size of instars 1–3, and there was no consistent effect on the length of time between molts. However, second instars exposed to 5 and 10% WSF, especially at salinities of 10 or 20 psu, had higher rates of oxygen consumption, suggesting that they were metabolically stressed. In a study examining the effects of Bunker C oil, Strobel and Brenowitz (1981) found that the percent survival of trilobites was high (>75%) at concentrations up to the maximum tested, 3.5 mg l^{-1}. However, there was a significant lengthening of the intermolt period between the trilobite and the second instar stages, and between the second and the third instar stages. Under field conditions, the persistence and probable ecological impact of an oil spill event would be influenced by the composition of the oil, sediment type, the temperature, and nitrogen composition, which are known to influence the rate of microbial biodegradation (Venosa et al. 1996).

2.3 Organic Compounds

Halogenated hydrocarbons are widespread and highly persistent pollutants in the marine environment. Neff and Giam (1977) investigated the effects of Aroclor® (a polychlorinated biphenyl, PCB) and Halowax® (a polychlorinated naphthalene, PCN) on second through fourth instar *L. polyphemus*. Short-term (96 h) mortality did not occur even at concentrations up to 80 ppb, but toxic effects were seen in the ensuing weeks of the experiment. Second instars were more sensitive than third instars to both PCB's and PCN's, and animals exposed to these pollutants had a reduced intermolt time as well as increased oxygen consumption relative to controls. Although equal concentrations of Aroclor® and Halowax® were administered in these bioassays, the final tissue concentrations of Aroclor® were much higher, corresponding to the greater toxicity found for this compound.

Weis and Ma (1987) studied the effects of the pesticide Dimilin® on larval *L. polyphemus*. This compound, a chitin synthesis inhibitor, had limited effects on mortality or molting at 5 ppm but severe mortality at 50 ppm. The mean prosoma width of surviving animals in the 50 ppm group was significantly lower than controls or the 5 ppm group.

In summary, the results of these bioassay studies suggest that the early developmental stages of *L. polyphemus* are highly tolerant of pollutants, often surviving and molting at concentrations that may be 1–2 orders of magnitude higher than other marine arthropods. Unfortunately, comparative studies on the three Asian species of horseshoe crabs are lacking.

3 Effects of Pollutants on Normal Embryonic Development and Limb Regeneration

Itow et al. (1998a) exposed sequential developmental stages of horseshoe crab embryos to heavy metals for 24 h and then assessed the condition of the surviving embryos. Even when percent survival was high, exposure to heavy metals at concentrations as low as 1 mg l^{-1} for 24 h induced developmental malformations (Fig. 1). Certain metals were associated with particular

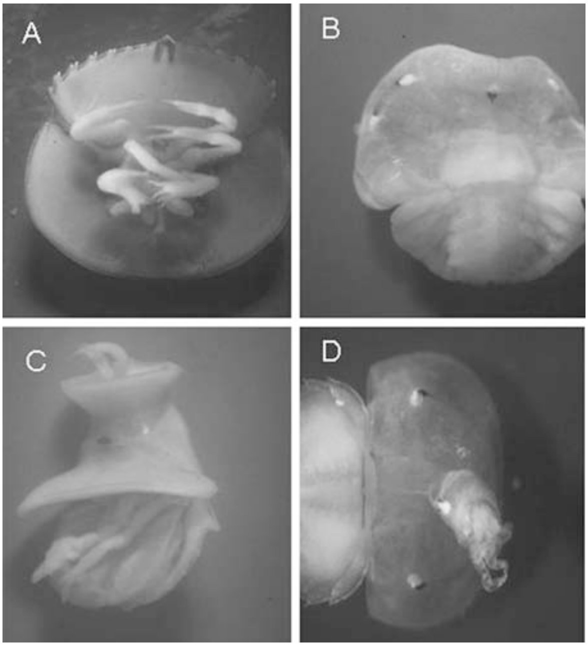

Fig. 1 Examples of developmental abnormalities found in horseshoe crab embryos. (**A**) Segment-defective embryo, (**B**) embryo with abnormal lateral eyes, (**C**) separated embryo, and (**D**) double embryo. All photographs by T. Itow

Table 2 Frequency of different embryonic abnormalities in horseshoe crabs induced by 24 h exposure to heavy metals. Experiments with zinc (as $ZnSO_4$, 100 mg l^{-1}) and tributyltin (1 mg l^{-1}) used *Limulus polyphemus* embryos; and experiments with mercury (as $HgCl_2$, 1 mg l^{-1}) used *Tachypleus tridentatus* embryos (modified from Itow et al. 1998a)

Type of developmental abnormality	Zinc	Mercury	Tributyltin
Double embryos	30 (26.8%)	0	0
No posterior embryos	22 (19.6%)	0	62 (33.7%)
Segment-defective embryos	21 (18.8%)	30 (62.5%)	5 (2.7%)
No anterior embryos	17 (15.2%)	6 (12.5%)	7 (3.8%)
Separate embryos	12 (10.7%)	12 (25.0%)	7 (3.8%)
Abnormal lateral eye embryos	8 (7.1%)	0	101 (54.9%)
No anterior and posterior embryos	2 (1.8%)	0	1 (0.5%)
Half embryos	0	0	1 (0.5%)
Total number of malformed embryos	112	48	184
Total number of embryos treated	650	160	650
Malformed embryos as % of total	17.2%	30.0%	28.3%

malformations (Table 2). For example, exposure of embryos (especially at stages 9–12) principally induced the formation of segment-defective embryos, whereas exposure to TBT (especially at stages 5–9) was primarily associated with abnormal lateral eyes. On the other hand, exposure to zinc during a broad period of embryonic development (stages 6–17) caused a range of developmental anomalies, including double embryos, no-posterior embryos, segment-defective embryos, no-anterior embryos, separate embryos, and abnormal lateral eyes. Although these abnormal animals were alive, they were not observed to develop beyond the first instar stage.

Itow et al. (1998b) studied the effects of heavy metals on the size and morphology of regenerated walking legs in late-stage embryos and trilobite larvae. Following amputation, the legs did not regenerate until molting occurred, and even in seawater controls, the regeneration was not fully completed in one molt. Continuous exposure to sublethal levels of heavy metals did not affect the rate of molting, but regeneration of the leg was partially or completely inhibited by 0.1 mg l^{-1} TBT, 1 mg l^{-1} mercury and cadmium, and 10 mg l^{-1} chromium and zinc. Exposure of embryos to 100 mg l^{-1} lead and copper had no effect on regeneration.

4 Field Studies on the Effects of Pollution

The frequency of occurrence of abnormal embryos in the natural environment can be used as an indicator of water quality. Using this criterion, it is evident that several populations of *T. tridentatus* have been severely impacted by coastal pollution, especially in the heavily industrialized areas within the Seto Inland Sea of Japan (Table 3). By contrast, few abnormal *L. polyphemus* embryos have been found in urban estuaries with high contaminant loads (Jamaica Bay, New York, and Sandy Hook Bay, NJ), as well as lower Delaware Bay, NJ (Itow et al. 1998a; Botton et al. 2006a) (Table 4). The frequency of occurrence of malformed embryos from Fushigoe, Shimizu City, and Fuji City in Japan far exceeds any location that we have examined in the United States. It would be useful to conduct simultaneous laboratory bioassays using *T. tridentatus* and *L. polyphemus* to determine if there is a differential susceptibility to pollution (and possibly to expand upon this study to include *T. gigas* and *C. rotundicauda*). We would note, however, that results from bioassays using a single pollutant do not replicate the complex mix of heavy metals, organic compounds, and other constituents of contaminated waters. It is possible that the high frequencies of embryonic malformations in coastal Japan could be due to the additive or synergistic effects of different contaminants.

Table 3 Occurrence of malformations among field-collected embryos of *Tachypleus tridentatus* from Japan (modified from Tsuchiya and Asano 1989 and Itow 1997)

Location	Embryos examined	Not developed	Normal	Malformed	Percent malformed
Natsume, Kasaoka City, Okayama Pref.	542	15	527	0	0.0
Fushigoe, Kasaoka City, Okayama Pref.	520	239	236	45	8.7
Port of Tsukama, Shimizu City, Shizuoka Pref.	149	16	112	21	15.8
Tagonoura Harbor, Fuji City, Shizuoka Pref.	148	33	47	34	42.0

Table 4 Occurrence of malformations among field-collected embryos of *Limulus polyphemus* from the United States. Animals from Jamaica Bay were collected as eggs or early embryos and cultured in the laboratory using water from either Jamaica Bay (JB) or lower Delaware Bay (DB). Modified from Itow et al. (1998) and Botton et al. (2006)

Location	No. embryos examined	Percent malformed (%)
Delaware Bay, NJ		
Cooks Beach	776	0.26
Cape Shore Lab Beach	1,263	0.48
Gandys Beach	1,191	0.59
Thompsons Beach	2,440	0.45
Reeds Beach	408	0.25
Sandy Hook Bay, NJ	1,268	0.16
Jamaica Bay, NY (in JB water)	2,428	0.95
Jamaica Bay, NY (in DB water)	1,466	1.16

5 Other Water Quality Issues: Temperature and Salinity

Global warming has the potential to affect horseshoe crab development by affecting temperature and salinity. The most direct influence that global warming may have on horseshoe crabs is on sea level rise and the likelihood of erosion of critical spawning areas. In principle, increases in water temperature and alterations of the salinity regime could have adverse impacts. However, the horseshoe crab embryos and larvae are well known for their ability to survive in an extremely broad range of both of these environmental factors. When *L. polyphemus* eggs were reared at constant temperatures, optimum development took place between 25 and 33°C. However, at constant 35°C temperature, embryos arrested at stage 20 and no development occurred at 40°C (Jegla and Costlow 1982; Ehlinger and Tankersley 2004). However, Botton et al. (2006b) noted that horseshoe crab eggs are unlikely to experience constant temperatures in their estuarine habitats. They found that *L. polyphemus* embryos that were acclimated to 13 or 22°C had close to 100% survival when exposed to a 3 h heat shock of 40°C, although a 3 h heat shock at 45°C was lethal. The ability to survive short-term exposure to temperatures up to 40°C is adaptive on sandy beach environments where temperatures are highly variable.

All four species of horseshoe crabs have embryos and larvae that are highly tolerant to a wide range of salinities. For *L. polyphemus*, the optimal salinity range was 20–40‰, although embryos survived but took longer to hatch at 10–20 and 40–60‰ (Jegla and Costlow 1982; Laughlin 1983; Ehlinger and Tankersley 2004). Trilobites survived in water between 10 and 70‰, encompassing the highly variable and occasionally hypersaline conditions in the Indian River Lagoon, Florida (Ehlinger and Tankersley 2004). Embryos (stage 12, germ band formation) of *T. tridentatus*, *T. gigas*, and *C. rotundicauda* had high viability in salinities ranging from 5 to 35‰, but early embryos (stage 3, appearance of cleavage nuclei) of *T. tridentatus* and *C. rotundicauda* had <20% survival at salinities below 15‰ (Sugita 1988). *T. gigas* trilobites survived all salinities between 10 and 40‰, but molting occurred 1 week sooner at 40‰ (Chatterji et al. 2004).

It would appear, then, that the main effects of global warming on horseshoe crab will probably be related to beach erosion rather than to increasing temperature or to alterations of the salinity regime.

6 Mode of Transfer of Pollutants into Horseshoe Crabs and Potential Effects on Consumers

Relatively little is known about the mechanisms by which contaminants enter horseshoe crabs, but several studies indicate the potential for pollutants to be incorporated into the eggs during vitellogenesis. In a study of *T. tridentatus* from Japan, Kannan et al. (1995) found that levels of pollutants were generally higher in hepatopancreas, gill, and eggs compared with other tissues. These authors did not find a consistent difference in the levels of heavy metals (iron, manganese, zinc, copper, lead, nickel, cadmium, cobalt, mercury) comparing eggs from a polluted location (Habu Bay, within the Seto Inland Sea) with a cleaner location (Hakata Bay). However, organotins (diphenyl and triphenyltin) in the hepatopancreas and eggs were much greater at Habu Bay. The concentrations of these pollutants within horseshoe crab hepatopancreas were much higher than in the sea water, indicating that there was a substantial bioconcentration process.

A number of heavy metals have also been detected in female *L. polyphemus* (Burger et al. 2002). Although there were significant differences between levels of various metals in the eggs, leg muscle, and apodeme (a portion of the exoskeleton to which muscles attach), there was no indication that eggs were consistently higher or lower than somatic tissue.

A summary of heavy metal levels found in female horseshoe crabs and eggs from Japan, Vietnam, and the United States is presented in Table 5. It is difficult to make comparisons among these studies because not all pollutants were examined in each study (Kannan et al. 1995, Boman et al. 2001, Burger et al. 2002). It would appear, however, that the concentrations of heavy metals in eggs and muscle from Japan were lower than in Vietnam and the United States, which is somewhat unexpected given the higher frequency of malformed embryos from Japan compared with the United States (Tables 3 and 4).

Given that *L. polyphemus* eggs are the primary food for migratory shorebirds in Delaware Bay (Botton et al. 1994; Tsipoura and Burger 1999; Baker et al. 2004; Haramis et al. 2007; Mizrahi and Peters 2009), there has been concern about the potential transfer of contaminants from eggs to these consumers. Levels of lead, cadmium, and chromium in horseshoe crab eggs from Delaware Bay females have declined over the period 1993–2000, while concentrations of mercury have remained stable (Burger et al. 2003). Heavy metals have been detected in feathers of red knots, semipalmated sandpipers, and sanderlings collected from Delaware Bay, but this is indicative of exposure to contaminants on the wintering grounds (Burger et al. 1993). A direct linkage between contaminants in horseshoe crab eggs and bird tissue has yet to be made. However, Burger et al. (2003) concluded that the current concentrations of metals in horseshoe crab eggs were unlikely to have adverse effects upon shorebirds during the 2–3 weeks of feeding in Delaware Bay.

Maternally derived egg contaminants can affect developmental success. Itow (1997) obtained eggs from different Japanese populations of *T. tridentatus*, fertilized them by artificial insemination, and then cultured the embryos in clean seawater to ensure that the only contaminants present were the result of vitellogenesis. Eggs taken from female crabs from a relatively clean region (Hakata Bay, Fukuoka Prefecture, on the northern coast of Kyushu) had about 90% development and only 0.2% malformed embryos (Table 6). In comparison, eggs from Okayama, a highly industrialized bay on the Seto Inland Sea, had only about 20–40% development and 12.2% of these were malformed.

Table 5 Trace metal concentrations (ppb) in female horseshoe crab tissues, *Tachypleus tridentatus* from southeast Asia, and *Limulus polyphemus* from the United States. ND = below detection limits; (–) = not included in study. Modified from Kannan et al. (1995), Boman et al. (2001), and Burger et al. (2002)

Tissue and metal	*T. tridentatus* (Hakata Bay, Japan)	*T. tridentatus* (Habu Bay, Japan)	*T. tridentatus* (Vietnam)	*L. polyphemus* (Maine, USA)	*L. polyphemus* (Delaware Bay, NJ, USA)	*L. polyphemus* (Gulf of Mexico, FL, USA)
Muscle						
Arsenic	–	–	–	5,620	12,300	41,800
Cadmium	0.02	0.01	–	9	19	146
Copper	9.9	12	37	–	–	–
Iron	1.8	3.4	21	–	–	–
Lead	ND	ND	ND	21	54	44
Mercury	0.08	0.04	–	15	75	60
Zinc	124	122	610	–	–	–
Eggs						
Arsenic	–	–	–	4,450	3,670	32,000
Cadmium	0.01	0.01	–	17	99	160
Copper	20	20	93	–	–	–
Iron	7.2	4.9	20	–	–	–
Lead	ND	ND	ND	92	48	15
Mercury	0.05	0.02	–	4	22	33
Zinc	32	32	76	–	–	–

Table 6 Occurrence of malformations among horseshoe crab eggs obtained by artificial insemination and raised in the laboratory in clean ocean water (modified from Itow 1997)

Species and location	Embryos developed	Percent development (%)	Percent malformed (%)
Tachypleus tridentatus			
Fukuoka (northern Kyushu, Japan)	4,647	~90	0.2
Ohita (northern Kyushu, Japan)	1,393	~30–40	4.2
Yamaguchi (Seto Inland Sea, Japan)	558	~30–40	2.7
Okayama (Seto Inland Sea, Japan)	6,481	~20–40	12.2
Carcinoscorpius rotundicauda (Thailand)	2,565	~80–90	0.2
Limulus polyphemus (Woods Hole, USA)	6,131	~90	1.8

Further evidence for the transfer of toxins from females into eggs has come from cases of food poisoning in Southeast Asia. The consumption of *C. rotundicauda* in countries such as Thailand, Bangladesh, and Cambodia is considered to be quite risky because of the potential contamination by tetrodotoxin (TTX), a potent neurotoxin (Kungsuwan et al. 1987, Ngy et al. 2007). The toxicity of *C. rotundicauda* from Cambodia was not clearly correlated to the size of the animal or the time of year, but assayed TTX dosages were sometimes high enough that a single meal of 100 g of eggs could be fatal to a person (Ngy et al. 2007). TTX appears to be produced by bacteria (*Vibrio* spp.) which live in the intestine and transferred to the eggs during the maturation process (Kungsuwan et al. 1988).

Once the eggs are deposited on the beach and begin their development, there may be additional mechanisms for pollutants to enter, but none of these has been directly demonstrated. Based on previous studies (e.g., Botton et al. 1998a, b; Botton 2000; Itow et al. 1998a), diffusion of heavy metals is probably taking place through the chorion and the inner egg membrane. Following hatching, intake across permeable surfaces such as the gills could take place. Once the crabs begin feeding at the second instar stage, the ingestion of food and sediment particles could provide another route of entry for contaminants.

7 Physiological Mechanisms for Environmental Tolerance

Horseshoe crab embryos and larvae are very well adapted to survive over a broad range of temperatures and salinities, and they are able to tolerate much higher levels of pollutants than other marine arthropods. However, the specific biochemical and physiological mechanisms that enable horseshoe crabs to survive environmental stresses have received relatively little attention. Botton et al. (2006b) studied the role that stress proteins (also known as heat shock proteins or Hsps) might have in the ability of *L. polyphemus* embryos and larvae to withstand temperature stresses. Hsps are "molecular chaperones" that help to insure the structural integrity of cellular proteins that have been distorted by heat shock or other kinds of environmental stresses (Feder and Hofmann 1999; Lewis et al. 1999; Sørensen et al. 2003). In many organisms, stress initiates the synthesis of "inducible" Hsps, elevating total Hsp relative to the baseline level of "constitutive" Hsps found in unstressed individuals. Botton et al. (2006b) acclimated stage 20 embryos and trilobite larvae to 13 or 22°C and then subjected them to a heat shock at 35°C for 3 h. Levels of Hsp70 (molecular weight of 70 kDa) were very similar among heat-shocked animals and controls kept at constant temperature, which suggested that the ability of horseshoe crabs to tolerate temperature shock was not related to the synthesis of inducible Hsp70. Rather, *L. polyphemus* appears to possess a high level of constitutive Hsp70 (Hsc70), which may be adaptive given that the changes in temperature on an intertidal beach occur frequently because of the tidal and day/night cycles. It may simply be more efficient to maintain a high level of Hsc70 than to frequently up-regulate and down-regulate the genes responsible for inducible Hsp70 synthesis.

The importance of Hsp's in enduring temperature stresses does not prove that Hsp's are the basis of pollution tolerance; further experimentation in this area is warranted. Also, there are many other possible physiological mechanisms of pollution tolerance which have been demonstrated in other marine organisms (e.g., Viarengo 1985; George 1990; Amiard et al. 2006), all of which remain to be studied in horseshoe crabs. For instance, metallothioneins are low molecular weight proteins (6–7 kDa) that have a very high affinity for heavy metals. Complexing of metals with metallothioneins presumably means that fewer metal molecules are left to cause toxicity. It has also been shown that heavy metals accumulate in lysosomes, where they may be immobilized within the cross-linked matrix of lipofuscins. These may be expelled by exocytosis. Finally, small (0.5–2.0 µm diameter) intracellular concretions are commonly found in crustaceans and mollusks. These structures contain calcium and magnesium phosphates may be rich in heavy metals and are thought to be important in accumulation and detoxification of heavy metals.

8 Summary, Conclusions, and Implications for Horseshoe Crab Conservation

Based on laboratory studies, *L. polyphemus* embryos and larvae are capable of surviving over a wide range of temperatures, salinities, and contaminant levels. Moreover, embryos collected from urban estuaries in the United States showed few developmental abnormalities. Hence, we do not believe that the recent declines in horseshoe crab populations in the United States are related to pollution. In contrast, studies in Japan suggest that polluted water is having a far greater impact on *T. tridentatus*. We do not know the reason for the difference; *T. tridentatus* could be more sensitive to pollutants than *L. polyphemus*, but it is also possible that the types and levels of contaminants in Japan are especially harmful. The glaring lack of data on *T. gigas* and *C. rotundicauda* prevents us from making definite conclusions about the influence of contamination on their populations, though some of them certainly occur in polluted embayments (e.g., Chiu and Morton 2003).

For the future survival of horseshoe crabs, there must be enough suitable spawning habitat (Botton 2001). Some good spawning beaches may be recreated from eroded or degraded shorelines that have bulkheads or other man-made structures. For beach replenishment to be successful, careful consideration must be paid to sediment quality (Avissar 2006), but to restore beaches without assuring that water quality is adequate is only a partial solution. Tsuge and Washida (2003) found that the Japanese people living along the highly industrialized Seto Inland Sea placed a very high economic value on the restoration of their coastline, especially when they viewed it in the context of a habitat that was essential for threatened species such as the horseshoe crab. We are hopeful that this sense of optimism can translate into the successful recovery of the horseshoe crab in Japan.

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Heavy Metal Concentration in Horseshoe Crab (*Carcinoscorpius rotundicauda and Tachypleus gigas*) Eggs from Malaysian Coastline

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Abstract The level of trace elements (Cu, Zn, Fe, Ni, Pb, and Cd) was measured in eggs of horseshoe crabs, *Carcinoscorpius rotundicauda*, and *Tachypleus gigas,* from Malaysia. The concentrations (µg/g wet weight) of these elements in *C. rotundicauda* eggs ranged from 18.84 to 65.44 for Cu, 34.65 to 104.08 for Zn, 4.497 to 75.95 for Fe, 1.88 to 11.17 for Ni, 0.52 to 3.64 for Cd, and non-detectable for Pb. The level of these elements in *T. gigas* eggs was from 30.54 to 120.32 for Cu, 46.34 to 88.96 for Zn, 21.88 to 88.96 for Fe, 4.71 to 7.82 for Ni, 0.02 to 4.11 for Cd, and 10.00 to 25.84 for Pb. *C. rotundicauda* eggs showed significantly higher amounts of trace elements except for Ni and Cd. The heavy metals analyzed were higher than the range of permissible limit for human consumption.

1 Introduction

The discoveries on the contamination of horseshoe crabs by chemical pollutants emerged from 2003 in an investigation of species distribution in Peninsular Malaysia. There are only four living species of horseshoe crab, *Limulus polyphemus*, *Tachypleus tridentatus*, *T. gigas*, and *Carcinoscorpius rotundicauda* (Sekiguchi, 1988). *T. gigas* and *C. rotundicauda* can be found in Malaysia, where they spawn throughout the year. Adult horseshoe crabs migrate from the offshore continental shelf to spawn on intertidal sandy (*T. gigas*) and mud-sandy beaches and mangrove area (*C. rotundicauda*) at every full and new moon (Hajeb et al*.,* 2005a). Horseshoe crabs inhabit shallow marine waters, generally on sandy bottoms where they move about or burrow just beneath the surface, preying on other animals.

The sensitivity of horseshoe crab embryos toward chemical pollution and impacts on their developments has been showed by few studies (Botton, 2000;

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Botton et al., 1998; Itow et al., 1998a). Itow et al. (1998b) showed that tributyltin (TBT), Hg, Cd, Cr, and Zn inhibited the regeneration of walking legs in horseshoe crabs. Botton (2000) provided evidence of negative impacts of long exposure to Hg and Cd on embryonic and larval development of American horseshoe crab. However, in comparison to early developmental stages of other arthropods, horseshoe crab embryos, and trilobite larvae showed a high tolerance to Hg and Cd (Connor, 1972; Kraus et al., 1988; Bat et al., 1998). Botton et al. (1998) reported greater tolerance of larvae to Cu and Zn than embryos.

Rapid economic growth in Malaysia has resulted in increasing production and usage of toxic chemicals such as trace metals (Agusa et al., 2005). The Strait of Malacca (Fig. 1) is one of the most important habitats for *Tachypleus gigas* and *Carcinoscorpius rotundicauda* (Christianus et al., 2004). The Strait of Malacca is subjected to a great variety of pollutants due to its strategic location as a major international shipping lane and the concentration of agriculture, industry and urbanization on the west coast of Peninsular Malaysia (Abdullah et al., 1999).

Fig. 1 Map showing sampling locations of horseshoe crabs, *T. gigas,* and *C. rotundicauda* in the Straits of Malacca, Malaysia. (PD = Port Dickson; JB = Johor Bahru)

Levels of heavy meals in coastal Malaysia have been reported by Law and Singh (1991), Ismail et al. (1995), and Yap et al. (2002, 2004). Moreover, the Strait of Malacca is one of the most vulnerable areas to contamination by oil spills (Eng et al., 1989). Harmful substances released by human activities will be accumulated in marine organisms through the food web. Consequently, there may be human health risks caused by consumption of contaminated seafood. Horseshoe crab eggs are consumed by local fishermen and coastal residents in Malaysia. However, no studies have been conducted on contaminant loads in horseshoe crab eggs, or the potential risk to human health in this country.

To investigate the present contamination levels, patterns of accumulation, and possible toxic effects of pollution, the levels of trace metals (Cu, Zn, Fe, Ni, Pb, and Cd) were measured in eggs of horseshoe crabs from the coast of Malaysia. This investigation can act as a beginning for future research on horseshoe crabs in order to conserve them and to prevent them from being lost in this country. It is important to establish whether heavy metals contaminants are contributing to changes in horseshoe crab populations.

2 Materials and Methods

Samples of female horseshoe crabs collected from two sites along the Strait of Malacca during full moon (Fig. 1). Samples were transferred to the laboratory; prosomal length and body weight were measured for each. Horseshoe crab eggs were extracted and kept in –20°C until using for analysis.

All samples were digested in concentrated HNO_3 in the hot block digester in low temperature (40°C) for 1 hour and high temperature (135°C) for at least 3 hours. Digested samples were then cooled and subsequently diluted in deionized water into 40 ml. After filtration, samples were analyzed for Cd, Cu, Pb, Fe, Ni, and Zn using a Perkin–Elmer Model 4100 air–acetylene flame atomic absorption spectrophotometer.

To avoid contamination, all the glassware used was soaked in detergent solution overnight, then rinsed, and soaked in 10% (v/v) HNO_3 overnight. To check for contamination, procedural blanks were analyzed in every ten samples. Quality control samples, made from standard solutions of Cd, Cu, Pb, Fe, Ni, and Zn, were analyzed in every ten samples to check for the metal recoveries. The percent recoveries were 99% for Cd, 97.5% for Cu, 96% for Pb, 98.3% for Fe, 92.8% for Ni, and 99% for Zn. Detection limits were 1 ng/g for Cu and Fe, 5 ng/g for Cd, 7 ng/g for Zn, and 8 ng/g for Ni and Pb.

The description of samples and sampling locations are presented in Table 1. The Pearson correlation coefficient was used to measure the strength of the

Table 1 Description of horseshoe crab samples and sampling locations

Species	Number of samples	Location	Habitat	Human activity in location	Prosomal width (cm) mean (range)	Female body weight (g) mean (range)
T. gigas	11	Port Dickson (PD)	Sandy beach	Fishing boat traffic, Recreation	19.87 (19.10–21.50)	600 (520–740)
	16	Johor Bahru (JB)	Sand-muddy beach	Fishing boat traffic, urban waste release	20.81 (19.40–22.60)	731.43 (570–910)
C. rotundicauda	–	Port Dickson (PD)	–	–	NF	NF
	14	Johor Bahru (JB)	Mangrove	Industrial and urban waste release	12.39 (10.60–13.50)	158.75 (120–200)

NF, not found.

association between trace element concentration and prosomal width. Differences among trace element concentrations were tested by ANOVA. A *P* value of less than 0.05 was considered to indicate statistical significance. T-tests were conducted to compare the data obtained from two collection sites. All statistical analysis was done using MINITAB Statistical Software (Release 14).

3 Results

The levels of trace metals in the eggs of *T. gigas* and *C. rotundicauda* are presented in Table 2. In general, Zn concentration was the highest followed by Fe and Cu. *T. gigas* showed significantly higher levels of Cu, Zn, and Fe than *C. rotundicauda* collected from Johor Bahru (JB). Pb was not detected in the egg samples of either horseshoe crab species from JB. In terms of Cd, content, there was no significant difference between the two species, while *C. rotundicauda* had higher amounts of Ni compared to *T. gigas* ($P < 0.005$). There were higher levels of Cu, Zn, Fe, Ni, Pb, and Cd in *T. gigas* samples from JB than PD ($P < 0.005$). Comparison between two sampling locations demonstrated higher level of four elements, Cu, Zn, Fe, and Cd, in *T. gigas* samples from JB site ($P < 0.005$).

There was a size difference between adult female *T. gigas* from two sites; samples from PD were larger and heavier ($P < 0.005$) (Table 1). There were significant positive correlations between prosomal width and levels of Cu, Zn, Fe, and Ni for *C. rotundicauda* (Fig. 2). Conversely, heavy metal levels in *T. gigas egg* samples were not significantly correlated with the size of the female.

4 Discussion

T. gigas egg samples from PD site showed higher levels of Ni and Pb than JB, but the reverse trend was seen for Cu, Zn, and Fe (Table 2). The concentrations of metals in *C. rotundicauda* eggs from JB were generally similar to T. gigas from the same site (*C. rotundicauda* was not found at PD). Comparing the current data with Kannan et al. (1995) on concentration of heavy metal in *T. tridentatus* in Japan, Malaysian horseshoe crabs showed higher levels of all the metals analyzed. These two species of horseshoe crab also showed higher levels of Pb and Cd than reported by Burger (1997) and Burger et al. (2002) in American horseshoe crab eggs. The high correlation for Cd and Pb in both species can be explained by their bioaccumulation by age (Burger et al., 2002).

Higher levels of Cu, Zn, Fe, and Cd in *T. gigas* eggs from JB and Ni and Pb from PD sites can suggest that there are some sources of these metals in these areas. These contaminants possibly arose from industrial, agricultural, and shipping activities in the Strait of Malacca (Agusa et al., 2005; Yap et al., 2004). Horseshoe crab eggs showed higher levels of trace elements than other

Table 2 Heavy metal concentration (μg/g wet wt.) in horseshoe crab egg samples from the Straits of Malacca

Species	Location	*N*	Cu mean (range)	Zn mean (range)	Fe mean (range)	Ni mean (range)	Pb mean (range)	Cd mean (range)
T. gigas	Port Dickson PD	11	57.27 (44.83–70.02)	62.94 (53.08–71.62)	44.35 (32.45–61.68)	5.93 (4.71–7.82)	18.26 (10.00–25.84)	0.29 (0.02–0.52)
	Johor Bahru (JB)	16	78.45 (30.54–120.32)	100.21 (46.34–150.53)	60.26 (21.88–88.96)	1.02 (0.05–4.8)	ND	3.59 (1.38–4.11)
C. rotundicauda	Johor Bahru (JB)	14	36.79 (18.84–65.44)	63.33 (34.65–104.08)	36.42 (4.49–75.95)	4.47 (1.88–11.17)	ND	2.28 (0.52–3.64)

ND, non-detectable.

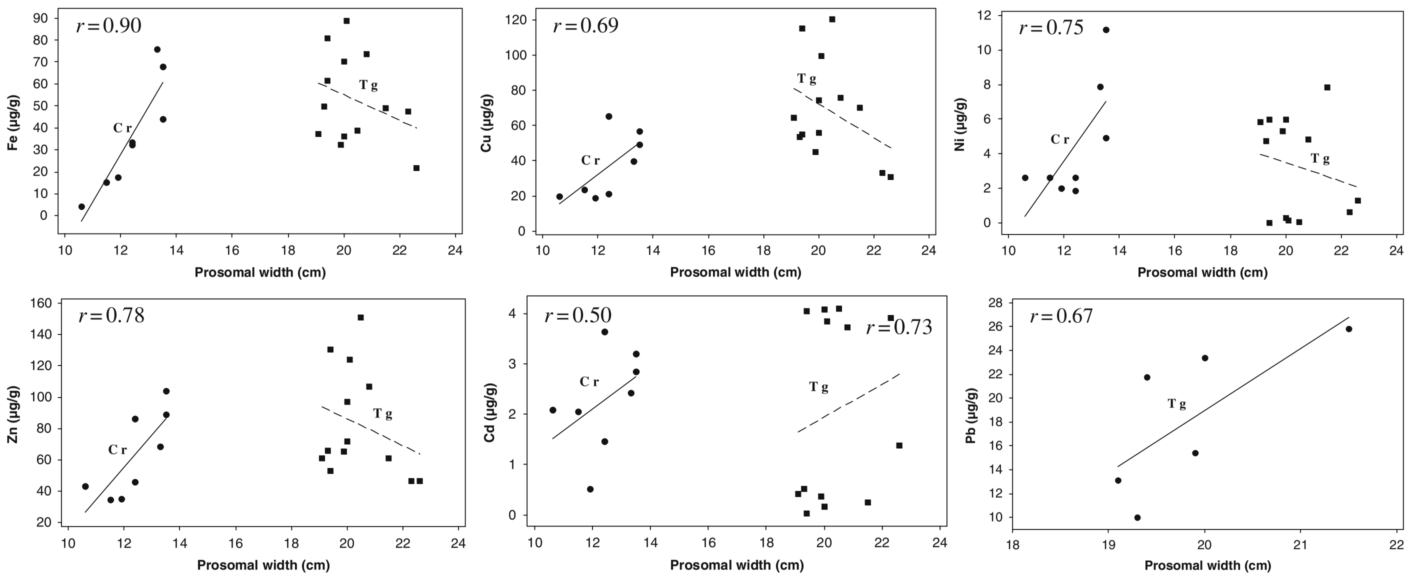

Fig. 2 Correlation between metal levels (μg/g) and prosomal width (cm) in horseshoe crab samples (circles: *C. rotundicauda;* squares: *T. gigas*)

Table 3 Comparison of heavy metal concentrations (μg/g) with other biota in the Straits of Malacca

Biota	Weight base	Cu	Zn	Pb	Cd	Reference
Green liped mussle (*Perna viridis*)	Wet	1.00–3.00	10.8–30.0	0.50–5.90	0.10–1.80	Ismail (1993)
Fish	Wet	0.51–1.05	5.50	0.10–0.29	–	Law and Singh (1991)
Fish	Wet	–	2.30–6.48	0.21–32.00	0.03–0.05	Babji et al. (1979)
Fiddler crab *(Uca annulipes)*	Wet	8.02–19.91	10.63–19.10	3.65–9.41	1.56–2.54	Ismail et al. (1991)
Hermit crab (*Clibanarius sp)*	Wet	22.34–86.00	23.55–42.87	4.02–12.67	1.43–2.33	Ismail et al. (1991)
Mollusks	Wet	6.00–15.00	18.00–47.00	7.00–17.00	0.10–2.50	Ismail and Ramli. (1997)
Sediments	Wet	4.00–670.00	4.00–550.00	3.40–46.50	0.10–2.10	Ismail and Ramli. (1997)
Prawn	Wet	12.80–159.00	5.00–16.00	0.06–5.90	009–0.80	Ismail et al. (1995)
C. rotundicauda	Wet	18.84–70.02	34.65–150.53	ND	0.52–4.11	Current study
T. gigas	Wet	44.83–70.02	53.08–71.62	10.00–25.84	0.02–0.52	Current study

ND, non-detectable.

marine biota in this area of Malaysia (Table 3). High levels of heavy metals in the eggs of horseshoe crabs could impair development (Burger, 1997; Itow et al., 1998a). Based on Zhou and Morton (2004), horseshoe crabs are benthic predators, feeding mainly on bivalve and mussels. Data from Yap et al. (2004) showed lower levels of Cu, Cd, Pb, and Zn in sediments and green-lipped mussel, *Perna viridis,* from the Strait of Malacca compared to the metal content of horseshoe crab. This may be explained by accumulation of these metals in horseshoe crab body and its sequestration from the female during egg formation. The positive correlations between metal concentrations in the eggs and female body size (especially in *C. rotundicauda*, Fig. 2) can be partially related to the longer exposure of the larger horseshoe crab to the polluted areas, assuming that size and age are correlated (Hajeb et al., 2005b).

The ability of horseshoe crab embryos and larvae to survive in the presence of heavy metals implies the potential for these minerals to be passed on to shorebirds and other predators (Botton, 2000). Levels of contaminants in eggs of horseshoe crabs are also of interest because they are being consumed by fishermen and some local people as a delicacy. The level of these metals is considered to be high when compared to permissible limits set by Malaysian Food Regulation (1985) for Cu (30.0 mg/kg ww), Cd (1.00 mg/kg ww), Zn (100 mg/kg ww), and Pb (2.00 mg/kg ww). However, the consumption of these eggs is not that high to be consider as serious health risk to the population.

In conclusion, the level of heavy metals in the eggs of horseshoe crabs is of interest as a bioindicator of pollutant levels in the Strait of Malacca and an indicator of potential problems for developing horseshoe crabs. On a world-wide basis, reduction of horseshoe crab populations has been attributed to over harvesting (Burger, 1986; Botton, 2000; Botton and Loveland, 2001), the use of eggs as food (Kungsuwan et al., 1987), and inorganic and organic contaminants (Burger et al., 2002; Burger, 1997; Kannan et al., 1995). This study showed that two horseshoe crab species may be affected by heavy metal pollutants in the Strait of Malacca.

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A Discussion of Horseshoe Crab Management in Five Countries: Taiwan, India, China, United States, and Mexico

Jim Berkson, Chang-Po Chen, Jayant Mishra, Paul Shin, Braddock Spear, and Jaime Zaldívar-Rae

Abstract A panel of five international experts was convened during the International Symposium on the Science and Conservation of Horseshoe Crabs to compare and contrast horseshoe crab management in their countries. The panel members each responded to a series of questions prepared by a facilitator. All five speakers stated that habitat degradation and destruction were a major threat to the horseshoe crab population(s) in their country. Pressure for economic development often hindered efforts to preserve and protect coastal habitats. Public education was viewed as an important step toward the implementation of effective management actions. The urgent need for conservation was viewed to be a strong, motivating factor to strengthen international management efforts.

1 Introduction

The International Symposium on the Science and Conservation of Horseshoe Crabs, which took place in June of 2007 in New York, was the first meeting to bring together horseshoe crab researchers, managers, educators, activists, and enthusiasts from over nine countries to meet and share information. The majority of the nations where horseshoe crabs are extant were represented by individuals who gave presentations about their work.

While horseshoe crabs have been able to survive over 200 million years without being managed, that is no longer the case. Horseshoe crab populations are facing stressors which are impacting their survival at a magnitude and on temporal and spatial scales unlikely experienced by the species at any time in its history. These stressors are human-caused. Horseshoe crab conservation now requires the management of humans causing these stressors.

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During the Symposium, speakers discussed the capture and use of horseshoe crabs for purposes including fisheries bait, Limulus Amebocyte Lysate (LAL) production, and human consumption. Speakers discussed the horseshoe crab's important role in the complex and rapidly changing coastal ecosystem. There were also presentations about the horseshoe crab's dependence on specific types of habitat for critical life stages. It was pointed out that achieving and maintaining a sustainable catch, conserving an ecosystem under stress, and maintaining essential habitat all require effective management.

The Symposium's Steering Committee thought it is important to include a discussion of management-related issues presented by an international panel of horseshoe crab experts both at the Symposium and in this book developed from the Symposium. The format was set up as a facilitated panel. Jim Berkson took the role of facilitator and developed a list of questions for the panelists. Chang-Po Chen (Taiwan), Jayant Mishra (India), Paul Shin (China – Hong Kong), Braddock Spear (United States), and Jaime Zaldívar-Rae (Mexico) were asked to serve on the panel. Although other countries with extant horseshoe crab populations were represented at the Symposium, not all were invited to participate on the panel due to time constraints.

This chapter has been written to summarize the participants' answers to the discussed questions. Both the panel discussion at the Symposium and this Chapter allow us to learn about management-related issues in each country and, more importantly, to compare and contrast the situations among the countries.

2 Questions

Panelists were asked a series of questions to better understand the role management plays or has the potential to play in their countries. Panelists were first asked about the status of the horseshoe crabs in their countries and the greatest threats to the populations. Next, a series of questions was asked about management. Finally, panelists were asked to address whether they were optimistic or pessimistic about the future of the horseshoe crab populations in their countries.

2.1 What is the Current Status of the Horseshoe Crab Population(s) in Your Country?

Chang-Po Chen, Taiwan: Horseshoe crab populations are largely thought to be in decline. Adults are only occasionally caught by trawling and spawners are not seen on beaches as in the past. Juvenile populations in nursery areas have also declined.

Jayant Mishra, India: Horseshoe crabs in India are found along the north-eastern coast of India in good numbers. Two species of horseshoe crabs are found in India: *Tachypleus gigas* and *Carcinoscorpius rotundicauda*. No research has been done to determine the actual population density.

Paul Shin, China (Hong Kong): Anecdotal records from local villagers reveal the presence of adult horseshoe crabs coming to the shores for spawning some 40–50 years ago. However, no mating pairs have been recorded or observed in recent years. We have no information on adult horseshoe crab populations in Hong Kong waters. At times, fishermen obtain adults (one or two animals) as bycatch from their trawling activities in Hong Kong as well as mainland Chinese waters. Horseshoe crabs can still be found in markets and restaurants. We do have juvenile horseshoe crabs (*Tachypleus tridentatus* and *Carcinoscorpius rotundicauda*) on some of the shores in the western waters of Hong Kong. At present, it is noted that even the juvenile horseshoe crab populations on the shores in Hong Kong are declining, for both species.

Braddock Spear, United States: Currently, there is no serious threat to the population of horseshoe crabs as a whole, but depletion in areas of heavy, localized harvest remains a concern. The overall status of horseshoe crab populations is largely dependent on localized or regional dynamics (e.g. harvest pressure, water pollution, habitat availability). Many of the populations along the Atlantic coast of the U.S. faced heavy harvest pressure from the late 1980s through the 1990s. Since then harvest has been restricted through regulation allowing the populations to stabilize or grow.

Jaime Zaldívar-Rae, Mexico: There are no estimates of population sizes in Mexico. However, Dr. Samuel Gómez of the Institute of Biology, National Autonomous University of Mexico, stated in 1979 and 1993 that Mexican *Limulus* populations were declining rapidly based on his experience of some 30 years with the species. This was probably the key opinion which resulted in the horseshoe crab being included in the Mexican list of species at risk (Official Norm 059-SEMARNAT-2001). *Limulus polyphemus* is listed as being “in danger of extinction”.

2.2 What Are the Greatest Threats Facing the Horseshoe Crab Population in Your Country?

Chang-Po Chen, Taiwan: Horseshoe crabs are negatively affected in Taiwan by habitat loss and water pollution. The lack of knowledge among administrators and policymakers about horseshoe crabs and the threats against them is a major contributor to the problem. This is occurring on a national scale.

Jayant Mishra, India: The greatest threat to horseshoe crab populations in India is the destruction of beaches where the adults spawn. Although there are no major threats to the populations from the biomedical industry or from large-scale fisheries, spawning animals are taken off the beaches in some areas, causing a serious concern. Both the habitat destruction and the removal of spawning animals are localized problems which can be managed by increasing the awareness and involvement of the people who are directly or indirectly involved with the coastal environment. In recent times global climate change may also be playing a major role in the form of an increasing number and/or intensity of natural calamities in the form of super cyclones and tsunamis, which destroy the coastal environment and breeding beaches.

Paul Shin, China (Hong Kong): Urbanization, land reclamation, and other forms of economic development leading to habitat loss along the coast are the greatest threats to horseshoe crabs in Hong Kong. This all stems from the large number of people in Hong Kong. These disturbances mainly affect the juvenile horseshoe crab nursery grounds and spawning activities. Habitat degradation affects not only horseshoe crabs but also the entire ecosystem. Note that there is no target fishery on horseshoe crabs in Hong Kong waters. However, adult horseshoe crabs are served as special dishes in some seafood restaurants.

Braddock Spear, United States: Currently, the greatest threat to horseshoe crabs is loss of suitable habitat. The crabs depend on particular beach types to spawn successfully. As coastal development and erosion reduce spawning habitat, horseshoe crabs have reduced opportunities to reproduce. Harvest or exploitation of horseshoe crabs is less of a threat, as it is believed to be under control. Habitat degradation affects a suite of species and threatens inter-tidal and coastal ecosystems. Many different species of fish, shellfish, crustaceans, birds, and others depend on beaches and near shore waters at some point in their lifecycle.

Jaime Zaldivar-Rae, Mexico: The greatest threats to horseshoe crabs are anthropogenic habitat modification including urbanization of pristine coastline, destruction of mangrove forests, and filling in of coastal lagoons; natural habitat modification such as the impacts of hurricanes and increasing sea levels due to climate change; potential impacts of the shrimp trawl fishery on bottom conditions in the shallow portions of the continental shelf; and pollution generated by the oil industry and agricultural activities in the Southern portion of the Gulf of Mexico. Different regions are affected by different threats.

2.3 Do Pathways for Management Exist at the Present Time and What Are the Biggest Obstacles to Management?

Chang-Po Chen, Taiwan: Many pathways exist for the government to take management action, but it takes a long time and strong lobbying to convince the policymakers to take action. The main obstacle for helping horseshoe crabs in Taiwan is convincing the government that conservation issues are as important as economic development.

Jayant Mishra, India: Horseshoe crabs inhabit 620 km of coastline in India. The greatest obstacle to their management is their large range of distribution. Addressing threats across such a large area may require the involvement of others outside the government, such as Non-Governmental Organizations (NGOs) and local bodies, to make the management successful.

Paul Shin, China (Hong Kong): In addition to legislation under the Environmental Impact Assessment Ordinance, there is one other pathway for management. In Hong Kong, horseshoe crab spawning and nursery grounds can be designated as a Site for Special Scientific Interest (SSSI). The aim of a SSSI designation is to ensure that full account is taken when development or change in land use is proposed. Economic development often out-weighs environmental concern in Hong Kong, and like other areas, it is difficult to balance the diverse interests.

Braddock Spear, United States: The Atlantic States Marine Fisheries Commission (ASMFC) has set up an effective process to manage the harvest of horseshoe crabs. Pathways for protecting spawning habitat are more localized at the county and township level. The biggest obstacles now are the competing interests that exist in coastal areas. State and local planning and zoning authorities must balance the heavy pressure to develop coastal lands with the need to preserve ecologically important and unique areas

Jaime Zaldívar-Rae, Mexico: Pathways exist in that fairly adequate legislation is in place, and several important populations are within federal and state protected areas. Problems also exist, however, in that funding is often limited to carry out the different management actions required in protected areas, staff in government agencies is limited, and law enforcement is often weak and inconsistent. The biggest obstacle to management is the strong economic interests associated with coastal areas (the tourism and oil industries, fisheries, real estate development) which are critical for the Mexican economy, and, as a result, have strong political and financial influence. State governments are under strong pressure to find options for economic development and the creation of jobs. Thus, their priorities frequently oppose those of conservation agencies, including those of the Federal Government. In addition, the efforts of governmental agencies for economic development and conservation are often not coordinated and even opposed. There needs to be more local involvement in the creation and implementation of management plans.

2.4 *What Management Actions Are Currently in Place to Deal with the Threats?*

Chang-Po Chen, Taiwan: To combat the threats to horseshoe crabs in Taiwan, a small protected area has been set up in Kinmen. An NGO is also doing work to restore the horseshoe crab populations.

Jayant Mishra, India: No proper management plans are in place to deal with the threats to horseshoe crabs.

Paul Shin, China (Hong Kong): No management plans are in place directly for horseshoe crabs, but other management plans address the threats to horseshoe crabs. We have four marine parks and one marine reserve in Hong Kong, but their primary functions are for other purposes such as protection of corals, the Chinese white dolphins, and/or biodiversity. The Environmental Impact Assessment Ordinance (EIOA) requires any large development projects to undergo an environmental review, and ecologically sensitive areas, such as horseshoe crab nursery grounds, will be examined carefully so as to minimize any adverse impact resulting from such development projects.

Braddock Spear, United States: The Atlantic States Marine Fisheries Commission implemented a state-by-state quota management system in 2000. Since then the Commission has taken further action to restrict harvest in the most important region for the species, Delaware Bay. In 2001, the National Marine Fisheries Service created the first marine reserve of a known horseshoe crab habitat area spanning the continental shelf outside the mouth of Delaware Bay. It outlawed harvest of crabs migrating to the continental shelf before returning to the Bay to spawn. Also, individual states have protected areas of particular importance to the horseshoe crabs along their coasts.

Jaime Zaldívar-Rae, Mexico: No management plans are in place directly aimed at horseshoe crabs. However, the Mexican Commission for Protected Natural Areas (CONANP) administers several coastal protected areas that encompass horseshoe crab habitats along coasts of the Yucatan Peninsula. These areas include monitoring and recovery programs particularly geared toward improving the health of coastal water bodies and certain organisms such as shorebirds, waterfowl, and sea turtles. Horseshoe crab populations can potentially benefit from these protected areas as well. At the present time the protected areas are under-funded and under-staffed, but budgets are improving steadily.

2.5 What Role Will Public Education Play in the Management of Your Country's Horseshoe Crab Population(s)?

Chang-Po Chen, Taiwan: Education will be a key factor in changing the people's value systems needed for horseshoe crab conservation. Only a large force working together will achieve change and convince the government that ecosystems, such as those crucial to horseshoe crabs, are worth protection.

Jayant Mishra, India: Education of the public is the best possible way to protect horseshoe crabs. At this time, it is also the only way.

Paul Shin, China (Hong Kong): Public education will play a significant role to make people aware of the threats and plight of horseshoe crabs in Hong Kong and/or mainland Chinese waters. Of particular importance is informing the younger generations of the situation of horseshoe crabs and why we should protect and conserve their populations. Once educated, people can move forward to voice their concerns to the local legislators. An official webpage aimed at informing the public on the importance of conserving horseshoe crabs has been set up by the government. Public education must be an ongoing effort.

Braddock Spear, United States: Public education can help local residents understand the importance of horseshoe crabs and protecting their habitat. The residents can then become empowered and pressure state and local decision makers to make management choices that will, at the very least, not threaten horseshoe crab populations.

Jaime Zaldívar-Rae, Mexico: Public education is critical and still not widespread. With the exception of coastal cities, coastal communities are becoming smaller and their influence and capacity are becoming weaker. Thus, pressure on governmental agencies and the large interests in the area will increasingly have to come from urban societies, which are geographically, socially, and environmentally distant from the coasts. The only way to involve urban and coastal societies, and hopefully, turn them into strong advocates for the conservation of coastal ecosystems will be to make them aware of the relevant issues and their potential role in addressing such issues. With poor coastal communities, it is not enough to instill a sense of awe and fascination with horseshoe crabs and their habitat. We must find ways to convincingly demonstrate that conservation can mean financial benefits to these communities.

2.6 What Can Other Scientists and/or Organizations Do to Help You Achieve Your Management Goals?

Chang-Po Chen, Taiwan: Scientists banding together and supporting one another will help. We need to include NGOs and as many people as possible. The key is to just keep moving forward and do something, rather than contemplating or planning alone.

Jayant Mishra, India: Scientists can help by sharing their knowledge and experiences to develop a global management plan.

Paul Shin, China (Hong Kong): They can help in providing advice from their experience on the conservation and management of horseshoe crab populations, collaborating on research studies, and sharing data and information. They can also join together to exert a concerted international effort to conserve these animals. One specific international effort could be to work together with many organizations to help list horseshoe crabs (especially for the Asian species) on the IUCN or CITES endangered species list. This could help stop the import/export of horseshoe crabs between countries, such as between Malaysia and Thailand, and could raise the status of horseshoe crab species worldwide. This will require the collection of data, in terms of population status, etc., to support the case.

Braddock Spear, United States: Other scientists and organizations can continue to conduct research and monitor the species. More research can focus on the little known juvenile life stage on the Atlantic coast of the United States. Others can also help to educate the public and decision makers about the importance of the species in the ecosystem, as well as the species' habitat needs.

Jaime Zaldívar-Rae, Mexico: Efforts in Mexico will greatly benefit from close cooperation with scientists and managers working with horseshoe crabs in the United States, where most of the work on *Limulus polyphemus* has been done. Mexico also needs the help of international agencies, NGOs, and individuals to develop research and management programs, and to find the financial resources to carry them out.

2.7 Do You Have Any Suggestions as to Where the Funding Can Be Found for Additional Research, Monitoring, and Public Education?

Chang-Po Chen, Taiwan: The pharmaceutical companies who produce lysate from the blood of horseshoe crabs receive tremendous profits from the species and should be willing and able to provide the funding.

Jayant Mishra, India: Funding from government sources for monitoring and public education is crucial. However, more participation should be requested from private bodies as well.

Paul Shin, China (Hong Kong): In Hong Kong, there is the government Environment and Conservation Fund which supports projects such as conservation of horseshoe crabs locally. The Ocean Park Conservation Foundation, a non-profit organization, also funds projects locally as well as in the Asia Pacific region.

Braddock Spear, United States: Funding agencies or organizations are becoming increasingly focused on supporting research and education that encompasses more than one species. Research and monitoring that covers an ecosystem or parts of a system tend to be more attractive. Scientists and researchers may have more success at obtaining funding if they incorporate horseshoe crabs research into more comprehensive studies.

Jaime Zaldívar-Rae, Mexico: We will have to explore the "traditional" national and international sources, because so far no one has tried to attract these financial resources to the study and management of horseshoe crab populations in Mexico. However, we will also have to be creative to find new and original sources of funding. This includes joining forces with other organizations that deal with the same geographic areas, ecosystems, and habitats where horseshoe crabs live, to gain access to large funds and optimize their use. We will also have to involve other elements of society such as large companies, the government, and the general public in funding programs aimed at supporting conservation.

2.8 *Are You Optimistic or Pessimistic About the Future of the Horseshoe Crab Population(s) in Your Country?*

Chang-Po Chen, Taiwan: The troubling situation of horseshoe crabs in Taiwan does not lend itself to optimism. However, we cannot afford to be sit back and be pessimistic. We must work together to ensure that horseshoe crabs can survive.

Jayant Mishra, India: I am very optimistic about the future of horseshoe crabs in India, in general. However, I am cautious because of the possibility of the exploitation of horseshoe crabs for lysate and because of the likely effects of global climate change.

Paul Shin, China (Hong Kong): I am pessimistic about the future of the horseshoe crab populations in Hong Kong as continuing economic developments and pressure for urbanization will inevitably affect the habitats of the spawning and/or nursery grounds for horseshoe crabs. We have very little information on where the animals are living or on their abundance.

Braddock Spear, United States: I am optimistic. The progress we have made in managing horseshoe crabs over the past 10 years shows much promise for the species. A species that has been on this planet for 300–400 million years must have unique capabilities to adapt to a changing environment.

Jaime Zaldívar-Rae, Mexico: I am optimistic in some sense. There is still time to thwart some of the biggest threats, and efforts toward that goal are becoming more common and are beginning to involve many sectors of society. However, both the magnitude and the pace of some of the threats are alarming and likely to surpass any efforts for conservation and management. Rather than leading to pessimism, this should instill a good dose of realism and a sense of urgency among conservationists, managers, and our society as a whole.

3 Summary

While the status of horseshoe crab populations varies from country to country, there is reason for serious concern for the populations in several countries including Taiwan, China, and Mexico.

A majority of the speakers talked about how the basic data required to assess the status of the horseshoe crab populations in their countries have not been collected. The primary source of information on population status currently comes from anecdotal information, information that does not lend itself to the development of sound, scientifically based, stock assessments, or effective management strategies. Given the need for management, development and implementation of research and monitoring plans to collect basic population data should be a high priority.

Horseshoe crabs continue to be caught for human consumption in Asia, but the catch numbers are not documented. In some countries they are caught solely as bycatch because their numbers are too small for a directed catch. The US population continues to be caught for use as bait and the production of Limulus Amebocyte Lysate.

Habitat degradation and loss was mentioned by all five of the speakers as the primary threat facing the horseshoe crab populations in their countries. This threat is not unique to horseshoe crab populations, but impacts the entire ecosystem. Managing horseshoe crab populations will ultimately come down to effectively managing the ecosystems in which they live.

The majority of the speakers mentioned that pathways existed for management in their countries, but the actual implementation of effective management was hindered or blocked due to interests related to economic development. The conflict between conservation and development is not new, nor unique to horseshoe crabs. The intensity of this conflict may be greatest for coastal communities, which in many parts of the world are seeing the highest levels of human population growth.

Protecting spawning and nursery habitat must be a primary focus of management actions internationally. This is underway to some degree in most of the countries through the creation of protected areas, although the current level does not appear to be sufficient in most cases. With regard to economically challenged coastal communities, win–win solutions must be found in which conservation measures will benefit the local communities financially, to give them an incentive to conserve. For example, protected areas can bring in substantial tourism revenue if planned appropriately.

All of the speakers agreed that public education must take place in order to make the citizenry and policymakers understand the importance of horseshoe crab persistence. It is hoped that as the public becomes more aware, their influence may more effectively counter the economic interests hindering or opposing conservation efforts. Given that the problems facing horseshoe crab conservation are ecosystem in nature, education should focus at least partially on the importance of ecosystem health and services. Education cannot be limited to coastal communities, as many people including existing and potential conservationists live in inland cities and, as a group, have a great deal of political clout.

The speakers stressed that information transfer is needed from countries with more experience to countries with less experience in topics such as horseshoe crab research, management, and public education. The creation of more international collaborations among scientists and organizations is also desired. A priority should be placed on holding regularly scheduled meetings, like this Symposium, and identifying and securing the funding to allow for maximum participation.

Speakers brought up a diverse set of potential funding sources for future work. It was pointed out that we need to be more creative in our efforts to find funding and that we may want to look for international sources, sources that would be interested in efforts across national boundaries, and/or sources more interested in funding ecosystem-level work.

In the end, speakers' outlooks for the future of horseshoe crab conservation ranged from optimistic to pessimistic. It is likely that a number of factors were involved in each speaker's outlook. These may include the following: the current status of the population, the magnitude and pace of the stresses on the population, the amount of research and monitoring in place available for the development of scientifically based management policies, and the presence of management policies. For example, Braddock Spear from the United States expressed optimism regarding the large US horseshoe crab population, where considerable monitoring and research is in place, as are commercial fishery harvest restrictions. In contrast, Paul Shin from China expressed pessimism regarding the population in Hong Kong, where historical populations can no longer be found, no information exists on abundance or distribution, and the pressures of urbanization and economic development are overwhelming the ability to conserve. Effective management brings with it optimism about the future.

Ultimately, we cannot rely on the horseshoe crab and its historical resilience to guarantee its continued survival. As with so many other species, the impacts of our society on the natural world are occurring at a pace with which many of the most resilient species have not been able to adapt. As we are observing in countries such as China and Taiwan, there is no substitute for suitable spawning and nursery habitat. When populations can be found in local restaurants but not local waters, there is serious cause for concern. If there is a battle that needs to be waged to see necessary management implemented, horseshoe crab enthusiasts do not need to wage it alone. The concern is at a larger scale. Those concerned with the plight of the horseshoe crab, who recognize the need for research, monitoring, public education, funding, and ultimately, sound management practices, can join forces with the myriad of individuals and groups calling for the conservation of coastal ecosystems.

We should be encouraged by the calls of our international speakers to not let the current state of affairs dissuade our efforts, but rather encourage us to work harder, with a sense of urgency to find ways to move forward toward effective management.

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Part IIB
Culture and Captive Breeding

Fig. 2b Artificial insemination begins with the collection of eggs (*top*) and sperm *(bottom)* from animals during the breeding season (photographs by M. Botton)

Clinical Evaluation, Common Diseases, and Veterinary Care of the Horseshoe Crab, *Limulus polyphemus*

Michael W. Nolan and Stephen A. Smith

Abstract The American horseshoe crab, *Limulus polyphemus*, can be maintained in a wide variety of systems ranging from glass aquaria to fiberglass tanks with various types of mechanical and biological filtration. Adult horseshoe crabs are tolerant of a wide range of environmental conditions, with temperatures ranging from –5 to 35°C and salinities from 5 to 35 ppt, with optimal conditions between 15 and 21°C and 27 ppt salinity. Horseshoe crabs should be fed good-quality dead fish, squid, small crabs, clams, frozen brine shrimp, and artificial shrimp/fish diets. Clinical evaluation of a horseshoe crab can be problematic as the hard carapace makes examination and sample collection difficult; however, non-lethal clinical assessment can include external examination, radiology, and hemolymph chemistries and cultures. Biochemical parameters of the horseshoe crab's hemolymph parallel those seen in many other marine species, but several parameters are notably different from ambient seawater, i.e., calcium, magnesium. Survey and contrast radiographic studies of the cardiovascular and gastrointestinal systems of the horseshoe crab can be undertaken using conventional and fluoroscopic techniques. Infectious etiologies include algae, fungus, colonial and filamentous cyanobacteria, Gram-negative bacteria, and a variety of protozoan and metazoan parasites. Non-infectious problems range from water quality problems to developmental syndromes and traumatic injuries. Little is known of the therapeutic options for treatment of horseshoe crab diseases; however, a few treatments have been suggested for the removal of ectocommensals and external parasites, and the pharmacokinetics of oxytetracycline following intracardiac and oral dosing have recently been investigated.

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1 Introduction

Although the horseshoe crab is not a commonly kept laboratory animal, this species is an important one. Accordingly, the captive maintenance of these animals has proven invaluable to researchers using horseshoe crabs as experimental models in such varied fields as conservation biology, hematology, and ocular research. As with other laboratory species, proper husbandry is key to horseshoe crab health. But mastery of husbandry techniques is not enough; successful rearing of captive horseshoe crabs also relies on the scientist's ability to detect and manage disease. This chapter reviews the clinical approach to assessing the health of individual adult horseshoe crabs, describes some of the more commonly encountered diseases of captive animals, and concludes with a discussion of both preventative medicine and therapeutic veterinary care of the horseshoe crab.

2 Clinical Evaluation

2.1 Clinical History

The clinical history is the most basic, and often the most informative part of the clinical evaluation. The clinical history has three basic components: signalment, chief complaint, and patient history.

Signalment defines the individual patient's identification. In the case of horseshoe crabs, the species should be defined as being *Limulus polyphemus*, *Tachypleus gigas*, *T. tridentatus*, or *Carcinoscorpius rotundicauda*. Although there are no specified breeds of horseshoe crabs, there are distinct genetic strains defined by the physical environment from which the animals are derived. As such, the physical (e.g., wild vs. captive, brackish vs. saltwater) and geographical (e.g., Atlantic vs. Pacific, subtropical vs. temperate) environment where the crab lives should be described. The age of the animal should be estimated (or if raised in captivity, the age can be specified), and the sex of adult animals should be noted. Finally, the identity of the individual animal should be defined (i.e., tank number, tag number).

The chief complaint is defined by the client or researcher and should become the focal point of the comprehensive clinical evaluation; it is the main reason the animal is being presented to the clinician. The chief complaint should include description of the manner of disease onset, clinical signs noted, and duration of those signs. The chief complaint is more clearly identified by the clinician as the evaluation progresses, with the eventual goal being identification of an etiology (or a combination of etiologies) that explains the chief complaint.

The patient history should include description of diseases (and any treatments) which have previously affected the individual, its family, or its cohorts (in the wild this would be defined as diseases which previously affected the individual's population; in captivity this is defined as pathologies that have affected tankmates). The patient history should also include description of the physical microenvironment, including time in captivity, housing, water quality (salinity, temperature, nitrogenous waste, etc.), diet, water filtration, light cycle, etc.

2.2 Physical Examination

After acquisition of a clinical history, the physical examination should be initiated. The examination should be systematic and thorough; all body systems should be investigated. The examination should begin with a "hands-off" examination, consisting of visual observation of respiration, ambulation, feeding behavior, etc. The examination should then progress to the "hands-on" portion of the examination. The entire carapace should be visually inspected, then palpated. Note any obvious problems such as crush injuries, fractures, and epibiont fouling, but also note abnormal coloration, texture, and hardness. Be sure to evaluate the joints between the prosoma and opisthosoma, opisthosoma and telson, and opisthosoma and spines; manipulate the joints to assess range of motion and to identify untoward resistance to manipulation of the joint and to identify abnormal flexion/extension. In inspecting these joints, evaluate the color, texture, and general integrity of the arthrodial membranes. Next, inspect the two lateral compound eyes; one should be on each side of the prosoma, and the lenses should be free of lacerations and/or ulcerations. Turn the horseshoe crab over and examine the ventrally located mouth at the center of the base of the legs and feeding appendages. Moving to the gills, inspect the operculum and individual book gills. Note any emphysema, hemorrhage, parasitic infestation, traumatic injury, etc., to the gill leaflets. Finally, examine the anal slit and genital pores; distal patency of these tubular tracts can be confirmed with passage of a blunt probe into the orifices.

The goal is to compile data from the history and physical examination in order to formulate a list of differential diagnoses. These differentials should guide the clinician in arriving at a diagnosis. Before resorting to postmortem diagnostic evaluation, the diagnostic plan should employ non-lethal techniques capable of ruling out differentials low on the list and/or confirm (or at least increase the suspicion of) a specific disorder.

2.3 Antemortem Diagnostics

2.3.1 Tissue and Fluid Sampling

Clinical pathology includes analysis of body fluids and tissue samples. Carapace and gill booklet scrapings, gill leaflet biopsies, fecal analysis, and hemolymph sampling are among the most rewarding of pursuits in clinical pathology of the horseshoe crab.

Carapace and gill booklet scrapings involve scraping the surface of these structures with a glass coverslip and making a wet mount preparation of the mucus, isolated cells, and debris. This technique can be utilized to identify parasites, fungi, and certain bacteria (namely those of the genus *Flexibacter*). Taking a gill leaflet biopsy is a simple method by which either a wet mount can be made to examine gross morphology and cytology or tissues can be prepared for histopathologic examination (refer to Section 2.4.2).

Fecal analysis is most often performed by direct smear and/or fecal flotation and is used to identify gastrointestinal parasites. Feces can be collected directly from the animal by insertion of a small fecal loop into the posterior end of the intestine; if this is not possible, an alternative, but suboptimal, method involves collecting feces from the tank.

Hemolymph can be non-lethally sampled from the interdigitating membranes of the legs or from the cardiac sinus, the latter location providing larger volumes of hemolymph and for ease of access is often the preferred site of sampling. Hemolymph can be used for bacterial culture if such an infection is suspected or to evaluate for hemoparasites. Clinical chemistries may be obtained by analysis of horseshoe crab serum and can be used to gain specific information regarding the function of various body systems. Serum is obtained by centrifuging whole hemolymph in a sterile glass vial and pipetting the serum away from the cellular components which pellet at the bottom of the vial. Serum samples can be analyzed using automated clinical chemistry systems found in human or veterinary diagnostic laboratories. Reference intervals have been reported for healthy adult horseshoe crabs (*L. polyphemus*) (Smith et al., 2002).

It is a common misconception that horseshoe crabs are resistant to development of systemic bacterial infections; this theory arose because horseshoe crabs have a specific lysate found within amebocytes that demonstrates anti-endotoxin properties. The presence of endogenous antibacterial compounds reduces the incidence of sepsis, but cannot prevent septicemia from developing, as is demonstrated each time a vertebrate animal dies of sepsis despite chemical and cellular mechanisms for destruction of bacteria within their blood. Therefore, it is important not to discount the potential for bacterial sepsis in the case of a lethargic, anorectic animal. If septicemia is suspected, culture and identification of bacteria from the hemolymph should be attempted. Whole hemolymph can be streaked onto marine agar (1% NaCl in any standard media, such as brain heart infusion, trypticase soy, or Luria-Bertani) and incubated at 25°C for 1–4 days. Alternatively, whole hemolymph can be passed through a sterile, stainless steel syringe filter holder and over an encased piece of sterile filter paper; the filter paper can be used to directly inoculate marine agar, which is then incubated at 25°C for 1–4 days.

2.3.2 Diagnostic Imaging

Various modalities exist by which the living horseshoe crab can be imaged. Among these are conventional radiology, contrast radiology, fluoroscopy, ultrasound, computed tomography, and MRI. To date only radiographic/ fluoroscopic techniques have been described in the literature (Melchior et al., 1995; Spotswood and Smith, 2007).

Conventional radiography can be performed to obtain films of living animals. In the event that the animal is active, sedation may be needed to minimize motion artifacts. Sedation can be achieved, without chemicals and without apparent physiologic harm, by removing the horseshoe crab from water for 5–15 minutes prior to handling and imaging.

The simplest type of radiographic imaging study is accomplished by taking at least two survey films; the minimum of two films includes both a lateral and a dorsoventral radiograph (Fig. 1). Because the majority of the internal viscera of the horseshoe crab have similar radiodensities, it is difficult to ascertain morphologic pathology from survey films alone. Positive contrast radiography has been reported; included were results from gastrointestinal and angiographic studies (Melchior et al., 1995; Spotswood and Smith, 2007). To perform a positive contrast gastrointestinal series, either static radiographs or fluoroscopy can be employed (Fig. 2). If conventional radiography is used, the time series should include a pre-injection radiograph and images immediately, 6, 18, and 30 minutes after administration of the contrast medium. Spotswood and Smith (2007) demonstrated similar results when either iodinated contrast media (15 mL of 300 mg/mL iohexol or 15 mL of 370 mg/mL sodium amidotrizoate/ meglumine) or barium sulfate (15 mL of a 30% solution) was used to perform the study. These media can be administered via gavage using a #8 French polyvinyl catheter. Because non-ionic iodinated contrast media is preferred for angiographic studies (Fig. 3), it may prove more cost-effective to keep only one contrast medium in stock and use a compound such as iohexol for both gastrointestinal and angiographic studies. Spotswood and Smith (2007) injected 12 mL/kg iohexol into the cardiac sinus over a 10 second period. Using fluoroscopy, computerized digital subtraction was used to improve

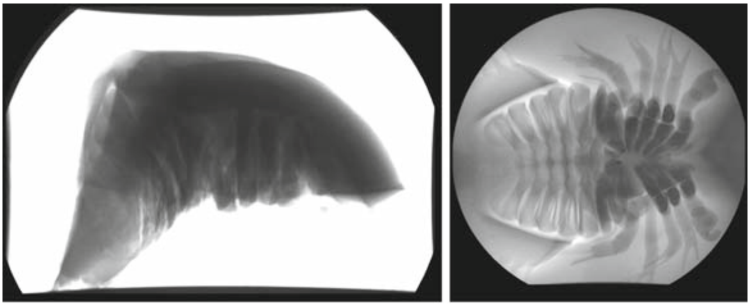

Fig. 1 Survey radiographs. These survey films are fluoroscopic still images (*left* = lateral; *right* = dorsoventral); similar information could be obtained with conventional survey radiographs

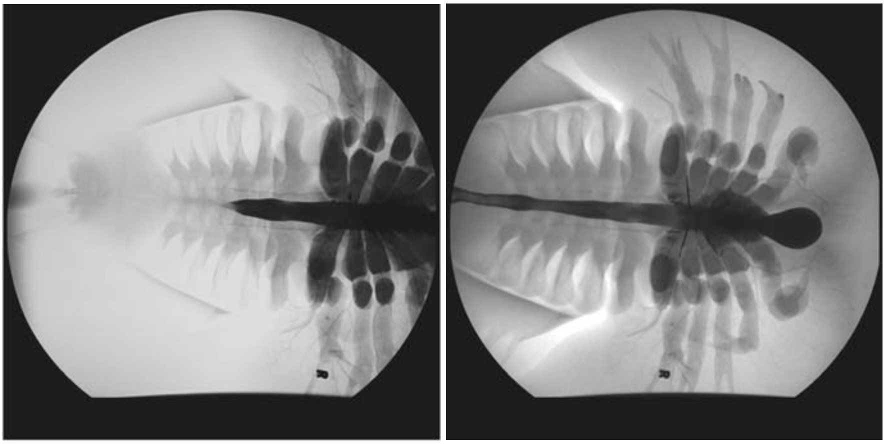

Fig. 2 Still images from a fluoroscopic positive contrast gastrointestinal series (*left* = dorsoventral, 6 minutes; *right* = dorsoventral, 18 minutes); iohexol was used as the contrast medium

visualization of peripheral vasculature. However, if fluoroscopy is not available, similar data can be collected by taking several static radiographs at timed intervals following injection of the contrast media.

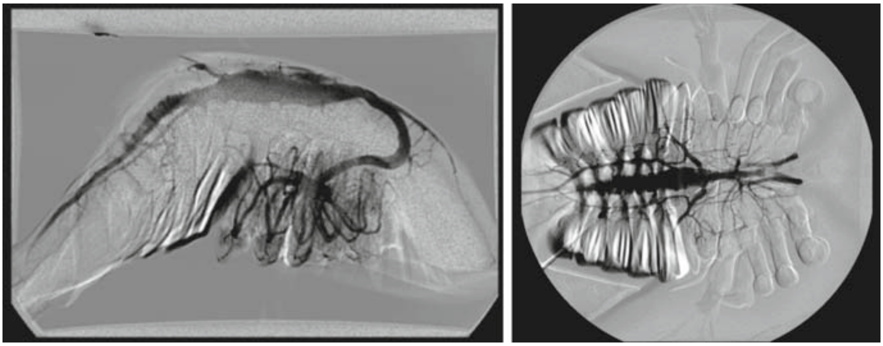

Fig. 3 Still images from a fluoroscopic positive contrast angiogram (*left* = lateral, 4.5 seconds; *right* = dorsoventral, 1.0 second)

2.4 Postmortem Diagnostics

2.4.1 Necropsy

As with physical examination, a necropsy (the veterinary equivalent of an autopsy) should include a systematic and thorough review of all body systems. The goal of a necropsy is evaluation and identification of gross pathologies not identifiable via non-lethal methods and acquisition of tissue samples for histopathologic analysis. Because internal viscera rapidly autolyze, the necropsy should always be performed as soon after death as possible. If the animal is alive, euthanasia can be achieved by injection of pentobarbital (390 mg per animal) into the cardiac sinus. Cardiac, respiratory, and cerebral arrest should occur within approximately 30 seconds of injection of the euthanasia solution.

A necropsy is begun with a full, non-invasive physical examination. Internal examination involves dissection of the animal, starting with removal of the dorsal half of the prosoma. Use bone cutters or heavy-duty shears to trim a few millimeters proximal to the free margin at the union of dorsal and ventral prosoma. Use a scalpel to connect the two ends of this incision by cutting through the dorsal prosomal exoskeleton a few millimeters cranial to the joint between the prosoma and opisthosoma. A curved probe is then used to bluntly separate the dorsal exoskeleton from any underlying tissues. Once the carapace is freed from underlying soft tissue connections, scissors may be used to cut the optic nerve. Lift the dorsal prosomal exoskeleton away from the body, exposing the hepatopancreas, gonadal tissue, brain, and cardiac sinus. At this point, blunt dissection can be used to access other internal viscera, including those of the digestive, circulatory, excretory, nervous, and reproductive systems. Tissues should always be collected as atraumatically as possible to minimize artifact on histopathology.

2.4.2 Histopathology

In the clinical setting, histopathologic evaluation of tissue specimens is most often used to characterize the microscopic details of a lesion. Alone, results of such analysis cannot be used to make a clinical diagnosis. Rather, formulation of a descriptive diagnosis should arise from correlation of histopathologic findings with other results from the comprehensive clinical evaluation.

Several obstacles exist which may hinder the utility of histopathology in horseshoe crab diagnostics. The first problem encountered is often the investigator's lack of knowledge regarding proper methods for tissue collection and preservation. But perhaps the weightiest hindrance to histopathologic analysis of horseshoe crab tissues is a lack of available reference materials that describe normal histologic anatomy in the horseshoe crab. This section attempts to remedy that problem, providing histological descriptions of normal, clinically relevant horseshoe crab tissues.

Postmortem tissue sampling for histologic analysis can be performed using the approaches described in Section 2.4.1. Antemortem sampling can be more challenging, and surgical biopsies are possible with the assistance of proper instrumentation. Aseptic technique should be employed; use alcohol swabs to cleanse the carapace and sterile surgical equipment for all invasive procedures. Starting from the outside and working inward, the first tissue which can be sampled for histologic analysis is the carapace. If the target piece of carapace is located at the lateral border of the prosoma, bone cutters or shears can be employed to remove a small sample; otherwise, carapace biopsies should be cut, using a #22 sterile, stainless steel surgical scalpel blade, as small squares (ideally $<1\ \text{cm}^2$) in the carapace. Remove the square of chitinous carapace by gently undermining the tissue with a probe to remove it from the attached soft tissue structures (i.e., vasculature, nervous structures, organ capsules). If biopsy of internal viscera is desired, surgical access to those organs must be attained. The previously described biopsy technique used to sample exoskeletal tissue should be used to make a small window for surgical access to underlying tissues. The simplest place to make such a window is just anterior to the legs on the ventral prosoma, as the carapace is thinnest and only minimally mineralized in this anatomic region. Once a window is prepared, blunt dissection can be used to visualize superficial organs. If the target tissue is deeper, introduction of a laparoscope (with saline, not gas insufflation) may be necessary. After collection of all necessary biopsy specimens, the body wall (carapace) must be closed. If access to the body was attained by making a surgical window in well-mineralized carapace, use a small amount of surgical epoxy to close the wound. If access was gained in the region of relatively less mineralized chitin and if the window was small enough, sutures may provide sufficient closure. It should be noted that, as in most other veterinary species, mucosal biopsy samples can also be obtained non-lethally from the gastrointestinal tract by means of endoscopy.

For standard histopathology, tissues should be immersed in buffered 10% formalin; the volume of formalin in which these tissues are placed should exceed the volume of the specimen by at least ten times. And although ultrastructural morphology will not be discussed, tissues can be collected for electron microscopy (scanning or transmission) by immersion in 5% glutaraldehyde, 4.4% formaldehyde, and 2.75% picric acid in 0.05 M sodium cacodylate buffer at pH 7.36. Whether preparing tissues for light or electron microscopic evaluation, the tissues should be immersed in fixative for no less than 24 hours.

Once the tissues have been properly preserved in formalin they can be prepared for sectioning and staining. Soft tissues can be processed using standard histological techniques (Luna, 1968). For optimal results, chitinous structures (namely the carapace, gills, and anterior portion of the gastrointestinal tract) should be demineralized prior to sectioning. Rinse formalin-fixed tissues in phosphate-buffered saline, then in deionized water. Place rinsed tissues in a solution containing 30 mL 0.5 M EDTA (pH 8.0), 70 mL ddH_2O and 1.2 mL 6 N HCl with agitation, at room temperature, for 2 or 3 days. Remove tissues and rinse with water several times before proceeding to the paraffin embedding process (Moore et al., 2002). Standard histologic stains such as hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), silver stains, and trichrome stains can be employed.

The chitinous carapace (Fig. 4) is variable in thickness and is defined by three distinct layers. The outermost layer, the epicuticle, is quite thin, refractory to staining, and has a slight greenish coloration. It is acellular and lacks chitin. It provides a hard, waterproof surface to protect the horseshoe crab's body.

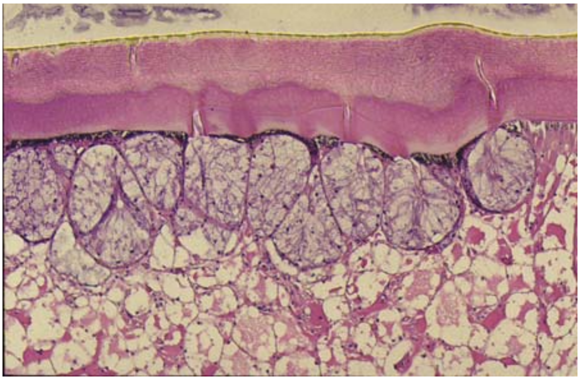

Fig. 4 Normal histology: carapace (H&E; 10×)

The middle layer of carapace, the exocuticle, is typically much thicker than the epicuticle. It is chitinous, with a pale, eosinophilic, and largely acellular appearance. The deepest layer is the endocuticle which is more eosinophilic than the epicuticular layer; it has a laminated appearance, which results from layering of chitin within a scant protein matrix. Beneath these layers of the exoskeleton lies the epidermal layer; the epidermis is composed of a single layer of columnar cells that contains small "packets" of black pigment near the apex. The epidermal cells produce chitinous matrix. Intertwined within the epidermal layer are dermal glands and trichogen cells. The dermal glands are large and globoid in shape; they are secretory in nature and are just deep to channels called dermal gland ducts. The ducts extend to the outer surface of the carapace and allow for release of products, such as pheromones, from the gland cells. The trichogen cells are vacuolated and basophilic and have a striking resemblance to nerve bundles; from these cells, bristles are projected through channels in the carapace and to the surface of the horseshoe crab.

Each book gill (Fig. 5) is composed of numerous gill leaflets. Each leaflet is made of two parallel lamellae, which are connected by chitinous pillars. The

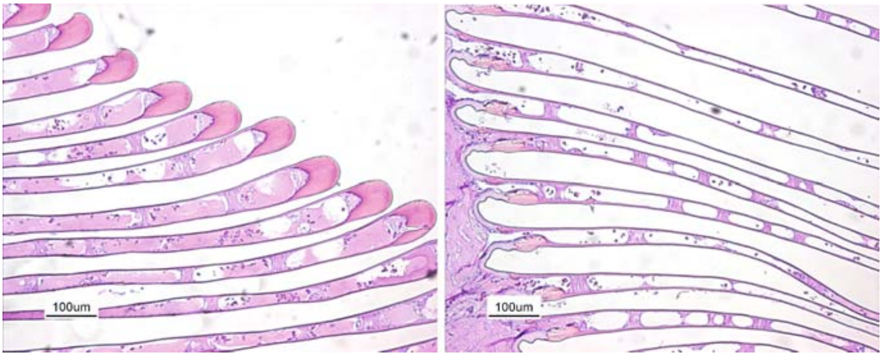

Fig. 5 Normal histology: gills (*left* = distal gill tips; *right* = proximal gill attachments; H&E)

pillars provide structural support; spaces between the pillars serve as vascular channels, which presumably provide the horseshoe crab with large amounts of surface area for both respiration and osmoregulation. The tips of the leaflets are blunt and composed of a thick layer of proteinaceous matrix. Cytoplasmic structures of the gills stain darkly with eosin while nuclei stain more basophilic. The proximal gill attachment is highly muscular, and the caudal half of each leaflet has a small muscle bundle between the proximal attachment and the first pillar; contraction of this bundle allows for gill movements responsible for both movement of water across the gills (aiding in respiration) and ambulation. The distal tip of each gill leaflet displays a thick, blunt, and acellular cap which connects the two lamellae of the individual leaflet and protects the leaflet from physical damage.

The hepatopancreas (Fig. 6) is a large accessory digestive organ. Its tubules are lined with simple columnar cells, allowing the tubules to act as secretory acini. Interstitial cells are large, angulated, and arranged in a loose cord-like structure. The interstitium occupies more of the organ than does the tubular network.

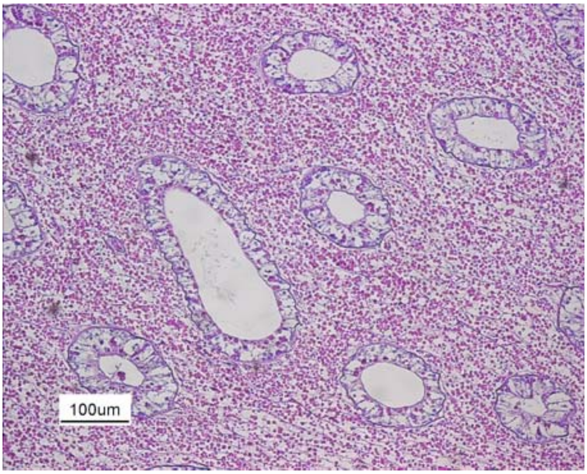

Fig. 6 Normal histology: hepatopancreas (H&E)

Based on gross morphology, the gastrointestinal tract (Fig. 7) of horseshoe crabs can be divided into four segments: esophagus, proventriculus, ventriculus, and intestines. The basic morphology of the tubular tract is similar to that of vertebrates, with each segment displaying several distinct layers, including a mucosal layer, mucosa muscularis, submucosa, and tunica muscularis externa. The most anterior segment is the esophagus, which begins at the mouth and extends caudally to the proventriculus. Identifying characteristics of this segment include epithelial crypts which produce the sclerotized lumenal surface of the esophagus. These epithelia are well perfused, as is evidenced by the innumerable

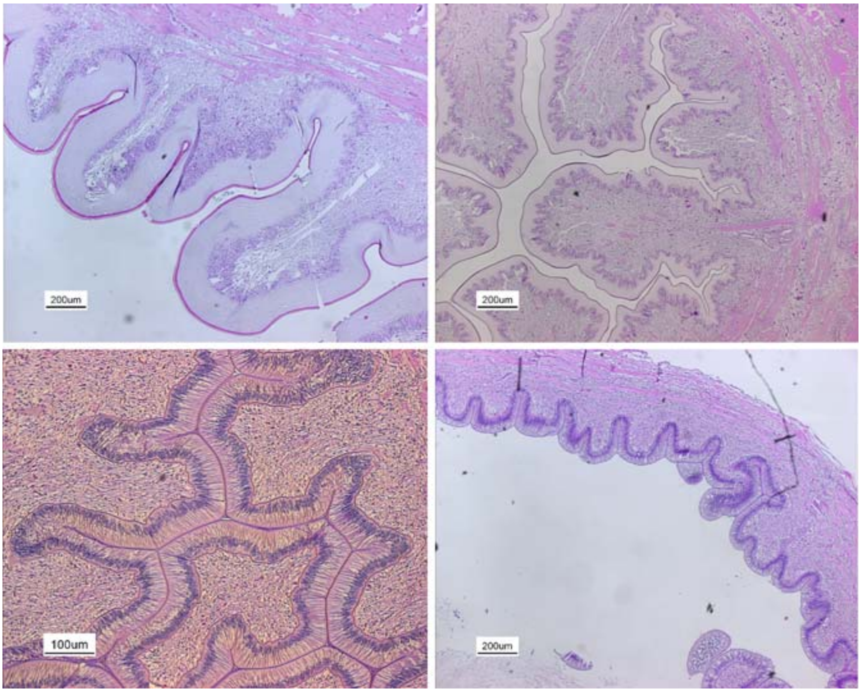

Fig. 7 Normal histology: gastrointestinal tract (*top left* = esophagus; *top right* = proventriculus; *bottom left* = ventriculus; *bottom right* = intestinal tract; H&E)

vascular channels in the submucosa. The proventriculus is also sclerotized, but its protective coating is of much greater thickness than can be found in the esophagus. The tunica muscularis externa is also far thicker in the proventriculus than in the esophagus, ventriculus, or intestines. It should be noted that the proventriculus is highly plicated, which allows this organ to expand when accepting digesta. Moving distally, the ventriculus is the first digestive segment which is not sclerotized. It is composed of a tall columnar epithelium lying atop a thick submucosal layer; the muscularis layers are thin in the ventriculus. Continuing to move distally, the next and final segment is a short, straight intestinal tract with multibranched digestive diverticulae interdigitating into the hepatopancreas. The intestines are similar to the large intestines of mammals in that they are lined with a columnar epithelium which displays crypts but not villi. The submucosal and muscular layers are intermediate in thickness.

Muscle tissue (Fig. 8) in the horseshoe crab is also much like that of vertebrates. Skeletal muscle is striated, with eccentric nuclei, a few satellite cells, and occasional vascular channels. The cardiac sinus is located in a dorsomedial position just deep to the prosomal carapace. The horseshoe crab has an open circulatory system; the heart pumps hemolymph into a well-defined arterial system which delivers and baths organs with hemolymph; hemolymph is

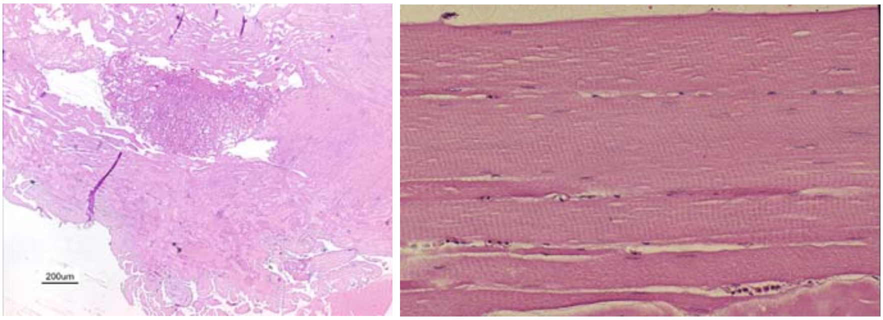

Fig. 8 Normal histology: muscle (*left* = cardiac muscle; *right* = skeletal muscle; H&E)

returned to the heart via venous shunts. Like skeletal muscle, the cardiac muscle is striated and has eccentric nuclei; however, the muscular bundles are less organized and tend to have a more pronounced interstitial matrix than appears in skeletal muscle bundles. Smooth muscle cells are fusiform and have central nuclei and, in comparison with skeletal muscle, are not highly organized.

The testes (Fig. 9) have a thin tunica albuginea lined with islands of spermatogonia. The male gonadal primordia have large basophilic nuclei, with sparse basophilic cytoplasm. The primordial cells migrate toward the lumen as they mature, allowing the cells to move into the lumen for storage; mature spermatocytes residing within the testicular lumen are tailless and stain deeply basophilic.

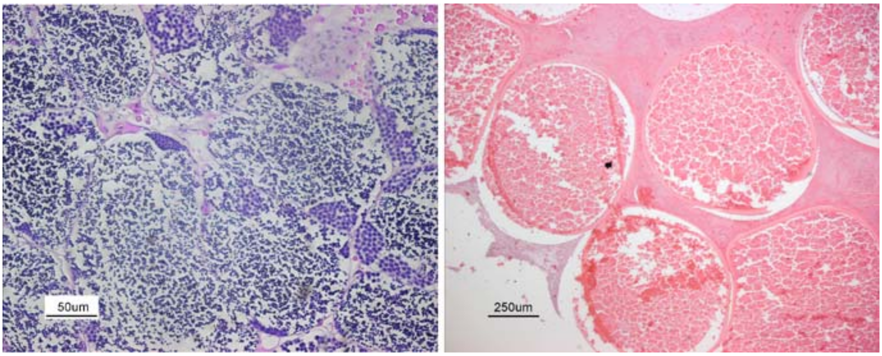

Fig. 9 Normal histology: gonads (*left* = male; *right* = female; H&E)

Ovaries are the largest organ in the body of a gravid adult female. Histologic description of this organ is challenging, as the eggs are encased within a thick shell which make sectioning and staining of the ovarian tissues difficult. In contrast to the testes, ovarian tissue (Fig. 9) retains far more eosin than hematoxylin. The ovaries are lined with columnar epithelium which appears to be oogenetic. The eggs are large (>500 μm) and surrounded by a thick cuticle; within the cuticle lies granular cytoplasm and a nucleus which varies in size depending upon stage of egg maturity. And unlike other invertebrates, the individual eggs of the horseshoe crab do not lie within their own follicles. A tunica propria lies outside the ovogenetic epithelium; it is between this connective tissue layer and the oogenetic tissue where eggs mature and are stored (Kingsley, 1892).

Neuroanatomy and ocular anatomy of the horseshoe crab have been described at length by other investigators and for sake of brevity will not be discussed here (Chamberlain and Barlow, 1980; Chamberlain and Wyse, 1986; Fahrenbach, 1981; Weiner and Chamberlain, 1994).

Amebocytes are the only cellular constituent of hemolymph. These cells are nucleated and granulated; the granules contain coagulogen and are variable in number and size. Amebocytes first appear within the hemocoel cavity of the embryonic horseshoe crab at the fifteenth embryonic stage in *T. tridentatus* (Liang et al., 1990) and during the eighteenth embryonic stage in *L. polyphemus* (Coursey et al., 2003). Although amebocytes are often seen in tissues and within vascular channels, to date, a hematopoietic organ has yet to be identified.

3 Common Diseases

3.1 Non-infectious Diseases

A significant non-infectious cause of morbidity and mortality in captive adult horseshoe crabs is panhypoproteinemia. A specific etiology has yet to be identified, but suggested causes include (in order of descending likelihood) nutritional imbalance/deficiency, protein-losing enteropathy, hepatic insufficiency, and protein-losing nephropathy. This syndrome appears to affect all adult animals in captivity and generally results in 100% mortality. The total protein levels in the hemolymph of these animals begin to fall within 3–4 weeks of wild harvest and commencement of captive maintenance. Protein levels drop below the reference interval (3.4–11.7 g/dL; Smith et al., 2002) within 3–4 months. Morbidity is not noted until about 5 weeks before an individual horseshoe crab ultimately succumbs to the disease; clinical signs include anorexia and lethargy. Despite this syndrome having nonspecific signs, progressive worsening of panhypoproteinemia can be monitored by measuring the total protein concentration using a clinical refractometer. As the disease progresses, changes in hemolymph protein concentrations will also become grossly observable; the hemolymph clotting time will increase significantly, and the serum will be clear and colorless rather than the normal opaque and blue (Fig. 10). At this point it is not known whether the primary cause of death in affected animals is respiratory failure (resulting from loss of hemocyanin), secondary infection (due to compromised innate immunity arising from loss of acute phase proteins), or distributive shock (or relative hypovolemia, resulting from increasingly severe edema due to loss of oncotic pressure).

Fig. 10 Gross appearance of hemolymph from a hypoproteinemic horseshoe crab; the tube on the *left contains* clear, colorless hemolymph from a hypoproteinemic animal, while the tube on the *right contains* opaque, blue hemolymph from a normal animal

Other non-infectious problems of captive horseshoe crabs range from water quality problems of ammonia toxicity, gas supersaturation and high turbidity, to molting problems of the shell, legs, or telson. In addition, traumatic injuries such as puncture wounds, fractures of the carapace, and crushing of the exoskeleton have been documented. Hemorrhage from these lesions can often appear significant but is rarely fatal. Wound repair in horseshoe crabs is facilitated by the migration of amebocytes from the hemolymph followed by wound healing (Bursey, 1977; Clare et al., 1990).

3.2 *Infectious Diseases*

Only scattered reports of infectious diseases affecting horseshoe crabs exist in the literature. These include algae, fungi, colonial and filamentous cyanobacteria, Gram-negative bacteria, and a variety of parasites (Bang, 1956; Leibovitz and Lewbart, 2004, Smith, 2006). Shell disease is probably the most common problem in both wild and captive horseshoe crabs. This syndrome is usually manifested by discoloration of the carapace or erosion of the exoskeleton (Bullis, 1994). Chlorophycophytal (green algal) infection of the surface of the prosoma is probably the most common pathogen identified from the horseshoe crab (Leibovitz and Lewbart, 1987, 2004). Infections may manifest as a greenish to grayish discoloration of the superficial surface and deeper tissues of the exoskeleton.

Fungal infections of the horseshoe crab appear to be limited to reports from captive individuals. Adult horseshoe crabs with branchial mycosis were reported by Leibovitz and Lewbart (2004), and mycotic infection of juvenile horseshoe crabs has been reported in captive individuals (Densmore, pers com).

Horseshoe crabs are also commonly infected with blue-green cyanobacteria (Leibovitz, 1986). These filamentous organisms (*Oscillatoria* spp.) colonize and penetrate the chitinous surfaces of the gill tissue. The disease can progress to involve deeper tissues of the gill and vasculature sinuses, sometimes resulting in tissue necrosis, swollen and ruptured gills leaflets, and death. A similar bacteria (*Beggiatoa* spp.) also colonizes the surface of the gill leaflets, but does not appear to be as invasive as *Oscillatoria* spp. (Leibovitz and Lewbart, 2004). Other bacteria identified from shell and gill lesions of the horseshoe crab included *Leucothrix* sp., *Vibrio* sp., *Flavobacterium* sp., *Pseudomonas* sp., and *Pasteurella* sp.

A number of parasites have been reported and include a variety of protozoa, a digenetic trematode, a couple of nematodes, and several turbellarid worms. Debilitated horseshoe crabs are often affected with protozoan species belonging to the ciliate genera *Pananophrys* spp., the flagellate genera *Hexamita* spp., or amoeba of the family Paramoebidae (Leibovitz and Lewbart, 2004). An unidentified protozoan was also reported from the hemolymph of an Asian species of horseshoe crab (Chen et al. 1989). The digenetic trematode, *Microphallus limuli*, of the herring gull (*Larus argentatus*) uses the horseshoe crab as a second intermediate host for its life cycle (Stunkard, 1950, 1951, 1953, 1968). The encysted metacercarial stage can be found in the connective tissue, muscle, brain, and eye of juvenile and adult horseshoe crabs. Though it has been postulated that these parasites may interfere with normal body functions, clinical significance has not been reported. Nematodes (i.e., *Monhysteria* spp. and *Grathponema* spp.) have been reported to invade the carapace of the horseshoe crab (Leibovitz and Lewbart, 2004) and several species of triclad turbellarid worms have been described from the horseshoe crab (Groff and Leibovitz, 1982; Kawakatsu, 1989; Ryder, 1882; Wheeler, 1894). The most significant of these, *Bdelloura candida*, commonly resides between the gill leaflets, on the ventral appendages, and on the external surface of the ventral carapace and obtains some of its nutrition from hemolymph acquired from lesions on the gill tissue. In addition, stalked cocoons of these parasites are located on the surface of the gill leaflets where they may interfere with respiratory activity of the gills.

Finally, there are a number of ectocommensals that frequent the external surfaces of the exoskeleton. These include bryozoans, sponges, barnacles, blue mussels, lady slippers, snails, oysters, whelks, and a variety of coelenterates, annelids, and free-living nematodes (Botton, 1981; Turner et al., 1988; Deaton and Kempler, 1989; Grant, 2001). Rarely do any of these organisms cause harm to the horseshoe crab, except when they directly interfere with normal functions such as mobility or respiration.

4 Veterinary Care

4.1 Preventative Medicine

4.1.1 Husbandry

Appropriate husbandry of adult horseshoe crabs requires knowledge and understanding of the housing, water quality, and nutritional requirements of the animal. Routine health checks are a good way of monitoring the overall health of the captive animals.

A recent review detailed housing and water quality requirements for the adult horseshoe crab (Smith and Berkson, 2005). To summarize their findings, tank choice is largely dependent upon the user's preference; anything from glass aquaria to fiberglass tanks, depending on the size and number of horseshoe crabs, may be used with success. Substrate may include sand or crushed coral, but are often forgone without sacrificing animal health. Culture systems must incorporate both biological and mechanical filtration; use of protein skimmers, UV filters, and ozonators are optional additions to the filtration system. The ultimate goal of filtration is to remove and maintain low concentrations of nitrogenous and organic wastes and minimize turbidity. Water quality should be monitored daily to assure that proper filtration is occurring. Of importance are temperature (15–21°C), salinity (25–27 ppt), ammonia (<1.2 mg/L), nitrite (though of less importance in saltwater than freshwater, nitrites should be kept below 0.15 mg/L), and pH (7.4–8.0). Saltwater can be either obtained from natural water sources and filtered to remove potential contamination and infectious agents or made using commercially available artificial marine salts.

In the wild, horseshoe crabs consume a variety of foods including marine mollusks and worms (Walls et al., 2002, Smith, 2006). The goal of feeding captive animals is to mimic the nutritional composition of diets of wild animals and maintain health. Larval stages of the horseshoe crab readily feed on new hatched brine shrimp. Captive adult horseshoe crabs are typically fed good-quality raw fish fillets, squid, crabs, clams, and shrimp or artificial diets such as commercially extruded shrimp diets and sinking, bottom-feeder pellets. Feeding is typically done at a rate of 0.75% body weight every 2–3 days. Unfortunately, the aforementioned problems with panhypoproteinemia in captive adult horseshoe crabs seem to suggest that these diets may be nutritionally incomplete.

Appropriate monitoring of horseshoe crab health and performing frequent "health checks" is essential. These examinations are a miniature version of the complete physical examination and should include monitoring weight gain/loss, observing behavior (feeding, ambulation, sexual, etc.), and completing a non-invasive hands-on physical examination. Measurement of total serum proteins is an inexpensive adjunct to the health check and provides important information pertaining to the long-term health of the horseshoe crab (refer to Section 3.2.1).

4.1.2 Biosecurity

Equally important as providing adequate husbandry and regularly assessing horseshoe crab health is establishment and enforcement of strict biosecurity protocols for disease prevention. Such protocols should describe requirements for animal identification, protocols for disinfection and quarantine, and other procedures for limiting spread of pathogens.

Identification of individual animals is an important aspect of disease prevention, as it provides a means by which individual medical records can be maintained. It would be impossible to track a single animal's health without individual identification; in turn, this would make it impossible to institute preventative measures such as repeated treatments and quarantines. Tagging is a simple means of attaching numerical identifiers to individual animals. Two simple and effective methods for attaching tags to the adult horseshoe crab are drilling the carapace and using marine epoxy (Fig. 11). In drilling the carapace, use a small diameter drill bit and make the holes as close to the lateral border of the prosoma as possible. This helps to avoid the hemolymph-filled body cavity and prevents trauma to internal organs. Affix the tag to the animal by threading plastic cable ties through the hole in the tag and then through the freshly drilled holes in the carapace. A less invasive method for affixing tags to the carapace involves using marine epoxy. Choose a quick-setting epoxy that is minimally exothermic; dry the carapace and place enough epoxy on the exoskeleton to allow for adhesion of the tag. Although slightly more labor intensive and more invasive, these authors prefer the drilling method, as epoxy-adhered tags tend to fall off the carapace after a couple of months. Note that drilling through the carapace to affix a permanent identification marker should only be performed in the adult animal; anecdotal evidence suggests drilling of the carapace of juvenile animals could interfere with growth and molting (Gore et al., 2006).

Fig. 11 Individual identification can be achieved by tagging (*left* = drilled carapace; *right* = marine epoxy)

It is also important to have established quarantine measures. These must be predetermined in order to ensure that all staff members of a facility are familiar with the protocol and can institute them in the face of an outbreak of infectious disease. Individual quarantine protocols can be developed for different diseases, but this is a cumbersome task and can become problematic in the event of initial misdiagnosis or delay in proper diagnosis. Instead, it is recommended that a single, conservative protocol be developed that provides suitable isolation for an infected animal or population of animals. These authors recommend quarantining sick animals in a designated "hospital" tank located at least 8 m from any other horseshoe crab tank; this distance minimizes risk of waterborne or aerosol transfer of disease. The hospital tank should have its own set of nets, water quality testing equipment, etc. There should also be a suitable perimeter around the hospital tank; anyone entering this buffer zone should be required to step through a footbath containing an appropriate disinfectant. The final step in setting up a quarantine protocol is deciding on length of quarantine. Any sick animal which enters quarantine should remain in isolation for a minimum of 45 days after the cessation of clinical signs of disease; this amount of time should be adequate to ensure the animal has cleared the infection and is not harboring an infectious pathogen. Additionally, a preemptive 60 day quarantine for any new animals entering a facility is recommended; this allows ample time for recrudescence of latent disease following the stress of shipment to the new facility.

As was alluded to earlier, when discussing the development of quarantine protocols, limiting the spread of potential pathogens between animals is an essential part of disease prevention. Quarantine can aid in limiting spread of disease from an individual to all its tankmates. Limiting spread of pathogens between tanks should be approached in a similar manner; each tank should have its own set of accessory equipment (nets, water quality equipment, etc.), and tanks should be physically separated by a suitable distance to minimize the risk of spreading pathogens through the air or through splashing water.

In the event that disease does occur, there should be a standardized disinfection protocol to help limit the spread of disease to other animals in the facility and to animals that will occupy the affected tank at some point in the future. Nets and other tank-side accessories can be disinfected by submersion in Roccal (1:256 dilution; Roccal-D Plus disinfectant, Pfizer Animal Health) or another similar water-based disinfectant. Saltwater tanks can be disinfected by draining the saltwater and rinsing with freshwater. After rinsing, fill the tank with 50,000 ppm bleach and expose for at least 30 minutes; drain the tank, spray down with 70% ethanol. Allow the tank to dry, and sit for at least 24 hours before refilling with freshwater. After refilling, sodium thiosulfate (2.8% per 1 ppm chloride) can be added for the purpose of inactivating chlorine in the water. After 2 hours, saltwater can be added to the system and animals can be reintroduced (Mainous and Smith, 2005).

4.2 Therapeutics

Little information is available in the literature to guide drug therapy in the horseshoe crab. Successful treatment of microbial disease (algal, bacterial, and fungal) has not been reported. As such, attempts at using antimicrobial drugs in the horseshoe crab should be attempted only after reviewing reports of drugs used in other invertebrate species. The only antimicrobial which has FDA approval for use in an invertebrate is oxytetracycline, which is approved for treatment of gaffkemia in *Homarus americanus*, the American lobster. These authors have studied the oral and intracardiac pharmacokinetics of oxytetracycline in the horseshoe crab (Nolan et al., 2007). They found that intracardiac administration of 25 mg/kg oxytetracycline sustains a plasma concentration over 10 g/mL for at least 5 days and has a terminal half-life of 128.3 hours. Intracardiac administration of oxytetracycline is simpler for the user and less stressful for the animal; together with the fact that the aforementioned plasma concentrations are higher and maintained longer than is accomplished with oral administration, we suggest that the intracardiac route of drug administration in the horseshoe crab is more appropriate than the oral route.

Three treatments have been suggested for external parasites; these include a 3–12 minute freshwater bath, a 15–60 minute formalin bath (1–1.5 ppt formalin), and a 60 minute acetic acid bath (3–5% acetic acid) (Bullis, 1994; Landy and Leibovitz, 1983). Because formalin is not highly soluble in saltwater, formalin treatment should be combined with a freshwater bath treatment whenever attempted. Animals should be monitored for adverse reaction for the duration of any of these bath techniques; at the first sign of distress, the animals should be removed from the bath, quickly rinsed in freshwater, and returned to their saltwater tanks.

The final available disease management option in horseshoe crabs is euthanasia. There are no AVMA-approved techniques for terminating invertebrate species; however, the most rapid and atraumatic method reported involves injection of pentobarbital (390 mg/animal) into the cardiac sinus of an adult horseshoe crab. As noted in Section 2.4.1, cardiac, respiratory, and cerebral arrest should occur within approximately 30 seconds of injection of the euthanasia solution.

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Aquaculture Methods and Early Growth of Juvenile Horseshoe Crabs (*Limulus polyphemus*)

Martin P. Schreibman and Chester B. Zarnoch

Abstract Current knowledge of horseshoe crabs (HSC) has been derived, in large part, from field studies. Comprehending the biology and conservation of HSC could be facilitated and augmented by understanding and improving their culture methods. Although many researchers and even lay people are capable of getting animals to the early stages of development, very few are successful in getting them to survive for longer periods of time. The Aquatic Research and Environmental Assessment Center (AREAC) has been successful in rearing HSC in annual cohorts, some for more than 7 years. We have used indoor recirculating aquaculture systems (RAS) containing medium prepared with artificial sea salts. Animals have been given various diets, including both natural and specially formulated feeds. We have traced the earliest developmental stages through juvenile development in animals that were derived from eggs fertilized in the field and laboratory. This chapter will discuss the problems and successes of culturing adult and developing HSC in RAS, methods of fertilization, feed regimes, growth, and survivorship and observations on HSC development from egg to juvenile.

1 Introduction

We have witnessed, in past years, dramatic reductions in the population of horseshoe crabs in most parts of the world where they have existed – some almost to extinction. Although not as severe in the Far East, populations of the Atlantic horseshoe crab, *Limulus polyphemus*, have also declined. There is a concerted effort to address this problem; however, the various stakeholders including pharmaceutical companies, commercial fishermen, shorebird advocates, and environmentalists have largely differed in estimating the significance of the problem and the method of approaching its solution (Berkson and Shuster 1999;

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Odell et al. 2005). It is certain that whichever method is adopted in its conservation, there will continue to be a harvest demand. Sustainable harvests of a species serving such a vital role in ecosystem processes may only be achieved by augmenting wild populations with cultured individuals. The ultimate goal of this form of stock enhancement and restoration must be to produce large numbers of healthy young horseshoe crabs that can be returned to the ecosystem with an optimistic anticipation of their survival. In this chapter we discuss horseshoe crab aquaculture methodologies using land-based, indoor, intensive production of animals in recirculating (water reuse) aquaculture systems (RAS).

Growth in horseshoe crabs is generally slow taking 9–12 years to reach maturity (Shuster and Sekiguchi 2003). Therefore, aquaculture production of mature animals is clearly not feasible. However, production of juveniles to be released into the environment for stock enhancement can be an effective management strategy as observed by its use in salmonid and bivalve fisheries. Botton et al. (2003) estimated the survival rates of trilobites transitioning from the planktonic phase to the epibenthic form to be 2.5%. The authors then estimated a log-fold decrease in the density of juveniles occurring with each molt from the second instar to the fourth instar at the end of their first summer. Although, data describing the survival of fourth instars to maturity are limited, it is believed that increasing the strength of the first-year class through aquaculture stock enhancement may lead to improved viability and stronger adult-year classes.

Horseshoe crabs, including adults and embryos, have been maintained in captivity largely for research purposes. Brown and Clapper (1981) described their methodologies for obtaining gametes and culturing embryos for studying the earliest stages of development. More recently, Smith and Berkson (2005) presented their work on system design, water quality, health issues, and nutritional demands. They described a chronic mortality that occurs in juvenile and adult HSC when they are held in captivity for greater than 6 months. They believe that the mortality is a result of a hypoproteinemic deficiency that is associated with inappropriate diet formulation. Clearly, nutritional requirements must be further studied in order for HSC aquaculture to be successful.

The growth of juvenile horseshoe crabs has been studied in field (Rudloe 1981; Botton et al. 2003) and laboratory experiments (Sekiguchi et al. 1988; Lee and Morton 2005). Typically, growth measurements are expressed in prosomal width. However, the quantity of feed distribution in aquaculture is calculated on a per weight basis. Lee and Morton (2005) fed *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda*, a mixture of fresh seafood in excess at 50% per wet weight for 5½ months. Both species increased approximately 24 and 71% in wet weight and prosomal width, respectively, after each molt. Similar studies have been lacking for the American horseshoe crab. In addition, further studies need to identify a cost-effective feed type and feeding regime. Lastly, another important consideration is the ability to produce large numbers of healthy juveniles. Therefore, all future studies should be directed toward production on a large scale.

We at the Brooklyn College (City University of New York) Aquatic Research and Environmental Assessment Center (AREAC) have instituted a program to study the captive breeding and maintenance of HSC in specially designed systems. It has comprised more than 7 years of intensive field and laboratory studies. What follows is a report of our successes and failures in the aquaculture of HSC and our recommendations for future applications and continuing research.

2 Aquaculture Methods

Our annual production schedule is summarized in Fig. 1. This timetable has been followed, with some modification, for almost 8 years, so that our oldest animals are now more than 7 years old. Our annual activities begin in the months of May and June, a period that is the spawning season for HSC in the New York City region.

Eggs are collected and fertilized in the field or in AREAC. The fertilized eggs are maintained in McDonald jars (Aquatic Eco-Systems Inc.; Fig. 2) and

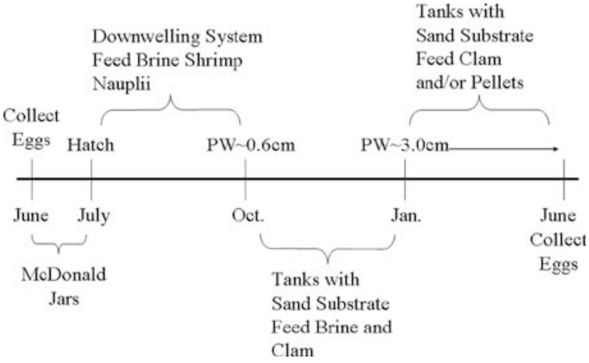


Fig. 1 An annual horseshoe crab aquaculture production schedule at AREAC (PW, prosomal width)

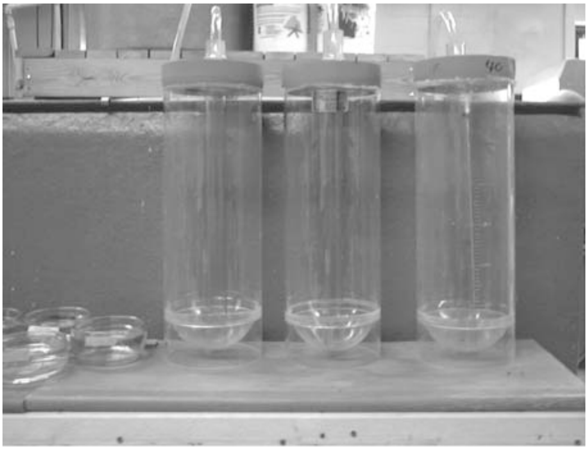


Fig. 2 Egg culture units; finger bowls are on the left and McDonald jars are on the right

transferred to downwelling systems (see below) between July and September. In October animals are transferred to larger recirculating aquaculture systems (RAS) and fed brine shrimp nauplii and finely chopped clam meat. In January, the chopped clams are supplemented with commercial fish food pellets with high protein content (35–40%). The next annual cycle is repeated (as it has in nature for 440 million years!!) in ensuing Mays.

2.1 Egg Collection and Culture

The major source of our animals is derived from Plumb Beach (N45° 35.002′ W073° 55.311′), a sandy beach on the north side of the Jamaica Bay inlet (Brooklyn, NY, USA). Plumb Beach, which generally has a robust spring spawn of HSC, is part of an urban US National Park – Gateway National Recreation Area. Fertilized eggs were derived from field collections in the sediment of spawn sites formed the previous evening. Gametes were also obtained by manual stripping in the field or in animals brought to AREAC. In this procedure slight pressure is applied anterior to the genital operculum (French 1979). We have also utilized a method of electrical stimulation, using a two pronged wire that is connected to a 3–4 V dry cell. The probes are placed below the gonopores and current applied in short intervals to release the gametes (Brown and Clapper 1981). The released eggs are collected from the gonopores by hand and the sperm is collected with a 1 ml pipette. Eggs and sperm are immediately placed together in a fingerbowl with sea water. After 30–60 min the water is changed in the fingerbowl or the eggs are placed in a McDonald jar (Fig. 2). Neither of these methods of artificially stripping HSC will produce sufficient numbers of eggs for large-scale production. Prolonged or repeated stripping will result in lesions below the gonopores and/or damage to the genital operculum. Collection of naturally spawned eggs appears to be a better strategy for aquaculture, at least until methodologies of induction of spawning are identified. We have also been concerned that egg quality may decrease if eggs are manually collected from HSC that have already completed spawning for the season. To examine this, we sampled four individual crabs from the shallow subtidal zone at Plumb Beach in late July and compared egg dry weights ($n = 30$) between the crabs (Fig. 3). There was no significant difference ($P > 0.05$) in egg quality as determined by egg dry weight between the crabs. Furthermore, we have also compared the dry weight of larvae produced from natural spawns ($n = 120$; pooled from four individuals) and artificial spawns ($n = 120$; pooled from four individuals) and found that there was no significant difference ($P > 0.05$) between the two groups (Fig. 4). Therefore, it appears as though methods to induce spawning will not produce inferior eggs and larvae.

We have experimented with the culturing of eggs and have compared hatch successes of eggs placed in small (12.5 cm in diameter) finger bowls and

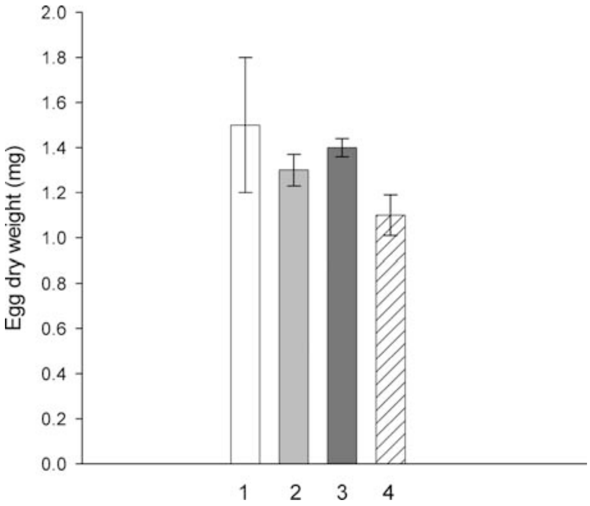


Fig. 3 The egg dry weights (n = 30) of four artificially spawned horseshoe crabs collected in July (error bars; ±SE)

McDonald jars. The eggs in the finger bowls are not agitated except during water changes which are typically done once per week. The greater hatch success (>75%) occurs when we use the McDonald jars where the eggs are constantly agitated, while our results with the finger bowls are far more variable. Typically, we will keep 300–500 eggs in a McDonald jar with a flow of 40 l min^{-1}. This density and flow rate are adequate for the number of crabs we have been producing. These values would have to be increased for large-scale production to be efficient, as these jars are 6 l in capacity and can hold 100,000 trout eggs. The use of static finger bowls is also adequate for culturing eggs. However, in our experience the eggs are more prone to fungal infection. Regardless of egg culture methodology, fertilized eggs generally hatch within 1 month.

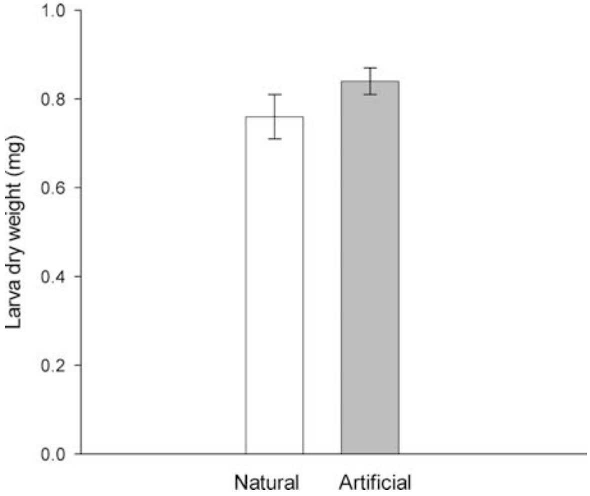


Fig. 4 The mean dry weight (±SE) of larva (n=120) from natural and artificially spawned horseshoe crabs

2.2 Larvae and Juvenile Culture

In all phases of animal rearing, recirculating aquaculture systems (RAS) are employed. The capacities of these systems range from 6 l (McDonald jars) to 2,000 l (our larger RAS are used to house adult HSC). The saltwater we utilize is constituted from artificial sea salts (Instant Ocean®) and filtered New York City tap water. Table 1 summarizes the water quality parameters that we have determined to be effective. For the most part, they reflect the characteristics of the water in the animal's natural habitat (Jamaica Bay, NY). These conditions are maintained throughout the year. Water quality is measured thrice weekly.

Table 1 Water quality parameters maintained in recirculating aquaculture systems for horseshoe crab culture

Temperature	20–25°C
Salinity	27–33 ppt
Dissolved oxygen	>5.0 mg l^{-1}
pH	7.5–8.8
Ammonia	<1.0 ppm
Nitrite	<0.6 ppm

When the HSC eggs hatch and the larvae emerge, they are moved to downwellers where our best survival rates have been achieved. These downwellers are typically used in our bivalve aquaculture programs. Generally, our downwelling systems are composed of a 320 l rectangular fiberglass trough (Fig. 5) with several PVC cylinders approximately 30 cm in diameter covered at one end with a 180 μm Nitex® screen. Each downweller is created by adding an airlift, which enters the top of the cylinder and forces seawater from the trough into the cylinder. A flow of 1.5–2.0 l min^{-1} is maintained into the cylinder. This flow rate helps minimize

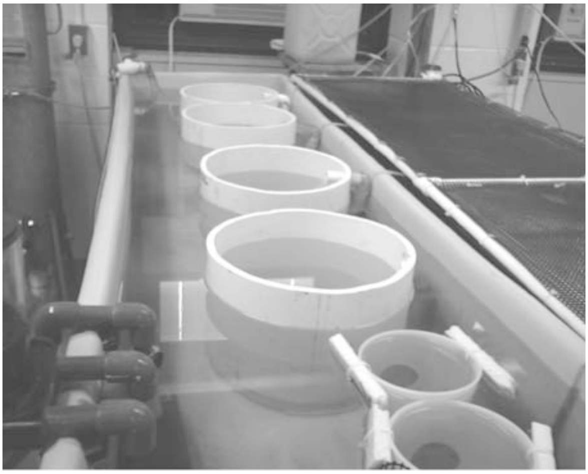


Fig. 5 An example of the downwelling systems used in the culture of horseshoe crabs through the second or third instars

the occurrence of external epiphytes and fungus. The use of the downwelling system does not conflict with our annual bivalve production cycles since the young bivalves are moved to field grow-out sites prior to HSC egg hatching. Therefore, this provides an additional use for these systems and could be used by bivalve aquaculturists interested in producing HSC for stock enhancement.

We compared the percentage of total lipid and carbohydrates occurring in eggs (24 h post-fertilization) and larvae (24 h post-hatch) to observe changes in these energy substrates. There was no significant difference between the lipid content of eggs and larvae. However, we did observe a significant reduction in carbohydrates from egg to larvae (Fig. 6). The larvae do not have to be fed until after the first post-hatch molt (Brown and Clapper 1981). Following the first post-hatch molt the HSC are fed brine shrimp nauplii (~450 μm). The 180 μm screen at the bottom of the downwellers serves to retain the brine shrimp for HSC consumption. The downwellers are removed from the system three times per week and gently sprayed with seawater to remove the organic material that has accumulated on the mesh screen, as well as on the HSC. The downwellers are sprayed with freshwater and scrubbed, after removing HSC, on an irregular basis. The HSC are cultured in the downwelling system through September and have at that time a mean prosomal width (PW) of 0.6 cm.

By October of the first year (see production schedule, Fig. 1), animals are moved to RAS tanks of various dimensions (see Schreibman and Zarnoch (2005) for details on RAS). In all cases a layer of sand is placed at the bottom of the tank. Larger juveniles (>1.0 cm PW) utilize a fine substrate for burrowing which also minimizes epiphyte accumulation.

These HSC are fed chopped clam meat thrice each week at approximately 1.5% of their body weight. They will also accept, and are offered periodically,

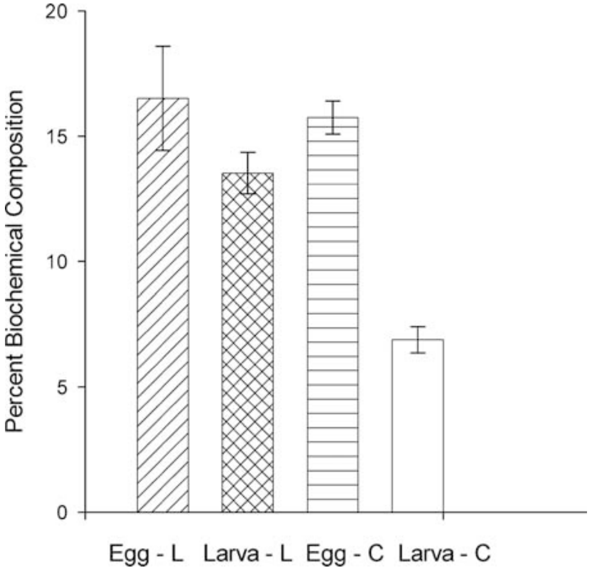


Fig. 6 The change in total lipid and carbohydrate content in eggs (n = 30) to newly hatched trilobites (n = 30). Units are given as a percentage of the biochemical constituent per unit dry weight (DW) of the tissue (L, lipids; C, carbohydrates)

high protein (35–40%) commercial fish pellets. We have instituted a program of nutritional studies in an attempt to improve and accelerate growth rates. In a recently completed study (Tzafrir-Prag et al., in prep.) juvenile HSC were fed increasing levels of energy and protein (0, 1, 2, 3% BW^{-1}) through pelleted feeds composed of fish meal or clam meat. This resulted in a linear gain in protein and energy. However, the partial efficiency of utilization, which accounts for losses (i.e., heat) in protein and energy when food energy is transformed into usable forms, was low (0.13 for protein and 0.16 for energy) indicating that there was poor assimilation of the feed. This is probably related to molting dynamics and should be further investigated. For example, Barthel (1974) described a quiescent period of 5–6 days prior to molt for instar 3 HSC and 1 day of inactivity after the molt.

2.3 *Molting and Growth*

Molting and concomitant growth in HSC is an exciting phenomenon and has been a major interest of our laboratory. Shuster and Sekiguchi (2003) have eloquently reviewed the current knowledge of molting and growth. Here we describe our observations in an aquaculture setting. Several molts occur in the first year while only two or three occur in the ensuing 18 months (Fig. 7);

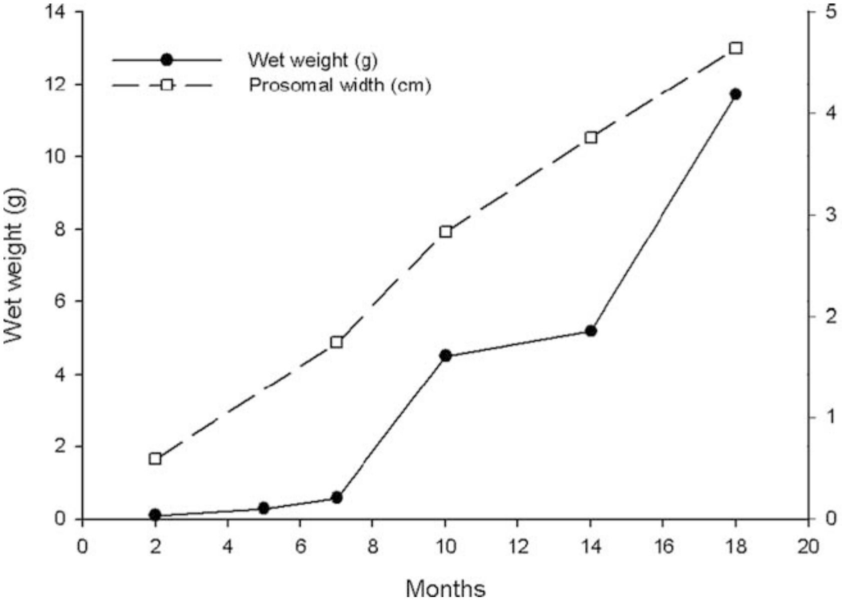
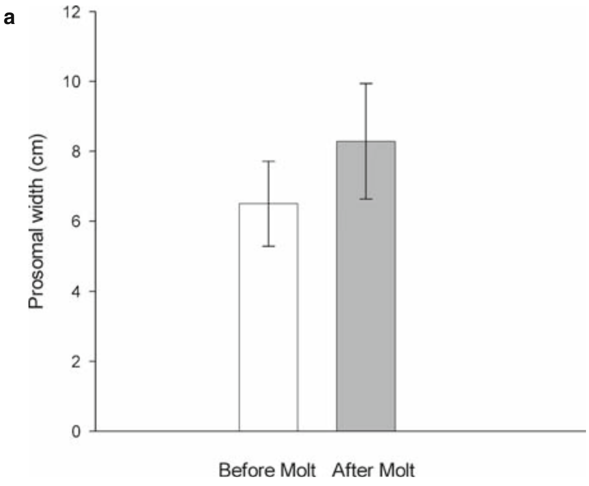


Fig. 7 The mean growth of successive cohorts of horseshoe crabs cultured from trilobites in recirculating aquaculture systems at the Aquatic Research and Environmental Assessment Center, Brooklyn College, NY, USA



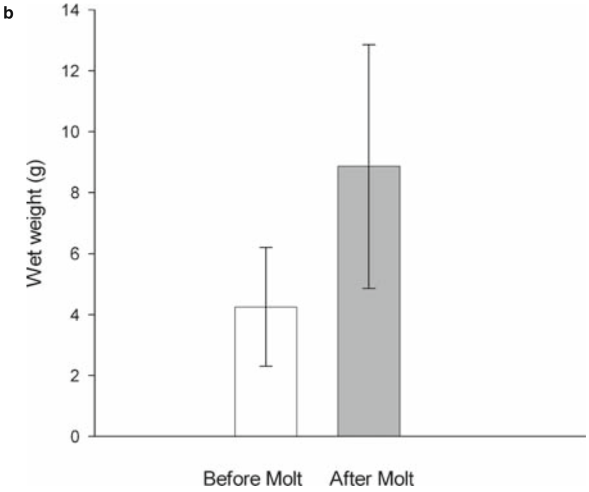


Fig. 8 **a** (*Top*) The increase in the prosomal width of a juvenile horseshoe crab after molting. **b** (*Bottom*). The increase in wet weight of a juvenile horseshoe crab after molting

this is similar to the molting schedule described by Shuster and Sekiguchi (2003). In each molt there is a major increase in the wet weight and prosomal width of the emerging animal. As depicted in Fig. 8a, b, there was a 15–20% increase in prosomal width and a 100% increase in weight as it emerged from the molt. An examination of the growth supports the notion of stepwise growth (Sekiguchi et al. 1988) with each molt. We have also determined (Fig. 9) that the percentage of HSC that molt increases with increasing light duration (20% in 0 h of light, 70% with 12 h of light, and 80% with 24 h light). However, 10% mortality occurred during the molting process in the

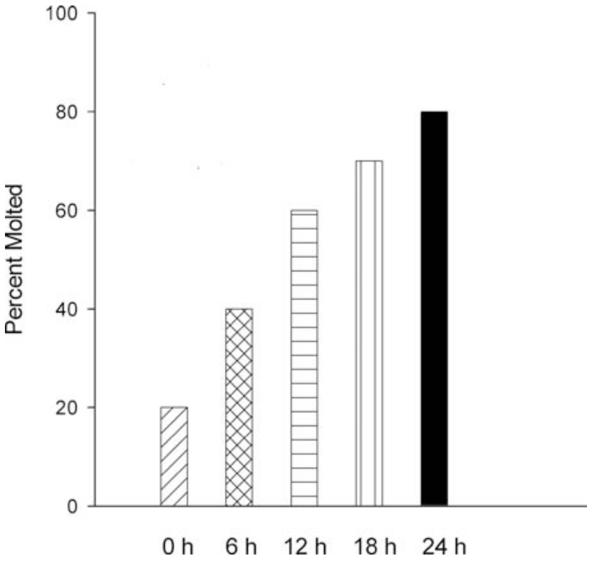


Fig. 9 The influence of light cycle on the molting of juvenile horseshoe crabs

groups held at 18 and 24 h of light, while no mortality occurred in the 0 and 12 h treatments. Therefore, we have adopted a 12 h/12 h light–dark cycle at AREAC.

3 Conclusions

We have developed or modified methods that permit the successful culture of HSC from fertilization to 7-year (and counting)-old juveniles. The growth observed in our studies seems to follow what has been described from field observations (Shuster and Sekiguchi 2003). This work should provide a basis for further studies which examine large-scale production of HSC for stock enhancement/restoration purposes. However, this is only the beginning of a new and exciting field of study in aquaculture and much more research is needed for large-scale production.

As described, AREAC utilizes RAS systems that permit long-term experiments in which environmental variables can be controlled year round. However, future research efforts may consider utilizing land-based systems that are nearshore allowing for the use of natural seawater (with its organic material) which may provide an additional feed type, particularly for the first and second instars. There is also a definite need for additional studies on induction of spawning including the influence of environmental factors, diet, social interactions, and physiological mechanisms. Furthermore, research should address the influence of molting hormones (i.e., 20-hydroxecdysone) on the acceleration of growth rates.

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Larval Culture of *Tachypleus gigas* and Its Molting Behavior Under Laboratory Conditions

J.K. Mishra

Abstract Horseshoe crab populations along the northeast coast of India are under threat due to degradation of the breeding beaches. To augment the trend, attempts were made to culture the larvae of *Tachypleus gigas* and study its growth rate by enhancing the molting pattern in the laboratory condition. Trilobites of *T. gigas* were cultured on a controlled diet of brine shrimp (*Artemia*) and diatom (*Chaetoceros gracilis*) at 26–28°C and $32–34^{0}/_{00.}$

Trilobites could molt up to the fourth posthatched juvenile stage within a period of 180 days from the day of hatching of trilobite from the egg membrane as free swimming larval stage. The molting behavior was faster from the first to the third posthatched juvenile stage, i.e., within a period of 90 days. The average growth rate in terms of total body length from the first to second posthatched juvenile was about 63%, and from the second to third posthatched larva was about 38%. The growth rate was found to be about 25% from the third to fourth posthatched juvenile stage, and molting took place 180 days after the day of hatching of trilobite. All the posthatched juveniles had similar morphological features to the adults. The fourth posthatched juveniles exhibited more prominent morphological features with fully grown legs, spines, and segmentation, with a total body length of 45 mm.

Further studies on food-dependent molting patterns of juvenile instars may help to establish a standardized aquaculture method to grow horseshoe crabs in captivity. Sea ranching of these reared animals can be carried out regularly in the holding areas to increase horseshoe crab populations and conserve these precious organisms from the brink of extinction.

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1 Introduction

Horseshoe crab populations over the world are now under threat as never before due to several known and unknown factors. Attempts are also being made to restore their populations (Rudloe 1982, Berkson and Shuster 1999, Widener and Barlow 1999, Botton 2001, Tanacredi 2001, Carmichael et al. 2004). Two of the four extant species, *Tachypleus gigas* and *Carcinoscorpius rotundicauda*, are found along the northeast coast of India (Sekiguchi et al. 1976, 1978, Sekiguchi 1988, Chatterji et al. 1988). Their distribution extends from Sunderbans in the extreme northern coast of Bay of Bengal, associated with Gangetic estuarine system (Itow et al. 2004), to the coast of Andhra Pradesh through the coast of Orissa, where they are found in high numbers (Fig. 1). *T. gigas* uses the intertidal zones of Orissa coast, associated with estuaries and creeks for breeding. This is due to favorable conditions such as beach characteristics, estuarine and creek environments, and suitable sand grain size at the breeding beaches (Mishra 1991). The highest number of breeding pairs are found at Balaramgari (Lat. 19^0 16 ' N; Long. 84^0 53' E) in the vicinity of Budhabalanga estuary (Mishra 1991, 1994, Mishra et al. 1992).

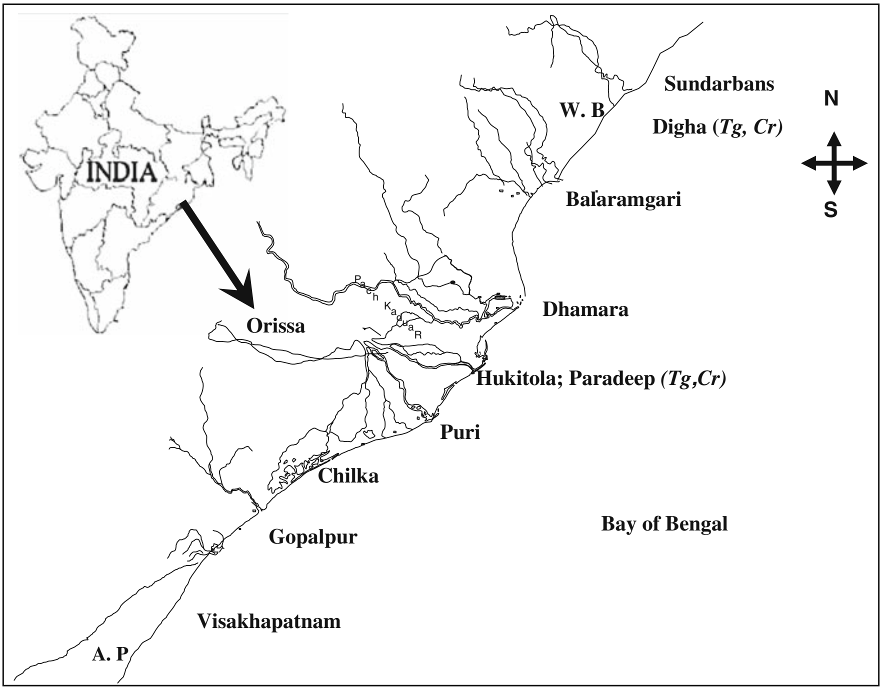

Fig. 1 Map showing the distribution of horseshoe crabs along the northeast coast of India

In recent years, the number of horseshoe crab pairs at Balaramgari has declined, which may be mainly attributed to the destruction of breeding beaches due to various anthropogenic activities as in the case of *Limulus polyphemus* (Botton et al. 2006), fisheries-related activities, and disturbance of the beach sand due to natural calamities. These factors affect the breeding activity and successful recruitment of *T. gigas* along the coasts of India. Though these environmental threats are encountered mainly at a local level, it may become a potential threat in the future and inhibit shoreward migration of the horseshoe crabs for breeding.

Several studies on the developmental biology and culture of juvenile horseshoe crab species have already been carried out at different environmental conditions (Sekiguchi 1973, Brown and Clapper 1981, Jegla 1982, Laughlin 1983, Sekiguch et al. 1988, Mishra 1991, Chatterjee et al. 2004, Lee and Morton 2005). However, there are no reports on the juvenile molting behavior of *T. gigas* from the trilobite (first instar) stage to different posthatched juvenile instars in response to food and culture conditions under laboratory conditions.

The present study was carried out to culture the larvae of *T. gigas* on a supplemented mixed diet of brine shrimp (*Artemia*) and the diatom (*Chaetoceros gracilis*) and study their molting behavior under laboratory conditions. Attempts were also made to observe the growth rate of the juveniles starting from trilobite to posthatched juvenile instars up to 180 days. The objective was to standardize the technique of rearing of *T. gigas* juveniles in the laboratory. Eventually, sea ranching of horseshoe crab juveniles in the holding areas is planned as a means of restoring natural populations. Therefore, it is important to develop laboratory culture methods that can grow fertilized eggs through the early juvenile stages in an environment that protects them from scavengers and excess human activities in the breeding zones.

2 Materials and Methods

Two hundred freshly deposited fertilized eggs of *T. gigas* were collected from the nests made by breeding pairs at Balaramgari beach immediately at the low tide on 24th-Feb-2002. Eggs were brought to the laboratory and incubated in the laboratory at room temperature of 26–28°C by keeping them in a plastic tray with sand, moistened with seawater. Trilobites hatched out 38–40 days after fertilization. These freshly hatched trilobites were transferred to fiberglass tanks with cartridge-filtered natural seawater of salinity 32–34%. Larvae were reared at a density of two larvae per liter of seawater at room temperature, and the culture water was aerated continuously. A thin sand bed was created in the tank for providing shelter for the larvae and to minimize epiphytic growth (French 1979). Larvae were fed with a substitute diet of *Artemia* and diatom, *C. gracilis*, during the culture period. The larvae were checked daily, and 40% of the culture water was changed. The growth rate was studied by measuring the total length of the molted individual at each stage by random sampling; total length (TL) of the larvae (starting from the anterior end of the prosoma to the tip of the telson at the posterior end) was measured by using vernier calipers. Simultaneously, prosoma width of the juvenile instars after each molting was also measured.

3 Results

The average total length of the freshly hatched trilobite larvae, which do not posses any tail, was 8 mm (Table 1). These larvae were very active and swam upside down in the culture tank. Trilobites could molt up to the fourth post-hatched juvenile stage (fifth instar) within a total period of 180 days from the day of hatching as free swimming trilobites (Table 1). The rate of molting was faster from trilobite (without tail) to the third posthatched juvenile stage, i.e., within a total period of 90 days from the day of hatching of trilobite. In case of the first juvenile molt, the average growth rate (TL) from trilobite to first posthatched juvenile (first-tailed stage) with the appearance of fully grown tail was found to be 100% within a period of 30 days. The average growth rate was 63% from the first to second posthatched stage, and 38% from the second to third posthatched stage. However, there was a slower growth rate of about 25% from the third to fourth posthatched juvenile stage, which took 180 days after the day of hatching of trilobite. All the posthatched juveniles exhibited similar morphological features to that of the adults. The fourth posthatched juveniles exhibited more prominent morphological features with fully grown legs, spines, and appendages with a total body length of 45 mm. Prosoma width increased

Table 1 Growth increment of larval stages of *T. gigas*

Stages	Days for development	Total body length (mm)	Prosoma width (mm)	Percentage of growth increment (total body length)
Trilobite	38 days after fertilization	8	7	–
First posthatched juvenile	30 days after reached trilobite	16	11	100
Second posthatched juvenile	45	26	16	63
Third posthatched juvenile	90	36	20	38
Fourth posthatched juvenile	180	45	24	25

from 7 mm at the trilobite stage to 24 mm at fourth posthatched juvenile (Table 1). However, the mortality rate during this study period was observed to be very high with an average survival rate of about 30% at the end of the culture. Although there was 100% survival between trilobites and first posthatched juvenile stage, it was about 67% from the first to second posthatched stage. It further declined to about 62% from the second to third posthatched stage, and 30% from the third to fourth posthatched juvenile stage.

4 Discussion

In the present investigation, it was observed that the trilobites exhibited rapid molting under laboratory condition in comparison to the juvenile molting of *Limulus*, which took about 7 months to reach the fourth posthatched juvenile (Brown and Clapper 1981). In all the stages, there was a positive correlation between the total length and the prosoma width (Fig. 2), indicating that the growth takes place in terms of increasing both length and width. It has been reported that salinity influences larval growth in *T. gigas* (Chatterji et al. 2004). In this experiment, natural seawater with a salinity range of 32–34% was found to be suitable for larvae culture. In this region of India, trilobites migrate to the sea immediately after their hatching with the receding tide and grow as juveniles in the seawater with little variation in salinity regime. Under experimental culturing conditions, the food given to larvae may have influenced the molting behavior in juvenile horseshoe crabs. However, further studies on food-dependent molting pattern of juvenile instars can help to establish a standardized culture method of this species.

The intermolt period increased with stage, from 30 days between trilobite and first juvenile to about 90 days between the third and fourth juvenile stage (Table 1). Though the reason for this is unknown, the role of ecdysones as molt-promoting hormones (Jegla 1982; Sekiguchi et al. 1988; Shuster and Sekiguchi 2003) deserves further study. But the involvement of a molting protein in

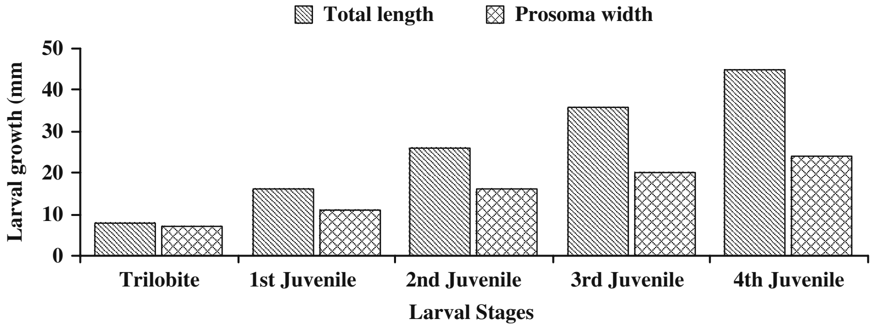

Fig. 2 Total length and prosoma width of different larval stages of *T. gigas*

T. gigas, as in the case of other crustaceans, particularly in barnacles (Satuito et al. 1996) should also be investigated. It may be termed as horseshoe crab molting protein (HCMP). As molting takes place, I hypothesize that the HCMP in the larva may be used, causing depletion in its concentration. Again it may take some time to generate the protein within the body to the required level, which is capable of inducing the molting process. However, it requires further study to clarify the involvement of any such protein in horseshoe crabs.

Sea ranching of laboratory-reared *T. gigas* was carried out by the author on the World Environment Day in the year 2002 at Visakhapatnam (northeast coast of India). The sea ranching of laboratory-reared horseshoe crab juveniles was done in the holding areas as a step to compensate the loss of horseshoe crab population, due to the loss of breeding grounds, and to restore natural populations. Simultaneously, an awareness campaign among the coastal population was conducted to highlight the threats encountered by horseshoe crabs in their natural habitat due to environmental degradation with a view to conserve these precious organisms from the brink of extinction at national and international levels.

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Diet Composition of Juvenile Horseshoe Crabs: Implications for Growth and Survival of Natural and Cultured Stocks

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Abstract Horseshoe crabs are valued for economic, ecological, and educational purposes. These values have raised interest in managing natural stocks and culturing crabs for conservation, research, and education. To inform these efforts, we used N and C stable isotopes to define the natural diet of juvenile horseshoe crabs and then assessed effects of different diets on growth and survival of juveniles in culture. In the natural environment, N and C isotope ratios in juvenile horseshoe crabs changed as crabs grew, with larger crabs consuming larger prey. Linear mixing analyses suggested young crabs were supported by high quantities of benthic and suspended particulate organic matter (POM), shifting between marine algae and salt marsh-based food webs, depending on size. In culture, we tested the relative importance of algae, as a proxy for POM, in horseshoe crab diet by feeding juvenile crabs different percentages of algae and prey animals. Initially, juvenile crabs showed a significant increase in size when fed diets >70% protein, but showed a decrease in survival compared to algae-rich diets. Overall, growth rates and survival declined through time during the 128-day study, regardless of diet composition. These data suggest horseshoe crabs require foods from a combination of plant and animal sources. Successful culture or conservation of horseshoe crabs will depend on understanding the relative importance of different food sources at different life stages as well as discerning the balance between factors that increase growth, but reduce survival.

1 Introduction

Interest in culture of American horseshoe crabs, *Limulus polyphemus*, has increased in recent years due to increasing concern over use and conservation of natural stocks. American horseshoe crabs are harvested primarily as bait for the conch and eel fishery, for scientific research, and to produce Limulus

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amoebacyte lysate (LAL), which is used to test medical supplies and equipment for pathogens (Berkson and Shuster 1999, Botton 2002). Horseshoe crabs also are ecologically important as a food source to migratory shorebirds, which fuel a significant ecotourism industry in some regions (Botton 2002). More recently, horseshoe crabs have gained popularity as part of a worldwide movement to integrate science, management, and policy into public education (Botton 2001, Chen et al. 2004, ERDG 2007, O'Connell et al. in press). These interests have prompted curiosity in programs to culture horseshoe crabs for use in classrooms, the aquarium trade, controlled research settings, and emerging aquaculture enterprises to supplement natural stocks (Botton 2002, Carmichael et al. 2003, Chen et al. 2004, Degener 2007, ERDG 2007, O'Connell et al. (2009), Carmichael unpublished data).

Research on horseshoe crab development has provided basic information to establish the physical conditions for culture. Field and laboratory research has defined the basic tolerances of horseshoe crabs to culture conditions, including salinity, temperature, and dissolved oxygen (DO) concentrations (Sekiguchi 1988, Shuster and Sekiguchi 2003). In particular, this work has defined the effects of variation in these parameters on growth and survival of early life stages (Jegla and Costlow 1982, Sekiguchi 1988). There also is a growing body of information on health management of horseshoe crabs in captivity (Smith and Berkson 2005). Despite these advances, however, there are few published reports to guide culture enterprises in the United States, particularly with regard to diet and nutrition.

To successfully rear horseshoe crabs in culture and aid conservation of natural populations, we must understand the diet composition that yields the highest rates of growth and survival. Gut content analyses and limited choice tests suggest that protein sources, particularly bivalves, may be a main component of adult horseshoe crab diet (Shuster 1960, Botton 1984, Botton et al. 2003a). Gut content analyses, however, are limited to detecting the most recent diet and may be biased toward identification of species with hard skeletons or shells that remain in the gut (Hyslop 1980, Alexander et al. 1996). Stable isotope analyses provide an alternative method to discern foods that are nutritionally important by identifying items that were not only consumed but also assimilated (Carmichael et al. 2004). Recent studies among horseshoe crabs suggest a variety of foods, including particulate organic matter (POM) such as algae, may play a significant role in the diet of horseshoe crabs (Gaines et al. 2002, Carmichael et al. 2004, Carmichael and Valiela 2005). Measuring growth and survival of horseshoe crabs fed diets that vary in proportion of animal protein and components of POM, such as algae, may help define artificial diets and improve culture success.

In this study, we sought to inform culture of juvenile horseshoe crabs by better defining their natural diet and using this information to guide development of artificial diets. To do this we took a two-part approach. First, to better define the natural diet of juvenile horseshoe crabs, we analyzed N and C stable isotope ratios in horseshoe crabs and their potential foods in the field. Second, to test the relative importance of POM to horseshoe crab diet, we used marine algae as a proxy for POM and measured growth and survival of juveniles in culture when fed diets that differed in percentages of algae and prey animals.

2 Methods

2.1 Field Sampling and Stable Isotope Analyses

Methods for the field component of this study are described in detail in Gaines et al. (2002) and Carmichael et al. (2004). Hence, we briefly describe the methods here. Juvenile and adult horseshoe crabs and potential prey items were sampled by hand from Nauset Beach in Massachusetts. Crabs were measured to the nearest 0.1 mm prosomal width and assigned to instar stages according to Carmichael et al. (2003). Benthic and suspended POM were sampled using modified syringe corers and by filtering 1 L of near-bottom water, respectively. All animals, sediments, and water filters were dried to a constant weight at 60°C. Animals and sediments were ground to a powder. All samples were packed in tin capsules and sent to the U.C. Davis stable isotope facility for analysis by mass spectrometry. A linear mixing model was applied to estimate the maximum possible contribution of different components to horseshoe crab diet (Phillips 2001).

2.2 Laboratory Culture

Juvenile horseshoe crabs were cultured at the Downeast Institute of Applied Marine Research (DEI) on Great Wass Island, Maine. Horseshoe crabs were obtained as eggs from Delaware Bay and as juveniles from Pleasant Bay, MA.

2.2.1 Culture Conditions and Apparatus

Crabs were kept in a closed tank under ambient conditions for 2 weeks before the start of the study. Crabs ranging in prosomal width from 2.4 to 9.7 mm were sorted into cohorts based on known instar stages (Sekiguchi 1988, Carmichael et al. 2003) and distributed by prosomal width so that each treatment bin received a total of 13 crabs; one crab (8.90 ± 0.10 mm), eight crabs (6.72 ± 0.03 mm), two crabs (4.69 ± 0.05 mm), and two crabs (3.05 ± 0.04 mm). We selected these smallest sized horseshoe crabs because they are likely to grow quickly and have similar expected molting rates (typically 2- to 3-week intervals; Sekiguchi 1988, Carmichael et al. 2003). The number of individuals within each size (prosomal width) class depended on availability during field collections.

Horseshoe crabs were reared in 24 plastic bins of two different sizes; 12 (15.4 cm × 24.2 cm) and 12 (19.8 cm × 28.6 cm size), without covers (Fig. 1, Bin).

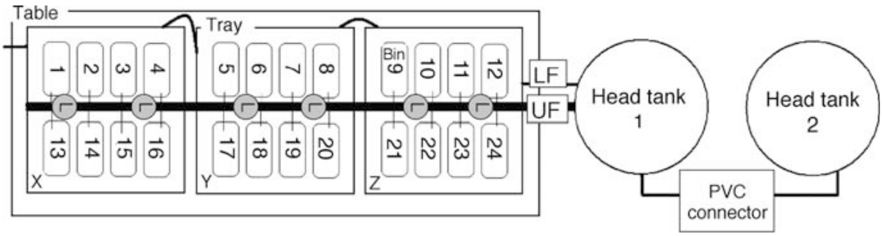

Fig. 1 The horseshoe crab culture apparatus consisted of two head tanks connected by PVC pipe to a table containing three trays (X, Y, Z) of eight bins (1–24). LF = Lower flow from head tanks to trays. UF = Upper flow from head tanks directly to bins. L = location of lights on the upper PVC pipe

The bottom of each bin was removed and replaced with 700 μm mesh, and plastic feet were added to the bottom of each bin to allow water movement into and out of bins from below. The bottom of each bin was covered with 1 l of pre-washed natural sand (700 μm–2 mm), collected from Roque Bluffs Beach, Maine.

Bins were randomly placed into three large, flat trays in which they remained throughout the study (Fig. 1, Tray X, Y, Z). Two large head tanks were elevated above the level of the trays to provide a gravity fed system of continuous water flow. Head tanks were filled daily with ambient seawater at salinity of 34%. Water was serially pre-filtered to 10 μm and heated to 25°C to ensure lower trays maintained temperatures ranging from 17 to 23°C. DO concentration was maintained at 6.0–7.6 mg l^{-1} by using two air stones in each tray, with one additional air stone in the head tank. Trays were supplied by a continuous flow of water at approximately 1 cm s^{-1}, which kept sand moist but held water levels just below the surface of the sand in each bin (Fig. 1, LF). In addition to this continuous flow, each bin was supplied with water from an overhead hose (Fig. 1, UF). This overhead supply of water was regulated by a timer and raised the water level in the bins to ~ 6–10 cm by flowing from 11 pm to 1 am, 3 am to 5 am, and again from 11 am to 1 pm, 3 pm to 5 pm to simulate two 6-h periods of high tide. To reduce temperature variation and likelihood of hypoxia in the bins, we opted to use these two shorter periods of continuously flowing “high tide”, spanning 6 h, rather than one longer period of slack high water. Water did not drain completely during the 1-h break, representing peak high tide in our system. To simulate ambient light conditions, we mounted six 60-W natural-sun lights (GE) above the bins (Fig. 1, L). Lights were set on a timer to maintain 12-h light and dark cycles.

To account for potential differences in culture conditions among experimental bins and trays, we regularly measured DO, temperature, and salinity in each head tank and tray, and in two randomly selected bins in each tray. DO and salinity were measured every 2 weeks using a model EDO (LaMotte #7414) DO kit and using an optical refractometer, respectively. Water temperature was measured daily with a thermometer.

To reduce fouling by silt, algae, and *Pseudomonas* sp. (fouling bacteria common to culture facilities), the culture apparatus was cleaned on a regular basis. Head tanks were cleaned with mild soap every week. Every 2 weeks, bins and large trays were scrubbed with a mixture of diluted bleach and detergent, rinsed with fresh water, and re-rinsed with copious quantities of salt water. Sediments from each bin were also washed over a 3 mm sieve with fresh ambient salt water.

2.2.2 Experimental Diets

To determine the relative importance of algae to horseshoe crab diet, we tested four diets differing in ratio of algae to animal matter (referred to as "protein" for simplicity); 90%:10%, 60%:40%, 30%:70%, and 100% protein. Test algae included *Ulva* var., *Enteromorpha* spp. (macroalgae), and *Tetraselmis* sp. (microalgae). Animal protein sources included soft-shelled clams (*Mya arenaria*), blue mussels (*Mytilus edulis*), and polychaetes (*Neanthes virens* and *Glycera* spp.). These species were chosen because they represent a combination known to be part of horseshoe crab diet and are locally available in Downeast Maine and elsewhere along the Atlantic coast to supply natural and cultured stocks of crabs (Gaines et al. 2002, Carmichael et al. 2004, Carmichael pers. obs.). All species were obtained by hand from local beaches in Downeast Maine except *Mya* and polychaetes, which were obtained from local commercial fishermen.

To create diet mixtures, each species was washed thoroughly with clean seawater, patted dry with paper towels, and for bivalves, soft tissues were separated from shell. To ensure equal proportions of each test species contributed to the final algae and protein mixtures used to create the test diets, we pureed each species separately (polychaetes were combined) in a commercial grade blender. Purees were then combined by volume, using batches of 50 ml of each species, to create final "algae" and "protein" stock mixtures, which were subsequently mixed at the test ratios. Food stocks (pastes) were prepared at once for the entire sampling period and frozen at –20°C so that small batches of food could be thawed for feeding as needed. Horseshoe crabs were fed 2.0 g (wet weight) of the appropriate test mixture per bin. We determined this quantity of test diet during week 1, by offering crabs 5.0 g of food mixture per bin and weighing the amount uneaten after 24 h. Food paste was mixed into the sediment every 2 weeks after cleaning but before horseshoe crabs were reintroduced to their bins. Any visible pieces of uneaten food were removed after 24 h to minimize fouling.

2.2.3 Measuring Growth and Survival

To measure growth and survival, juvenile horseshoe crabs were recovered from bins by hand sieving over a 2 mm mesh screen every 2 weeks. Crabs were counted, along with shed molts, and prosomal width was measured (by one person throughout the experiment) to the nearest 0.1 mm with vernier calipers. We used change in prosomal width as the unit for determining growth because this unit has been widely used (allowing comparison to other studies) and shows relatively little variation within instars among populations (Riska 1981, Sekiguchi 1988). Growth and survival were measured as the change in prosomal width and number of crabs, respectively, through time for the first 56 days of the study and through day 120. After day 56 growth and survival were measured every 4 weeks.

2.3 *Statistics*

Analyses of variance (ANOVA) were performed to test for treatment effects, including differences in horseshoe crab size and survival between trays, treatments, bin size, and bin location for each date. Regression analyses were used to determine significant differences in growth, survival, or culture conditions (DO, salinity, temperature) through time. Where significant relationships were found, we further performed analyses of covariance (ANCOVA), preceded by a test for homogeneity of slopes (Sokal and Rohlf 1987). This approach was used to determine differences among trays (for changes in temperature through time), treatments (for growth and survival rates), and to compare the initial days of study (through day 56) to the entire study (day 120).

3 Results

3.1 *Field Study*

3.1.1 Available Foods and Adult Horseshoe Crabs

To provide a context for analysis of stable isotope ratios in juveniles, we first measured ratios in available foods and adult horseshoe crabs. We identified three isotopically distinct dietary groups available to horseshoe crabs at our field sites. These groups included (1) bivalves and gastropods, which clustered near δ^{13}C values for marine algae (Fig. 2; Bi, Ga circle and dark gray line), (2) crustaceans and polychaetes, which clustered near δ^{13}C values for salt marsh grass (Fig. 2; Cr, Po circle and light gray line), and (3) POM, which showed depleted δ^{15}N% compared to other dietary groups and had δ^{13}C% that reflected a mixture of marine algae and salt marsh sources. N and C stable isotope ratios in tissues of adult horseshoe crabs were roughly 9 and –15%, respectively, showing the appropriate 2–4% enrichment compared to the two animal-based dietary groups and reflecting a mixture of marine algae and salt marsh-supported food webs (Fig. 2, A). Refer to Gaines et al. (2002) for the species-specific stable isotope ratios within each dietary group presented here (Fig. 2).

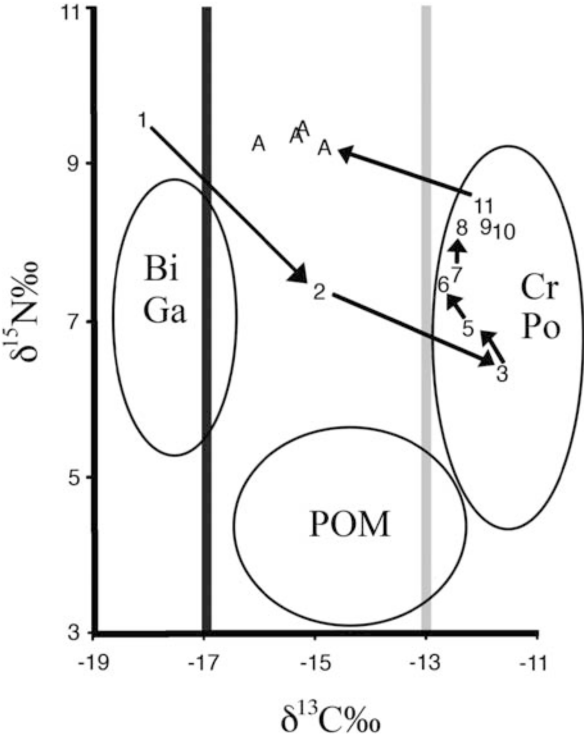

Fig. 2 δ^{15}N compared to δ^{13}C values in juvenile (indicated as instars 1–11) and adult (A) horseshoe crabs and dietary groups (defined in Table 1). Ovals indicate the general region in which isotope values clustered for each dietary group. The gray lines show expected δ^{13}C values for food webs supported by marine algae (–17%) and salt marsh grass (–13%). Arrows depict the isotopic shift in horseshoe crab tissues with growth. Modified from Gaines et al. (2002)

3.1.2 Juvenile Horseshoe Crabs

N and C stable isotope ratios in juvenile horseshoe crabs changed as crabs grew, with young crabs depending primarily on small particles of organic matter (Fig. 2 and Table 1). First instars had stable isotope ratios similar to adult horseshoe crabs (Fig. 2; 1 and A). Second and third instars showed relatively depleted δ^{15}N values compared to other instars (Fig. 2; 2 and 3), and their δ^{13}C values reflected benthic and suspended POM (Fig. 2; POM circle). δ^{15}N% in tissues of instars 5 through 11 increased from ~6% to nearly 9% and maintained δ^{13}C % similar to salt marsh sources (Fig. 2; gray line). Accordingly, stable isotope values of these instars clustered near values for crustaceans and polychaetes (Fig. 2; Cr, Po circle). Linear mixing analyses further confirmed that young crabs fed on high quantities of benthic and suspended particulate organic matter, shifting from POM to animal-based food sources as they grew (Table 1). In fact, instars 2–7 appeared to feed almost exclusively on POM. Among instars 8–11, diets shifted to a combination of primarily benthic POM, crustaceans and polychaetes (Table 1; shaded area).

Table 1 Estimated percent contribution of dietary components to diet for juvenile horseshoe crabs (instar stages 2–11). Data were not available for instar 4. Bi = bivalves, Ga = gastropods, Cr = crustaceans, Po = polychaetes, POM = particulate organic matter. Shaded area shows the components estimated to be most important at each instar (modified from Gaines et al. 2002)

	Instar								
Diet component	2	3	5	6	7	8	9	10	11
Bi	–	–	11	22	20	22	16	11	17
Ga	–	–	14	27	24	27	20	13	21
Cr/Po	–	–	44	56	63	79	87	84	95
Benthic POM	21	<100	100	100	99	74	77	64	70
Suspended POM	100	<100	23	42	34	31	18	9	16

3.2 Laboratory Culture

3.2.1 Growth

In culture, we tested the relative importance of marine algae, a major component of benthic and suspended POM, to horseshoe crab diet by feeding juvenile horseshoe crabs different mixtures of algae and potential prey animals. Mean prosomal width of horseshoe crabs in all treatments increased during the study, showing distinctive jumps in growth, characteristic of molting (Fig. 3; top, between days 14 and 28). Despite this stepped pattern of growth, linear regression best fit these data and explained a majority of variation in horseshoe crab size through time (Fig. 3). Hence, the slopes of the regression lines in Fig. 3 were used to calculate mean growth rates (Table 2).

We also observed significant differences in growth between the first half and second half of the study (Fig. 3). First, during the initial days in culture (through day 56), mean prosomal width increased significantly through time among horseshoe crabs fed diets >70% protein, but not among crabs fed diets higher in algae content (Fig. 3 and Table 2). By day 120, however, all treatments showed significant increases in mean prosomal width through time except crabs fed 40% protein (Fig. 3, bottom). Second, growth rates slowed significantly during the final weeks in culture (test for homogeneity of slopes, d56 vs. d120: $F_{1,27} = 5.15$, $P = 0.03$; Fig. 3, comparison of slopes between top and bottom panels), decreasing from a mean value of 0.10 mm week^{-1} through day 56–0.06 mm week^{-1} through day 120 (Table 2). Overall, mean growth rates also were generally slower among crabs fed primarily algae diets (Table 2), but they did not significantly differ among treatments (test for homogeneity of slopes, d56: $F_{1,6} = 0.01$, $P = 0.91$; and d120: $F_{1,15} = 0.46$; $P = 0.81$).

3.2.2 Survival

The number of juvenile horseshoe crabs in all treatments decreased significantly through time during the study (Fig. 4), with rates of decline slower during initial weeks in culture (test for homogeneity of slopes, d56 vs. d120:

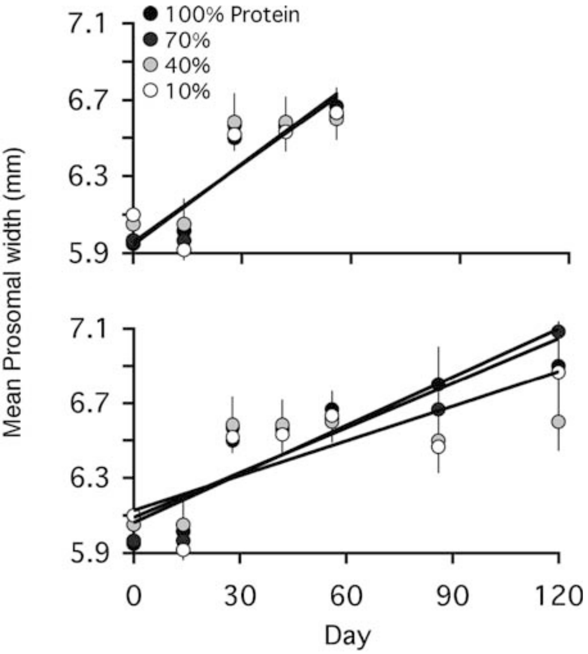

Fig. 3 Mean prosomal width (mm) of juvenile horseshoe crabs in each diet treatment during the first 56 days of the study (*top*) and throughout the 120-day experiment (*bottom*). Percent (%) protein refers to the % of diet mixture comprised of prey animals relative to algae. Error bars show standard error. Where no bars are visible, error was smaller than the symbol. (*Top*, days 0–56: 100%: $y = 0.01x + 5.94$, $R^2 = 0.89$, $F_{\text{reg }1,3} = 25.93$, $P = 0.01$; 70%: $y = 0.01x + 5.96$, $R^2 = 0.79$, $F_{\text{reg }1,3} = 11.17$, $P = 0.04$; *bottom*, days 0–120: 100%: $y = 0.01x + 6.09$, $R^2 = 0.82$, $F_{\text{reg }1,5} = 22.94$, $P < 0.01$; 70%: $y = 0.01x + 6.07$, $R^2 = 0.82$, $F_{\text{reg }1,5} = 23.03$, $P < 0.01$; 10%: $y = 0.01x + 6.13$, $R^2 = 0.65$, $F_{\text{reg }1,5} = 9.40$, $P = 0.03$)

Table 2 Growth and survival rates (±se) of juvenile horseshoe crabs in each dietary treatment, as of days 56 and 120. Percent (%) protein refers to the % of diet mixture comprised of prey animals relative to algae. Growth and survival rates were calculated from the slopes of the regression lines plotted in Figs. 3 and 4. *indicate values from regression lines in which $P_{reg} > 0.05$ and not included in comparisons (tests for homogeneity of slopes) among treatments or between days 56 and 120

	Growth rate (mm week^{-1})		Survival rate (no. week^{-1})	
	Day			
Treatment (% protein)	56	120	56	120
100	0.10±0.02	0.06±0.01	–0.45±0.11	–0.53±0.05
70	0.10±0.02	0.06±0.01	–0.43±0.08	–0.61±0.06
40	0.08±0.03*	0.03±0.01*	–0.39±0.07	–0.47±0.04
10	0.08±0.03*	0.04±0.01	–0.27±0.05	–0.55±0.07

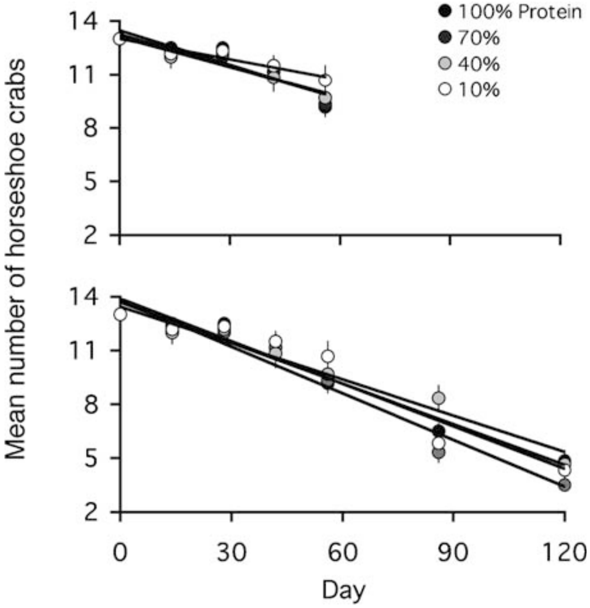

Fig. 4 The mean number of juvenile horseshoe crabs surviving in each diet treatment during the first 56 days of the study (*top*) and throughout the 120-day experiment (*bottom*). Percent (%) protein refers to the % of diet mixture comprised of prey animals relative to algae. Error bars show standard error. Where no bars are visible, error was smaller than the symbol. (*Top*, days 0–56: 100%: $y = -0.06x + 13.47$, $R^2 = 0.84$, $F_{\mathrm{reg}\ 1,3} = 15.50$, $P = 0.03$; 70%: $y = -0.06x + 13.27$, $R^2 = 0.91$, $F_{\mathrm{reg}\ 1,3} = 28.52$, $P = 0.01$; 40%: $y = -0.06x + 13.10$, $R^2 = 0.91$, $F_{\mathrm{reg}\ 1,5} = 30.15$, $P = 0.01$; 10%: $y = -0.04x + 13.00$, $R^2 = 0.91$, $F_{\mathrm{reg}\ 1,5} = 28.99$, $P = 0.01$; *bottom*, days 0–120: 100%: $y = -0.08x + 13.68$, $R^2 = 0.96$, $F_{\mathrm{reg}\ 1,5} = 126.23$, $P < 0.001$; 70%: $y = -0.09x + 13.81$, $R^2 = 0.96$, $F_{\mathrm{reg}\ 1,5} = 119.98$, $P < 0.001$; 40%: $y = -0.07x + 13.42$, $R^2 = 0.96$, $F_{\mathrm{reg}\ 1,5} = 132.54$, $P < 0.001$; 10%: $y = -0.08x + 13.88$, $R^2 = 0.92$, $F_{\mathrm{reg}\ 1,5} = 59.72$, $P < 0.001$)

$F_{1,44} = 6.94$, $P = 0.01$; Fig. 4, comparison of slopes between top and bottom panels). On average, survival rates were –0.39 ± 0.07 through day 56 and dropped to –0.54 ± 0.02 through day 120 (Table 2). Survival rates did not differ among treatments (test for homogeneity of slopes, d56: $F_{3,12} = 0.98$, $P = 0.43$; and d120: $F_{3,20} = 1.05$, $P = 0.39$). Smallest size classes (mean prosomal width 3.1–6.7 mm) showed greatest loss during initial weeks in culture, while largest sized crabs (mean prosomal width 8.9 mm) experienced 100% survival until week 6. In the initial weeks in culture, we also found a pattern of lower survival rates when growth rates were highest (Table 2, d56). Because growth did not change significantly through day 56 for the primarily algae treatments (Fig. 3; top, 10 and 40% protein), however, we could not establish a significant correlation between growth and survival rates during this time (Table 2, d56).

3.2.3 Culture Environment

Despite minor variation in water temperature and DO among trays, we found no relationships between these variables and growth or survival of juvenile horseshoe crabs during our study. Hence, we briefly discuss this variation here, but for simplicity, we excluded these data. Water temperature in each tray (Fig. 1; X, Y, and Z) decreased from an average of 23 to 17°C during the time of the study, as ambient air and water temperature declined. This decrease was significant for trays X and Y. DO concentration in each tray increased during the study, as water temperature declined, but also showed no effect on growth. Mean salinity did not differ among trays during the study. We also found no treatment effects in relationships between growth or survival and tray location or bin size and location within the culture apparatus.

4 Discussion

Stable isotope data provided insight to help identify the components of available diet, which were nutritionally important in the natural environment. These data indicated that juvenile horseshoe crabs used a diet of mixed composition, which changed with horseshoe crab size, and showed reliance on potentially high concentrations of POM. Similarity between δ^{15}N values in first instars and adult horseshoe crabs is consistent with the fact that this life stage does not have a gut and was living off nutrients provided to the egg by adult crabs. Reliance of youngest crabs (instars 2–7) on a diet supported largely by POM is consistent with their small size (roughly 3–22 mm), at which most available prey species were too large to eat (Gaines et al. 2002). The apparent greater use of suspended POM by second instars ($\sim$5 mm) suggests this life stage may be resuspended in near shore waters, while instars 3+ (>7 mm) remain settled in the benthos (Shuster 1982, Botton et al. 2003b). It also is possible that smallest instars select a component of pelagic POM such as phytoplankton, either because it is a suitable size or more readily available compared to benthic sources in some estuaries. Instars 8–11 (roughly 30–60 mm) were large enough to begin eating many of the smaller amphipod, isopod, and polychaete species available in the study area (Gaines et al. 2002, Carmichael et al. 2004). Accordingly, the N stable isotope ratios in these larger instars increased as much as 3%, indicating their shift to a higher trophic level and reflecting a mixed diet including prey animals as well as POM (Fig. 2). These data point out that different-sized crabs depend on different food sources, and POM may have a significant influence on horseshoe crabs during their earliest life stages.

Use of marine algae as a proxy for POM, however, did not significantly enhance growth and survival of juvenile horseshoe crabs in culture. The significant increase in size observed first among horseshoe crabs fed higher protein diets suggests that an animal-based diet promoted more rapid molting, at least among our test diets. It is important to consider that POM includes detritus and living material from a variety of sources including bacteria, salt marsh grasses (which stable isotope analyses also identified as potentially important), terrestrial plants, and aggregates of mixed composition, as well as marine macro- and microalgae (Lee et al. 2004). We tested only two of the many groups of marine macroalgae and microalgae, which may contribute to POM in the natural environment. Although the species we tested are commonly available in areas where horseshoe crabs live and feed, they may not represent the groups most abundant within POM or most nutritionally important. It also is conceivable that POM provides a unique combination of molecules that are not available in any single component. These molecules may include amino sugars, which are building blocks for chitin and common in marine sediments, and astaxanthin, an antioxidant in marine algae that enhances growth and survival in crustaceans (Carmichael et al. 2004, Chien and Shiau 2005). Additional research is needed to better define components of POM consumed by horseshoe crabs and test these as supplements to artificial diets for culture.

It is difficult to evaluate the overall effectiveness of our culture approach because there are few published reports on growth and survival rates of horseshoe crabs in culture with which to make comparisons. The growth increments, time between molts, and survival rates we found were generally consistent with previous lab studies (Sekiguchi 1988, K. Tsuchiya pers. comm.). Our culture growth rates were slower and survival was higher, however, compared to crabs studied in the natural environment (Carmichael et al. 2003, Botton et al. 2003, Chen et al. 2004, Carmichael pers. obs.). This latter finding is not surprising since culture conditions eliminate predation, which is a primary cause of mortality among early instars in nature (Carmichael et al. 2003; Botton et al. 2003). Additional study on survival of juveniles in the natural environment, in the absence of predation, would provide a better context to assess survival in culture. These findings also suggest that our culture conditions lacked some component necessary to support maximum growth, and this component may be available (but not yet identified) among natural POM in the benthos and near-bottom waters.

Despite these challenges, our data provide guidance for refinement of culture conditions for juvenile horseshoe crabs. For example, although not conclusive, our data relating increased growth rates to reduced survival raise concern regarding a possible link between increased molting and mortality during initial days in culture (Table 2). Molting is dangerous business in the natural environment and may be more so in culture. Handling practices may increase physiological stress, and limited space or insufficient water flow may somehow encumber molting or increase exposure to and infection by bacteria or other parasites (Smith and Berkson 2005). We observed ongoing colonization of cages and occasionally horseshoe crabs by *Pseudomonas* sp. among our culture tanks and bins. This group of bacteria has been associated with external lesions on the surface of adult horseshoe crabs (Smith and Berkson 2005). We did not find a clear relationship between *Pseudomonas* and growth or survival of juvenile horseshoe crabs, but exposure of newly molted crabs to these bacteria may have contributed to reduced survival. Furthermore, the significant decrease in survival rates in the second half of the study suggests that stressors in culture could have a greater effect through time (Figs 3 and 4, bottom).

Considered together, our stable isotope and culture data indicate that a diet comprised of a combination of foods from plant and animal sources is needed to best support growth and survival of horseshoe crabs. Successful culture or conservation of horseshoe crabs will depend on understanding the relative importance of different food sources at different life stages as well as discerning the balance between factors that increase growth, but reduce survival. Discerning the appropriate composition of artificial diet, including particle size and the appropriate mixture of particles derived from animal and vegetation sources, may be particularly important among early life stages.

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Effect of Sediment Type on Growth and Survival of Juvenile Horseshoe Crabs (*Tachypleus tridentatus*)

Shuigen Hong, Xian Zhang, Yingjun Zhao, Yongzhuang Xie, Yunwu Zhang, and Huaxi Xu

Abstract In China, horseshoe crabs have economic and biomedical value. However, the abundance of horseshoe crabs has declined markedly because of uncontrolled exploitation and habitat loss. Artificial breeding and release of hatchery reared horseshoe crabs might be a useful method for protecting and recovering the horseshoe crab population. To determine successful methods for artificial propagation, juvenile horseshoe crabs (*Tachypleus tridentatus*) were experimentally maintained in simulated sea environments using sand and mud. The experimental results show that juveniles grow and survive better in the presence of sand compared to mud, and juveniles living in sea water with sediment (either sand or mud) will grow and survive better than those living in sea water without sediment. These results provide some guidelines for artificial propagation of juvenile horseshoe crabs.

1 Introduction

Horseshoe crabs belong to the phylum Arthropoda, class Merostomata, order Xiphosurida, and family Limulidae. These creatures are sometimes called “living fossils” because they have existed over 400 million years. There are four species of the horseshoe crabs in existence in the world today. The species are *Limulus polyphemus*, *Tachypleus tridentatus*, *T. gigas*, and *Carcinoscorpius rotundicauda*. The Chinese or Japanese horseshoe crab, *T. tridentatus*, is mainly distributed along the coast of the Changjiang River, the east of the Indo-china, and the west of the Sea of Japan (Hong et al. 1995; Hong and Huang 1999; Weng and Hong 2001; Hong 2004).

Horseshoe crabs have economic and biomedical value, but the abundance of horseshoe crabs has declined rapidly in part because of uncontrolled exploitation (Hong et al. 1998, 1999; Hong 2004). In addition, horseshoe

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crabs have lost their habitat, and their survival is being directly threatened by increased development and utilization of marine resources, increased economic and tourist-related development, decreased beach habitat, and degraded sediments of sandy beaches. Moreover, the few remaining sandy beaches are heavily polluted. Meanwhile, impacts from human activity, such as sewage and trash from the industrial and household waste, caused coastal eutrophication and pollution further threatening horseshoe crab habitat and survival. If these trends continue, the native horseshoe crabs resource will be exhausted in China. Now that it is recognized that the native horseshoe crab resource could be exhausted in China, authorities are taking the horseshoe crab's living condition seriously.

Artificial breeding and release of hatchery reared horseshoe crabs might be a useful method for protecting and recovering the horseshoe crab population. To understand how juvenile horseshoe crabs use habitat and to provide a basis for artificial propagation of juveniles for release to the ocean, we conducted experiments to determine the effect of sediment type on growth and survival of juvenile horseshoe crabs.

2 Materials and Methods

Adult horseshoe crabs for artificial fertilization were purchased from a commercial market (i.e., the 8th Market) in Xiamen. There were 20 female and 40 male adult horseshoe crabs used in the experiments.

Sand and mud were taken from the beach of Huandao Lu, Xiamen. Seawater used in experiments was filtered to have a specific density = 1.02 (30‰). Artificial fertilization and breeding were carried out at the Station for Extension of Aquatic Technology of Xiamen from July 2004 to September 2004. The experiments involving juvenile rearing were carried out for 152 days from October 14, 2004 to March 15, 2005.

Sexually mature female horseshoe crabs were selected for collection of eggs. After cutting the ventral prosoma of the horseshoe crab, a large amount of eggs were taken out of the abdominal cavity, and the eggs were placed into plastic boxes containing seawater. Liver, yellow connective tissue, and bad eggs were removed. The eggs were cleaned with filtrated seawater several times until the seawater was not turbid, and then the eggs were maintained in the clean plastic boxes with seawater.

To collect spermatozoa, the prosomal appendages of male horseshoe crabs were removed with a scalpel. Body fluid mixed with spermatozoa was collected in large beakers containing seawater and stirred with a glass rod. Blood clots and tissue were removed by filtration with a multilayer cotton cloth.

The body fluid of male horseshoe crabs was poured into the plastic boxes where eggs were tiled. The eggs and body fluid containing spermatozoa were stirred lightly with a glass rod until they mixed sufficiently. After standing for a few hours, fertilization was completed.

The eggs were incubated at 28–30°C with salinity = 35 ppt, pH = 8.2, and air supplied by an oxygen booster pump. Green necrotic eggs were removed. The hatched first-instar juveniles were maintained in plastic containers at room temperature with oxygen supplied by a booster pump. The juveniles were fed rotifers every day until their telson appeared, and then they were fed brine shrimps. From the second day after fertilization, seawater was replaced with filtrated fresh seawater every morning and night.

The juveniles were divided into three groups. Each group of 500 first-instar larvae was maintained in an identical plastic box (60 cm × 50 cm × 40 cm). Each box was maintained under the same environmental conditions, such as water level = 45 cm, water temperature = 28°C, salinity = 35 ppt, and pH = 8.2. Water was refreshed one time every day and air was supplied by the oxygen booster pump. Juveniles were fed with 2 g of feed per day.

To compare the effect of sediment on juvenile behavior and development, each plastic boxes contained different sediments. One box was paved with fine sand to height of 25 cm. Another box was paved with mud to a height of 25 cm. The remaining box had no sediment to serve as a control. Behavior and developmental condition of the juveniles were observed and recorded every day. The length of the juvenile was measured from the anterior edge of the prosoma to the end of the telson. The width of the juvenile was the maximum width of the prosoma.

3 Results

The larvae maintained with sandy and muddy sediment mostly hid themselves by burying in the sediment. The larvae maintained without sediment could not burrow and mostly gathered and stayed in bottom of boxes together. Some juveniles in all groups crawled on the surface and swam in the seawater. Swimming dorsal side down was typical.

Among the larvae maintained with muddy sediment, we observed a pore above the place where the juvenile had burrowed. If we dug along the pore, we would find the hidden juvenile. Also, excretion around the pore was observed, which seemed to indicate that the juvenile had been burrowed in the mud for a long time. Also, the body color of the juveniles reared in mud was close to those reared without sediment.

Juveniles tended to grow largest in sand and smallest without sediment (Table 1). Growth in mud was intermediate. Juveniles tended to molt more quickly in sand and mud than those reared without sediment (Table 2). Also, survival rate was much lower among the juveniles reared without sediment (Table 2).

Table 1 Change in body size for juveniles reared in different sediments. The sediment types were sand and mud. No sediment was used as a control. Measurements are means, and standard errors are shown in parentheses. The numbers of juveniles measured were 300. Length was measured from the anterior edge of the prosoma to the end of the telson. The width of the juvenile was the maximum width of the prosoma

	Sand sediment			Mud sediment			No sediment (control)		
Morphology	Initial measurement (mm)	Final measurement (mm)	% change	Initial measurement (mm)	Final measurement (mm)	% change	Initial measurement (mm)	Final measurement (mm)	% change
Length	6.6 (±0.3)	12.1(±0.6)	83.3	6.6 (±0.2)	10.8 (±0.5)	63.6	6.6 (±0.3)	9.7 (±0.4)	47.0
Width	5.8 (±0.2)	7.8 (±0.3)	34.5	5.8 (±0.2)	7.5 (±0.3)	29.3	5.8 (±0.2)	7.2 (±0.2)	24.1
Telson	0	3.2 (±0.1)	.	0	3.0 (±0.1)	.	0	2.8 (±0.1)	.

Table 2 Days from start of the experiment until second-instar molt and survival rates for juveniles reared in different sediments. Sediment types were sand and mud. No sediment was used as a control. Days are shown as means with standard errors in parentheses. There were 500 juveniles within each group at the start of the experiment

Sediment type	Days until second-instar molt	Survival rate (%)
Sand	18.5 (2.3)	96
Mud	19.6 (2.1)	94
No sediment (control)	21.1 (2.5)	54

4 Discussion

Some research from home and abroad suggests that artificial breeding and releasing might be a useful method for protecting and recovering the horseshoe crab population (Hong et al. 2002). A few experts questioned whether artificial releasing is useful for the horseshoe crab (Hong et al. 2002). Some predict that a large percentage of released juveniles would be consumed by predators. Also, some predict that the released horseshoe crabs could migrate to areas other than the point of release. In either case, artificial propagation would have little effect on the recovery of a local horseshoe crab population. Moreover, it is difficult to estimate the survival rate of artificially propagated horseshoe crabs, which makes it difficult to gauge the success of programs for artificial breeding and releasing.

According to the results of our experiment, we could see that the larvae burrowed in sand and mud. Furthermore, the growth and survival of larvae were higher for those who lived in environments containing sand and mud compared to those reared without sediment. The survival rate of the juveniles was lowest when reared without sediment. These juveniles lacked suitable habitat, so they had to keep swimming during day and night. The experiment suggests the type of sediment where juveniles should be released. Artificially propagated juveniles should be released at coastal beaches with sandy or muddy sediment which fits growth of the juveniles.

The juveniles of the horseshoe crab live on intertidal beaches where the ocean wave undulates sharply up and down. Their living environment is different from the subtidal environment where adult horseshoe crabs live. At the low tide juveniles crawl on the beach with sand or mud, and at high tide they burrow into the sand or mud to protect against being washed away by the waves or consumed by predators in the sea.

An experiment by Chabot et al. (2004) indicated that juvenile the *L. polyphemus* reared in the lab had a higher activity during the night. During the daytime, they burrowed into the sand or mud, and at the same time of the night they got out of the sand, but at a certain time of the early morning they burrowed into the sand again. This behavior may be induced by light. Moreover, Chabot et al. (2004) found that the neonatal juvenile can bypass obstacles, which indicates that the juvenile has visual ability and therefore has the ability to evade the danger.

We studied the effect of different sediments on growth and survival of juvenile horseshoe crabs under lab conditions. To obtain more faithful and more persuasive results, several characteristics of sandy beaches and tidal flats need to be studied, such as influence of particle size, abundance of carbon and nitrogen, water temperature, and salinity. These experiments help determine if artificial breeding and releasing of juvenile horseshoe crab back to the ocean are important and effective measures for saving and recovering horseshoe crab populations.

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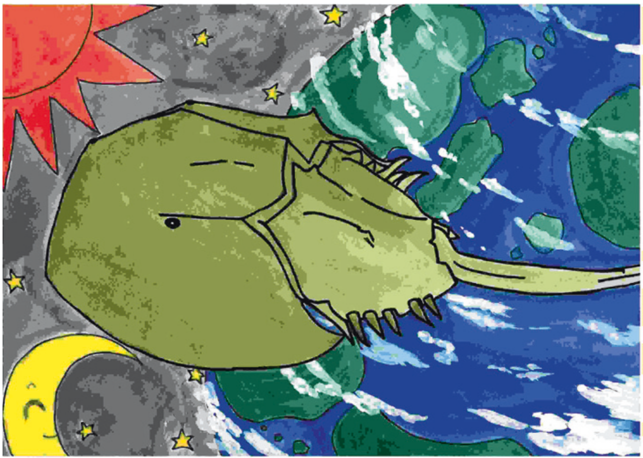
Part IIC
Public Awareness and Community-Based Conservation



Watercolor by Momoko Shimizu, 6th grade, Kasaoka-Chuo Elementary, Kasaoka, Japan

The Samurai

By Ashley Tomasello, 9th grade, Lambertville, New Jersey, USA, 2003

Once the great shores,
were guarded and protected,
by warriors fierce and strong.
Adorned, these were
by mantles of fine armor,
with helmets set upon their brow.

No more are these shores watched,
by those unflagging sentinels.
Their bodies have returned,
to the sand, from whence they came.
Their people now fall to shadow,
with no guardian remaining.

But in the moonlight,
upon the sand,
comes to shore,
the embodiment of their spirit.
Waves billow, and on them ride,
those helmets that once adorned the brow,
of warriors of old.
Their souls are in these creatures,
of those fierce and strong.
And in each drop of blood they give, their people go on.

Fig. 2c Examples of children's artwork and poetry commissioned by The Horseshoe Crab Conservation Network™, as part of a worldwide effort to promote awareness and conservation of the world's four living species of horseshoe crabs (courtesy of ERDG, Inc.)

The Conservation Network of Horseshoe Crab *Tachypleus tridentatus* in Taiwan

C.-P. Chen, H.-L. Hsieh, A. Chen, H.-Y. Yeh, P.-F. Lin and W. Wang

Abstract The horseshoe crab *Tachypleus tridentatus* once thrived in the west coast of Taiwan. We started our research on the horseshoe crabs when we discovered a few juveniles crawling on mudflats in Kinmen Island in 1996. However, after a decade's effort, the status of the horseshoe crab is still dangerously poor in Taiwan. The whole horseshoe crab conservation project could basically be described as a process of searching for missing links, not only in scientific understanding but also in many other social perspectives in order to construct the conservation network. The horseshoe crab has great value not only in economics but also in biodiversity, ecology, and the local culture of Taiwan. Thus, it adequately serves the role as a flagship species in coastal environments. An integrated approach to the conservation network has been formulated as a triadic framework, i.e., life history study, habitat requirement study, and community-based conservation action, along with six principles, including cultural concern, build-up of scientific knowledge, stakeholder organization, localization, input of younger generations, and industrialization (i.e., ecotourism).

1 Horseshoe Crabs in Taiwan History

In Taipei city there is a famous temple named Lungshan Temple. At the base of its left column at the front gate is a sculpted horseshoe crab (Fig. 1). In the Hokin dialect, the pronunciation for the word horseshoe crab is the same as that of "filial piety." Ancient Chinese have used this wise way to educate people, since filial piety is the foundation of their society. Moreover, in northern Taiwan, modern, local people still call Keelung harbor the horseshoe crab harbor, which at one time was home to many horseshoe crabs. Here is a poem describing the beautiful scene around this harbor:

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Fig. 1 This is a famous temple named Lungshan temple. At the base of its left column at the front gate is a carved horseshoe crab. In the Hokin dialect, the pronunciation for the word horseshoe crab is the same as that of "filial piety" as the foundation of our tradition

> "Crisp light illuminates rooster hill after the rain,
> Gentle sun shimmers on horseshoe-crab beach in the morning".

Many interesting facts were found concerning horseshoe crabs in history, literature, colloquial language, and daily usage in Taiwan. For example, some places are named after the horseshoe crabs, such as horseshoe crab hill or village. Its shell was commonly used as a water ladle and as a large scoop for frying pans (Fig. 2). These utensils were named after the horseshoe crab, and even though the shell itself is no longer in use, the utensils still bear the name of the horseshoe crab. In Kinmen, shells were hung at the tops of doors and walls to protect the house from evil, or they were painted with the face of a tiger or with the faces of Chinese opera characters. The ova and meat were often consumed, and were even used to fertilize crops. There are several expressions using the Chinese word for horseshoe crabs to describe the close bond between husband and wife both in ancient Chinese poems and daily language. It is indeed an intimate connection between this organism and the lives of people.

Based on this information, although solid numbers of horseshoe crabs have not been counted, we believed that the horseshoe crab *Tachypleus tridentatus* must once have thrived on the west coast of Taiwan, including Kinmen and

Fig. 2 Horseshoe crabs have been extensively used in Taiwan. Its shell was commonly used as a water ladle or as a large scoop for frying pans

Peng-Hu Islands. Now, adults are only occasionally caught by trawling nets in the Taiwan Strait and people see them mostly in poor condition with broken book gills or torn legs in aquariums.

In 1996 we started our research on the horseshoe crabs when we discovered a few juveniles crawling on mudflats on Kinmen Island. After a decade of effort, the status of the horseshoe crab is still dangerously poor in Taiwan.

2 An Integrated Approach to the Conservation

The whole horseshoe crab conservation project could basically be described as a process of searching for missing links, not only in scientific understanding but also in many other social perspectives in order to construct the conservation network. Acknowledging the poor status of horseshoe crabs in Taiwan is not enough while conducting scientific research of horseshoe crabs. A difference has to be made in the real world, meeting with people, talking to local communities, and initiating some kind of social consciousness. Conservation cannot be achieved without the recognition and participation from local communities.

In order to make this difference, a scientist must first change his own beliefs and behavior in order to make changes in other people's minds and actions. Here is the way to do it based on the concept of 4P/4C (people, paper, publish, perish; community, communicate, cooperate, collapse). Many scientific researchers have been trained to publish scientific papers. People keep telling them “you will have to publish papers or you will perish from scientific research.” In order to meet this demand, some scientists try to avoid dealing with people as much as they can. But in reality, we are people living together, forming a community and within this community, we should communicate with each other. In order to survive, all have to cooperate otherwise all will collapse. Based on this concept, a researcher can more easily reduce paper work and start to work with people in real life. Indeed, the concept in academia “Publish or Perish,” is too narrow and limited. In regard to conservation, it should be changed to “Cooperation or Collapse.” All of us have to try to cooperate or we may face the collapse of the ecosystem and human society.

In the natural ecosystem, there are three flows known as energy flow, matter flow, and information flow; however, when humans enter this virgin ecosystem, two flows are added: the money flow and the vote flow. Now the whole system is composed of these five flows. Natural science and social science should not be separated, but should be together; otherwise environmental problems cannot be solved. These days, the uneven distribution of money flow is the key issue in environmental protection. The purpose of the community empowerment is to let local people decide how to use their natural resources. By doing so, a consensus on environmental issues can be achieved. This is the vote flow.

2.1 *Flagship Species*

The horseshoe crab has great value, not only in economic terms but also in biodiversity, ecology, and the local culture of Taiwan. Thus, it adequately serves the role as a flagship species (Zacharias and Roff 2001) in coastal environments so that as long as its habitat is preserved or conserved, the entire community including the target flagship species and species dependent on that habitat are secured.

2.2 *An Operating Triadic Framework Along With Six Principles*

Conservation of the species habitat has been acknowledged to be much more effective than conservation of the species itself. The horseshoe crab needs three types of habitats: a high tidal zone consisting of coarse sand for spawning, an intertidal mudflat to be used as a nursery, and a shallow coastal zone for maturation. Therefore, integration of the marine coastal ecosystem with the socioeconomic system is demanded. Based on these habitat requirements, an operating framework for conducting conservation projects has been formulated. The framework is composed of three elements: life history, habitat, and community along with six principles: cultural concern, build-up of scientific knowledge, stakeholder organization, localization, input of younger generations, and industrialization (i.e., ecotourism). This triad is indispensable to the conservation of the horseshoe crab because success obviously depends upon a healthy habitat, a participative community, as well as the knowledge of how to manage the conservation area and how to sustain economic growth resulting from the practices. The contents of these six principles are summarized in Table 1.

- Cultural concern: When we try to change people’s minds and persuade them to act within their community we must take into consideration the local culture and customs. In Chinese culture, we respect the elderly and the scholars, so I must present myself as an old scholar. I (C-PC) am only 62 but I act as if I am an older person so I can gain an advantage when dealing with them. Next, I dress in a more ragged, casual manner, so that they do not recognize me as the typical academic type. This allows me to fit in with the people more easily; as the saying goes, “birds of a feather flock together.” is the culture concern. There are many different cultures within each country so you have to be mindful of them and adapt accordingly.
- Scientific research is of course one fundamental necessity which prepares the ground work for any meaningful effort and discourse in the business of environmental protection, natural resources management, and other relevant activities.

Table 1 Six principles of promoting conservation projects

Principles	Contents
Cultural concern	To trace back the relationship between horseshoe crabs and Taiwanese ancestors To link people’s daily life to the health of the horseshoe crabs
Localization	To get local organization, both official and unofficial, and get communities involved To initiate public education programs
Stakeholder organization	To organize resources, manpower, expertise, and financial support from different sectors To form an organization or to help existing organizations to conduct relevant activities
Scientific knowledge	To carry out relevant research and surveys and to formulate management knowledge To transfer scientific knowledge to the locals To learn tacit knowledge from the local culture and traditional wisdom
Involvement of younger generations	To attract and encourage a younger generation to participate To instill an environmental awareness in the minds of students
Economic incentives	To discover and explore the potential attraction for ecotourism To assist the locals to develop an ecotour industry To formulate management plans

- Stakeholder organization: We have to get as many of the stakeholders together as we can. These include governmental sections, legislators, educators, researchers, local residents and so on. When they fight among themselves we have to let them fight so that we can listen and find solutions to the problems. If they do not talk then we do not have a chance to find out what the real problems are.
- Localization: The whole action should take place in the local community. Community has a broad definition as a gathering of people, like today we come together and sit here in a kind of community. We also empower each other; we share knowledge and concerns, then we try to solve the problems together. Yet here we emphasize on the local space-based ones. In order to go into the community you must take the people's lives into account. You must consider the economy in the area otherwise the people in the community may not be able to survive. You must also educate them so that they may empower themselves later. There are several aspects to empowering the people in the community: awards, patience, and action. From my experience awards are easy, being patient with them has worked so far, but getting them to take action has been the hard part. The skill is not easy to imprint onto the local community.
- Input of the younger generation: We need more young people involved in this kind of conservation work. Only with the constant engagement of fresh troops, it is able to keep sustainable management of these tasks that required long-term effort.
- Economic incentives: How to get the money while doing the conservation. Ecotourism is one way but not the only way. There are many different methods. One of the difficulties in promoting ecotourism is caused by several factors: it is less profitable and more time-consuming. This highlights the need for public education and propaganda. Only when people understand their health depends on a healthy environment, will they be willing to compromise their short-term interests for their long-term welfare. It is also important to inform people that the current pattern consumption and utilization of natural resources, construction and economic development will have devastating consequences on the natural environment irrespective of the expensive cost to repair, restore and rehabilitate the natural environment.

Horseshoe crab conservation is a means and not an end in itself. Hence, the three-stage goals have been formulated, as shown in Table 2. The goal was more than the conservation of horseshoe crab population itself, but the protection of environment as a whole. The ultimate goal is biodiversity conservation, rather than protection of a single flagship species. With the ultimate goal in mind, even though the primary role in the early stages is still restricted to the realm of scientific research, particularly to the biology of horseshoe crabs, many outreach and educational programs have been conducted. These have contributed to the public understanding of the relationship between living beings and their environment as well as to establishing local cooperation.

Table 2 The goals of promoting conservation in horseshoe crab project

- The immediate goal: species conservation
  - To understand its basic biology
  - Juvenile aquaculture
  - Habitat features study
  - Set up a protected area in Kinmen
  - To formulate an initial conservation plan
- The intermediate goal: habitat conservation
  - Promoting horseshoe crab conservation in Kinmen as a flag species representing coastal zones
  - To develop an environmental monitor framework for horseshoe crab conservation in Kinmen
  - To launch trials for the reintroduction of horseshoe crabs in Taiwan with the help from the local community
  - To complete their life history survey
  - Public education: conservation propaganda
  - Ecotourism promotion
  - To list them as conserved animals under the *Wildlife Conservation Law*
- The ultimate goal: biodiversity conservation
  - To encourage a culture of naturalism and establish land ethics

3 The Processes of Developing Actions

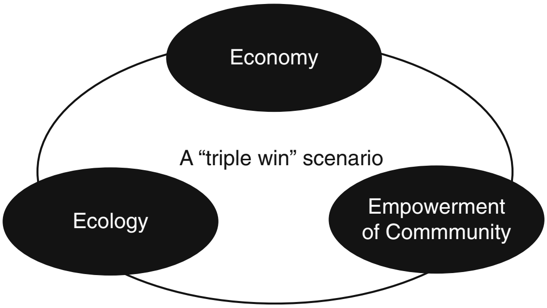
It is very challenging to translate research findings into more general community knowledge, and to influence management with regard to environmental conservation. In this sense, a breakthrough has to be made in the case of the conservation of horseshoe crabs by transforming the research project into an action plan with basically three steps: research, education, and coordination.

3.1 Formulating the Workshop Processes

People perceive nature in different ways. Some see its economic potential to develop, some see the abundant resources to exploit, some see its wonder to discover, some see its underlying dangers to fight against, and some just see the great beauty it possesses in its own right. All of these are valid. The problems and contentions come only when people from different backgrounds see different possibilities for the same area and begin to put their ideas in practice. Which ideology should take priority? The answer is neither quick nor simple. The problem has to be considered and assessed from multiple perspectives.

When it comes to conservation, scientists and researchers are basically left with a scientific–cultural–socio-economic–political complex. However, they are not environmentalists, tourism promoters, social reformers, or politicians. Apart from offering information and knowledge on a scientific basis, they cannot transform their roles into any of the other kinds. So, how can we make the conservation programs work? In the Kinmen case, the idea of an “environmental

Fig. 3 To reach sustainability Kinmen relies on the 3E dimensions. With the 3E the environmental monitoring framework creates a triple win stage to work upon



monitoring framework" was proposed aiming to incorporate the "3 E" dimensions, which are ecology, economic growth, and empowerment of the community, into one system. On the basis of these three aspects, it is more feasible and meaningful to talk about sustainable development (Fig. 3). In order to make the practices follow the above six principles, the notion of a "workshop" protocol was adopted into this working model (Fig. 4). The characteristics of this model are that you start from yourself, and reach out to those close to you, just like a ripple extending farther and farther.

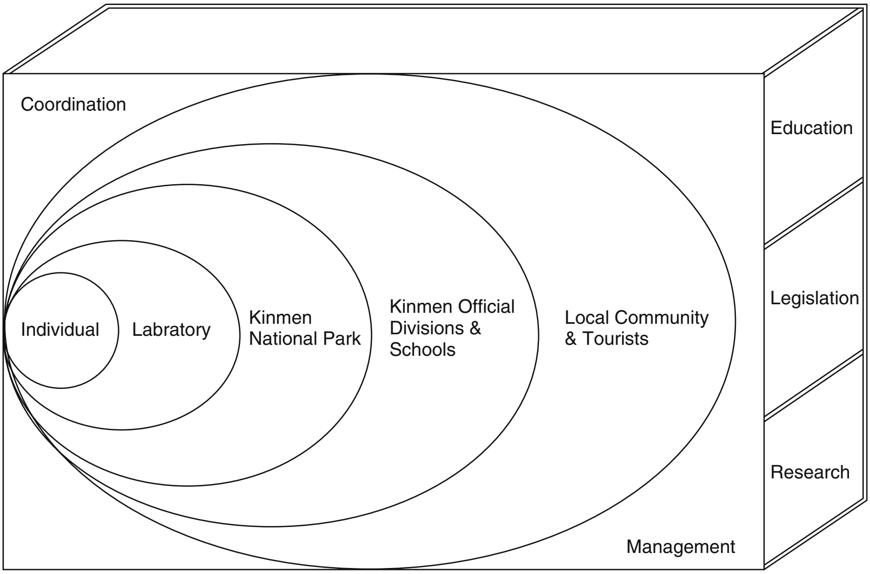


Fig. 4 The workshop working model. In the case of horseshoe crab conservation, the workshop contains people from research units, official units, schools, the local community, and tourists. Through various conferences, workshops, and meetings, ideas, opinions, and information can be collected and integrated. All of these efforts have to be built on the basis of education, legislation, and research

It is commonly recognized that societies cannot avoid certain levels of construction if they want to progress. There will always be construction, and some degree of impact is inevitable. The question is what level of impact the environment can tolerate and what restrictions we are willing to accept. In order to answer these, both scientific research and societal participation are required. That is why the workshop working model is so important here. The workshop model provides "interfaces" or platforms for different sectors, organizations, and special interests groups, to identify questions, to decide priorities, and to work out solutions under the same purpose of a "sustainable Kinmen."

By means of the workshop platform, each organization could provide an essential contribution to the services, expertise and energy needed to advance the overall effort of conservation as well as disseminating and ingraining an awareness of information on and practice of conservation in the public consciousness.

The workshop model of horseshoe crab conservation includes people from research units, official units, conservation units, schools, local communities, and even tourists. Through various kinds of workshops, knowledge, experiences, ideas, opinions, and information can be collected, shared, and integrated. The workshop has reciprocal benefits. While it facilitates the process of cross-sector communication, community empowerment, and the interaction with various sectors, it contributes to formulating and improving the framework of the conservation program itself.

3.2 Conservation as an Action Plan

Usually, as much as a decade may pass before research findings become part of practical conservation programs and common knowledge. Therefore, solving the problem of how to "synchronize" scientific research in academia with management practices and general public knowledge is an important task for scientists, particularly those in conservation science. Success requires recognition and support from various sectors in society. Working as a team in this manner, scientific findings, in one aspect, could be adopted, delivered, and shared within and among different sectors, and in another aspect, could be integrated into the existing management system as well as become common knowledge.

4 Creation of Key Rings in the Conservation Network

Basically, the whole horseshoe crab conservation project could be described as a process of searching for missing links, not only in scientific understanding but also in many other social perspectives. So far several rings have been formed.

4.1 Protected Areas

In 1995, Kinmen County wanted to build a harbor at Shuitou Bay, which is the best place for horseshoe crabs due to the fact that the three required habitats occur nearby. The project for protecting the horseshoe crabs gained public attention due to the proposed changes to this bay. This bay is the only place suitable for building the harbor; however, juveniles occur along the west coast of Kinmen, so a tradeoff was made to create a protected area. With our scientific and local government's support, 800 ha of coastal zone were designated as "The Horseshoe Crab Protected Area" at Gunnintou in Kinmen in December 1999 (Chen et al. 2004). Many conservation efforts took off then, although the function of this protected area needs further improving. Several research projects are now ongoing.

4.2 Population Genetic Analyses

Another line of horseshoe crab study is based on population genetic analyses. With the tools of molecular biology, it is possible to identify a given population's status and connectivity among local populations. The genetic features of local populations also provide information which can be used for implementing different conservation strategies. For example, population subdivisions of the horseshoe crab in the Taiwan Strait were studied (Yang et al. 2007, 2009). The initial results of the genetic study highlight the importance of population genetic analyses toward conservation efforts in Taiwan as well as Southeast Asia and Japan. The higher genetic diversity and larger census population size found in Kinmen provide a reservoir of horseshoe crab genetic variation. Translocation of horseshoe crabs from nearby populations might be initiated to increase the genetic variability and to help prevent extinction of the local population.

4.3 Micro-Habitat Study of Nursing Grounds

What is the role of mud flats in the conservation of horseshoe crabs? The occurrence of juveniles in this habitat is a good indicator of the health of the mud flat, and its population size is also a good indicator of the status of the horseshoe crabs (for detailed results, see Hsieh and Chen 2009). Based on this study we look for potential nursing grounds for the juvenile horseshoes crabs in Taiwan.

4.4 Inducing Spawning of Horseshoe Crabs at Beaches in Taiwan

Horseshoe crab adults are easy to pair and spawn in a large aquarium. However, rearing larvae to the size suitable for releasing into the field is another story. So we tried to develop induced spawning on the beach. In 2001, spawning was successfully induced at a beach in Kinmen (Chen et al., 2004). Eggs were deposited, but whether or not they developed into juveniles is unknown. Since then, we began studies to restore the population in Taiwan. We went through the literature to find the best place to do the restoration. We found that the coastal area of Tung-Hsiao once had a large population of horseshoe crabs. Assisted by the staff of local "Tung-Hsiao Beach Resort (West-Ocean Educational Sea World)," we started the restoration in the resort in 2002. The spawning experimental area enclosed both original substrate mud and restored coarse zone with fences. In accord with the tidal amplitude at the site, adult crabs were released for spontaneous mating and spawning. The experiment was very successful. Several pairs of horseshoe crab spawned in the experimental area. We found at least 10 nests. Every nest houses hundreds of eggs. About 50 days later, we found several newly hatched first instars. The details of this study are described in this book (Hsieh and Chen 2009).

We spent time cooperating with a local company and they created an exhibit so that students have the chance to see what horseshoe crab looks like and learn something about them. The owner has good connections with the TV stations, so there was lot of publicity and we were able to make the community aware of the horseshoe crab's situation. The resort invited the national media to report the story. Even Taiwan's vice president came to the resort. Horseshoe crabs became the subject of a special series of TV programs and the general public got to know about these living fossils.

In the end we gave up Tung-Hsiao for three reasons: it did not have a strong community base, there was a shortage of man power, and the natural tide energy is too low so the hatched trilobite larvae cannot be washed out to the mud flats by the natural tide. Our efforts are now concentrated on Chiayi (see Section 4.7).

4.5 Public Awareness

Many kinds of public education programs have been carried out. Symposia are academic meetings, but for the local community, the topic and the language must be appropriate to the audience. The workshop is really useful. Humans have hands as well as eyes and the hands need to be satisfied. During and after the workshop, participants have many learning opportunities to work by their hands. This not only makes them happy but also solves many problems.

Publication, the science book, and the television coverage are all parts of the public education programs. We published a Chinese book with cartoons so that it is easier for the young people to read. There has also been considerable media interest in horseshoe crab conservation. From October 1999 onwards, more than six TV stations have voluntarily contacted the team and with their help produced programs introducing horseshoe crabs and highlighting the importance of its conservation to the public.

In 2007, two elementary school teachers from Taichong located near the middle of Taiwan compiled information on horseshoe crabs onto a CD which is now used as a teaching tool. This CD has already been passed on to other elementary schools helping to spread the message. The most intriguing part of this story is that neither of the two teachers attended any of our workshops but one of their mothers had participated frequently in Chiayi. Under her mother's suggestion and encouragement, the young teacher made this CD. This seed was planted and germinated in a place without our direct input or knowledge.

4.6 Tourism Industry in Kinmen

Ecotourism seems to bring a promising future to Kinmen, where there is a potential to develop this industry because of several unique features in its history, geography, culture, and natural environment. Kinmen hosts many endemic species, such as horseshoe crabs, amphioxus, and otters, and is also a stop for migratory birds. In addition, Kinmen had been famous for its Min-Nan style historical architectures, military sites, and agricultural products such as peanut tribute candy, Kao Liang liquor and even common products like the kitchen knife.

The problem of the impacts of tourism on the natural environment is sizable but not really new. In anticipation of the problems brought by tourism, the process of promoting and developing ecotourism management systems has been conducted in Kinmen since 2000. At the end of 2001, the team had already published a report, "A Strategic Plan for Ecotourism Management in Kinmen," urging the Kinmen government to develop a robust, science-based ecotourism management program. The report illustrated Kinmen's natural scenery, historical objects, and wildlife, and offered the framework to conduct and promote ecotourism in Kinmen.

The Kinmen government has already designed many souvenirs based on the shape of horseshoe crab, which means they already consider it one of the icons of Kinmen. Other measures are still awaiting implementation.

Furthermore, with the purpose of enhancing the ecotourism business, we published the English and the Japanese book in Taiwan, yet it did not work as well as hoped. In 2006, one airline company, UNI Air, provided a special tour offer with the emphasis of guiding tourists to see the horseshoe crab, a living fossil. This means their promotion efforts at least made some impact.

4.7 Community-Based Conservation Actions in Chiayi

In 2005, one local ecotourism operator discovered a small, natural juvenile population in the wetlands of Chiayi County. The owner made a small fence around the juvenile horseshoe crabs for tourists to see them more easily. Although that specific population has occurred only once so far, that is enough to give us hope that natural populations can still be recovered. With our help, the NGO, Chiayi Ecological Environment Conservation Association, had formed the Horseshoe Crab Restoration Group of Chiayi, and a workshop for training the basic techniques of juvenile horseshoe crab monitoring and surveying was held in 2006. This group is under the Ecological Conservation Association of Chiayi. Apparently, the efforts of the team have been highly appreciated there and have contributed to the process of empowerment of the local community. "This restoration program makes me feel I've gained so much in the process," Yin Tian Su, the chair of the Association, said while interviewed on PTV. He went on to say, "There are three aspects I'd like to mention. Firstly, I am so moved and excited that I could join a restoration project of a living fossil. Secondly, I've gained an adequate knowledge of horseshoe crab ecology and learned restoration techniques. Thirdly, this program has attracted many local people to take part in the environmental protection. The ecological environment has become one of the main concerns of our community."

During 2006–2007, the team members bought about 10 adult crabs, which is one-third of landed on the local fish market. This summer (2007), we will help the members to induce adult horseshoe crabs to spawn along Budai coastal zone. We have built a spawning ground close to that area and we had a workshop gathering, measuring the slope using the camera as a horizontal measurement. The principal of an elementary school gathered students to clean out the sand beach. They have really done wonderful field work. This area in Chiayi County became the horseshoe crab restoration area. The government made a big advertising sign with the message "鱟起之秀," (Fig. 5), which can have one of several meanings such as an up-and-coming youngster, a budding young talent, a promising young person, or a boy becoming a man. You just need to have the chance to see the small ones become the big ones. Here we see the Kinmen experience now has been duplicated in Chiayi in a much more efficient and effective way.

4.8 Cooperation with Scientists and Administrators Locally and Internationally

In 2000, 2001, and 2005, Japanese scholars and horseshoe crab conservationists led by Dr. Tomio Itow and Keiji Tsuchiya (Chairman, Japanese Horseshoe Crab Preservation Society) came to Kinmen to observe the status of horseshoe crab juveniles and share their experiences in conservation and aquaculture.

Due to the increasing construction in the coastal area in Xiamen, China, sand procurement has increased. Dredge boats that collect sand in the strait between Kinmen and Xiamen have become a common sight. Their activities could be contributing to the erosion of beach which in turn damages the

Fig. 5 A billboard put up in Chiayi County meant to increase awareness in the community of the dire situation the horseshoe crab faces if it is not protected and its habitat not preserved. The sign reads, "鱟起之秀," which can be translated to be an up-and-coming youngster, a budding young talent, a promising young person, or a boy becoming a man, which encourages the reader to see the potential in the young horseshoe crabs

intertidal area of Gunnintou inhabited by the horseshoe crab juveniles. The other problem is that although the Kinmen Coast Guard can expel the boats from mainland China, the area of their jurisdiction is only within 2 miles of the shore. Outside this area they have no authority. Also, the establishment of invasive species from Xiamen is also a concern. Recently, smooth cordgrass (*Spartina alterniflora*) invaded into Kinmen from Xiamen. All of these issues highlight the urgent needs to cooperate with Xiamen. In 2006, an informal forum on horseshoe crab conservation was held in Xiamen, signaling the start of communication and cooperation across the strait; moreover, attending the ISSCHC 2007 is a great asset to the members of the conservation network in Taiwan.

5 Further Works

- After this meeting, our take-home actions are these: petition the government to list *T. tridentatus* as an endangered species. In the difficult task of conserving horseshoe crabs in Taiwan we need more efforts such as legislative protection on the species using the *Wildlife Conservation Law*.

- Cooperation with scientists and administrators across the Taiwan Strait, tri-region (Taiwan, China, and Hong Kong), and the Indo-Pacific region.
- Create the Blue-Palm Award (this name is takeoff of the green-thumb for people who are skilled at gardening) for the people who rear the horseshoe crabs from juveniles to sub-adults. This has three purposes: to learn about the aquatic ecosystem, and through this action gain passion and patience, and to gather important scientific data.
- Utilize the talents of young artists as horseshoe crabs are highly stimulating creatures for our imagination, especially for children who are so pure and naïve that their creations such as poems, stories, and drawings are heartfelt. We will encourage Taiwan students to attend the contest offered by the Ecological Research & Development Group (ERDG) or we may even create a Taiwan contest of our own. The work can be viewed online under the "Poems, Tales & Images" category of ERDG's website at (www.horseshoecrab.org).

By creating a network of volunteers, the program has empowered people at the grass-roots level. These active individuals and/or groups are a great benefit to the conservation program because they help coordinate outreach programs, education, and training in their respective areas. However, it is hard to sustain this network with merely a volunteer force. Legislation and political power relating to the practices of conservation and public education related programs are required.

6 Conclusions

The conservation network of horseshoe crabs in Taiwan is workable but not easy to maintain. Before the ISSCHC 2007, we said that "Do nothing, and you gain absolutely nothing; Do it, and you have no guarantee of gaining it. Do it often and you may gain some of it, so you must do it." Now, we say "We are on the right track, so just do it, and don't worry too much."

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The History of Horseshoe Crab Research and Conservation in Japan

Keiji Tsuchiya

Abstract Scientific studies of the Japanese horseshoe crab, *Tachypleus tridentatus*, from the Seto Inland Sea have been published for nearly a century, beginning with the pioneering work of Owhatari (1913). Studies by Matsunari, Asano, Oka, Nishii, Sekiguchi, and many others established much of the basic reproductive biology of the species in the vicinity of Kasaoka City. Oe-hama beach was designated as a "Horseshoe Crab Spawning Ground Natural Monument" in 1928. In spite of this formal recognition, and in the face of opposition by various local conservation organizations, the Kasaoka Bay Land Reclamation Project began in 1969. Horseshoe crab abundance since then has declined, which has stimulated efforts to raise horseshoe crabs in captivity. The success of such projects, though small in scale, affords some hope that horseshoe crab populations might experience recovery.

1 Introduction

Until very recently, the majority of the articles about the Japanese horseshoe crab (*Tachypleus tridentatus*) have been published in Japanese, and consequently, the rich history of horseshoe crab research in Japan is little known to the rest of the world. In particular, Kasaoka City, on the Honshu coast of the Seto Inland Sea, has been a focal point for horseshoe crab science, public awareness, and formal conservation activities for nearly 100 years. More recently, it has been the location of several efforts which may someday re-establish horseshoe crab populations using captive breeding methods. The 2007 International Symposium on the Science and Conservation of Horseshoe Crabs, upon which this book is based, allowed me the opportunity to discuss some of these past and present research and educational activities with a broad audience.

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2 Horseshoe Crabs in Kasaoka and Vicinity up to 1961

2.1 Report of Professor Chutarou Owhatari

A report on “Habits of the Horseshoe Crab” was one of the articles in the July 1913 edition of the *Japanese Journal of Zoology* (no. 298); the author was Chutarou Ohwatari, at the time a teacher at the Dai-roku High School. The article is a summary of the results of on-site surveys and collection of horseshoe crab specimens carried out by Ohwatari during the summer of 1912 at several sites in Okayama prefecture. These locations included Katakami, Hinase, Hachi-hama, Banda, Kogushi, and Sanban along the Honshu coast of the Seto Inland Sea as well as in Marugame and Takamatsu in Kagawa prefecture on the Shikoku coast of the Seto Inland Sea. Thus, this report is extremely valuable in understanding the natural history of horseshoe crabs in the Seto Inland Sea.

At the time, many people believed that horseshoe crabs made good agricultural compost, and if a few horseshoe crabs were incidentally caught in fishing nets, they were brought home and added to the compost heap. Questioning local people in Banda in Kojima county and around Kogushi yielded the information that, until a few years previously, boats out of In-no-shima and Takashima would come every summer to specifically catch horseshoe crabs. Several hundreds to several thousands would be caught, and set out to dry. Local residents found it hard to bear the terrific stench from the rotting horseshoe crabs, and so they called for a halt to this practice.

Even assuming a demand for horseshoe crabs as compost, it was not easy to catch a large number of them at one time, and so there was really no fishery specifically aimed at catching them. On both the Honshu and Shikoku coasts, they were caught incidentally by hand trawl (Danish seine), sailing drag seine, and drift net fisheries aimed at catching small shrimp, ordinary crabs or finfish, but generally only one or two horseshoe crabs were caught per night per fisherman. Most crabs were caught between May and July, but by early September, they had moved into deeper water, where they were more difficult to catch.

On the night of August 30, 1912, Ohwatari contracted with 12 hand-trawl fishing boats working out of Sanban Bay in Jodo county to bring him all the horseshoe crabs incidentally caught in their nets; a total of 33 horseshoe crabs were collected with prosoma widths ranging from 9.2 to 28.7 cm. This averages out to a little less than three horseshoe crabs per boat per night. In waters off of Atsu and Kogushi in Kojima Bay, not a single horseshoe crab was caught by a boat fishing the bottom with a drag net from 10 pm on August 29 to 5 am the following morning. However, 10 juveniles with anterior shells measuring from 4.6 to 8.6 cm wide were found in the mud at low tide on the tideflat at the eastern extremity of Kogushi beach.

This information allows us to confirm that horseshoe crabs inhabited a wide area, not only on the Okayama (Honshu) side but also around Shodoshima Island, Takamatsu, and Marugame on the Shikoku side.

2.2 The"Horseshoe Crab Field Guide" (1929), by Tsurukichi Matsunari

Tsurukichi Matsunari (1851–1940) carried out long-term field studies of horseshoe crabs at Oe-hama beach in Kasaoka starting in 1917, and published the "Horseshoe Crab Field Guide" in 1929. This book includes an account of how the horseshoe crab spawning areas of Kasaoka came to be designated as a national "Natural Monument."

Matsunari's interest in the horseshoe crab followed a visit in March 1917 by Kotaro Amamori of the national Ministry of Agriculture, Forestry and Fisheries' Seafood Production Experimental Station, who had come to the Oe-hama Beach Fishermen's Cooperative to conduct surveys related to half-crenate ark shell (*Scapharca subcrenata*) aquaculture experiments. He happened to see a 1-year-old horseshoe crab juvenile and mentioned that this was an extremely mysterious creature seldom found anywhere in the world.

This motivated Ohwatari to visit the site repeatedly in order to carry out research. Professors Shouzaburo Watase and Seitaro Goto, as well as scientists from the United States and Russia, also visited the site. Starting in late June 1926, Osamu Hattori of Tokyo Teikoku University conducted surveys here for about 4 weeks. Matsunari provided information and served as a guide to all these visiting scientists, and this aroused his interest in the horseshoe crab.

Following surveys in 1927 by Yaho Atari (Ministry of Interior, Department of Geography) and Sei-ichi Inoue (Ministry of Okayama prefectural office), and a visit by the prefectural school inspector stationed at Tsuda, Okayama, the "Horseshoe Crab Spawning Ground Natural Monument" was created in March 1928, under the "Law for the Protection of Historic, Scenic and Natural Monuments" (1919). In Japan, both sites and species can be designated as natural monuments, and in this case a site, not the species, was designated.

Matsunari also detailed the methods and results of his independent observations of horseshoe crab habitat, spawning and feeding behavior, etc. He estimated that approximately 1500 horseshoe crabs were caught in hand-trawl nets between April and November every year and dried for use in compost.

2.3 Studies by Daigoro Moriwake, Eitsu Oka, and Uichiro Asano (1936–1945)

In the November 1936 issue of *Kagaku Toppiku* ("Science Topics") magazine, there appeared an article entitled "Horseshoe Crabs," by Yoshitaka Imai, Taito Kodama, and Daigoro Moriwake. This article noted how Moriwake, at the time a freshman at Tokyo University, stopped at Kasaoka on his way home to Iwakuni (Yamaguchi prefecture) to call on Osamu Hattori of Tokyo University's Department of Science, who was doing research at Kanaura Bay. Moriwake gives an account of horseshoe crab spawning events in sandy areas along rock walls bordering Yo-nasu (literally “west beach”) and Oe-hama in Kanaura Bay, although apparently these are not eyewitness accounts. He also described horseshoe crabs stuck into cracks in rock walls around fishermen’s homes to dry, looking like “upside-down frying pans.”

Also in 1936, Ikio Sato published a report on “A Naturally Deformed Horseshoe Crab with Two Tails.” This document describes a mature male horseshoe crab caught in late July off of Yorishima-cho in Asaguchi county, Okayama prefecture that had a completely formed “second tail” growing out of the right side of the shaft of the telson.

Around that time, Eitsu Oka of Tokyo University’s Department of Science spent every summer in Kasaoka to conduct experimental embryology studies using horseshoe crab eggs. Despite its dry title, his report of his experiences, “Collecting Horseshoe Crab Eggs” (1940), provides a lively, readable, and comprehensive account of the horseshoe crab situation at Oe-hama beach and Kasaoka at the time. It described overall horseshoe crab distribution and spawning behavior, reviewed the existing literature, provided a guide to spawning sites in the Kasaoka area, and even listed horseshoe crab-related souvenir items available at the time. Particularly relevant was his comment that, “...Okayama prefecture in particular is a major spawning area. Within Okayama, Kanaura Bay is especially famous as horseshoe crab breeding habitat. Thus, when collecting horseshoe crab eggs, one invariably heads for Kanaoka Bay or its environs.”

Uichiro Asano, a teacher at Kasaoka Girl’s High School, also carried out long-term field studies of horseshoe crab spawning and embryology, and his “Natural History of the Horseshoe Crab” (1942) can be regarded as a summary of nearly a decade of effort. In particular, its section on the growth process remains an extremely valuable work even today.

2.4 Studies by Hiroyuki Nishii (1945–1961)

The next person to become deeply involved with the horseshoe crabs of Kasaoka was Hiroyuki Nishii, M.D. After graduating from Okayama University School of Medicine, he took a post at what was at the time the Okayama Prefecture/Kanaura Township/Oda County Hospital in June 1934. On his daily trips by Jinrikisha to and from the hospital, Nishii passed the stone monument at Oe-hama beach marking the “Horseshoe Crab Spawning Grounds Natural Monument,” which aroused his interest in horseshoe crabs. Most local people, however, were not only uninterested in horseshoe crabs but in fact also treated them as a nuisance. The sight of people drying horseshoe crabs by sticking them tail-first into cracks in stone walls and other cruel treatment astounded Nishii and aroused his righteous indignation. He became acquainted with Matsunari (see Section 2.2), who became his guide, and in time Nishii started his own studies of horseshoe crab ecology and embryology during the scarce moments when he was not busy seeing patients.

After the long hiatus of the war, Nishii returned to his studies in Kasaoka, and by virtue of his strong leadership and outspoken commitment, a major movement to protect horseshoe crabs grew up in the Kasaoka area. When the Ministry of Agriculture, Forestry and Fisheries proposed the Kasaoka Bay Land Reclamation Project, Nishii worked to obtain funds earmarked for horseshoe crab protection, and his success led to the construction of a globally unparalleled horseshoe crab protection center. Nishii also organized the existing literature, both Japanese and foreign, which he published together with his own research results as the “Horseshoe Crab Encyclopedia” (first edition 1973, expanded edition 1975). This work remains an essential source for understanding the literature, in particular on the history of horseshoe crab protection.

Nishii also put considerable effort into training the next generation, including the author. Soon after starting my job as a science teacher at East Kasaoka Municipal Middle School in 1961, I was introduced to Dr. Nishii and, inspired by his enthusiasm, I began my continuing involvement in the horseshoe crab protection movement, the history of which is dealt with in the next section.

3 Surveys by the East Kasaoka Middle School Horseshoe Crab Research Club (1961)

The East Kasaoka Middle School Horseshoe Crab Research Club was established in 1961, and, guided by Dr. Nishii, immediately embarked on surveys of horseshoe crab spawning.

In the 2 years following the Club’s establishment, its work included studies of horseshoe crab spawning and embryology. Dissection of adults revealed that females have about 13,000 eggs in the subfrontal area of the prosoma. It was found that spawning occurred from mid-June to the end of August, generally in the middle of the night during spring tides, from about midnight until 3 am. On average, each pair of horseshoe crabs spawned at five to six sites in succession along an arc-like path and each nest contained about 500 eggs. After about a month, embryos started to rotate, and hatching took place on about the 50th day. It appeared that juvenile (third molt) hatchlings hibernate in the sand from about October to about March of the following year.

In the Club’s third year (1963), spawning surveys confirmed an increased number of nests all along the eastern shore, and also that horseshoe crabs spawned at sites located progressively eastwards, starting at Oe-sawa and moving to Irie, Koh-no-shima inlet, Ohshima beach, and so on.

In 1964, the club organized the results of its 4 years of research to enter in the annual Japan Student Science Awards, sponsored by the Yomiuri Shimbun newspaper, and won first prize for Okayama prefecture. The work of the Horseshoe Crab Study Club continued, and I devoted myself to protection activities as well as field studies with my students, literally, in the mud. The result of these efforts was the publication of my book “The Horseshoe Crab of

Seto." Excerpts of this book appeared nationwide in Japanese textbooks for fourth graders under the titles "The Horseshoe Crab" and "Protecting the Horseshoe Crab" for 22 years.

It should also be noted that in 1964, an abnormal proliferation of the naticid gastropod *Neverita didyma*, a bivalve predator, occurred along a stretch of coast where the concentration of juvenile horseshoe crabs was the greatest, centering on the tideflats at Natsume and Torinoe beach on western Ohshima Island and extending about 6 km from Yokoshima to Nagahama. No direct link between this snail and juvenile horseshoe crabs was identified, but it seems unlikely that horseshoe crabs would not be affected by this event's impact on the local food chain.

4 Surveys for the Kasaoka Bay Land Reclamation (1966)

Major construction work on the Kasaoka Bay Land Reclamation Project was to have commenced in 1966, but because this project involved landfilling the Horseshoe Crab Spawning Ground Natural Monument site at Oe-hama beach, surveys were required to identify an alternative site. Professor Shiro Kawaguchi of Okayama University carried out surveys of adult spawning density and the characteristics of adult and juvenile habitat from 1966 to 1971.

Most of these field surveys were performed by the main research team members, assisted by five field researchers and many graduate and undergraduate students. Because they were conducted just before the land reclamation project began, their results constitute an extremely valuable reference for understanding the status of horseshoe crab habitation in Kasaoka Bay before it was reclaimed. The reports based on the 1966 surveys in particular describe a landscape that is unimaginable now. Thus, at the risk of waxing long-winded, I would like to quote from these reports at some length here.

4.1 The Oe-Hama Beach Area in Kasaoka City

Habitation status: Rather extensive areas of tidal flats extended along both the east and west shores of Kanaura Bay including the area at the mouth of the bay, most of this was fine, silty mud so deep that one sank in to the thighs even when wearing rubber-soled boots. Many juvenile horseshoe crabs with prosoma widths from 2–3 to 7–8 cm were found at low tide on these areas of fine, silty mud. Because it was so difficult to walk here, it was nearly impossible to measure numbers of individuals distributed over a wide area, but concentrations of several individuals per square meter were frequently observed. Thus, we can surmise that quite a considerable number of horseshoe crabs inhabited this area. However, large individuals with shells wider than 10 cm were very seldom seen on the areas of tidal flat exposed at low water, but rather were seen in the shallowest parts of boat channels running through the area. The mud flat area where the greatest numbers of horseshoe crab juveniles were seen was located on the east side of the mouth of Kaneura Bay. The juveniles, including some very small individuals less than 1 cm wide, were normally found completely submerged in the fine, silty mud. Horseshoe crab juveniles were also found on tidal flats on the Oe-hama side and at other locations around the mouth of the bay, but not quite as many as on the east side of the bay entrance.

Spawning sites: Going eastwards from the mouth of Kanaura Bay there was a sandy beach 20–30 m long near Kanazaki, but otherwise the bottom is muddy right up to the seawall. Every time we visited this beach to determine the presence of nesting, we invariably failed to find any nests. However, on the west side, that is, on the Oe-hama beach side, some sandy areas remained in the Kanaura-hashizume area, and the more landward beaches provided particularly suitable spawning sites. Here we were able to observe adult pairs engaged in spawning, and we also found many indentations in the sand where spawning had taken place. Numerous horseshoe crab juveniles were observed on the mud flats in the Oe-hama beach area, and the larger adults were also observed in boat channels there. Of particular significance was our observation of adults spawning on the sandy areas of the beach, because it had been said that no more horseshoe crabs were to be found on Oe-hama beach due to various construction projects that had been going on, including a seawall, a bridge, and a road. It was thought that the Oe-hama beach area had lost its practical value as a Horseshoe Crab Spawning Ground Natural Monument, and for this reason as well as in reference to the land reclamation project, it was said that an alternative site needed to be designated. Another spawning site was thought to have existed toward the south side of Oe-hama beach, but this survey was unable to confirm any spawning at this site.

4.2 Katashima and Vicinity

An extensive horseshoe crab habitat site was found in Katashima and vicinity; in particular, it was confirmed that great numbers of horseshoe crabs inhabited a large muddy/sandy area on the north side of the site. Spawning was confirmed over an extensive area of sandy beach even during the 1968 surveys.

4.3 The Yokoshima Coast

Yokoshima had an extensive area of tidal flat, and large numbers of juveniles were found. There were also large, sandy spawning sites, and the area was well known as a place where numerous pairs came to spawn, but in fact during this survey spawning was confirmed at only two spots.

4.4 The Irie Vicinity and the Natsume Coast

There was a deep inlet in the vicinity of Irie, with an extensive area of tideflats inhabited by a large number of horseshoe crab juveniles. There were sandy areas between the small boat harbor and Ohdonsu, and numerous instances of spawning were confirmed, especially in sandy pools. Although there was no sand along the seawall at Shinden in west Ohshima, between the Ohshima River and the Natsume coast there were sandy areas along the road embankment, and many instances of spawning were confirmed here. The presence of horseshoe crabs was also confirmed along the shoreline continuing southwards from the Natsume coast; also, although the beach was rather narrow, the presence of many horseshoe crab juveniles and spawning was confirmed along Ohdonsu beach near the Youth House.

4.5 The Koh-No-Shima/Uchiura Vicinity and the Furue Coast

There were extensive tidal flats in the vicinity of Koh-no-shima/Uchiura, at Katashima and in the Tenjin area. Many horseshoe crab juveniles were also found on these tidal flats, and many rather large horseshoe crabs were also seen in the small boat channels and along the edge of the tidal flats. The seawall built for the land reclamation project extends to a considerable distance into the muddy area at this site, and almost no sandy areas were seen along the coast here. Even so, many nests were found in the small areas of sand still remaining and in spaces among the gravel and rocks. Along the Furue coast south of here there are also sandy beaches and extensive tidal flats, where the presence of horseshoe crab juveniles was also confirmed.

5 The Kasaoka Bay Land Reclamation Project (1969) and Formation of the “Kasaoka Association for the Protection of Horseshoe Crabs” (1970)

These surveys by Kawaguchi and his group gave additional credence to the research carried out by Nishii, Tsuchiya, and the East Kasaoka Middle School Horseshoe Crab Research Club. However, in September 1969, while Kawaguchi’s studies were still under way, an opening ceremony was held to commemorate the start of construction on the Kasaoka Bay Land Reclamation project, slated for completion in 1975. Taking his warning from this, Nishii submitted a statement to the Mayor of Kasaoka that clearly and concretely explained the need to take measures to conserve the horseshoe crabs and secure funds specifically for this purpose. While calling on the government for a swift resolution to this issue, by putting pressure on the Kasaoka Rotary Club, Lion’s Club, Jaycees, Women’s Association, Parent/Teacher Organization (PTA), Youth Organization, and other groups, Dr. Nishii gained the agreement of about 13,000 Kasaoka residents in favor of horseshoe crab conservation. With their support, the “Kasaoka City Association for the Protection of Horseshoe Crabs” was formed, and a “Horseshoe Crab Emergency Declaration” was published on December 1, 1970, calling on the relevant authorities to expedite protection measures. Dr. Nishii also submitted a statement to the Ministry of Agriculture suggesting that an opinion on horseshoe crab protection measures by Dr. Koichi Sekiguchi of Tokyo University of Education should be sought, and based on this suggestion, in May of the following year (1971) Sekiguchi provided the detailed and practical “Opinion Statement on Measures to Protect the Horseshoe Crab.” Based on this statement, in May 1972, the ministry elected to provide a subsidy of 24.8 million yen to Kasaoka City earmarked for horseshoe crab protection measures.

In the meantime, the Mayor of Kasaoka had submitted an application for a supplementary “Horseshoe Crab Breeding Site Natural Monument” including the entire Koh-no-shima channel; based on this application, then Culture Minister Sakata Michita designated this site in 1971.

In July 1971, the “Kasaoka Horseshoe Crab Protection Youth Group” was set up on the suggestion of the Kasaoka City Board of Education, and the activities of the East Kasaoka Middle School Horseshoe Crab Research Club were taken over by this group, which included 54 students from 3 middle schools. All the surveys and research noted above were continued by this new group.

At about this time, a new problem arose, namely how to save the horseshoe crabs living in and around Oe-hama beach once construction started in earnest on the Kasaoka Bay Land Reclamation Project. One problem was the fine, silty, and very deep mud of the tideflats off of this beach; there was a lot of excited discussion about the best way to gather the horseshoe crabs on these flats, for example, by using platform-type geta clogs, rowing out on the flats in large tubs, or even by utilizing special frequency electromagnetic waves. However, none of these methods actually ended up being implemented before construction began. The desperate efforts of the Horseshoe Crab Youth Group from Kanaura Middle School only managed to save several hundred horseshoe crab juveniles, which were released mainly at Natsume and Torinoe beaches.

Professor Sekiguchi, who had been using horseshoe crabs collected in Kasaoka Bay in his research at the Tokyo University of Education Shimoda Marine Research Facility (now the University of Tsukuba Shimoda Marine Research Center), became concerned that the Kasaoka Bay Land Reclamation Project and other factors would deprive Kasaoka Bay of its value as horseshoe crab habitat. He released about 20,000 artificially inseminated eggs and hatchlings at Koh-no-shima in September 1969. This activity was repeated annually for some years afterwards, though the release site was changed to Natsume beach, and in recent years has been continued by Professor Tomio Itow of Shizuoka University.

6 Breeding and Raising Japanese and American Horseshoe Crabs in Captivity

6.1 Rationale

As detailed above, the Oe-hama shore in Kasaoka was designated as a protected breeding place of horseshoe crabs in 1928. Unfortunately, the shallow sea was increasingly reclaimed after World War II, and the horseshoe crabs disappeared. Efforts to propagate horseshoe crabs through captive breeding have been conducted as one possible measure to protect the species from the threat of extinction.

6.1.1 Studies by Tokiko Mitsueda

Ms. Tokiko Mitsueda directs the horseshoe crab breeding program at the Kasaoka Municipal Horseshoe Crab Museum. She has successfully raised both American and Japanese horseshoe crabs from the egg stage to adulthood.

On September 1, 1998, Ms. Mitsueda started raising fertilized eggs of American horseshoe crabs that she received from Dr. Sekiguchi. The fertilized eggs were spread upon a plastic frame (30 × 20 × 7 cm). Eggs were placed on the top of the tray and the seawater was changed twice a day. Four days later, all the eggs hatched, and these newly hatched horseshoe crabs began to develop into the first molting stage. During the first stage, these larval horseshoe crabs do not need to feed because they have their embryonic yolk sacks. From the second stage, juveniles were raised in sand and fed mixed food such as brine shrimp, TetraMin, and Tetra Krill-E in a laboratory dish up to the fifth stage. They were returned to their containers after being fed. From the sixth stage, worms (tubifex) were added as live food. It was noted that these juvenile horseshoe crabs habitually molted during the night.

On November 30, 1990, these juvenile *Limulus* attained the full length of 16.2 cm of the 13th stage. They were moved to a water tank of 45 × 30 × 20 cm. The bottom of the water tank was spread with coral sand, which doubled the height. On March 10, 1991, they reached the 14th stage of larval horseshoe crabs after molting for the 13th time. On June 18, 1991, they matured to the 15th stage and preferred to eat live food such as polychaetes (lugworms), littleneck clams, and ark shells. After eating in a separate container for about an hour, they were returned to the water tank.

On September 6, 1991, these horseshoe crabs molted for the last time, and finally became adult male horseshoe crabs, 3 years since they were raised from the egg stage. These male horseshoe crabs had been bred to study how many years they could live afterward; however, they died in 2006.

Ms. Mitsueda has also had success in raising the Japanese horseshoe crab in captivity. In 1998, eggs were gathered from the spawning pond of the Horseshoe Crab Protection Center and hatched in the laboratory. A selectivity experiment determined that young horseshoe crabs preferred mud rather than sand, therefore mud was gathered from the tidal flats and put on the bottom of the containers.

Sea water was exchanged about once a month, but depending on water temperature and the number of feedings, about half of the sea water was replaced about every 20 days after the crabs reached the second instar stage. Fresh water was added as needed to maintain the salinity.

Ms. Mitsueda developed a method of feeding that reduced the fouling of the aquarium. Horseshoe crabs were taken out of their aquarium and moved to another container in which they were allowed to feed. After feeding, the horseshoe crab was washed with sea water, and returned to its aquarium. In this way, the water in their aquarium remained clear and less susceptible to rotting. The sea water in the aquarium was replaced with 50% new sea water during feeding.

Tachypleus juveniles were fed a similar regimen to *Limulus* as described previously, i.e., brine shrimp for second instars, brine shrimp and TetraMin for third and fourth stages, brine shrimp, TetraMin, and tubifexes for fifth stages, and TetraMin, chopped clams and worms for sixth stages and beyond.

Unfortunately, there was only surviving horseshoe crab left by 1997. On September 28, 1999, it finally became an adult female crab after the last molting. This individual became an adult female in its 11th year.

6.1.2 Studies by Oshige Yoshinori

Mr. Oshige Yoshinori, a junior high school science teacher, began to raise 50 Japanese horseshoe crab eggs from the Kasaoka Municipal Horseshoe Crab Museum in September 1993. He also obtained another 50 larval horseshoe crabs the following year which he raised and studied with his students at Konoshimasoto Junior High School. In April 1995, he was transferred to another school, yet he was able to take 38 of the original 100 horseshoe crabs to continue raising and studying by himself at home. The following report is based on the progress of that group of 38 crabs beginning in April 1995 until the present time.

Mr. Yoshinori's method of raising the crabs was to spread mud from a tidal flat at the bottom of a water tank. The mud contains plentiful natural food for horseshoe crabs; in nature, the mud also provides a protective refuge from enemies and predators. Also, in captivity, the bacteria in the mud help to purify the seawater to the benefit of the young horseshoe crabs. Eleven larval horseshoe crabs were placed in a water tank (30 × 90 × 40 cm). The sea water is changed once every half year. The tank is placed next to a bright window which receives plentiful sunlight, and under these conditions the water remains clear.

From the first stage to the fifth stage, the larval horseshoe crabs are fed brine shrimp and those larval horseshoe crabs past the sixth stage are moved to another container and fed. Despite the fact that there are individual differences, these larval horseshoe crabs generally molt three times following the year when they hatch and become the fourth stage of larval horseshoe crabs. Furthermore, in the second year, these larval horseshoe crabs molt again and attain the sixth developmental stage of larval horseshoe crabs, and after the third year, they molt once a year. After that, these larval horseshoe crabs molt only once a year, and assuming they become adult horseshoe crabs when they reach the 15th stage, they become adults 11 years after they hatch.

It is likely that the growth rates under these conditions are faster than those experienced by natural individuals. Conditions in artificial breeding are thought to be better than nature, and the survival rates of bred crabs are high as well. Additionally, when water temperature is high, horseshoe crabs in tanks move aggressively and can consume all their food in around 30 min. In contrast, in the case of wild populations, individuals must search for food and are adversely affected by their predators and competitors. In all likelihood, then, both growth rates and survival may be higher among horseshoe crabs raised in laboratory aquaria.

7 Future Directions

The projects conducted by Ms. Mitsueda and Mr.Yoshinori are small-scale successes, but for future larger scale projects on captive breeding, some refinements and additional data will be necessary. As they grow, horseshoe crabs increasingly require larger aquaria and this is a problem that may constrain aquaculture facilities. It is extremely difficult to breed horseshoe crabs from egg to adulthood, because of the slow growth rate and the importance of providing juveniles with the proper nutrition at each stage. Additional studies should be conducted on influence that sediment type (e.g., mud vs. sand) has on the frequency of molting and growth increment. If the bred horseshoe crabs are released at a relatively small size, we must be able to clarify their survival relative to natural horseshoe crab populations. Follow-up investigations should include studies of post-release survival, and whether these individuals survive to breeding age.

Public Awareness and Community-Based Conservation for the Horseshoe Crab at Saikai National Park in Nagasaki Prefecture, Japan

Chikako Iwaoka and Toshinao Okayama

Abstract Formerly horseshoe crabs (*Tachypleus tridentatus*) were commonly distributed in Japan. However, the horseshoe crab population has been decreasing due to habitat loss caused by coastline development. The Saikai Pearl Sea Center Aquarium (SPSCA) has been conducting many activities for horseshoe crab conservation at Kujukushima Islands in Japan. In SPSCA, we attach great importance to cooperation with local fishermen for public awareness. Because there is so much by-catch of horseshoe crabs in the Kujukushima area, local fishermen bring us much useful information on distribution and habitat. Basically, horseshoe crabs are a burden to fishermen. We make much effort to deepen their understanding of horseshoe crabs. Recently local fishermen are recognizing that horseshoe crabs are endangered and valuable animals. We also exhibit living horseshoe crabs at SPSCA, give lectures to local schools, conduct excursions to observe the habitat of horseshoe crabs, engage in cooperation with local government, and publicize our work in newspapers and TV. For research activities, we measure morphology for all individual by-caught horseshoe crabs and submit blood samples for DNA analysis in Kyushu University. We report on the public awareness and research activities at SPSCA.

1 Introduction

1.1 Background

The horseshoe crab (*Tachypleus tridentatus*) is well known as a “living fossil” in Japan. Formerly horseshoe crabs were commonly distributed from the northern part of Kyushu Island to the Inland Sea of Japan (Sekiguchi, 1998). However, the population has recently decreased due to habitat loss caused by coastline development and environmental degradation including water

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pollution (Itow, 1997; Itow et al., 1998). The populations of the Inland Sea of Japan in particular, once containing the largest habitats in Japan, have decreased dramatically and consequently the Inland Sea population is almost extinct now (Nagasaki Prefecture, 2001). As a result, obvious spawning sites are now observed only at Yamaguchi, Fukuoka, Saga, Oita, and Nagasaki in Japan. The horseshoe crab is listed as an endangered species in many red data books in Japan (Nagasaki Prefecture, 2001; Sasebo City, 2001; Ministry of the Environment of Japan, 2006).

The Saikai Pearl Sea Center Aquarium (SPSCA) is located in Nagasaki Prefecture in the northwestern part of Kyushu Island. Nagasaki Prefecture is famous for horseshoe crabs in Japan. The Saikai Pearl Sea Center Aquarium has been undertaking various conservation activities such as education, raising public awareness, carrying out research and surveys of the horseshoe crab population for about 8 years. In this chapter, we report our activities, especially about raising public awareness among the local population and research activities on the horseshoe crab at the SPSCA.

1.2 The Distribution of Horseshoe Crabs in Nagasaki Prefecture

Nagasaki Prefecture (Fig. 1a, b) is not so large in area (4,094 km^2) but its coastline is very complicated, having what is called a “saw-tooth coastline.” Because of this, Nagasaki Prefecture has the longest coastline (4,137 km) in all the administrative divisions of Japan. The notable horseshoe crab habitat feature in Nagasaki is that many small habitats are scattered along the saw-tooth coastline. Because of its complicated coastline, there are many undiscovered habitats where surveys have not yet been conducted so there is the possibility of finding new habitats and spawning sites in the future. Because the habitats of the Inland Sea of Japan are almost all gone, the main horseshoe crab habitat now is Kyushu Island. Conservation activities in Nagasaki Prefecture are especially important for the survival of horseshoe crabs in Japan.

1.3 The Kujukushima Islands in the Saikai National Park

The field work undertaken in this study takes place in the Kujukushima Islands (Figs. 1c and 2) in Nagasaki Prefecture. The Kujukushima Islands are located within the Saikai National Park and consist of many small islands which have saw-tooth coastlines. Though “Kujukushima” means “99 islands” in Japanese, there are in fact 208 islands in the area. The Kujukushima Islands have a relatively large number of natural coastlines and various environments like rocky beaches, sand beaches, tidelands, and algae sea beds. Such diverse environments seem suitable for the survival of the horseshoe crab.

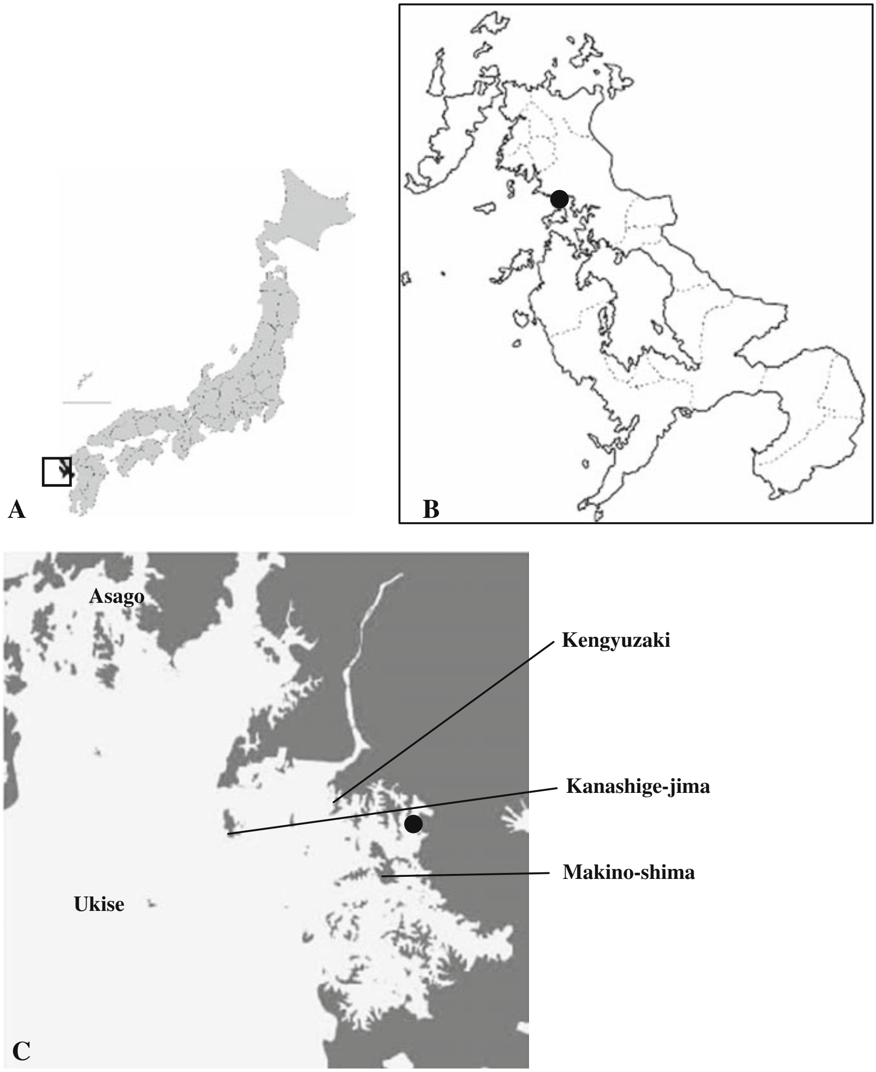

Fig. 1 Location of Nagasaki Prefecture (A: a map of Japan, B: a map of Nagasaki Prefecture, C: a map of study area). Nagasaki Prefecture is located at west end of mainland of Japan (*square* in **A**). *Black circle* indicates the location of SPSCA (**B** and **C**)

2 Public Awareness Activities

2.1 Cooperation with Local Fishermen

The fishing industry is very active in the Kujukushima Islands. The local fishermen bring us much useful information on distribution and habitat

Fig. 2 Kujukushima Islands in the Saikai National Park

and also specimens of horseshoe crabs. Therefore, we attach great importance on cooperation with local fishermen. Many horseshoe crabs are by-caught with gill nets and trawl nets from spring to autumn in the Kujukushima Islands. However, for the fishermen, horseshoe crabs are simply a burden because they disturb fishing and sometimes their spine breaks the fishing nets. Consequently, many fishermen have just disposed of by-caught horseshoe crabs.

We frequently visit local fishermen and sometimes help them in their work to deepen their understanding of horseshoe crabs and we ask them to inform us when they catch horseshoe crabs. As a result, awareness of the horseshoe crabs among local fisherman has been rising. Recently, local fishermen have been recognizing that the horseshoe crab is an endangered and valuable animal. For example, in 2006, we were able to collect a total of 132 by-caught horseshoe crabs based on information from local fishermen. These individuals were used for research activities (see below).

But of course not all fishermen recognize the importance of the horseshoe crab. Occasionally we find carcasses of horseshoe crabs that have most likely been discarded by fishermen. Enlightenment activities concerning the local fishermen are still important and should be continued.

2.2 *Exhibition at SPSCA*

The SPSCA has a permanent water tank for horseshoe crabs to display the living animals. The animals inside the tank are also by-caught crabs from around the Kujukushima Islands by local fishermen. Explanation boards, touchable stuffed specimens, and other materials are also exhibited in SPSCA so that visitors can study the importance and significance of horseshoe crabs. Recently visitors from abroad from areas such as Korea, China, and Taiwan also have been increasing.

2.3 *Environmental Education Activities at the SPSCA*

The SPSCA has a regular environmental education program which is entrusted by the Sasebo Municipal Office. All the third grade elementary school children in Sasebo City attend this program every year. The total number of children is around 2,300 every year. The main purpose of this program is to study not only horseshoe crabs but also the total rich environment of the Kujukushima Islands in which horseshoe crabs are brought up. This environmental education program has been going on for 10 years. Children who took this program the first year will enter the 12th grade of high school this year. Such a large-scale environmental education program should be very effective for local children.

2.4 *Delivering Lectures at Elementary and Junior High Schools*

The SPSCA has an original lecture program for local elementary and junior high schools. The lectures delivered by the SPSCA staff comply with the requests from each school and the local government. The requests for this program have been increasing recently. We held 15 such classes in 2006. This reflects the rising awareness of horseshoe crabs and the natural environment of the Kujukushima Islands.

Sometimes we bring living horseshoe crabs and dried specimens to the lectures. Usually, most students are willing to take the class. We also enjoy holding such classes but it is a little difficult for us to manage both ordinary aquarium tasks and giving lectures. Because members of the aquarium have limited time, how to deal with classes is a problem. It is expected that trained volunteer staff and local NGOs will hold such classes in the future.

2.5 *Field Excursions*

Education activities at SPSCA and delivering the lectures mentioned above are the so-called ex situ programs. The SPSCA also has an in situ activity program.

In this program, people can see wild horseshoe crabs and their eggs on the beach (Fig. 3). Recently, there are many children and citizens who have not had the experience of walking around tidelands and who do not know about the horseshoe crab. It is a good opportunity for them to go to the field to feel the rich environment and observe horseshoe crabs in their own environment. We conducted such field excursions 10 times in 2006. The problem is the same as with holding classes. The demand to hold such field excursions exceeds our capacity. To meet such demand, it is necessary to increase trained volunteer staff and local NGOs who can act as a moderator of the excursion.

Fig. 3 Field excursion activities at the SPSCA

2.6 Using Mass Media

The SPSCA frequently issues press releases to mass media outlets when we have some new topics related to horseshoe crabs. After the broadcasting of TV programs or news on horseshoe crabs, inquiries and information input to the aquarium increase. There are many local people that become aware of the importance of horseshoe crabs through TV and newspapers. Such news also becomes a cue for requests for delivering lectures and field excursions. Mass media is very effective for broadening the dissemination of information.

2.7 Collaboration with Local Government

For conservation of local natural resources, including horseshoe crabs, public awareness activities as mentioned above are very important on one hand and collaboration with local government is necessary on the other. The SPSCA maintains a close relationship with the local government. Many classes and environmental education at SPSCA are carried out by request from the Sasebo Municipal Government and the Nagasaki Prefectural Government.

Some SPSCA staff are members of the Natural Environment Council of Nagasaki Prefecture. We can make interventions for both conservation and development policy to the government through the council. We gave advice on protective levee construction and coastline conservation to the Nagasaki Prefectural Government and the Ministry of Land, Infrastructure and Transport through the Council in 2006.

3 Research Activities

3.1 Materials and Methods

As mentioned above (Section 2.1), many by-caught horseshoe crabs have been brought to SPSCA mainly by local fishermen. These individuals were captured by gill net and trawl net in the Kujukushima Islands. The capture date, locations, and the sizes of these individuals were recorded. Measured regions for each individual were total length including tail (ToL), body length (combined prosoma and opisthosoma) (BL), tail length (TaL), and carapace (prosoma) width (CW). Tail ratio (TR) and flatness of carapace (FC) were calculated ($TR = TaL/ToL$, $FC = CW/BL$). The TR and CW were compared with those of individuals from the Kitakyushu population (Hayashi, personal communication). Blood samples from these animals were sent to Kyushu University to study genetic structure of Japanese horseshoe crab populations using mitochondrial DNA analysis.

The total number of by-caught individuals was 82 from May 2000 to November 2005. In this chapter we report on the capture locations for all 81 individuals and morphological data for 32 individuals (13 adult males, 9 adult females, and 10 subadults) that were brought to the SPSCA in 2005.

3.2 Results

Main by-caught locations were (1) between Ukise and Kanasige-jima, (2) off Asago, (3) off Kengyuzaki, and (4) south of Makino-shima (Fig. 1c). Though fishermen set their nets all through the year, many horseshoe crabs were by-caught between May and August. May was the month for the highest number captured (Table 1).

Table 1 Monthly number of by-caught horseshoe crab in Kujukushima area from 2000 to 2005

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
2000					2	1				2			5
2001					3	2		1	1				7
2002					5	2		2					9
2003		1	1	3	2	3	1	3	2	1			17
2004					2	1		3		2		3	11
2005				4	7	7	3	3	4	1	3		32
total	0	1	1	7	21	16	4	12	7	6	3	3	81

The ToL of the biggest individual was 600 mm for males and 800 mm for females. The average ToL was 537.8 mm for males and 626.3 mm for females (Table 2). Both ToL and CW of Kujukushima individuals were larger than those of Kitakyushu individuals.

Table 2 Comparison of ToL and CW of the horseshoe crab from Kujukushima and those from Kitakyushu. The numbers without parentheses indicate average size. The numbers in parentheses indicate the range

	Kujukushima (n = 19 for ToL, n = 22 for CW*)	Kitakyushu (n = 85)
ToL (♂)	537.8 mm (445–600 mm)	514.7 mm (430–581 mm)
ToL (♀)	626.3 mm (530–800 mm)	589.9 mm (511–680 mm)
CW (♂)	252.9 mm (240–270 mm)	242.9 mm (225–267 mm)
CW (♀)	297.5 mm (250–355 mm)	281.3 mm (250–305 mm)

* CW were measured for all the adult individuals (n = 22) that were brought to the SPSCA in 2005. Three individuals whose tails were broken were excluded from ToL.

The individuals for which the TR was 0.86 or less were all female, individuals for which the TR was 0.87 or over and 1.00 or less were a mixture of males and females, and individuals for which the TR was 1.08 or over were all males (Table 3). Males have a tendency to have longer tails than females. The individuals for which the FC was 0.89 or less were all female, individuals for which the FC was 0.90 or over and 0.95 or less were a mixture of males and females, and individuals for which the FC was 0.96 or over were all males (Table 4). Males have a tendency to have a flatter shape than females. These data suggested that males have proportionately longer tails and a more flattened shape than females.

Table 3 Tail Ratio (TR) of the horseshoe crab (ToL: Total length, BL: Body length, TaL: Tail length)

No	sex	ToL (mm)	BL (mm)	TaL (mm)	TR
104	♀	570	335	235	0.70
132	♀	625	350	275	0.79
125	♀	590	320	270	0.84
128	♀	590	320	270	0.84
134	♀	530	285	245	0.86
110	♂	504	269	235	0.87
127	♂	520	270	250	0.93
112	♀	630	325	305	0.94
114	♀	675	348	327	0.94
129	♂	530	270	260	0.96
135	♀	800	400	400	1.00
108	♂	520	260	260	1.00
122	♂	520	260	260	1.00
131	♂	600	300	300	1.00
126	♂	520	250	270	1.08
117	♂	505	240	265	1.10
136	♂	537	252	285	1.13
109	♂	570	260	310	1.19
118	♂	590	260	330	1.27

3.3 Discussion

There were a large number of by-caught horseshoe crabs in the late spring (May and June) and the late summer (September and August) in the Kujukushima area. The horseshoe crabs in this area spawn on flood tide days from June to September. The horseshoe crabs may be by-caught before and after the spawning season when they go back and forth between their usual habitat (seabed) and spawning sites (sand beach). Most of the by-catch was observed from May to October in Omura Bay which is located southeast of the Kujukushima area (Kai and Morikawa, 1999), which is almost in accord with the results in this study for the Kujukushima area. However, the most abundant by-catch was observed in August which is the middle of the spawning season in Omura Bay (Kai and Morikawa, 1999), while the most by-catch for the Kujukushima area was observed before spawning season (May). This may be because of the difference in their range of activity and location of fishing gear between the Kujukushima area and Omura Bay.

Two individuals were by-caught off Asago and one individual was by-caught off Ukise in November 2005. Three individuals were by-caught off Ukise in December 2004. The horseshoe crabs stop their activities beneath the sand or mud of the seabed. It is suggested that off shore of Asago and Ukise are wintering places in the Kujukushima area.

Both the ToL and CW of the individuals of the Kujukushima population were larger than those of the Kitakyushu population for both males and females. The

Table 4 Flatness of carapace (FC) of the horseshoe crab (BL: Body length, CW: Carapace width)

No	sex	BL (mm)	CW (mm)	FC
132	♀	350	300	0.86
112	♀	325	280	0.86
128	♀	320	280	0.88
134	♀	285	250	0.88
135	♀	400	353	0.88
125	♀	320	285	0.89
104	♀	335	300	0.90
130	♂	290	260	0.90
131	♂	300	270	0.90
127	♂	270	250	0.93
119	♀	325	310	0.95
114	♀	348	332	0.95
113	♂	250	240	0.96
126	♂	250	240	0.96
136	♂	252	242	0.96
109	♂	260	250	0.96
118	♂	260	250	0.96
122	♂	260	253	0.97
129	♂	270	265	0.98
108	♂	260	260	1.00
117	♂	240	240	1.00
110	♂	260	262	1.01

largest female measured at Kasaoka City in Okayama Prefecture which fronts on the Inland Sea of Japan was 630 mm in ToL (Kasaoka City Horseshoe Crab Museum, personal communication), which is a little larger than the female average size of the Kujukushima population. The largest individual from Kujukushima was an 800 mm female which was captured off Asago on November 19, 2005. There are no previous records of such large horseshoe crabs in Japan. This individual was reported on by many news outlets as being the largest horseshoe crab in Japan. It is suggested that the horseshoe crabs of the Kujukushima population are larger than those of other regions in Japan.

It is known that female horseshoe crabs are larger than males (Yamasaki et al., 1988). In addition to the total body size, our data suggest that the body shape of horseshoe crabs is different for males compared to females. Males have shorter tails and a flatter body and females have longer tails and an elongated body. Statistical analysis for the sex difference for the TR and the FC could not be conducted because of the limited sample size. To ensure the results of the sex differences in horseshoe crabs it is necessary to increase the number of measured individuals.

The DNA analysis done at Kyushu University indicated there are some population subdivisions among Japanese horseshoe crabs (Nishida and Koike, 2006, 2009). Such information is very useful to help make a conservation plan for the horseshoe crab in Japan.

4 General Discussion

4.1 Population Status of Horseshoe Crabs in Nagasaki Prefecture

Although formerly horseshoe crabs were commonly distributed in the northern part of Kyushu Island and the Inland Sea of Japan, the population of the Inland Sea of Japan is almost extinct now (Nagasaki Prefecture, 2001). The major horseshoe crab populations in Kyushu Island are located at the Sone-Tideland in Kitakyushu, Imari-Bay in Saga and Morie-Bay in Kitsuki (Sekiguchi, 1993; Sato, 2000). Kita-Matsuura, Sasebo, Nishishonogi, Hirado, Omura Bay, Iki Island, and Tsushima Island are reported as being horseshoe crab habitats in Nagasaki Prefecture (Ikezaki, 1980; Yamaguchi, 1989, 1993; Ikezaki, 1977, 1995; Kai and Morikawa, 1999; Sekiguchi, 1999; Hisano et al., 2005). A feature of the horseshoe crab populations in Nagasaki Prefecture is that many small populations are scattered along the coastlines. This is simply because Nagasaki Prefecture has a very complicated coastline. Consequently, it is very difficult to find all the horseshoe crab populations in Nagasaki, and there are still many possible habitats and spawning sites where scientific surveys have not yet been undertaken. Searching out new habitats and spawning sites should be continued.

4.2 The Population Status of Horseshoe Crabs in the Kujukushima Area

The Kujukushima Islands are located in the northwestern part of Kyushu Island and within the Saikai National Park which consist of many small islands which have long saw-tooth coastlines (Fig. 2). The Kujukushima area has still many natural coastlines and various environments such as rocky beaches, sand beaches, tideland, and algae sea beds. Consequently, the Kujukushima area has rich fauna and flora including other endangered species such as mollusks (*Onchidium hongkongensis*, *Anomalodiscus squamosa*, *Meretrix lusoria*), sea grasses (*Zostera marina*, *Z. japonica*), seed plants (*Hibiscus hamabo, Mucuna sempervirens*) in addition to horseshoe crabs (Otani, 2004; Hashiguchi, 2004; Otani, 2005; Kawakubo et al., 2005). The diverse and rich environment seems suitable for the survival of the horseshoe crab.

4.3 Public Awareness for Horseshoe Crab Conservation

How can a local aquarium like the SPSCA contribute to local environmental conservation? Public awareness for local people is one of the answers. Conducting awareness raising activities for the public concerning the local environment is one of the most important tasks for a local aquarium. To conserve horseshoe crabs and their habitats in the Kujukushima area, it is essential for local people to realize the importance of their local environment and be proud of it. Based on this point of view the SPSCA has been conducting such activities described above.

Because horseshoe crabs are famous as a living fossil in Japan and most of the Japanese know that it is an endangered species now, horseshoe crabs can become a good "tool" for public awareness. To continue public awareness activities through horseshoe crabs it is necessary to collect more information, to explore new habitats, and to conduct further study and research.

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Public Participation in Studies on Horseshoe Crab Populations

Carl N. Shuster Jr.

Abstract This chapter describes two ways in which public awareness about horseshoe crabs can be increased. First, a modification of the largely volunteer-based spawning surveys, as exemplified by the Delaware Bay effort, can provide information on the "health" of the spawners. This would add an important dimension to spawning survey. There are probably 10 year-classes of spawners and it is obvious that all are not at the same level of health. Second, the conservation community and the public in general should be aware that populations of horseshoe crabs that are small in numbers are more at risk than large populations.

1 Introduction

This chapter explores two ways in which to increase public awareness about horseshoe crabs: (1) through monitoring the physical condition, i.e., "health" of individual spawners, and (2) emphasizing differences between populations with large and small numbers.

Beginning with Lockwood (1870) and amply illustrated by Sekiguchi (1988), Shuster et al. (2003), and this book, there is a basic knowledge about the natural history and ecology of horseshoe crabs. Whether the species is under impact or just certain populations is less well known. A review of the status of populations of the American horseshoe crab, *Limulus polyphemus*, reveals a singular fact that all populations are not equally capable of surviving habitat loss, over-harvesting, or other serious impacts. Perhaps this is merely a reflection of the relative abundance of animals and habitat – that the more of each, the more resilient the population may be to change.

The recent impetus for conservation of the crabs has come from observations that shorebirds feeding upon horseshoe crab eggs in Delaware Bay during their

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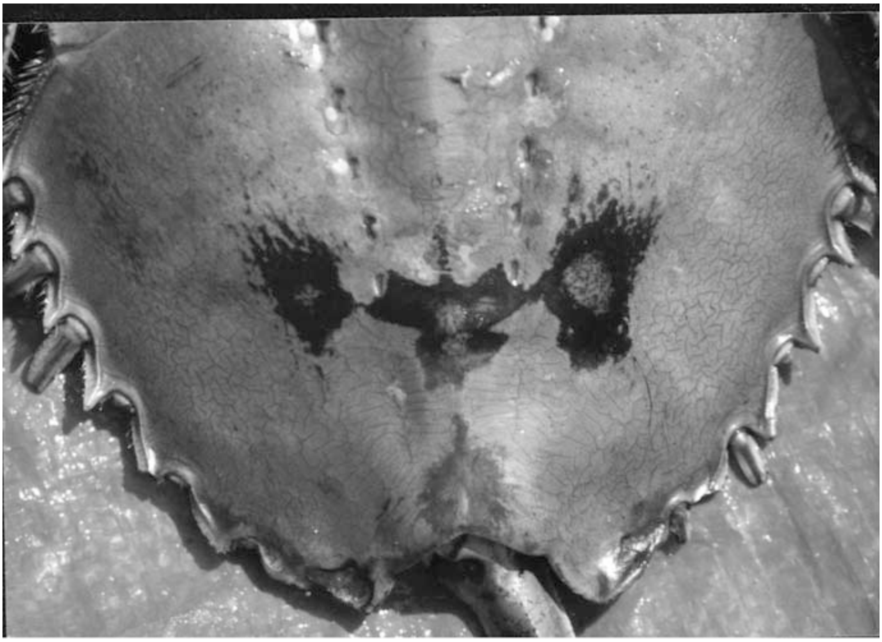

Fig. 1 Opisthosoma of a multioviparous female *Limulus polyphemus* with mating marks. The "butterfly spawning scar" indicates prolonged amplexus, especially since the depressions caused by the male pedipalps on the terminal projections of the opisthosoma are deep

annual migration from winter grounds in South America to breeding areas in Canada have been adversely impacted by a marked reduction in the *Limulus* population. This has created a unique situation: the management of a fisheries resource to protect birds by the Atlantic States Marine Fisheries Commission and member states.

2 Monitoring Physical Appearance of Spawners

A mechanism already exists through which the physical appearance of spawning horseshoe crabs can be monitored. Since 1990, an annual survey at Delaware Bay has involved a large team of volunteers. Revised by Smith et al. (2002), it gathers data that provide indices on spawning activity that can be compared temporally and spatially. Because horseshoe crab eggs are necessary to sustain sizeable flocks of migratory shorebirds during their stopover each spring at Delaware Bay, monitoring the physical condition of the female horseshoe crabs would seem to be an essential activity. Data on the condition, i.e., "health" of the females, in addition to their numbers, would add an important dimension to an understanding of the crab population. It would also involve more of the public, i.e., volunteers, to gather the additional data. The following describes what additional information could be obtained to evaluate the health of that or any population during that survey or in a separate one. If nothing else is done, an annual album should be assembled of horseshoe crabs spawning at selected sites during the spawning season.

Several studies have used the appearance of crabs, such as old versus young, aggressive versus docile, but there has not been an effort to use these criteria to monitor the relative ages and physical condition of the adult population. Because the quantity of eggs available to the migratory shorebirds is an overriding concern, I have interjected here a short description of the role of female horseshoe crabs in reproduction.

2.1 Reproduction by Female Horseshoe Crabs

Horseshoe crabs are *oviparous* (i.e., they deposit eggs that develop and hatch outside the maternal body). The older, large-sized female horseshoe crabs can be defined according to their reproductive status: *immature* = large juveniles, as large as or approaching the size of adults; *nullioviparous* = mature, having molted in an autumn month and not yet spawned, hence without mating scars; *primioviparous* = first-year spawners (adults with small mating scars and a lustrous carapace on which a fine-tracing of irregular black lines are prominent); and *multioviparous* = those that have spawned several years, i.e., with multiple mating scars and eroded carapaces, often bearing epibionts (Fig. 1).

Understanding the reproductive system of female *L. polyphemus* began with the epic monograph by Munson (1898). He described the vast ovarian network within which the eggs develop and then are moved through increasingly muscular tubules into the main oviducts leading to the genital pores. After adding information from a few salient studies, we can begin to postulate what occurs during spawning. Of the four extant species, *Limulus* has the most complex and extensive reproductive system (Makioka 1988). Perhaps this is related to the size (1.6–1.8 mm in diameter) and quantity (90,000) of eggs produced (Shuster and Botton 1985, Shuster and Sekiguchi 2003). The eggs mature within the ovarian network which is composed of fine tubules that occupy the lateral portion of the prosoma. At the time of spawning *Limulus* females bear up to 100,000 mature eggs with at least two clutches of smaller, undeveloped eggs visible in the ovaries (0.1–0.3 mm in diameter and 0.5–0.7; mature eggs are not found in juvenile crabs: Shuster 1955).

Conceptually, it is possible for a female to lay all of her mature eggs during a few days. Using Munson (1898) and personal observations as guides, it is apparent that all those eggs (perhaps about 20,000) that can be laid during one tide are in the large oviducts. When a female has laid those eggs she leaves the beach, usually returning on subsequent high tides, each time with the oviducts refilled with mature eggs. Thus, in increments, a female can complete spawning all her mature eggs within the window of 5 or so days through a period of the higher tides during the new and full phases of the moon.

While we are equally interested in the early life stages, the older females – those that are nearing or have reached reproductive maturity – are usually more closely studied because they are more available, as during spawning. Indeed, the ecological role of horseshoe crab eggs in the diets of migratory shorebirds was the major criterion for the establishment of the horseshoe crab reserve off the mouth of Delaware Bay. The sanctuary was established in 2001 by the National Marine Fisheries Service to protect the heart of the female reproductive population of Delaware Bay, notably large juveniles, young adults (i.e., those that molted in late fall of the year), and the first- and second-year spawners.

2.2 Possible Indices to Evaluate the Health of a Population

Historically, the analysis of the abundance of spawners and their sex ratios has been the basic tool in evaluating the status of a population. When an age component is added, it would be possible to begin to estimate the health of the population. Additional data could evaluate the extent of disease and the abundance and species of epibionts.

The methodology developed by Smith et al. (2002) is the basic approach used in obtaining indices on the relative abundance of horseshoe crab spawners within a 1-m band along a beach. A methodology for sampling specimens for health criteria could be added to the spawning survey methodology.

In addition to the information on the reproductive status of both sexes (a comparable approach could be applied to the males), other indices of spawning could include tabulation on individual size, condition of the carapace, and epibionts. Visual guides should be provided. This approach should aid in estimating the health of individuals and the population as a whole. The following criteria are based on personal observations and the recent paper by Duffy et al. (2005).

2.2.1 Size

The most common size measurement is the prosomal width (PW) in millimeters. The distance between the spines above the lateral compound eyes (IO, interocular distance) is becoming more useful as it is often the only measurement that can be made without disturbing the spawning females. Because the sizes of individuals vary within as well as between populations and there is an overlap in sizes between large immature specimens and adults, it is necessary to establish a baseline of sizes for each population.

2.2.2 Activity

The activity of individuals differs due to several factors: time out of water (dehydration or overheated), temperatures less than 15°C, and amount of epibionts and disease. Depending upon these factors, they exhibit different levels of activity when handled (H) or released on a beach near high water line (R):

- 0 = (H) aggressive; vigorously thrusts appendages against handling, with nipping action by claws and flexing of the opisthosoma; (R) continuous movement, reaching foot of beach or water.
- 1 = (H) activity ranging from obvious movement of legs and book gills, in at least several second bursts, to feeble, occasional movements; (R) crawling slowly down beach, may move up to a meter before stopping.
- 2 = (H) none; morbid; (R) no movement.

2.2.3 Viability Analysis

The response of the stranded crabs when physically stimulated at the time of collection:

- 0 = very active, thrust telson in attempting to turn over.
- 1 = responded but with little attempt to get away; folded up.
- 2 = very little to no response.

2.2.4 Appearance (Aging) of Carapace

Because the initial stage in the aging of the carapace of an adult is by abrasion, signs of erosion of the shell are the first characteristic to look for. After the protective, lustrous outer shell layer has been abraded, invasion of microscopic organisms can begin (Leibovitz and Lewbart 2003). Assigning years of adult age is more subjective as each crab usually has its own history of exposure to biological and environmental conditions:

- 0 = Ranging from a lustrous shell, as if lacquered, with a fine mosaic of clearly visible black lines all over the carapace with no scratch marks to a shell with a dull surface and usually mating scars (adult age 1–2 years).
- 1 = Shell more than half to totally black; multiple mating scars.
- 2 = Shell eroded down to innermost, cream-colored layer; often with flesh exposed.

2.2.5 Relative Age

Information provided by an examination of the carapace (size, amount of abrasion of the shell, epibionts, etc.):

- 0 = young adult; no mating scars (virgin); carapace very clean; mosaic patterns clearly visible.
- 1 = early middle age; mating scars present but not deep; carapace still clean with no erosion.

- 2 = middle age; mating scars are more defined; some erosion; mosaic pattern visible in most areas.
- 3 = late middle age; mating scars dark and large; some pitting of carapace; mosaic pattern less visible; carapace at least one-half blackened.
- 4 = old adult; mating scars very deep and cover large area of opisthosoma; carapace very blackened and eroded; deep pitting and mosaic pattern hardly visible.

2.2.6 Determination of Stage of Fecundity

The eggs of a gravid female *Limulus* can be categorized as mature or immature, based on size and color (Shuster 1955):

- 0 = mature eggs measure 1.0 mm or more and are typically green in color.
- 1 = immature eggs have a diameter less than 1.0 mm and are cream-colored.

2.2.7 Telson Length and Abnormal Condition

The telson is the most likely body part to be injured or malformed (e.g., swollen or forked); its length and strength are most critical when stranded, upside down (Botton and Loveland 1989):

- 0 = no injuries to telson; long (about as long as body length); telson fully mobile with no evident damage to musculature.
- 2 = worn or broken but still longer than prosomal length.
- 4 = worn or broken, forked or swollen, shorter than prosoma length; telson dangling (musculature obviously damaged) or forked, bent, swollen, etc.

2.2.8 Quantity of Epibionts (Especially *Bdelloura*)

Limulus is a “walking museum” (Allee 1922, Grant 2001) carrying many species of sessile organisms as well as mobile ones such as sea stars and snails. Most of the epibionts may be commensals but some are pathogenic (Leibovitz and Lewbart 2003), particularly triclad flatworms such as *Bdelloura*. Others, especially the fast growing blue mussel, *Mytilus edulis*, by sheer weight on the dorsal surface of the shell or by position around the appendages may be a burden and immobilize a crab (Hillman 1959, Botton 1981):

- 0 = free of epibionts.
- 2 = epibionts present but neither weighty nor immobilizing; few triclads and few of their egg cases.
- 4 = crab overburdened, sometimes immobilized by epibionts; or many triclads and their egg cases, resulting in erosion of the book gills.

2.2.9 Summary Score

A tabulation of the observations on horseshoe crabs, such as suggested above, could lead to an index of the condition or relative health of an individual and, cumulatively, to a population. The scoring, as indicated below, would depend on how many observations should be made or are feasible:

- 0–3 = excellent condition.
- 4–13 = ranging from good (4) to fair (12) condition.
- > 14 = poor condition.

3 Protecting Populations

It is clear that discrete populations, rather than the entire species, should be the units of conservation and management. Why this is so can be illustrated by briefly comparing small populations with the largest one, Delaware Bay. Problems usually arise soon when large populations are over-managed, to the extent that fishing pressure on a large population is transferred to smaller populations. This is especially possible because the lesser populations usually exist in smaller habitats and have restricted ranges.

3.1 Fragility of a Small Population

No one should believe that horseshoe crabs are indestructible due to their long geologic history. The virtual elimination in recent years of a well-studied population of *Limulus* in Buzzards Bay, Massachusetts, should be a warning of what can happen when small, relatively discrete populations are intensively harvested (Widener and Barlow 1999). Because there are many discrete populations as defined both morphometrically (Say 1818, Shuster 1955, 1979, Riska 1981) and genetically (King et al. 2005), individual populations are important. Small populations may be more vulnerable because they do not wander far. There was no mixing of crabs between spawning populations that existed in separate sub-embayments less than 4 km apart apparently in a small estuary in northern New England (Moore and Perrin 2007). Clearly, a population of *Limulus* should be the unit of study rather than the species as a whole. It should receive particular focus in the conservation and management of the species.

3.2 Resiliency of a Large Population

In sharp contrast to elsewhere, the Delaware Bay area population of horseshoe crabs has an extraordinary resilience and abundance. This resilience and great abundance appears to be due to the combination of a favorable hydroclimate with the juxtaposition of ample habitats. For over 100 years, on average, 1 million crabs were harvested annually for fertilizer (Shuster and Botton 1985, Shuster 2001, 2003). The highest recorded harvest was over 4 million in 1 year in the 1870s. The harvest decreased in a parabolic curve from over 2 million in the 1910s to about 100,000 per year in the 1950s and 1960s. Not all the decline was due to lack of horseshoe crabs; part of the decline was due to the increasing use of commercial nitrogen and phosphate fertilizers. During the 1950s and 1960s, the Delaware Bay population was at its lowest abundance, compared with any subsequent year, including during the late 1990s into the 2000s.

At the same time that the crab population was slowly rebounding in the 1960s and particularly in the 1970s, it began to be cropped by an increasing fishery for bait (Shuster 1997). The number of crabs spawning reached a peak by 1990 and 1991 (Shuster 2003) but the next year decreased to less than 50% of their former level. At first this was attributed to inclement, crab-adverse weather during the 1992 spawning season but, in ensuing years, it was evident that those lower numbers would be around for some time, due to the 10-year span for crabs to grow up.

From personal experience, it appears that small numbers of horseshoe crabs can exist in small habitats. In contrast, a favorable hydroclimate and the juxtaposition of extensive spawning and other habitats in Delaware Bay are the keys to its large population. However, due to the many changes that have occurred in the beaches of that bay over the past 50 years and the sensitivity of the crabs to beach conditions (Botton et al. 1988), part of the decrease in its horseshoe crab population could have been due to decreasing quality and extent of some beaches. Despite rumors that it was threatened, the horseshoe crab population of Delaware Bay never has been challenged as the world's largest!

4 Conclusions

In the comparison above, the number of crabs involved should certainly be the key factor in conservation and management decisions. When informing the public we should make clear what kind of population of horseshoe crabs is being discussed, including the quality and quantity of its habitats, its hydroclimate, impacts on the population – in essence, those local conditions germane to that population.

In reporting research results we should determine or otherwise be cautious about the universal application of the information – is it always interchangeable between populations? In a nutshell, the population is the unit of study and we have to be careful not to imply that all characteristics of one population or their habitats or the prevailing hydroclimate may also pertain to others.

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Green Eggs and Sand: A Collaborative Effort of Scientists, Teachers, Resource Managers, and Stakeholders in Educating About *Limulus polyphemus*

Katy O'Connell, Cindy Etgen, Gary Kreamer, and Michael Oates

Abstract The highly successful *Green Eggs & Sand* (GE&S) project is a joint effort of educators, scientists, resource users, and managers engaged in building a unique workshop/field experience, a wealth of educational video, and a series of associated activity-based learning modules, centered on the current horseshoe crab/shorebird phenomenon and management controversy on Delaware Bay. Over the past 8 years, more than 800 teachers, non-formal educators, scientists, resource users, and managers from 20 states and 3 foreign countries have taken part in GE&S workshops, which are now offered up and down the Atlantic coast each spring around lunar event peak times for horseshoe crab spawning. These workshops are intensive, spanning 2–3 days, and feature a mix of hands-on field experiences, presentations by experts, and demonstration of curricular components. This article provides a case-study overview of how GE&S was developed and implemented, with an eye to how this approach might be adapted and modified for use elsewhere.

1 Introduction

1.1 Background and History

Green Eggs & Sand was initiated in 2000, in response to an interest in developing educational modules around the mid-Atlantic horseshoe crab/shorebird phenomenon and the complex and challenging management issues it presents.

A steering committee was subsequently formed, comprising a unique coalition of environmental educators, natural resource agencies, and nonprofit organizations from Delaware, New Jersey, and Maryland. Over the next several months, this group planned and presented a special workshop for the spring 2000 spawning season, inviting 14 master teachers from the three states. The objectives of the first Green Eggs & Sand (GE&S) workshop were to (1) immerse educators

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in experiencing this phenomenon first hand; (2) expose them to the issues and experts: the scientists, managers, and other individuals who are directly involved in these resources; and (3) explore the feasibility of, and chart directions for, developing educational modules around the phenomenon and issues (Fig. 1).

Along with the steering committee, the 14 teachers who convened at the Mallard Lodge in Smyrna, Delaware for that weekend became the "think-tank" for GE&S. The initial tasks were to respond to surveys, exchange countless emails, and meet to hash out priorities, plan strategies, and ultimately form the module writing teams that produced the draft/pilot curriculum. These same teachers then assisted in planning and presenting the second GE&S workshop in May 2001 as a vehicle for exposing a new group of educators to the phenomenon and issues. The second workshop also served as a venue for distributing draft curriculum modules to the collective group for piloting during the school year ahead. Feedback on the draft curriculum was used to develop a more finished product for 2002.

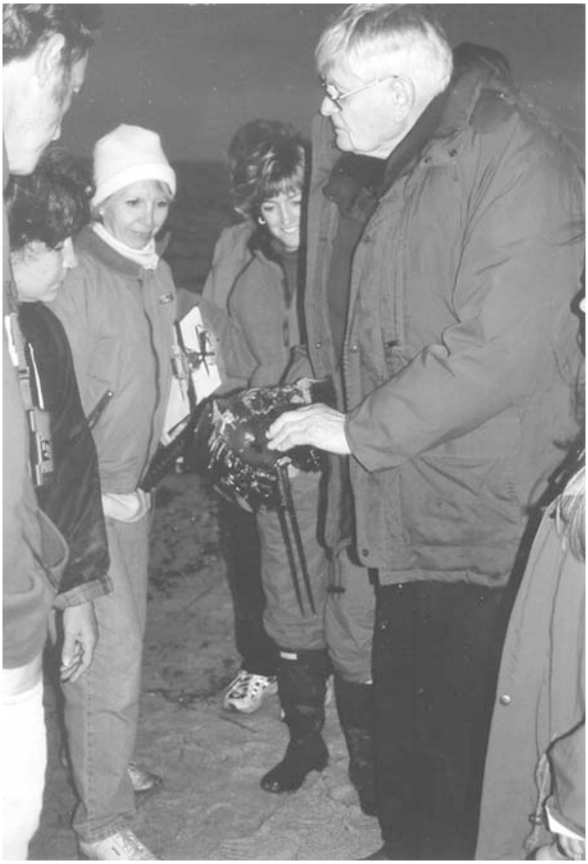


Fig. 1 Horseshoe crab expert and GE&S contributor, Dr. Carl N. Shuster, shares his knowledge of horseshoe crab anatomy with teachers at the pilot Delaware Bay workshop

To distribute the curriculum to a wider audience, two workshops per year were held on Delaware Bay in 2002 and 2003, using the same structure as in past workshops: presentations by experts, field trips to view shorebirds and horseshoe crabs, and orientation to the educational modules.

As awareness of and interest in GE&S grew, the project team began to receive requests to offer additional workshops at locations beyond the Delaware Bay. To meet this demand, in 2004, along with the traditional workshop in Delaware, the project team organized special 1-day sessions in Massachusetts, New Jersey, and Virginia. These workshops still offered presentations by local experts, but did not include much of the field component that was key to the Delaware workshop. Nevertheless, the 1-day sessions were positively received by participants.

In 2005, the full weekend Delaware workshop was supplemented by 1-day sessions in New Jersey, Massachusetts, Virginia, and Georgia, due to continued requests for workshops. Upon completion of those five spring workshops, the team re-designed the GE&S program. It was decided that in 2006, three regional workshops would be offered, but that each of these would span a full weekend to include field experiences comparable to the Delaware sessions. So, in addition to the traditional Delaware weekend workshop 2-day workshops were scheduled for 2006 for Cape Cod, Massachusetts, and the Georgia Coast to offer a regional focus because each area has unique horseshoe crab/shorebird issues.

In 2005, the GE&S team started to be recognized for the program's success. In April 2005, the team received the Northeast Association of Fish and Wildlife Agencies "Communicator of the Year" Award for "exemplary leadership in conservation communication." Later that year, "Green Eggs & Sand: The Horseshoe Crab/Shorebird Education Project" was awarded first place in the curriculum category at the National Association for Interpretation's annual conference in Mobile, Alabama. The National Association for Interpretation (NAI) is dedicated to the advancement of the profession of natural and historic interpretation, including all aspects of environmental communication and education. Its membership encompasses 4,500 professionals from the United States, Canada, and 30 other nations. With such stiff competition as Yellowstone National Park, Bandelier National Monument, and others, the project team, needless to say, was very proud.

Green Eggs & Sand has continued to expand its reach. As of August 2007, GE&S team has hosted 18 workshops reaching over 800 participants from 20 states and 3 foreign countries. More than 50 expert presenters have contributed to those sessions, many returning year after year to share their respective areas of expertise. Along the way, the project team has presented GE&S at conferences far and wide, from Japan to Puerto Rico, and all over the United States.

1.2 Educational Design

Green Eggs & Sand is composed of four educational modules, which systematically guide students through an understanding of the horseshoe crab, its connection to a larger ecosystem (through shorebirds), its use by man, and human's attempts to manage this resource given limited science and multiple stakeholders. Yet, while GE&S offers multi-faceted learning opportunities, it has been designed by teachers to be flexible and adaptable for use at various grade levels in both the classroom and the field. Hence, the four educational modules can be taught as sequential learning blocks or independently of one another (Fig. 2). For example, a teacher of the lower grades interested primarily in science and math can use Modules 1 and 2 to engage children with a focus on anatomy, ecology, and hands-on activities about the crabs and birds. Conversely, those interested in higher level exploration of the issues and impacts on the human use and management side of this topic may wish to focus their studies on the material provided in Modules 3 and 4.

Video segments are used to introduce all four modules and to supplement specific lessons where appropriate. In the earlier modules, these clips provide background, context, and a "feel" for what it is like to be out on the beaches experiencing the birds and the crabs. In Module 4, video segments are infused throughout the lessons.

Since there is still much more to understand about this phenomenon, educators are encouraged to adapt these exercises or develop their own as new scientific data is published.

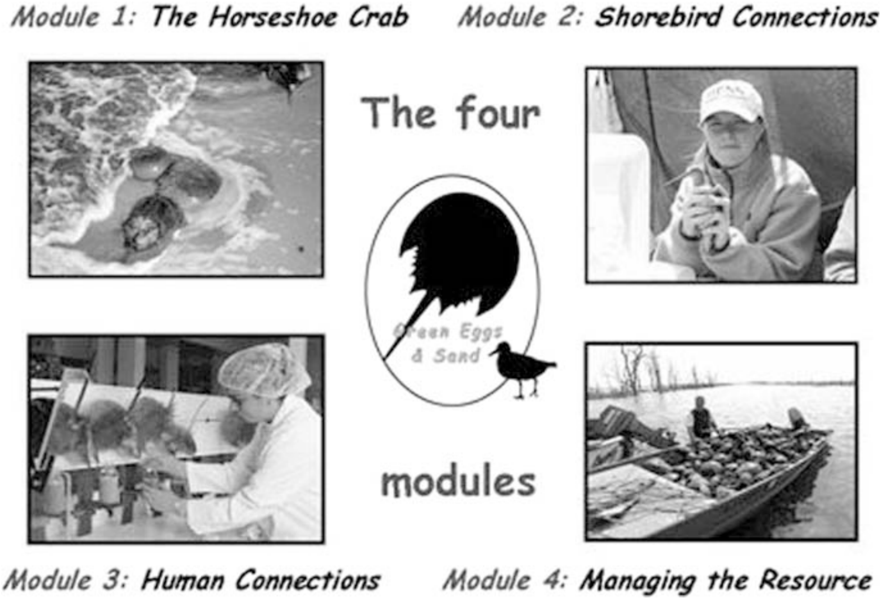


Fig. 2 The four Green Eggs & Sand learning modules

1.3 Module Highlights

Module 1, “The Horseshoe Crab”, provides a strong foundation for the curriculum. In addition to interactive lessons on horseshoe crab anatomy, life history, and ecology, one of the highlights of this module is the companion “Horseshoe Crabs in the Classroom” piece provided by the Maryland Aquatic Resources Education Program. As part of this project, teachers attend a 1-day workshop to receive training in the methods and materials needed to care for juvenile horseshoe crabs. In the classroom, students raise juveniles throughout the year, feeding them, collecting water quality data, and recording observations. Experiments are conducted on topics such as substrate, salinity, lighting, and feeding. Students are required to submit data from their experiments to a project manager. Maryland is currently the only state with a formalized program on rearing juvenile horseshoe crabs. Although the lessons can be conducted in any classroom, teachers must check with their individual states regarding regulations on the collection of eggs.

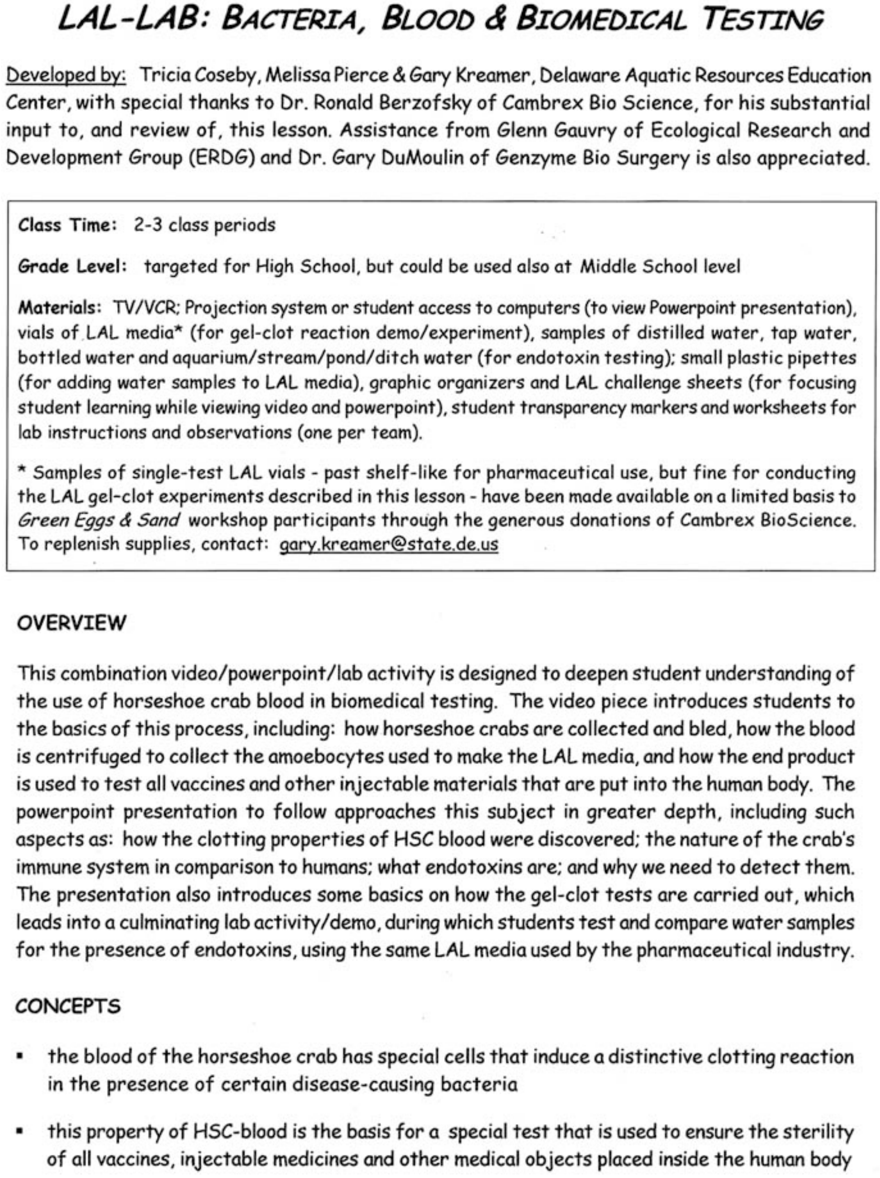
Module 2, “The Shorebird Connection”, introduces shorebirds into the curriculum. Several stimulating lessons on shorebird biology, natural history, and interactions with horseshoe crabs are offered. One of these, called the Red Knot Olympics, combines physical activities and mathematical word problems to promote numerous kid to bird comparisons and provides students with a more at-their-level grasp of the magnificent feats and amazing adaptations these migratory shorebirds exhibit during their long-distance flights

Module 3, “Human Use,” introduces man’s relationship to this natural phenomenon while also introducing social science activities such as history, economics, and statistics. It makes clear mankind’s increasing dependence on an animal that the larger ecosystem depends upon.

Module 3 is highlighted by a lesson entitled “Limulus Amebocyte Lysate Lab: Bacteria, Blood, & Biomedical Testing.” An accompanying video, power point presentation, and lab activity engage students in the use of a gel-clot test for detecting endotoxins in various waters. The lab uses past-shelf-life test vials of LAL donated by a biomedical company whose employees have served as expert presenters and contributors to the curriculum (Fig. 3).

Module 4 addresses how human use of a resource is managed when there is limited science and politics influences management. This module is noteworthy because the real data sets that fisheries managers grappled with to develop the management plan and the insights of actual stakeholders, scientists, and managers were used as material on the lessons on the horseshoe crab harvesting controversy. Ultimately, students are asked to examine their own values and beliefs regarding this resource by developing and justifying their own management plan.

The strong point of Module 4, “Managing a Resource”, is the wealth of video it uses to chronicle the horseshoe crab management controversy in the mid-Atlantic region over time. Video segments go hand-in-hand with lesson plans, weaving a story-line that plays off the various activities provided. An example



LAL-LAB: BACTERIA, BLOOD & BIOMEDICAL TESTING

Developed by: Tricia Coseby, Melissa Pierce & Gary Kreamer, Delaware Aquatic Resources Education Center, with special thanks to Dr. Ronald Berzofsky of Cambrex Bio Science, for his substantial input to, and review of, this lesson. Assistance from Glenn Gauvry of Ecological Research and Development Group (ERDG) and Dr. Gary DuMoulin of Genzyme Bio Surgery is also appreciated.

Class Time: 2-3 class periods

Grade Level: targeted for High School, but could be used also at Middle School level

Materials: TV/VCR; Projection system or student access to computers (to view Powerpoint presentation), vials of LAL media* (for gel-clot reaction demo/experiment), samples of distilled water, tap water, bottled water and aquarium/stream/pond/ditch water (for endotoxin testing); small plastic pipettes (for adding water samples to LAL media), graphic organizers and LAL challenge sheets (for focusing student learning while viewing video and powerpoint), student transparency markers and worksheets for lab instructions and observations (one per team).

* Samples of single-test LAL vials - past shelf-like for pharmaceutical use, but fine for conducting the LAL gel-clot experiments described in this lesson - have been made available on a limited basis to *Green Eggs & Sand* workshop participants through the generous donations of Cambrex BioScience. To replenish supplies, contact: gary.kreamer@state.de.us

OVERVIEW

This combination video/powerpoint/lab activity is designed to deepen student understanding of the use of horseshoe crab blood in biomedical testing. The video piece introduces students to the basics of this process, including: how horseshoe crabs are collected and bled, how the blood is centrifuged to collect the amoebocytes used to make the LAL media, and how the end product is used to test all vaccines and other injectable materials that are put into the human body. The powerpoint presentation to follow approaches this subject in greater depth, including such aspects as: how the clotting properties of HSC blood were discovered; the nature of the crab's immune system in comparison to humans; what endotoxins are; and why we need to detect them. The presentation also introduces some basics on how the gel-clot tests are carried out, which leads into a culminating lab activity/demo, during which students test and compare water samples for the presence of endotoxins, using the same LAL media used by the pharmaceutical industry.

CONCEPTS

- the blood of the horseshoe crab has special cells that induce a distinctive clotting reaction in the presence of certain disease-causing bacteria
- this property of HSC-blood is the basis for a special test that is used to ensure the sterility of all vaccines, injectable medicines and other medical objects placed inside the human body

Fig. 3 Opening page of the biomedical use-focused LAL-Lab lesson plan, from Module 3 of the GE&S curriculum

of this is the "Identifying the Stakeholders" lesson, where after watching "talking head" clips of 10 real-world stakeholders in the horseshoe crab resource, students are challenged to use an accompanying worksheet to identify the role, values, and points of view that each of those people represent. Module

4 video clips include (1) interviews with scientists, stakeholders, and managers; (2) chronological highlights of the management process and the challenges it entailed; and (3) a closing piece that showcases approaches that are helping address the conservation need.

2 Elements of Success

2.1 A Management Module

A major strength of the GE&S curriculum, which sets it apart from many other environmental education materials, is that one module is dedicated specifically to the management aspects of a natural resource issue. In doing so, GE&S does not advocate or instruct its users to follow the views of a certain "side" of the story, but rather presents the differing viewpoints of all stakeholders and stresses the importance of gathering rigorous scientific data to base decisions. The management module addresses the challenges faced by managers seeking to balance the needs of diverse user groups and encourages students and teachers to understand and become involved in public processes that provide input to the management of natural resources.

2.2 Expert Involvement

One of the key elements of GE&S has been the ongoing interaction of scientists, stakeholders, and educators. Biomedical professionals, commercial fishermen, fisheries and natural resource managers, environmental educators, and horseshoe crab and shorebird researchers have all contributed to GE&S. They made presentations at workshops and contributed to video pieces. They have provided data and information useful to lesson plan development and have reviewed lessons for accuracy and fairness. Their dedication, passion for their field, and willingness to share their expertise with educators continues to play a vital role in the success of GE&S.

2.3 Using "Real" Data

Hand-in-hand with the expert involvement came the utilization of "real" scientific data in the curriculum. Students use data provided by the experts to learn about trends, look for flaws in data and data collection, and learn how data are used to guide management strategies. The same data sets managers struggled with in developing the fisheries management plan for the horseshoe crab are used for these exercises – we do not "fix" the data to meet our needs or show a certain story.

2.4 Training Workshops

From its inception, the GE&S project team has held that the only way to obtain a complete copy of the GE&S curriculum is to attend a GE&S workshop endorsed by the team. This is due in large part to the complexities of the horseshoe crab/shorebird phenomenon and controversy. Teachers who are immersed in the issues through field experiences, get hands-on training in the use of the curriculum and hear from topic experts are much better suited to teach this curriculum in an accurate and balanced way. The curriculum is not sold. A modest registration fee covers some of the costs, and the balance is covered with operational funds from the GE&S project team's respective agencies. Workshops are open to classroom teachers and non-formal environmental educators, with a focus on middle and high school levels.

2.5 The Importance of Video

The GE&S curriculum is rich in video material. Each of the four modules opens with an introductory video clip, followed by multiple supplementary segments that complement, enrich, and provide focus for the respective lessons within the modules. Video segments are particularly important in Module 4, where specific clips capture the real-life chronology, stakeholder perspectives, and numerous factors that have and continue to drive efforts to manage the horseshoe crab resource. In crafting the curriculum, its developers realized the challenges that many classroom educators have with arranging field trip opportunities. Video allows students to see the horseshoe crab/shorebird phenomenon first hand and "meet" some of the players involved in the issues. In certain lessons, video is used to simulate real-world field experience, such as in Module 2: "Be Shore About Your Birds" where students identify and quantify flocks of shorebirds using a dichotomous key, while watching video footage of shorebirds on the beach. While not a primary objective of using video, the developers have now recognized the value of students seeing "real" scientists in action, steering them away from the stereotypical lab coat scientist, to one who might go to work in sneakers and cutoff shorts.

2.6 Curriculum Standards

In today's standards-and-testing-focused educational landscape, the curriculum developers recognized the importance of addressing education standards in the lesson plans. Due to the regional/cross-states interest in this curriculum, the decision was made to forego making correlations with individual state curriculum standards in favor of using the national ones. These were completed by an independent consultant with expertise in applying the standards and are presented in easy-to-view formats at the beginning of each module section, as well as in the appendix to the overall curriculum. The lessons were correlated to National Education Standards for Math, Science, Social Studies, and Language Arts for the middle and high school levels. Individual states have begun to correlate the curriculum to their standards as funding has allowed.

2.7 Tracking, Assessment, and Evaluation

Tracking, assessment, and evaluation help to improve GE&S and to document success and effectiveness to current and potential funding sources. Pre- and post-tests are given to training workshop participants to assess their level of knowledge at the start of the workshop and document change through the workshop experience. These participants also complete a workshop evaluation to comment on presentations, field experiences, and logistical items. The curriculum includes evaluations for each individual lesson and for the module as a whole. In 2005 and 2006, the GE&S project team conducted a survey of past workshop participants to determine the extent of the use of the curriculum. The 2006 survey of persons trained in the GE&S curriculum showed that in that year alone GE&S activities reached over 22,000 students.

2.8 Supporting Pieces

The success of GE&S has spawned several opportunities to offer enriching pieces to the curriculum. In 2004, special grant funding from the US Fish and Wildlife Service "Eco-Teams" enabled the GE&S team to produce a high-quality, information-packed horseshoe crab poster. The quality of this effort was much enhanced by partnership with the Ecological Research Development Group (ERDG), a nonprofit dedicated to the conservation of the world's four remaining species of horseshoe crab. Thousands of copies of this poster were reproduced and have been distributed through special events, educational conferences, the ERDG web site, and other outreach venues. Also during 2004, the curriculum and video segments were converted to a CD/DVD, product which is now distributed to workshop participants in lieu of a notebook and VHS tapes.

In 2006 the project team produced a special learning and tribute piece featuring Dr. Carl N. Shuster, Jr., who was instrumental in the development of the project, participating in all but one of the GE&S training workshops. The piece entitled "Dr. Carl Shuster and the Horseshoe Crab" is distributed as a learning tool for teachers who attended a workshop, met Carl, and want to share his extensive knowledge of horseshoe crabs with their students.

In the spring of 2007, a GE&S Sampler CD was created to spark additional interest in the curriculum and to give interested persons a "taste" of the lessons. Background information about the curriculum and a sample lesson from each of the first three modules is included on the Sampler CD. The intent is that the Sampler CD will encourage educators to attend a full GE&S workshop.

3 Looking Toward the Future

The GE&S project team has tentatively set three workshops for the spring of 2008: Georgia, Delaware, and New York. The curriculum is now 7 years old and the group is looking to update and add to the modules in the years ahead. Opportunities for partnerships across the United States will also continue to be explored as a vehicle for strengthening and expanding the project's reach. For more information on any aspect of the Green Eggs & Sand workshop and curriculum, contact any of the GE&S team members/authors.

Community Building: An Integrated Approach to Horseshoe Crab Conservation

Glenn Gauvry

Abstract Throughout the world, many marine species that come to shore to breed face increased risks from human activity, ranging from coastal development, pollution, and human-induced erosion to the harvesting of individual animals at a time when they are most vulnerable.

The regulatory process can mitigate a portion of this risk, but it is difficult to regulate human behavior, particularly when those being regulated are not in agreement over the necessity of such regulations and when such regulations are perceived to interfere with economic growth or individuals' livelihoods. In addition, the regulatory process often alienates the very communities that are most able to assist in the stewardship of the natural resource.

From its inception in 1995, the Ecological Research and Development Group (ERDG) has recognized the importance of building an engaged community to achieve wildlife conservation. An engaged community, if given the chance, will far surpass the unimaginative "conservation through regulation" approach. An engaged community is more aware of the changes in the environment; is in the best position to educate neighbors and visitors; and is more likely to assist stranded or injured animals, help with scientific analysis, shape public opinion, and report infractions of regulatory statutes.

This chapter describes the tools and strategies ERDG has developed to promote the conservation of the world's four horseshoe crab species at a community level. These tools can be adapted to other situations around the world to help to develop community-based conservation initiatives that engender a spirit of cooperation and inclusion.

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1 Introduction

The world's four horseshoe crab species, with few exceptions, do not spawn within protected habitats. They come ashore where humans live, play, and work. Sometimes they are accepted, but more often they are exploited or even reviled; numerous horseshoe crabs die from stranding on beaches during the spawning season each year, and their carcasses create an odorous and untidy shoreline, often in areas where the value of luxury homes and vacation destinations depend on a "pristine" environment.

It is here, within these coastal communities, that horseshoe crab conservation can be most effective. Driven by science as well as economic considerations and strengthened by compassion for both the natural resource and the community, a conservation framework can be established.

Building on the belief that conservation is an informed individual's responsibility, which establishes the foundation for community-based conservation, the Ecological Research and Development Group (ERDG), a 501(c)(3) nonprofit organization, created *The Horseshoe Crab Conservation Network*^{TM}. This chapter outlines the network's multifaceted, cross-cultural initiatives designed to inform and engage individuals and organizations in the conservation of the world's four horseshoe crab species.

ERDG's Web site, www.horseshoecrab.org, forms the network's core, providing information about horseshoe crab species to hundreds of thousands of visitors annually. ERDG's exhibits and classroom tools reach thousands more. The power of expression from our young environmental stewards is vastly underutilized. ERDG's *Young Voices, Horseshoe Crabs and the Arts*™ program unites these voices throughout the world.

ERDG's *Just flip 'em!*® program was designed to bring attention to the thousands of spawning horseshoe crabs that die each year from being stranded upside down. ERDG's *Practical Conservation* program has been responsible for a substantial reduction in the use of horseshoe crabs as bait in the US whelk ("conch") fishery.

ERDG's horseshoe crab sanctuary program encourages coastal communities to declare their shared habitat a horseshoe crab sanctuary and promote species awareness to visitors and neighboring communities. To support community-based horseshoe crab conservation initiatives around the world, ERDG created *The Horseshoe Crab Conservation Fund*™.

2 The Programs

> *Never doubt that a small group of thoughtful, committed citizens can change the world. Indeed, it is the only thing that ever has.* Margaret Mead (Warner 1992)

Although fascination and admiration of a species may not form the base for good science or resource management, it is the primary motivator of community-based conservation. ERDG developed *The Horseshoe Crab Conservation Network*$^{\text{TM}}$ to spark admiration and fascination of the horseshoe crab and build community concern and conservation efforts for the species.

The Horseshoe Crab Conservation Network$^{\text{TM}}$ is a multifaceted, cross-cultural suite of seven integrated initiatives designed to inform and engage the broadest coalition of individuals, communities, organizations, and scientists in the conservation of the world's four horseshoe crab species.

2.1 Education and Outreach: Reaching Students, Communities, and Researchers on the World Wide Web

The power of the Internet to move information around the world is unequaled. Through ERDG's award-winning Web site (www.horseshoecrab.org), information and educational tools are placed in the hands of individuals, communities, and organizations who are actively engaged in the science and conservation of horseshoe crabs. The success of ERDG's site is measurable: in 2007, it had over 350,000 visitors and was linked to 1,500 other Web sites (Google 2007).

Topics addressed on the site include evolution, natural history, anatomy, medical uses, research, conservation, global distribution, and news. A special section of poems, tales, and images is also featured. ERDG's goal is to provide the world with the most comprehensive source of peer-reviewed information on the world's four horseshoe crab species.

The Web site also provides a platform for community interaction and cultural exchange. To further facilitate the exchange of information and ideas, ERDG is working to make the site multilingual. Although this will take some time, the growing support from ERDG's international partners makes this task more manageable.

A comprehensive Web site that offers tools to educators, students, and researchers can be a critical step to raising awareness and involvement, even when the individual citizens in a community of concern do not have access to the Internet themselves. A number of species (Bengal tigers, mountain gorillas) have local conservation groups committed to their protection, but worldwide attention for these species has been raised through the media, including the Internet, creating a *global* community effort for their conservation.

2.2 Expanding Beyond the Internet – Tools for Education

Although ERDG believes in the power of the Internet to move information around the world, information alone will only engage the most committed student. To reach the broadest possible audience, it is important to transform this information into compelling learning tools. To that end, ERDG has assisted in the development of interpretive centers and has designed exhibits, signage, and printed information. The world's only life-size teaching model that reveals the horseshoe crab's internal anatomy was designed by ERDG. These museum-grade models are used by schools, nature centers, and aquariums throughout the United States and Japan.

The DuPont Nature Center at the Mispillion Reserve, located at the north end of Slaughter Beach, Delaware, is situated on one of the most prolific horseshoe crab spawning beaches and migratory shorebird stops in the world. Thousands of visitors annually will learn about horseshoe crabs and shorebirds from the center's information. In addition to providing information content for the center's exhibits, ERDG assisted in the center's design and development.

ERDG continues to explore opportunities to transform its Web-based information and conservation message into compelling learning tools for all ages. Education through non-electronic means, such as public programs, can be tailored to any age group.

2.3 Young Voices™, Horseshoe Crabs, and the Arts

It is important, as focus is placed on the science of this remarkable mariner, that we do not lose sight of our compassion for these living beings, for it is compassion that lies at the heart of conservation. *Young Voices* is a program designed to explore, express, and nurture the compassionate heart. The power of expression from our young environmental stewards is a vastly underutilized resource.

Through ERDG's in-school program, annual juried art competition, traveling art exhibition, and Web-based digital art gallery, these voices are united throughout the world in celebration of the horseshoe crab. ERDG's in-school program uses the science of horseshoe crabs as a means to evoke artistic expression, creating a bridge to the annual juried art competition. Thousands of students have contributed to this collective voice, sharing their heartfelt expression through poems, stories, images, and musical compositions.

ERDG is presently developing online lesson plans to help teachers to replicate this program in areas beyond the reach of ERDG's staff. Through ERDG's traveling art exhibition and digital art gallery, these *Young Voices* and the conservation message that lies within them can be heard around the world.

2.4 Practical Conservation: Reducing Human Impact and Engaging the Broadest Coalitions

This initiative is designed to build conservation partners, not enemies, out of conflicting user groups. ERDG seeks common ground and innovative solutions to reduce the detrimental effects of human activity on the world's four horseshoe crab species. This is accomplished by creating an interface between the various interest groups and the science and technology associated with the issue at hand.

It is essential to the success of any conservation plan that the concerns of the community regarding its role as a conservation partner be addressed. These concerns can be as simple as providing community-wide education and assisting in the development of conservation plans, land use issues, logistics, etc. or as complex as finding economic incentives and/or solutions to address the loss of income to communities that may depend on the resource for all or part of their livelihood.

ERDG was responsible for initiating the first study to test the effectiveness of bait bags in reducing demand on horseshoe crabs as bait in the US whelk fishery (ASMFC 1999). Working with the Virginia Institute of Marine Science (VIMS), it was shown that bait needs could be reduced by half if placed in a bait bag (Fisher and Fisher 2000). To promote this initiative, ERDG manufactured and distributed, free of cost, over 15,000 bait bags to whelk fishermen along the Atlantic Coast of the United States (Germano 2003). In addition, ERDG is actively facilitating dialog between various interest groups toward the development of alternative bait.

In 2004, ERDG organized an alternative bait and gear workshop, which was held in Baltimore, Maryland, and sponsored by the Atlantic States Marine Fisheries Commission (ASMFC). The purpose of the 2-day meeting was to bring together watermen, fisheries managers, researchers, distributors, and *Limulus* amebocyte lysate (LAL) manufacturers from the Atlantic Coast to share ideas, designs, and strategies that would reduce the need for horseshoe crabs in the conch and eel fishery. The net result of the meeting was an outline containing many sound ideas in terms of gear and bait alternatives that, if financed, could lessen the pressure on the horseshoe crab resource without the need for additional regulatory restrictions (ASMFC 2004).

Although it is easy for people to take sides in any conservation issue, it is far more effective and productive if all sides can work together to find ways to solve a problem. It requires more time, effort, and innovation, but in the end, it helps to build a cooperative community of problem solvers who will readily accept the solutions they themselves have had a hand in creating.

2.5 Backyard Stewardship™: Coastal Communities Define Their Shared Habitat as a Horseshoe Crab Sanctuary

The future survival of the world's four horseshoe crab species will ultimately depend upon the preservation of spawning habitat – a challenging prospect in light of the ever-increasing human density along the same beaches horseshoe crabs rely on for propagation. ERDG's community-based sanctuary program was designed to encourage coastal communities around the world to declare their shared habitat a horseshoe crab sanctuary and promote awareness of the species to visitors and neighboring communities.

Since the program's inception in 1998, ERDG has discovered that regardless of what initially motivates a community to become a horseshoe crab sanctuary, as they begin to embrace this idea of a shared habitat, a paradigm shift occurs. It begins on an individual level, as residents become educators to their friends and neighbors who visit them, and while they walk along the beach and come in contact with this remarkable animal. Eventually, the community as a whole begins to embrace the idea that they are stewards of their environment and this is one species that should be included in that stewardship.

Compassion for all living beings lies at the heart of ERDG's conservation philosophy. *Backyard Stewardship* is a continuation of the process begun with *Young Voices*. Both are designed to explore, express, and nurture the compassionate heart. As of 2007, six Delaware communities are participating in ERDG's horseshoe crab sanctuary program: Broadkill Beach, Prime Hook Beach, Fowlers Beach, Slaughter Beach, Kitts Hummock, and Pickering Beach. To date, ERDG's community-based horseshoe crab sanctuary program has protected over 15 miles of some of the most productive horseshoe crab spawning beaches in the world. In addition, ERDG has just finished a documentary designed to help coastal communities establish horseshoe crab sanctuaries in the state of New Jersey. Ultimately, ERDG plans to expand this documentary to assist coastal communities throughout the spawning range of horseshoe crabs worldwide.

Any community can decide that it wants to protect habitat for one or more target species; all it takes is one person or group to be the first. In some areas, an economic benefit must be shown before a community agrees that a habitat is worth saving, but the recent boom in ecotourism is proof that many habitats have enormous value.

2.6 Just Flip 'em!®

This program is designed to bring attention to the hundreds of thousands of horseshoe crabs (*Limulus polyphemus*) that die each year from stranding during their yearly spawning ritual. It encourages individuals, through a simple act of compassion, to take the time to assist and appreciate these remarkable creatures, which will not survive public indifference.

Studies have shown that as much as 10% of the spawning horseshoe crab population dies each year from being stranded upside down during spawning (Botton and Loveland 1989). The success of this program is a measure of how effectively it engages individuals and communities behind the conservation of their shared resource.

Sometimes, all it takes is one simple action to make a difference, and if one person can do it instead of walking by with a blind eye, then others will also see that they can do it, too.

2.7 The Horseshoe Crab Conservation Fund^{TM}

To build upon the success of the horseshoe crab sanctuary program and to find a vehicle to expand the program beyond ERDG's physical reach, ERDG created the *Horseshoe Crab Conservation Fund*$^{\text{TM}}$, which is an endowment fund designed to support community-based horseshoe crab conservation initiatives around the world. Through partnership with the scientific community, *The Horseshoe Crab Conservation Network*$^{\text{TM}}$ has the potential to inspire and assist individuals, communities, and organizations to solve problems, change behaviors, and promote sound decisions in the conservation of the species. Although the fund is operational, its financial resources are small. Currently, ERDG is using the funds to assist communities who have declared themselves horseshoe crab sanctuaries in creating interpretive signs and educational materials and in developing plans to protect the community's natural resources. Ultimately, several mini-grants each year will be awarded to communities around the world who are engaged in the conservation of their horseshoe crab species.

While fund-raising can be one of the most difficult challenges for any organization, even small amounts of money used for community education can have an enormous impact.

3 Summary

Throughout the world, marine species that come to or near the shore to breed, be they horseshoe crabs, sea turtles, seals, fish, or pelagic birds, are at an increased risk from human activity. Many of these species suffer simply from the lack of awareness borne from ignorance, indifference, and/or intolerance. Their survival has yet to be elevated to a level of concern, much less a level of regulation. In many parts of the world, the human struggle for survival, which is often dependant on these species, overshadow conservation concerns. For these animals, community-based conservation efforts represent their best hope for continued existence.

In community building, the key to success is advocacy from within the community. One respected and committed individual is all that is required to build a movement. It is not necessary to begin with the community most strategically placed in relationship to the natural resource, for as the movement grows, it will eventually encompass neighboring communities and, eventually, the full range of the habitat.

There is a considerable amount of detail associated with *The Horseshoe Crab Conservation Network*$^{\text{TM}}$ that cannot be explored in the body of this document. However, it is a growing and viable network with the capability of serving the global community committed to the science and conservation of the horseshoe crab. Although specifically designed to promote the conservation of the world's four horseshoe crab species, we believe these tools and strategies can be tailored to other species and communities around the world where humans and wildlife share the same habitat, particularly – as in the case of the horseshoe crab – species that lack the visual aesthetics and behaviors to awaken the compassion of their potential stewards.

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Abstracts of Additional Papers and Posters Presented

Horseshoe Crabs in New Haven Harbor: An Initial Study of Reproductive Biology and Beach Quality

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We studied the reproductive biology of horseshoe crabs during the 2006 breeding season in New Haven Harbor (NHH), an urban estuary on the Connecticut shore of Long Island Sound. Three principal spawning areas in NHH were identified. About 82% of the animals were found at Sandy Point, with much lower breeding activity at Long Wharf (13%) and Morris Cove (4%). Extensive bulkheading of the inner harbor precludes any horseshoe crab spawning, while the eastern shore of NHH (except for Morris Cove) is unsuitable because it is too rocky. Peaks in spawning activity were not linked to lunar phase, in contrast to several previous studies of horseshoe crabs. Overall, females outnumbered males by a 2.6:1 ratio on NHH beaches. Females were always part of a mated pair, and most of the unpaired males were single individuals patrolling the shoreline in search of females. Tagging provided evidence for cross-harbor movements of horseshoe crabs, and for migrations between NHH and other embayments in Long Island Sound. Males (mean carapace width = 19.9 cm) and females (25.9 cm) were similar in size to populations in western Long Island Sound and the New York Bight, but significant larger than individuals north of Cape Cod. Sediments at all three spawning beaches were comprised primarily of medium to coarse sands with <1% organic carbon (measured as loss on ignition), suggesting that they are probably suitable for successful egg development. Future studies will be necessary to determine if petrochemicals or other environmental contaminants in NHH might affect developmental success.

Use of a Baywide Radio Telemetry Array to Study Horseshoe Crab *(Limulus polyphemus)* Spawning Behavior

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We studied the distribution and spawning behaviors of horseshoe crabs throughout Delaware Bay by tracking radio-tagged horseshoe crabs using 14 receiving/data logging stations arrayed along 125 km of shoreline. A total of 437 horseshoe crabs were tagged during April and early May of 2004 (160 females, 60 males) and 2005 (217 females). Digitally encoded radio transmitters had a battery capacity of approximately 2 years. Transmitters were attached to the dorsal surface of the prosoma via a hook and loop harness and quick-set adhesive (cyanoacrylate). Seventy-five percent of tagged individuals were relocated within each spawning season, and 44% of the individuals tagged in 2004 were also tracked during the 2005 season. We compared timing and duration of signal receptions to tide schedules to classify behavior as spawning, staging, stranding, or mortality. Regardless of sex, animals with lightly worn carapaces tended to start spawning later and to finish earlier in the season than those with moderately or heavily worn carapaces. Between seasons, females were likely to return to the same side of the bay (85% returned, $p = 0.0001$), but males returned to the same side of the bay at a rate not significantly different from random mixing (60% returned, $p = 0.85$). Only 34% of females returned to spawn within telemetry range of the previous year's receiver station, but females were five times more likely to do so than males. Horseshoe crabs tagged with radio transmitters were recaptured at a high rate, were tracked continuously throughout the spawning season, and provided valuable insight into spawning behavior in Delaware Bay.

The Relevance of Environmental Cues to the Temporal Partitioning of Behavior in the American Horseshoe Crab, *Limulus polyphemus*

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While American horseshoe crabs have long been known to be tidal breeders, only recently have they been shown to exhibit endogenous circatidal rhythms. We have used both activity chambers and "running wheels" to address the conditions under which these circatidal rhythms are expressed and the cues

that are relevant for the synchronization of these rhythms. In the lab, expression of tidal activity rhythms is largely dependent on temperature: these rhythms are most apparent when water temperatures are above, but not below, 15°C. The expression of the rhythms appears to be independent of photoperiod: neither “winter” nor “summer” photoperiods allow expression of circatidal rhythms when temperature is below 15°C. Water level changes delivered at periods of 12.1–12.4 h are very effective in synchronizing these rhythms while temperature cycles and/or current changes delivered at similar periods have little apparent impact on the rhythms. In the field, animals housed in running wheels in an estuary and exposed to rising and falling water levels expressed clear tidal rhythms while animals that were exposed to the identical conditions, but without tidal changes in water depth, were largely diurnal, with little evidence of tidal periodicity. Thus, seasonal mating during high tides is likely to result from an endogenous tidal clock that is activated above a certain temperature and is synchronized by changes in water depth coincidental with naturally occurring tidal cycles.

Artificial Breeding of Horseshoe Crabs in Hong Kong

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Results of a recent territory-wide survey showed that the density of juvenile horseshoe crabs (*Tachypleus tridentatus*) on nursery shores in Hong Kong has significantly reduced since 2002. To enhance the survival and re-stock the population density of the juveniles, an artificial breeding study using the eggs and sperms from dissected mating pairs was conducted in the laboratory in 2004. Successful hatching of horseshoe crab trilobites was achieved. However, mortality after hatching varied over the time, with occasional high mortality even under administration of antibiotics. By comparing the effect of a series of combinations of four salinities (15, 20, 25, and 30%) and three temperatures (20, 25, and 32°C) on the survival and hatching rates of horseshoe crab eggs, results showed that horseshoe crab eggs can tolerate a wide range of temperatures (20–32°C), and salinities (20–30%). No hatching, however, could be observed at the salinity of 15%. Although the survivorship at low temperatures and low salinities were relatively high within the 90-day study period, developmental and hatching rates were reduced at these low temperature and salinity ranges. Thus, in considering the successful hatching rate of the horseshoe crab eggs, 32°C and 30% would be the optimal environmental conditions for artificial breeding practices. Preliminary experiments on alternative artificial breeding methods using electrical stimulation for collection of horseshoe crab eggs and sperms were also performed. Several successful trials were undertaken and further studies are planned to ascertain the applicability and repeatability of this electrical stimulation method.

Traditional Uses of Horseshoe Crabs in Malaysia and Thailand

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Three species of horseshoe crabs, *Carcinoscorpius rotundicauda, Tachypleus gigas,* and *T. tridentatus* are found in Malaysia. While only the first two species are found in Thailand. Commonly known as “Belangkas” (Malaysia) and “Mengda Thalle” (Thailand), these horseshoe crabs are being utilized mainly as food. Usually the females are consumed for their eggs, even though there are times that males are also consumed for the little meat they had on their appendages. In Malaysia, one female cost RM2 (USD 0.50), while it can fetch as high as Baht120 (USD 3) at the local market in Thailand. Local fishermen through many generations believe that consuming horseshoe crabs eggs and blood will bring much good to one’s health and can cure some diseases. Death cases were reported upon consumption of one particular species, the mangrove horseshoe crab, *C. rotundicauda*, but it does not deter the locals from consuming these invertebrates. Other usages of horseshoe crabs are as baits for fish traps and their helmet-shaped carapace as decorative ornament.

Captive Spawning and Juvenile Culture of *Limulus polyphemus*

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The *Limulus* populations along the central and northeastern Atlantic Coast of the United States have undergone a decline in recent years, while the demand for the organism for biomedical and bait purposes increases. Studies were conducted testing whether or not it was possible to induce *Limulus* adults to spawn in captivity. Various factors were tested for their importance, including sediment grain size, tidal height, light, tidal regime, diet, and male:female sex ratios. These studies resulted in the successful mating of *Limulus* adults and the production of thousands of eggs. The eggs were allowed to develop under normal conditions in a laboratory setting and had an initial hatching rate of approximately 60% over a 2-month period.

Additional studies were undertaken to determine and evaluate the effects of different food sources on the growth and survival of post-hatch *Limulus polyphemus*. Post-hatch molted *Limulus* juveniles were fed one of three food items (newly hatched brine shrimp, rotifers, or concentrated dried food flakes). Juveniles were checked everyday and secondary molts or deaths were noted. It was observed that second and third post-hatch molt and survivorship varied with diet. The results from these initial studies suggest that food quality and type have only a slight effect on the two earliest post-hatch molt stages of *Limulus polyphemus* juveniles but a strong effect upon the third post-hatch molt.

Results and Benefits of a Coordinated Atlantic Coast Tagging Program for Horseshoe Crabs

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A coast-wide horseshoe crab (*Limulus polyphemus*) tagging program was established by the US Fish and Wildlife Service in 1999. Over 80,000 external disc tags have been attached to horseshoe crabs along the US Atlantic Coast from South Carolina to Massachusetts. Tags were applied by horseshoe crab researchers (61.9%), biomedical companies (35.5%), and volunteer tagging programs (2.5%). Over 8,000 horseshoe crabs have been recaptured (10.0%), with individuals being reported up to 13 times and crabs also being reported up to 7 years after release. Most recaptures are reported by researchers working with horseshoe crabs (55.0%) and by the general public encountering crabs while on the beach (33.9%), followed by biomedical companies (5.7%), and commercial fishermen (5.5%). Data from recaptures have indicated that there are several discrete spawning populations of horseshoe crabs along the Atlantic Coast. Directed studies using tagging information have also provided information on spawning frequency, spawning site fidelity, and population size. Reporting rates for horseshoe crabs are unusually high for an aquatic organism, which may be related to the ability to tag and recapture horseshoe crabs while spawning on the beach without specialized or expensive sampling equipment. One quarter of all tagging (24.7%) and most recaptures (88.0%) occurred with spawning horseshoe crabs while they were on the beach. The annual cost to manage the tagging program (not including expenses related to tagging crabs in the field) is about $20,000 USD. This estimate includes staff time, purchasing tags, and sending rewards (cost ~$5 USD) to individuals reporting tagged crabs.

Discovering the Differences in Heat Shock Protein Expression in the Development of *Limulus polyphemus*

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Heat shock proteins are proteins involved in a diverse array of chaperone functions in cells. Such functions include the folding of newly made proteins and the refolding of proteins denatured by changes in temperature or by other stresses. We followed the expression of a superfamily of the heat shock protein Hsp70 through the developmental stages of *Limulus polyphemus*: ova before and after fertilization, the first instar (trilobite), and second instar stages. We tested the hypothesis that a greater amount of Hsp70 was needed in the later developmental stages of *L. polyphemus*. Proteins were extracted by a non-ionic detergent, and Western blotted from SDS-PAGE gels. The membranes were probed with an antibody that detects both constitutive and induced Hsp70's. Many bands of varying intensity (170, 125, 82, 66, 41, and 34 kDa) were recognized by the antibody, but the most intense band at ca. 70 kDa was labeled in both unfertilized and fertilized ova in both the disulfide–reduced samples and the "unreduced" samples. There was an increase in the amount of Hsp70 in the fertilized eggs as compared to the unfertilized eggs. Another striking difference was the appearance of a 21 kDa band following reduction of the proteins of the unfertilized egg sample. The significance of these changes still has to be investigated, as well as possible changes in the first and second instar stages.

The Use of Bait Bags to Reduce the Need for Horseshoe Crab as Bait in the Virginia Whelk Fishery

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The preferred and most effective bait in the Virginia whelk (conch) trap fishery is the horseshoe crab (*Limulus polyphemus*). Virginia fishermen alone used 1.4–1.5 million crabs in 2000 for bait in the whelk fishery. Leading producing states of horseshoe crabs for bait established harvesting quotas for crabs due to concerns of a declining population, which limited the number of crabs available for the whelk fishery. Measures were taken to reduce the fisheries reliance on horseshoe crabs, which included the testing of bait holding devices which could potentially allow for less horseshoe crab used per trap. A bait holding device (bait bag) constructed of rigid, plastic aquaculture mesh was tested in the

Virginia commercial whelk pot fishery. The theory was that if scavenger animals and trapped whelk could be kept from consuming bait placed in bait bags, then less bait would be needed without impacting catch. Horseshoe crabs were cut into halves, thirds, and quarters (treatment groups), representing reduction of one half, one third and one quarter of the traditional bait usage (control treatment). Three hundred and forty-six treatment traps, and 341 control traps were tested. No significant differences ($P>.05$) were observed in the number of whelk caught per pot using half the amount of bait traditionally used. Bait reductions of thirds and quarters demonstrated an overall significant ($P<.05$) loss of catch, however, in areas of low whelk densities catch was more equal to whole crab(s). The results suggest that less horseshoe crab bait could be used in the Virginia whelk trap fishery without a significant loss in catch, but overall catch declines with bait reductions below one half.

Preliminary Results on the Reproductive Activities of Horseshoe Crabs in Tampa Bay, Florida

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Many beaches suitable for horseshoe crab spawning exist in southwest Florida, yet no life history studies on horseshoe crabs have been conducted there. We began a study of reproductive activities of horseshoe crabs in Safety Harbor, Tampa Bay in April 2006. We sampled twice per week during April and May 2006; beginning in June 2006, we sampled on 8 days distributed through the lunar cycle. On most sampling days we started at the time of the daytime high tide; we have also conducted limited nighttime sampling. During peak abundance we placed 1-m^2 quadrats at the waterline equal distance apart; at other times we sampled the entire beach. Within each sampling area, we counted the number of horseshoe crabs, and for each individual we recorded sex, size, mating position, and carapace condition. We tested the effects of date, lunar day, tide height, water temperature, and wind speed and direction on abundance. We calculated sex ratios and compared sizes of individuals in different mating positions. We also initiated a tagging program to study repeat spawning, site fidelity, and switching of mating strategies within a season. Results indicate that lunar cycle, tide height, and some abiotic factors affect abundance; males are more abundant, but smaller than females; attached and satellite males do not differ in size or carapace condition; the number of satellite males is not correlated with female size; males return to the same beach more often than do females; and males generally do not change mating position within a season.

Bycatch Associated with the Horseshoe Crab Trawl Survey

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Fishery Ecosystem Plans (FEPs) incorporate the impacts of harvest on all organisms including bycatch. The horseshoe crab (*Limulus polyphemus*) fishery along the eastern coast of the United States currently has no FEP and little is known about bycatch. A trawl survey was designed to monitor horseshoe crab populations along the Atlantic coast. Using commercial benthic trawl gear, this survey can provide data to identify bycatch within the horseshoe crab fishery. Randomly selected sites were sampled for bycatch composition from New York to Virginia during the fall of 2005 ($n = 73$) and 2006 ($n = 83$). Fifteen-minute tows were conducted and all taxa were identified and counted. Little and winter skates (*Leucoraja* spp.) and horseshoe crabs made up the majority of the total biomass (86% in 2005, 91% in 2006). Clearnose skates (*Raja eglanteria*), horseshoe crabs, spider crabs (*Libinia* spp.), summer flounder (*Paralichthys dentatus*), and windowpane flounder (*Scophthalmus aquosus*) were most common throughout the survey area, present in more than 50% of tows. Preliminary cluster analyses indicate that bycatch composition varied between northern and southern sites. At the northern sites, horseshoe crabs were associated with little and winter skates, summer flounder, and windowpane flounder. At the southern sites, horseshoe crabs were predominately found with clearnose skates, knobbed (*Busycon carica*) and channel whelks (*Busycotypus canaliculatus*), and spider crabs. It is not known to what extent the horseshoe crab trawl fishery impacts bycatch species; however, identifying bycatch is the first step to developing an FEP and managing at an ecosystem-level.

Learning with Limulus – Tools for Teaching about Horseshoe Crabs in New Jersey

D. GRANT

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The Ocean Institute serves over 8,000 K-12, college and adult students each year in its marine science program at Sandy Hook, NJ. One of their most effective teaching tools is the horseshoe crab. Aspects of the 150-page project guide include: Horseshoe crab biology, embionts, teaching techniques, and a DVD of teachers participating in workshop and training activities.

Horseshoe Crab Research at Virginia Tech University

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The horseshoe crab (*Limulus polyphemus*) is an ecologically and economically important species in near-shore Atlantic coast ecosystems. Recent population declines can impact the biomedical industry, a bait fishery, ecotourism, and migratory shorebirds. The Horseshoe Crab Research Center performs research supporting science-based decision making, thereby promoting sustainability of the living natural resource. An annual, fishery-independent trawl survey conducted at selected sites along the Atlantic coast has indicated a stable population, although numbers of sub-adults may be too low to support population expansion at historic fishing levels. Patterns of genetic differentiation show distinctiveness of populations north of Cape Cod, from Cape Cod to Cape Hatteras, from Cape Hatteras to Atlantic Florida, along the Gulf coast, and on the Yucatan Peninsula of Mexico, suggesting that these populations will have to be managed as separate units. Analyses of additional spawning assemblages and commercial harvests are underway. Imperiled red knots and other shorebirds heavily exploit, but do not deplete horseshoe crab eggs on beaches of the Delaware Bay. Research results are regularly shared with the Atlantic States Marine Fisheries Commission and state marine resources management agencies to support regulatory decision making.

Factors Affecting Heat Shock Protein (Hsp70) Levels in Horseshoe Crab (*Limulus polyphemus*) Embryos: Effects of Temperature and Exposure to Copper

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We have examined the combined effects of two forms of stress, temperature shock and copper (a common pollutant in coastal environments) on Hsp70 in horseshoe crab embryos. Hsp70 was assayed in Western blots of SDS-PAGE gels with a mouse monoclonal antibody against bovine Hsp70 that recognizes both constitutive and induced Hsp70s. Embryos were exposed to 10 mg/L $CuSO_4$ for 24, 48, and 78 h at 22°C, and subsequently heat shocked at 35°C for 3 h. They were allowed to recover in the same solution for 0, 4, or 24 h at room temperature. Embryo viability was high in all heat shock and $CuSO_4$ combinations. Proteins were extracted in a non-ionic buffer with added protease inhibitors. The prominent band at ca. 70 kDa was preceded by a poorly resolved "shoulder" of lower molecular weight.

In all cases the highest total Hsp70 levels were found as an initial response (i.e. in the zero hour recovery groups) with lower levels in the 4 and 24 h recovery groups. A preliminary 2-D analysis of proteins of trilobite (first instar) larvae illustrated the complexity of the Hsp70 "family" in *Limulus*. It showed many poorly resolved spots with pI values between 5 and 6, but the same mass. It is possible that, in the 1-D analysis, two effects were masked by changes in different Hsp species.

The Investigation on the Resources of Horseshoe Crab in Fujian Province of China

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Several decades ago, there were plenty of horseshoe crabs distributed along the seashore of Fujian Province. The horseshoe crabs did not seem to require strict habitat. They had related to all aspects of civilian life. During 2005–2006, we conducted a project to investigate the resources of horseshoe crab in Fujian Province. We interviewed the native fishermen, observed the spawning habitats, and investigated the shipside and restaurants. The results showed that the numbers of horseshoe crabs are decreased sharply. In many places such as Qianqi in Fuding County, Shandu Island in Ningde City, the horseshoe crabs had disappeared for several years. We cannot see the horseshoe crab climb up the beach to spawn in Huangcuo Beach of Xiamen City, Meizhou Island of Putian City, and Pingtan Island. Now people cannot catch any horseshoe crab on the beaches, only catch a small quantity of horseshoe crab by trawling in shallow ocean. The resources of horseshoe crabs are becoming rare, so we have put forward a proposal to the government to set up conservation zone to protect horseshoe crab in situ.

Summary of Past and Future Horseshoe Crab Research on Cape Cod, MA (USA)

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In 1999, a collaborative among the University of Rhode Island, National Park Service, US Fish and Wildlife Service, and Massachusetts Audubon Society initiated research on the spawning populations of horseshoe crabs (*Limulus*

polyphemus) on Cape Cod, MA (USA). This 3-year effort (2000–2002) produced the first recent information on spawning densities, egg densities, and movement patterns (from tag-recapture data) within four Cape Cod embayments (Cape Cod Bay, Nauset Estuary, Pleasant Bay, and Monomoy National Wildlife Refuge). Additionally, movement patterns of bled and unbled female crabs were tracked using sonar tags during the summer of 2001 in Nauset Estuary. Throughout Cape Cod, spawning densities were lower than one crab 25 m^{-2} although certain locations had higher densities (three crabs 25 m^{-2}). Spawning indices ranged from 0 to 1.3 females 25 m^{-2}. Spawning sex ratios varied from 1 to 1.6 to 1 to 3.1 (females to males), except within Pleasant Bay where highly skewed ratios (1 to 5.8) were observed. Egg densities were less than 1 egg cm^{-2} and were higher in deeper sediments at most locations. Horseshoe crabs tended to remain within each embayment and most traveled less than 2 km from the tag location. Rate of movement between bled and unbled crabs was similar; however, bled crabs exhibited random movements compared to directional movements of unbled crabs suggesting bled crabs experienced more disorientation. Future spawning studies are planned for 2008–2010 and will involve an intensified telemetry effort within Pleasant Bay. This research has been published in Estuaries and Marine and Freshwater Behavior and Physiology.

Genetic Diversity in the Horseshoe Crab (*Limulus polyphemus*) and Implications for Management

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The horseshoe crab, *Limulus polyphemus*, is an important ecological and economic resource throughout the Atlantic coast of North America. Concern over perceived declines in abundance and consequent impacts to shorebird communities has prompted a need for management plans that will preserve this ecosystem relationship. Because effective management regulations require an understanding of population structure, we characterized temporal and spatial genetic variation of spawning *L. polyphemus* adults sampled from six beaches in Delaware and New Jersey. We used 12 microsatellite loci to compare allele frequencies of adults sampled during three peak spawning events corresponding to major lunar events during the spawning season (March–June 2004). Preliminary results indicate substantial genetic variation over all samples (average $H_o = 0.7659$, mean number of alleles per locus = 11.8), however, minimal (non-significant) differences were observed temporally or spatially among sampling periods or beaches. Correct classification to population of origin by maximum likelihood based assignment tests was only slightly greater than expected by chance alone (56% to state, 55% to sampling time period). Lack

of evidence for genetic population structure suggests that horseshoe crabs within Delaware Bay represent a panmictic population suitable for regulation as a management unit regardless of state and time of spawning.

Regional Differentiation and Sex-biased Dispersal among Populations of the Horseshoe Crab *Limulus polyphemus*

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Fourteen microsatellite DNA loci were surveyed in 1342 horseshoe crabs (*Limulus polyphemus*) collected from 27 spawning populations and 3 dredge or trawl samples from near- or off-shore locations sites ranging from Franklin, ME to the Yucatan Peninsula, Republic of Mexico. This range-wide survey revealed a high degree of genetic diversity (up to 50 alleles per locus) and heterozygosity (up to 97.2%). There appears to be substantial gene flow between each population and its nearest neighbors. The correlation of genetic distance and geographic distance supports isolation-by-distance as a mechanism underlying structure of *L. polyphemus* along the Atlantic coast of North America. Two statistical descriptors, mean assignment percentage and assignment variance, suggested the presence of male-biased dispersal throughout the study area. Within the continuum of isolation-by-distance and in the presence of male-biased dispersal, clustering of specific collections on a tree of genetic distances and hierarchical gene diversity analyses demonstrate discrete regional units. These results suggest the definition of five management units within the United States: Gulf of Maine (Maine and New Hampshire collections), Mid-Atlantic (Massachusetts to Virginia), Southeast (North Carolina, South Carolina, and Georgia collections), Florida-Atlantic, and Florida-Gulf. Multilocus assignment tests indicate a high probability of correctly assigning individuals back to the proposed management unit from which they were collected. Preliminary results suggest that the unique multilocus genotypes serve as a useful tool for identifying the region of origin of *L. polyphemus* caught at sea.

Clinical Applications of *Limulus* Amebocyte Lysate

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Bacterial endotoxin (bacterial lipopolysaccharide, LPS) is a component of the cell walls of all gram-negative bacteria. Endotoxin has a wide range of biological effects. The pathophysiological effects of endotoxin are believed to be associated

with many infections caused by gram-negative bacteria. Endotoxin has been shown to activate the cascade of components which constitute the blood coagulation, fibrinolytic, complement, and inflammatory cytokine systems. Endotoxin can produce fever and hypotension, the latter leading to shock. Therefore, there is potential benefit from determining if endotoxin is present in the blood and other body fluids of humans during infection. Until the development of the *Limulus* amebocyte lysate (LAL) test for bacterial endotoxins, no feasible and practical test for bacterial endotoxin was available. Multiple studies using the LAL test have demonstrated a relationship between the detection of endotoxin in blood and outcome in patients with sepsis, e.g., presence of hypotension and increased mortality. Studies of patients with the hemolytic–uremic syndrome have demonstrated a correlation between endotoxemia, activation of the complement system, and mortality. Extensive investigations of patients with systemic meningococcemia (caused by *Neisseria meningitidis*) have shown excellent correlations between the levels of endotoxin in blood and activation of blood coagulation, complement activation, and mortality. Measurements of endotoxin in blood have been used to evaluate the return of liver function in patients who have undergone liver transplantation, since the liver normally filters endotoxin from blood. Therefore, the *Limulus* test has been a valuable tool for better understanding of the role of bacterial endotoxin in human disease.

A Comparison of Sandy Beaches with Marginal Habitats for Spawning Horseshoe Crabs (*Limulus polyphemus*) in Lower Delaware Bay, New Jersey

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Sandy beaches are the dominant coastal feature of lower Delaware Bay, NJ. Open beaches within this range have been regarded as optimal habitat for spawning horseshoe crabs. The combination of global ocean rise and sinking landscape is causing dramatic changes in beach structure, thus creating a more heterogeneous bay shore. Marginal habitats (viz., other than open sandy beaches) have not previously been recognized as important for spawning. In this study, we examined a variety of shoreline types during the 2001 spawning season with the objective of characterizing their value to horseshoe crabs. Using egg density as an index of habitat suitability, we compared open bay beaches with nearby marginal habitats, such as tidal creeks and adjacent sandbars, erosional and bulkheaded beaches, and over-wash areas of sand landward of peat banks. Lowest egg densities were found on beaches that were strongly sand starved. Horseshoe crab egg densities in areas where sand was accreting, such as mini-deltas and embankments along tidal creeks, were generally comparable or superior to adjacent open bay beaches. Some of the highest egg densities in 2001 were found at two widely separated tidal creeks and their associated sandbars. Previous studies have indicated that tidal creek areas are hot spots for migratory shorebirds. We suggest that these habitats are deserving of special consideration by agencies concerned with the conservation and management of horseshoe crabs and shorebirds.

Identification of Horseshoe Crab (*Limulus polyphemus*) Migratory Pathways Using Telemetry and Archival Tags

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In this study, we developed methods to determine migratory pathways used by adult horseshoe crabs (*Limulus polyphemus*) as they move within Delaware Bay to spawning beaches. Thirty-four female horseshoe crabs were tagged with radiotelemetry and archival tags throughout Delaware Bay just prior to the spawning season of 2005. Six of the tagged females were recaptured physically when they came onto beaches to spawn and four of those had recoverable archival data. Archival tags recorded pressure/depth and temperature while the crabs were at large. Radio-tagged females were relocated repeatedly by fixed-station and manual receivers. So, release and spawning locations were known for each tagged female based on telemetry tracking. Archival-depth profiles along with known release and spawning locations were compared to bathymetric maps of Delaware Bay to determine likely migratory pathways. Two GIS-based models were applied. The first model determined a least-cost pathway where percent bathymetric slope was used as the cost function. The second model created a set of plausible pathways by comparing archival depth to bathymetric depth starting at the release point and moving toward spawning locations. Based on telemetry observations, movement rate was assumed to be on average <30 m per h. Migratory pathways identified using these methods provide new insight and understanding of habitat use during horseshoe crabs spawning migration in Delaware Bay.

Breeding Behavior and Movement Patterns of *Limulus polyphemus* in Long Island Sound: Results From a 10-year Tagging Study

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The breeding behavior of *Limulus polyphemus* was investigated over the past 10 years through a mark-recapture study conducted at Milford Point, Connecticut in Long Island Sound. A total of 10,376 crabs were tagged of which 40% were female. Six percent (663/10,376) of the total tagged have been recaptured. Of the total recaptures, 57% (379/663) were found on the beach within 2 weeks of tagging. Approximately 1% of the total individuals tagged at Milford Pt. were recaptured between 1 and 7 years later. Animals originally tagged at Milford were recaptured as far west as Brooklyn, NY, east to New London, CT, and across the sound at Eaton's Point. Sonar tracking data revealed that tagged horseshoe crabs remain offshore from where they originally spawned for several months. By November all 20 signals were lost from the search area. The following summer five sonar tagged individuals returned to the waters around Milford but were not recaptured on the beach. Horseshoe crabs do not exhibit strong site fidelity for particular beaches within or among seasons, however, there is evidence that some females lay more than one clutch within a season. The sex ratios of tagged and recaptured horseshoe crabs are both skewed toward males (1:1.5 and 1:1.8, females to males, respectively). Ninety-nine percent of spawning horseshoe crabs were found mating in pairs. This behavior contrasts with what is typically found in Delaware Bay where 44% of spawning females are in clusters of two or more males.

Fishery Management of Horseshoe Crab in New York

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Due to concerns about exploitation of horseshoe crabs (*Limulus polyphemus*), the Atlantic States Marine Fisheries Commission (ASMFC) developed a Horseshoe Crab Fishery Management Plan (FMP) in 1998. Addendum I was developed in 2000 to control exploitation. The Addendum established state-by-state caps on horseshoe crab bait landings which are 25% below reference period landings. Horseshoe crabs are harvested commercially primarily for use as bait in pot fisheries for whelks and eels. Small numbers are also used for biomedical purposes. New York's ASMFC quota was established in 1990 at 366,272 horseshoe crabs. Harvest greatly exceeded the quota in 1990, and the fishery was further restricted for 2 years while paying back the overage. In 2004, New York Department of Environmental Conservation (NY DEC) voluntarily reduced the horseshoe crab quota to 150,000 crabs. This voluntary quota has been exceeded during the past 2 years, coincident with increased restrictions on Delaware Bay harvest. NY DEC relies on a variety of fishery independent surveys to assess the status of horseshoe crab populations in New York waters. A number of these surveys were used for the 2004 ASMFC horseshoe crab stock assessment. In addition, NY DEC in conjunction with Cornell Cooperative Extension is developing a spawning survey to collect better information.

Delaware Bay Horseshoe Crab Spawning Survey

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The Delaware Bay horseshoe crab (*Limulus polyphemus*) spawning survey has been implemented annually since 1999 to provide a reliable index of spawning activity for comparing baywide spawning among years, estimating beach-level spawning within the Delaware Bay, and examining the spatial and temporal distribution of spawning crabs. The survey also strives to increase the understanding of the relationship between environmental factors and spawning activity and to promote public awareness of the ecological and economic importance of horseshoe crabs. Baywide spawning has remained stable since 1999 (slope = 0.01, SE = 0.013, 90% CI = –0.01 to 0.04), and annual estimates of spawning activity have been precise ($CV < 14\%$). Trends in spawning activity have differed by state with a decline in Delaware (slope = –0.03, SE = 0.013, $p = 0.04$) and an increase in New Jersey (slope = 0.06, SE = 0.023, $p = 0.05$). These state-specific trends were compensatory and could be a response to differences in state harvest strategies or a shift in spatial distribution. The percent of May spawning (important to migratory shorebirds) has been consistently higher in New Jersey than Delaware and associated with water temperature ($r_{DE} = 0.77$ and $r_{NJ} = 0.83$). The survey relies on a large contingent of volunteers who are instructed on survey methodology and the importance of the resource. The survey is an extremely valuable tool for monitoring the Delaware Bay horseshoe crab spawning population and highlighting the importance of this valuable resource.

The Ecology of *Carcinoscorpius rotundicauda* and *Tachypleus gigas* in Singapore

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Two species of horseshoe crab occur in Singapore: *Carcinoscorpius rotundicauda* and *Tachypleus gigas*. Over the last 200 years, mangrove forest cover, where *C. rotundicauda* occurs, has been reduced from ~13 to ~0.5% of Singapore's total land area. It has been suggested that abundances of *C. rotundicauda* have decreased over the last two decades while *T. gigas* is listed as vulnerable. However, to date, little research has been carried out on the ecology of either species in

Singapore. Preliminary observations have shown that horseshoe crabs occur in Kranji mangroves and Mandai mudflats, with juveniles being found in streams, mating pairs along the coastline in mangroves and eggs laid in the mangroves. Spawning aggregations have been observed at night at Sungei Buloh. Both species are present at Chek Jawa, Pulau Ubin, which was scheduled for reclamation in 2001, but which, at present, is being protected for as long as it is not required for development. The aim of the present proposed project is to (1) quantify the abundance, population structure, and sex ratios of each species in Singapore; (2) examine the timing and level of breeding, egg densities and identify any significant nursery areas; (3) study the behavior, in particular feeding activity, of juveniles and adults during low tide; (4) to carry out growth and tagging experiments; and (5) assess the role of horseshoe crabs in the food webs in Singapore mangroves. Ideally this research would be part of an integrated pan-Asian project, carrying out simultaneous parallel research, using the same methodologies.

Genome Analysis in the Ancient Marine Arthropod, *Limulus polyphemus* (horseshoe crab)

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Limulus polyphemus (North Atlantic horseshoe crab) occupies a unique phylogenetic position within the metazoan tree of life. It is one of the last surviving species in the class Merostomata and is often referred to as a “living fossil”. We have initiated a student-based analysis of the *Limulus* genome using a number of different experimental strategies. A cDNA library has been prepared from *Limulus* hemocytes and number of clones sequenced and submitted to the NCBI database. A total of 134 genes revealed that 28% were known *Limulus* genes, 18% had a significant match to genes from other species in the GenBank database, and 54% had no significant match (unknowns). A moderate-sized Bacterial Artificial Chromosome (BAC) library has been constructed and a number of end sequences have been generated from clones. Preliminary data reveal an average GC content of 40% and the presence of a number of microsatellite sequences. To investigate repetitive DNA organization in the *Limulus* genome we have identified a novel 350 bp tandemly repeated element (Hd350) that makes up approximately 5% of the *Limulus* genome. Significant sequence variation and clustering are seen within the Hd350 element family. To investigate gene structure in *Limulus*, we determined the genomic sequence for the antimicrobial peptide gene polyphemusin 1. The 2100 bp gene contains 4 exons, typical eukaryotic splicing signals and a significant GC% bias between coding and non-coding DNA. Analysis (~700 bp) of the 5' region of the polyphemusin gene reveals a 40 base purine–pyrimidine repeat 400 bp upstream of exon I.

Estimation of Nutrient Requirements for Atlantic Horseshoe Crabs (*Limulus polyphemus*; Linnaeus) Grown in Captivity

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Population declines of the Atlantic horseshoe crab (HSC) have led to conflict regarding management strategies among commercial fishermen, biomedical companies, and shorebird activists. These concerns and conflicts have also led to increased interest in determining the potential for horseshoe crab aquaculture. In this study, feeding trials were performed with juvenile HSC to quantify dietary energy and protein requirements in order to optimize feed formulation and feeding regimes. Adult horseshoe crabs were collected from Jamaica Bay, New York, induced to spawn, and the offspring were raised in recalculating aquaculture systems at AREAC. Initial trials were conducted to measure digestibility of two formulated feeds based on clam meal or fish meal. Juveniles were fed 3% of their body weight daily, and the fecal matter was collected hourly. Chemical analysis of the feces and feed included protein, lipid, and energy and ash. Acid insoluble ash was used as a non-absorbed reference substance to determine digestibility of the feeds. Apparent digestibility of protein was found to be 93.2 and 90.8% and energy digestibility was 83.7 and 87.5% for the clam meal and fish meal, respectively. Growth trials were conducted with HSC offering the diets at increasing feeding levels daily in order to estimate their requirements for energy and protein –information essential for successful horseshoe crab aquaculture.

Analysis of Adult Sizes and Mate Preferences in the Mexican Populations of Horseshoe Crab, *Limulus polyphemus*

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The Mexican Horseshoe Crab lives in the Yucatán Peninsula in the states of Campeche, Yucatán, and Quintana Roo. Unfortunately, human activity is

reducing its breeding habitat. In the adult stages, there is an evident sexual dimorphism: the females are larger than the males. Also, the males have his first pair of pedipalps modified into a grasping claw, which they use to grasp the female during amplexus. In the United States, an adult size latitudinal gradient of *Limulus polyphemus* has been observed. In order to study if this behavior also occurs in Mexico, we compare the size of adults in five populations along the Yucatán Peninsula: (1) Laguna de Términos, Campeche; (2) Champotón, Campeche; (3) -Yucalpetén, Yucatán; (4) Río Lagartos, Yucatán; and (5) Chiquilá, Quintana Roo. For both, male and females, we compare the ED = eye distance and the GD = genal distance. Our analyses show that the adult size varies depending on the locality, but there is no clear latitudinal correlation. Adults in Champotón are significantly larger than in the rest of the localities. This is the only population that is directly near the sea, while the others are inside lagoons. We also conducted a biometric analysis using mates in order to test whether there are or not mating preferences related to size. This analysis shows that there are no evident preferences for sizes during matting. These works hope to contribute to the knowledge of the populations of *Limulus* in Mexico in an effort to ascertain why the species has been dwindling and determine how we can better protect it.

Contribution to the Understanding of the Biology of the Horseshoe Crab *Limulus polyphemus* in the Yucatán Peninsula, Mexico

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Limulus polyphemus is currently endemic to North America, only present in the United States and Mexico. The populations of the United States have been well studied. They occur from Maine to Florida with the greatest reproductive activity occurring May–July. In contrast, little has been done to understand the biology of the Mexican populations. In this work, we present information about the Mexican populations, including a historical overview since the Pre-Columbian period, localities were *Limulus* can be found and some notes about its biology and reproductive strategies. In Mexico, *Limulus* was known by the Mayas, who depicted it in Pre-Columbian paintings. There are records reporting that the eggs were a food source to them. Today, *Limulus* can be found along the coast of the Yucatán Peninsula, in the states of Campeche, Yucatán and Quintana Roo. We present all the known localities from expeditions during 1985 and 1988. We analyze the possible mating localities and seasonal reproductive behavior, comparing them with the available information for the US populations. We present the results of our observations of reproduction and development in

captivity, including SEM pictures of different instars, describing our laboratory observations of a successful coupling incident at 60 cm depth (when a female released the ovules and a male fertilized them). These results add insight to the knowledge and understanding of the biology of the Horseshoe crab in Mexico.

Environmental Factors Triggering Spawning in the American Horseshoe Crab (*Limulus polyphemus*) in Northern New England: a Regression Analysis

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The American horseshoe crab, *Limulus polyphemus*, is a commercially and ecologically valuable species that recently became regulated by the Atlantic States Marine Fishery Commission. Species management requires monitoring that frequently includes population surveys during spring spawning. Data from such counts are highly variable and a variety of environmental factors are anecdotally believed to influence peak spawning events. Multivariate regression analyses were conducted to evaluate the role of water temperature, time of high tide, lunar phase, tide height, daily weather, and cumulative weather patterns on both the onset of spawning and on daily counts throughout spawning season. A model resulted that explained 60% of the variability in the onset of spawning ($R^2 = 0.6045$; adjusted $R^2 = 0.5550$). Statistically significant factors ($P<0.0000$) influencing the seasonal onset of spawning include lunar phase (a full moon or new moon), a minimum water temperature of 13°C, time of high tide (later in the day), tide heights that are less than the highest monthly heights, and cumulative weather patterns that have been sunny or mostly sunny for 3 or more days. Another model was developed to explain the variation in daily counts *throughout* the season that was statistically significant ($P<0.0000$), but only explained 35% of the daily variation in counts ($R^2 = 0.3589$; adjusted $R^2 = 0.3265$). Factors associated with peak spawning throughout the season were cumulative days of clear, sunny weather ($P<0.0000$), the time of high tide ($P = 0.0122$), a minimum water temperature of 13°C ($P = 0.0759$), and the height of the tide ($P = 0.0819$).

Intraspecific Variation in the Effects of Trace Metals on Late-Stage *Limulus polyphemus* Embryos

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Intraspecific variation in tolerance to multiple environmental pressures has been observed among several different species of marine organisms, including the horseshoe crab *Limulus polyphemus*. In order to determine the variation in response to heavy metal stress in the horseshoe crabs, the effects of short-term exposure to copper and zinc were investigated in stage 20–1 embryos from three different estuaries: Delaware Bay (DE), Sandy Hook Bay (NJ), and Seahorse Key in the Gulf of Mexico (FL). Animals were exposed to nominal metal concentrations from 0.01 to 1000 mg/L for 48 h and observed continuously until successful molting or death occurred. The population structure of *Limulus* was investigated in animals from nine estuaries spanning their habitat range along the Atlantic coast. Sequence data were obtained from three different regions of the mitochondrial genome, a 700 bp region of cytochrome oxidase 1, a 500 bp region of NADH dehydrogenase 6, and a 400 bp region of 12 S and the AT-rich non-coding region and will be used to determine the degree of connectivity between the estuaries. In addition, the affects of short-term exposure to sub-lethal concentrations of the metals on gene regulation will be investigated at the molecular level.

An Evaluation of a Horseshoe Crab (*Limulus polyphemus*) Beach Spawning Survey for Long Island, NY: Is One Sampling Method Suitable for the Region?

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We initiated a program to assess the relative abundance of spawning horseshoe crabs on Long Island (LI) beaches. LI has a large coastline with a diverse set of coastal estuaries, which presents a challenge for such sampling. We classified LI into three sampling zones: North shore (NS), South shore (SS), and Peconic estuary (PE) and employed a modified version of the Delaware Bay spawning survey. Site coordinators were assigned to six beaches and worked with volunteers on 15 nighttime, new/full moon high tides from May 11–July 14, 2005 and 2006. Horseshoe crabs were counted along a fixed 100 m transect in the “swash-zone”, sexed, and measured for size (mm). The survey protocol was effective at the SS beaches, but was not suitable for the NS and PE sites because sub-tidal spawning behavior prevented effective counting. Differences in tidal amplitudes and sediment composition (e.g., SS tidal amplitude $\leq$ 1 m, fine-grain sand: NS tidal amplitude $\geq$ 2.0 m, cobble & sand) could be causing differences in spawning behavior. Jamaica bay (SS) had the highest spawning counts (Avg. = 325), and counts were consistent between years with peak spawning in late May and early June. Sex ratios were 3:1 female to male. We conclude that the existing methods are effective in the SS, and with increased spatial coverage and possibly, tagging methods, should produce

reliable measures of relative abundance. The NS and PE both require exploration of other sampling methods that can produce more reliable data.

Conservation Planning for Endangered Species of Horseshoe Crab in Terms of Geomorphology of Habitats and Life History

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The relationship between the habitat conditions of the horseshoe crab *Tachypleus tridentatus*, one of the endangered species in Japan, and the artificial changes in a bay was investigated, taking Moriye Bay in Oita Prefecture as an example. A mitigation method for restoring the spawning sites of the horseshoe crab was proposed. Since the tidal flat has a vast area and is exposed for only a short period during the ebb tide, comprehensive field observation is difficult. Therefore, an investigation method using high-resolution aerial photographs taken at low altitude was developed. A precise classification of the surface of the tidal flat in terms of its micro-geomorphology was carried out. The impact of river flooding on the tidal flat was investigated by detailed surveys of the tidal flat, and the habitat conditions of the horseshoe crab were classified. In the lower Yasaka River, the straightening of the meandering part was carried out in 2000, causing an increase in the flood velocity at the river mouth bar, where the spawning site of the horseshoe crab was located, resulting in its disappearance. An appropriate alternative location of the spawning site was selected, and beach nourishment was carried out using the materials obtained from the nearest rivers to minimize the environmental impact due to the mixing of different species from other regions. New conservation and investigation methods for the horseshoe crab were proposed, taking their whole life history into account. The survey methods of the habitat were reconsidered, focusing on the fact that large-scale landform changes have entirely altered the geomorphologic features of the habitat.

Protection and Artificial Incubation of Horseshoe Crab *Tachypleus tridentatus*

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Horseshoe crabs are rare living fossils which are a marvel of nature and an important source of scientific study, as they have over 50 active biological materials in their blood. But they are facing a set of serious common and growing threats to their survival, including the erosion and man-made alteration of essential spawning habitat, coastal pollution, and overfishing. In China, the quantity of horseshoe crab, *Tachypleus trdentatus* is declining very rapidly.

After research of several years, it would be shown that artificial incubation and releasing of juvenile horseshoe crab, *Tachypleus tridentatus* back to the ocean are important and effective measures for saving and recovering the quantity of horseshoe crab's population. A substantial amount of research on conservation, developmental biology, and pharmaceutical value for horseshoe crabs in China has been carrying out since 1970s.

Sexual Size Dimorphism in Horseshoe Crabs (*Limulus polyphemus*): A Test of Competing Hypotheses

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Sexual size dimorphism is prominent in horseshoe crabs (*Limulus polyphemus*), but unexplained. We proposed and tested three competing hypotheses to explain how females become larger than males. The delayed-maturity hypothesis proposes that females remain as juveniles longer than males. The differential-growth hypothesis proposes that females grow at greater increments than males. The indeterminate-molting hypothesis proposes that females continue to molt as adults while males undergo a terminal molt. During 2003 to 2005 in Delaware Bay, we measured 9075 juveniles (out of 31,719 caught) and 36,273 adults. We analyzed size and estimated age frequencies to test consequences of the competing hypotheses. Females matured later than males. For juveniles that survived to age 9, the proportions of juvenile females that matured or migrated from Delaware Bay by age 10, 11, and 12 were 0.24 (0.00, 0.58), 0.54 (0.00, 0.86), and 0.98 (0.71, 1.0), respectively. Those proportions for males were 0.39 (0.09, 0.66), 0.99 (0.98, 1.0), and 1.0. Differential growth failed to remove lack of fit between female and male size frequencies ($P < 0.0001$). Modeling size-frequencies as finite mixture distributions indicated that one size distribution encompassed $\geq 90\%$ of adult female prosomal widths (PW). The ratio of median PW for adult females to males was 1.26, and the ratio of PW for the largest adult to juvenile female was 1.28. Both ratios are near the expectation for 1 molt increment. We conclude that sexual size dimorphism in horseshoe crabs is predominantly due to delayed maturity of females. However, because of a low frequency of females in the 200–240 mm size range (i.e., 11 year old), we hypothesize that females, but not males, migrate from Delaware Bay as juveniles and reach maturity in the ocean.

Evolution of Body Size in *Limulus polyphemus*

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Adult body size is an important organismal trait because it is a key determinant of the ecology, physiology, and life-history of an individual. Despite this, patterns of body-size distribution and the selection pressures (e.g., temperature, predation) underlying them are not well understood. For example, an increase in size with latitude (Bergmann's rule) is commonly seen in some species, while the reverse pattern (converse Bergmann's rule) is seen in others. Temperature and season length are thought to influence these patterns (respectively) but evidence is still equivocal. *Limulus polyphemus* shows an unusual pattern of size change with latitude: the largest animals are in the center of the distribution with smaller animals to the north and south. What selective pressure account for this unique distribution? Here we present the size–latitude pattern of *L. polyphemus* in North America and possible influences underlying this pattern. Understanding influences on body size is important because size is related to fecundity and number of eggs laid in a bout of nesting, and insight into such fitness parameters may aid conservation efforts of horseshoe crabs.

Survey and Contrast Radiographic Studies of the Horseshoe Crab (*Limulus polyphemus*)

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Survey and contrast radiographic studies of the cardiovascular and gastrointestinal systems of the "American" horseshoe crab (*Limulus polyphemus*) were undertaken using conventional and fluoroscopic techniques. Survey films easily identified the three main body sections of the exoskeleton: the frontal prosoma (cephalothorax); the hindbody opisthosoma (abdomen), and the posterior telson (tail). The book gills and appendages (a single pair of modified chelicera, six pairs of segmented legs and brachial appendages) were also easily distinguished, but the internal organs could not be resolved. Contrast angiography readily opacified the tubular cardiac sinus, the single frontal artery, the pair of anterior arteries, and four pairs of lateral arteries. Real-time fluoroscopic imaging demonstrated rhythmical contractions of the cardiac sinus of

approximately one beat per 1.5 seconds. By the second heartbeat, contrast media could be seen at the periphery of the appendages, and opacification of the gills occurred by the third heartbeat. The venous circulatory phase, however, was poorly visualized. The gastrointestinal system was also well visualized with contrast imaging. The three contrast agents (Omnipaque®, Gastrografin® and barium sulphate) used for the gastrointestinal studies varied minimally in their radiographic quality and transit times. In conclusion, angiography and gastrointestinal contrast studies were easily performed in this species, and no adverse effects from the radiographic procedures were observed in any of the horseshoe crabs.

Automated Acoustic Tracking of the Endangered Horseshoe Crab: Monitoring Trial in a Small-scale Bay

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In order to conserve the endangered horseshoe crab, *Tachypleus tridentatus*, we examined the migratory behavior in a small-scale bay at Tsuyazaki, Fukuoka, Japan, using an automated acoustic telemetry. The telemetry tracking was conducted from the breeding season to hibernation period for more than 9 month in 2006–2007. It is commonly believed that adult *T. tridentatus* migrate into shallow coastal area to mate and spawn during the breeding season from late June to early August, then overwinter out of the bay area at about 20 m depth. In this study, a total of 10 coded acoustic transmitters were attached with mature males, and monitored with 4 receivers placed along the coastal area. Five of them were attached to the individuals released near the place of capture (i.e., out of the bay) before the breeding season, and the remaining five were used for males with paired female in the bay during the period. The subsequent acoustic monitoring showed that eight males remained at the inner bay over winter after coming into the coastal area for the reproduction. Moreover, the analysis of tracking data revealed that a few individuals moved around the inner bay after the breeding period, but most of them were predicted to be present at the head of the bay during the hibernation period. Our results suggest that this study area would best suited to monitor and track the behaviors of the species, indicating that some adult horseshoe crabs spend in a small-scale bay, including sand beach, mud flat, and seagrass bed, through the year.

Dissociation between Circadian Rhythms of Visual Sensitivity and Circatidal Rhythms of Locomotion in the Horseshoe crab, *Limulus polyphemus*

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Horseshoe crabs have a circadian rhythm of visual sensitivity and also express both circadian and circatidal rhythms of locomotion in the laboratory. The major goal of this investigation was to determine if the clocks that influence expression of locomotor rhythms also modulate the eye, and visa versa. We developed a technique that enabled us to simultaneously record changes in visual sensitivity (electroretinograms, ERGs) and locomotion. Every animal tested ($n = 37$) expressed consistent circadian rhythms of visual sensitivity. In contrast, activity rhythms were more variable and rarely coincided with ERG rhythms. When water levels in the experimental chambers were stable and animals were exposed to a 14:10 LD cycle, 58% of them expressed a tidal rhythm of activity ($\text{period} = 13.44 \pm 2.6$ h), 17% a daily rhythm, and the rest (25%) expressed patterns of activity that were too variable to classify. Of the 7 animals that expressed daily rhythms, 1 was diurnal and 6 were nocturnal. When exposed to fluctuating changes in water depth that mimicked a tidal cycle, the majority of the animals synchronized their activity to the imposed rhythm (12.76 ± 0.36 h). With imposed tides, ERG rhythms remained stable (synchronized to the L:D cycle) and were not influenced by the tidal rhythm of locomotion, even during subsequent exposure to constant light or dark conditions. These results indicate that horseshoe crabs are likely to possess at least two biological clocks; one circadian clock primarily concerned with modulating visual sensitivity and one or more clocks that control patterns of locomotion.

Study on the Construction and Management of the Protected Area for *Tachypleus tridentatus* Leach in Xiamen

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The horseshoe crabs (*Tachypleus tridentatus*) have been over-catched severely, and their habitats have been polluted or destroyed, which have made the resource of *T. tridentatus* in Xiamen nearly exterminated. So, it is necessary to

establish a nature reserve for protection the resources of *T. tridentatus* in Xiamen. Based on the biological characteristics of *T. tridentatus* and the inshore features of Xiamen coast, a protected area (120 m^2), which lies in southeast of Xiamen City should be set aside. We formulated the scientific planning for constructing the nature reserve in Xiamen, and further to discuss the pattern of management and operation of the nature reserve. In order to get sustainable development and exploiture of the nature reserve of *T. tridentatus* properly, it not only needs to draw up some relevant policies and regulations but also needs to carry out scientific investigation and environment monitoring work, and to cooperate with international organization as well.

Public Education and Social Actions for Conservation of the Horseshoe Crab, *Tachypleus Tridentatus*, in Taiwan

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The horseshoe crabs, *Tachypleus tridentatus*, have been a part of daily lives of local residents in Taiwan. People use them as food, tools, medicines, even symbols to protect their houses from evil spirits. Nowadays, due to habitat loss and pollution, they have largely disappeared. To raise public awareness of the value of horseshoe crabs, we developed and implemented a series of public education programs, including symposiums, workshops, published pamphlets in three languages and a book in Chinese, and television programs. We even invited a folk musician to serve as a spokesperson. In 1996, we started long-term researches of the horseshoe crab's habitat in Kinmen Island, the only natural habitat left around Taiwan. Because of our efforts and government's recognition, 800 ha of the coastal zone on Kinmen were designated as "The Horseshoe Crab Protected Area" in December 1999. During a campaign in 2000, juvenile crabs were released to this protected area by local elementary school students. In 2002, we began to restore the population in Taiwan's main island by introducing adult horseshoe crabs to their potential spawning grounds in central Taiwan. Assisted by the staff of local Tung-hsiao Beach Resort, the restoration was successful. In addition, we were pleased to discover a natural juvenile population in Chiayi County in 2005 and prompted local residents to establish "Team for Conservation and Restoration of the Horseshoe Crab in Budai." Our works on conservation of horseshoe crabs have been fruitful and the key of success is changing people's attitude through public education.

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