12-2014

Metamorphosis Of Limulus Polyphemus Trilobite Larvae: Role Of Chemical And Structural Cues, Competency, And The Cost Of Delayed Metamorphosis

Stephanie Marie Kronstadt

METAMORPHOSIS OF *LIMULUS POLYPHEMUS* TRILOBITE LARVAE: ROLE OF CHEMICAL AND STRUCTURAL CUES, COMPETENCY, AND THE COST OF DELAYED METAMORPHOSIS

by

STEPHANIE MARIE KRONSTADT

B.S., Florida Institute of Technology

A thesis submitted to the Department of Biological Sciences of Florida Institute of Technology in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGICAL SCIENCES

Melbourne, Florida
December 2014

METAMORPHOSIS OF *LIMULUS POLYPHEMUS* TRILOBITE LARVAE: ROLE OF CHEMICAL AND STRUCTURAL CUES, COMPETENCY AND THE COST OF DELAYED METAMORPHOSIS

A THESIS

by

STEPHANIE MARIE KRONSTADT

Approved as to style and content by:

_____
Richard A. Tankersley, Ph.D., Chairperson
Professor
Department of Biological Sciences

_____
Robert van Woesik, Ph.D., Member
Professor
Department of Biological Sciences

_____
John Trefry, Ph.D., Member
Professor
Department of Marine and Environmental Systems

_____
Richard Aronson, Ph.D.
Professor and Head
Department of Biological Sciences

December 2014

ABSTRACT

METAMORPHOSIS OF *LIMULUS POLYPHEMUS* TRILOBITE LARVAE: ROLE OF CHEMICAL AND STRUCTURAL CUES, COMPETENCY, AND THE COST OF DELAYED METAMORPHOSIS

by Stephanie Marie Kronstadt, B.S., Florida Institute of Technology

Chairperson of Advisory Committee: Richard A. Tankersley, Ph.D.

Many marine and estuarine arthropods exhibit complex life cycles that include planktonic larval stages and benthic juvenile and adult phases. Chemical and structural cues associated with juvenile habitats often induce settlement and metamorphosis, thereby shortening the duration of the larval phase. These cues can trigger metamorphosis only after larvae reach competency, or developmental maturity. The point at which larvae reach this competency period and the ability to retain competency is highly species specific. Once competency is attained, a decrease in the time to metamorphosis (TTM) can decrease dispersal potential but may increase the chance of settling in a suitable habitat. Alternatively, an increase in TTM (delayed metamorphosis) may enhance dispersal and the possibility of finding a more suitable habitat. However, delaying metamorphosis may reduce energy stores, affecting growth and survival in later life stages.

The American horseshoe crab (*Limulus polyphemus*) inhabits estuarine and coastal habitats from the Yucatan Peninsula to northern Maine. It possesses a single lecithotrophic larval stage (trilobite) that molts, or metamorphoses, to a benthic juvenile stage. Metamorphosis is accelerated in the presence of chemical cues from several habitat-associated cues, including conspecifics and the seagrass *Halodule wrightii* (Boleman 2011). This thesis research further examined the effect of these two habitat-associated sources (i.e., conspecifics and *H. wrightii*) on metamorphosis of *L. polyphemus.* The first set of experiments tested the hypotheses that (1) the effect of both cues on time to metamorphosis (TTM) is dose-dependent, (2) the molecule(s) responsible for inducing metamorphosis is (are) a thermally-stable, low molecular weight compound(s), similar to those found to induce metamorphosis in other marine invertebrate species, and (3) *L. polyphemus* larvae also respond to structural cues by reducing the TTM.

When larvae were exposed to conspecific- and *H.* wrightii-exudate water at concentrations between 0.3-30 g L^{-1}, TTM declined in all treatments, even at the lowest concentration tested (0.3 g L^{-1}). The observed dose-dependent effect on TTM suggests that the chance of metamorphosis increases as larvae approach a chemical source (i.e., juvenile population or seagrass bed). Heating and cooling (-70 °C or 100 °C) exudate water did not alter or reduce the potency of the molecules responsible for inducing metamorphosis, suggesting that the molecule(s) are thermally stable. Trilobite larvae exposed to exudate water dialyzed through membranes of different pore sizes (0.5-1.0 kDa, 8.0-10 kDa, and 100 kDa)

responded similarly to all size fractions, suggesting the effective molecules in both source waters were relatively small (< 0.5 kDa) compounds. Finally, artificial *H. wrightii* structure decreased TTM in *L. polyphemus* trilobite larvae, indicating that larvae respond to both chemical and structural cues. There was no additive or synergistic effect when *H. wrightii* structural and chemical cues were combined, suggesting that there is a hierarchy of cues in which the chemical cue takes precedence (at a concentration of 30 g L^{-1}).

In order to determine the effect of timing of exposure to chemical cues on the metamorphosis of *L. polyphemus*, a second series of experiments tested the hypotheses that (1) trilobite larvae become competent within a few days in the plankton, and (2) delaying exposure to cues (i.e., delaying metamorphosis) negatively impacts post-metamorphic size, shape, and survival of *L. polyphemus* juveniles. The beginning of the competency period was determined by measuring time required for 25% of larvae to metamorphose (TTM_{25}) after exposure to a known inducer of metamorphosis (conspecific exudate). To determine the effect of delayed metamorphosis on competency, larvae were exposed to conspecific cues either immediately following hatching (control) or at delay intervals of 7, 14, 21, and 28 days post-hatching. Larvae in the control and 7-day delay treatment had similar patterns of metamorphosis. In both treatments, TTM_{25} was 16 days, suggesting that larvae become competent about 16 days post-hatch. The effect of delayed metamorphosis on post-metamorphic size and survival was examined by measuring survivorship (%), the molt-stage duration (MSD), prosoma length (PL),

prosoma width (PW), and the shape (PL: PW) for the first three juvenile instars (J1-J3) of crabs in the control, 21-day, and 28-day delay treatments. Delaying metamorphosis had no significant effect on survivorship, MSD, and PL for any of the treatments. However, third juvenile instars (J3) that were in the 28-day delay treatment were significantly narrower than those in the control. This difference resulted in individuals that were slightly more circular in shape (close to a 1:1 PL: PW ratio) when compared with juveniles in the control (close to a 1:2 PL: PW ratio). This study demonstrated that delaying metamorphosis of trilobite larvae had no lethal effects, and minimal sublethal effects, on later life stages. Therefore, extending the larval phase in order to find a suitable habitat may be an adaptive advantage for *L. polyphemus*.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DEDICATION

I would like to dedicate this thesis to my father, Samuel, and my mother, Sandra, who both have provided me with words of encouragement and support throughout my entire educational career. I am forever grateful to you for helping me achieve my academic goals and pursue my dreams.

ACKNOWLEDGMENTS

My advisor, Dr. Richard A. Tankersley, has always pushed me to become a better scientist and student. I am forever grateful for his guidance, support, and patience throughout my educational career at Florida Tech. Without his influence I would certainly not be the person I am today. I would also like to thank my committee members, Drs. Robert van Woesik and John Trefry, for their thought-invoking feedback and support.

I would like to thank Meghan Buckley for introducing me to collection sites and keeping me company during long research days, all the while putting a smile on my face. To my lab mate, Philip Gravinese, and colleague, Robert Fidler III, for their invaluable advice and eagerness to edit my writing. I am forever thankful to the following people for their advice and help with fieldwork and animal care: Captain Al, Casey Butler, Christopher Bridgeman, Christian Custer, Alexandra Folcik, Samuel Kronstadt, Kathy Kronstadt, Kelly McCaffrey, Joe Pilla, Sandra Pope-Kronstadt, Daniel Rice, Aabha Sant, Courtney Shadik, Katie Skura, Steven Tankersley, Ann Wassick, and Sean Vandedrinck. I would like to thank Florida Tech's InSTEP Program for funding me through a Graduate Teaching Fellowship (GK-12) from the National Science Foundation under grant No. DGE 0638702 and Florida Tech for providing me funding through a teaching assistantship.

CHAPTER I

EFFECT OF CHEMICAL AND STRUCTURAL CUES ON THE METAMORPHOSIS OF THE AMERICAN HORSESHOE CRAB, *LIMULUS POLYPHEMUS*

INTRODUCTION

Many marine and estuarine arthropods possess complex life cycles that include early planktonic larval forms followed by benthic juvenile and adult stages (reviewed by Pawlik 1992, Pechenik 1999, Pechenik 2010). Before they settle to the benthos, late-stage larvae are often able to recognize suitable settlement sites and subsequently orient themselves towards these habitats (Pawlik 1992). Identification and localization of settlement habitats typically involves behavioral and physiological responses to cues originating external to the organism (i.e., exogenous cues). Such cues include gravity, light, pressure, water-soluble chemicals, structures (i.e., substrate complexity and substrate texture), water flow, magnetic fields, sounds, and visual cues (reviewed by Sulkin 1984, Kingsford et al. 2002, Arvedlund and Kavanagh 2009).

In particular, chemical and structural cues associated with suitable benthic habitats may mediate larval behaviors, such as changes in activity (e.g., kineses) or the direction of swimming or orientation (e.g., taxes) (Sulkin 1984, Rodriguez et al. 1993, Abelson and Denny 1997, Metaxas 2001, Kingsford et al. 2002, Jeffs et al. 2005). Additionally, these environmental cues may either accelerate or delay the

physiological transition from the plankton to the benthos, also known as metamorphosis (Burke 1986, Forward et al. 2001, Hadfield and Paul 2001, Hay 2009), and are therefore termed 'metamorphic cues'.

Studies of metamorphic cues in marine and estuarine arthropods have focused on a variety of ecologically and economically important groups, including brachyuran crabs and barnacles (reviewed by Burke 1986, Forward et al. 2001, Hadfield and Paul 2001). These studies investigated how chemical and structural cues may affect time to metamorphosis (TTM), also known as the duration of the last larval phase. Cues that shorten TTM (i.e., positive cues) decrease the time spent in the plankton, which increases the probability of remaining near a suitable benthic habitat (Pawlik 1992, Pechenik et al. 1993, Hadfield and Paul 2001). Known positive cues of arthropod larvae include estuarine water (Forward et al. 1994, Fitzgerald et al. 1998), humic acids (Forward et al. 1997), biofilms (reviewed by Hadfield 2001, Khandeparker and Anil 2006), aquatic vegetation (Gebauer et al. 1998), prey items (Rodriguez and Epifanio 2000), conspecifics (O'Connor and Gregg 1998, Andrews et al. 2001), and specific substrates (Krimsky and Epifanio 2008, Steinberg et al. 2008). Cues that delay TTM (i.e., negative cues) of arthropod larvae typically extend the duration of the planktonic larval phase, increasing the chance that the larvae will be transported away from an unsuitable habitat (Pechenik et al. 1999). These cues are typically associated with adverse or suboptimal environmental conditions, including high ammonia/ammonium (Forward et al. 1997), predators (Welch et al. 1997), extreme salinity and/or

temperature (Fitzgerald et al. 1998, Anil et al. 2001), and hypoxia (Tankersley and Wieber 2000).

Settling and metamorphosing in a suitable habitat may reduce post-settlement mortality caused by physiological stress, predation, or competition (Hunt and Scheibling 1997, Andrews et al. 2001, Stevens and Swiney 2005). Describing chemical cues that induce settlement and metamorphosis may provide insight into how marine invertebrates interact with the environment and why these interactions are triggered.

HABITAT CHEMICAL CUES

Chemical cues influencing TTM are water-soluble compounds exuded by organisms or substrates associated with potential settlement habitats (i.e., habitat-associated cues). These chemical cues emanate from various sources, including conspecifics, benthic vegetation, biofilms, predators, and prey species (Burke 1986, Forward et al. 2001). Estuarine water contains a mixture of these habitat-associated cues and therefore reduces TTM in several arthropods, including *Callinectes sapidus* (Forward et al. 1994), *Limulus polyphemus* (Boleman 2011), and *Rhithropanopeus harrisii* (Fitzgerald et al. 1998).

Commonly identified cues for selection of settlement sites are chemical exudates released from conspecifics and habitat substrate (i.e., benthic vegetation). For example, *Pagurus maclaughlinae* (Harvey 1996), *Hemigrapsus sanguineus* (Kopin et al. 2001, O'Connor 2007), *Chasmagnathus granulata* (Gebauer et al. 1998), *Panopeus herbstii* (Andrews et al. 2001), *Sesarma curacoense* (Gebauer et

al. 2002), and *L. polyphemus* (Boleman 2011) molt more quickly to the juvenile stage in the presence of conspecific exudates. The presence of juveniles and adults may signal the availability of a habitat conducive to post-metamorphic growth and survival. Furthermore, exudates from benthic vegetation, including marsh grasses (e.g., *Phragmites australis* and *Spartina alterniflora*), seagrasses (e.g., *Halodule wrightii, Ruppia maritima,* and *Zostera marina*) and macroalgae species (e.g., *Bryropsis plumose, Cladophora* spp., *Fusus vesticulosus, Ulva lactuca*, and *U. rotunda*) accelerate the TTM of many estuarine arthropods, such as *C. sapidus* (Forward et al. 1994, 1996), *C. granulata* (Gebauer et al. 1998), *P. herbstii* (Weber and Epifanio 1996), and *L. polyphemus* (Boleman 2011). Benthic vegetation can serve as suitable habitat by protecting early life stages of marine invertebrates, which are particularly susceptible to predation (reviewed by Gosselin and Qian 1997). For instance, predation of *C. sapidus* megalopae and *L. polyphemus* trilobite larvae decreases significantly in seagrass beds when compared with adjacent open-sand habitats (Heck et al. 2001, Boleman 2011). Complex structure provided by seagrass blades may make detecting prey challenging for predators, creating protection for the inhabitants (Hovel and Lipcius 2001).

Time to metamorphosis has been shown to decrease with increasing concentration of habitat-associated chemical cues (Forward et al. 2001). The lowest concentration which elicits a decrease in TTM is known as the threshold of sensitivity. A dose-dependent effect can increase the likelihood that larvae will

metamorphose in close proximity to an abundant source of the cue (i.e., close to an established juvenile population or dense patch of seagrass).

THE NATURE OF CHEMICAL CUES

The induction of settlement and metamorphosis in marine invertebrates can be affected by various compounds within the habitat-associated chemical cues, including but not limited to carbohydrates, fatty acids, peptides, and proteins (reviewed by Zimmer and Butman 2000, Hadfield and Paul 2001, Steinberg et al. 2002) that are frequently soluble in water (Steinberg et al. 2002). For instance, peptides induce metamorphosis of larval abalone (Morse 1992, Roberts and Lapworth 2001), oysters (Zimmer-Faust and Tamburri 1994), nudibranchs (Lambert et al. 1997), jellyfish (Fleck et al. 1999), and sand dollars (Burke 1984), whereas larger insoluble proteins induce settlement of barnacles (Clare and Matsumura 2000).

Laboratory studies of the nature of chemical cues responsible for inducing metamorphosis have focused on a variety of marine and estuarine organisms, including polychaetes, cnidarians, molluscs, ascidians, echinoderms, and crustaceans (reviewed by Zimmer and Butman 2000, Hadfield and Paul 2001, Steinberg et al. 2002, Fusetani 2004). These studies typically involve partial characterization of molecules that induce metamorphosis, which defines specific chemical or physical properties of a molecule such as the thermal stability and molecular size via filtration, dialysis, or enzyme digestion techniques. Loss of potency after treatment with low or high temperatures suggests that the inducing

molecule is vulnerable to denaturation (i.e., loss of three-dimensional structure), which may be the result of disruption of hydrogen bonds, non-polar hydrophobic interactions, salt bridges, or disulfide bonds. Molecules susceptible to thermal denaturation includes some proteins, fatty acids, and carbohydrates (Wu and Wu 1925, Kunugi and Tanaka 2002). However, some inducing molecules are known to be thermally stable (Boettcher and Targett 1996, Andrews et al. 2001). Thermal stability is typically associated with globular proteins, some of which are capable of renaturation (regaining structure and function) or a type of peptide or carbohydrate (Mishra 2011).

STRUCTURAL CUES

Metamorphosis of planktonic larvae can also be induced by structural cues associated with benthic habitats. To test the effect of structure on TTM separately from the effect of chemical cues emanating from the source, sterile substrata or artificial mimics are commonly employed. The effects of the artificial mimics are compared with those of the natural substratum and a control (artificial or filtered seawater) (Forward et al. 2001). Studies by O'Connor (2007) and Steinberg et al. (2008) found that when exposed to both sterile rocks and natural rocks, TTM of the varunid crab *H. sanguineus* was similar, indicating that the presence of the substrate, not the chemical cue or any associated biofilms, were responsible for shortening TTM. For other species, the cues (i.e., chemical and structural) must be detected simultaneously for them to have an effect on TTM. For instance, TTM of the xanthid crab *Menippe mercenaria* decreases when exposed to a combination of

chemical and structural cues of the brown alga *Sargassum fluitans*. However, neither cue alone is capable of reducing TTM (Krimsky and Epifanio 2008). Additionally, chemical and structural cues can have synergistic and additive effects on TTM (Bao et al. 2007, Steinberg et al. 2008).

STUDY ORGANISM

American horseshoe crabs, *Limulus polyphemus,* commonly occur in estuarine and coastal habitats from the Yucatan Peninsula to northern Maine (reviewed by Botton et al. 2003). Adults in most areas reproduce during spring and summer months (Rudloe 1980, Cohen and Brockmann 1983). During reproduction, male crabs clutch females using modified pedipalps and fertilize the eggs that are deposited by the female in nests 5-20 cm beneath the sediment surface near the high tide mark on sandy beaches (Rudloe 1979, Shuster 1982, Brockmann and Penn 1992). During incubation within the sediment, embryos proceed through 21 stages and four embryonic molts (reviewed by Penn and Brockmann 1994) over 14-28 days (Jegla and Costlow 1979, Sekiguchi 1988, Shuster et al. 2003). During the fourth and final embryonic molt, the embryo hatches into a lecithotrophic, trilobite larva. At spring high tide, the nest is flooded, triggering hatching in the remaining developed embryos, and releasing trilobite larvae into the water column (Rudloe 1979, Botton et al. 2010).

At least two weeks after hatching, trilobite larvae metamorphose into the juvenile stage (first instar) and resemble adult horseshoe crabs (Sekiguchi 1988, Botton and Loveland 2003, Shuster et al. 2003). Juvenile *L. polyphemus* remain on

the benthos and bury themselves in the sediments of near-shore habitats (Rudloe 1979, Rudloe 1981) and may remain on intertidal flats for several molts (Loveland 2002, reviewed by Botton et al. 2010). As juveniles mature, they move into deeper waters and do not return to the upper beach area until they are ready to spawn 9-10 years later (Rudloe 1979, Shuster 1982, Brockmann and Penn 1992, Chiu and Morton 1999).

As with other coastal and estuarine arthropods, larval horseshoe crabs are able to detect and respond to habitat-associated chemical cues (Medina and Tankersley 2010, Boleman 2011). Chemical cues, including exudates from conspecifics, the seagrass *Halodule wrightii,* the mummichog *Fundulus grandis*, and estuarine water, elicit changes in swimming and orientation and induce metamorphosis in *L. polyphemus* (Medina and Tankersley 2010, Boleman 2011). In a study on the effect of chemical cues on rheotactic behavior (response to water flow), Boleman (2011) found that posthatch (< 2 days posthatching) and premetamorphic (10 days posthatching) trilobite larvae swim upstream (positive rheotaxis) in estuarine water and in water containing chemical cues from *H. wrightii*. However, the larvae exhibit no rheotactic response to chemical cues from conspecifics. Medina and Tankersley (2010) found that similar habitat-associated chemical cues alter the orientation of *L. polyphemus* larvae. In the presence of chemical cues from conspecifics and from the seagrasses *H. wrightii* and *Syringodium filiforme*, larvae tend to orient away from visual targets (Medina and Tankersley 2010). Chemical cues that evoke positive rheotactic behavior (i.e., *H.*

wrightii and *F. grandis*) along with conspecific cues also significantly reduce TTM in *L. polyphemus* trilobite larvae (Boleman 2011). These results suggest that *L. polyphemus* trilobite larvae are capable of detecting chemical cues as they approach coastal and estuarine habitats.

Chemical cues that induce metamorphosis in *L. polyphemus* (i.e., conspecifics, *H. wrightii*, and *F. grandis*) have not been described in detail. I tested the hypothesis that the TTM of *L. polyphemus* larvae decreases with increasing concentration of the inducing chemical cue from conspecifics and from *H. wrightii*. To determine the nature of the chemicals responsible for accelerating metamorphosis, I examined the thermal stability and size (molecular weight) of the inducing molecule in both exudates. Finally, to investigate if *H. wrightii* provides multiple cues that induce metamorphosis, I tested the hypothesis that seagrass structure reduces TTM.

MATERIALS AND METHODS

COLLECTION AND MAINTENANCE OF LARVAE

Limulus polyphemus eggs were collected from December 2012 to October 2013 near the Sunshine Skyway Bridge, Tampa Bay, FL (27°35´5.51" N, 82°36´44.70" W) and brought back to the laboratory at Florida Institute of Technology. Developing embryos and trilobite larvae were reared in 19 cm diameter × 6 cm glass bowls containing 1 L of filtered (< 5 µm) seawater. Larvae from different nests were maintained separately at 26 °C and at a salinity of 35. Water used to incubate embryos was collected ~ 50 m off the beach near the Florida Institute of Technology's Vero Beach Marine Laboratory (VBML), Vero Beach, FL (27°40´31.83" N, 80°21´50.69" W). Seawater within the glass bowls was replaced daily and embryos were maintained on a light-dark cycle that approximated the natural photoperiod at time of collection.

EXPERIMENTAL DESIGN

All experiments were conducted using similar procedures and environmental conditions (26 °C, salinity of 35, 14:10 hour light-dark cycle). Immediately following hatching, individual trilobite larvae (replicates) were placed in separate chambers (5 cm × 5.5 cm × 4 cm) of a compartmentalized plastic container (Wollcott and De Vries 1994, Fitzgerald et al. 1998, Krimsky and Epifanio 2008). Each treatment or treatment combination was replicated at least 30 times using larvae from at least 5 different nests (cohorts). Each compartment contained 40 mL of either offshore water (control) or treatment water. Offshore

water was collected 15 km seaward of Ft. Pierce Inlet, FL, well beyond the estuarine plume. Thus, offshore water was assumed to be free of any estuarine or coastal chemical cues that may affect metamorphosis.

All chemical sources used to create treatment (exudate) water were collected from the Indian River Lagoon (Brevard County, FL). Exudate water was prepared by incubating the chemical source (e.g., *L. polyphemus* or *Halodule wrightii*) in glass aquaria with 1 L of offshore water for 24 hours. Before incubation, the chemical source was gently rinsed with coastal water, blotted dry with a paper towel, and weighed. After 24 hours, the offshore water was then assumed to contain any chemical cues produced, or exuded, by the source (i.e., *L. polyphemus* or *Halodule wrightii*). Fresh exudate water was prepared daily and was filtered (< 5 μm) prior to being used in experiments.

Trilobite larvae were checked daily at 16:00 hours for metamorphosis. Time to metamorphosis (TTM) was calculated as the duration of the trilobite phase (in days). Water was changed daily. Plastic containers were rinsed twice with deionized water and allowed to air-dry overnight. Unless otherwise noted, experiments lasted 40-50 days or until all larvae metamorphosed to the juvenile stage.

HABITAT CHEMICAL CUES

To examine the effect of conspecific and *H. wrightii* chemical cue concentration on *L. polyphemus*, newly hatched trilobite larvae were placed in one of six concentrations of exudate water ranging from 0 g L^{-1} (offshore water) to

30 g L^{-1}. A stock solution of each chemical cue (30 g L^{-1}) was prepared by incubating 30 g of the chemical source (live horseshoe crabs or fresh *H. wrightii*) in 1 L of offshore water for 24 hours. Intermediate concentrations of test waters were produced by diluting the stock solution with offshore water to produce half-log unit concentrations of 9.0, 3.0, 0.9, and 0.3 g L^{-1}. Sixty larvae from six different nests were randomly assigned to one of the six treatments (10 larvae from each nest per treatment; n=60).

THE NATURE OF CHEMICAL CUES

To test the thermal stability of the metamorphic cue, conspecific- and *H. wrightii*-exudate waters (30 g L^{-1}) were either frozen (-70 °C for 12 hours) or heated (100 °C for 10 minutes) prior to being used in experiments (Steinberg et al. 2007). In addition to the two experimental treatments (cold and heat), larvae were exposed to a positive control (untreated exudate water at 25 °C) and offshore water. Sixty larvae from six different nests were randomly assigned to one of the seven treatments (10 larvae from each nest per treatment; n=60).

To estimate the molecular size of the molecule(s) responsible for inducing metamorphosis, conspecific- and *H. wrightii*-exudate waters were fractionated by diffusion using dialysis membranes with known molecular weight cut-offs (MWCO): 0.5-1.0 kDa, 8.0-10 kDa, 100 kDa (Andrews et al. 2001) (Biotech Cellulose Ester dialysis tubing). All dialysates (treatment water) were obtained from the same exudate water sample that was prepared daily. Exudate water

(0.016 L; concentration of 350 g L^{-1}) was then placed into pieces of dialysis tubing (11 cm x 3.1 cm) of different MWCOs. The pieces of tubing were then sealed and each was placed in separate dialysate containers containing 0.6 L of offshore water. The treatments were allowed to dialyze for 16 hours (Steinberg et al. 2007, Khandeparker and Kumar 2011). Assuming that complete diffusion of the active molecule occurred across the tubing membrane, the final concentration of the dialysate water was considered to be 9.0 g L^{-1}. Two control treatments were also tested. The positive control was exudate water that was not fractionated (9.0 g L^{-1}) and the negative control was offshore water. Thirty larvae from five different nests were randomly assigned to one of the five treatments (6 larvae from each nest per treatment; n = 30).

STRUCTURAL CUES

Two experiments were conducted to determine whether the physical structure of *H. wrightii* influences TTM. In the first experiment, flat, polypropylene ribbon (~1 mm wide) was used as artificial seagrass mimic. Whereas, in the second experiment, glass rods (~1 mm in diameter) were used instead of ribbon. In both experiments, larvae were exposed to two treatments: water type and structure type. Levels of water type were offshore water and natural *H. wrightii*-exudate water (30 g L^{-1}). Levels of structure type were no structure and artificial *H. wrightii* structure. In the ribbon experiment, larvae were also exposed to an additional control that consisted of exudate water from the artificial seagrass (30 g L^{-1}). Artificial seagrass was cut into lengths of 2.5 cm and placed directly

into containers with the larvae. Sections of ribbon were replaced daily and glass rods were cleaned daily by soaking in a mild HCl acid solution. Sixty larvae from six different nests were randomly assigned to one of the six treatment combinations (10 larvae from each nest per treatment; n=60).

DATA ANALYSIS

The effect of different treatments on metamorphosis was determined using failure-time analysis (Cox Proportional Hazards Model) (Muenchow 1986), with time to metamorphosis, or the duration of the trilobite larval stage, serving as the "time until an event occurs" in the analysis. The resulting hazard function for each treatment was the probability that a given larva would metamorphose during the next time interval (Δt= 1 day) (Muenchow 1986, Kleinbaum and Klein and Klein 1996). To control for potential nest effects on metamorphosis rates, cohort group or nest was added as a covariate in the analysis. Comparisons of the TTM for larvae exposed to different treatments and controls were made using a Log-Rank (LR) test (Kleinbaum and Klein and Klein 1996). All analyses were performed using SPSS 20.0 and SigmaPlot 11.0.

RESULTS

HABITAT CHEMICAL CUES

When larvae were placed in one of six concentrations of conspecific exudate (0 g L^{-1} to 30 g L^{-1}), metamorphosis to the juvenile stage (J1) was significantly accelerated in all treatments relative to the offshore water control (LR = 274.5, d.f. = 5, $P < 0.001$; Fig. I-1), even at the lowest concentration tested (Fig. I-1 and Table I-1). Time to metamorphosis was shortest at the highest concentration of conspecific exudate (Fig. I-1 and Table I-1), with a median time to metamorphosis (TM_{50}) of 13.5 days ± 0.4 (±SE) (Fig. I-1). The TM_{50} increased with decreasing concentration of conspecific exudate (Fig. I-1). The TTM was similar for larvae in the 3.0 g L^{-1} and 9.0 g L^{-1} exudate treatments (LR = 0.11, d.f. = 1, $P = 0.735$; Fig. I-1). Comparison of the hazard ratios revealed that larvae in the highest concentration treatment (30 g L^{-1}) were 65 times more likely to metamorphose at any given time during the experiment than larvae in offshore water (Table I-1). The likelihood that larvae would metamorphose decreased proportionally with the concentration of conspecific exudate (Table I-1). The cohort (nest covariate) had a significant effect on TTM (Wald $\chi^2 = 49.1$, d.f. = 6, $P < 0.001$).

The pattern of the response of larvae to *H. wrightii* exudate was similar to that of the conspecific exudate. Metamorphosis was accelerated in all concentration treatments of *H. wrightii* exudate relative to the offshore water control (LR = 206.7, d.f. = 5, $P < 0.001$; Fig. I-2), even at the lowest concentration

(Fig. I-2 and Table I-2). The TTM was shortest at the highest concentration of *H. wrightii* exudate (Fig. I-2 and Table I-2), with a TM_{50} of 20.5 days ± 0.6 (±SE) (Fig. I-2). The TM_{50} increased with decreasing concentration of *H. wrightii* exudate (Fig. I-2). Also, TTM of larvae was not significantly different in the 0.3 g L^{-1}, 0.9 g L^{-1}, and the 3.0 g L^{-1} *H. wrightii* exudate treatments (0.3 g L^{-1} vs. 0.9 g L^{-1}: LR = 0.46, d.f. = 1, $P = 0.500$; 0.9 g L^{-1} vs. 3.0 g L^{-1}: LR = 3.3, d.f. = 1, $P = 0.068$; Fig. I-2). Trilobite larvae exposed to 30 g L^{-1} of *H. wrightii* exudate were 26 times more likely to metamorphose at any given time than larvae maintained in offshore water (Table I-2). The likelihood that larvae would metamorphose decreased proportionally with the concentration of *H. wrightii* exudate (Table I-2). Again, the cohort (nest covariate) had a significant effect on TTM (Wald $\chi^2 = 71.7$, d.f. = 6, $P < 0.001$). Only 35% of the larvae maintained in the offshore-water control metamorphosed by the end of the 40 day observation period (Figs. I-1 and I-2).

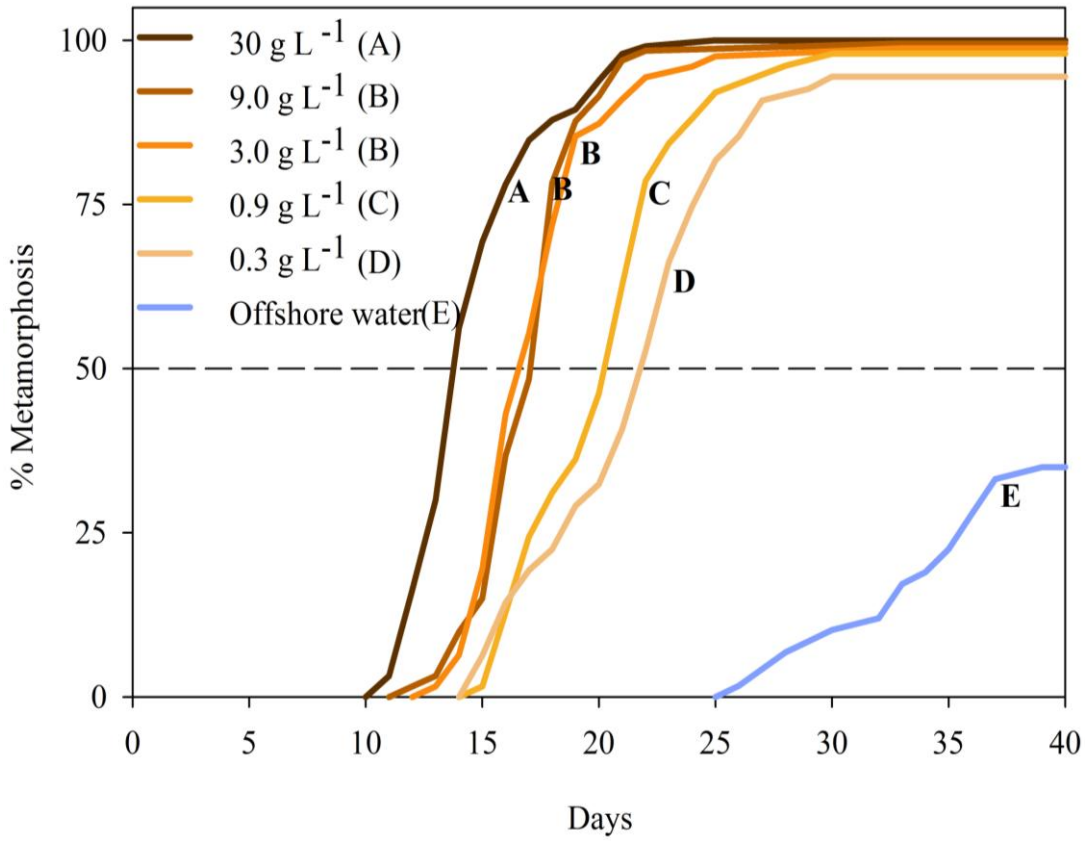

Figure I-1. Cumulative percent metamorphosis of *Limulus polyphemus* larvae during a 40-day exposure period to water containing one of six different concentrations of conspecific exudate (n=60). Curves with the same letters are not significantly different at $\alpha = 0.05$ (Cox Proportional Hazards Model).

Table I-1. Log-rank pairwise comparisons of time to metamorphosis of *Limulus polyphemus* trilobite larvae to the juvenile (J1) stage in the presence of increasing concentrations of conspecific exudate (n = 60). Each concentration is compared to the offshore water control. Degrees of freedom for all comparisons are equal to 1.

Concentration (g L^{-1})	**Log-Rank Test Statistic**	***P*-value**	**Hazard Ratio**
0.3	90.1	< 0.001	9
0.9	107.0	< 0.001	13
3.0	108.1	< 0.001	25
9.0	116.4	< 0.001	32
30	136.5	< 0.001	65

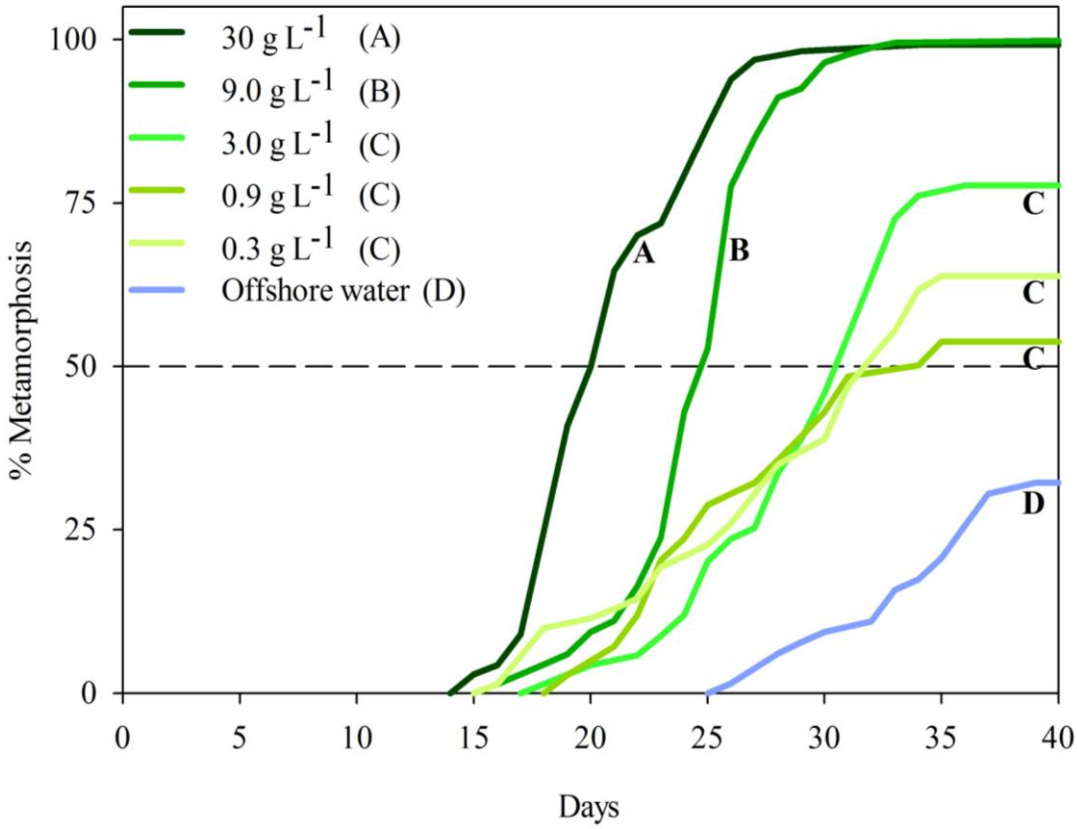

Figure I-2. Cumulative percent metamorphosis of *Limulus polyphemus* larvae during a 40-day exposure period to water containing one of six different concentrations of *Halodule wrightii* exudate (n=60). Curves with the same letters are not significantly different at $\alpha = 0.05$ (Cox Proportional Hazards Model).

Table I-2. Log-rank pairwise comparisons of time to metamorphosis of *Limulus polyphemus* trilobite larvae to the juvenile (J1) stage in the presence of increasing concentrations of *Halodule wrightii* exudate (n = 60). Each concentration is compared to the offshore water control. Degrees of freedom for all comparisons are equal to 1.

Concentration (g L^{-1})	**Log-Rank Test Statistic**	***P*-value**	**Hazard Ratio**
0.3	13.6	< 0.001	3
0.9	8.1	< 0.001	3
3.0	31.8	< 0.001	4
9.0	102.3	< 0.001	13
30	106.4	< 0.001	26

THE NATURE OF CHEMICAL CUES

Time to metamorphosis of larvae maintained in conspecific exudate that had previously been frozen (-70 °C) or heated (100 °C) was not significantly different from the TTM of larvae in the untreated conspecific exudate (positive control) (Fig. I-3 and Table I-3). All conspecific treatments had a similar TM_{50} (positive control: 15.5 ± 0.4; frozen: 17.5 days ± 0.2; heated: 16.5 days ± 0.3; Fig. I-3). At any given time, larvae in the previously frozen treatment were 36 times more likely to metamorphose than those maintained in offshore water, whereas larvae in the previously heated treatment were 61 times more likely to metamorphose than those in offshore (Table I-3). The cohort (nest covariate) had a significant effect on TTM (Wald $\chi^2 = 28.5$, d.f. = 5, $P < 0.001$).

Similar to the conspecific exudate, freezing and heating *H. wrightii* exudate had no effect on TTM of larvae (Fig. I-4 and Table I-4). All *H. wrightii* treatments had a similar MT_{50} (positive control: 21.5 ± 0.6; frozen: 22.5 days ± 0.6; heated: 22.5 days ± 0.7; Fig. I-4). Larvae in the previously frozen treatment were 28 times more likely to metamorphose when compared with those in offshore water, whereas larvae in the previously heated treatment were 24 times more likely to metamorphose (Table I-4). And again, the cohort (nest covariate) had a significant effect on TTM (Wald $\chi^2 = 20.1$, d.f. = 5, $P = 0.001$). Only 19% of the larvae maintained in the offshore water metamorphosed by the end of the 40 day observation period (Figs. I-3 and I-4).

To determine the size of the inducing molecule(s), exudate water was dialyzed through membranes of three sizes (0.5-1.0, 8.0-10, and 100 kDa). Conspecific exudate dialyzed through each of the three membranes had a similar effect on TTM when compared with the untreated conspecific exudate (positive control) (Fig. I-5 and Table I-5). All treatments had a similar TM_{50} (positive control: 29.5 ± 2.6 (±SE); 0.5-1.0 kDa: 17.5 days ± 2.7; 8.0-10 kDa: 21.5 days ± 2.4, 100 kDa: 31.5 days ± 2.2; Fig. I-5). Comparison of the hazard ratios showed that larvae in the 0.5-1.0 kDa-dialysate treatment were 14 times more likely to metamorphose at any given time than larvae kept in offshore water (Table I-5). The larvae in the 0.8-10 kDa- and 100 kDa-dialysate treatments were 14 and 9 times more likely to metamorphose, respectively, when compared with those larvae in the offshore control (Table I-5). Additionally, the cohort (nest covariate) had a significant effect on TTM (Wald $\chi^2 = 46.9$, d.f. = 10, $P < 0.001$).

Similar to the conspecific exudate, *H. wrightii* exudate dialyzed through each of the three membranes had a similar effect on TTM when compared with the untreated *H. wrightii* exudate (positive control) (Fig. I-6 and Table I-6). All *H. wrightii* treatments had a similar TM_{50} (positive control: 32.5 days ± 1.9 (±SE); 0.5-1.0 kDa: 35.5 days ± 1.8; 8.0-10 kDa: 33.5 days ± 2.2; 100 kDa: 37.5 ± 1.9; Fig. I-6). At any given time, larvae in the 0.5-1.0 kDa-dialysate treatment were 8 times more likely to metamorphose than those larvae in offshore water (Table I-6). The larvae in the 0.8-10 kDa- and 100 kDa-dialysate treatments were both 6 times more likely to metamorphose when compared with larvae in offshore water (Table

I-6). Once again, the cohort (nest covariate) had a significant effect on TTM (Wald $\chi^2 = 89.5$, d.f. = 10, $P < 0.001$). Only about 20% of the larvae in the offshore-water control metamorphosed by the end of the 50 day experiment (Figs. I-5 and I-6).

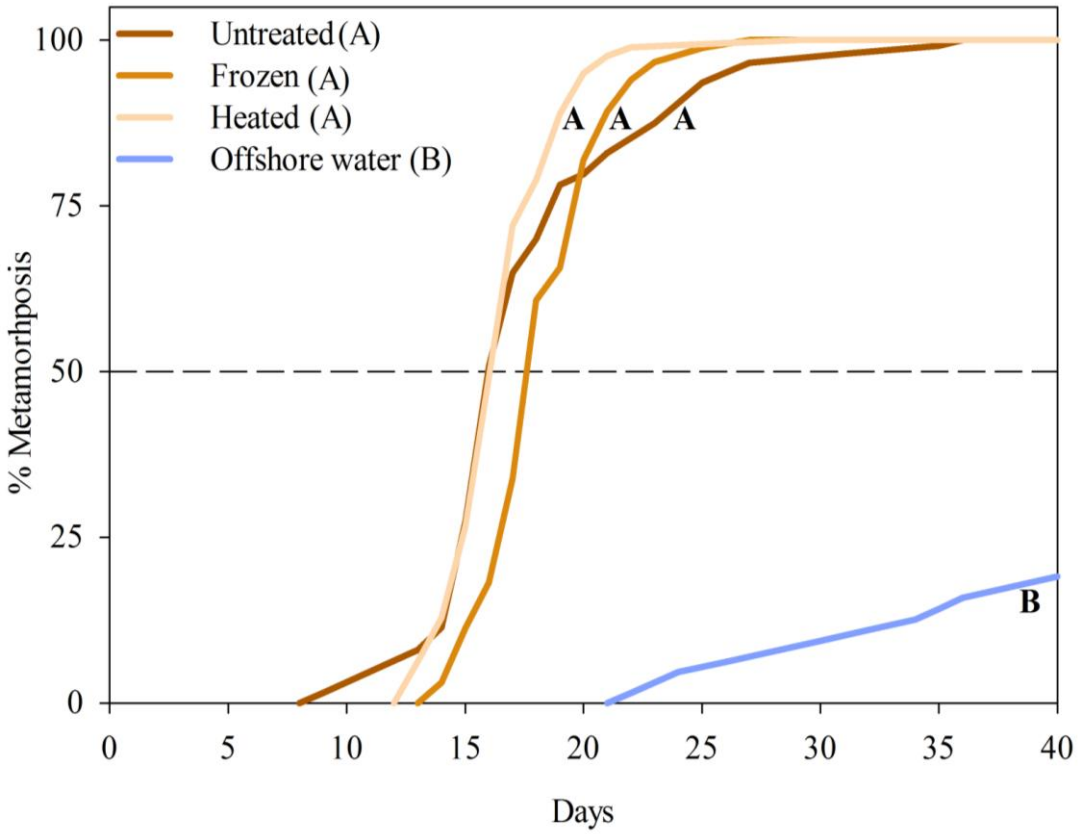

Figure I-3. Cumulative percent metamorphosis of *Limulus polyphemus* larvae during a 40-day exposure period to conspecific exudate that was untreated or previously subjected to freezing and heating (n = 60). Curves with the same letters are not significantly different at $\alpha = 0.05$ (Cox Proportional Hazards Model).

Table I-3. Log-rank pairwise comparisons of time to metamorphosis of *Limulus polyphemus* trilobite larvae to the juvenile (J1) stage in the presence of conspecific exudate that had been untreated or previously subjected to freezing and heating (n = 60). Degrees of freedom for all comparisons are equal to 1.

Temperature Treatment	**Comparison**	**Log-Rank Test Statistic**	***P*-value**	**Hazard Ratio**
Untreated	Offshore	129.9	< 0.001	40
Frozen	Offshore	136.5	< 0.001	36
Heated	Offshore	138.4	< 0.001	61
Frozen	Untreated	0.09	0.768	-
Heated	Untreated	3.7	0.053	-

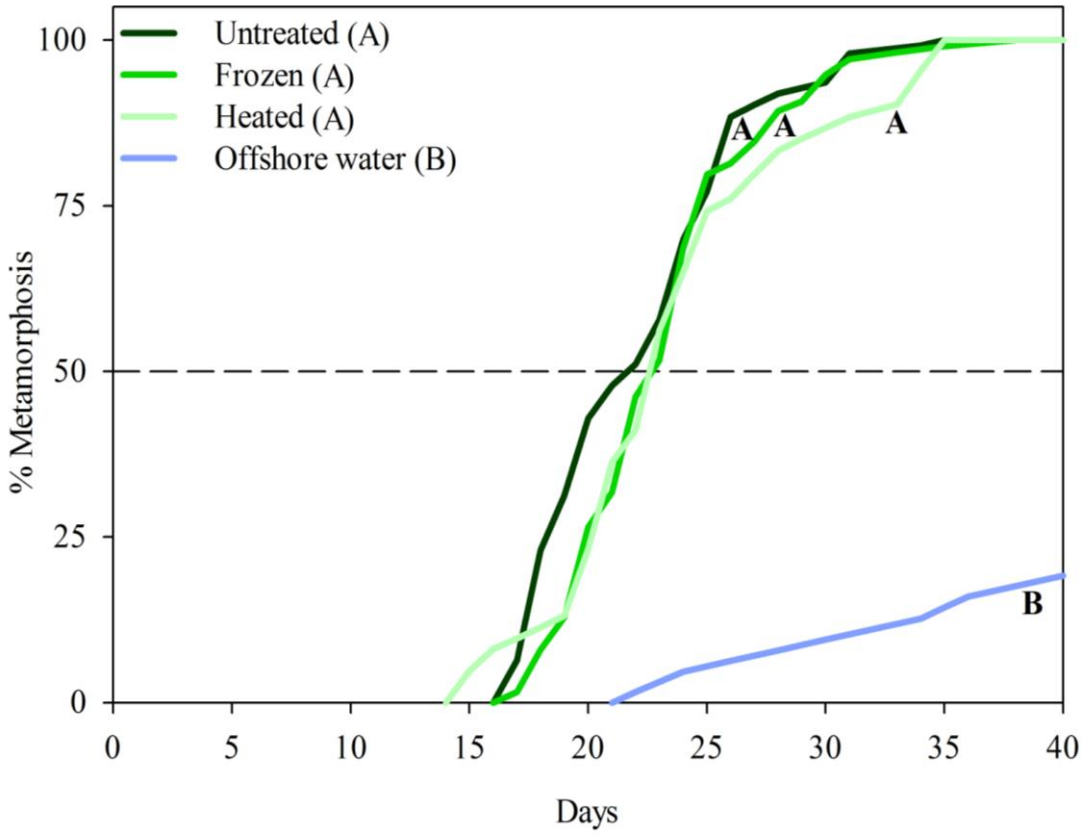

Figure I-4. Cumulative percent metamorphosis of *Limulus polyphemus* larvae during a 40-day exposure period to *H. wrightii* exudate that was untreated or previously subjected to freezing and heating (n = 60). Curves with the same letters are not significantly different at $\alpha = 0.05$ (Cox Proportional Hazards Model).

Table I-4. Log-rank pairwise comparisons of time to metamorphosis of *Limulus polyphemus* trilobite larvae to the juvenile (J1) stage in the presence of *Halodule wrightii* exudate that had been untreated or previously subjected to freezing and heating (n = 60). Degrees of freedom for all comparisons are equal to 1.

Temperature Treatment	**Comparison**	**Log-Rank Test Statistic**	***P*-value**	**Hazard Ratio**
Untreated	Offshore	120.3	< 0.001	32
Frozen	Offshore	117.0	< 0.001	28
Heated	Offshore	110.1	< 0.001	24
Frozen	Untreated	1.6	0.206	-
Heated	Untreated	2.7	0.103	-

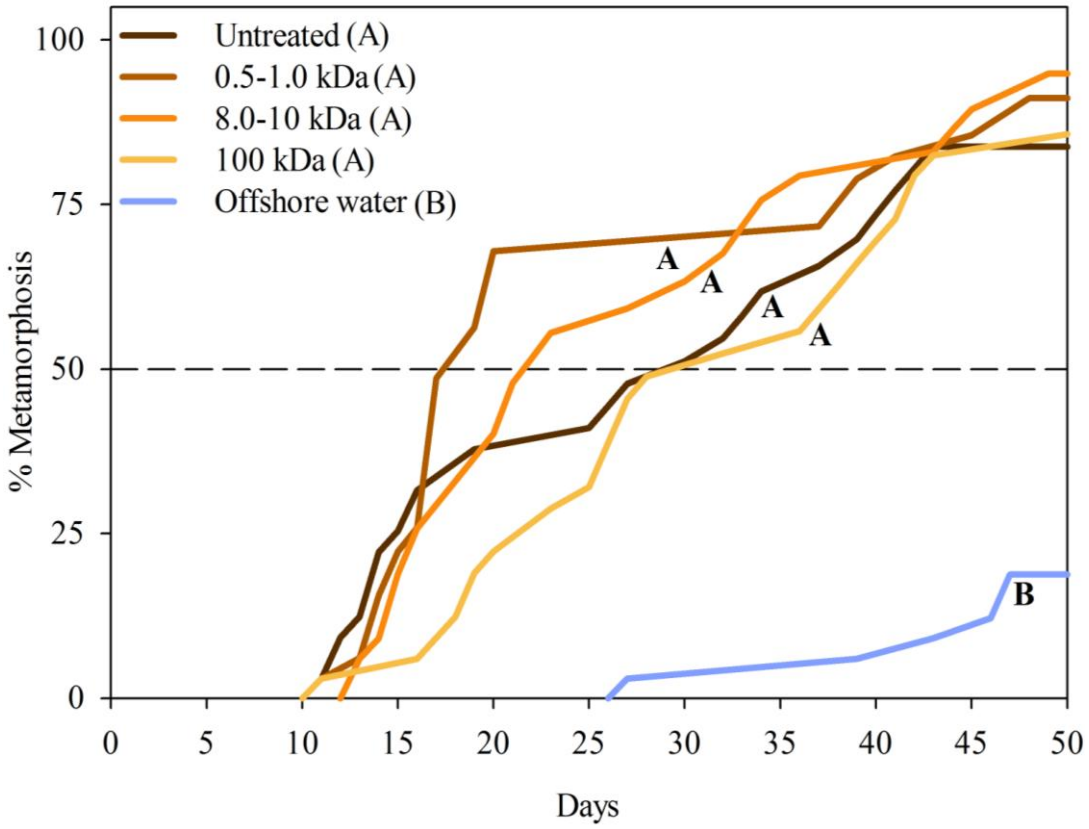

Figure I-5. Cumulative percent metamorphosis of *Limulus polyphemus* larvae during a 50-day exposure period to conspecific exudate either untreated or dialyzed through membranes of different molecular weight cut-offs (0.5-1.0 kDa, 8.0-10 kDa, and 100 kDa) (n = 30). Curves with the same letters are not significantly different at $\alpha = 0.05$ (Cox Proportional Hazards Model).

Table I-5. Log-rank pairwise comparisons of time to metamorphosis of *Limulus polyphemus* trilobite larvae to the juvenile (J1) stage in the presence of conspecific exudate dialyzed through membranes of different pore sizes (n = 30). Degrees of freedom for all comparisons are equal to 1.

Membrane Pore Size	**Comparison**	**Log-Rank Test Statistic**	***P*-value**	**Hazard Ratio**
Untreated	Offshore	32.8	< 0.001	10
0.5-1.0 kDa	Offshore	35.1	< 0.001	14
8.0-10 kDa	Offshore	21.8	< 0.001	14
100 kDa	Offshore	35.5	< 0.001	9
0.5-1.0 kDa	Untreated	0.08	0.784	-
8.0-10 kDa	Untreated	0.09	0.765	-
100 kDa	Untreated	0.12	0.726	-

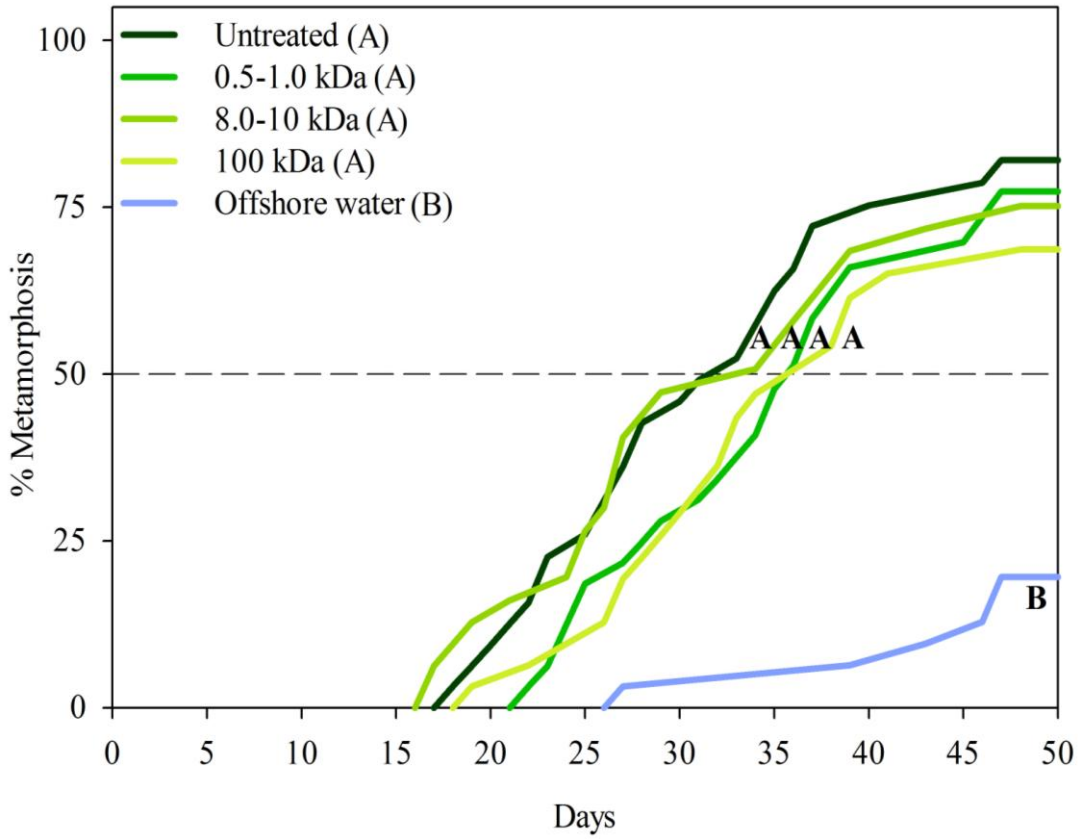

Figure I-6. Cumulative percent metamorphosis of *Limulus polyphemus* larvae during a 50-day exposure period to *H. wrightii*-exudate water either untreated or dialyzed through membranes of different molecular weight cut-offs (0.5-1.0, kDa, 8.0-10 kDa, and 100 kDa) (n = 30). Curves with the same letters are not significantly different at $\alpha = 0.05$ (Cox Proportional Hazards Model).

Table I-6. Log-rank pairwise comparisons of time to metamorphosis of *Limulus polyphemus* trilobite larvae to the juvenile (J1) stage in the presence of *Halodule wrightii* exudate dialyzed through membranes of different pore sizes (n = 30). Degrees of freedom for all comparisons are equal to 1.

Membrane Pore Size	**Comparison**	**Log-Rank Test Statistic**	***P*-value**	**Hazard Ratio**
Untreated	Offshore	31.5	< 0.001	9
0.5-1.0 kDa	Offshore	23.7	< 0.001	8
8.0-10 kDa	Offshore	21.8	< 0.001	6
100 kDa	Offshore	16.3	< 0.001	6
0.5-1.0 kDa	Untreated	1.2	0.278	-
8.0-10 kDa	Untreated	0.54	0.461	-
100 kDa	Untreated	2.9	0.088	-

STRUCTURAL CUES

Times to metamorphosis of larvae in all treatments containing artificial seagrass structure (flat, polypropylene ribbon) were significantly reduced when compared with larvae in offshore water (LR = 152.0, d.f. = 3, $P < 0.001$; Fig. I-7). The seagrass exudate plus structure treatment had the strongest effect on TTM (Fig. I-7 and Table I-7), with a TM_{50} of 19.5 days ± 0.6 (±SE) (Fig. I-7). These larvae were 32 times more likely to metamorphose than those maintained in offshore water (Table I-7). Metamorphosis of larvae maintained in both the exudate-only and the structure-only treatments was accelerated when compared with those in offshore water (Fig. I-7 and Table I-7). There was no significant difference in TTM of larvae exposed to exudate-only and those larvae maintained in structure-only treatments (Fig. I-7 and Table I-7), with both treatments having a similar TM_{50} (exudate-only: 22.5 days ± 0.7; structure-only: 22 days ± 0.9; Fig. I-7). At any given time, larvae in the exudate-only treatment were 18 times more likely to metamorphose than those in the offshore water (Table I-7). Larvae in the structure-only treatment were 19 times more likely to metamorphose than those in the offshore water (Table I-7). Furthermore, the cohort (nest covariate) in this experiment had a significant effect on TTM (Wald $\chi^2 = 30.5$, d.f. = 5, $P < 0.001$). Only 16% of the larvae in the offshore-water control metamorphosed by the end of the experiment (Figs. I-7 and I-8).

For the experiment in which glass rods were used to mimic seagrass structure, all treatments significantly decreased TTM of larvae when compared

with those in the offshore water (LR = 64.8, d.f. = 3, $P < 0.001$; Fig. I-9). The exudate-only treatment and the exudate plus structure (glass rods) treatment had the strongest effect on TTM (Fig. I-9 and Table I-8). There was no significant difference in TTM of larvae between the two exudate treatments (with and without structure) (Fig. I-9 and Table I-8). Larvae in both treatments had similar TM_{50} (exudate-only: 27.5 days ± 1.2 (±SE); exudate plus structure: 29.5 days ± 1.3; Fig. I-9). Comparison of the hazard ratios indicate that larvae maintained in the exudate-only treatment were 15 times more likely to metamorphose, at any given time, than larvae in offshore water (Table I-8). Larvae exposed to the exudate plus structure treatment were 19 times more likely to metamorphose than those in the offshore water (Table I-8). Additionally, both exudate treatments (with and without artificial structure) significantly accelerated metamorphosis when compared with larvae in the structure-only treatment (Fig. I-9 and Table I-8). Finally, larvae exposed to only structure had significantly shorter TTM when compared with larvae maintained in offshore water (Fig. I-9 and Table I-8), with an TM_{50} of 39.5 days ± 0.9. At any given time during the experiment, larvae exposed to only structure were 10 times more likely to metamorphose than those in offshore water (Table I-8). The cohort (nest covariate) did not have a significant effect on TTM (Wald $\chi^2 = 10.8$, d.f. = 10, $P = 0.055$). Only 8% of the larvae maintained in the offshore water control metamorphosed by the end of the observation period (Fig. I-9).

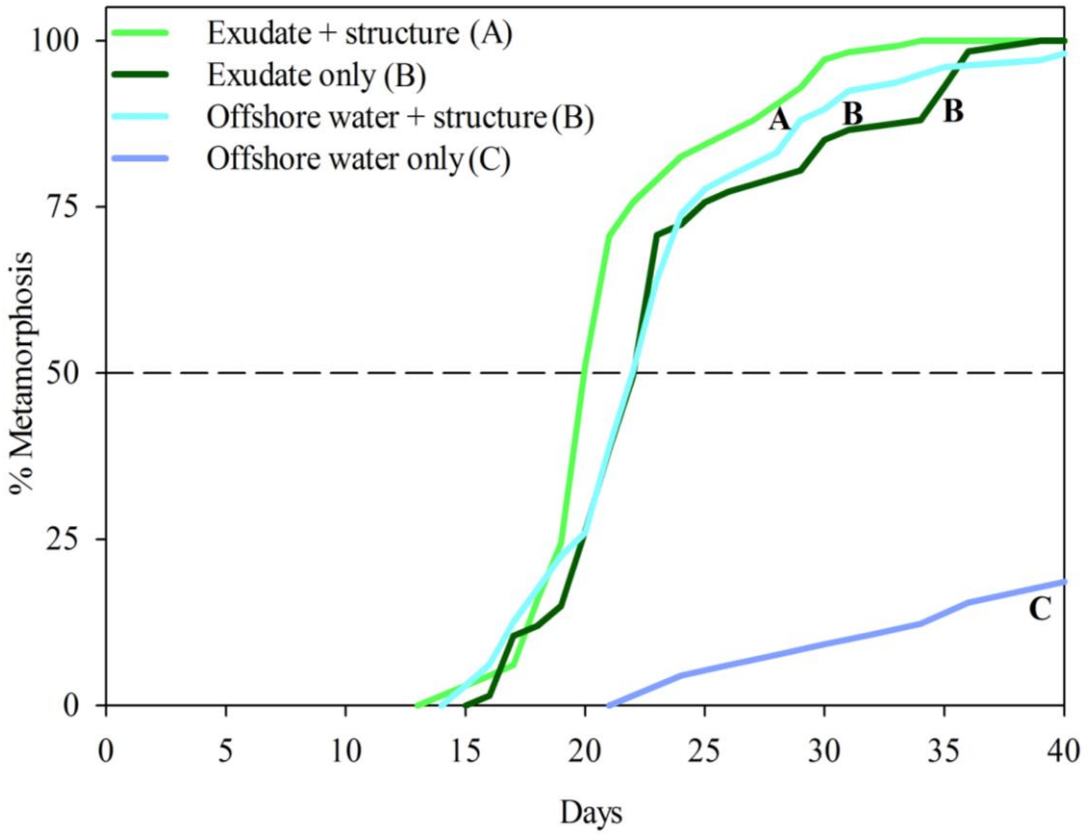

Figure I-7. Cumulative percent metamorphosis of *Limulus polyphemus* larvae during a 40-day exposure period to different levels of artificial seagrass structure (none or artificial ribbon) in water containing no chemical cues (offshore water) and seagrass exudate (n = 60). Curves with the same letters are not significantly different at $\alpha = 0.05$ (Cox Proportional Hazards Model).

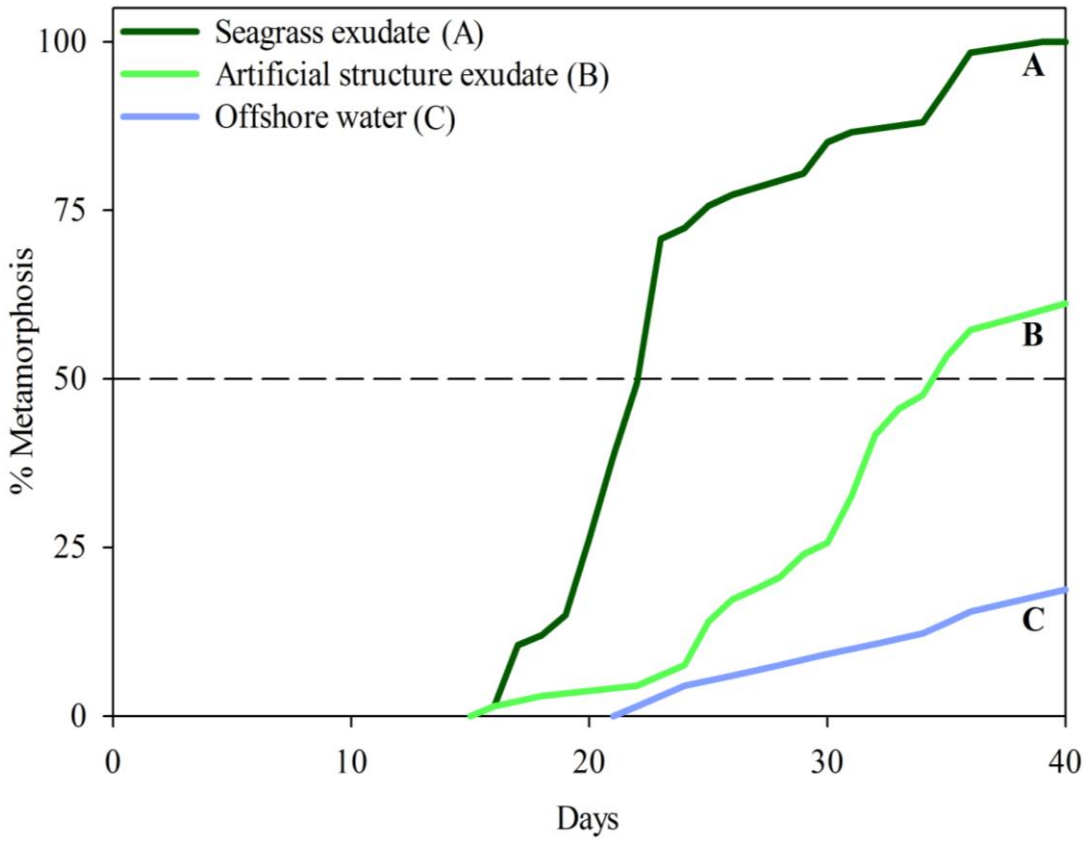

Figure I-8. Cumulative percent metamorphosis of *Limulus polyphemus* larvae during a 40-day exposure period to different exudates (none or offshore water, artificial structure exudate, and seagrass exudate) (n = 60). Curves with the same letters are not significantly different at $\alpha = 0.05$ (Cox Proportional Hazards Model).

Table I-7. Log-rank pairwise comparisons of time to metamorphosis of *Limulus polyphemus* trilobite larvae to the juvenile (J1) stage in the presence of different levels of artificial *H. wrightii* structure (none and ribbon) in water containing no chemical cues (offshore water), natural *H. wrightii* exudate (exudate), and artificial *H. wrightii* exudate (artificial exudate) (n = 60). Degrees of freedom for all comparisons are equal to 1.

Treatment	**Comparison**	**Log-Rank Test Statistic**	***P*-value**	**Hazard Ratio**
Offshore + structure	Offshore	96.0	< 0.001	19
Exudate only	Offshore	112.6	< 0.001	18
Exudate + structure	Offshore	124.4	< 0.001	32
Exudate only	Offshore + structure	0.41	0.525	-
Exudate + structure	Exudate only	7.6	0.006	-
Exudate + structure	Offshore + structure	7.0	0.008	-
Artificial exudate only	Offshore	19.0	< 0.001	4

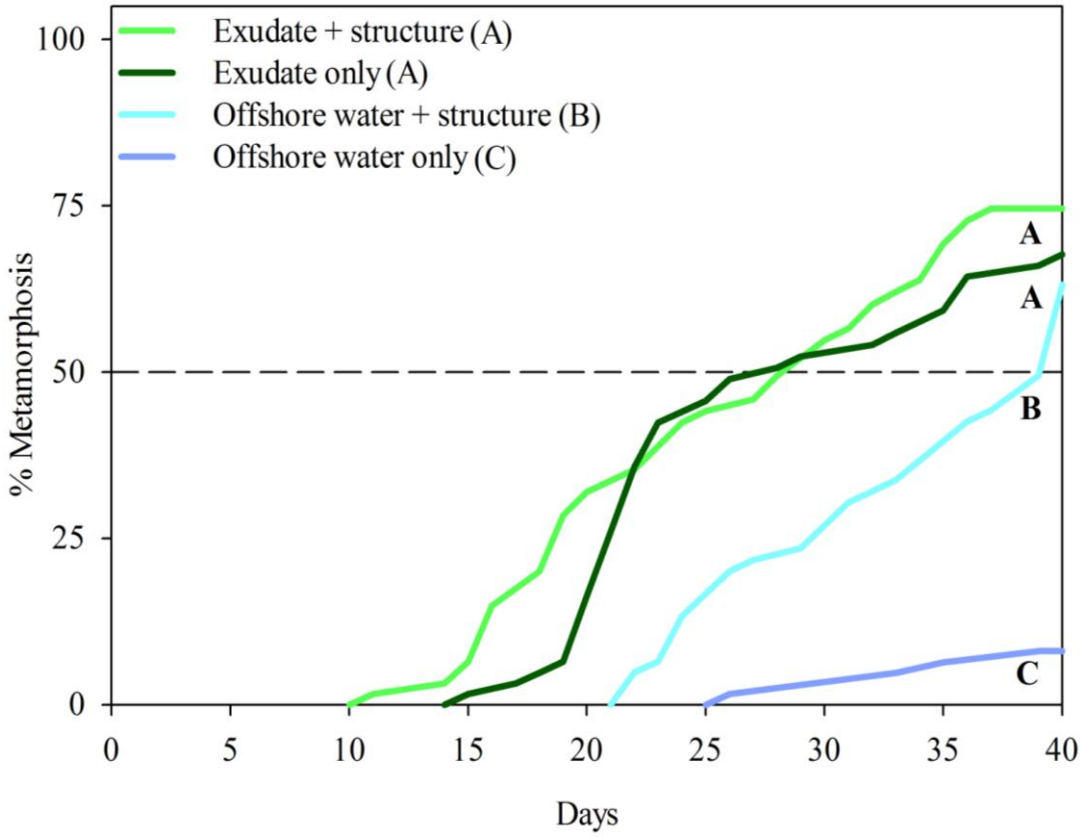

Figure I-9. Cumulative percent metamorphosis of *Limulus polyphemus* larvae during a 40-day exposure period to different levels of artificial seagrass structure (none or glass rods) in water containing no chemical cues (offshore water) and seagrass exudate (n=60). Curves with the same letters are not significantly different at $\alpha = 0.05$ (Cox Proportional Hazards Model).

Table I-8. Log-rank pairwise comparisons of time to metamorphosis of *Limulus polyphemus* trilobite larvae to the juvenile (J1) stage in the presence of different levels of artificial *H. wrightii* structure (none and glass) in water containing no chemical cues (offshore water) and natural *H. wrightii* exudate (exudate) (n = 60). Degrees of freedom for comparisons are equal to 1.

Treatment	**Comparison**	**Log-Rank Test Statistic**	***P*-value**	**Hazard Ratio**
Offshore + structure	Offshore	37.6	< 0.001	10
Exudate only	Offshore	50.7	< 0.001	15
Exudate + structure	Offshore	58.2	< 0.001	19
Exudate only	Offshore + structure	4.5	0.034	-
Exudate + structure	Exudate only	0.56	0.454	-
Exudate + structure	Offshore + structure	7.9	0.005	-

DISCUSSION

Chemical cues known to reduce time to metamorphosis (TTM) in *Limulus polyphemus* trilobite larvae are exudates from conspecifics and the seagrass *H. wrightii* (Boleman 2011). However, the effect of concentration on TTM and partial characterization of these cues have, until the current study, never been described. Exposure to conspecific- and *H. wrightii*-exudate water at concentrations ranging from 0.3 g L^{-1}- 30 g L^{-1} significantly reduced TTM in trilobite larvae (Figs. I-1 and I-2). The ability of the exudate water to significantly reduce TTM at the lowest concentration (0.3 g L^{-1}) indicated that the minimum concentration to affect metamorphosis is below 0.3 g L^{-1}. This is consistent with studies of other arthropods, which have also demonstrated low sensitivity thresholds in the presence of metamorphosis-inducing chemical cues. For instance, larvae of the estuarine crab *R. harrisii* (Fitzgerald et al. 1998) and the common mud crab *P. herbstii* (Andrews et al. 2001) possess a sensitivity threshold to conspecific exudate that is below 0.1 g L^{-1}. Under natural conditions, turbulence associated with tides and currents dilutes chemical cues to undetectable concentrations (reviewed by Pawlik 1992). However, marine invertebrates (e.g., *C. sapidus*) have been shown to respond to chemical cues in the natural habitat (Welch et al. 1997) and metamorphic chemical cues can be found at effective concentrations up to 2-3 cm from a chemical source (Hadfield and Scheuer 1985). Furthermore, when exposed to estuarine water collected from the field, TTM of *L. polyphemus* larvae is significantly reduced (Boleman 2011), indicating that horseshoe crabs have the

ability to detect chemical cues in the natural environment. Additionally, the ability of trilobite larvae to detect low concentrations may increase the chance of metamorphosing in a suitable habitat, despite any dilution of the chemical cue.

Moreover, TTM of *L. polyphemus* larvae decreased with increasing concentrations of conspecific and *H. wrightii* exudates, supporting the hypothesis that the effect of chemical cues is dose-dependent. These results are similar to those reported for other estuarine arthropods. For instance, TTM of the portunid crab *C. sapidus* decreases with increasing concentration of estuarine water (Forward et al. 1997). Also, the TTM of the panopeid crabs *R. harissii* (Fitzgerald et al. 1998) and *P. herbstii* (Andrews et al. 2001) decreases as the concentration of adult exudate increases. Furthermore, the effect of *H. wrightii* exudate on rheotactic behavior and visual orientation of *L. polyphemus* is concentration-dependent, theoretically increasing the likelihood of larvae finding the habitat-associated source (Medina and Tankersley 2010, Boleman 2011). Collectively, these results suggest that as trilobite larvae approach coastal and estuarine habitats, there is an increased probability of locating and metamorphosing close to the chemical source (i.e., a seagrass bed or conspecifics). The presence of juveniles and adults signals a habitat conducive to post-metamorphic growth and survival whereas seagrass may provide refuge from predation.

Thermal stability testing and filtration techniques can provide clues to the identity of the molecules present in exudates that induce metamorphosis. The freezing and heating (-70 °C or 100 °C) of conspecific- and *H. wrightii*-exudate

water had no effect on the potency of the molecules, indicating that the compounds were thermally stable (Figs. I-3 and I-4). Similarly, trilobite larvae exposed to exudate water dialyzed through membranes of different pore sizes (0.5-1.0 kDa, 8.0-10 kDa, and 100 kDa) responded similarly to all size fractions, suggesting that the effective molecule(s) in both source waters are relatively small (< 0.5 kDa) compounds (Figs. I-5 and I-6). Similar results for both exudate waters demonstrate that the same compound(s) responsible for inducing metamorphosis is (are) produced by both sources, but perhaps in different concentrations. In addition, these results indicate that *L. polyphemus* may be able to detect a range of similar cues, or a general class of cues (e.g., proteins, peptides, or carbohydrates).

The molecules that affect metamorphosis in *L. polyphemus* share characteristics with metamorphosis-inducing molecules of other marine invertebrates. Inducing molecules for *Haliotis rufescens* (Morse et al. 1984), *Crassostrea virginica* (Zimmer-Faust and Tamburri 1994), and *Echinarachnius parma* (Pearce and Scheibling 1990) have been found to be < 1.5 kDa in size. Additionally, chemical cues from conspecifics responsible for reducing TTM in *Hemigrapsus sanguineus* are between 3.5- 14 kDa in size (Steinberg et al. 2007), while molecules in estuarine water that induce metamorphosis in *C. sapidus* are < 10 kDa (Forward et al. 1996). Furthermore, the inducing molecules for the common mud crab *P. herbstii* and the queen conch *Strombus gigas* are both small in size (< 1 kDa) and resistant to thermal denaturation (Boettcher and Target 1996, Andrews et al. 2001). The similarity of characteristics of metamorphosis-inducing

molecules among a variety of species indicates that metamorphosis may be regulated by a similar compound or compounds across taxa. Moreover, similarity across taxa suggests that some marine invertebrate larvae may have the ability to respond to a variety of chemical sources (reviewed by Chia 1989, Steinberg et al. 2002).

Organisms that respond to very specific chemical sources are typically those that have narrow habitat ranges or narrow geographic distributions, such as the Australian endemic echinoid *Holopneustes purpurascens* (Williamson et al. 2000) and the marine herbivore *Alderia modesta* (Krug and Manzi 1999). *Limulus polyphemus* is considered an ecological generalist that exists in a variety of coastal habitats throughout its range (reviewed by Botton et al. 2003, Sekiguchi and Shuster 2009). Additionally, all chemical cues tested thus far induce metamorphosis in *L. polyphemus*, including conspecific and *H. wrightii* exudates and exudates from a known predator (the mummichog *Fundulus grandis*) (Boleman 2011). Together, these results suggest that the horseshoe crab responds to a wide range of chemical cues. The ability to respond to multiple habitat-associated chemicals should increase the probability of metamorphosing in a suitable habitat.

Various molecules within chemical exudates have been shown to induce metamorphosis in marine invertebrates, including, but not limited to, fatty acids, peptides/proteins, and carbohydrates (reviewed by Hadfield and Paul 2001, Steinberg et al. 2002). Metamorphosis-inducing molecules are often described as

small, heat-stable peptides ending in arginine or lysine structures (reviewed by Rittschof and Bonaventura 1986, Rittschof 1989, Hadfield and Paul 2001, Steinberg et al. 2002). The small size (< 0.5 kDa) and thermal stability of the molecule(s) in the present study suggests that the inducing molecule for *L. polyphemus* may be a low molecular weight, heat-stable compound, possibly an amino acid. Amino acids and peptides may be effective cues for marine organisms because the background level of peptides in the marine environment is low, making them easy to detect (Rittschof and Bonaventura 1986). Furthermore, many organisms already use amino acids and peptides or their derivatives in internal signal transduction systems, making the receptors required to respond to these cues readily available to many taxa (Steinberg et al. 2002).

Habitat-associated chemical sources may also provide structural or textural cues that induce metamorphosis. In the present study, mimicking seagrass structure (polypropylene ribbon) significantly reduced TTM of *L. polyphemus* larvae in the absence of seagrass exudate, supporting the hypothesis that structure has a significant effect on metamorphosis (Fig. I-7). However, the ribbon exuded a chemical that also reduced TTM (Fig. I-8). This result demonstrates the need to carefully choose artificial mimics when conducting structural experiments. When ribbon was replaced with sterilized and biologically inert glass rods, TTM was still significantly reduced in the absence of chemical cues, emphasizing the importance of structure for *L. polyphemus* (Fig. I-9). Metamorphosis of other marine arthropods has been found to be influenced by structural cues. For instance,

Gebauer et al. (1998) found that metamorphosis of the postlarval stage of *C. granulata* is accelerated in the presence of artificial filamentous algae. Additionally, TTM in *H. sanguineus* is reduced when exposed to nylon mesh of certain pore sizes (Steinberg et al. 2008).

The TTM of larvae in the seagrass exudate with structure was not significantly different from the TTM of larvae maintained in the exudate-only treatment (Fig. I-9). This result indicates that there was no additive or synergistic effect when the cues were combined. Therefore, at a concentration of 30 g L^{-1}, the chemical cue may be the primary trigger of metamorphosis and therefore takes precedence over the structural cue. Chemical cues are often the primary trigger for metamorphosis because they are typically water-soluble and detectable from a distance (Steinberg et al. 2002). Furthermore, in the marine environment, clean structure is never encountered because structures are frequently covered by biofilms and epibionts that release chemical exudates (reviewed by Qian et al. 2007).

However, early life stages of marine and estuarine arthropods are often abundant in structurally complex microhabitats, such as mussel beds (e.g. *Carcinus maenas*: Klein-Breteler 1976), seagrass beds (e.g., *C. sapidus*: Heck and Orth 1980), cobblestones (e.g., *Homarus americanus*: Wahle and Steneck 1992), and macroalgae (e.g., *Panulirus argus*: Herrnkind and Butler 1986). These structured habitats may provide the inhabitants with refugia from predation. For instance, when compared with open-sand habitat, predation rates on marine arthropods tend

to be lower in vegetative habitats (e.g., *C. sapidus*: Perkins-Visser et al. 1996; *C. maenas*: Moksnes et al. 1998; *L. polyphemus*: Boleman 2011) and on substrates which provide three-dimensional structure, such as cobblestone and mussel beds (e.g., *Homarus americanus*: Barshaw and Lavalli 1988, Wahle and Steneck 1992; *C. maenas*: Moksnes et al. 1998). The results in the present study indicate that structure may be important in inducing metamorphosis of *L. polyphemus* in habitats that provide protection from predation and therefore increase post-metamorphic survival.

Today, *L. polyphemus* is primarily harvested as bait for fishing industries, for the production of a bacterial endotoxin indicator (Berkson and Shuster 1999, Kreamer and Michels 2009), and for research in vision and endocrinology (Berkson and Shuster 1999, Rutecki et al. 2004). Ecologically, horseshoe crabs are an important part of benthic food webs (Botton 2009). Additionally, horseshoe crab eggs supply a food source to shorebirds that migrate along the east coast of the United States (Carmichael and Brush 2012). However, *L. polyphemus* populations have declined (Carmichael and Brush 2012), particularly in the New York and New England areas (Sweka et al. 2013). Population declines, along with the importance of horseshoe crabs both economically and ecologically, have prompted a growing interest in the captive rearing of *L. polyphemus* to supplement natural populations (Carmichael and Brush 2012). Characterizing molecules within exudates that affect metamorphosis determining their effective concentrations may assist aquaculture programs in enhancing depleted populations of *L. polyphemus*

(reviewed by Qian et al. 2007). Avoiding a delay in metamorphosis to the first juvenile stage can increase post-metamorphic growth and survival in some species (Pechenik 1990), therefore increasing aquaculture yield (Qian et al. 2007).

Furthermore, determining and analyzing cues that induce metamorphosis may lead to the identification and protection of potential settlement sites for *L. polyphemus*. For instance, previous research has demonstrated the importance of *H. wrightii* exudate in the metamorphosis of horseshoe crabs (Boleman 2011). Therefore, the implementation of management plans geared towards the conservation of seagrass beds will be important in restoring horseshoe crab populations. Substrates that exude chemical cues that influence metamorphosis (i.e., *H. wrightii*) also provide refuges. Typically, structure is thought to be important for animals that are incapable of burying themselves to avoid predators (e.g., *H. americanus*: Wahle and Steneck 1992). However, as indicated by the present study, seagrass structure can also significantly affect TTM of horseshoe crabs, demonstrating that structure may be more important for burying animals than previously thought. The response of *L. polyphemus* larvae to both chemical and structural cues provided by *H. wrightii* indicates that seagrass habitats are essential to a wide range of larval marine taxa. These results highlight the importance of restoring seagrass habitats as an essential part of marine conservation programs and the need for taxon-specific studies on the effect of structure on metamorphosis of marine larvae.

CHAPTER II

METAMORPHIC COMPETENCY AND COST OF DELAYED METAMORPHOSIS IN THE AMERICAN HORSESHOE CRAB *LIMULUS POLYPHEMUS*

INTRODUCTION

The larval phases of many benthic marine and estuarine invertebrates are planktonic, undergoing development in the pelagic environment (reviewed by Pechenik 1999), and are typically classified as either planktotrophic (feeding) or lecithotrophic (nonfeeding). The transition to the benthos typically occurs at the time of metamorphosis and is often triggered by exogenous cues, including water-soluble chemical cues and structural cues associated with the settlement habitat (reviewed by Pechenik et al. 1998, Hadfield 2000, Forward et al. 2001, Hadfield and Paul 2001). Exogenous cues known to affect (accelerate or delay) metamorphosis include exudates from conspecifics and aquatic vegetation and structural components of potential settlement or nursery habitats (reviewed by Forward et al. 2001). When exogenous cues signaling suitable habitat (positive cues) are absent or cues indicating an unsuitable habitat (negative cues) are present, both planktotrophic and lecithotrophic larvae can postpone metamorphosis (reviewed by Forward et al. 2001, Pechenik 2006).

Following hatching, there is usually a period of early development when larvae are unable to settle and/or metamorphose (reviewed by Hadfield et al. 2001,

Gebauer et al. 2003). This phase is typically referred to as the "pre-competency" period. Once competency is reached, larvae become physiologically and morphologically able to undergo metamorphosis (reviewed by Hadfield et al. 2001, Gebauer et al. 2003). Competency is a developmental trait shared by many marine invertebrates that enables larvae to (1) remain viable in the plankton until a suitable habitat is found, and (2) metamorphose rapidly in response to exogenous cues (reviewed by Hadfield 2001). After competency is reached, larvae often develop juvenile components before losing larva-specific structures (reviewed by Hadfield et al. 2001). Competency can last for a few hours to several weeks, with some aquatic invertebrate species, such as the sea hare *Aplysia juliana* and the coral *Pocillopora damicornis*, retaining the ability to metamorphose for more than 100 days (reviewed by Hadfield et al. 2001). However, after a certain period of time, competency, and the adaptive advantages associated with it, is lost (reviewed by Hadfield et al. 2001, Pechenik et al. 2006). The ability to retain competency differs among species and is correlated with the specificity of the juvenile habitat (i.e., the greater the habitat specificity, the greater the ability to retain competence) (reviewed by Bishop et al. 2006).

An extended competency period (i.e., delaying metamorphosis) increases the time in the plankton and may increase the chance of finding a habitat which is favorable to juvenile growth and survival (Pechenik 1990, Anger 2001, reviewed by Gebauer et al. 2003). Lengthening the pelagic phase may also increase transport and dispersion, therefore increasing genetic exchange between spatially separated

populations, reducing competition between parents and offspring, lessening habitat saturation, and assisting in range expansion and settlement in new habitats (Pechenik and Cerulli 1991, reviewed by Pechenik 1999). However, the transition from the plankton to the benthos is often metabolically and energetically costly (Shilling et al. 1996, Thiyagarajan et al. 2003). Therefore, delaying metamorphosis may have negative effects on the growth and survival of the consequent developmental stages because of nutritional stress (especially for lecithotrophic larvae), which could reduce an animal's ability to compete for space, food, and mates (i.e., reduce fitness) (Woollacott et al. 1989, Pechenik et al. 1998, Gebauer et al. 2003, Marshall and Keough 2005, Pechenik 2006). Additionally, remaining in the plankton can increase the chance of predation, slow development by increasing the molt-stage duration (MSD), decrease metamorphic success, and delay the onset of sexual maturity (Lucas et al. 1979, Hunt and Scheibling 1997, Pechenik et al. 1998, Gebauer et al. 1999, Pechenik et al. 1999).

The American horseshoe crab, *Limulus polyphemus,* possesses a lecithotrophic trilobite larva that develops in the plankton before settling in benthic habitats and metamorphosing to the juvenile stage (Jegla and Costlow 1979, Sekiguchi 1988, Shuster et al. 2003). Trilobite larvae are known to delay metamorphosis for at least 40 days in the absence of chemical cues (Boleman 2011; Chapter 1). Yet, it is unknown when they become competent to metamorphose, and if there is any impact of delayed metamorphosis on post-metamorphic growth and survival. In the current study, I tested the hypotheses that trilobite larvae

undergo a brief pre-competency period after hatching and that delayed metamorphosis negatively impacts post-metamorphic size and survival.

MATERIALS AND METHODS

COLLECTION AND MAINTENANCE OF LARVAE

Limulus polyphemus eggs were collected in August 2013 near the Sunshine Skyway Bridge, Tampa Bay, FL (27°35´5.51" N, 82°36´44.70" W) and brought back to the laboratory at Florida Institute of Technology. Developing embryos and trilobite larvae were reared in 19 cm diameter × 6 cm glass bowls containing 1 L of filtered (<5 µm) seawater. Larvae from different nests were maintained separately at 26 °C and at a salinity of 35. Embryos were exposed to a 14:10 hour light-dark cycle. Water used to incubate the embryos was collected ~50 m off the beach near the Florida Institute of Technology's Vero Beach Marine Laboratory (VBML), Vero Beach, FL (27°40´31.83" N, 80°21´50.69" W). Seawater within the glass bowls was replaced daily.

EXPERIMENTAL DESIGN

All experiments were conducted at 26 °C, at a salinity of 35, and under a 14:10 hour light-dark cycle. Immediately following hatching, individual trilobite larvae (replicates) were placed in separate chambers (5 cm × 5.5 cm × 4 cm) of a compartmentalized plastic container. Each compartment contained 40 mL of either offshore water or conspecific-exudate water (30 g L^{-1}). Offshore water was collected 15 km seaward of Ft. Pierce Inlet, FL, well beyond the estuarine plume, and was considered to be devoid of any chemical cues that may affect metamorphosis.

Each treatment level was replicated 60 times using larvae from six different nests (cohorts). Treatment water was prepared daily and was filtered (< 5 µm) prior to being used in experiments. Plastic containers were rinsed twice with deionized water and allowed to air-dry overnight.

COMPETENCY

To investigate when *L. polyphemus* larvae become competent to metamorphosis, trilobite larvae were exposed to one of five treatments in which metamorphosis was delayed by increasing lengths of time: no delay (i.e., immediate exposure to conspecific exudate; 0 day control) and delays of 7 days, 14 days, 21 days, and 28 days post-hatch. In the delay treatments, larvae were placed in compartments containing offshore water (40 mL) for the length of the delay period (7, 14, 21, or 28 days) before being placed in water containing conspecific exudate (40 mL) for the remainder of the experiment.

Offshore water was collected 15 km seaward of Ft. Pierce Inlet, FL, well beyond the estuarine plume, and was assumed to be free of estuarine or coastal chemical cues that are known to accelerate metamorphosis. *L. polyphemus* juveniles used to create exudate water were collected from the Indian River Lagoon (Brevard County, FL). Exudate water was prepared by incubating juveniles in a glass aquarium with 1 L of offshore water for 24 hours. Before incubation, juveniles were gently washed with coastal water, blotted dry with a paper towel, and weighed.

Trilobite larvae were checked daily at 16:00 hours for metamorphosis and mortality. The effect of different treatments on time to metamorphosis (TTM) was determined using failure-time analysis (Cox Proportional Hazards Model) (Muenchow 1986), with TTM or the duration of the trilobite larval stage serving as the "time until an event occurs" in the analysis. The resulting hazard function for each treatment was the probability that a trilobite larva would metamorphose during the next time interval (Δt= 1 day) (Muenchow 1986, Kleinbaum and Klein 1996). To control for potential nest effects on metamorphosis rates, cohort group or nest was added as a covariate in the analysis. Comparisons of the TTM for larvae exposed to different treatments and controls were made using the Log-Rank (LR) test (Kleinbaum and Klein 1996). The onset of competency was arbitrarily defined as the time for 25% of the larvae to metamorphose to the first juvenile stage (TTM_{25}). All analyses were performed using SPSS 20.0 and SigmaPlot 11.0.

COST OF DELAYED METAMORPHOSIS

To detect any costs of delayed metamorphosis, larvae in the competency experiment that experienced the longest delay periods (21 and 28 days) were compared with larvae immediately exposed to the inducing chemical cue (30 g L^{-1} of conspecific exudate; 0 day control). These crabs were monitored through the third juvenile stage (J3) for the effects of delayed metamorphosis on post-metamorphic size, shape, and survival. This was investigated by comparing survivorship (%), molt-stage duration (MSD, days), and size and shape of individuals in the three treatments to the third juvenile instar (J3). Size

measurements included prosoma length (PL) and prosoma width (PW). Prosoma shape was analyzed by computing PL: PW ratios (J1-J3).

Following metamorphosis to the first juvenile instar, all larvae (regardless of the treatment) were placed in filtered seawater (< 5 µm) and fed concentrated *Artemia* nauplii daily (50 nauplii per 40 mL). The stage and condition of larvae and juveniles were recorded daily at 16:00 hours. To determine and compare prosoma size and shape, digital images (Nikon Coolpix 995) of individual crabs were taken after each molt (J1-J3). Measurements of individuals to the nearest 0.01 mm were made by analyzing the digital images using ImageJ software.

The effect of each delay treatment on survivorship was investigated using failure time analysis (Cox Proportional Hazards Model) (Muenchow 1986), with time to death serving as the "time until an event occurs". To control for potential nest effects on death rates, cohort group or nest was added as a covariate in the analysis. Additionally, the effect of delay on MSD was analyzed using a repeated measures ANOVA with one between-subjects factor (delay treatment) and one within-subjects factor (juvenile instar). Measurements of PL, PW, and PL: PW ratios were compared among treatments using a Kruskal-Wallis test and post-hoc comparisons were made using Dunn's test. All statistical analyses were performed using SPSS 20.0 and SigmaPlot 11.0.

RESULTS

COMPETENCY

Delaying the time to exposure to conspecific exudate had a significant impact on the time to metamorphosis (TTM) (Fig. II-1 and Table II-1). Time to metamorphosis of larvae in the 14 day, 21 day, and 28 day delay treatments were significantly longer when compared with larvae immediately exposed to conspecific exudate (no delay; control) (Fig. II-1 and Table II-1). However, TTM of larvae in the 7-day delay treatment was not significantly different from the control (Fig. II-1 and Table II-1). Median time to metamorphosis (TM_{50}) increased with the increase in delay interval (control: 18 days ± 0.84 (±SE); 7 day delay: 18 days ± 0.56; 14 day delay: 22 days ± 0.53; 21 day delay: 29 days ± 0.55; 28 day delay: 36 days ± 0.92; Fig. II-1). If the initiation of the competency period is defined as the time when 25% of the larvae metamorphose (TM_{25}) then larvae in both the control and 7 day delay treatment began to reach competency at day 16 (Fig. II-2). Time for larvae to reach competency in the remaining treatments increased as delay interval increased (Fig. II-2). Moreover, the cohort (nest covariate) had a significant effect on TTM (Wald $\chi^2 = 32.2$, d.f. = 5, $P < 0.001$).

When TTM was measured relative to the day of exposure, TTM differed significantly among treatments (Fig. II-3 and Table II-2). Metamorphosis of larvae in each delay treatment was significantly accelerated in comparison to the control (Fig. II-3 and Table II-2). The TTM of larvae in the 7 day delay treatment was significantly longer than those in the remaining delay treatments (Fig. II-3 and

Table II-2). However, there was no significant difference in TTM among larvae in the 14, 21, and 28 day delay treatments (Fig. II-3 and Table II-2). Median time to metamorphosis relative to the day of exposure (MT_{50}) decreased as delay interval increased from 0 to 14 days and leveled off at about 8 days in the 21 and 27 day delay treatments (Fig. II-3). Again, the cohort (nest covariate) had a significant effect on TTM (Wald $\chi^2 = 17.5$, d.f. = 5, $P = 0.004$).

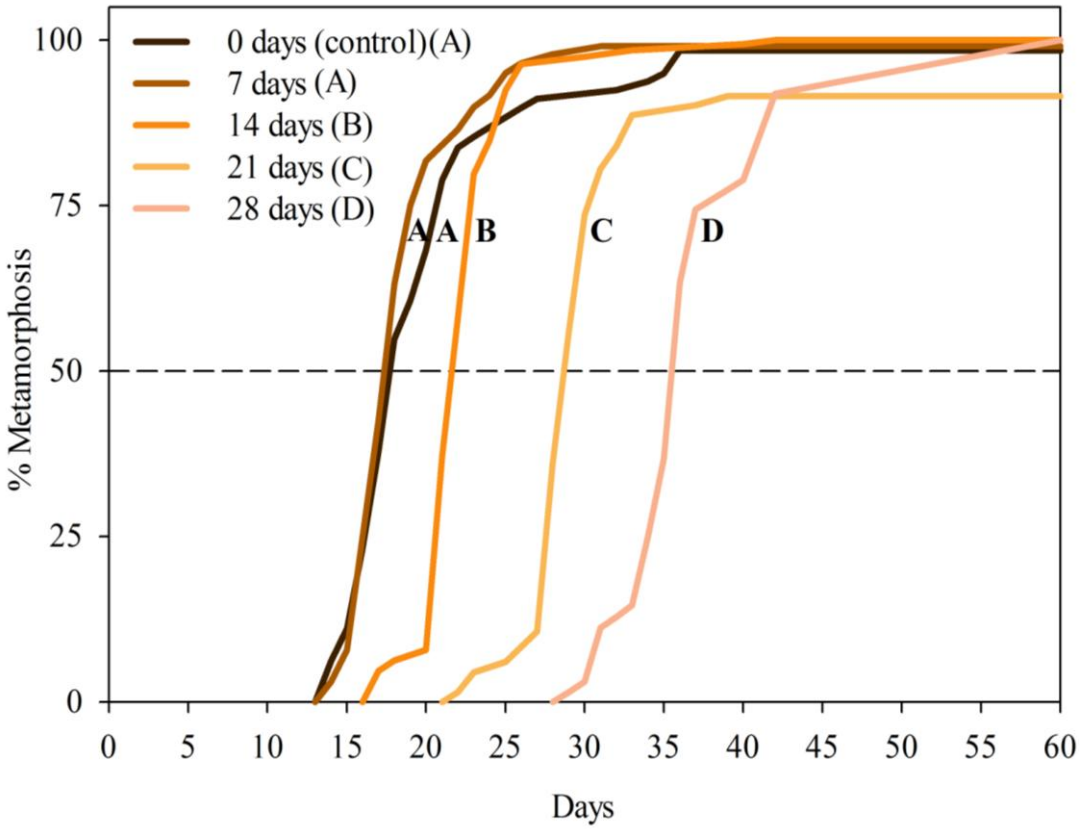

Figure II-1. Cumulative percent metamorphosis of *Limulus polyphemus* larvae following exposure to conspecific exudate (30 g L^{-1}) either immediately following hatching (0 days) or at delay intervals of 7, 14, 21, and 28 days post-hatch (n = 60). Curves with the same letters are not significantly different at $\alpha = 0.05$ (Cox Proportional Hazards Model).

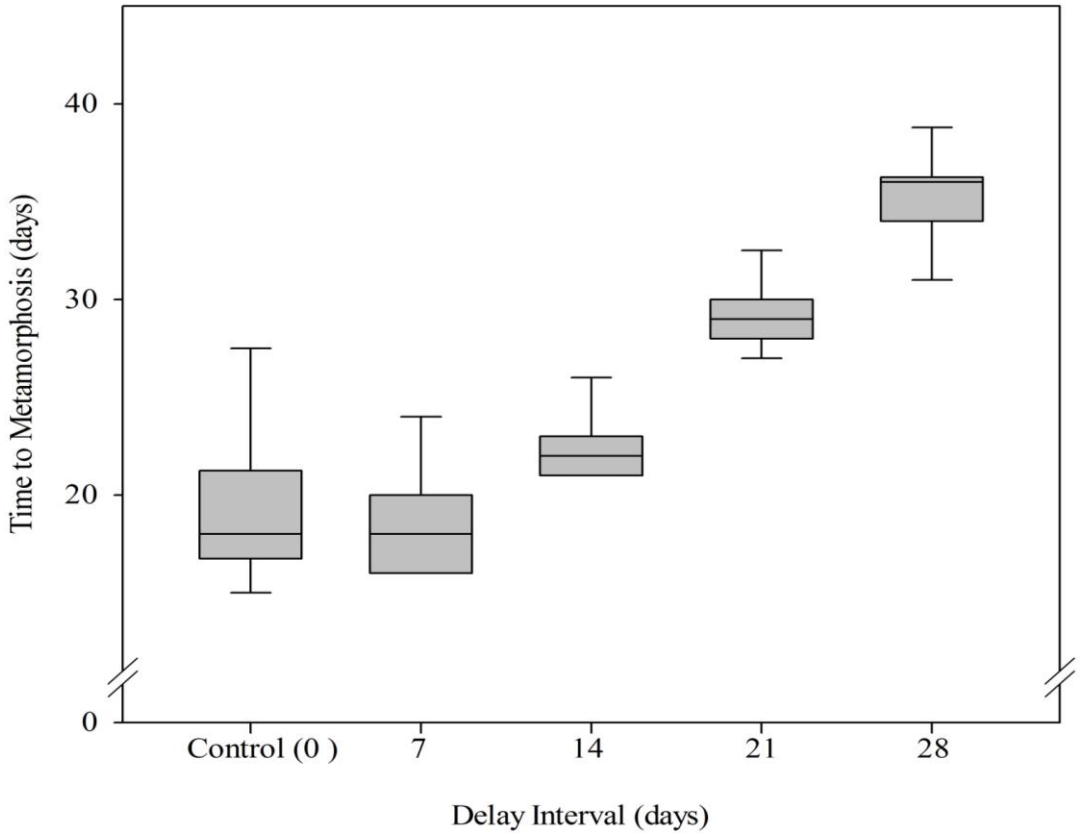

Figure II-2. Box plot of time to metamorphosis of *Limulus polyphemus* larvae after exposure to conspecific exudate (30 g L^{-1}) either immediately following hatching (0 days) or at delay intervals of 7, 14, 21, and 28 days post-hatch (n = 60).

Table II-1. Log-rank pairwise comparisons of time to metamorphosis of *Limulus polyphemus* trilobite larvae to the juvenile (J1) stage after exposure to conspecific exudate (30 g L^{-1}) either immediately following hatching (control) or at delay intervals of 7, 14, 21, and 28 days post-hatch (n = 60). Degrees of freedom for all comparisons are 1.

Delay Interval (days)	**Comparison**	**Log-Rank Test Statistic**	***P*-value**
7	Control	2.5	0.113
14	Control	7.2	0.007
21	Control	39.8	< 0.001
28	Control	77.1	< 0.001
7	14	26.1	< 0.001
14	21	59.3	< 0.001
21	28	34.2	< 0.001
14	28	96.4	< 0.001

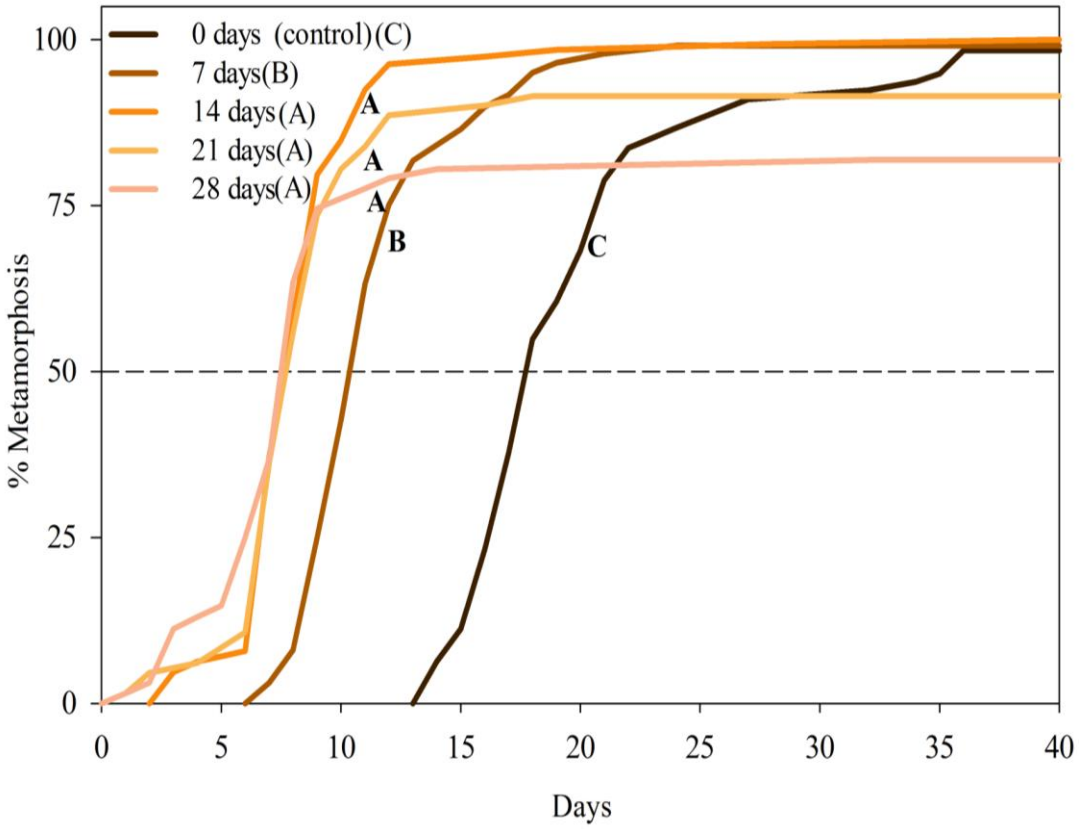

Figure II-3. Cumulative percent metamorphosis of *Limulus polyphemus* larvae relative to the day of exposure to conspecific exudate (0, 7th, 14th, 21st, 28th day post-hatch) (n = 60). Curves with the same letters are not significantly different at $\alpha = 0.05$ (Cox Proportional Hazards Model).

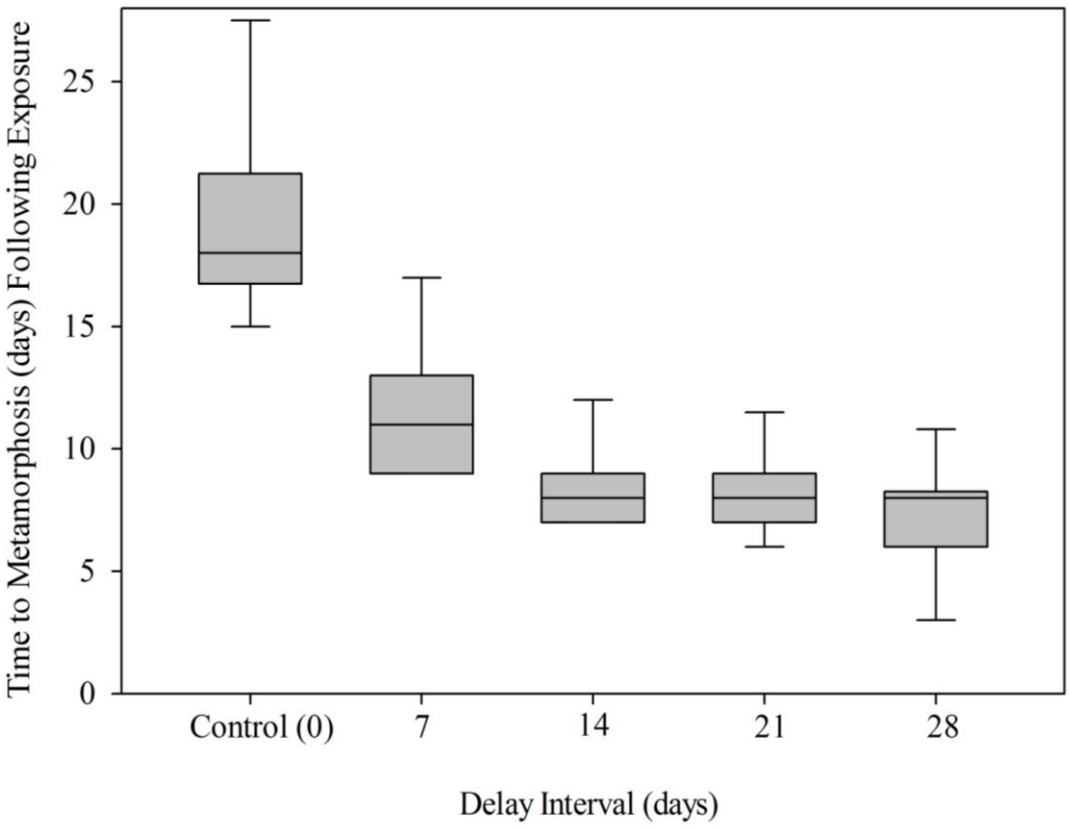

Figure II-4. Box plot of time to metamorphosis of *Limulus polyphemus* larvae relative to the day of exposure to conspecific exudate (0, 7th, 14th, 21st, 28th day post-hatch) (n = 60).

Table II-2. Log-rank pairwise comparisons of time to metamorphosis of *Limulus polyphemus* trilobite larvae to the juvenile (J1) stage relative to the day of exposure to conspecific exudate (0, 7th, 14th, 21st, 28th day post hatch) (n = 60). Degrees of freedom for all comparisons are 1.

Delay Interval (days)	**Comparison**	**Log-Rank Test Statistic**	***P*-value**
7	Control	55.4	< 0.001
14	Control	71.0	< 0.001
21	Control	34.3	< 0.001
28	Control	14.4	< 0.001
7	14	17.5	< 0.001
14	21	0.72	0.396
21	28	0.01	0.935
14	28	0.69	0.407

COST OF DELAYED METAMORPHOSIS

Delaying metamorphosis had no significant effect on survivorship of *L. polyphemus* individuals to the third juvenile instar (J3) (LR = 0.64, d.f. = 2, P = 0.726; Fig. II-5). Also, the cohort (nest covariate) had no significant effect on TTM (Wald χ^2 = 0.127, d.f. = 5, P = 0.127). Likewise, molt-stage duration (MSD, days) did not differ significantly among treatments (Fig. II-6 and Table II-3). First juvenile instars (J1) had an MSD of about 14 days, whereas second juvenile instars (J2) had an MSD of about 15 days (Fig. II-6 and Table II-3). There was no significant interaction between the delay treatment and juvenile instar on MSD (Table II-3).

Furthermore, there was no significant effect of delay treatment on the prosoma length (PL) of any juvenile instars (J1-J3) (Figs. II-7a – II-9a and Table II-4). However, delaying metamorphosis had a significant impact on prosoma width (PW). Older crabs (J3) in the longest delay treatment (28 days) were significantly narrower than younger individuals (J1 and J2) in the control and 21 day delay treatments (Fig. II-9b and Table II-4). Additionally, delaying metamorphosis for 28 days had a significant effect on prosoma shape (ratio of PL: PW) (Fig. II-12 and Table II-4), producing third instar juveniles that were more circular in shape (Fig. II-12 and Table II-4) when compared with similar stage juveniles maintained in the control (no delay).

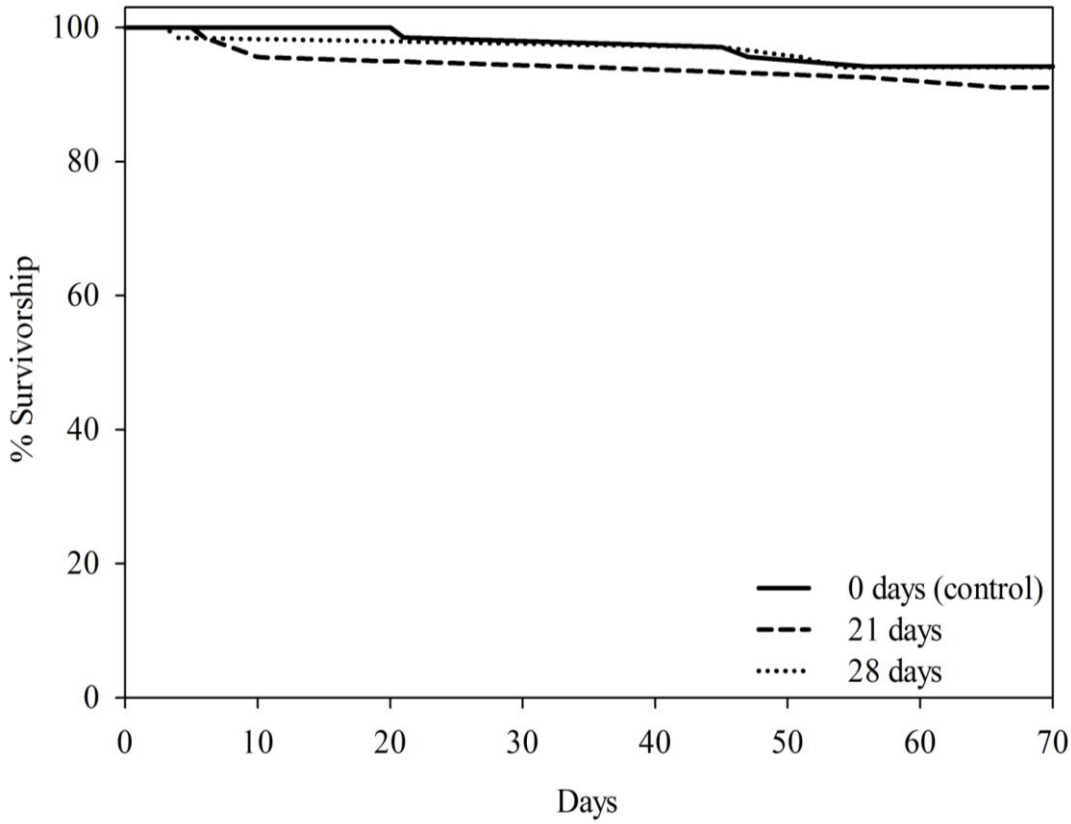

Figure II-5. Cumulative percent survival of *Limulus polyphemus* larvae to the third juvenile (J3) stage following exposure to conspecific exudate (30 g L^{-1}) either immediately following hatching (0 days) or at delay intervals of 21 and 28 days post-hatch (n = 60) (Cox Proportional Hazards Model).

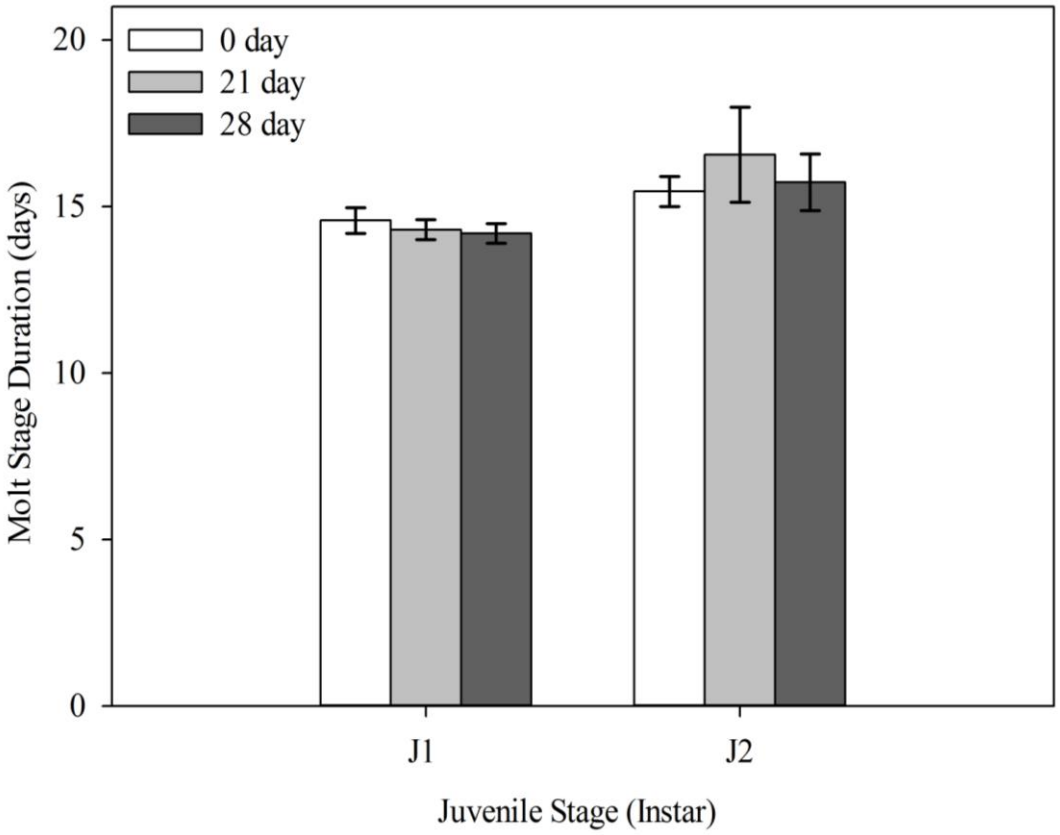

Figure II-6. Mean (± 95% CI) molt-stage duration (days) for the first and second *Limulus polyphemus* juvenile instars (J1 and J2) in control (0 day delay) and delay treatments (21 and 28 day delays) (n = 45 - 53).

Table II-3. Results of a repeated measures analysis of variance (ANOVA) on molt stage duration (MSD, days) of *Limulus polyphemus* juveniles (J1 and J2) exposed to conspecific exudate immediately after hatching (control; 0 day delay) or exposed 21 and 28 days post-hatch (n = 45-53).

Source of Variation	**F**	**d.f.**	***P*-value**
Delay	0.65	2, 144	0.524
Juvenile Instar	26.8	1, 144	< 0.001
Interaction- Juvenile Instar × Delay	1.7	2, 144	0.194

Table II-4. Results of Kruskal-Wallis tests on size and shape measurements of *Limulus polyphemus* juveniles (J1-J3) exposed to conspecific exudates immediately after hatching (control; 0 day delay) or exposed 21 and 28 days post-hatch (n = 44- 52). Degrees of freedom for all comparisons is equal to 2. Post-hoc comparisons of delay treatments were made using Dunn's test. NS = not significant at $P = 0.05$.

Size/Shape Measurement	Juvenile Instar	χ^2	*P*-value	Post-Hoc Comparison	*P*-value
Prosoma Length (PL)	J1	2.6	0.278	--	--
	J2	0.83	0.660	--	--
	J3	1.7	0.433	--	--
Prosoma Width (PW)	J1	4.4	0.113	--	--
	J2	4.3	0.117	--	--
	J3	8.6	0.013	0 vs. 21	NS
				0 vs. 28	S
				21 vs. 28	NS
Ratio (PL: PW)	J1	0.4	0.805	--	--
	J2	3.5	0.173	--	--
	J3	6.7	0.036	0 vs. 21	NS
				0 vs. 28	S
				21 vs. 28	NS

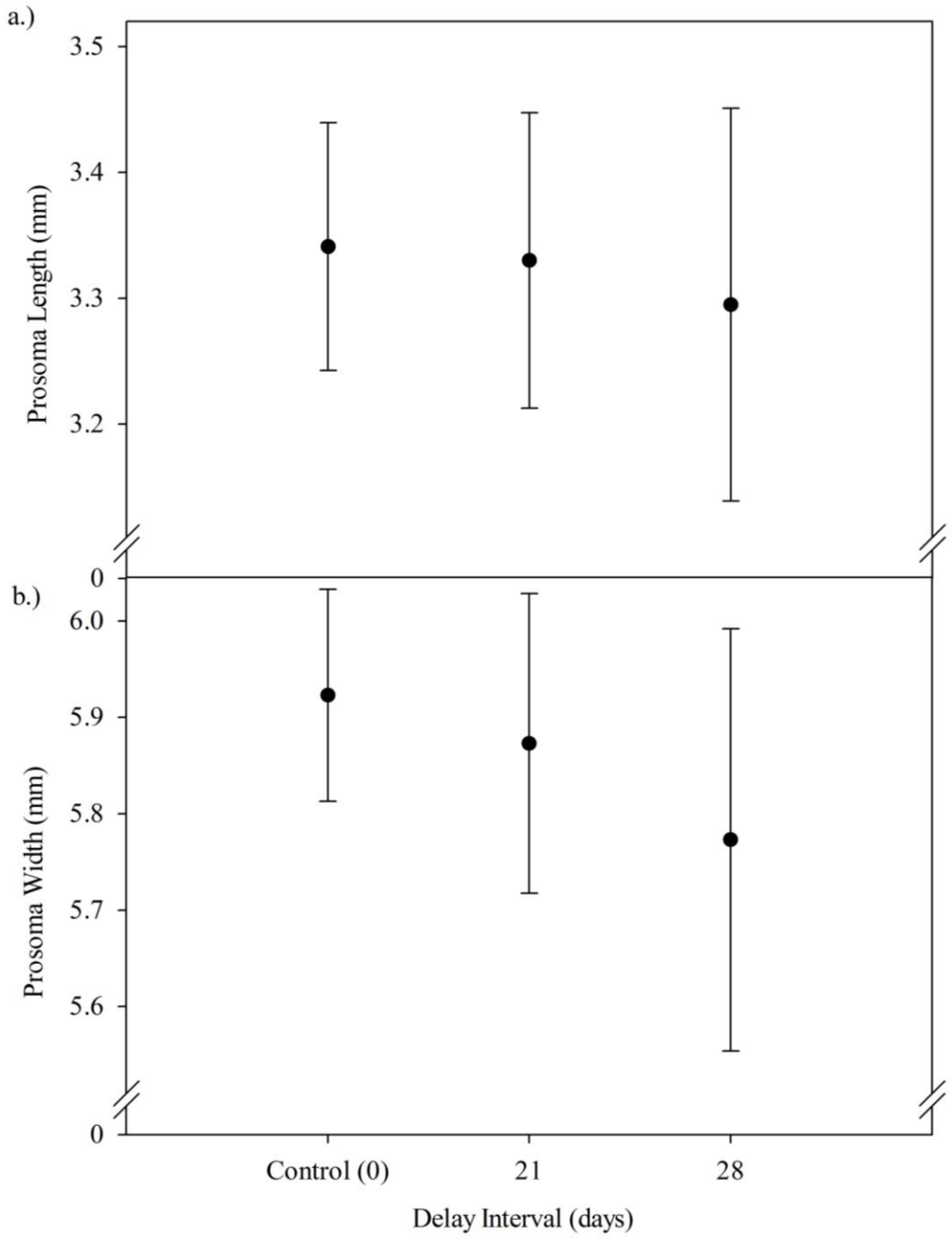

Figure II-7. a). Median (± MAD) prosoma length (mm) and (b.) median (± MAD) prosoma width (mm) of *Limulus polyphemus* first juvenile instars (J1) exposed to each delay treatment: 0 (control), 21, and 28-day delay intervals (n = 44-52).

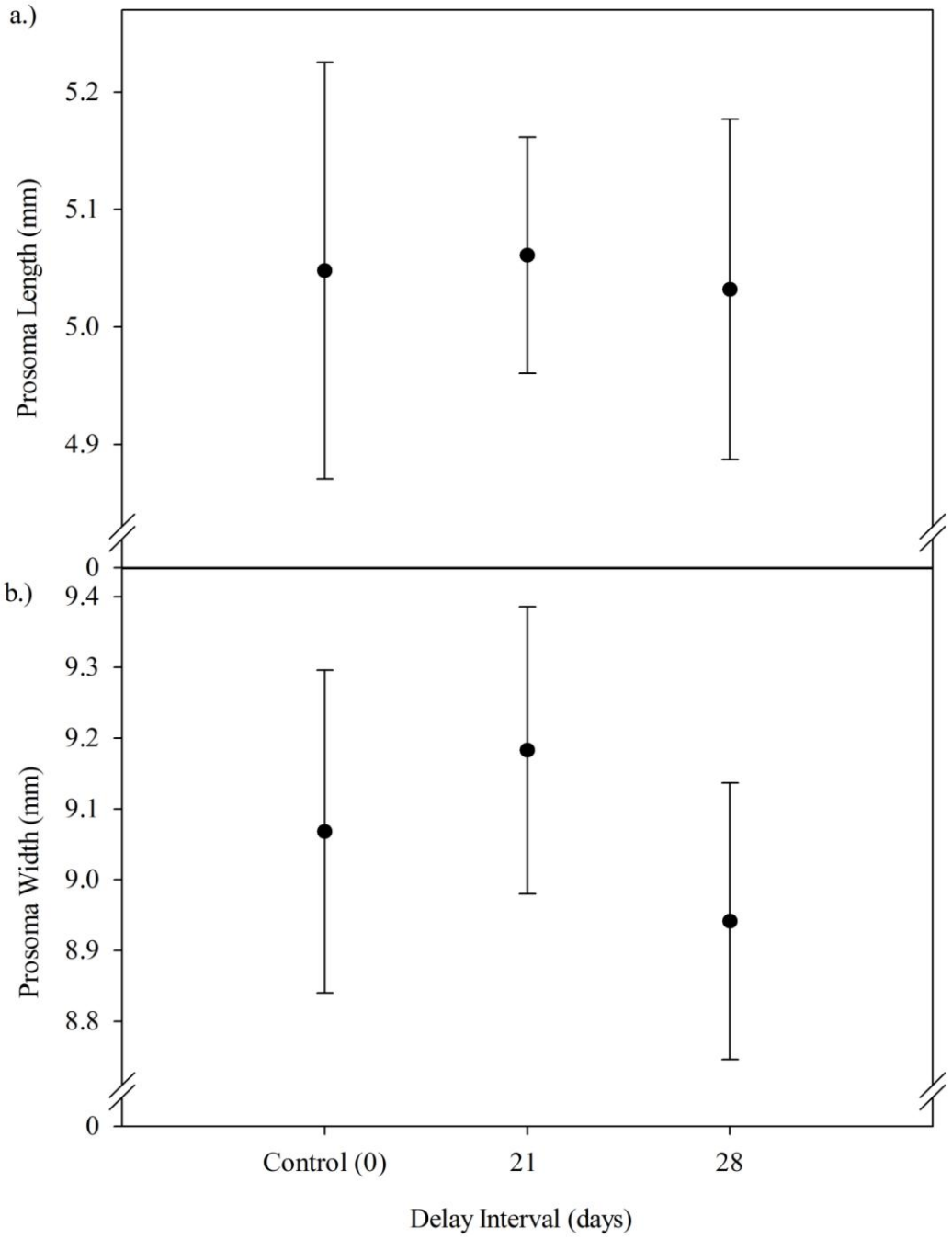

Figure II-8. a). Median (± MAD) prosoma length (mm) and (b.) median (± MAD) prosoma width (mm) of *Limulus polyphemus* second juvenile instars (J2) exposed to each delay treatment: 0 (control), 21, and 28-day delay intervals (n = 44-52).

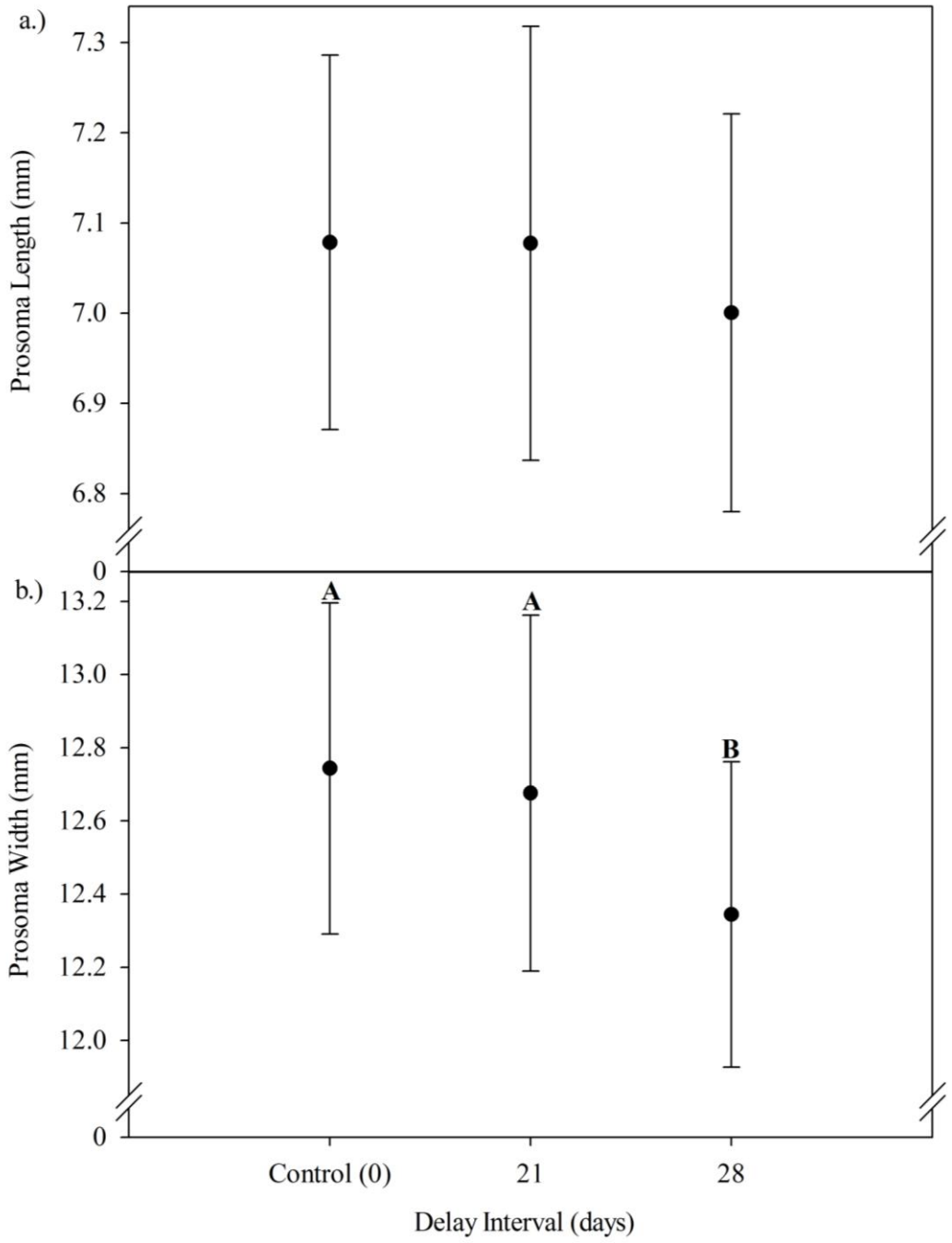

Figure II-9. a). Median (± MAD) prosoma length (mm) and (b.) median (± MAD) prosoma width (mm) of *Limulus polyphemus* third juvenile Instars (J3) exposed to each delay treatment: 0 (control), 21, and 28-day delay intervals (n = 44-52). Treatments with the same letters are not significantly different at $\alpha = 0.05$ (Kruskal-Wallis test).

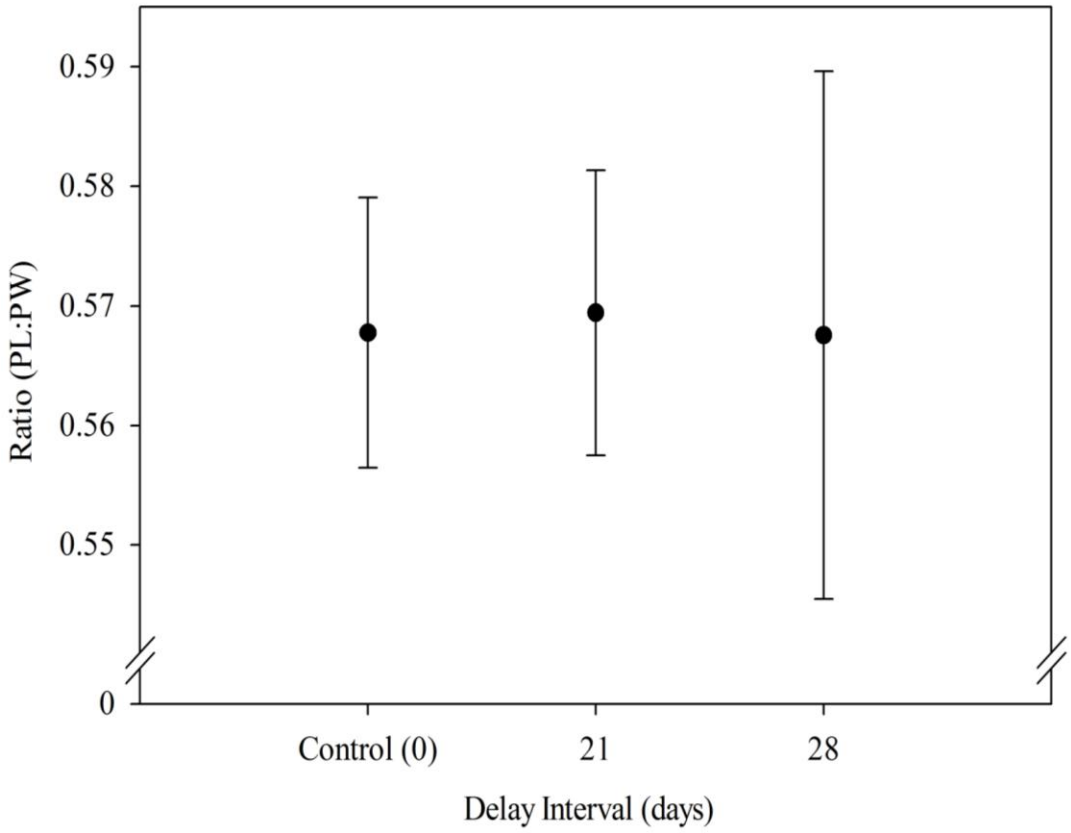

Figure II-10. Median (± MAD) ratio (PL: PW) of *Limulus polyphemus* first juvenile instars (J1) exposed to each delay treatment: 0 (control), 21, and 28-day delay intervals (n = 44-52).

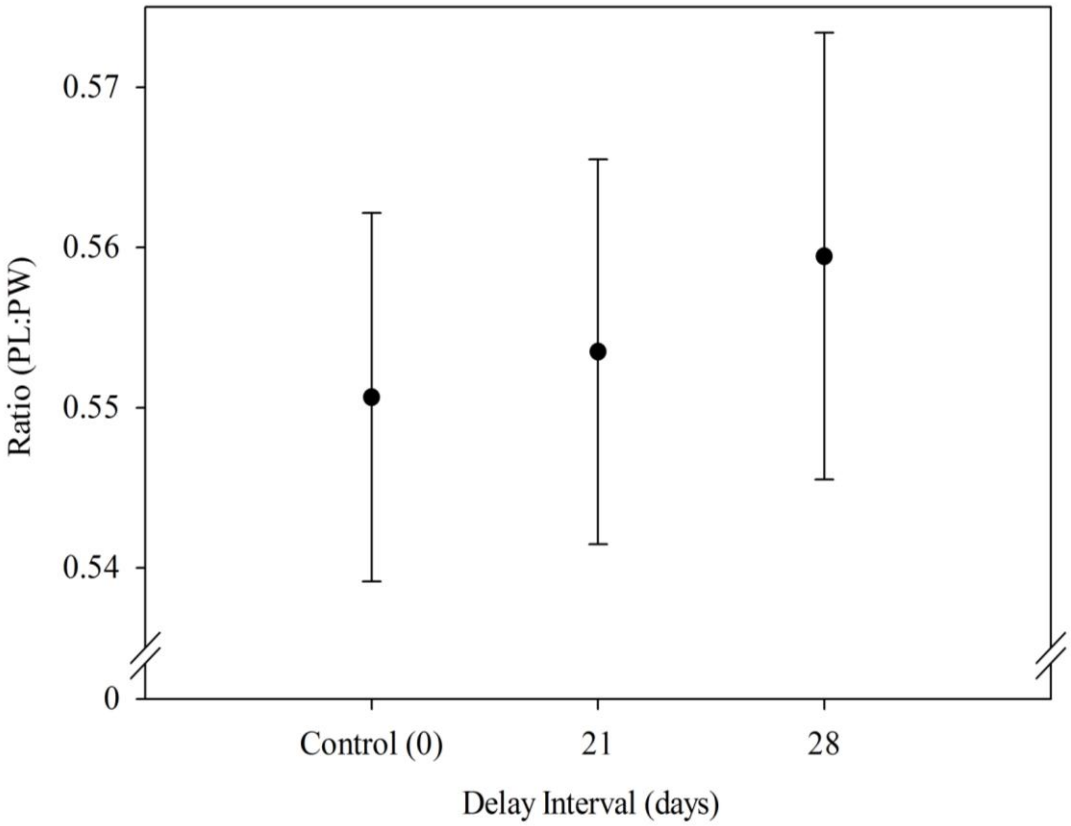

Figure II-11. Median (± MAD) ratio (PL: PW) of *Limulus polyphemus* second juvenile instars (J2) exposed to each delay treatment: 0 (control), 21, and 28-day delay intervals (n = 44-52).

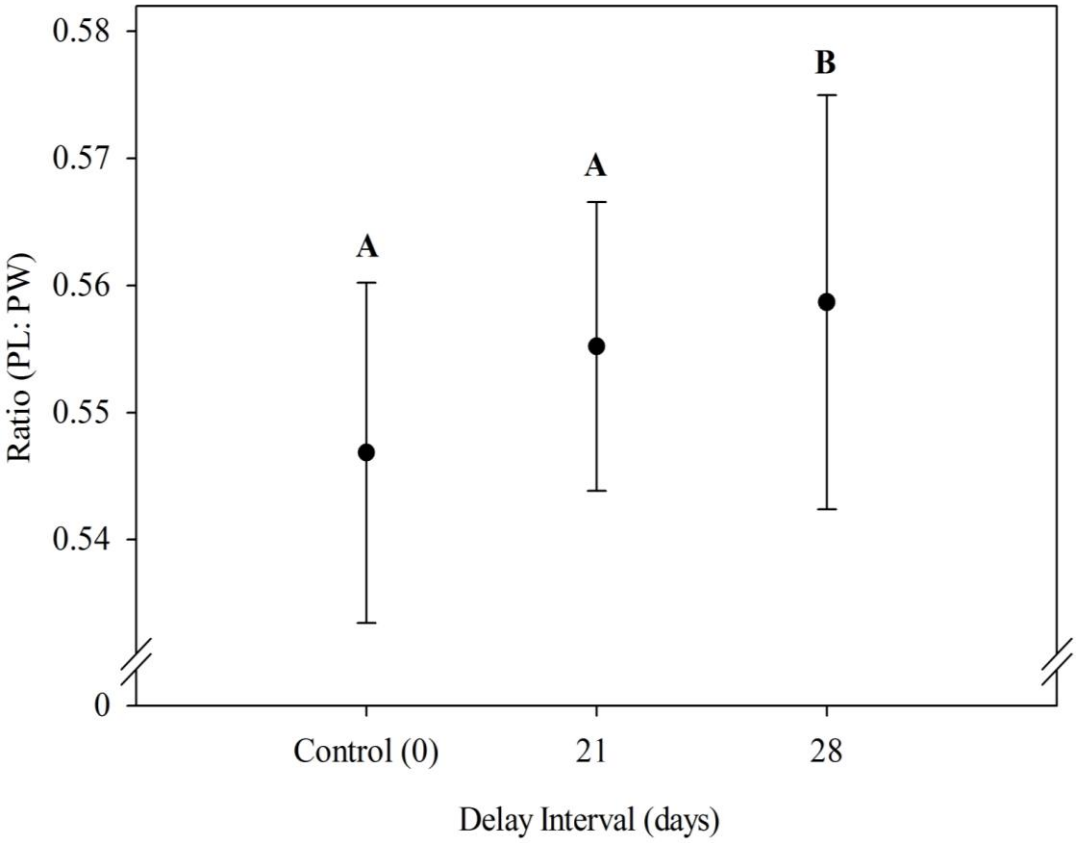

Figure II-12. Median (± MAD) ratio (PL: PW) of *Limulus polyphemus* third juvenile instars (J3) exposed to each delay treatment: 0 (control), 21, and 28-day delay intervals (n = 44-52). Treatments with the same letters are not significantly different at $\alpha = 0.05$ (Kruskal-Wallis test).

DISCUSSION

After a period of time in the plankton, many marine and estuarine invertebrate larvae attain competency, or the ability to metamorphose, and are then capable of extending the larval phase in order to increase the chances of locating a suitable habitat (reviewed by Gebauer et al. 2003, Pechenik 2006). In the present study, *L. polyphemus* exhibited a brief pre-competency period with minimal costs of delaying metamorphosis. Time to metamorphosis of larvae in the control and 7 day delay treatment were similar (Figs. II-I, II-2 and Table II-1). In both of these treatments, 25% of the larvae had metamorphosed by day 16 (Fig. II-2), indicating that competency, or the ability to metamorphose, occurs about 16 days post-hatch.

Time to metamorphosis (TTM) of *L. polyphemus* significantly increased as the delay interval extended ≥ 14 days (14, 21, and 28 day delay treatments) (Fig. I-1). However, when larvae were delayed 14, 21, or 28 days post-hatch, time to metamorphosis relative to the day of exposure to the conspecific cue did not differ significantly (Fig. II-3 and Table II-2). In these three treatments, 25% of larvae metamorphosed within 6-7 days after exposure compared with 9-11 days for those in the control and 7 day delay treatments (Fig. II-4). Therefore, once competency is reached, and the appropriate cue is available, metamorphosis proceeds over a similar timeframe. This contrasts with other marine invertebrates, such as the abalones *Haliotis discus hannai* (Takami et al. 2002) and *H. rufescens* (Barlow 1990), and the oyster *Crassostrea gigas* (Coon et al. 1990), in which the

time to metamorphosis relative to the day of exposure decreases with an increase in delay interval.

Although delaying metamorphosis affects the timing of metamorphosis, it does not affect the ability of *L. polyphemus* to successfully metamorphose. Trilobite larvae retained the ability to metamorphose after a delay period of 28 days. Additionally, many crabs in the offshore-water controls metamorphosed at 40 days. This suggests that *L. polyphemus* has the capacity to delay metamorphosis for several weeks. In contrast, many marine invertebrates have a window of competency in which, after a certain number of days post-hatch, the ability to metamorphose is lost (reviewed by Pechenik 1990, Hadfield et al. 2001). For instance, *Haliotis iris* loses competency after 26 days (Roberts and Lapworth 2001), *H. rufescens* loses competency after 2-4 days (Searcy-Bernal 1999), and the tubeworm *Hydroides elegans* loses the ability to metamorphose in the presence of adult exudate after only 3 days (Pechenik and Qian 1998). Being able to retain competency for as long as possible can increase the chances of locating potentially suitable habitats and increases dispersal potential, which in turn can increase gene flow among populations and decrease extinction rates (reviewed by Thorson 1950, Scheltema 1971, 1986, Hedgecock 1986, Jablonski 1986, Hadfield et al. 2001).

Delaying metamorphosis of *L. polyphemus* had no significant effect on post-metamorphic survival, with ~93% surviving in each treatment to the third juvenile instar (J3) (Fig. II-5). These results are consistent with studies showing that metamorphosis can be delayed in other non-feeding marine invertebrates, such

as the barnacles *Balanus amphitrite* (Pechenik et al. 1993) and *B. balanoides* (Lucas et al. 1979), and the ascidian *Styela plicata* (Thiyagarajan and Qian 2003), without affecting survivorship. Additionally, delayed metamorphosis did not significantly affect MSD with the first juvenile instar stage (J1) lasting ~14 days and the second juvenile instar stage (J2) lasting ~15 days (Fig. II-6 and Table II-3). Extending the larval phase produces similar results in the fiddler crab *Uca pugilator* (O'Connor 1991). However, while survival and MSD may not be affected, extending the larval phase can negatively affect the size and shape of organisms throughout subsequent juvenile stages because of energy constraints (Pechenik 1990, Pechenik et al. 1993, Forward et al. 1996, Pechenik and Rice 2001).

In *L. polyphemus* juveniles, delaying metamorphosis did not affect the prosoma length (PL) of any juvenile instar stage (J1-J3) (Fig. II-7a—II-9a and Table II-4) or the prosoma width (PW) of the first two stages (J1 and J2) (Figs. II-7b, II-8b, and Table II-4). Only the longest delay period (28 days) affected the PW of third instar juveniles (J3), producing narrower individuals (Fig. II-9b). Juvenile crabs in the 28-day-delay treatment were ~3% narrower than crabs in the control treatment (Fig. II-9b). Similar to the present study, delayed *C. sapidus* individuals exhibited reduced size (carapace length) only in late juvenile stages (J4 and J5) (Gravinese and Tankersley, pers. obs.). In contrast, studies of other marine invertebrates, including the hermit crab *Clibanarius longitarsus* (Harms 1992) and the grapsid crab *Chasmagnathus granulata* (Anger 1991, 2001), have found an

effect of delayed metamorphosis on the size of newly metamorphosed juveniles. Simith and colleagues (2013) found that body size (carapace width) of delayed *U. cordatus* juveniles was significantly reduced in comparison with non-delayed individuals throughout all stages observed (J1-J5), suggesting that the cost of delayed metamorphosis can occur early and persist throughout development.

The difference in PW exhibited by *L. polyphemus* juveniles exposed to the longest delay treatment (28 days) resulted in a significantly different body shape (PL: PW) in these individuals when compared with the non-delayed crabs (Fig. II-12). These juveniles exhibited a slightly more circular shape after molting to the third juvenile instar (closer to a 1:1 PL: PW ratio). However, the body ratio exhibited by the delayed individuals was only ~2% larger than the non-delayed juveniles. These results differ from other studies that found no statistically significant influence of delayed metamorphosis on body ratios of the grapsid crab *Chasmagnathus granulata* (Gebauer et al. 1999) and the blue crab *C. sapidus* (Gravinese and Tankersley, pers. obs.).

The current study indicates that delaying metamorphosis has no lethal effect and minimal sublethal on *L. polyphemus* juveniles. A significant effect was expected because trilobite larvae are lecithotrophic (i.e., non-feeding) and therefore subsist on a maternal yolk during the larval stage. Delayed metamorphosis is thought to deplete maternal reserves and therefore can have latent effects on juvenile stages (Pechenik et al. 1998, 1999, Bishop et al. 2006, Pechenik 2006). But some non-feeding marine invertebrate larvae, such as bivalves (Fankboner and

deBurgh 1978, Rice et al. 1980, Manahan 1990), echinoderms (Fontaine and Chia 1968), and polychaetes (Bass et al. 1969), have the ability to uptake dissolved organic matter (DOM) to supplement maternal reserves. Furthermore, Wendt and Johnson (2006) found that post-metamorphic costs of delayed metamorphosis (decreased survival and growth) in the bryozoan *Bugula neritina* were offset when DOM was made available to the larvae. Measurements of amino acid synthesis via autoradiographic, biochemical, and kinetic experiments (Manahan and Crisp 1982) are needed to determine if *L. polyphemus* individuals utilize DOM during early developmental stages. Some marine invertebrates, such as the marine worm *Sipunculus nudus,* have also been shown to downregulate their metabolism to conserve energy during periods of environmental stress (Reipschläger and Pörtner 1996). Oxygen consumption during periods of delayed metamorphosis must be monitored to determine if *L. polyphemus* is capable of downregulating metabolic activities.

The differences in size (PW) and shape of *L. polyphemus* juveniles exposed to the 28 day delay treatment and those crabs in the control treatment were statistically significant, but small (2-3% change). Therefore, these differences in size and shape may not be biologically significant. However, these slight morphological differences (i.e., reduced size/change in shape) may indicate physiological stress experienced by the last larval stage (Gebauer et al. 1999). Effects of physiological stress experienced during the larval stage may hinder the ability to compete for food, mates, and refuge (Hines 1986, Pechenik 1990, Stearns

1992, Williams 1994, Bernado 1996, Marshall et al. 2006). Therefore, these differences in morphology may be important in explaining natural variability in survival and reproduction (Phillips 2002, Jarrett 2003, Marshall and Keough 2005), which may lead to a better understanding of recruitment processes, population dynamics, and temporal/spatial variability in distribution and abundance (reviewed by Giménez 2006).

The absence of post-metamorphic consequences of delayed metamorphosis on survival, molt-stage duration (MSD), and morphology after a delay of 28 days suggests that the ability of *L. polyphemus* trilobites to extend the larval phase for at least 28 days may be adaptive for *L. polyphemus.* However, larvae may experience other sublethal effects. For instance, delayed metamorphosis has been shown to slow reproductive maturation (e.g., the bryozoan *Bugula neritina*) and decrease fecundity of adult marine invertebrates (e.g., the polychaete *Polydora ligni*) (Pechenik et al. 1998). Additionally, delayed metamorphosis may affect the ability of some marine invertebrate juveniles (e.g., the echinoid *Dendraster excentricus*) to tolerate environmental stressors (Highsmith and Emlet 1986). More studies on post-metamorphic costs of delayed metamorphosis should be conducted on *L. polyphemus* to determine if it truly is an adaptive trait.

The absence of post-metamorphic consequences of delayed metamorphosis for *L. polyphmeus* juveniles delayed for 28 days, suggest that horseshoe crab populations may be more affected by other factors, such as anthropogenic exploitation and the destruction of adult spawning areas, than previously thought.

Additionally, the present research supports the notion that horseshoe crab larvae are resilient in the absence of metamorphic cues. Previous research has also shown that the horseshoe crab is capable of tolerating a wide range of environmental conditions (e.g., hypoxia/anoxia, extreme variations in salinity and temperature, and pollutants) (Laughlin 1983, Carlsson and Gäde 1986, Botton et al. 1998a, Botton et al. 1998b, Ehlinger and Tankersley 2004). This information may force conservation programs to implement new preservation strategies and alter research aims in order to determine the factors that are affecting adult populations of *L. polyphemus.*

LITERATURE CITED

Abelson A, Denny M. 1997. Settlement of marine organisms in flow. Annu Rev Ecol Syst 28:317-339.

Andrews WR, Target NM, Epifanio CE. 2001. Isolation and characterization of the metamorphic inducers of the common mud crab, *Panopeus herbstii*. J Exp Mar Biol Ecol 261: 121-134.

Anger K. 1991. Effects of temperature and salinity on the larval development of the Chinese mitten crab *Eriocheir sinensis* (Decapoda: Grapsidae). Mar Ecol Prog Ser 72: 103-110.

Anger K. 2001. The biology of decapods crustacean larvae. Crust Iss 14:1-420.

Anil AC, Desai D, Khandepark L. 2001. Larval development and metamorphosis in *Balanus amphitrite* Darwin (Cirripedia; Thoracica): significance of food concentration, temperature and nucleic acids. J Exp Mar Biol Ecol 263: 125-141.

Bao WY, Satuito CG, Yang JL, Kitamura H. 2007. Larval settlement and metamorphosis of the mussel *Mytilus galloprovincialis* in response to biofilms. Mar Biol 150: 565-574.

Barlow LA. 1990. Electrophysiological and behavioral responses of larvae of the red abalone (*Haliotis rufescens*) to settlement-inducting substances. Bull Mar Sci 46(2): 537-554.

Barshaw DE, Lavalli KL. 1988. Predation upon postlarval lobsters *Homarus americanus* by cunners *Tautogolabrus adspersus* and mud crabs *Neopanope sayi* on three different substrates: eelgrass, mud and rocks. Mar Ecol Prog Ser 48: 119-123.

Bass N, Chapman G, Chapman JH. 1969. Uptake of leucine by larvae and adults of *Nereis*. Natue 211: 476-477.

Berkson J, Shuster, Jr. CN. 1999. The horseshoe crab: the battle for a true multiple use resource. Fisheries 24(11): 6-10.

Bernado J. 1996. The particular maternal effect of propagule size, especially egg size: patterns, models, quality of evidence and interpretations. Amer Zool 36(2): 216-236.

Bishop CD, Huggett MJ, Heyland A, Hodin J, Brandhorst BP. 2006. Interspecific variation in metamorphic competence in marine invertebrates: the significance for comparative investigation into the timing of metamorphosis. Integr Comp Biol 46(6): 662-682.

Boettcher AA, Targett NM. 1996. Induction of metamorphosis in queen conch, *Strombus gigas* Linnaeus, larvae by cues associated with red algae from their nursery grounds. J Exp Mar Biol Ecol 196: 29-52.

Boleman CL. 2011. Role of chemical cues and predation in determining the post settlement distributions of larval and juvenile *Limulus polyphemus* [thesis]. Melbourne (FL): Florida Institute of Technology. 46 p.

Botton ML. 2009. The ecological importance of horseshoe crabs in estuarine and coastal communities: a review and speculative summary. In: Tanacredi JT, Botton ML, Smith D, editors. Biology and Conservation of Horseshoe Crabs. US: Springer. p 45-63.

Botton ML, Hodge M, Gonzalez TI. 1998a. High tolerance to tributyltin in embryos and larvae of the horseshoe crab, *Limulus polyphemus*. CERF 21(2): 340-346.

Botton ML, Johnson K, Helleby L. 1998b. Effects of copper and zinc on embryos and larvae of the horseshoe crab, *Limulus polyphemus*. Arch Environ Contam Toxicol 35(1): 25-32.

Botton ML, Loveland RE. 2003. Abundance and dispersal potential of horseshoe crab (*Limulus polyphemus*) larvae in the Delaware estuary. Estuaries 26(6): 1472-1479.

Botton ML, Loveland RE, Tiwari A. 2003. Distribution, abundance, and survivorship of young-of-the-year in a commercially exploited population of horseshoe crabs *Limulus polyphemus*. Mar Ecol Prog Ser 265: 175-184.

Botton ML, Tankersley RA, Loveland RE. 2010. Developmental ecology of the American horseshoe crab *Limulus polyphemus.* Curr Zoo 56: 550-562.

Brockmann HJ, Penn D. 1992. Male mating tactics in the horseshoe crab, *Limulus polyphemus*. Anim Behav 44(4): 653-665.

Burke RD. 1983. The induction of metamorphosis of marine invertebrate larvae: stimulus response. Can J Zool 61(8): 1701-1719.

Burke RD. 1984. Pheromonal control of metamorphosis in the Pacific sand dollar, *Dendraster excentricus.* Science 225(4660): 442-443.

Burke RD. 1986. Pheromones and the gregarious settlement of marine invertebrate larvae. Bull Mar Sci 39(2): 323-331.

Carlsson KH, Gäde G. 1986. Metabolic adaptation of the horseshoe crab, *Limulus polyphemus*, during exercise and environmental hypoxia and subsequent recovery. Biol Bull 171: 217-235.

Carmichael RH, Brush E. 2012. Three decades of horseshoe crab rearing: a review of conditions for captive growth and survival. Rev Aqua 4: 32-43.

Castille Jr. FL, Lawrence AL. 1979. The role of bacteria in the uptake of hexoses from seawater by postlarval penaeid shrimp. Comp Biochem Phys A 64(1): 41-48.

Chia FS. 1989. Differential larval settlement of benthic marine invertebrates. In: Ryland JS, editor. Reproduction, genetics and distributions of marine organisms. Fedensborg (Denmark): Olsen & Olsen. p 3-12.

Chiu HMC, Morton B. 1999. The distribution of horseshoe crab (*Tachypleus tridentatus* and *Carcinoscorpius rotundicauda*) in Hong Kong. AsianMar Biol 16: 185-196.

Clare AS, Matsumura K. 2000. Nature and perception of barnacle settlement pheromones. Biofouling 15: 57-71.

Cohen JA, Brockmann HJ. 1983. Breeding activity and mate selection in the horseshoe crab, *Limulus polyphemus.* Bull Mar Sci 33(2): 274-281.

Coon SL, Walch M, Fitt WK, Weiner RM, Bonar DB. 1990. Ammonia induces settlement behavior in oyster larvae. Biol Bull 179(3): 297-303.

Eggleston DB, Armstrong DA. 1995. Pre-and post-settlement determinants of estuarine Dungeness crab recruitment. Ecol Monogr 65(2): 193-216.

Ehlinger GS, Tankersley RA. Larval hatching in the horseshoe crab, *Limulus polyphemus*: facilitation by environmental cues. J Exp Mar Biol Ecol 292(2): 199-212.

Ehlinger GS, Tankersley RA. 2006. Endogenous rhythms and entrainment cues of larval activity in the horseshoe crab *Limulus polyphemus*. J Exp Mar Biol Ecol. 337(2): 205-214.

Ehlinger GS, Tankersley RA. 2009. Ecology of horseshoe crabs in microtidal lagoons. In: Tanacredi JT, Botton ML, Smith D, editors. Biology and Conservation of Horseshoe Crabs. US: Springer. p 149-162.

Fankboner PV, De Burgh ME. 1978. Comparative rates of dissolved organic carbon accumulation by juveniles and pediveligers of the Japanese oyster *Crassostrea gigas* Thunberg. Aquaculture 13(3): 205-212.

Fernández M, Armstrong D, Iribarne O. 1993. First cohort of young-of-the-year Dungeness crab, *Cancer magister*, reduces abundance of subsequent cohorts in intertidal shell habitat. Can J Fish Aquat Sci 50(10): 2100-2105.

Fitzgerald TP, Forward, Jr. RB, Tankersley RA. 1998. Metamorphosis of the estuarine crab *Rhithropanopeus harrisii*: effect of water type and adult odor. Mar Ecol Prog Ser 165: 217-223.

Fontaine AR, Chia FS. 1968. Echinoderms: an autoradiographic study of assimilation of dissolved organic molecules. Science 161: 1153-1155.

Forward Jr. RB, DeVries MC, Rittschof D, Frankel DAZ, Bischoff JP, Fisher CM,Welch JM. 1996. Effects of environmental cues on metamorphosis of the blue crab *Callinectes sapidus.* Mar Ecol Prog Ser 131: 165-177.

Forward Jr. RB, Frankel DAZ, Rittschof D. 1994. Molting of megalopae from the blue crab *Callinectes sapidus:* effects of offshore and estuarine cues. Mar Ecol Prog Ser 113: 55-59.

Forward Jr. RB, Tankersley RA, Blondel D, Rittschof D. 1997. Metamorphosis of the blue crab *Callinectes sapidus*: effects of humic acids and ammonium. Mar Ecol Prog Ser 157: 277-286.

Forward Jr. RB, Tankersley RA, Rittschof D. 2001. Cues for metamorphosis of brachyuran crabs: an overview. Amer Zool 41: 1108-1122.

Fusetani N. 2004. Biofouling and antifouling. Nat Prod Rep 21: 94-104.

Gebauer PK, Paschke K, Anger K. 1999. Metamorphosis in a semiterrestrial crab, *Sesarma curacaoense*: intra-and interspecific settlement cues from adult odors. J Exp Mar Biol 268: 1-12.

Gebauer P, Paschke K, Anger K. 2002. Metamorphosis in a semiterrestrial crab, *Sesarma curacaoense*: intra-and interspecific settlement cues from adult odors. J Exp Mar Biol Ecol 268: 1-12.

Gebauer P, Paschke K, Anger K. 2003. Delayed metamorphosis in decapod crustaceans: evidence and consequences. Rev Chil Hist Nat 76: 169-175.

Gebauer P, Walter I, Anger K. 1998. Effects of substratum and conspecific adults on the metamorphosis of *Chasmagnathus granulata* (Dana) (Decapoda: Grapsidae) megalopae. J Exp Mar Biol and Ecol 223: 185-198.

Giménez L. 2006. Phenotypic links in complex life cycles: conclusions from studies with decapod crustaceans. Integ Comp Biol 46(5): 615-622.

Gosselin LA, Qian PY. 1997. Juvenile mortality in benthic marine invertebrates. Mar Ecol Prog Ser 146: 265-282.

Hadfield MG. 2000. Why and how marine-invertebrate larvae metamorphose so fast. Semin Cell Dev Biol 11(6): 437-443.

Hadfield MG, Carpizo-Ituarte EJ, Del Carmen K, Nedved BT. 2001. Metamorphic competence, a major adaptive convergence in marine invertebrate larvae. Amer Zool 41: 1123-1131.

Hadfield MG, Paul VJ. 2001. Natural chemical cues for settlement and metamorphosis of marine-invertebrate larvae. In: McClintock JB, Baker BJ, editors. Marine chemical ecology. Boca Raton, FL: CRC Press. p 431-561.

Hadfield MG, Scheuer D. 1985. Evidence for a soluble metamorphic inducer in *Phestilla*: ecological, chemical and biological data. Bull Mar Sci 37(2): 556-566.

Harms J. 1992. Larval development and delayed metamorphosis in the hermit crab *Clibanarius erythropus* (Latreille) (Crustacea, Diogenidae). J Exp Mar Biol Ecol 156: 151-160.

Harvey AW. 1996. Delayed metamorphosis in Florida hermit crabs: multiple cues and constraints (Crustacea: Decapoda: Paguridae and Diogenidae). Mar Ecol Prog Ser 141: 27-36.

Heck. KL, Coen LD, Morgan SG. 2001. Pre- and post-settlement factors as determinants of juvenile blue crab *Callinectes sapidus* abundance: results from the north-central Gulf of Mexico. Mar Ecol Prog Ser 222: 163-176.

Heck. KL, Orth RJ. 1980. Seagrass habitats: the roles of habitat complexity, competition and predation in structuring associated fish and motile macroinvertebrate assemblages. In: Kennedy VS, editor. Estuarine Perspectives. New York (US): Academic Press. p 449-464.

Hedgecock D. 1986. Is gene flow from pelagic larval dispersal important in the adaptation and evolution of marine invertebrates? Bull Mar Sci 39(2): 550-564.

Herrnkind WF, Butler IV MJ. 1986. Factors regulating postlarval settlement and juvenile microhabitat use by spiny lobsters *Panulirus argus*. Mar Ecol Prog Ser 34: 23-30.

Highsmith RC, Emlet RB. 1986. Delayed metamorphosis: Effect on growth and survival of juvenile sand dollars (Echinoidea: Clypeasteroida). Bull Mar Sci 39: 347-361.

Hines AS. 1986. Larval problems and perspectives in life histories of marine invertebrates. Bull Mar Sci 39: 506-525.

Hines AH, Ruiz GM. 1995. Temporal variation in juvenile blue crab mortality: nearshore shallows and cannibalism in Chesapeake Bay. Bull Mar Sci 57(3): 884-901.

Hovel KA, Lipcius RN. 2001. Habitat fragmentation in a seagrass landscape: patch size and complexity control blue crab survival. Ecology 82(7): 1814-1829.

Hunt HL, Scheibling RE. 1997. Role of early post-settlement mortality in recruitment of benthic marine invertebrates. Mar Ecol Prog Ser 155: 269-301.

Jablonski D. 1986. Larval ecology and macroevolution in marine invertebrates. Bull Mar Sci 39(2): 565-587.

Jackson D, Leys SP, Hinman VF, Woods R, Lavin MF, Degnan BM. Ecological regulation of development: induction of marine invertebrate metamorphosis. Int J Dev Biol 46: 679-686.

Jarrett JN. 2003. Seasonal variation in larval condition and postsettlement performance of the barnacle *Semibalanus balanoides*. Ecology 84(2): 384-390.

Jeffs G, Montgomery JC, Tindle CT. 2005. How do spiny lobster post-larvae find the coast? New Zeal J Mar Fresh 39(3): 605-617.

Jegla TC, Costlow JD. 1979. The *Limulus* bioassay for ecdysteroids. Biol Bull 56: 103-114.

Khandeparker L, Anil AC, Raghukumar S. 2006. Relevance of biofilm bacteria in modulating the larval metamorphosis of *Balanus amphitrite*. FEMS Microbiol Ecol 58(3): 425-438.

Khandeparker L, Kumar SK. 2011. Significance of biofilm proteins in modulating cyprid metamorphosis of *Balanus amphitrite* (Cirripedia: Thoracica). Mar Ecol 32(4): 509-520.

Kingsford MJ, Leis JM, Shanks A, Lindeman KC, Morgan SG, Pineda J. 2002. Sensory environments, larval abilities and local self-recruitment. Bull Mar Sci 70(1): 309-340.

Kleinbaum DG, Klein M. 1996. Survival Analysis. NewYork (US): Springer Verlag. p 86-128.

Klein-Breteler WCM. 1976. Settlement, growth and reproduction of the shore crab, *Carcinus maenas*, on tidal flats in the Dutch Wadden Sea. Neth J Sea Res 10(3): 354-376.

Kopin CY, Epifanio CE, Nelson S, Stratton M. 2001. Effects of chemical cues on metamorphosis of the Asian shore crab *Hemigrapsus sanguineus*, an invasive species on the Atlantic Coast of North America. J Exp Mar Biol Ecol 265(2): 141-151.

Kreamer G, Michels S. 2009. History of horseshoe crab harvest on Delaware Bay. In: Tanacredi JT, editor. Biology and Conservation of Horseshoe Crabs. New York (US): Springer. p 299-313.

Krimsky LS, Epifanio CE. 2008. Multiple cues from multiple habitats: effect on metamorphosis of the Florida stone crab, *Menippe mercenaria.* J Exp Mar Biol Ecol 358: 178-184.

Kunugi S, Tanak N. 2002. Cold denaturation of proteins under high pressure. Biochim Biophys Acta 1595: 329-344.

Lambert WJ, Todd CD, Hardege JD. 1997. Partial characterization and biological activity of a metamorphic inducer of the dorid nudibranch *Adalaria proxima* (Gastropoda: Nudibranchia). Invert Biol 116(2): 71-81.

Laughlin R. 1983. The effects of temperature and salinity on larval growth of the horseshoe crab *Limulus polyphemus*. Biol Bull 164: 93-103.

Loveland RE. 2002. The life history of horseshoe crabs. In: Tanacredi JT, editor. *Limulus* in the Limelight: A Species 350 Million Years in the Making and in Peril? New York (US): Springer. p 93-101.

Lucas MI, Walker G, Holland DL, Crisp DJ. 1979. An energy budget for the free swimming and metamorphosing larvae of *Balanus balanoides* (Crustacea: Cirripedia). Mar Biol 55(3): 221-229.

Manahan DT. 1990. Adaptations by invertebrate larvae for nutrient acquisition from seawater. Amer Zool 30(1): 147-160.

Manahan DT, Crisp DJ. 1982. The role of dissolved organic material in the nutrition of pelagic larvae: amino acid uptake by bivalve veligers. Amer Zool 22(3): 635-646.

Marshall DJ, Cook CN, Emlet RB. 2006. Offspring size effects mediate competitive interactions in a colonial marine invertebrate. Ecology 87: 214-225.

Marshall DJ, Keough MJ. 2005. Complex life cycles and offspring provisioning in marine invertebrates. Integr Comp Biol 46(5): 643-651.

Medina JM, Tankersley RA. 2010. Orientation of larval and juvenile horseshoe crabs *Limulus polyphemus* to visual cues: effects of chemical odors. Curr Zool 56(5): 618-633.

Metaxas A. 2001. Behaviour in flow: perspectives on the distribution and dispersion of meroplanktonic larvae in the water column. Can J Fish Aquat Sci 58: 86-98.

Mishra A, Loo Y, Deng R, Chuah YJ, Hee HT, Ying JY, Hauser CAE. 2011. Ultrasmall natural peptides self-assemble to strong temperature-resistant helical fibers in scaffolds suitable for tissue engineering. Nano Today 6: 232-239.

Moksnes PO, Pihl L, van Montfrans J. 1998. Predation on postlarvae and juveniles of the shore crab *Carcinus maenas*: importance of shelter, size and cannibalism. Mar Ecol Prog Ser 166: 211-225.

Morse DE. 1992. Molecular mechanisms controlling metamorphosis and recruitment in abalone larvae. Abalone of the world: biology, fisheries and culture. Oxford (UK): Fisheries News Books. p 107-119.

Morse ANC, Froyd CA, Morse DE. 1984. Molecules from cyanobacteria and red algae that induce larval settlement and metamorphosis in the mollusc *Haliotis rufescens*. Mar Biol 81(3): 293-298.

Muenchow G. 1986. Ecological use of failure time analysis. Ecology. 67: 246-250.

O'Connor NJ. 1991. Flexibility in timing of the metamorphic molt by fiddler crab megalopae *Uca pugilator*. Mar Ecol Prog Ser 68(3): 243-247.

O'Connor NJ. 2007. Stimulation of molting in megalopae of the Asian shore crab *Hemigrapsus sanguineus*: physical and chemical cues. Mar Ecol Prog Ser 352: 1-8.

O'Connor NJ, Gregg AS. 1998. Influence of potential habitat cues on duration of the megalopal stage of the fiddler crab *Uca pugnax.* J Crustac Biol 18: 700 709.

Pawlik JR. 1992. Chemical ecology of the settlement of benthic marine invertebrates. Oceanogr Mar Biol Annu Rev 30: 273-335.

Pearce CM, Scheibling RE. 1990. Induction of settlement and metamorphosis in the sand dollar *Echinarachnius parma*: evidence for an adult-associated factor. Mar Biol 107: 363-369.

Pechenik JA. 1990. Delayed metamorphosis by larvae of benthic marine invertebrates: does it occur? is there a price to pay? Ophelia 32(1-2): 63-94.

Pechenik JA. 1999. On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles. Mar Ecol Prog Ser 177: 269-297.

Pechenik JA. 2006. Larval experience and latent effects- metamorphosis is not a new beginning. Integr Comp Biol 46(3): 323-333.

Pechenik JA. 2010. Biology of the invertebrates. 6th edition. McGraw-Hill. New York (US) pgs. 373-378.

Pechenik JA, Cerulli TR. 1991. Influence of delayed metamorphosis on survival, growth, and reproduction of the marine polychaete *Capitella* sp l. J Exp Mar Biol Ecol 151(1): 17-27.

Pechenik JA, Rice ME. 2011. Influence of delayed metamorphosis on postsettlement survival and growth in the sipunculan *Apionsoma misakianum*. Invert Biol 120(1): 50-57.

Pechnik JA, Qian PY. 1998. Onset and maintenance of metamorphic competence in the marine polychaete *Hydroides elegans* Haswell in response to three chemical cues. J Exp Mar Biol Ecol 226: 51-74.

Pechenik JA, Rittschof D, Schmidt AR. 1993. Influence of delayed metamorphosis on survival and growth of juvenile barnacles *Balanus amphitrite*. Mar Biol 115: 287-294.

Pechenik JA, Wendt DE, Jarrett JN. 1998. Metamorphosis is not a new beginning. BioScience 48(11): 901-910.

Penn D, Brockmann HJ. 1994. Nest-site selection in the horseshoe crab, *Limulus polyphemus.* Biol Bull 187: 373-384.

Perkins-Visser E, Wolcott TG, Wolcott DL. 1996. Nursery role of seagrass beds: enhanced growth of juvenile blue crabs *Callinectes sapidus* Rathbun. J Exp Mar Biol Ecol 198(2): 155-173.

Phillips NE. 2002. Effects of nutrition-mediated larval condition on juvenile performance in a marine mussel. Ecology 83(9): 2562-2574.

Qian PY, Lau SCK, Dahms HU, Dobretsov S, Harder T. 2007. Marine biofilms as mediators of colonization by marine macroorganisms: implications for antifouling and aquaculture. Mar Biotech 9: 399-410.

Reipschläger A, Pörtner HO. 1996. Metabolic depression during environmental stress: the role of extracellular versus intracellular pH in *Sipunculus nudus*. J Exp Biol 199: 1801-1807.

Rice MA, Wallis K, Stephens GC. 1980. Influx and net flux of amino acids into larval and juvenile European flat oysters, *Ostrea edulis* (L.). J Exp Mar Biol Ecol. 48: 51-59.

Rittschof D. 1989. Peptide analogs of the mud crab pumping pheromone; structure function studies. Chem Senses 14(1): 137-148.

Rittschof D, Bonaventura J. 1986. Macromolecular cues in marine system. J Chem Ecol 12(5): 1013-1023.

Roberts RD, Lapworth C. 2001. Effect of delayed metamorphosis on larval competence, and post-larval survival and growth, in the abalone *Haliotis iris* Gmelin. J Exp Mar Biol Ecol 258:1-13.

Rodriguez RA, Epifanio CE. 2000. Multiple cues for induction of metamorphosis in larvae of the common mud crab *Panopeus herbstii.* Mar Ecol Prog Ser 195: 221-229.

Rodriguez SR, Ojeda FP, Inestrosa NC. 1993. Settlement of benthic marine invertebrates. Mar Ecol Prog Ser 97: 193-207.

Rudloe A. 1979. Locomotor and light response of larvae of the horseshoe crab, *Limulus polyphemus* (L.). Biol Bull 157: 494-505.

Rudloe A. 1980. The breeding behavior and patterns of movement of horseshoe crabs, *Limulus polyphemus*, in the vicinity of breeding beaches in Apalachee Bay, Florida. Estuaries 3(3): 177-183.

Rudloe A. 1981. Aspects of the biology of juvenile horseshoe crabs, *Limulus polyphemus.* Bull Mar Sci 31: 125-133.

Rutecki D, Carmichael RH, Valiela I. 2004. Magnitude of harvest of Atlantic horseshoe crabs, *Limulus polyphemus*, in Pleasant Bay, Massachusetts. Estuaries 27(2): 179-187.

Scheltema RS. 1971. Larval dispersal as a means of genetic exchange between geographically separated populations of shallow-water benthic marine gastropods. Biol Bull 140(2): 284-322.

Scheltema RS. 1986. On dispersal and planktonic larvae of benthic invertebrates: an eclectic overview and summary of problems. Bull Mar Sci 39(2): 290-322.

Searcy-Bernal R. 1999. Settlement and postlarval ecology of the red abalone *Haliotis rufescens* in culture systems [dissertation]. San Diego (CA): San Diego State University. 129 p.

Sekiguchi K. 1988. Post-embryonic development of the horseshoe crab. Biol Bull. 174:337-345.

Sekiguchi K, Shuster CN. 2009. Limits on the global distribution of horseshoe crabs (Limulacea): lessons learned from two lifetimes of observations: Asia and America. In: Tanacredi JT, Botton ML, Smith D, editors. Biology and Conservation of Horseshoe Crabs. New York (US): Springer. p 5-24.

Shuster CN. 1982. A pictorial review of the natural history and ecology of the horseshoe crab *Limulus polyphemus*, with reference to other Limulidae. Prog Clin Biol Res 81: 1-52.

Shuster CN. 2001. Two perspectives: horseshoe crabs during 420 million years, worldwide, and the past 150 years in the Delaware Bay area. In: Tanacredi JT, editor. *Limulus* in the Limelight. New York (US): Kluwer Academic/Plenum Publishers. p 17-40.

Shuster CN, Barlow Jr. RB, Brockmann HJ. 2003. The American horseshoe crab. Cambridge: Harvard University Press. 427 p.

Shilling FM, Hoegh-Guldberg O, Manahan DT. 1996. Sources of energy for increased metabolic demand during metamorphosis of the abalone *H. rufescens* (Mollusca). Biol Bull 191: 402-412.

Simith DJB, Diele K, Abrunhosa FA. 2013. Carry-over effects of delayed larval metamorphosis on early juvenile performance in the mangrove crab *Ucides cordatus* (Ucididae). J Exp Mar Biol Ecol 440: 61-68.

Smith DR, Pooler PS, Swan BJ, Michels SF, Hall WR, Himchak PJ, Millard MJ. 2002. Spatial and temporal distribution of horseshoe crab (*Limulus polyphemus*) spawning in Delaware Bay: implications for monitoring. Estuaries 25(1): 115-125.

Souza JCR, Strüssmann CA, Takashima F, Satoh H, Sekine S, Shima Y, Matsuda H. 2010. Oral and integumental uptake of free exogenous glycine by the Japanese spiny lobster *Panulirus japonicus* phyllosoma larvae. J Exp Biol 213(11): 1859-1867.

Soylemez SC, Murakami K, Strüssmann CA, Yokota M, Watanbe S. 2010. Uptake of dissolved free amino acids by spiny lobster *Panulirus japonicus* phyllosoma larvae. Fish Sci 76(3): 437-444.

Stearns SC. 1992. The evolution of life histories. Vol. 248. Oxford: Oxford University. 249 p.

Steinberg PD, De Nys R, Kjelleberg S. 2002. Chemical cues for surface colonization. J Chem Ecol 28(10): 1935-1951.

Steinberg MK, Epifanio CE, Andon A. 2007. A highly specific chemical cue for the metamorphosis of the Asian shore crab, *Hemigrapsus sanguineus.* J Exp Mar Biol Ecol 347(1-2): 1-7.

Steinberg MK, Krimsky LS, Epifanio CE. 2008. Induction of metamorphosis in the Asian shore crab *Hemigrapsus sanguineus*: effects of biofilms and substratum texture. Estuar Coast 31: 738-744.

Stevens BG, Swiney KM. 2005. Post-settlement effects of habitat type and predator size on cannibalism of glaucothoe and juveniles of red king crab *Paralithodes camtschaticus.* J Exp Mar Biol Ecol 321: 1-11.

Sulkin SD. 1984. Behavioral basis of depth regulation in the larvae of brachyuran crabs. Mar Ecol Prog Ser 15: 181-204.

Sweka JA, Klopfer M, Millard M, Olszewski S, Smith D, Sysak R, Wong R. 2013. 2013 horseshoe crab stock assessment update. ASMFC (Atlantic States Marine Fisheries Commission) Stock Assessment Report,Washington, DC. 68 p.

Takami H, Kawamura T, Yamashita Y. 2002. Effects of delayed metamorphosis on larval competence, and postlarval survival and growth of abalone *Haliotis discus hannai*. Aquaculture 213(1): 311-322.

Tankersley RA, Wieber MG. 2000. Physiological responses of postlarval and juvenile blue crabs *Callinectes sapidus* to hypoxia and anoxia. Mar Ecol Prog Ser 194: 179-191.

Thiyagarajan V, Harder T, Qiu JW, Qian PY. 2003. Energy content at metamorphosis and growth rate of the early juvenile barnacle *Balanus amphitrite*. Mar Biol 143: 543-554.

Thiyagarajan V, Qian PY. 2003. Effect of temperature, salinity and delayed attachment on development of the solitary ascidian *Styela plicata* (Lesueur). J Exp Mar Biol Ecol 290(1): 133-146.

Thorson G. 1950. Reproductive and larval ecology of marine bottom invertebrates. Biol Rev 25(1): 1-45.

Wahle RA, Steneck RS. 1992. Habitat restrictions in early benthic life: experiments on habitat selection and in situ predation with the American lobster. J Exp Mar Biol Ecol 157: 91-114.

Weber JC, Epifanio CE. 1996. Response of mud crab (*Panopeus herbstii*) megalope to cues from adult habitat. Mar Biol 126: 655-661.

Welch JM, Rittschof D, Bullock TM, Forward, Jr. RB. 1997. Effects of chemical cues on settlement behavior of blue crab *Callinectes sapidus* postlarvae. Mar Ecol Prog Ser 154: 143-153.

Wendt DE, Johnson CH. 2006. Using latent effects to determine the ecological importance of dissolved organic matter to marine invertebrates. Integr Comp Biol 46(5): 634-642.

Williams TD. 1994. Intraspecific variation in egg size and egg composition in birds: effects on offspring fitness. Biol Rev 69(1): 35-59.

Williamson JE, De Nys R, Kumar N, Carson DG, Steinberg PD. 2000 Induction of metamorphosis in the sea urchin *Holopneustes purpurascens* by a metabolite complex from the algal host *Delisea pulchra*. Biol Bull 198: 332-345.

Wollcott DL, DeVries MC. 1994. Offshore megalopae of *Callinectes sapidus*: depth of collection, molt-stage and response to estuarine cues. Mar Ecol Prog Ser 109: 157-163.

Woollacott RM, Pechenik JA, Imbalzano KM. 1989. Effects of duration of larval swimming period on early colony development in *Bugula stolonifera* (Bryozoa: Cheilostomata). Mar Biol 102(1): 57-63.

Wu H, Wu DY. 1925. Nature of heat denaturation of proteins. J Biol Chem 64: 369-378.

Zimmer RK, Butman CA. 2000. Chemical signaling processes in the marine environment. Biol Bull 198: 168-187.

Zimmer-Faust RK, Tamburri MN. 1994. Chemical identity and ecological implications of a waterborne, larval settlement cue. Limnol Oceanogr 39: 1075-1087.