PHENOTYPTIC PLASTICITY IN LARVAL AND JUVENILE MARINE INVERTEBRATES:

EFFECTS OF PREDATORS, FOOD, GRAVITY, AND SUNLIGHT

by

JENNA ROSE VALLEY

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Student: Jenna Rose Valley

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This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Biology by:

Barbary (Bitty) Roy	Chair
Richard Emlet	Advisor
Alan Shanks	Core Member
Kelly Sutherland	Core Member
William Orr	Institutional Representative

and

Scott L. Pratt	Dean of the Graduate School

Original approval signatures are on file with the University of Oregon Graduate School.

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DISSERTATION ABSTRACT

Jenna Rose Valley

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Title: Phenotypic plasticity in larval and juvenile marine invertebrates: Effects of predators, food, gravity, and sunlight

Phenotypic plasticity, the ability of a single genotype to be expressed as a range of phenotypes in response to environmental variation, is a widespread phenomenon. Documented increasingly among the larval stages of marine organisms, phenotypic plasticity in the veliger larvae of the marine snail *Littorina scutulata* was investigated in response to predatory, nutritional, and gravitational stimuli.

Veligers developed rounder shells, smaller apertures, and reinforced aperture margins in response to water-borne cues from predatory crab larvae. The nature and degree of the induced-morphologies depended on cue composition and conferred decreased vulnerability to predation.

Food-limited veligers developed larger feeding and swimming structures (vela) with longer cilia relative to shell size compared to larvae raised with high food. This inducible offense corresponded with a decrease in vertical swimming speed, an unexpected result possibly reflecting behavioral manipulation of individual velar components. A cell proliferation assay indicated that growth of the larger structure was achieved partially by a steady rate of cell division over a longer period of time; an initially higher level of cell proliferation in veligers raised on high food dropped off sharply.

Velar lobe asymmetry, where one lobe is larger than the other, may exist to offset an asymmetry in weight distribution due to how the larval shell is carried. The larger velar

lobe overlies the protruding spire of the larval shell. Bi- and multi-lobed vela get bigger with shell size but follow different rules with regards to the relationship between velar asymmetry and shell asymmetry. Experimental alternations of mass distribution of the larval shell caused changes in the ratio of area between each side of the velum and total velar growth for larvae of *L. scutulata.*

Following settlement and metamorphosis, juveniles of intertidal marine invertebrates are subject to additional stressors that can manifest as phenotypic variation. Color differences between juvenile and adult *Strongylocentrotus purpuratus* were shown to be caused by variation in light exposure. Green juveniles raised in sunlight turned purple (due to more pigment) and showed decreased susceptibility to artificial UVR than urchins kept in the dark, which remained green (due to less pigment).

This dissertation includes previously unpublished co-authored material.

CURRICULUM VITAE

NAME OF AUTHOR: Jenna Rose Valley

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene, OR
Appalachian State University, Boone, NC
University of North Carolina at Wilmington, Wilmington, NC

DEGREES AWARDED:

Doctor of Philosophy, Biology, 2016, University of Oregon
Master of Science, Biology, 2011, Appalachian State University
Bachelor of Science, 2008, University of North Carolina at Wilmington

AREAS OF SPECIAL INTEREST:

Phenotypic plasticity
Larval biology and ecology
Marine biology

PROFESSIONAL EXPERIENCE:

Graduate Research Fellow, Department of Biology, Oregon Institute of Marine Biology, University of Oregon, Eugene, 2011 – 2016

Graduate Teaching Fellow, Department of Biology, Oregon Institute of Marine Biology, University of Oregon, Eugene, 2011 – 2016

Administrative Outreach Coordinator, Department of Biology, Oregon Institute of Marine Biology, University of Oregon, Eugene, 2012 – 2013

Graduate Research Assistant, Department of Biology, Appalachian State University, Boone, NC, 2010 – 2011

Graduate Teaching Assistant, Department of Biology, Appalachian State University, Boone, NC, 2008 – 2010

GRANTS, AWARDS, AND HONORS:

National Academies Education Fellow in the Life Sciences, 2015

Oregon Society of Conchologists Scholarship, 2014

Oregon Society of Conchologists Scholarship, 2013

Oregon Sea Grant Neil Richmond Memorial Fellowship, 2013

Inducted into the Cratis D. Williams Society, Appalachian State University, 2011

Graduate Teaching Assistantship Award for Introductory Biology – Majors, Appalachian State University, 2010

Graduate Student Association Senate Grant, Appalachian State University, 2010

Office of Student Research Grant, Appalachian State University, 2010

Office of Student Research Grant, Appalachian State University, 2009

Charlotte Mangum Student Support Grant, Society for Integrative and Comparative Biology, 2009

Graduate Student Association Senate Travel Grant, Appalachian State University, 2009

Office of Student Research Travel Grant, Appalachian State University, 2009

Graduate School Provost Fellowship, Appalachian State University, 2009-2010

Office of Student Research Grant, Appalachian State University, 2008

Graduate Student Association Senate Grant, Appalachian State University, 2008

Graduate Student Research Grant, Appalachian State University, 2008

Graduate School Alumni Scholarship, Appalachian State University, 2008-2009

Summa cum laude, University of North Carolina at Wilmington, 2008

PUBLICATIONS:

Valley, J. and T. C. Hiebert. 2015. Littorina plena. In *Oregon Estuarine Invertebrates: Rudys' Illustrated Guide to Common Species, 3rd ed*., T. C. Hiebert, B. A. Butler, and A. L. Shanks, eds. University of Oregon Libraries and Oregon Institute of Marine Biology, Charleston, OR. http://hdl.handle.net/1794/19870

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TABLE OF CONTENTS

Chapter Page

Chapter Page

Chapter Page

LIST OF FIGURES

Figure Page

Figure Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

The relationship between organism and environment is most often thought of in the context of natural selection, whereby individuals with traits that increase fitness in an environmental setting (such as having a thicker coat in a cold climate) are more likely to survive and pass these favorable traits onto their offspring by genetic inheritance. In this context, the expression of a phenotypic trait is determined solely by an organism's genetic make-up and the environment simply provides the framework within which the adaptive qualities of the trait are assessed via natural selection. Evolutionary change by natural selection is based around variation between individuals or even between populations or species whereby a trait evolves to be better adapted to an environment over multiple generations (between-generation variation). However, the temporal scale of environmental variation often outpaces the rate of genetic change. While a trait may have evolved in response to a particular set of environmental conditions, changes in these environmental conditions may disrupt that match, thereby reducing fitness. In this situation it is beneficial for an organism to be able to adjust its phenotype during its lifetime in response to its surroundings (within-generation variation).

This ability, environmentally-dependent 'intra-individual' variation, is referred to as phenotypic plasticity and encompasses changes in behavior, physiology, morphology, or life history of an organism (e.g., Schlichting and Pigliucci, 1998; Pigliucci, 2001; West-Eberhard, 2003; Whitman and Agrawal, 2009, Kelly *et al*., 2012). In other words, one genotype (the individual) is capable of producing a range of phenotypes reflective of environmental variation. This phenotypic range (representing the relationship between environmental and phenotypic variation of a trait) is referred to as a reaction norm, a term coined by Woltereck (1909 – as cited by Gottlieb, 2003) in describing the development of 'helmets' in freshwater

crustaceans in response to varying food levels. Environmentally-influenced traits are referred to as 'plastic' whereas traits that are produced irrespective of external environmental variation are said to be 'fixed', 'canalized', or 'constitutive' (e.g., Pigliucci *et al*., 2006)

Whether a trait is constitutive or plastic heavily depends on the nature of the environmental heterogeneity, specifically the relationship between the spatial and temporal 'grain' of variation (e.g., Berrigan and Scheiner, 2004; Hollander, 2008; Scheiner, 2013; Hollander *et al*., 2014). If the scale of spatial or temporal variation is large (coarse-grained), exceeding a species' dispersal potential or lifespan, it is most beneficial for a population to evolve a fixed adaptation to the local set of conditions. Alternatively, spatial or temporal variation that is too fine-grained (conditions vary over a much smaller area or duration) may also favor a fixed phenotype, a generalist that is only intermediately matched to each set of conditions but exhibits higher fitness overall (a jack-of-all-trades, master of none). Plasticity should be favored only when an organism can change quickly relative to the scale of environmental change, otherwise it may be constantly lagging behind – this is especially relevant for morphological plasticities that require more time to change as opposed to physiological or behavioral changes (e.g., Padilla and Adolph, 1996; Whitman and Agrawal, 2009). It is also important to remember that plasticity and its attributes (e.g., reactions norms and underlying genetic variability) are themselves traits subject to natural selection, heritability, or canalization (e.g., Schlichting and Pigliucci, 1995, 1998; Pigliucci *et al.,* 2006; Whitman and Agrawal, 2009).

The value of phenotypic plasticity relies on the translation of environmental variation into the appropriate phenotypic response. The environmental stimulus (e.g., temperature, nutrition, light) for the production of altered phenotypes needs to be a reliable indicator and, to some degree, predictor of an organism's surroundings (e.g., Reed *et al*.,

2010; Scheiner and Holt, 2012). One of the most well-studied areas of phenotypic plasticity surrounds the relationship between predators and prey, such as the ability of an individual to respond to the threat of predation (e.g., chemical cues from the predator, from damaged conspecific prey, or from a combination of the two) via a change in behavior or morphology that confers decreased susceptibility (e.g., Tollrian and Harvell, 1999). For example, chemical cues from a nudibranch predator induce the formation of spines in colonies of a marine bryozoan (Harvell, 1986). In another instance, a species of freshwater cladoceran produces increased spine and body length in response to elevations in temperature but not to cues from predatory fish; in this case, predator cues are an unreliable indicator of predation risk because the fish are always present but predation risk increases over the summer months as juvenile fish grow in size – therefore, temperature is a reliable proxy of predation risk (Miehls *et al*., 2013). Inducible defenses can extend beyond threats of predation; for example, the ability to enhance melanin production in response to sun exposure is an inducible defense against subsequent damage by ultraviolet radiation (e.g., Friedmann and Gilchrest, 1987; Miyamura *et al*., 2006). Alternative to these 'inducible defenses' are 'inducible offenses', plastic traits enacted by consumers that confer an increased acquisition of resources (Padilla, 2001).

In order to merit a phenotype being expressed plastically as opposed to constitutively, theory dictates there should also be a trade-off in costs and benefits (e.g., Callahan *et al*., 2008). The most common type of cost cited with regards to inducible traits, such as an inducible defense, is the existence of some sort of direct or indirect trade-off associated with the defense (e.g., allocation costs resulting in a reduction in growth rate or reproductive output). Net fitness should increase when the trait is expressed in the appropriate environment (in the presence of a predatory threat) but decreases when the trait is expressed in an inappropriate environment (in the absence of predation). For

example, predator-induced thickening of snail shells often co-occurs with reduced somatic growth (e.g., see Brookes and Rochette, 2007).

The normalcy of environmental variation begets a ubiquity of phenotypic plasticity. The environment of many marine ecosystems is particularly variable with temporal and spatial heterogeneity of factors such as nutrients, temperature, salinity, light, predators, and hydrodynamics. Many marine organisms, such as intertidal invertebrates with indirect lifecycles, spend at least a portion of their ontogeny suspended in the water column and are subject to the influences of these parameters on their growth and development (e.g., Thorson, 1950; Young and Chia, 1985; Rumrill, 1990; Hoegh-Guldberg, 1995; Vaughn and Allen, 2010). Following settlement and metamorphosis into a benthic habitat, the organism remains subject to many of the same stressors but with added complications that come with an intertidal lifestyle (e.g., risk of desiccation and more extreme fluctuations in sun exposure, temperature, and wave action; Helmuth and Hofmann, 2001; Tomanek and Helmuth, 2002).

The subsequent three chapters of this dissertation encompass case-studies of plasticity in the larval stage of a local marine snail, *Littorina scutulata*, in response to three very different but important environmental variables: predators, food, and gravity. The fifth chapter provides an additional example of an inducible trait (in response to sun exposure) but in post-settlement juveniles of another intertidal invertebrate, *Strongylocentrotus purpuratus* (Stimpson, 1857), a local urchin species. All chapters were written as independently publishable units and should be referred to for additional pertinent background information.

Littorina scutulata (Gould, 1849) is a marine snail with planktotrophic development, meaning that the planktonic larvae need to feed on phytoplankton in order to develop to the point at which they can settle and metamorphose into juveniles (e.g., Thorson, 1950;

Strathmann, 1985). Adult *L. scutulata* can be found in the high intertidal and splash zone of rocky coast habitats (Hohenlohe, 2002, 2003) and release pelagic egg capsules containing varying numbers of embryos from early April to early October (Strathmann, 1987a; Hohenlohe, 2002). After approximately nine days at 12-14°C, these embryos hatch into swimming larvae called veligers, superficially resembling miniature snails (Buckland-Nicks *et al*., 1973; Hohenlohe, 2002). Veliger larvae, found in gastropod and bivalve mollusks, are named for the ciliated structure called a velum that is used to both swim and feed (Garstang, 1966; Strathmann, 1987b).

While examples of phenotypic plasticity are well-known, especially in the context of predator-prey interactions, the evidence of inducible morphological defenses is scarce in marine larvae. Chapter II of this dissertation is entitled "Predator-induced morphologies and cue specificity in veliger larvae of *Littorina scutulata*" and is co-authored with Richard Emlet. In order to better understand morphological plasticity in the larvae of the gastropod *Littorina scutulata*, we exposed veligers to predatory zoeae of *Hemigrapsus nudus*. The treatments included growing veliger larvae in the presence of predators and growing veliger larvae in the presence of predators consuming conspecific veliger larvae. The larvae were then evaluated for the presence of defensive morphologies including changes in shell shape, aperture area, and reinforcement of aperture margins. Control veligers and those that had been raised in the presence of predators consuming conspecific veliger larvae were directly paired with predators to compare vulnerability to predation.

Chapter III, "Morphological plasticity in response to variable food concentrations in veligers of *Littorina scutulata* with analyses of swimming speed and velar growth" explores the development of a phenotypically plastic response that confers a greater acquisition of resources (such as the change in size of a feeding structure), called an inducible offense, and its effect on swimming performance. Veligers of *Littorina scutulata* raised in either high or

low food conditions were measured for changes in velar size and prototrochal cilia length, differences in vertical swimming speed, and velum-specific proliferative activity over development.

The ability to orient in relation to gravity is an important component of planktonic life for the larvae of many marine invertebrates, including gastropod veligers. The helically-coiled shells of veligers are carried in such a way that the proportion of weight distributed under each velar lobe is unequal. There is often a corresponding asymmetry between the two sides of the velum, with the larger side overlying the protruding spire of the shell. Chapter IV, "Life in the balance: distribution and development of asymmetric vela", explores the prevalence of this phenomenon and its potential as a plastic trait subject to manipulation. I investigated the relationship between asymmetries in weight distribution and the relative sizes of overlying velar lobes as well as the relationship of total shell area vs. total velar area by measuring these attributes from veliger figures or photos in the literature. To test the plasticity of velar growth and velar symmetry, artificial weights were attached to the shells of veligers of *Littorina scutulata* and results were interpreted in the context of literature findings.

The purple sea urchin, *Strongylocentrotus purpuratus*, is a local urchin commonly found in the mid- to lower intertidal of rocky shores exposed to moderate to strong wave action (Durham *et al*., 1980; Pearse and Mooi, 2007). Like *Littorina scutulata*, *S. purpuratus* exhibits indirect development with a pelagic larval stage. Although this species is known for its purple color, newly-settled individuals and juveniles are often green in color and are found under rocks adjacent to exposed urchin beds where purple juveniles are found. Chapter V is titled "Sunlight and coloration in the purple urchin *Strongylocentrotus purpuratus*" and represents work done in collaboration with Laurel Hiebert and Bailey Counts. In this chapter, plasticity is explored in the context of color variation and function:

many marine invertebrates display intrapopulation variation in coloration. Based on the habitats in which the two ecotypes can be respectively found, we hypothesized that light exposure, and specifically ultraviolet radiation (UVR), may explain the color variation in juvenile urchins and that pigment production is a phenotypically-plastic response, presumably for photoprotection. While UVR and its damaging effects have been studied in echinoderms, most studies have focused on embryonic and larval stages. To test the role of sunlight in urchin coloration, field-collected green juvenile *S. purpuratus* were raised under ambient sunlight, UVR-filtered sunlight, or in darkness for over 100 days. Pigment production was monitored over time via photographic assessment of color and at the end of the study by spectrophotometric measurement of dermally-extracted pigment levels. Photoprotective properties of the pigment were also evaluated at the end of the study by exposure to artificial UVR.

CHAPTER II

PREDATOR-INDUCED MORPHOLOGIES AND CUE SPECIFICITY IN VELIGER LARVAE OF *LITTORINA SCUTULATA*

All work in this chapter was planned, implemented, and analyzed by the author. The written material is co-authored by Richard Emlet.

INTRODUCTION

Many environmental stimuli induce phenotypic responses such as those resulting from competitors (e.g., Relyea, 2002; Relyea and Auld, 2005; Todd, 2008), conspecific density (e.g., Kemp and Bertness, 1984), nutrition (e.g., Strathmann *et al*., 1993; Walls *et al*., 1993), light (e.g., Sultan, 2000; Todd, 2008), temperature (e.g., Stelzer, 2002; Atkinson *et al*., 2003), wave exposure/water flow (e.g., Trussell, 1997; Marchinko, 2003; Todd, 2008), etc. Studies on predator prey interactions abound with examples of phenotypic plasticity where predatory cues such as chemical, visual, auditory, or mechanical stimuli are capable of inducing behavioral, morphological, physiological, and life history changes in prey that are otherwise not displayed and which often result in decreased vulnerability to predation (Harvell, 1990; Lima and Dill, 1990; Kats and Dill, 1998; Tollrian and Harvell, 1999; Lass and Spaak, 2003; Bernard, 2004; Ferrari *et al*., 2010; Miner *et al*., 2010; Rundle *et al*., 2011). Tollrian and Harvell (1999) outline the requisite conditions for the evolution of an inducible (as opposed to constitutive) defense: exposure to the response-inducing cue needs to be variable, the cue must be a reliable indication of danger, the induced phenotype must be effective in lessening predation risk, and there should be a trade-off in costs and benefits of the response that warrants conditional implementation.

Predator-induced phenotypic plasticity in freshwater planktonic environments is well known with behavioral and morphological responses demonstrated in algae, rotifers, ciliates, and crustaceans (for reviews see Kats and Dill, 1998; Tollrian and Harvell, 1999; Lass and Spaak, 2003). Examples of predator-induced plasticities in a marine planktonic environment are scarce with limited examples including behavioral responses in crustaceans (e.g., Bollens and Frost, 1989; Neill, 1990; Bollens *et al*., 1994; Cieri and Stearns, 1999; Hamrén and Hansson, 1999), larval cloning in echinoderms (Vaughn, 2010), colony formation and cell wall thickening in phytoplankton (for a review see Van Donk *et al*., 2011), and modification of shell morphology in gastropod veligers (Vaughn, 2007). The studies by Vaughn (2007, 2010) are the only two examples of predator-induced morphological changes in marine larvae, the former determining that veligers of *Littorina scutulata* developed smaller apertures and rounder shells when exposed to cues from a larval decapod predator, zoeae of *Cancer* spp., and that these alterations enhanced survival.

The occurrence of smaller apertures and changes in shell shape are well-documented in benthic examples of predator-induced defenses of both marine and freshwater adult gastropods (e.g., Kitching and Lockwood, 1974; Appleton and Palmer, 1988; Palmer, 1990; DeWitt *et al*., 2000; Krist, 2002; Cotton *et al*., 2004; Hoverman, 2007; Rochette *et al*., 2007; Brönmark *et al*., 2011; Moody and Aronson, 2012; Hoverman *et al*., 2014). One additional defense, particularly prevalent in marine snails, is shell thickening (Kitching and Lockwood, 1974; Appleton and Palmer, 1988; Palmer, 1990; Trussell, 1996; Trussell and Smith, 2000; Delgado *et al*., 2002; Trussell and Nicklin, 2002; Dalziel and Boulding, 2005; Brookes and Rochette, 2007; Rochette *et al*., 2007; Lakowitz *et al*., 2008; Bourdeau, 2009, 2010a, 2010b, 2011, 2012; Moody and Aronson, 2012; Sepúlveda *et al*., 2012; Hoverman *et al*., 2014). The thickened shell reduces vulnerability to both shell-breaking and shell-entering predators (e.g., Vermeij, 1974; Hughes and Elner, 1979; Palmer,

1979; Bertness and Cunningham, 1981; Palmer, 1985; Bourdeau, 2009; Covich, 2010; Moody and Aronson, 2012). Gastropod veliger larvae are known to be able to survive predation attempts utilizing other altered features such as spiral sculpturing or changes in shell shape (Hickman, 1999; Vaughn, 2007), thus it is plausible that an inducibly-thickened shell may benefit both life stages of the snail in resisting mechanical damage. However, due to potential complications of a heavier shell in a planktonic environment, reinforcement might be limited to regions of the aperture opening, such as the apertural beak or velar notches, as observed in a variety of field-caught veligers (Hickman, 1999).

The composition of an environmental cue can influence the nature of the induced response. This has been investigated extensively in the inducible traits of adult snails (e.g., Appleton and Palmer, 1988; Palmer, 1990; Trussell and Nicklin, 2002; Bourdeau, 2010b). Many behavioral plasticities are known to occur in response to injured conspecifics (alarm cues; e.g., Snyder, 1967; Snyder and Snyder, 1971; Atema and Stenzler, 1977; Stenzler and Atema, 1977; Alexander and Covich, 1991; Vadas *et al*., 1994; Jacobsen and Stabell, 1999, 2004; McCarthy and Fisher, 2000; Grason and Miner, 2011). Isolated predator kairomones have also been shown to elicit behavioral and morphological modifications (Palmer, 1990; McCarthy and Fisher, 2000; Trussell and Nicklin, 2002; Marko and Palmer, 1991; Grason and Miner, 2011). The greatest range/extent of morphological and behavioral change has commonly been found only in response predators consuming conspecific snails (e.g., Alexander and Covich, 1991; Krist, 2002; Trussell and Nicklin, 2002; Jacobsen and Stabell, 2004; Bourdeau, 2010b). This combination likely provides the most accurate information about the perceived risk, which in turn influences the type and level of response (e.g., Schoeppner and Relyea, 2005, 2009). Others studies have proposed that this combined signal of predators and injured conspecifics 'labels' the predator as dangerous and that this information is able to persist through ingestion/digestion where it can be detected and

responded to post-feeding through predator excretions (diet cues; see Chivers and Smith, 1998 and Ferrari *et al*., 2010 for reviews).

In this study, we sought to expand our understanding of how different types of predatory treatments (and by inference the composition of the inducing cues) influence the shell characteristics of veliger larvae of *L. scutulata,* including the prospect of shell reinforcement. We exposed veliger larvae to predators that had been raised on food other than veligers, to predators consuming conspecific veliger larvae, and to a seawater control. Although the study by Vaughn (2007) tested the response to predators, it is important to note that the zoeae used in her predator treatment were fed veligers of *L. scutulata* prior to being placed in the experimental cages; therefore it is possible the experimental veligers were exposed not just to predator kairomones, but to post-digestive diet cues as well. Our choice of treatments allows us to distinguish the specificity of responses to different types of predatory cues. As predators, we used zoea larvae of *Hemigrapsus nudus* because zoea larvae of *Cancer* spp. (used by Vaughn, 2007) do not co-occur temporally with veligers of *Littorina* spp. in Oregon waters. Zoea larvae of *Hemigrapsus nudus* are likely to be encountered by the veligers in Oregon plankton, are in the same crustacean infraorder (Brachyura), are easily obtainable, are of similar size to those used by Vaughn (2007), and prey on veligers of *L. scutulata* via the aperture-chipping method typical of many zoea larvae (Hickman, 2001; Vaughn, 2007; J. Valley, personal observations).

If the larvae only respond to or respond in the greatest degree to the treatment of predators feeding on conspecifics, this will indicate the usage of combinatory information such as is often seen in adults that would likely alert the larvae to both conspecific injury as well as provide important information about the predator and its pertinent diet. Context specific signals such as the ones expected in this treatment are thought to exist so that prey can respond variably to different predators with different predation strategies (e.g.,

crushing vs. peeling) as has been seen in both behavioral and morphological responses in a variety of animals such as adult snails (e.g., Turner *et al*., 1999; DeWitt *et al*., 2000; Hoverman *et al*., 2005; Bourdeau, 2009), phytoplankton (Long *et al*., 2007) and vertebrates (e.g., Relyea, 2001, 2003).

We expected veligers to respond to the predator only treatment and to respond to a greater extent to the combinative treatment of predators consuming conspecific veliger larvae. We also expected the morphological alterations resulting from the latter treatment to enhance survival when subjected to direct predator contact.

MATERIALS AND METHODS

Predator collection and rearing

Adult *Hemigrapsus nudus* (Dana 1851) bearing eggs at different stages of development were collected locally from the boulder fields at the south-side of Sunset Bay (43°20'1.99"N, 124°22'37.36"W) or from the rocky shores lining Charleston mudflats, OR (e.g., 43°20'22.31"N, 124°19'5.87"W). The crabs were kept submerged in glass jars equipped with an air-stone. Upon hatching (usually early morning), approximately 200 zoeae were placed in up to four large finger bowls depending on the clutch size. The fingerbowls were stacked in one of two incubators kept at either 18°C or 13°C to hasten or slow development, thus ensuring a steady supply of zoeae of the desired stages for the duration of the study. Every other day, the zoeae were moved to fresh filtered seawater (FSW) of the appropriate temperature and were fed newly hatched *Artemia*, a tripartite algal mixture, and a diluted solution of artificial plankton (A.P.R.; Ocean Star International, Snowville, UT) filtered through 100 μm mesh. At this time, dead zoeae were counted, molts noted, and a portion of the zoeae was staged.

Prey adult and veliger collection

Snails in the genus *Littorina* (Gastropoda, Caenogastropoda, Littorinidae) are common intertidally worldwide (McQuaid, 1996) with five local Oregon species. Three of these species (*L. scutulata* Gould 1849, *L. plena* Gould 1849, and *L. keenae* Rosewater 1978) exhibit planktotrophic development. Snails of *L. scutulata* become reproductively mature at a shell height of 2-3 mm and the reproductive season spans from early April to early October (Strathmann, 1987; Hohenlohe, 2002) when pelagic egg capsules containing varying numbers of embryos are released. Veligers can become competent to settle after approximately three to five weeks (Buckland-Nicks *et al*., 1973; Hohenlohe, 2002; J. Valley personal observations).

To obtain veligers, several adult *L. scutulata* collected beside the jetty at OIMB beach (Charleston, OR; 43°20'58.85"N, 124°19'49.50"W) were placed in screen-bottomed tricorner plastic beakers suspended in FSW overnight. The following morning, the water was filtered through 350 μm mesh and egg capsules were collected and distributed into large glass jars filled with 3L FSW for a concentration of ~200 veligers/L upon hatching. During and following the ~eight days it takes the veligers to develop and hatch out of the capsules, the ~13°C water was changed every three days and gently stirred by paddles suspended from a motorized stir rack (Strathmann, 1987).

Experimental set-up

Three replicates of each of the three treatments (isolated predators, P; predators feeding on conspecific veligers, PV; and control, C) were randomly distributed in each of two sea tables, for a total of six replicates per treatment. The daily temperature of each sea table was ~13°C. Each replicate consisted of a Pyrex 1000 mL beaker containing 800 mL FSW and 50 newly-hatched veligers. Floating in each replicate container was a predator cage fashioned out of a 50 mL falcon tube with 100 μm mesh openings on the sides and

bottom (Figure 2.1a). Embedded in the lid of the cage was an open-bottomed eppendorf tube with a sealable lid. With this lid closed water was retained in the cage and zoeae remained submerged whenever the cage was moved. Just beneath the lid of the tube, the cage was outfitted with a circle of foam sheeting (4 mm thick) to allow the cage to float. Five stage 4 or stage 5 zoeae of *Hemigrapsus nudus* were placed in the cages of the six P and PV treatment replicates. Twenty food-veligers (~one week old, ~200 μm shell length) were also added to the cages of PV replicates for the zoeae to consume. Every three days, the water was changed, the veligers were fed a tripartite algal mixture (*Isochrysis galbana*, *Dunaliella tertiolecta*, *Chaetoceros gracilis*) of equal numbers of cells at a combined concentration of 10,000 cells/mL, and the treatments were renewed: the predators were removed (dead zoeae and molts were noted) and replaced with five new stage 4 or stage 5 zoeae. In the PV replicates, any remaining food-veligers were counted to confirm prey consumption and replaced with 20 new food-veligers (~one week old). After four weeks, the experimental veligers were collected, counted, and fixed in 80% ethanol buffered with sodium glycerophosphate (Turner, 1976) except for those used in the predation trials (see below).

Predation trials

The greatest response was expected from the veligers in the PV treatment; because of this and the number of control veligers that would have been needed to test both PV and P treatments, the predation trials only used veligers from the PV treatment. The methods for the predation trials were modeled after Vaughn (2007). Five veligers from each of the PV and control replicates were randomly selected to be used in predation/survival trials. Half of these 30 PV veligers and half of the 30 control veligers were randomly chosen to be stained for one hour in a 0.01% solution of Neutral Red. The veligers were then randomly assigned to one of two combinations, each with three replicates. The first combination

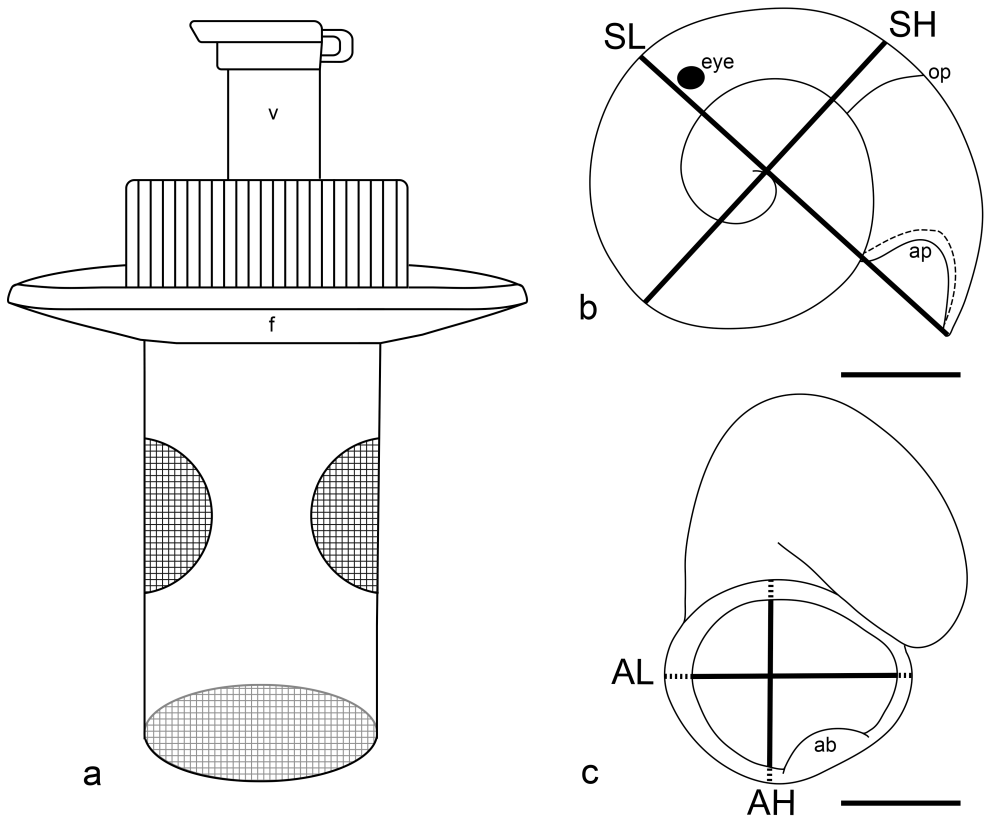


Figure 2.1. The cage used to contain the treatment cues has 100 μm mesh windows, a float, and a sealable vent to contain water when removed (a). The shell aspect ratio was calculated by dividing the shell length (SL) by the shell height (SH) (b). The elliptical area of the aperture was estimated using the aperture length (AL) and aperture height (AH) (c; solid lines). Marginal reinforcement was estimated using the average perceived thickness of the aperture margin at the periphery of the aperture length and height (c; dotted lines). Scale bars = 100 μm. ab = apertural beak, ap = aperture, f = float, op = operculum, v = vent.

consisted of five dyed control veligers + five undyed PV veligers. The second combination consisted of five dyed PV veligers + five undyed control veligers. Each replicate combination, along with two stage 5 zoeae of *H. nudus*, was placed in a randomly assigned well in a six-well plate. Every 30 minutes for 3.5 hours, the number of dyed vs. undyed veligers in each well was counted.

Measurement methods

To measure shell length, height, and aperture area, 15 randomly-selected, fixed veligers from each replicate were individually placed in the tapered base of a severed eppendorf tube, which enabled easy manipulation of the veligers into a profile position that

minimized the angular tilting seen when placed on a flat surface and allowed the veliger to also be easily positioned aperture-side up. The veligers were photographed and measurements from these images were later collected using ImageJ software (NIH, Bethesda, MD). Measurements of veliger length, height, shell aspect ratio, and aperture area were modeled after Vaughn (2007). Veliger length was measured as the largest distance from the tip of the apertural beak to the opposite side of the shell and shell height was measured as the greatest distance perpendicular to shell length (Figure 2.1b). A shell aspect ratio (SAR) was calculated for each veliger as an indication of overall shell shape (length/height). Aperture area was estimated using the formula for elliptical area: [(aperture length x aperture height x π)/4] (Figure 2.1c). The marginal reinforcement measurement consisted of the average perceived thickness of the aperture margin extending from either end of the aperture length and height measurements (Figure 2.1c). The marginal reinforcement appears to be a combination of thickening and curving of the apertural edge.

Statistical methods

An ANCOVA in SPSS was used to test the effects of the control (C), predator (P), and predator + injured conspecific + diet cues (PV) scents on resulting shell shape (SAR = shell aspect ratio; shell length/shell height), aperture area, and marginal reinforcement. To account for variance due to size, shell height was included as a covariate as it was not affected by treatment. There was no interaction between treatment and the covariate for any of the dependent variables therefore the interaction term was removed from the models. Subsequently, graphical inspection and data analysis indicated no statistical effect of beaker ($p \geq 0.362$), therefore it was removed from the models and all individuals within each treatment were pooled (Quinn and Keough, 2002). Normality of the standardized residuals for each treatment was demonstrated by Shapiro-Wilk's test, homogeneity of

variances was established by Levene's test, and all residuals were homoscedastic. Potential outliers were determined by looking for standardized residuals greater than ±3 standard deviations. One outlier was identified in the marginal reinforcement data but was left in because its removal did not change the outcome of the model. Post-hoc analyses were performed with a Bonferroni adjustment.

Survival of veligers from the control treatment and from the PV treatment in the paired-predation trials was analyzed using a life tables survival analysis in SPSS.

RESULTS

Initial pairing of different zoeal stages with various-sized veligers of *L. scutulata* showed that stage 3 zoeae (~2.2 mm total length) were capable of capturing and consuming medium-sized veligers (~200 µm shell length) and stage 4 zoeae (~2.6 mm) were capable of consuming veligers up to ~300 µm. Stage 5 zoeae (~3.3 mm) were capable of consuming veligers of all sizes. As has been described for other zoea predators (Hickman, 2001; Vaughn, 2007), once captured, the zoea rolls the veliger shell to where it can commence gradually chipping away at the aperture edges until the larval body is reached. Evidence of veliger consumption can be seen in the form of shell fragments on the bottom of the dish.

There was a significant effect of the covariate (shell height) on all three measured variables (p <0.0005) with the observed trend of a decrease in SAR and increases in aperture area and marginal reinforcement as shell height increased, regardless of treatment. Following adjustment by shell height, there was a statistically significant effect of treatment found for SAR ($F_{2, 266} = 20.471$, $p < 0.0005$), aperture area ($F_{2, 266} = 24.303$, $p < 0.0005$), and marginal reinforcement ($F_{2, 266} = 52.868$, $p < 0.0005$] (Table 2.1). Post-hoc analyses showed that veligers from the P group had a significantly smaller SAR (1.167 ± 0.004 SE) than veligers from either the control ($p < 0.0005$; 1.192 ± 0.004 SE) or PV ($p < 0.0005$; 1.188 ± 0.004 SE) groups, which were not significantly different from each other (p

= 0.108); in other words, veligers raised in the presence of predators alone developed shells that were significantly more round than those raised in the presence of predators consuming conspecifics or those from the control group (Tables 2.1 and 2.2, Figure 2.2).

Table 2.1. Summary of statistical results including ANCOVAs with post-hoc tests (SAR, aperture area, marginal reinforcement) and life tables survival analysis.

Variable	(df), Test Statistic	*p*
SAR	(2, 266), F = 20.471	<0.0005*
Control vs. P		<0.0005*
Control vs. PV		0.108
P vs. PV		<0.0005*
Aperture Area	(2, 266), F = 24.303	<0.0005*
Control vs. P		<0.0005*
Control vs. PV		<0.0005*
P vs. PV		1.000
Marginal Reinforcement	(2, 266), F = 52.868	<0.0005*
Control vs. P		<0.0005*
Control vs. PV		<0.0005*
P vs. PV		<0.0005*
Survival (Control vs. PV)	(1), W = 4.104	0.043*

Table 2.2. Summary of results from Vaughn (2007) and the present study for veligers raised in the presence of predators (P) or in the presence of predators consuming conspecifics (PV). SAR data are shell length/shell height (μm) ± SE. Aperture area and marginal reinforcement data are given in μm^2 and μm, respectively, ± SE.

Variable and treatments	Vaughn (2007)	This study
SAR		
Control	1.16 ± 0.41	1.192 ± 0.004
P	1.12 ± 0.32[1]	1.167 ± 0.004
PV	n/a[2]	1.188 ± 0.004
Aperture Area		
Control	21,151 ± 409.4	18,179 ± 127.1
P	19,075 ± 288.0[1]	17,042 ± 162.3
PV	n/a[2]	16,969 ± 184.6
Marginal Reinforcement		
Control		15.8 ± 0.2
P	n/a[2]	16.7 ± 0.2
PV		17.8 ± 0.2

[1]the predator kairomone treatment used by Vaughn (2007) may have also included diet cues (see text).
[2]this treatment and variable was not included in Vaughn (2007).

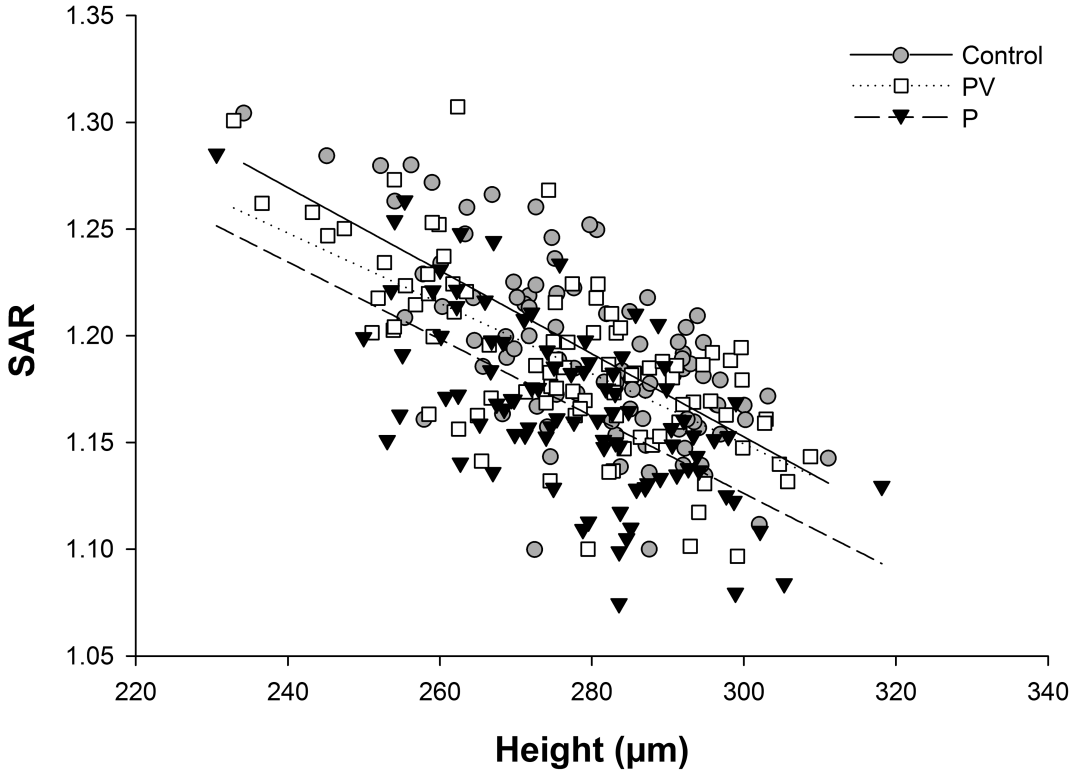


Figure 2.2. The relationship between shell aspect ratio (SAR) and shell height for veligers raised in a control environment (C), in the presence of predators (P), and in the presence of predators consuming conspecifics (PV). SAR = shell length (µm)/shell height (µm). As SAR decreases, the shell becomes more round. P<PV=C.

Veligers in the control group had significantly larger aperture areas (18,179 μm^2 ± 127.1 SE) than those raised in the presence of predators consuming conspecifics (p < 0.0005; 16,969 μm^2 ± 184.6 SE) and those raised in the presence of predators only (p < 0.0005; 17,042 μm^2 ± 162.3 SE), which were not significantly different from each other (p = 1.000) (Tables 2.1 and 2.2, Figure 2.3).

When raised in the presence of predators consuming conspecifics, veligers had significantly more marginal reinforcement (17.8 µm ± 0.2 SE) than those raised in the presence of predators only (p < 0.0005; 16.7 µm ± 0.2; both P and PV groups had significantly more marginal reinforcement than those in the control group (p < 0.0005; 15.8 µm ± 0.2 SE) (Tables 2.1 and 2.2, Figure 2.4).

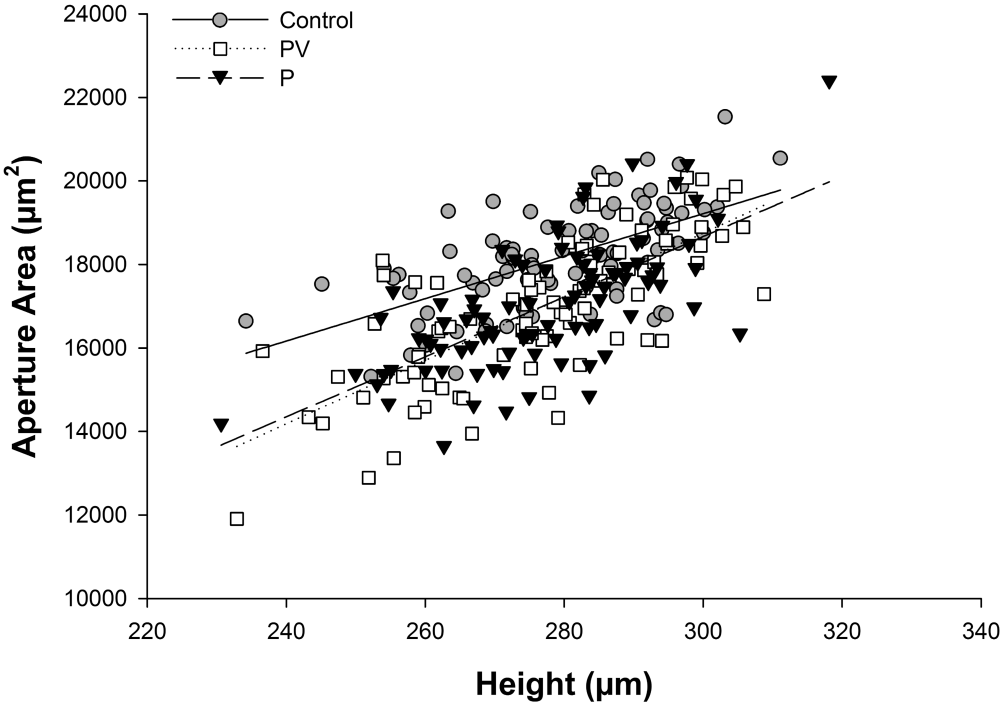


Figure 2.3. The relationship between aperture area (µm^2) and shell height for veligers raised in a control environment (C), in the presence of predators (P), and in the presence of predators consuming conspecifics (PV). PV=P<C.

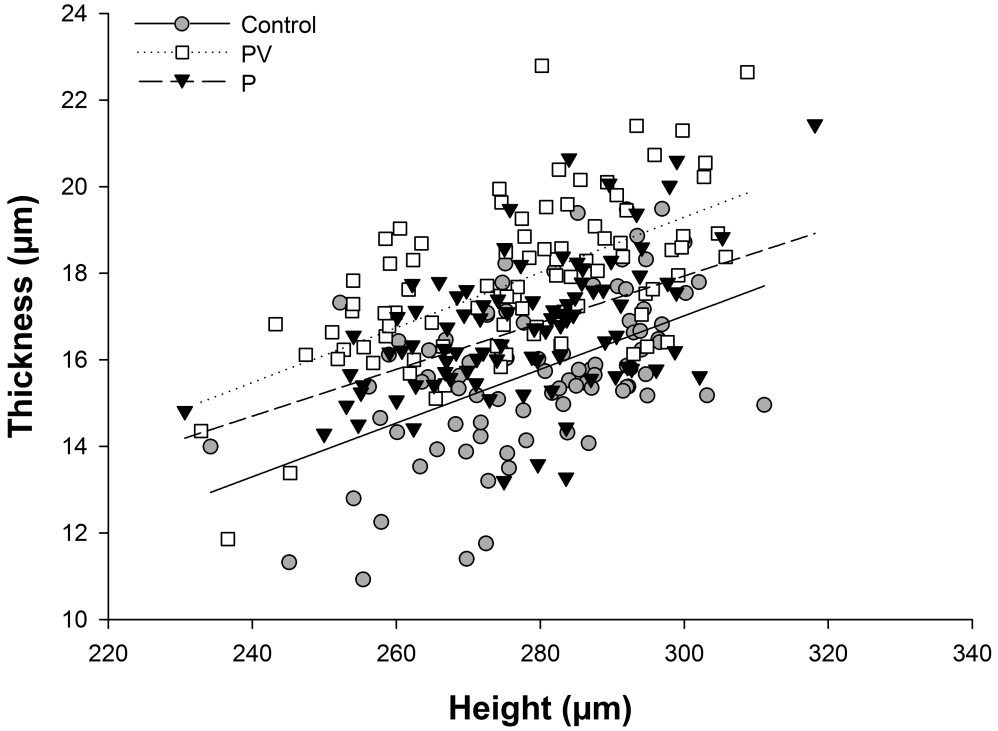


Figure 2.4. The relationship between marginal reinforcement (thickness; µm) and shell height for veligers raised in a control environment (C), in the presence of predators (P), and in the presence of predators consuming conspecifics (PV). C<P<PV.

Veligers from the PV treatment survived better than those of the control group. At 30, 120, and 210 minutes following the start of the experiment, the cumulative survival of PV veligers was 93%, 87%, and 74% while cumulative survival for control veligers was 83%, 70%, and 53%. The results of the Wilcoxon test used in the survival analysis show that experimental veligers from the PV treatment group survived significantly better than those from the control treatment (Table 2.1, Figure 2.5; W = 4.104, p = 0.043).

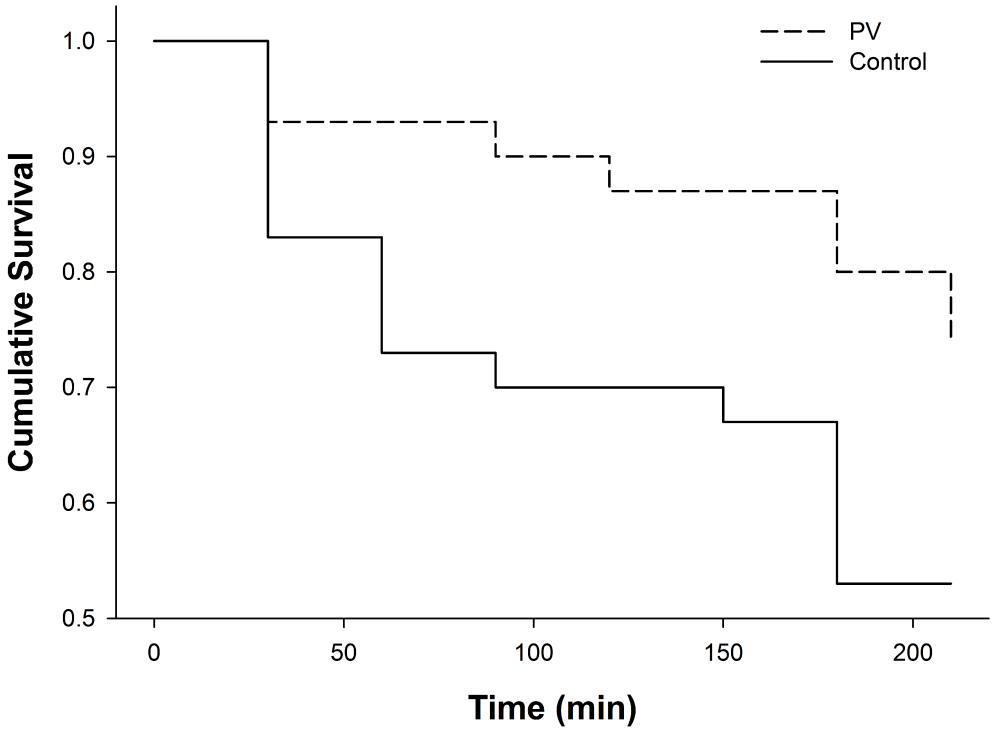


Figure 2.5. The survival of veligers raised in the presence of predators consuming conspecifics (PV) and veligers raised in a control environment (C) over time when directly exposed to zoea predators. C<PV.

DISCUSSION

Shell plasticity

Our results indicate that larval shells are altered in the presence of predators and these alterations confer increased survival against predation. This result is in agreement with findings by Vaughn (2007). Because we used a different species of crab zoea as the

predator than the one used by Vaughn, our results also indicate that more than one species of zoeal predator can induce phenotypic responses. We measured an additional morphological response, reinforcement of aperture margins, and found that there are differences in the responses dependent on the type of experimental treatment. Although the predator-only treatment induced changes in all three measured variables (shell aspect ratio, aperture area, and marginal reinforcement), the response to the treatment of predators consuming conspecifics consisted of alterations in two (shell aperture area and marginal reinforcement).

The experimental design in this study is most similar to the second experiment from Vaughn (2007) and it is to this study that results will be compared. Vaughn found that veligers developed smaller apertures and rounder shells in response to the presence of zoeae of *Cancer* spp. (Table 2.2). The results of the present study also show that aperture area is reduced in size and shells become more round in shape when veligers are raised in the presence of predators. When raised in the presence of predators consuming conspecifics (providing all three risk cues: predator kairomones, alarm cues, diet cues) the veligers also developed smaller apertures but shell shape was not significantly different from those in the control group. Differences between the two studies to keep in mind when applying juxtapositional interpretations include, first, the potentially intermediary intensity of cues used by Vaughn (2007) that likely contained both predator kairomones and diet cues but without alarm cues, and second, the predators used are from different brachyuran crab families and may have affected the veligers differently. Lastly, the inclusion of a reliable covariate was not clear in the analyses from Vaughn (2007) and thus there is the potential for misinterpretation due to differences in size. That being said, visuals and means ± SE from Vaughn (2007) suggest the veligers measured from each treatment had an

approximately equal range of sizes, and this range is similar to veligers measured in this study.

Veligers were able to develop smaller apertures without a change in shell shape, as seen in the response to the PV treatment. This lessens the likelihood of a correlation between shell aspect ratio and aperture area, as suggested by Vaughn (2007). These two features appear to be responses that can occur independently depending on the predatory cues present. It is possible, however, that a correlation exists between the reduction in aperture size and reinforcement of aperture margins since both P and PV treatments resulted in changes in both of these variables. Even if such a correlation exists, there still appears to be some autonomy of the response since veligers with similar aperture areas in the P and PV treatments had significantly different levels of marginal reinforcement.

Interestingly, the PV treatment did not elicit a response in SAR; the shell shape was not significantly different from the control group. Studies in adult gastropods have shown that when confronted with cues resulting from predators feeding on conspecifics or predator cues combined with crushed conspecifics, the measured response (whether it be escape, changes in shell shape, thickness, etc.) is more pronounced than with isolated cues, indicative of an additive effect that correlates to the intensity of predation risk (Atema and Stenzler, 1977; Appleton and Palmer, 1988; Alexander and Covich, 1991; Krist, 2002; Trussell and Nicklin, 2002; Dalesman *et al*., 2006; Bourdeau, 2010b). This was clearly the case for marginal reinforcement in the experimental veligers. It is possible that this larger degree of strengthening coupled with a reduction in aperture area becomes the preferential response over changes in shell shape when the information provided indicates a riskier environment where the predator in question is actually consuming conspecifics. Although roundness may increase predator mishandling, a reinforced margin and smaller entryway

are likely to be most effective when confronted with an aperture-entry predator such as a zoea.

The adult gastropod shell has long been considered a fundamental defense strategy against hungry predators with evidence coming from the fossil record (e.g., Vermeij *et al*., 1981) and studies on predator-prey interactions in extant species (e.g., Palmer, 1979; Bertness and Cunningham, 1981; Quensen and Woodruff, 1997; Rosin *et al*., 2013). In addition to serving as an innate protective refuge, damage from non-fatal encounters is repairable and certain features of the shell (e.g., thickness, aperture size, apertural teeth, sculptural reinforcement) can be altered to enhance its effectiveness. The putative purpose of the larval shell is also protective, although definitive evidence confirming this has been scant. Hickman (1999, 2001) has explored multiple mechanically-defensive features of larval gastropod shells, most of which are restricted to marginal structures such as reinforced apertural beaks, velar notches, and rapid repair of broken aperture margins. It was only around the aperture margins that evidence of reinforcement was observed in this study and is likely due to a combination of marginal thickening and curving of the aperture edges to add to the strength of the thickened margin without additional weight, although the extent of thickening throughout the shell is unknown. Hickman (1999) has proposed that reinforced marginal structures would have to be resorbed continually and re-secreted during growth to minimize the mass of a planktonic organism that relies on swimming and vertical adjustment for multiple aspects of larval ontogeny (e.g., Chia *et al*., 1984; Kingsford *et al*., 2002; Fuchs *et al*., 2004; Fiksen *et al*., 2007), although added weight could be potentially beneficial as an accelerated escape response upon retraction into the larval shell.

Cue considerations

This study focused on morphological responses of veligers to different predator treatments and did not investigate the nature of the cues but, consistent with the literature, has referred to them variously as kairomones, alarm cues, and diet cues (Ferrari *et al*., 2010). We do not know the chemical composition or concentration of the compounds or how they act to cause the changes we have demonstrated among the experimental treatments; the identification of cues underlying predator-prey interactions in general remains largely unexplored (Pohnert *et al*., 2007; Raguso *et al*., 2015).

The comparability of our results and those of experiment 2 of Vaughn (2007) to the larval response *in situ* is unknown. In marine systems the measure of importance of predation on planktonic larvae remains unclear and predation as a significant contributor to high larval mortality has been debated (e.g., Young and Chia, 1987; Rumrill, 1990; Morgan, 1995; Johnson and Shanks, 1997, 2003; Allen and McAlister, 2007; Pechenik and Levine, 2007; Vaughn and Allen, 2010). Natural encounters of predator and prey are difficult to estimate due to plankton patchiness resulting from physical and/or behavioral drivers (e.g., Omori and Hamner, 1982, Folt and Burns, 1999; Metaxas, 2001; Shanks *et al*., 2003). Concentrations of predators, meroplanktonic prey, and signals indicating predators or predation are unknown. The presence of background plankton has been shown to dramatically reduce predation (Johnson and Shanks, 1997, 2003; Johnson and Brink, 1998), therefore elevated predator concentrations may not accurately predict imminent risk.

In this study veliger larvae experienced predators or predators consuming conspecifics for the four week duration of the experiment. Both frequency and concentration are known to impact the intensity of predatory cues and prey response. Experiments concerning how predator density affects inducible morphologies have primarily been in closed freshwater systems or experimental units where physical

parameters affecting predator-prey interactions differ greatly from the marine environment. Despite the detectable effect of zoea predator cues on gastropod veligers, it is possible that the induced defenses were exaggerated under unnatural predator densities and exposure. Many inducible defenses have been shown to be positively correlated with predator density, size, or cue concentration (Barry and Bayly, 1985; Wiackowski and Starońska, 1999; Van Buskirk and Arioli, 2002; Kusch *et al*., 2004; Hölker and Stief, 2005; Ferrari *et al*., 2006, 2010) although some have been shown to reach a point where continued increases in stimulus concentration result in a lessened or lack of further morphological change (e.g., Palmer, 1990; Duquette *et al*., 2005) or the production of exaggerated phenotypic responses (Trussell, 1996). Most studies examining effects of predators on prey also provide constant exposure to predatory cues that can result in a more pronounced response than if the cues were temporally varied (e.g., Trussell, 1996; Sih and McCarthy, 2002; Chivers *et al*., 2008). Vaughn's (2007) study also included a separate experiment (exp. 1) with a treatment where larvae experienced transient exposure to predators (exposure to zoeae for four to six hours on one day each week for four weeks). This treatment was not fully replicated but showed veligers had smaller apertures and rounder shells than control larvae. This result was quite similar to her 2nd experiment and the present study where larvae had prolonged exposure to predators. The consistency of the results in Vaughn's two experiments and our own suggest that prolonged exposure to predators did not yield erroneous results.

There are many examples of the ability of prey to distinguish between predators and adjust their responses accordingly (e.g., Atema and Stenzler, 1977; Stibor and Lüning, 1994; Wicklow, 1997; Relyea, 2001, 2003); this is commonly observed in behavioral defenses (e.g., Turner *et al*., 1999, 2000; DeWitt *et al*., 2000) but induced-morphologies can also be specific to different predators, sometimes in opposite directions (e.g., Smith and

Jennings, 2000; Relyea, 2001, 2003; Hoverman *et al*., 2005; Long *et al*., 2007; Lakowitz *et al*., 2008; Bourdeau, 2009; Hoverman *et al*., 2014). When exposed to a combination of predators, most prey exhibit the response that reflects the most risky predator with the possibility of exaggerated (when the trait is reinforced by different predators in the same direction) and intermediate phenotypes (Relyea, 2003). The types of predatory threats to planktonic veligers relative to adult snails remain unclear. The aperture-entry method of some zooplankton predators is the only implicated, potentially survivable threat that could select for the observed defenses (Hickman, 2001; Vaughn, 2007, this study).

With only two studies having examined inducible morphological defenses in veliger larvae, it is unknown whether veligers can perceive differences between predator and non-predator zoeae (strictly herbivorous zoeae) or non-native zoeae. Adult snails have been shown to be able to distinguish between the effluents of predatory and non-predatory crabs (e.g., Marko and Palmer, 1991), between native and introduced predators (Edgell and Neufeld, 2008), and between effluents of damaged conspecifics vs. heterospecific or allopatric species in their responses (e.g., Jacobsen and Stabell, 2004; Dalesman *et al*., 2007; Bourdeau, 2010b).

Costs and trade-offs

Although the fitness benefit of the alterations found in the veligers are clear in the enhanced survival in the presence of predators, the potential costs are unknown as has been common in the study of inducible defenses (Tollrian and Harvell, 1999). In adult gastropods, a common observable and assumed cost of thickening or change in shell shape is reduced body mass, a feature clearly not feasibly measured in larval veligers, and reduced linear growth (e.g., Appleton and Palmer, 1988; Palmer, 1990; DeWitt, 1998; Trussell, 2000; Trussell and Nicklin, 2002; Brookes and Rochette, 2007). The shell height of experimental veligers was not significantly different and no reduction in shell length was observed in

veligers from the PV treatment exhibiting both smaller apertures and the greatest degree of marginal reinforcement. Other potential costs depend on the extent of reinforcement, its added weight, and the method by which the reinforcement is implemented.

Future considerations

Larval defenses in marine organisms continue to be an underexplored topic both in the discovery of novel examples and in further understanding of identified cases and their impacts in planktonic assemblages. Generalities concerning predator-prey interactions in adult organisms and in freshwater systems have generated a strong foundation with integrative potential in a marine planktonic environment. As we continue to better understand population dynamics and post-metamorphic plasticities in marine organisms, the capacity of these larvae to respond to their own set of environmental challenges should be an equally important consideration.

Bridge to Chapter III

The present study examined predator-induced morphological defenses in veligers of *L. scutulata*. However, these larvae deal with mortality risk due to starvation in addition to predation. Feeding by planktotrophic larvae such as veligers of *L. scutulata* is vital for their survival, development, and ultimate settlement. In addition to plasticity in shell morphology, structures used by veligers in order to feed and swim are also subject to environmentally-induced change.

CHAPTER III

MORPHOLOGICAL PLASTICITY IN RESPONSE TO VARIABLE FOOD CONCENTRATIONS IN VELIGERS OF *LITTORINA SCUTULATA* WITH ANALYSES OF SWIMMING SPEED AND VELAR GROWTH

INTRODUCTION

Two fundamental contributors to the fitness of an organism include its ability to evade predation and procure enough resources to grow and reproduce. Environmental variation in these and other aspects of life provides the evolutionary drive behind phenotypic plasticity, which is the ability of an organism to alter its behavior, morphology, physiology, or life history in response to changes in its surroundings (e.g., Schlichting and Pigliucci, 1998; West-Eberhard, 2003; Pigliucci *et al*., 2006). Inducible traits are the phenotypic changes that occur in response to particular environmental cues, such as those associated with predators, in which case the phenotype is called an inducible defense (Tollrian and Harvell, 1999). From a predator or grazer's point of view, plasticities that confer a greater acquisition of resources are referred to as inducible offenses (Padilla, 2001). In comparison to inducible defenses, inducible offenses are considered less common (Mougi *et al*., 2011); however, in the context of marine larvae, examples of morphological traits representing inducible offenses (see below) outnumber those of inducible defenses (Vaughn, 2007; Vaughn and Strathmann, 2008; see Chapter II).

These examples of inducible offenses have all been found in herbivorous planktotrophic larvae, meaning that the larvae need to feed on phytoplankton during their planktonic duration in order to develop to the point at which they are competent to metamorphose and settle as juveniles (e.g., Thorson, 1950; Strathmann, 1985). The highly variable spatial and temporal distribution of marine phytoplankton is well known (see

McManus and Woodson, 2012, Prairie *et al*., 2012 for recent reviews). Concentrated patches or layers resulting from oceanographic features such as pycnoclines, fronts, or internal waves can create sometimes extreme differences in phytoplankton concentrations between adjacent water masses that can persist for minutes to days (e.g., Shanks, 1983; Mackas *et al*., 1985; Davis *et al*., 1991; Franks, 1992; Cowles *et al*., 1993; Villafañe *et al*., 1995; Lennert-Cody and Franks, 1999; Seuront *et al*., 2001; Sosik *et al*., 2003; Menden-Deuer, 2008; Menden-Deuer and Fredrickson, 2010). This unpredictable but often strong variation in resource availability sets the framework for the usefulness of behavioral and morphological plasticities involved in the ability of marine larvae to improve feeding during their pelagic period.

Veliger larvae, like those of the indirectly-developing marine snail *Littorina scutulata*, are named after the characteristic ciliated structure, called a velum, which is used for both swimming and feeding (Strathmann, 1987b). Long compound cilia of the pre-oral prototrochal ciliary band line the edge of the velum and beat posteriorly, providing the current used to swim and feed. Food particles are trapped between the prototrochal cilia and the shorter cilia of a second post-oral ciliary band called the metatroch, which beat anteriorly in the opposite direction. The trapped food particles are then moved to the mouth along a ciliated food groove that lies between the two ciliary bands (Fretter, 1967; Strathmann, 1987b; Romero *et al*., 2010). The effectiveness of this 'opposed-band' feeding system (Strathmann *et al*., 1972; Riisgård *et al*., 2000; Pernet and Strathmann, 2011), and in the use of ciliary bands in general, depends on a number of parameters including the length of the ciliary bands that, in the case of veligers, means the size of the velum (e.g., Strathmann *et al*., 1972; Gallager, 1988; Hansen and Ockelmann, 1991; Strathmann and Grünbaum, 2006). All other parameters being the same, a longer ciliated band will allow for

a greater clearance rate, meaning that the veliger will be able to clear a greater volume of water of food particles over a certain period of time.

To lengthen the ciliated band, existing cells must increase in size and/or there must be cell proliferation; the underlying dilemma for lengthening via proliferation lies in the defining feature of the band – the presence of cilia. Cilia are thought to complicate a cell's ability to undergo mitosis due to the fact that the basal body anchoring a cilium is functionally interchangeable as a centriole, a vital component in the organization of the mitotic spindle during cell division (e.g., Plotnikova *et al*., 2008; Kobayashi and Dynlacht, 2011). Although uniciliated cells can resorb the cilium (thus freeing the basal body to return to its role as a centriole during cell division) and subsequently regrow it following mitosis (Rieder *et al*., 1979; Masuda and Sato, 1984), it is generally accepted that multiciliated cells of animals are terminally differentiated and cannot and do not divide since they possess multiple basal bodies (Dawe *et al*., 2007).

The prototroch of veligers is directly derived from the prototroch of the trochophore stage of development and the prototroch of the trochophore develops from specialized ciliated founder cells called 'trochoblasts' (Henry *et al*., 2007). Trochoblasts, like other ciliated cells, are generally believed not to divide yet there remains no explanation for the obvious increase in prototrochal nuclei in the often elaborate vela of many planktotrophic veligers (Nielson, 2004). Bird (2012) reported seeing a proliferative region in the prototroch located in the ventral velar notch just dorsal to the mouth in gastropod veligers of *Nassarius fossatus*; it was not clear that these cells were definitively prototrochal nuclei but progeny of this proliferative region may be intercalated into the prototrochal band. It is likely that the same mechanism affording velar growth in general is also utilized in the realization of plastically-induced growth.

A study by Strathmann *et al*. (1993) demonstrated that oyster larvae developed larger vela and longer cilia relative to shell size when raised in a low food environment. Similar studies have demonstrated increased growth of food-capturing larval structures in the veligers of gastropods (Estrella Klinzing and Pechenik, 2000; Phillips, 2011) and various echinoderm larvae when exposed to low food concentrations (Boidron-Mètairon, 1988; Strathmann *et al*., 1992; Fenaux *et al*., 1994; George, 1994, 1999; Hart and Strathmann, 1994; Miner, 2005, 2007; Podolsky and McAlister, 2005; Byrne *et al*., 2008; Soars *et al*., 2009; Adams *et al*., 2011; Sun and Li, 2013; Wolf *et al*., 2015). Having a small feeding structure in a low food environment impedes adequate levels of food capture, slowing larval growth. Alternatively, in situations where food is abundant, excess growth of the feeding structure provides more food than can be used and is therefore unnecessary.

The study by Strathmann *et al*. (1993) provided the taxonomic leap between echinoderms and mollusks in the successful search for a widespread existence of inducible offenses in marine larvae and this has since been extended to two gastropod mollusks (Calyptraidae: Estrella Klinzing and Pechenik, 2000; Vermetidae: Phillips, 2011). The purpose of this study was to provide an additional example of an inducible offense in a marine larva of a different gastropod family (Littorinidae) by testing the hypothesis that veligers of *L. scutulata* will develop larger vela relative to shell length when raised in low vs. high food concentrations. Veligers raised in low food are expected to develop at a slower rate since the inferred and demonstrated increases in clearance rate with inducibly lengthened ciliary bands have not been found to be able to fully compensate for the reduced food supply (Hart and Strathmann, 1994; Estrella Klinzing and Pechenik, 2000). I also attempted to explore a potential mechanism by which the expected difference in velar size is achieved through immunofluorescent labeling of dividing velar nuclei with the expectation that a higher number of dividing cells would be found in veligers raised in a low

vs. high food environment. Lastly, since veligers rely on the same structure to both feed and swim, I used vertical swimming speeds to examine how a larger velum might affect swimming performance.

MATERIALS AND METHODS

Adult and veliger collection

Adult *L. scutulata* were collected from the jetty at OIMB beach (Charleston, OR; 43°20'58.85"N, 124°19'49.50"W) and were placed overnight in mesh-bottomed tricorner plastic beakers suspended in FSW (filtered seawater). The following morning, egg capsules were filtered out of this water and distributed into large glass jars filled with 3L FSW. The ~14°C water was gently stirred by paddles suspended from a motorized stir rack (Strathmann, 1987a) and was replaced every three days over the ~eight days of capsule maturation. Newly-hatched veligers are approximately 195 μm in shell length (n= 15).

Study set-up

Upon hatching, 300 veligers were placed into each of six beakers (three replicate beakers per treatment) filled with 750 mL of FSW for a concentration of 0.4 larvae/mL. At set-up and immediately following each subsequent water change (every third day), the veligers were fed equal numbers of cells of *Isochrysis galbana*, *Chaetoceros gracilis*, and *Dunaliella tertiolecta* at a combined concentration of 1000 cells/mL for the low food treatment and 20,000 cells/mL for the high food treatment. Food and veliger concentrations were chosen based on culturing guidelines outlined in Strathmann (1987a); however, it should be noted that although the treatments are delimited as 'high' and 'low', both food concentrations and the veliger concentration exceed levels normally observed in the field (e.g., Allen, 1941; Bainbridge, 1956; Shanks and McCulloch, 2003a, 2003b; Shanks *et al.*, 2014). Following set-up and initial feeding, the veligers were allowed to acclimate to their respective environments for one day before any measurements were taken. The water level

at each water change and feeding was adjusted to reflect the number of veligers removed at that point (see below) so as to maintain the initial food concentration and veliger:food ratio established at the onset of the study. Veliger mortality was not followed. To account for differences in overall growth rates, as indicated by shell length, the low food treatment was sampled every fourth day for a total of seven times (28 days) and the high food treatment was sampled every other day for a total of seven times (15 days); this allowed me to obtain a comparable range of cumulative shell lengths for both treatments.

Velar and cilia measurements

At each time point for each treatment, four arbitrarily chosen veligers from each replicate beaker were filmed swimming upwards against a coverslip on a compound microscope. The veligers were placed within a square of window screening (~1 x 1 mm) to restrict their horizontal movement. Velar dimensions, cilia length, and shell length were later measured from video stills using ImageJ software (NIH, Bethesda, MD). The total velar circumference was approximated using the formula for calculating the circumference of an ellipse for each velar lobe $[2\pi *(((L/2)^2 + (W/2)^2)/2)^{1/2}]$, where L and W represent the lobe length and width (Figure 3.1). Regressions generated from the respective relationships of velar circumference and shell length for veligers raised at high and low food concentrations were used to obtain predicted velar circumferences for a given shell length from the swimming speed and cell proliferation data. Cilia length was measured as the average distance from the edge of the velum to the tip of a prototrochal cilium; two to four of the longest visible prototrochal cilia were measured for each veliger (Figure 3.1). Not all videos were adequate for measurement, therefore sample sizes per beaker and cumulative totals differ slightly.

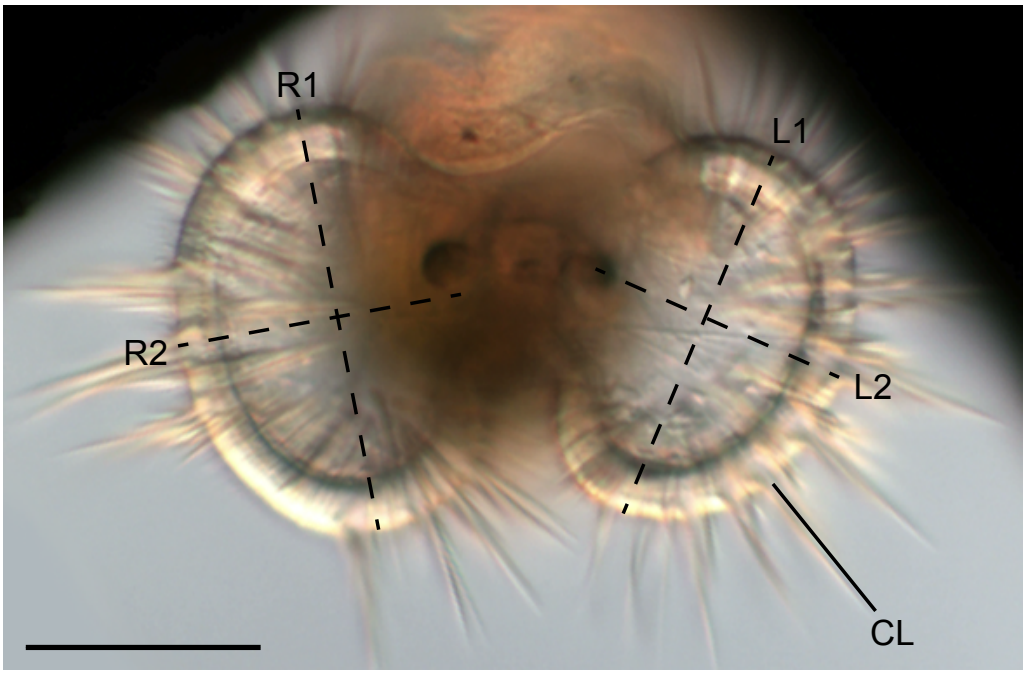


Figure 3.1. Velar and cilia measurements. The length and width of the right (R1, R2) and left (L1, L2) velar lobes were used to estimate velar circumference. Cilia length (CL) was measured from the edge of the velar lobe to the tip of the cilium. Scale bar = 100 μm.

BrdU incubation

BrdU (5-Bromo-2′-deoxyuridine) is a synthetic nucleoside that is incorporated into replicating DNA and can thus be used to identify proliferating cells (e.g., Moore *et al*., 1988). At each time point for each treatment, the 12 veligers used for velar measurements (see above) and 12 additional arbitrarily-chosen veligers (four from each replicate beaker) were incubated overnight in 50 μM BrdU (Sigma, St. Louis, MO; B5002) in FSW for 12 hours. The additional veligers were used to maximize the numbers of veligers for which BrdU-labeled cell counts could be obtained; not all veligers can be reliably relaxed/fixed with the velum outstretched and, even with high usable numbers following fixation, the harshness of the BrdU assay and unpredictability of mounting reduced the sample size. At the end of the incubation, veligers were relaxed in three 15-minute changes of 0.37 M $MgCl_2$ and fixed for 30 minutes in 4% paraformaldehyde in FSW. Following fixation, larvae were rinsed (one five-minute and three 10-minute rinses) in 1X TBS (Tris-buffered saline). Veligers were

stored in 1X TBS until processed. Following storage, it became apparent that the shells had dissolved, leaving behind what is presumed to be the organic matrix (the framework of the shell in which the calcareous concretions are embedded; e.g., Fretter and Pilkington, 1971; Eyster, 1986; Collin and Voltzow, 1998; Weiss and Schönitzer, 2006). Shell lengths were measured on a microscope with a calibrated ocular micrometer, although these were ~20 μm underestimates of actual shell size based on comparisons with shell lengths of veligers at equivalent time points measured prior to the BrdU exposure or from the swimming assay.

BrdU assay and antibody labeling

Following size approximation, the veligers were kept separate from this point on. First, veligers were incubated in 2 N HCl for 15 minutes to denature the DNA and allow the antibody access to the incorporated BrdU. The acid was then neutralized in several rinses (five for five minutes each) of 0.1 M $Na_2B_4O_7*10H_2O$. Next, veligers were permeabilized in three 10-minute rinses of TBT (1X TBS + 0.1% Triton X-100) followed by three 10-minutes rinses in 1X TBS. Veligers were then incubated in blocking serum (5% normal goat serum in TBT with 0.1% bovine serum albumin) for two hours at room temperature to block non-specific labeling and subsequently left overnight at 4°C in the primary antibody (mouse monoclonal anti-BrdU; Becton Dickinson, Franklin Lakes, NJ) diluted 1:1000 in blocking serum. The following day, the veligers were rinsed three times for 10 minutes each in TBT/BSA (0.1% bovine serum albumin in TBT) and incubated for two hours at room temperature in the secondary antibody (goat anti-mouse Alexa Fluor 488; Invitrogen) diluted 1:500 in TBT. All nuclei were simultaneously labeled with 2 μM Hoechst 33342 to be able to visualize the overall morphology. After being rinsed three times for 10 minutes each in 1X TBS, larvae were mounted in 75% glycerol in TBS velum-down on cover slips coated with poly-L-lysine to promote adherence. Preps were sealed with clear nail polish and

imaged on an Olympus IX81 inverted microscope with an Olympus FluoView 1000 laser scanning confocal system (Olympus America, Center Valley, PA). Confocal image stacks were processed using ImageJ software. In order to obtain a crude quantitative indication of proliferation levels in the vela of veligers raised in a high vs. low food environment, the number of labeled cells was counted in the region of the velum just above the mouth located along the bottom edge of the ventral velar notch (Figure 3.2); this was the only consistently countable region (primarily because of differences in or deformations due to mounting) and is the same region in which a previous study indicated velar proliferation in veligers of a different gastropod species (Bird, 2012).

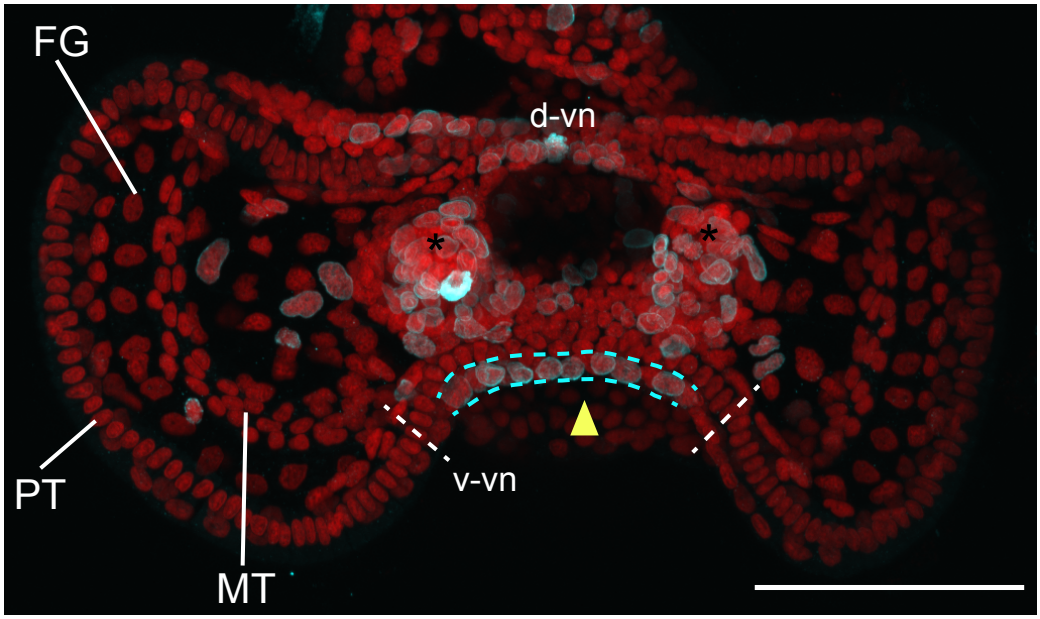


Figure 3.2. Confocal projection of BrdU-labeled nuclei (cyan) in a 3-day old veliger of *Littorina scutulata*. All cell nuclei are labeled with Hoechst (red). Following a 12 hr incubation period, BrdU-positive nuclei were found in the pre- and post-trochal epidermis, the cephalic tentacles (*), apical ganglia, food groove (FG), and metatroch (MT) with the highest concentrations of dividing velar nuclei located in the dorsal (d-vn) and ventral velar notches (v-vn, white lines). Several BrdU-labeled nuclei can be seen in close association with the prototroch (PT) within the enumerated region (cyan lines) located along the bottom of the ventral velar notch just above the mouth (yellow arrow head). Scale bar = 50 μm.

Swimming speed measurements

At each time point for both treatments, videos were taken from a horizontal view of upward-swimming veligers so a swimming speed could later be determined. To do this, five arbitrarily-chosen veligers at a time were gently introduced to the top of a container (5 cm wide X 5.5 cm tall X 2 cm deep) marked with horizontal lines 5 mm apart. The container sat in a temperature-controlled water-bath filled to the same level and set to 14°C. After sinking to the bottom, some veligers usually began swimming again after one or two minutes and the upward movement was filmed using a PointGrey camera and a Nikon AF Nikkor 35-70 mm f/3.3-4.5 lens mounted on a tripod at a set height and distance. The focal plane was in the center of the container between the front and back walls where wall effects would be minimized. Shell lengths of snails that swam were measured afterwards on a microscope with a calibrated ocular micrometer. Veligers that began to swim in less than 30 seconds were discounted to minimize any disturbance remaining from their introduction. Each set of five veligers was allotted 15 minutes to swim upwards before these were removed and the next set of five was added. This was repeated until recordings of upward swimming were obtained for ~four veligers per replicate beaker. All veligers were returned to their experimental beakers within 20 minutes of removal, regardless of whether or not they had cooperated. For each video, 5 mm of the most vertical portion of the swimming path was identified using ImageJ (start and stop points were overlaid and only paths where the veliger remained within two shell lengths of the vertical path were used so as not to be confounded by excessive helical coiling or angled swimming). The time it took the veliger to travel the 5 mm was measured with a stopwatch; if multiple acceptable paths were available, the fastest speed was the one recorded. Not all videos yielded a measurable path, therefore sample sizes per beaker and cumulative totals differed. The veligers generally swam upwards in the center of the container at a distance of 1 cm from both the front and

back walls. The 5 mm increments were marked on the front of the container, placing them between the camera and veliger; therefore, the swimming distance traveled by the veliger was actually 5.217 mm as opposed to 5 mm and swimming speeds were underestimated. For example, a veliger whose swimming speed was calculated to be 2.00 mm/s was actually swimming 2.09 mm/s.

Statistical methods

Linear regression models in R were used to compare the slopes and x-shifted intercepts of velar size relative to shell length, cilia length relative to shell length, cilia length relative to velar size, swimming speed relative to shell length, swimming speed relative to predicted velar size, cell proliferation relative to shell length, cell proliferation relative to predicted velar size, and cell proliferation relative to age for veligers raised in low vs. high food environments (Table 3.1). All y-intercepts were shifted to the maximum or minimum overlapping value of x (depending on at which value of x it made most sense to compare relative values of y between the two treatments) (Table 3.2). For comparisons of data other than at the y-intercept, all interpretations were made based on relative positions of the 95% confidence intervals of treatment regression lines. Normality of model residuals was determined by the inspection of Normal Q-Q plots and Shapiro-Wilk's tests. Normality was only approximate for velar circumference with respect to shell length ($p = 0.011$) and for cilia length with respect to shell length ($p = 0.056$) but these levels are acceptable considering the small p-values and should have little to no effect on the coefficient estimates and regression fit. The residuals of all other models were normally-distributed ($p \geq 0.2188$). All variances were homogeneous as determined by inspection of residual plots. There were no significant effects of beaker ($p \geq 0.097$) with Bayes factors (used in Bayesian model comparison and selection) ≥ 23.846, meaning that the models excluding beaker as a factor are at least 24 times more likely to explain the distribution of the data; therefore individuals

Table 3.1. Linear regression models performed, goodness of fit , and indication of treatment effects.

Model	Adjusted R^2	F statistic	df	*p*	Bayes factor
Velar circumference vs. shell length	0.93	675.2	3, 142	< 0.0005	$1.09e^{21}$
Cilia length vs. shell length	0.81	200.6	3, 142	< 0.0005	$1.49e^{6}$
Cilia length vs. velar circumference	0.77	161.5	3, 142	< 0.0005	$1.5e^{-1}$
Swimming speed vs. shell length	0.37	26.11	3, 123	< 0.0005	$6.54e^{2}$
Swimming speed vs. predicted velar circumference	0.39	26.11	3, 123	< 0.0005	$2.48e^{6}$
Proliferation vs. shell length	0.31	17.04	3, 102	< 0.0005	$3.05e^{2}$
Proliferation vs. predicted velar circumference	0.31	17.04	3, 102	< 0.0005	$3.60e^{2}$
Proliferation vs. age	0.31	16.47	3, 102	< 0.0005	$6.0e^{1}$

[1]Comparing models with and without treatment

Table 3.2. Comparisons between high and low food treatments for the slope and intercept of linear regressions, respective R^2 values, and sample sizes.

Model	High Food	Low food	t-value	*p*
Velar circumference vs. shell length				
• slope (± 95% CI)	4.55(± 0.31)x	6.19(± 0.48)x	6.671	< 0.0005
• intercept (± 95% CI) at X=335 µm	1124.46(± 25.03)	1349.60(± 37.78)	11.681	< 0.0005
• R^2	0.93	0.93		
• N	74	72		
Cilia length vs. shell length				
• slope (± 95% CI)	0.12(± 0.02)x	0.19(± 0.03)x	5.449	< 0.0005
• intercept (± 95% CI) at X=335 µm	71.15(± 1.32)	77.93(± 1.99)	6.671	< 0.0005
• R^2	0.77	0.82		
• N	74	72		
Cilia length vs. velar circumference				
• slope (± 95% CI)	0.025(± 0.00)x	0.029(± 0.01)x	1.657	0.0997
• intercept (± 95% CI) at X=1373 µm	76.91(± 2.23)	77.92(± 2.77)	0.718	0.4738
• R^2	0.73	0.79		
• N	74	72		

Table 3.2 continued…

Swimming speed vs. shell length				
• slope (± 95% CI)	0.0089(± 0.00)x	0.0038(± 0.00)x	-2.889	0.0046
• intercept (± 95% CI) at X=344 µm	2.64(± 0.19)	2.00(± 0.27)	-4.573	< 0.0005
• R^2	0.43	0.16		
• N	68	59		
Swimming speed vs. predicted velar circumference				
• slope (± 95% CI)	0.0020(± 0.00)x	0.00062(± 0.00)x	-3.995	< 0.0005
• intercept (± 95% CI) at X=1211 µm	2.73(± 0.21)	1.88(± 0.25)	-6.602	< 0.0005
• R^2	0.43	0.16		
• N	68	59		
Proliferation vs. shell length				
• slope (± 95% CI)	-0.054(± 0.02)x	-0.019(± 0.02)x	2.861	0.0051
• intercept (± 95% CI) at X=186 µm	9.04(± 1.29)	5.17(± 1.70)	-4.467	< 0.0005
• R^2	0.44	0.092		
• N	50	56		
Proliferation vs. predicted velar circumference				
• slope (± 95% CI)	-0.012(± 0.00)x	-0.0031(± 0.00)x	3.631	< 0.0005
• intercept (± 95% CI) at X=448 µm	9.04(± 1.29)	5.12(± 1.68)	-4.599	< 0.0005
• R^2	0.43	0.092		
• N	50	56		
Proliferation vs. age				
• slope (± 95% CI)	-0.54(± 0.19)x	-0.12(± 0.21)x	3.972	< 0.0005
• intercept (± 95% CI) at X=5 days	7.72(± 0.98)	5.27(± 1.50)	-3.194	0.0019
• R^2	0.41	0.10		
• N	50	56		

from replicate beakers were pooled within treatment for analysis (Quinn and Keough, 2002; Jarosz and Wiley, 2014). It was not feasible to keep veligers separated by beaker for the cell proliferation assay. To better visualize trends in the data and to assess the quality of a linear fit, locally weighted regression (LOESS) smoothing plots were also fitted for all data (Cleveland and Devlin, 1988; Cleveland *et al.*, 1992).

RESULTS

Veligers raised in a high food environment grew faster in shell length (Figure 3.3) and velar dimensions (Figure 3.4), reaching the pediveliger stage in ~two weeks as opposed to ~four weeks for veligers raised in a low food environment. In a high food concentration, veligers grew at rates of 11 µm and 51 µm per day in shell length and velar circumference, respectively. In a low food concentration, veligers grew at rates of 5 µm and 30 µm per day in shell length and velar circumference, respectively.

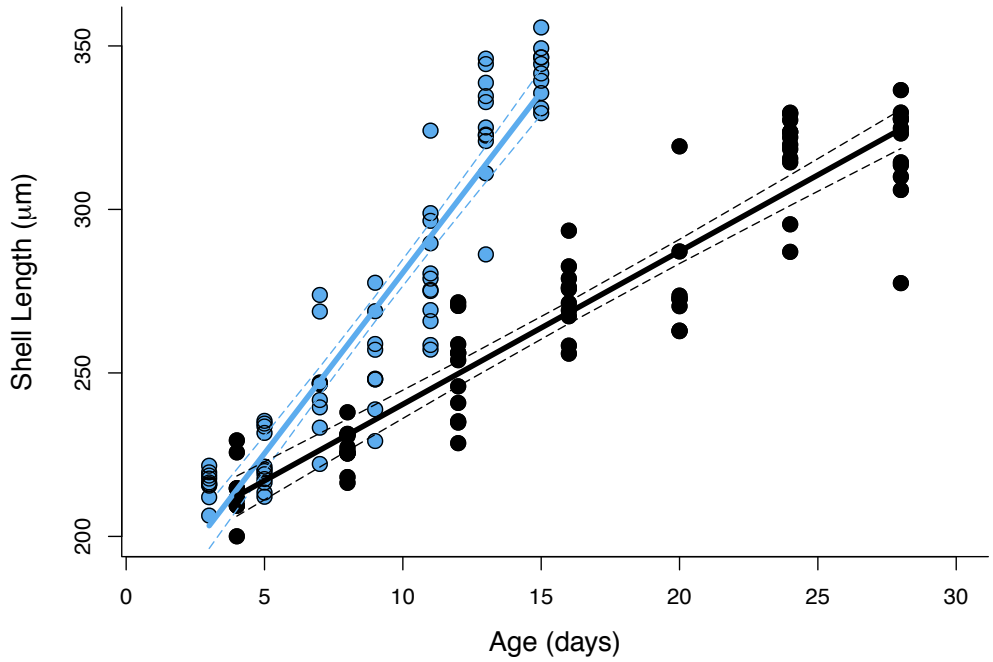


Figure 3.3. The relationship between shell size and age for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

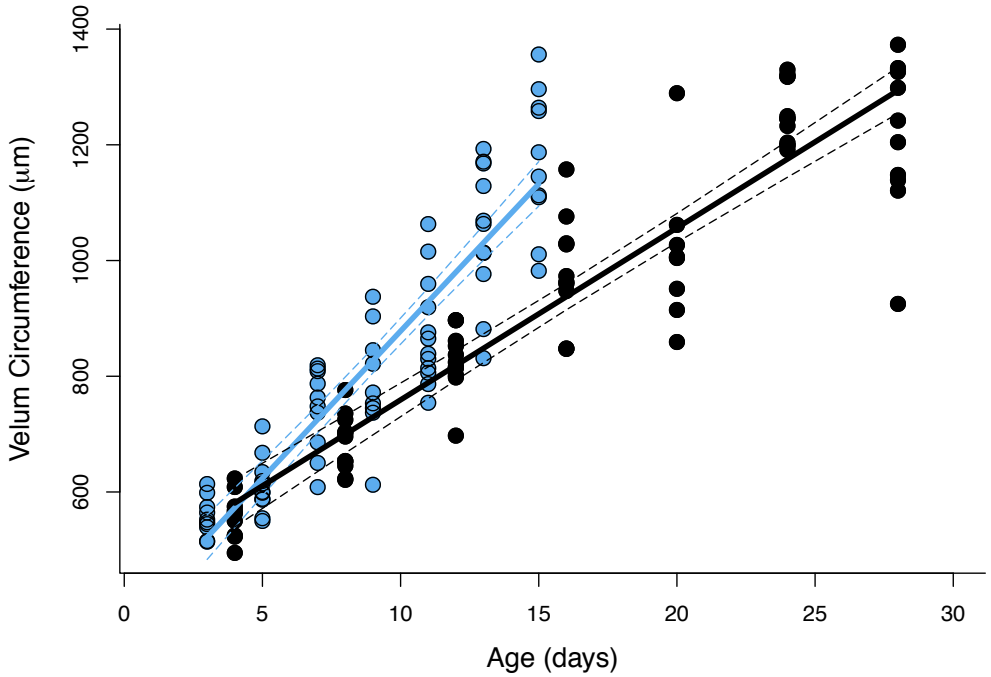


Figure 3.4. The relationship between velar size and age for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

Velar and cilia measurements

A linear regression of the relationship between velar circumference and shell length with treatment resulted in a statistically significant fit that explained 93% of variation ($F_{3, 142} = 675.2$, $R^2 = 0.93$, $p < 0.0005$; Table 3.1); a Bayes factor of $1.09e^{21}$ strongly favors a model inclusive of treatment effects (shell length*treatment). Although both treatment groups increased in velar circumference relative to shell length (Figures 3.5, 3.6), the increase was greater for veligers raised in a low food environment ($t_{142} = 6.671$, $p < 0.0005$; Table 3.2): for every micron increase in shell length, velar circumference increased by 4.55 μm and 6.19 μm in veligers from the high and low food treatments, respectively. In other words, veligers raised in a low food environment grew ~36% more in velar circumference for every micron increase in shell length. Velar circumference started off similar but became and remained significantly different between the two treatment groups at a shell length of ~230 μm. When shells were 230 μm in length, the average velar circumference of veligers

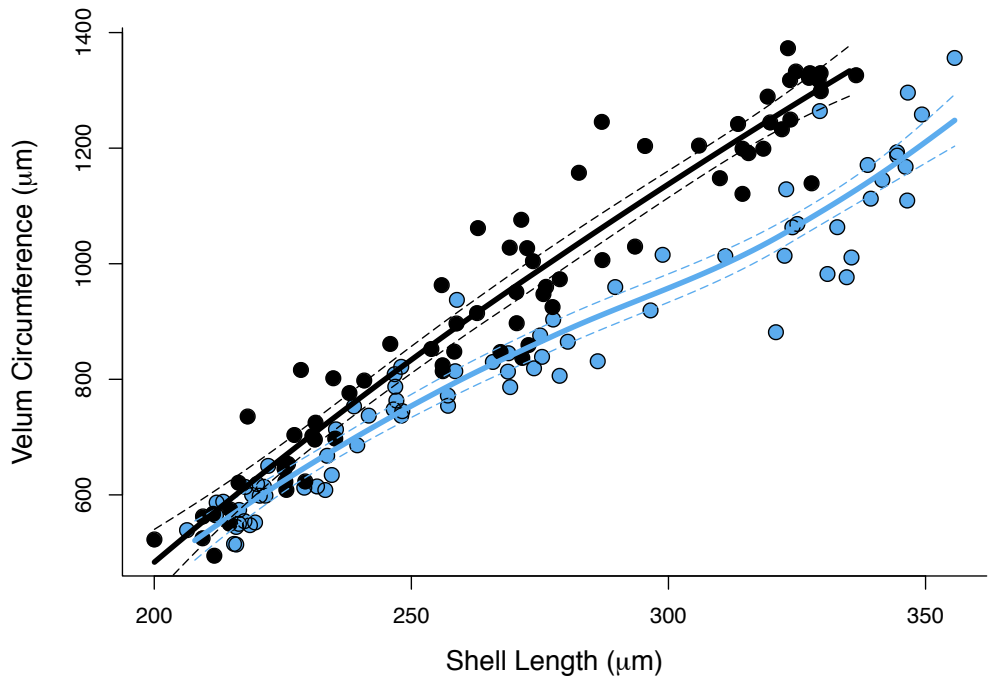


Figure 3.5. Loess smoothed regressions of the relationship between velar circumference and shell length for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

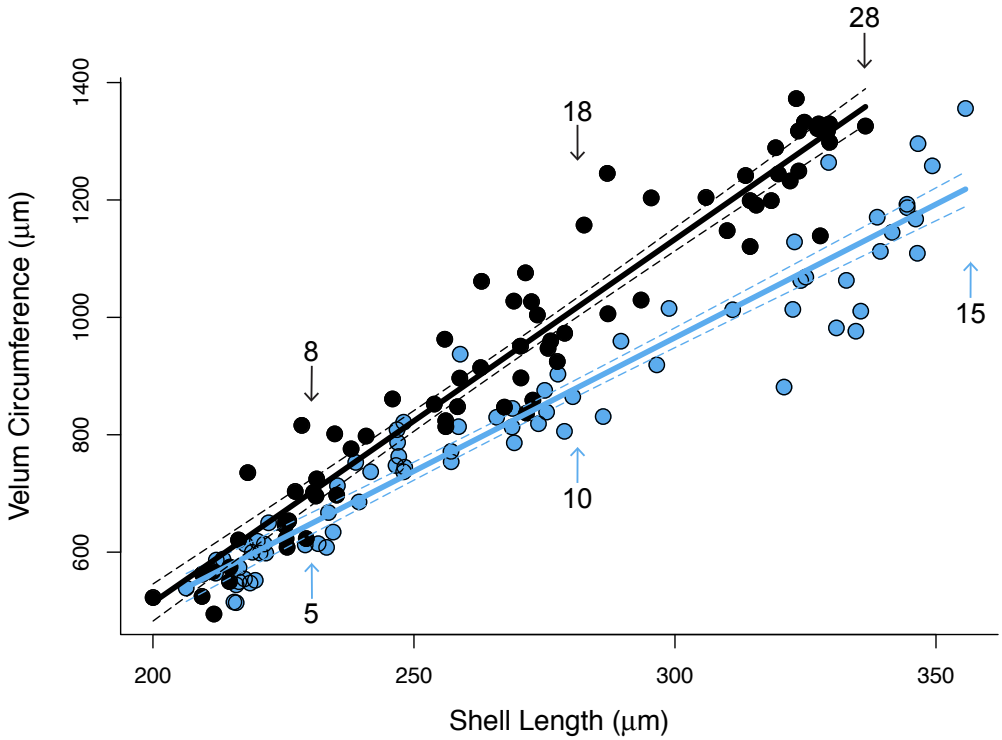


Figure 3.6. Linear regressions of the relationship between velar circumference and shell length for veligers raised in a high (blue) and low (black) food environment. The approximate age of each treatment group is shown for a shell size of 230 µm, 280 µm, and at the largest respective shell size attained. Error bars are 95% CI.

from high and low food treatments was ~645 μm and ~700 μm, respectively. At the maximum overlapping shell length of 335 μm, the average velar circumference for veligers from the high food treatment (1124 μm) was significantly less than for food-limited veligers (1350 μm; $t_{142} = 11.681$, $p < 0.0005$; Table 3.2). At a maximum attainable shell length of ~350 μm, the average velar circumference for veligers from the high food treatment and predicted velar circumference for food-limited veligers was ~1193 μm and ~1442 μm, respectively. The non-parametric fits indicate that while the increase in velar circumference with respect to shell length was very close to linear for veligers raised in the low food environment, growth of velar circumference in veligers raised in a high food environment appeared to slow down at the point of divergence (Figure 3.5).

A linear regression of the relationship between cilia length and shell size with treatment resulted in a statistically significant fit that explained 81% of variation ($F_{3, 142} = 200.6$, $R^2 = 0.81$, $p < 0.0005$; Table 3.1); a Bayes factor of $1.49e^6$ strongly favors a model inclusive of treatment effects (shell length*treatment). For every micron increase in shell length, cilia length increased 0.12 μm and 0.19 μm for veligers from the high and low food treatments, respectively; otherwise stated, the cilia length of food-limited veligers grew ~57% more for every micron increase in shell length ($t_{142} = 5.449$; $p < 0.0005$; Table 3.2). Like velar circumference, cilia length started off similar between the two groups but at a shell length of ~265 μm, became and remained significantly different (Figures 3.7, 3.8); at a shell length of ~265 μm, average cilia length for veligers from the high and low food treatments was ~63 μm and ~65 μm, respectively. At a maximum overlapping shell length of 335 μm, average cilia length for veligers from the high food treatment (71 μm) was

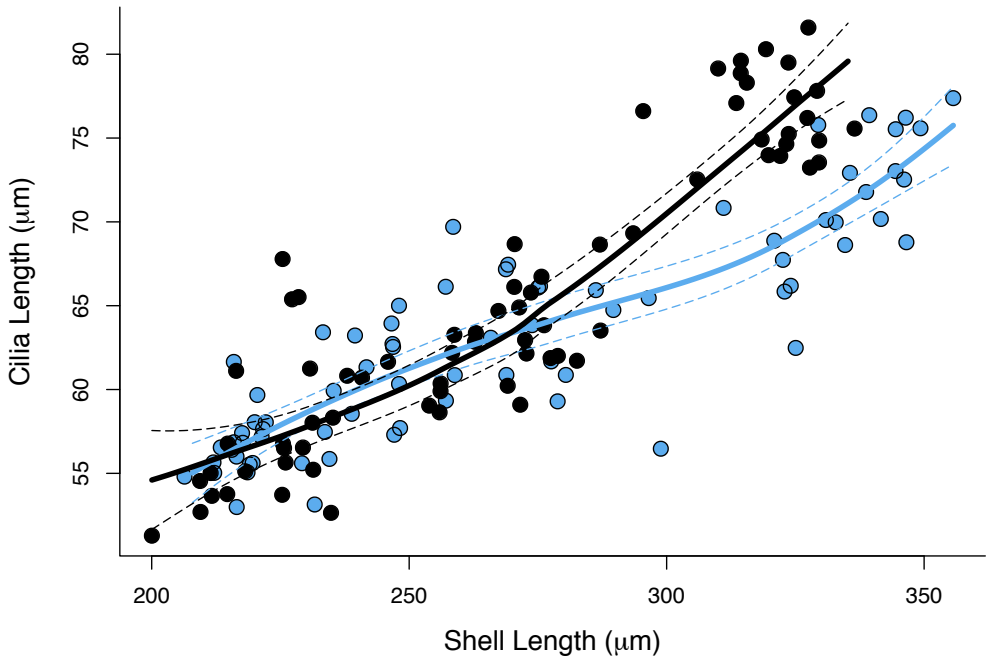


Figure 3.7. Loess smoothed regressions of the relationship between cilia length and shell length for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

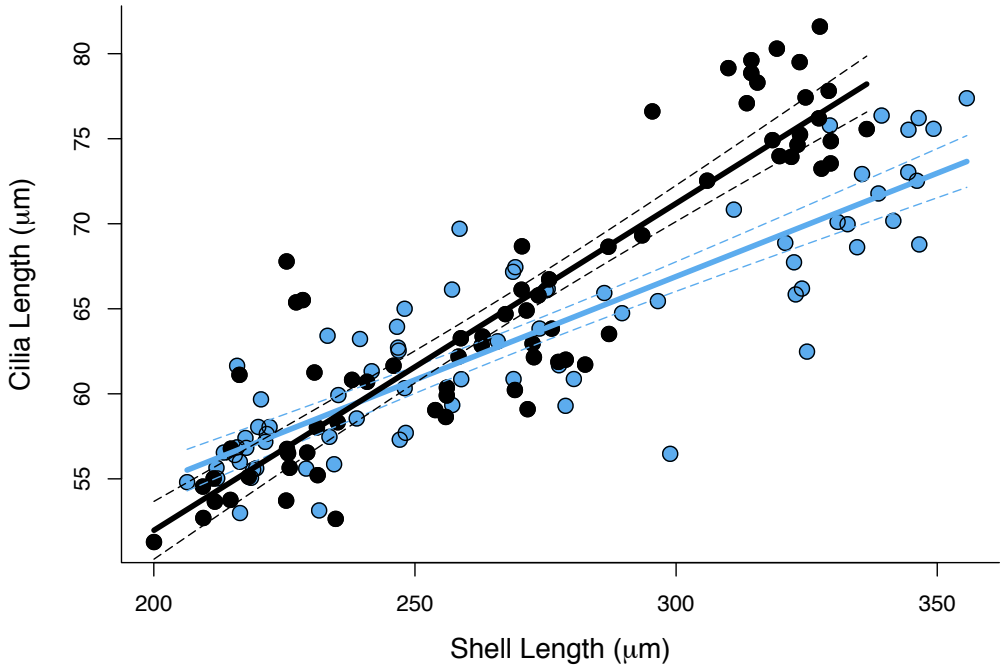


Figure 3.8. Linear regressions of the relationship between cilia length and shell length for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

significantly less than for food-limited veligers (78 μm; $t_{142} = 6.671$, $p < 0.0005$; Table 3.2). At the maximum attainable shell length of ~350 μm, average cilia length for veligers from the high food treatment and the predicted cilia length for food-limited veligers was ~73 μm and ~81 μm, respectively. The non-parametric fits indicate that at the point of divergence, cilia growth for veligers raised in a low food environment increased while cilia growth for veligers raised in a high food environment slowed down (Figure 3.7).

A linear regression of the relationship between cilia length and velar circumference with treatment resulted in a statistically significant fit that explained 77% of variation ($F_{3, 142} = 161.5$, $R^2 = 0.77$, $p < 0.0005$; Table 3.1); however, a Bayes factor of 0.15 favors a model without treatment effects (velar circumference*treatment). The increase in cilia length relative to velar size was similar regardless of treatment ($t_{142} = 1.657$, $p = 0.0997$; Table 3.2; Figures 3.9, 3.10): for every micron increase in velar circumference, average cilia length grew 0.025 μm and 0.029 μm for veligers from high and low food treatments, respectively. In other words, cilia length of veligers raised in a low food environment grew ~16% more for every micron increase in velar circumference, although this difference in growth was not significant. At a maximum overlapping velar circumference of 1373 μm, average cilia length for veligers raised in a high food environment (77 μm) was not significantly different from the cilia length for food-limited veligers (78 μm; $t_{142} = 0.718$, $p = 0.4738$; Table 3.2).

Swimming speed

A linear regression of the relationship between vertical swimming speed and shell size with treatment resulted in a statistically significant fit that explained 37% of variation ($F_{3, 123} = 26.11$, $R^2 = 0.37$, $p < 0.0005$; Table 3.1); a Bayes factor of $6.54e^2$ strongly favors a model inclusive of treatment effects (shell length*treatment). Swimming speed increased for both groups relative to shell size but the increase was greater for veligers raised

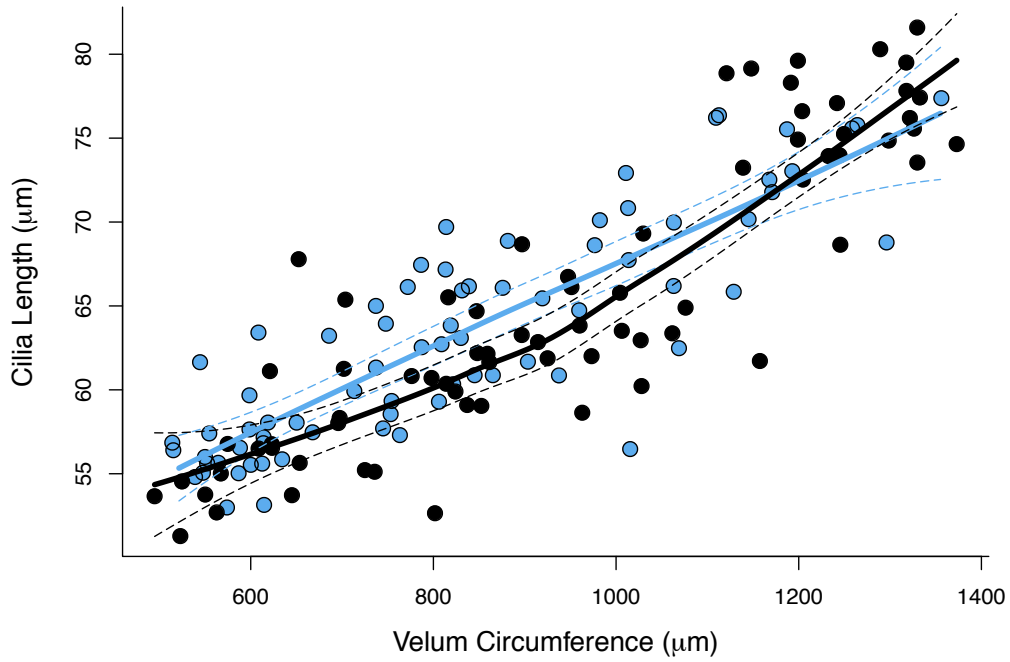


Figure 3.9. Loess smoothed regressions of the relationship between cilia length and velar circumference for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

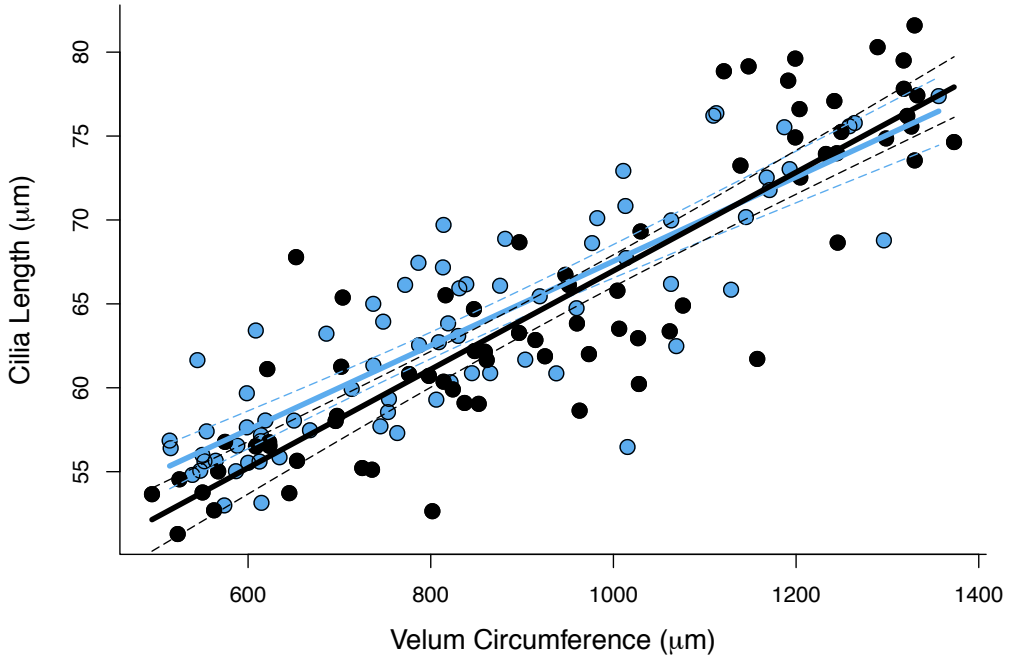


Figure 3.10. Linear regressions of the relationship between cilia length and velar circumference for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

in a high food environment (t_{123} = -2.889; p = 0.0046; Table 3.2; Figures 3.11, 3.12): for every micron increase in shell length, swimming speed increased by 0.0089 mm/s and 0.0038 mm/s for veligers from the high and low food treatments, respectively. In other words, the rate of increase per micron shell length for veligers raised in a high food environment was 2.3X the rate of increase by veligers raised in a low food environment. Swimming speed started off similar between the two treatment groups but became and remained significantly different when veligers were ~275 µm in shell length, at which point the average swimming speed for veligers from high and low food treatments was ~2.0 mm/s and ~1.7 mm/s, respectively. At a maximum overlapping shell length of 344 µm, the average swimming speed for veligers from the high food treatment (2.6 mm/s) was significantly greater than for food-limited veligers (2.0 mm/s; t_{123} = -4.573, $p < 0.0005$; Table 3.2).

Similar trends were observed when swimming speed was considered relative to predicted velar circumference. A linear regression of the relationship resulted in a statistically significant fit that explained 39% of variation ($F_{3, 142}$ = 26.11, R^2 = 0.39, $p <$ 0.0005; Table 3.1); a Bayes factor of $2.48e^6$ strongly favors a model inclusive of treatment effects (velar circumference*treatment). The increase in swimming speed relative to velar circumference was significantly greater for veligers raised in a high food environment (t_{123} = -3.995, $p < 0.0005$; Table 3.2; Figures 3.13, 3.14): for every micron increase in velar circumference, swimming speed increased by 0.0020 mm/s and 0.00062 mm/s for veligers from high and low food treatments, respectively. Stated otherwise, the rate of increase of swimming speed for veligers raised in a high food environment was ~3.2X the rate of increase by veligers raised in a low food environment. Swimming speed became and remained significantly different between the two treatment groups at a predicted velar circumference of ~800 µm; when velar circumference was 800 µm, the average swimming

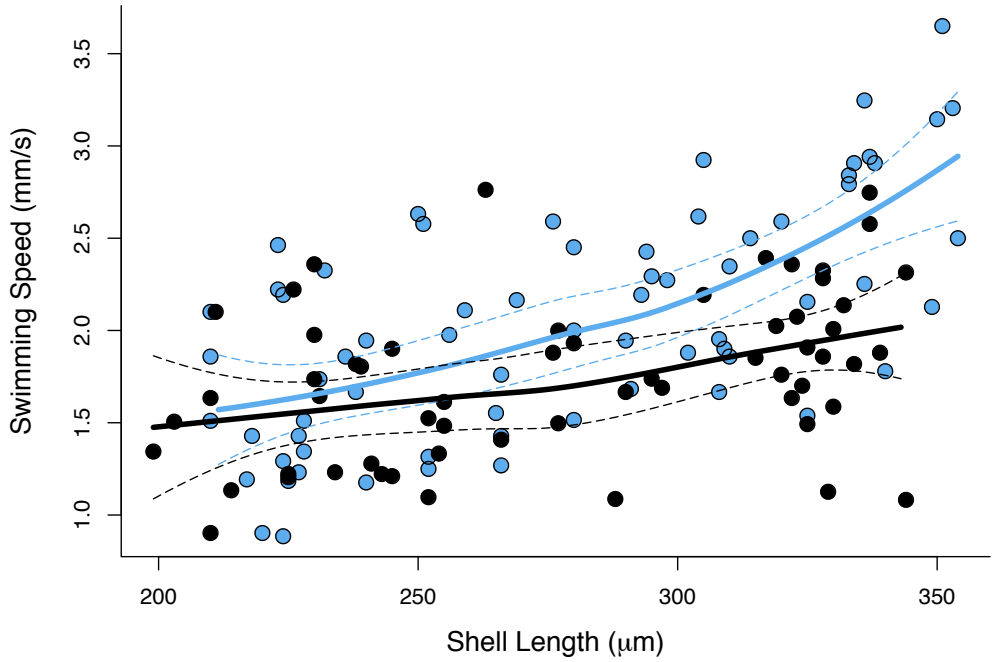


Figure 3.11. Loess smoothed regressions of the relationship between swimming speed and shell length for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

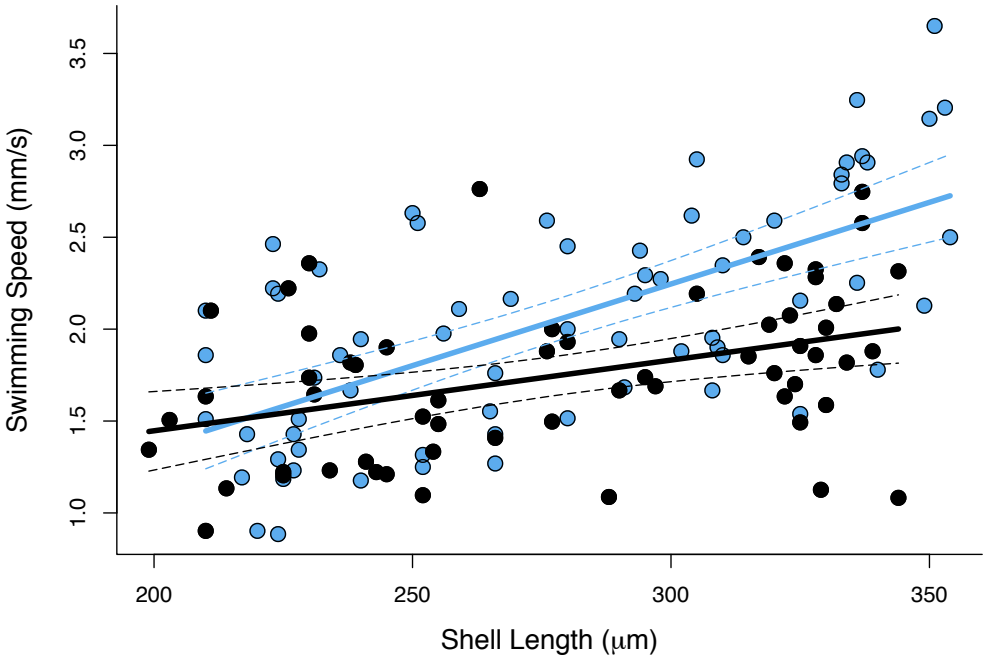


Figure 3.12. Linear regressions of the relationship between swimming speed and shell length for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

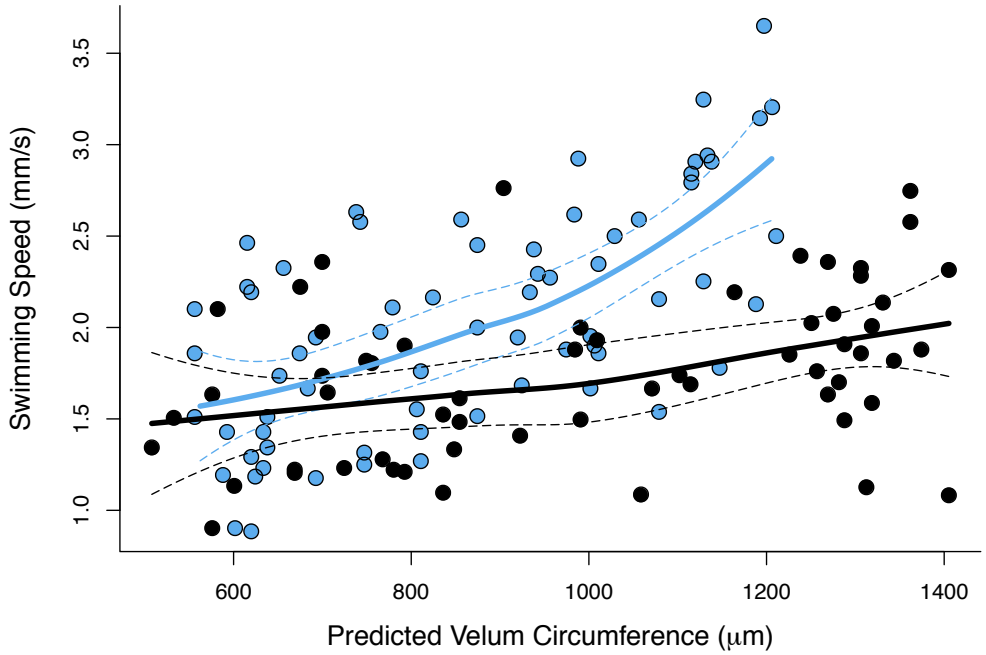


Figure 3.13. Loess smoothed regressions of the relationship between swimming speed and predicted velar circumference for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

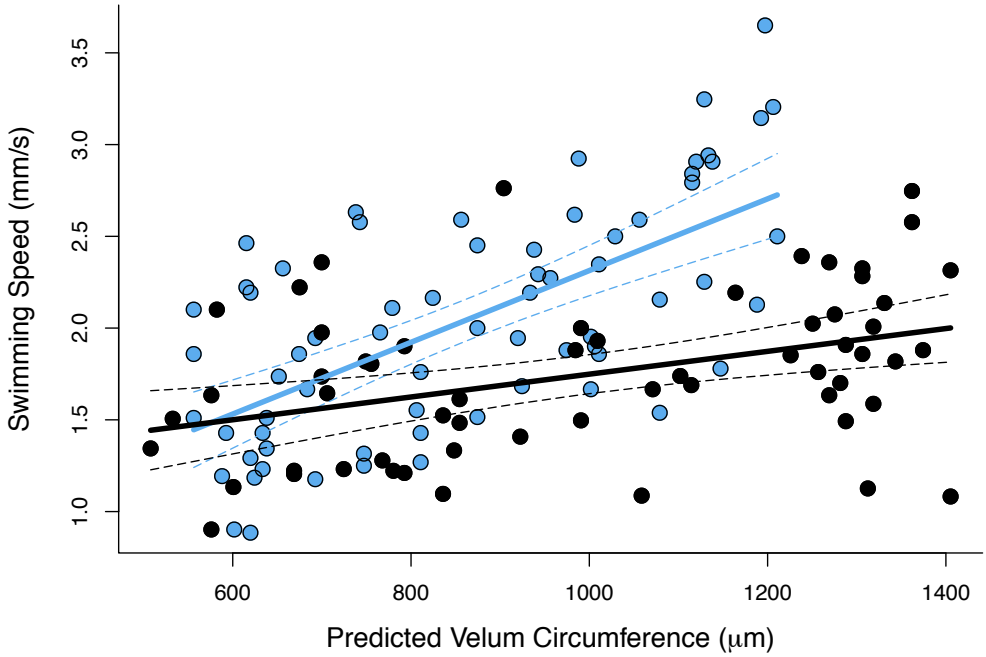


Figure 3.14. Linear regressions of the relationship between swimming speed and predicted velar circumference for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

speed for veligers from the high and low food treatments was ~2.0 mm/s and 1.6 mm/s, respectively. At the maximum overlapping predicted velar circumference of 1211 μm, average swimming speed for veligers from the high food treatment (~2.7 mm/s) was significantly greater than for food-limited veligers (~1.9 mm/s; $t_{123} = -6.602$, $p < 0.0005$; Table 3.2). At the maximum attained predicted velar circumference of ~1400 μm, the predicted swimming speed for veligers raised in a high food environment and average swimming speed for food-limited veligers was ~3.2 mm/s and ~2.0 mm/s, respectively. The non-parametric fits indicate that the increase in swimming speed with shell length and predicted velar circumference was close to linear for veligers raised in a low food environment. At the point of divergence, the increase in swimming speed with shell length and predicted velar circumference was much stronger for veligers raised in a high food environment.

Cell proliferation

A linear regression of the relationship between cell proliferation and shell length with treatment resulted in a statistically significant fit that explained 31% of variation ($F_{3, 102} = 17.04$, $R^2 = 0.31$, $p < 0.0005$; Table 3.1); a Bayes factor of $3.05e^2$ strongly favors a model inclusive of treatment effects (shell length*treatment). Trends of cell proliferation, as indicated by the number of BrdU-positive cells following 12 hour incubation periods (see methods and Figure 3.2), differed for veligers raised in high and low food environments relative to shell length ($t_{102} = 2.861$; $p = 0.0051$; Table 3.2; Figures 3.15, 3.16): for every micron increase in shell length, proliferation decreased by 0.054 and 0.019 cells/12 hrs for veligers from high and low food treatments, respectively. Put differently, the rate of decrease in cell proliferation for veligers raised in a high food environment was 2.8X the

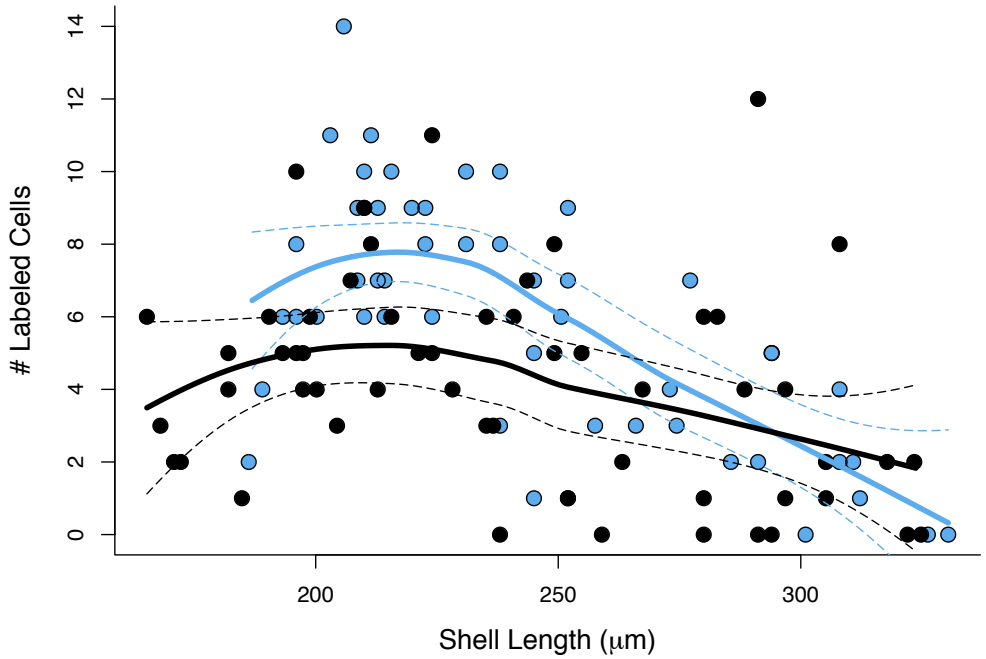


Figure 3.15. Loess smoothed regressions of the relationship between cell proliferation (# of labeled cells resulting from a 12 hour incubation in BrdU) and shell length for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

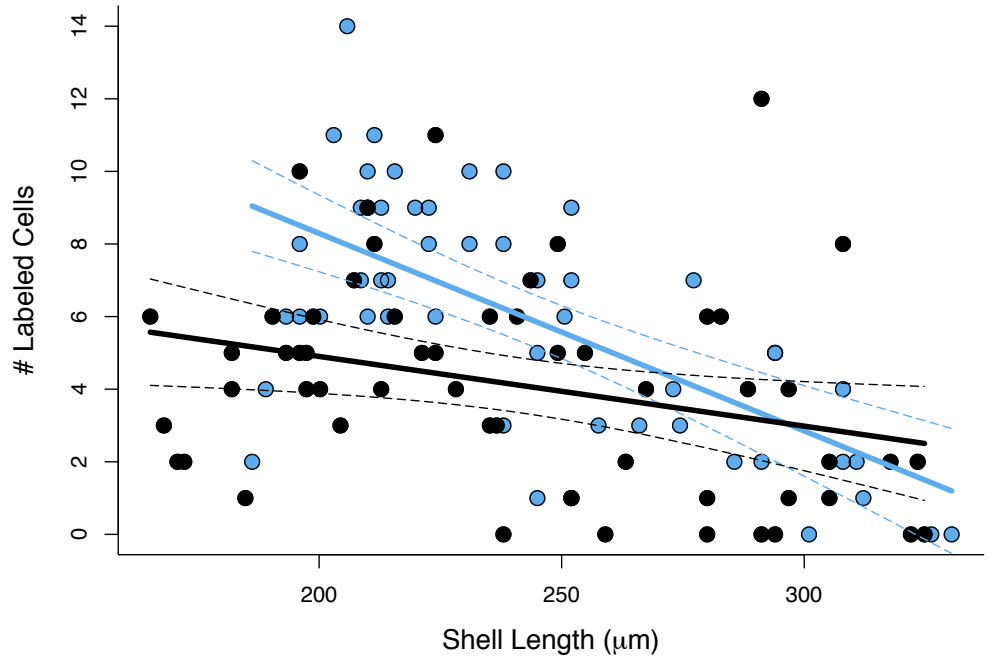


Figure 3.16. Linear regressions of the relationship between cell proliferation (# of labeled cells resulting from a 12 hour incubation in BrdU) and shell length for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

rate of decrease for veligers raised in a low food environment. However, average proliferation levels at the smallest overlapping shell size of 186 μm for veligers from the high food treatment (9.0 cells/12 hrs or 18 cells/day) were significantly greater than for food-limited veligers (5.2 cells/12 hrs or 10.4 cells/day) (t_{102} = -4.467, $p < 0.0005$; Table 3.2). Due to the difference in the rate of decrease, this initially significant difference appeared to be lost as shell size increased: by a shell length of ~300 μm, the average number of BrdU-positive cells for both groups was the same, at ~2.9 cells/12 hrs (or 5.8 cells/day). At the maximum shell size of ~325 μm, there was now a higher average number of dividing cells for food-limited veligers (~2.0 cells/12 hrs or 4 cells/day) vs. veligers from the high food treatment (~0.1 cells/12 hrs or 0.2 cells/day), although this difference did not appear to be statistically significant.

Comparable patterns exist when cell proliferation was considered relative to predicted velar size. A linear regression of the relationship resulted in a statistically significant fit that explained 31% of variation ($F_{3, 102}$ = 17.04, R^2 = 0.31, $p < 0.0005$; Table 3.1); a Bayes factor of $3.60e^2$ strongly favors a model inclusive of treatment effects (predicted velar circumference*treatment). The rate of decrease in proliferation levels for veligers from the high food treatment was significantly greater than for food-limited veligers (t_{102} = 3.631, $p < 0.0005$; Table 3.2; Figures 3.17, 3.18): for every micron increase in predicted velar circumference, the number of BrdU-positive cells decreased by 0.012 and 0.0031 cells/12 hrs for veligers from the high and low food treatments, respectively. In other words, the rate of decrease in cell proliferation for veligers raised in a high food environment was 3.87X the rate of decrease for veligers raised in a low food environment. Average proliferation levels for veligers from the high food treatment (9.0 cells/12 hrs or 18 cells/day) were significantly greater than for food-limited veligers (5.1 cells/12 hrs or 10.2 cells/day) at the smallest overlapping predicted velar circumference of 448 μm

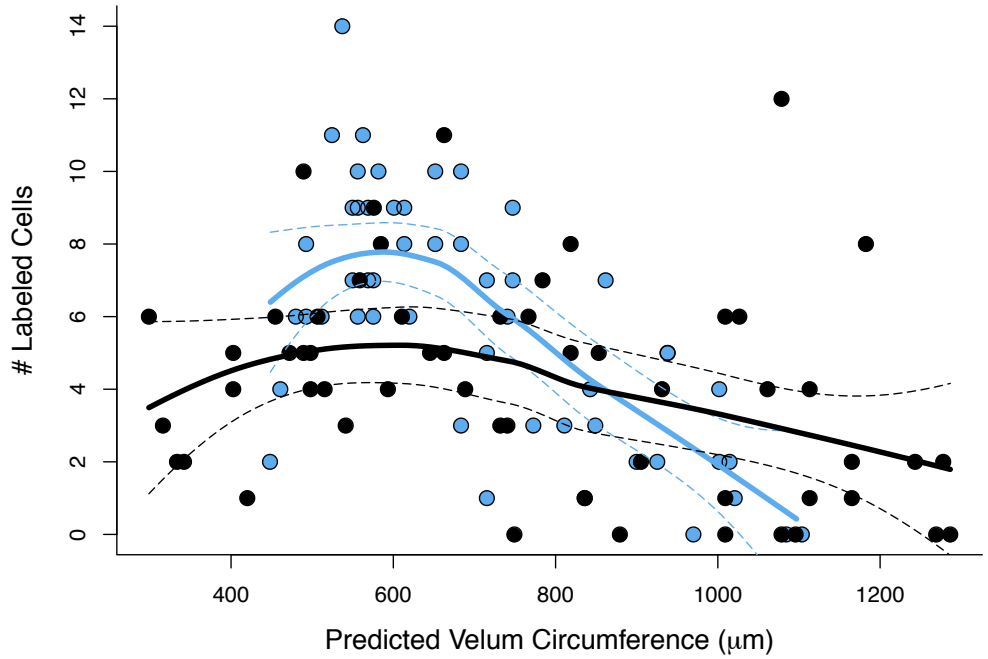


Figure 3.17. Loess smoothed regressions of the relationship between cell proliferation (# of labeled cells resulting from a 12 hour incubation in BrdU) and predicted velar circumference for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

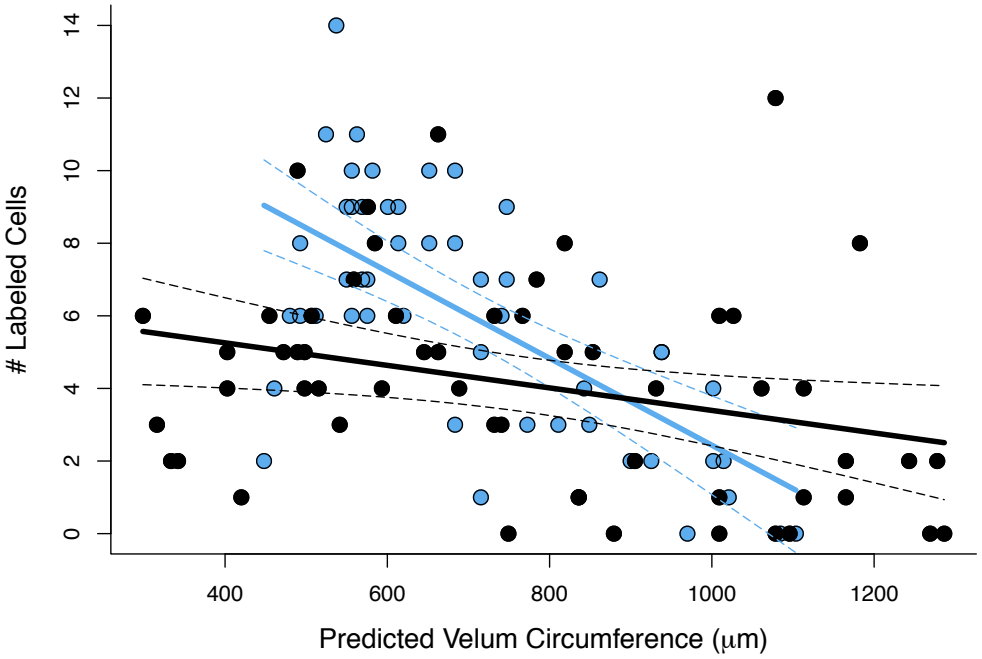


Figure 3.18. Linear regressions of the relationship between cell proliferation (# of labeled cells resulting from a 12 hour incubation in BrdU) and predicted velar circumference for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

(t_{102} = - 4.599; $p < 0.0005$; Table 3.2). Due to the difference in the rate of decrease, the initially significant difference was lost as velar circumference increased: by a circumference of ~900 μm, the average number of BrdU-positive cells for both groups was the same, at ~3.65 cells/12 hrs or 7.3 cells/day. At the maximum overlapping velar circumference of ~1000 μm, there was now a higher number of labeled cells for food-limited veligers (~3.4 cells/12 hrs or 6.8 cells/day) vs. veligers from the high food treatment (~2.4 cells/12 hrs or 4.8 cells/day), although this difference did not appear to be statistically significant. At the maximum velar circumference of ~1300 μm, the average number of dividing cells for food-limited veligers was ~2.5 cells/12 hrs (5 cells/day) and the number of predicted dividing cells for veligers from the high food treatment was <0. The non-parametric fits add more detail to the observed trends (Figure 3.17): there appeared to be an initial burst of proliferation earlier on, with a higher number of BrdU-positive cells found in veligers raised in a high food environment; following this initial peak, the number of labeled cells decreased in both groups albeit at a much faster rate for veligers from the high food treatment, resulting in a greater number of labeled cells in food-limited veligers at the largest shell and velar sizes.

A linear regression of the relationship between cell proliferation and age with treatment resulted in a statistically significant fit that explained 31% of variation ($F_{3, 102}$ = 16.47, R^2 = 0.31, $p < 0.0005$; Table 3.1); a Bayes factor of 60 favors a model inclusive of treatment effects (age*treatment). The rate of decrease in cell proliferation was much greater over the 15 days of growth for veligers raised in a high food environment vs. over the 28 days of growth for veligers raised in a low food environment (t_{102} = 3.972, $p < 0.0005$; Table 3.2; Figures 3.19, 3.20): as age increased, the average number of labeled cells decreased at a rate of 0.54 and 0.12 cells/12 hrs for veligers raised in high and low food environments, respectively. Stated otherwise, proliferation in the vela of veligers raised in a

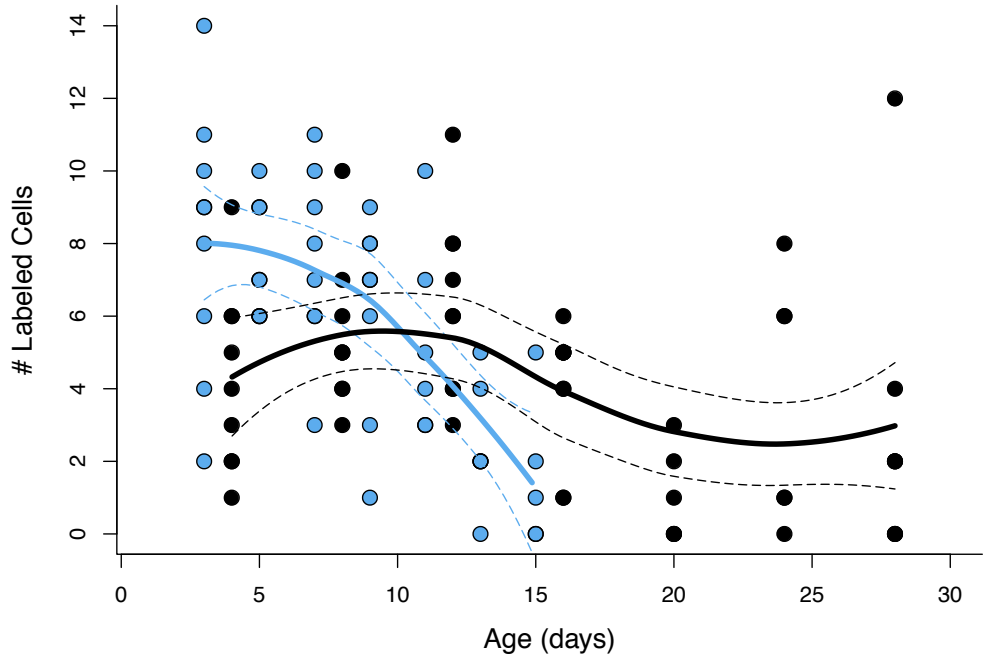


Figure 3.19. Loess smoothed regressions of the relationship between cell proliferation (# of labeled cells resulting from a 12 hour incubation in BrdU) and age for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

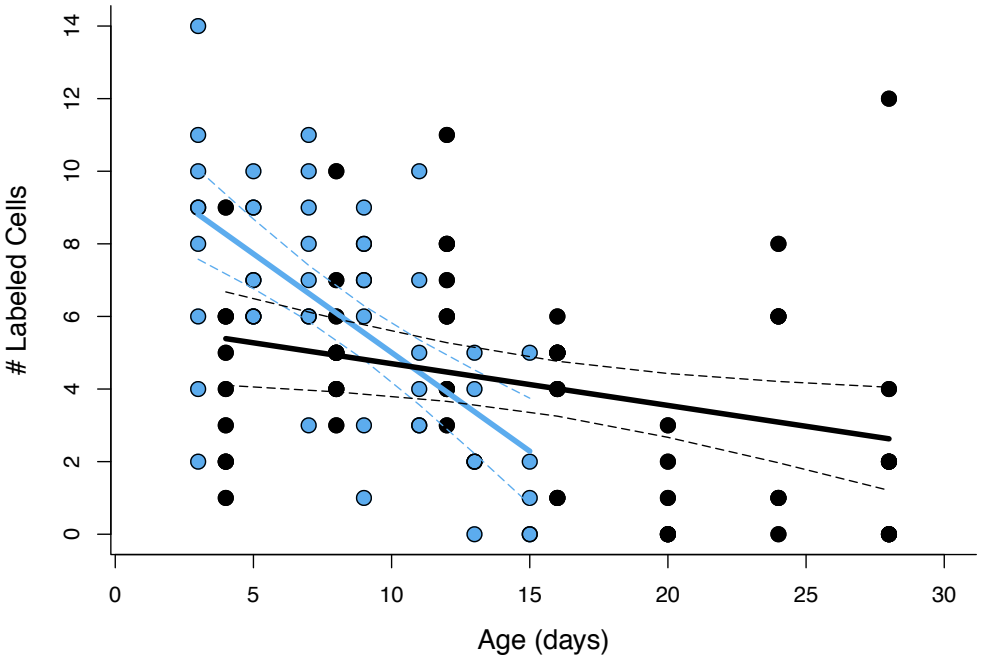


Figure 3.20. Linear regressions of the relationship between cell proliferation (# of labeled cells resulting from a 12 hour incubation in BrdU) and age for veligers raised in a high (blue) and low (black) food environment. Error bars are 95% CI.

high food environment decreased at 4.5X the rate of decrease for veligers raised in a low food environment. Average proliferation levels for veligers from the high food treatment (7.7 cells/12 hrs or 15.4 cells/day) were significantly greater than for food-limited veligers (5.3 cells/12 hrs or 10.6 cells/day) at the earliest overlapping age of 5 days (t_{102} = -3.194. *p* = 0.0019; Table 3.2). By 11 days of growth, the average number of BrdU-positive cells for both groups was the same, at ~4.5 cells/12 hrs (9 cells/day). After 15 days of growth (the final time point for the high food treatment), there was now a higher number of dividing cells for food-limited veligers (~4 cells/12 hrs or 8 cells/day) vs. veligers from the high food treatment (~2.3 cells/12 hrs or 4.6 cells/day), although this difference did not appear to be statistically significant. After 28 days (the final timepoint for the low food treatment and the end of the study), the average number of labeled cells for food-limited veligers was ~2.6 cells/12 hrs (5.2 cells/day) and the number of predicted dividing cells for veligers from the high food treatment was <0. The non-parametric fits indicate that during the first ~11 days of growth, there was a steady decrease in the number of dividing cells for veligers raised in a high food environment and an increase in the number of dividing cells for veligers raised in a low food environment. Past this point, the number of dividing cells for veligers from the high food treatment declined sharply and the number of dividing cells for food-limited veligers plateaued, decreased at a gradual level, and appeared to increase again over the final three days of growth (Figure 3.19). The average rates of proliferation (± 95% CI) for veligers raised in high and low food environments were 11.05 ± 3.79 cells/day and 7.83 ± 2.58 cells/day, respectively; therefore, food-limited veligers exhibited a total of ~220 BrdU-positive cells over 28 days of growth, 32% more than for veligers raised in a high food environment (~165 BrdU-positive cells over 15 days of growth).

DISCUSSION

Velar plasticity

Veligers raised at the low food concentration developed a greater velar circumference relative to shell length (Figures 3.5, 3.6). At the greatest overlapping shell length of 335 μm, the velar circumference of food-limited veligers was 20% bigger than for veligers from the high food treatment. Because clearance rates are known to increase with the length of ciliary bands (Strathmann *et al*., 1972, 1993; Gallager, 1988; Hansen and Ockelmann, 1991; Hart and Strathmann, 1994; Pernet and Strathmann, 2011), this increased velar circumference will improve feeding rates when resources are scarce, thus avoiding starvation and allowing for continued larval development. Veligers raised in a food-limiting environment also developed longer cilia relative to shell length (Figures 3.7, 3.8) but cilia length increased with velar size similarly independent of treatment (Figures 3.9, 3.10). This is similar to the results of Strathmann *et al*. (1993) for veligers of *C*. *gigas* and indicates that growth of cilia is closely coupled with lengthening of the ciliated band. Longer cilia, like those afforded by the larger velar sizes of food-limited veligers, allow for enhanced feeding rates by increasing the distance from the velar edge at which particles can be overtaken, increasing the volume of water that can be swept (because they are longer and have faster angular velocities), and may also allow for the capture of larger particles (Strathmann and Leise, 1979; Gallager, 1988; Strathmann, 1987b; Hansen and Ockelmann, 1991; Emlet and Strathmann, 1994; Riisgård *et al*., 2000, Pernet and Strathmann, 2011).

Larval mortality

Although larval mortality was not quantitatively followed, general observations did not indicate noticeable levels either within or between treatments. As noted by Strathmann *et al*. (1993), the possibility exists that the larger vela measured may simply reflect differential mortality of larvae resulting from genetic variations in velar size. However, if

larger velar sizes resulted only from natural genetic variation, I would have expected to also see greater variation of velar sizes in veligers raised in a high food environment: although there would be strong selection against having a small velum in a food-limited environment, no such selection would occur against veligers possessing a large velum when resources are unlimiting. It has also been posited that veligers in a low vs. high food environment are simply expanding the muscular velum to a greater or lesser extent, respectively (Strathmann *et al*., 1993). Although this would allow for a slightly greater coverage of the surrounding water, this explanation is unlikely in light of the observed increase in cilia length with velar size (Figures 3.9, 3.10). The differences in swimming speed also do not support this possibility - a larva whose velum is simply not fully extended is unlikely to be able to swim faster than one whose velum is expanded.

Costs and trade-offs

As with any example of an inducible trait, a stipulation underlying its conditionality is the existence of a trade-off in benefits vs. cost (e.g., Dodson, 1989; Moran, 1992; DeWitt *et al*., 1998; Tollrian and Harvell, 1999; Kopp and Tollrian, 2003; Auld *et al*., 2009); the most obvious trade-off for larval plasticities is in the allocation of resources toward the growth and development of structures that are maintained through metamorphosis vs. those that, although necessary to become metamorphically competent, ultimately do not. For example, longer larval arms in echinoderm larvae and larger vela in bivalve larvae enhance the ability to obtain food in nutrient-deficient conditions but come with a reduction in growth rates of the juvenile rudiment and shell/body, respectively (e.g., Strathmann *et al*., 1993; Hart and Strathmann, 1994). This is similar to what was observed for veligers of *L. scutulata*: enhanced growth of the velum corresponded with a reduced growth rate of juvenile structures (e.g., growth of the shell/body, development of the foot). In situations where at least some of the energy invested into velar growth is regained at metamorphosis (either

through partial/complete reabsorption or consumption of the velum), this would allow veligers to make up for some of the resources allocated to the ephemeral structure for both treatment groups but especially for the food-limited veligers that had to prioritize enhanced velar growth over growth of postlarval structures. Attempts to rear *Littorina scutulata* through metamorphosis has historically proven difficult (Buckland-Nicks *et al*., 1973; Hohenlohe, 2002) and, although Buckland-Nicks *et al*. (1973) reported seeing reduced velar lobes, these veligers failed to metamorphose and did not survive; Hohenlohe (2002) was able to rear a few individuals through metamorphosis but did not mention any specific changes leading up to or during metamorphosis.

Other potential trade-offs not investigated but that may exist include consequences of an extended pre-competent period (e.g., increased risk of planktonic mortality or dispersal away from favorable settlement habitats: Young and Chia, 1985; Pechenik, 1999; Cowen and Sponaugle, 2009; Vaughn and Allen, 2010) or influences of a low food environment on attaining metamorphic competence, delaying metamorphosis, or post-settlement success (e.g., Hart and Strathmann, 1994; Pechenik *et al*., 1998, Hadfield *et al*., 2001; Pechenik, 2006; Chiu *et al*., 2007; Pechenik and Tyrell, 2015). Only when veligers reached ~320-350 μm in size (regardless of treatment) did veligers appear to reach the pediveliger stage indicative of metamorphic competence (Hohenlohe, 2002; Vaughn, 2007; personal observations), suggesting that, unlike veligers of *Crepidula fornicata* (Pechenik *et al*., 1996a), there is a threshold size at which veligers of *L. scutulata* can become competent to settle. The benefits of a larger settlement/hatching size have been well-established (e.g., Moran and Emlet, 2001; Phillips, 2002; Marshall *et al*., 2003; Marshall and Keough, 2004; Marshall *et al*., 2006; Emlet and Sadro, 2006) but size alone does not necessarily guarantee juvenile parity. Several studies have indicated that it is also energy reserves that best predict post-settlement performance (e.g., Emlet and Hoegh-Guldberg, 1997; Phillips, 2002;

Thiyagarajan *et al*., 2003; Emlet and Sadro, 2006). Even though veligers raised in a high and low food environment likely settle at a similar size, veligers raised in the high food environment may possess superior lipid content that could give them a competitive edge in the ability to delay metamorphosis (e.g., Pechenik *et al*., 1996b; Hadfield *et al*., 2001), enhanced pre-metamorphic differentiation of other juvenile structures (e.g., radula; Lesoway and Page, 2011), and in early juvenile growth.

Veliger swimming speeds

All veligers swam upward more quickly as their velar circumference and shell length increased, but swimming speeds of those raised in the low food environment did not increase as rapidly as speeds of those raised in high food (Figures 3.11, 3.12). The inference in comparing the swimming rates of veligers of a certain shell length between the two treatment groups is that food-limited veligers will have a larger velum than those raised in high food. This suggests that swimming speed may be influenced negatively by velar size, a correlation that has been observed in veligers of *C. fornicata* (Chan *et al*., 2013). From a functional standpoint, since area increases faster relative to circumference, the increased drag imposed by a larger velar surface area could act as a partial tether that might enhance feeding (Emlet, 1990). However, when swimming speed is considered relative to predicted velar circumference, it is apparent that velar size alone is not responsible for the observed difference in speed. Swimming speed increases with velar circumference within each treatment group and, for the same velar circumference ~800 μm and above, veligers raised in a high food environment have a faster swimming speed than those raised in a low food environment (Figures 3.13, 3.14).

At a given velar circumference a veliger raised in a high food concentration will be carrying a proportionally larger shell and, likewise, for a given shell length, the velum of a food-limited veliger will be carrying a proportionally smaller shell. It has been proposed

that partial tethering by gravity due to the weight of the larval shell could increase feeding rates at a cost to swimming speed (Emlet and Strathmann, 1985; Emlet, 1990; Strathmann and Grünbaum, 2006). Although this concept may be true on an individualistic basis, when considering the swimming speeds of veligers from both treatments in relation to relative velar and shell sizes, the results appear contradictory. When simply considering only the weight of equal-sized shells, the swimming speed would be expected to be slower for veligers from the high food treatment that are carrying a proportionally larger shell size relative to velar size, but we see the opposite (Figures 3.11, 3.12). There are possible explanations for this discrepancy, each of which should be considered in a singular and additive context:

First, the slower swimming speed by food-limited veligers may be the trade-off of a behavioral alteration in prototrochal and/or metatrochal ciliary activity, synchronicity, or positioning (Strathmann and Grünbaum, 2006). The key to enhancing feeding is for the prototrochal cilia to move faster *relative* to the water; the increased shear allows the cilia to more easily overtake/intercept food particles (Emlet, 1990; Strathmann and Grünbaum, 2006). The metatroch is a structure whose function is thought to help capture food particles but also reduces the amount of water moved during the effective beat of prototrochal cilia: it serves not only as a barrier to corral food particles into the food groove, but its opposing current likely enhances particle collection by steepening the shear gradients of the prototrochal cilia. There is extensive evidence that veligers can exert fine control over ciliary beat and velar musculature (e.g., Mackie *et al*., 1976; Arkett *et al*., 1987; Kuang and Goldberg, 2001; Braubach *et al*., 2006; Strathmann *et al*., 2014) and a cessation or reduction in metatrochal beat in satiated veligers may explain the faster swimming speeds seen in larvae raised in a high food environment. Previous studies have indicated that in a highly

enriched food environment, the stomach of veligers can be filled in a few minutes (Fretter and Montgomery, 1968) and subsequent feeding would be unnecessary.

Alternatively, the reduced swimming activity seen in food-limited veligers compared to those from a high food environment may be purposeful in order to save energy when feeding efforts do not compensate for the costs of feeding/swimming (Strathmann, 1987b). It is also important to note that swimming speeds were measured on larvae that had been moved from their respective food environments into clean FSW; the especially stark change in food levels for the high food-acclimated veligers might have triggered faster swimming in an attempt to find their way back into a good foraging area.

Interestingly, swimming speed increased within both treatment groups with shell length and predicted velar circumference, which is contrary to previous studies indicating the fastest swimming speeds being found in early veligers and decreasing with age, possibly to bring veligers closer to settlement substrate (e.g., Bayne, 1964; Cragg, 1980; Chan *et al*., 2013). The observed increase in swimming speed may reflect interplay between gravity and nuances of veliger behavior. As part of normal swimming behavior, veligers regularly pull into their shell or arrest ciliary beating when disturbed and sink (e.g., upon contact with the air-water interface, in response to turbulence, or in response to predators; Garstang, 1929; Fretter, 1967; Mackie *et al*., 1976; Chia *et al*., 1985; Fuchs *et al*., 2004); the sinking velocity is in large part determined by the weight of the larval shell. Until a veliger is ready to settle (both in terms of competency and indication of favorable settlement habitat), net downward movement is to be avoided and larger veligers may need to increase upward swimming velocity to offset a faster sinking velocity during periods where swimming ceases (e.g., Cragg, 1980; Hidu and Haskin, 1978). That being said, larger veligers tended not to swim as *frequently* and may still accumulate closer to the substrate as settlement size is reached and/or in the presence of appropriate settlement cues as is commonly observed

(e.g., Bayne, 1964; Cragg, 1980; Cob *et al*., 2009), although this was not specifically followed in this study.

Sensation of food environments

An additional assumption implied with a plastic trait entails the existence of a reliable cue indicative of an organism's environmental conditions (e.g., Dodson, 1989; Moran, 1992; Tollrian and Harvell, 1999; Kopp and Tollrian, 2003). For a veliger, the types of cues to which they could be responding include physical contact with phytoplankton, chemical sensation of phytoplankton excretions, or cues related to ingestion and assimilation (i.e., a full stomach is an accurate predictor of food availability at that point in time and space) (e.g., Wilson, 1981; Shilling, 1995; Miner, 2007). Whether the cue is chemical or mechanical in nature, the plasticity is almost certainly realized via a neurosensory pathway. An apical cluster of neurons, often referred to as the "apical sensory organ" has been found in many gastropod veligers (e.g., Bonar, 1978; Chia and Koss, 1984; Uthe, 1995; Leise, 1996; Page and Parries, 2000; Hadfield *et al*., 2000; Ruthensteiner and Schaefer, 2002) and is believed to function as a sensory structure to detect both chemical and mechanical cues.

Cell division and velar growth

The prototrochal nuclei, food groove nuclei, and metatrochal nuclei are easily visualized in the vela of veligers of *Littorina scutulata* (Figure 3.2). Dividing cells were visible in the pre-trochal and post-trochal velar epidermis, in the food groove, metatroch, cephalic tentacles, apical sensory ganglion (located between the tentacles), lining the opening to the mouth, and in the viscera. The highest concentrations of labeled cells in association with the velum were found in the dorsal and ventral velar notches located just above the cephalic tentacles and just above the mouth, respectively. The anatomy of a veliger places the mouth between the pre-oral prototrochal band and post-oral metatrochal

band (e.g., Fretter and Montgomery, 1968; Strathmann and Leise, 1979; Strathmann, 1987b; Romero *et al*., 2010). Bird (2012) indicated a region of proliferative cells in the ventral velar notch just dorsal to the mouth in veligers of *Nassarius fossatus* and interpreted this to be mitotic prototrochal cells contributing to velar growth. The multiciliated cells of the prototroch are not believed to be able to divide, but the progeny of this proliferative zone may be intercalated into the cell bands of the prototroch, metatroch, and food groove, or may become accessory supporting cells intermittently bordering the prototroch (e.g., Mackie *et al*., 1976). Cells in the velar notches have features suggesting proliferative ability. 1) Many of the cells lack long compound cilia (seen in figures of veliger anatomy, e.g., Scheltema, 1962; Scheltema and Scheltema, 1963; Lin and Leise, 1996). 2) Although prototrochal nuclei are visible in these regions, they are more rounded in shape, possibly indicating recent progenies of the adjacent proliferative zone that have yet to develop the characteristic columnar shape seen lining the majority of the velum (Figure 3.2; Braubach *et al*., 2006; Gharbiah *et al*., 2013). There was clearly an increase in the number of prototrochal nuclei over the course of development, regardless of treatment, and the mechanism for velar growth in general is still not completely clear but likely involves the proliferative regions observed in the velar notches and may also consist of cellular growth (Nielson, 2004; Bird, 2012; this study).

Although the labeled cells that were counted represent only an intermittent snapshot of velar development in both space and time, the data indicate an initial spike of proliferation that decreased over time as shell length and velar circumference increased (Figures 3.15-3.20). This pattern was expected but the overall proliferative level was anticipated to be higher for food-limited veligers. Instead, a significantly higher initial level of proliferation was seen for veligers raised in a high food environment. As shell size and velar circumference increased, the level of proliferation decreased within both groups but

at a much faster decline for veligers from the high food treatment than for food-limited veligers, indicating that a more consistent level of cell division in this velar region is maintained as the velum grows for food-limited veligers and drops off quickly for those raised in a high food environment. These trends make sense in that the cells of veligers raised in an environment with greater resources are able to divide more rapidly in both the velum and body and the rate of division in the velum would be expected to decrease/drop off when it nears its maximum necessary, albeit smaller, size. The cells of veligers raised in a low food environment may not be able to divide as quickly in the beginning due to insufficient resources and, although the vela develop to be larger than those of veligers raised in high food conditions, they grow to this size over a longer period of time. Despite the cumulatively greater number of proliferative cells of food-limited veligers vs. veligers raised in a high food environment for the given developmental periods (realized by the relatively consistent and ultimately higher levels of proliferation observed in the low food treatment group over a longer developmental period), the initially higher rate of velar growth expected for food-limited veligers was not observed. It may be that if the proliferative activity could be quantified in other regions of the velum or in toto, the consistently higher level of cell division that was expected may have been found. Restricted bursts of excessive cell division in particular regions or points in time might also have been missed. As noted above, the growth of existing cells may also be a contributor to velar growth in addition to cell division, although the consistently tight spacing between prototrochal nuclei does not support this.

Future Considerations

Although the increase in feeding structure size has traditionally been considered the offensive response, a recent study by Adams *et al*. (2011) demonstrated that sea urchin larvae use dopamine signaling to inhibit feeding structure growth when food is abundant,

indicating that the food-mediated plastic response is to prevent unnecessary and costly growth that will otherwise occur unless signaled to stop. It would be interesting to determine if this same mechanism is used in veligers of *L. scutulata* and other larvae for which food-induced plasticities have been found as this would have important ecological and evolutionary implications.

Food quantity, in combination with other important factors such as food quality, genetic variation, and temperature, greatly influence the rate at which larvae develop in the plankton (e.g., Lima and Pechenik, 1985; Strathmann, 1987b; Olson and Olson, 1989; Pechenik, 1987; Hoegh-Guldberg and Pearse, 1995; Przeslawski *et al*., 2015); ambient food levels are rarely high enough to allow for maximum growth rates (e.g., Mullin and Brooks, 1976; Fenaux *et al*., 1994; Bos *et al*., 2006), such as that seen for veligers raised at 20,000 cells/mL. Although the developmental timeline of veligers raised at 1000 cells/mL better supports the minimum predicted planktonic period of three to four weeks (Buckland-Nicks *et al*., 1972; Hohenlohe, 2002), the low food concentration used is still higher than commonly observed ambient levels (e.g., Allen, 1941; Bainbridge, 1956; Shanks and McCulloch, 2003a, 2003b; Shanks *et al*., 2014); this indicates that the phenotypic range of velar sizes may be even wider than observed in the present study and merits further work. Ambient food levels are also much more temporally variable and taxonomically diverse than the constancy provided to the veligers during the study, two important variables rarely considered in the context of food-structure plasticity (e.g., see Estrella Klinzing and Pechenik, 2000; Miner and Vonesh, 2004).

The demonstrated ability of veligers of *Littorina scutulata* to develop larger vela and cilia relative to shell size when raised in a low food environment adds to a growing ubiquity of plasticity documented amongst marine larvae, a logical trend considering the variability of the planktonic environment. The effect of the differing morphologies on swimming speed

is interesting and may indicate behavioral complexities in tandem with the morphological changes. This study also contributes to our mechanistic understanding of how velar growth and the observed developmental plasticity is achieved.

Bridge to Chapter IV

The present study examined an inducible offense in veligers of *L. scutulata* in response to nutrient conditions. Veligers raised in a low food environment developed a larger feeding structure. In order to feed, however, these larvae need to swim. An important aspect of larval swimming is the ability to orient in relation to gravity in order to navigate the heterogeneity of the water column with regards to food and hydrodynamics. This ability is dependent upon a balance of propulsive and gravitational forces along the gravity vector. An asymmetric weight distribution, either natural or experimental, relative to the velar midline presents a problem that can be addressed in the proportions of velar area overlying each side of the shell and in velar area overall.

CHAPTER IV

LIFE IN THE BALANCE: DISTRIBUTION AND DEVELOPMENT OF ASYMMETRIC VELA

INTRODUCTION

Larvae whose swimming structures also function as the feeding structures (such as the velum of many gastropod and bivalve veliger larvae or ciliated arms of echinoderm plutei) may swim without feeding but have to swim in order to feed. Swimming by planktotrophic larvae is necessary not only for the collection of food, but is also important for the avoidance of unfavorable situations, remaining suspended in the water column, and for exploration and selection of settlement habitat (e.g., Chia *et al*., 1984; Pawlik, 1992; Kingsford *et al*., 2002; Koehl and Cooper, 2015). The small size and relatively slow swimming speeds of ciliated larvae compared to ocean currents (Chia *et al*., 1984) imply that larvae behave primarily as passive particles and have little control over their distribution; however, vertical swimming or sinking allow larvae to traverse a heterogeneous water column with concentrated patches or layers of food, and to take advantage of fluctuations in current speed and direction with depth. The latter allows some larvae to at least partially control their horizontal movement, which, for the larvae of many intertidal invertebrates, is equally important in limiting dispersal distance during planktonic development and for the return to favorable settlement habitats (e.g., Dekshenieks *et al*., 1996; Shanks *et al*., 2000; Metaxas, 2001; Kingsford *et al*., 2002; Shanks *et al*., 2003; Poulin *et al*., 2002; Metaxas and Saunders, 2009; Kunze *et al*., 2013; Fuchs *et al*., 2015a, 2015b). Vertical migratory behaviors have been demonstrated in a variety of zooplankton, including gastropod veligers (Richter, 1973; Barile *et al*., 1994; Kunze *et al*., 2013).

In order for an organism to be negatively gravitactic (move away from a gravitational field), as are the larvae of many marine invertebrates, there must be a way for the organism to orient itself relative to gravity (e.g., Chia *et al*., 1984; Mogami *et al*., 2001). Several passive mechanisms exist that are defined by the physical parameters of an organism such as differential drag (depends on shape asymmetry; Roberts, 1970) or an unequal density distribution along the anterioposterior axis (Verworn, 1889 as cited by Machemer and Bräucker, 1992). Having denser structures (such as the shell of a veliger) positioned posteriorly separates the centers of gravity and buoyancy. This creates a corrective, orienting torque along the gravity vector when the organism is tilted away from the passively upright orientation (e.g., Chia *et al*., 1984; Mogami *et al*., 2001; Grünbaum and Strathmann, 2003; Chan, 2012).

Although contributions of one or more of these passive gravitactic mechanisms are sufficient for some organisms to perform directed vertical swimming (e.g., Roberts, 1970; Pennington and Strathmann, 1990; Mogami *et al*., 2001; Hosoya *et al*., 2010), most mollusk larvae also possess paired internal gravity-sensing organs called statocysts. A statocyst consists of one (statolith) or multiple (statoconia) dense spherical masses suspended in a fluid-filled capsule that is lined with sensory cells (e.g., Budelmann, 1988; Wiederhold *et al*., 1990). When, for example, a veliger encounters rotational velocity gradients (vorticity) from turbulence or shear, tilt or tumbling of the larval body is translated into a similar effect within the statocyst; the stimulation of the peripheral sensory cells by the movement of the statolith or statoconia informs the larva of its orientation relative to gravity, allowing it to make corrective movements or turbulence-induced behaviors such as sinking or the active downward propulsion observed in oyster larvae, termed “dive-bombing” (e.g., Chia *et al*., 1981; Fuchs *et al*., 2004; Fuchs *et al*., 2013, 2015a, 2015b).

Bivalve and gastropod veligers possess an asymmetric density distribution (fulfilled by the posteriorly-positioned larval shell relative to the velum) and possess paired gravity-sensing statocysts for all or part of their larval duration. These characteristics speak to the importance of the ability to orient and swim relative to a gravitational field. The velum of most bivalve veligers consists of a single elliptical disk lined with cilia (Zardus and Martel, 2002) and is different from the velum of gastropod veligers, which is divided bilaterally into one or more pairs of velar lobes. Assuming the propulsive forces generated by the prototrochal cilia lining the velum are greater than the opposing force of gravity, the larva will move, and if these forces are balanced along the gravity vector, the net movement will be vertical. For a planispiral shell, the center of gravity will passively align parallel with the center of buoyancy along the gravitational vector and each velar lobe will be supporting an equal amount of weight. However, the shells of most larval gastropods are helically coiled, meaning that the spire of the shell protrudes out to the snail's right or left depending on whether the coiling is dextral or sinistral, respectively (Schilthuizen and Davison, 2005). In most adult snails, the shell is preferentially positioned at an angle so that its center of gravity is aligned with the bilateral midline of the underlying head and foot (the cephalopedal mass) (Linsley, 1977). Although it might make sense for larval snails to alter the positioning of the shell while swimming rather than the size or proportions of the velum, this is not often seen. Instead, the shell is carried so that its axis of coiling is more or less orthogonal to the cephalopedal axis with the spire of the shell conspicuously protruding out to one side of the larva. In this situation, the center of gravity is no longer aligned with the center of buoyancy along the gravity vector but is shifted toward the spire of the shell. Equally-sized velar lobes that produce equivalent swimming forces and carry unequal amounts of weight would cause upward propulsion to proceed instead along the angle imposed by the torque of an asymmetric weight distribution. In order to offset this

imbalance, there must be a corresponding asymmetry in propulsive forces – the velar lobe on the same side of the larva as the protruding spire of the shell should be larger in size than the velar lobe on the opposite side. This exact conformation has been reported, with the right lobe being the larger in dextrally-coiling species and the left lobe being larger in sinistrally-coiling species; in all cases, the larger lobe coincides with the spire of the larval shell (e.g., Lebour 1933a; Lebour, 1937; Lebour, 1945; Chukhchin, 1960; Scheltema and Scheltema, 1963; Pilkington, 1976; Thiriot-Quievreux, 1983; Tan and Morton, 1998; Page, 2011; Romero *et al*., 2004; Robertson, 2012; Page and Parries, 2000). A few authors even make mention of the putative purpose of the asymmetry or note its co-occurrence with an asymmetrical shell (Struhsaker and Costlow, 1968; Richter, 1973; Bandel *et al*., 1997; Hohenlohe, 2002); for example, Richter (1973) states that "species with a long turriculated shell always have an asymmetrical velum" and that the lobe positioned above the apex of the shell "is distinctly enlarged to equalize the counterweight of the shell".

In the first part of this study, I examined the patterns of velar asymmetry through measurements taken from literature figures of bi-lobed and multi-lobed veligers. The relationships between the amount of shell positioned underneath each side of the velum and corresponding velar proportions were considered along with the relationship between total shell area and total velar area. The results of this survey provide an appropriate foundational context for an experimental investigation into velar asymmetry.

As the shell grows and the torque imposed by the increasingly asymmetric weight distribution strengthens (as the spire becomes more prominent), a veliger would need to synchronize growth of the corresponding velar asymmetry to maintain the balance of gravitational and propulsive forces along the gravity vector. However, shell and velar growth is not always isometric (e.g., Strathmann *et al*., 1993; Estrella Klinzing and Pechenik, 2000; see Chapter III) and the center of gravity may be shifted by external causes such as

unsuccessful predation attempts (e.g., Hickman, 2001) or the growth of epibionts (Cedhagen and Middelfart, 1998). For these reasons, it is likely that velar asymmetry is not constitutive allometric growth but is a plastic response to gravitational cues. Plasticity of velar growth (in response to variable food conditions) is known in at least four gastropod larvae, including veligers of the local intertidal snail *Littorina scutulata* (Strathmann *et al*., 1993; Estrella Klinzing and Pechenik, 2000; Phillips, 2011; Chapter III). Building on this initial demonstration of velar plasticity and the aforementioned trends of velar asymmetry, the second intent of this study was to test the hypotheses that the purpose of an asymmetric velum is to offset the unbalanced weight distribution of a helically-coiled larval shell and that this asymmetry is a plastically-induced trait subject to manipulation. To do this, I glued artificial weights of varying size to the larval shells of *Littorina scutulata*. Veligers of *L. scutulata* develop a slight natural asymmetry in their velum (the right lobe grows to be ~30% larger in area than the left lobe (see results; Hohenlohe, 2002), with the larger lobe located above of the spire of the shell. I attempted to exacerbate this asymmetry by attaching weight to the spire side of the larval shell and expected to see an increase in the asymmetry of the right and left velar lobes with an increase in weight. This form of plasticity represents a shifting of growth between the two velar lobes (increased growth of the lobe on the weighted side with reduced/no growth of the other lobe). Alternatively or simultaneously, a veliger could also offset an unbalanced weight distribution by an increase in overall velar size (and concurrently, increased ciliary thrust), thereby decreasing the relative effect of the weight. Both the difference in size between the velar lobes as well as the total velar area were compared between unweighted and weighted veligers.

MATERIALS AND METHODS

Natural velar asymmetry in Littorina scutulata

As part of a previous study (see Chapter III), velar size was measured over time for veligers of *L. scutulata* raised in a low food environment (1000 cells/mL) over 28 days of growth; the same length and width measurements of each velar lobe used to calculate circumference in the aforementioned study were used here to calculate lobe areas in order to demonstrate the existence and development of a natural asymmetry in the size of the two velar lobes.

Literature review of velar asymmetry

The literature was searched both for mention of velar asymmetry and for photos or figures of veligers from which measurements of velar area and larval shell area could be obtained (see Tables 4.1 and 4.2). Figures representing a different stage of the same species were treated as individual data points since each stage represents velar parameters specific to the current shell size and areal distribution. Only swimming veligers photographed or depicted from an anterior view were used. A line was drawn between the two sides of the velum (velar midline); this almost always approximately corresponded with the cephalopedal axis (Figure 4.1). The area of each side of the velum and of each portion of the shell located on either side of the velar midline was measured using ImageJ software (NIH, Bethesda, MD). The appropriate scale was used in figures or photographs for which a scale bar or indication of size was provided. The ratio of shell area on either side of the veliger was calculated by dividing the area of the right side (S1) by the area of the left side (S2) unless the shell was sinistrally-coiled, in which case the larger left side was divided by the right side. The ratio of velar area on either side of the veliger was calculated by dividing the area of the side of the velum corresponding to S1 by the area of the side of the velum corresponding to S2 (V1/V2). For graphs in this study depicting total velar and shell areas,

Table 4.1. Literature measurements: the area of the right and left velar lobe(s) and area of the right and left sides of the shell were measured from figures and photos of the following veligers:

Specimen Name	Family	Obvious velar asymmetry (Y/N)	Nature of velar lobes[1]	Source (Figure or page #)
Aporrhais pespelicani	Aporrhaidae	N	M (6)	Lebour, 1937 (4H)
Atlanta inflata	Atlantidae	N	M (6)	Richter, 1973 (1)
Caecum imperforatum	Caesidae	N	B	Lebour, 1936 (2-1)
Crepidula lingulata	Calyptraeidae	N	B	Collin, 2000 (6C)
Zegalerus tenius	Calyptraeidae	N	B	Pilkington, 1976 (5B)
Cerithiid sp. 1	Cerithiidae	Y	B	Pilkington, 1976 (1F)
Cerithiid sp. 1 (later stage)	Cerithiidae	Y	B	Pilkington, 1976 (1G)
Cerithiid sp. 2	Cerithiidae	N	M (4)	Pilkington, 1976 (2B)
Cerithiid sp. 3	Cerithiidae	N	M (4)	Pilkington, 1979 (2D)
Cerithiopsis barleei (newly hatched)	Cerithiopsidae	N	B	Lebour, 1933a (2-3)
Cerithiopsis barleei (intermediate)	Cerithiopsidae	N	B	Lebour, 1933a (2-8)
Cerithiopsis barleei (late)	Cerithiopsidae	Y	B	Lebour, 1933a (2-14)
Cerithiopsis tubercularis	Cerithiopsidae	Y	B	Lebour, 1933a (1-9)
Cerithiopsis? sp. A	Cerithiopsidae	Y	B	Lebour, 1945 (10A)
Cerithiopsis? sp. B	Cerithiopsidae	Y	B	Lebour, 1945 (11)
Cerithiopsis? sp. D	Cerithiopsidae	Y	B	Lebour, 1945 (13A)
Anachis avara (early)	Columbellidae	Y	B	Scheltema and Scheltema, 1963 (1-6)
Anachis avara (intermediate)	Columbellidae	Y	B	Scheltema and Scheltema, 1963 (2-8)
Anachis avara (late)	Columbellidae	Y	B	Scheltema and Scheltema, 1963 (2-10)
Conus lividus	Conidae	Y	B	Page, 2011 (2A)
Conus mus	Conidae	N	M (6)	Lebour, 1945 (31C)
Eulima sp.	Eulimidae	Y	B	Richter, 1973 (1)
Haedropleura septangularis (?)	Horaiclavidae	N	M (4)	Lebour, 1936 (3-14)
Litiopa melanostoma	Litiopidae	Y	B	Lebour, 1945 (8C)
Echinella trochiformis	Littorinidae	N	B	Lebour, 1945 (7D)
Lacuna sp.	Littorinidae	N	B	Thiriot-Quievreux, 1983 (1A)
Littorina angulifera	Littorinidae	N	B	Lebour, 1945 (4B)
Littorina neritoides	Littorinidae	N	B	Chukhchin, 1960 (3B)
Littorina picta	Littorinidae	Y	B	Struhsaker and Costlow, 1968 (1F)
Littorina scutulata	Littorinidae	Y	B	This study
Littorina zigzac	Littorinidae	N	B	Lebour, 1945 (5C)
Risellopsis varia	Littorinidae	N	B	Pilkington, 1976 (1C)

Table 4.1 continued…

Specimen Name	Family	Obvious velar asymmetry (Y/N)	Nature of velar lobes[1]	Source (Figure or page #)
Cythara sp.	Mangeliidae	Y	M (4)	Chukhchin, 1960 (14)
Lora sp. (?)	Mangeliidae	N	M (4)	Lebour, 1936 (3-19)
Mangelia nebula (newly hatched)	Mangeliidae	N	B	Lebour, 1934b (1-9)
Mangelia nebula (intermediate)	Mangeliidae	N	MI (4)	Lebour, 1934b (2-4)
Mangelia nebula (late)	Mangeliidae	N	M (4)	Lebour, 1934b (2-7)
Mangilia sp. (?) (stage 1)	Mangeliidae	N	M (4)	Lebour, 1945 (30-1)
Mangilia sp. (?) (stage 2)	Mangeliidae	N	M (4)	Lebour, 1945 (30-2)
Mangilia sp. (?) (stage 3)	Mangeliidae	N	M (4)	Lebour, 1945 (30-3)
Modulus modulus	Modulidae	N	B	Lebour, 1945 (15E)
Rapana bezoar	Muricidae	N	B	Chukhchin, 1960 (12B)
Rapana venosa (early)	Muricidae	Y	B	Harding, 2006 (1B)
Rapana venosa (late)	Muricidae	Y	M (4)	Harding, 2006 (1H)
Thais (Stramonita) chocolata (early)	Muricidae	Y	B	Romero *et al*., 2004 (5A)
Thais (Stramonita) chocolata (late)	Muricidae	Y	M (4)	Romero *et al*., 2004 (5B)
Zeatrophon ambiguus	Muricidae	N	MI (4)	Pilkington, 1976 (10D)
Nassariid sp. 1	Nassariidae	N	M (4)	Pilkington, 1976 (10F)
Nassarius obsoletus	Nassariidae	Y	MI (4)	Scheltema, 1962 (1-5)
Nassarius trivittatus	Nassariidae	N	M (4)	Scheltema and Scheltema, 1965 (1-4)
Nassarius vibex	Nassariidae	N	MI (4)	Scheltema, 1962 (2-6)
Natica canrena	Naticidae	N	M (4)	Lebour, 1945 (17B)
Natica catena	Naticidae	N	B	Lebour, 1936 (2-27)
Natica poliana (?)	Naticidae	N	M (4)	Lebour, 1936 (2-28)
Naticid sp.	Naticidae	N	M (4)	Pilkington, 1976 (7D)
Olivella adelae	Olivellidae	Y	B	Thiriot-Quievreux, 1983 (1F)
~*Simnia patula*	Ovulidae (?)	N	M (4)	Lebour, 1945 (21)
Eulimella acicula[2]	Pyramidellidae	Y	B	Chukhchin, 1960 (17)
Pyramidellid larva[2]	Pyramidellidae	N	B	Lebour, 1936 (2-21)
Argobuccinum tumidum	Ranellidae	N	B	Pilkington, 1976 (7H)
Cymatiid sp. 4 (early)	Ranellidae	N	M (4)	Pilkington, 1976 (9E)
Cymatiid sp. 4 (late)	Ranellidae	N	M (4)	Pilkington, 1976 (9F)
Cymatium chlorostomum	Ranellidae	N	M (4)	Lebour, 1945 (24A)
Cymatium pileare (?)	Ranellidae	N	M (4)	Lebour, 1945 (23A)

Table 4.1 continued…

Specimen Name	Family	Obvious velar asymmetry (Y/N)	Nature of velar lobes[1]	Source (Figure or page #)
Cymatium sp.	Ranellidae	N	M (4)	Lebour, 1945 (25A)
Cymatoma kampyla	Ranellidae	N	MI (4)	Pilkington, 1976 (8D)
Fusitriton laudandus	Ranellidae	N	B	Pilkington, 1976 (8E)
Fusitriton oregonensis	Ranellidae	N	M (4)	Strathmann and Strathmann, 2007 (1)
Philbertia gracilis (early)	Raphitomidae	Y	M (4)	Lebour, 1933b (1-6)
Philbertia gracilis (late)	Raphitomidae	N	M (4)	Lebour, 1933b (1-8)
Philbertia leufroyi	Raphitomidae	N	M (4)	Lebour, 1934b (4-2)
Philbertia linearis (stage 1)	Raphitomidae	Y	B	Lebour, 1934b (3-4)
Philbertia linearis (stage 2)	Raphitomidae	Y	MI (4)	Lebour, 1934b (3-5)
Philbertia linearis (stage 3)	Raphitomidae	N	M (4)	Lebour, 1934b (3-6)
Philbertia linearis (stage 4)	Raphitomidae	Y	M (4)	Lebour, 1934b (4-3)
Alvania crassa	Rissoidae	N	B	Lebour, 1936 (1-11)
Alvania punctura	Rissoidae	Y	B	Lebour, 1934a (4-1)
Rissoa guerini	Rissoidae	N	B	Lebour, 1934a (3-12)
Rissoa sarsii	Rissoidae	Y	B	Lebour, 1934a (1-11)
Strombus alatus	Strombidae	N	M (6)	Thiriot-Quievreux, 1983 (1E)
Strombus canarium	Strombidae	Y	M (6)	Cob *et al*., 2009 (2C)
Struthiolaria papulosa	Struthiolariidae	N	MI (4)	Pilkington, 1976 (6C)
Tonna perdix	Tonnidae	N	M (4)	Lebour, 1945 (22A)
Notosinister huttoni (?)[2]	Triphoridae	Y	B	Pilkington, 1976 (2F)
Triphora perversa[2]	Triphoridae	Y	B	Lebour, 1933a (1-5)
Triphorid veliger[2]	Triphoridae	Y	B	Bandel *et al*., 1997 (8)
Triphoris sp.[2]	Triphoridae	Y	B	Lebour, 1945 (14)
Turridae sp.	Turridae	N	M (4)	Thiriot-Quievreux, 1983 (1H)
Turrid (?) veliger 1	Turridae (?)	N	B	Pilkington, 1976 (11E)
Turrid (?) veliger 2	Turridae (?)	Y	M (4)	Pilkington, 1976 (11F)
Turrid (?) veliger 3	Turridae (?)	Y	M (4)	Pilkington, 1976 (11J)
Maoricolpus roseus	Turritellidae	Y	B	Probst and Crawford, 2008 (6C)
Unknown veliger	Unknown	N	B	Emlet (personal communication)
Unknown veliger A	Unknown	N	M (4)	Lebour, 1945 (34A)
Unknown veliger B	Unknown	N	M (4)	Lebour, 1945 (35A)
Unknown veliger C	Unknown	N	M (4)	Lebour, 1945 (36A)

Table 4.1 continued…

Specimen Name	Family	Obvious velar asymmetry (Y/N)	Nature of velar lobes[1]	Source (Figure or page #)
Unknown veliger D	Unknown	N	M (6)	Lebour, 1945 (37)
Unknown veliger E	Unknown	N	M (4)	Lebour, 1945 (38)
Unknown veliger F	Unknown	N	M (4)	Lebour, 1945 (39)
Unknown veliger G	Unknown	N	M (4)	Lebour, 1945 (40)
Unknown veliger H	Unknown	N	MI (4)	Lebour, 1945 (41)
Unknown veliger J	Unknown	N	M (6)	Lebour, 1945 (42)
Unknown veliger K	Unknown	N	M (4)	Lebour, 1945 (43)
Veligers for which asymmetry of the velum was mentioned but no figure or photo was provided:				
Engina armillata	Buccinidae		B	Tan and Morton, 1998 (394)
Bittium reticulatum	Cerithiidae		B	Chukhchin, 1960 (9)
Amphissa versicolor	Columbellidae		B	Page and Parries, 2000 (389)
Epitoniid veligers	Epitoniidae		B	Bandel *et al*., 1997 (167)
Eulimid veligers	Eulimidae		B	Bandel *et al*., 1997 (170)
Boonea bisuturalis	Pyramidellidae		B	Robertson, 2012 (239)

[1]The different types of vela are: Bi-lobed (B) and multi-lobed (M) with either four or six lobes. MI indicates a velum that is intermediate between having two and four lobes.

[2]veligers described or depicted as having sinistrally-coiled shells.

Table 4.2. Literature measurements: shell area and velar area for bi- and multi-lobed veligers when scale was provided. Numbers shown here have been rounded to the nearest 1,000 μm^2.

Specimen Name	Family	Nature of velar lobes[1]	Shell area ($\mu m^2 \times 10^3$)	Velar area ($\mu m^2 \times 10^3$)	Source (Figure or page #)
Atlanta inflata	Atlantidae	M (6)	87	428	Richter, 1973 (1)
Caecum imperforatum	Caesidae	B	38	73	Lebour, 1936 (2-1)
Crepidula lingulata	Calyptraeidae	B	35	77	Collin, 2000 (6C)
Cerithiopsis barleei (newly hatched)	Cerithiopsidae	B	16	21	Lebour, 1933a (2-3)
Cerithiopsis barleei (late)	Cerithiopsidae	B	29	56	Lebour, 1933a (2-14)
Cerithiopsis tubercularis	Cerithiopsidae	B	83	69	Lebour, 1933a (1-9)
Cerithiopsis? sp. A	Cerithiopsidae	B	72	192	Lebour, 1945 (10A)
Cerithiopsis? sp. B	Cerithiopsidae	B	117	235	Lebour, 1945 (11)
Cerithiopsis? sp. D	Cerithiopsidae	B	71	128	Lebour, 1945 (13A)
Anachis avara (early)	Columbellidae	B	141	248	Scheltema and Scheltema, 1963 (1-6)
Anachis avara (intermediate)	Columbellidae	B	347	359	Scheltema and Scheltema, 1963 (2-8)
Anachis avara (late)	Columbellidae	B	424	612	Scheltema and Scheltema, 1963 (2-10)
Conus mus	Conidae	M (6)	219	1998	Lebour, 1945 (31C)
Eulima sp.	Eulimidae	B	137	300	Richter, 1973 (1)
Haedropleura septangularis (?)	Horaiclavidae	M (4)	392	1376	Lebour, 1936 (3-14)
Litiopa melanostoma	Litiopidae	B	163	335	Lebour, 1945 (8C)
Echinella trochiformis	Littorinidae	B	6	10	Lebour, 1945 (7D)
Lacuna sp.	Littorinidae	B	83	203	Thiriot-Quievreux, 1983 (1A)
Littorina angulifera	Littorinidae	B	9	12	Lebour, 1945 (4B)
Littorina picta	Littorinidae	B	8	8	Struhsaker and Costlow, 1968 (1F)
Littorina scutulata	Littorinidae	B	50	59	This study
Littorina zigzac	Littorinidae	B	3	5	Lebour, 1945 (5C)
Lora sp. (?)	Mangeliidae	M (4)	520	2367	Lebour, 1936 (3-19)
Mangelia nebula (newly hatched)	Mangeliidae	B	109	168	Lebour, 1934b (1-9)
Mangelia nebula (intermediate)	Mangeliidae	MI (4)	263	1591	Lebour, 1934b (2-4)
Mangelia nebula (late)	Mangeliidae	M (4)	405	1857	Lebour, 1934b (2-7)
Mangilia sp. (?) (stage 1)	Mangeliidae	M (4)	166	1080	Lebour, 1945 (30-1)
Mangilia sp. (?) (stage 2)	Mangeliidae	M (4)	229	1350	Lebour, 1945 (30-2)
Mangilia sp. (?) (stage 3)	Mangeliidae	M (4)	209	1643	Lebour, 1945 (30-3)
Modulus modulus	Modulidae	B	66	252	Lebour, 1945 (15E)
Rapana venosa (early)	Muricidae	B	90	129	Harding, 2006 (1B)
Rapana venosa (late)	Muricidae	M (4)	278	623	Harding, 2006 (1H)

Table 4.2. continued…

Specimen Name	Family	Nature of velar lobes[1]	Shell area ($\mu m^2 \times 10^3$)	Velar area ($\mu m^2 \times 10^3$)	Source (Figure or page #)
Thais (Stramonita) chocolata (early)	Muricidae	B	16	28	Romero *et al*., 2004 (5A)
Thais (Stramonita) chocolata (late)	Muricidae	M (4)	212	438	Romero *et al*., 2004 (5B)
Nassarius obsoletus	Nassariidae	MI (4)	258	430	Scheltema, 1962 (1-5)
Nassarius trivittatus	Nassariidae	M (4)	616	1319	Scheltema and Scheltema, 1965 (1-4)
Nassarius vibex	Nassariidae	MI (4)	214	294	Scheltema, 1962 (2-6)
Natica canrena	Naticidae	M (4)	148	947	Lebour, 1945 (17B)
Natica catena	Naticidae	B	159	266	Lebour, 1936 (2-27)
Natica poliana (?)	Naticidae	M (4)	442	2850	Lebour, 1936 (2-28)
Olivella adelae	Olivellidae	B	164	342	Thiriot-Quievreux, 1983 (1F)
~*Simnia patula*	Ovulidae (?)	M (4)	348	1494	Lebour, 1945 (21)
Pyramidellid larva[2]	Pyramidellidae	B	209	558	Lebour, 1936 (2-21)
Cymatium chlorostomum	Ranellidae	M (4)	4980	52076	Lebour, 1945 (24A)
Cymatium pileare (?)	Ranellidae	M (4)	7418	52109	Lebour, 1945 (23A)
Cymatium sp.	Ranellidae	M (4)	1634	8356	Lebour, 1945 (25A)
Fusitriton oregonensis	Ranellidae	M (4)	3054	11226	Strathmann and Strathmann, 2007 (1)
Philbertia gracilis (early)	Raphitomidae	M (4)	208	214	Lebour, 1933b (1-6)
Philbertia gracilis (late)	Raphitomidae	M (4)	938	2693	Lebour, 1933b (1-8)
Philbertia linearis (stage 2)	Raphitomidae	MI (4)	82	77	Lebour, 1934b (3-5)
Alvania crassa	Rissoidae	B	62	120	Lebour, 1936 (1-11)
Alvania punctura	Rissoidae	B	211	783	Lebour, 1934a (4-1)
Rissoa sarsii	Rissoidae	B	148	378	Lebour, 1934a (1-11)
Strombus alatus	Strombidae	M (6)	372	1711	Thiriot-Quievreux, 1983 (1E)
Strombus canarium	Strombidae	M (6)	142	452	Cob *et al*., 2009 (2C)
Tonna perdix	Tonnidae	M (4)	2882	32873	Lebour, 1945 (22A)
Triphora perversa[2]	Triphoridae	B	118	417	Lebour, 1933a (1-5)
Triphorid veliger[2]	Triphoridae	B	187	618	Bandel *et al*., 1997 (8)
Triphoris sp.[2]	Triphoridae	B	80	258	Lebour, 1945 (14)
Turridae sp.	Turridae	M (4)	263	827	Thiriot-Quievreux, 1983 (1H)
Maoricolpus roseus	Turritellidae	B	130	150	Probst and Crawford, 2008 (6C)
Unknown veliger A	Unknown	M (4)	3399	24450	Lebour, 1945 (34A)
Unknown veliger B	Unknown	M (4)	2862	27073	Lebour, 1945 (35A)
Unknown veliger C	Unknown	M (4)	316	3767	Lebour, 1945 (36A)

Table 4.2. continued…

Specimen Name	Family	Nature of velar lobes[1]	Shell area ($\mu m^2 \times 10^3$)	Velar area ($\mu m^2 \times 10^3$)	Source (Figure or page #)
Unknown veliger D	Unknown	M (6)	691	3822	Lebour, 1945 (37)
Unknown veliger E	Unknown	M (4)	133	802	Lebour, 1945 (38)
Unknown veliger F	Unknown	M (4)	3383	27021	Lebour, 1945 (39)
Unknown veliger G	Unknown	M (4)	302	2628	Lebour, 1945 (40)
Unknown veliger H	Unknown	MI (4)	138	1368	Lebour, 1945 (41)
Unknown veliger J	Unknown	M (6)	141	893	Lebour, 1945 (42)
Unknown veliger K	Unknown	M (4)	234	1722	Lebour, 1945 (43)

[1]The different types of vela are: Bi-lobed (B) and multi-lobed (M) with either four or six lobes. MI indicates a velum that is intermediate between having two and four lobes.
[2]veligers described or depicted as having sinistrally-coiled shells.

only data from veligers for which a size scale was provided were used (Table 4.2). The two data points representing veligers of *L. scutulata* used measurements generated from average values of the control (unweighted) group in the present study.

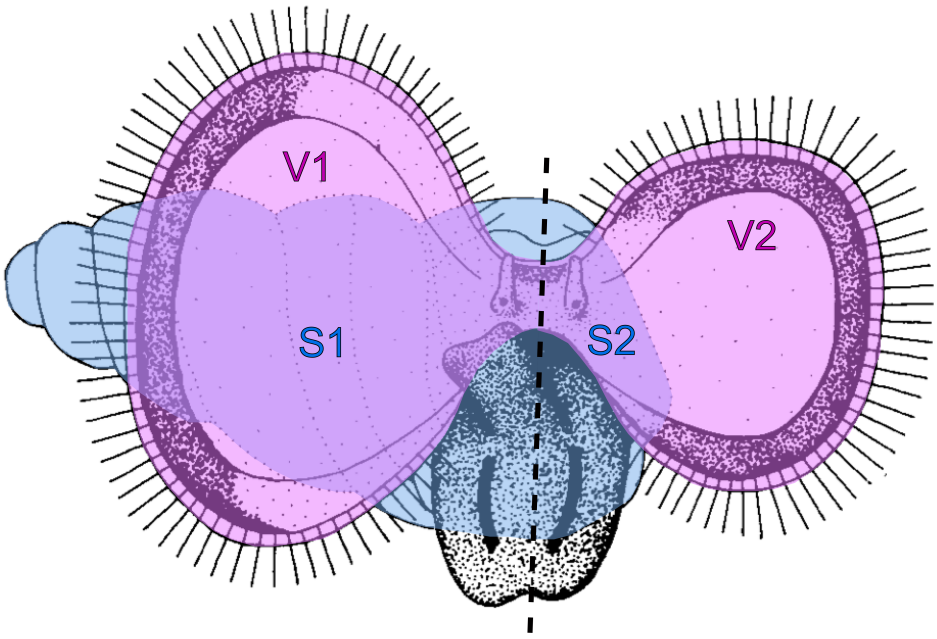


Figure 4.1. Measurements of veligers from the literature. The areas of the shell (S1 and S2) and corresponding areas of the velum (V1 and V2) were measured on either side of a central line (velar midline: dashed line) drawn between the two velar lobes and cephalic tentacles. Modified from Figure 1G, Cerithiidae sp. - Pilkington (1976).

Larval collection, rearing, and study set-up

Veligers were hatched from egg cases released overnight from adult *L. scutulata* and were reared for one week, during which time they were fed a tripartite algal mixture (*Isochrysis galbana*, *Dunaliella tertiolecta*, *Chaetoceros gracilis*) of equal numbers of cells at a combined concentration of 10,000 cells/mL and moved to fresh seawater every third day. After one week, the veligers had grown from a hatching size of ~190 µm to 250 µm in shell length; it takes ~three weeks for veligers to reach a size at which they are considered competent to settle, ~350 µm. Due to a difficulty of attaching weights to smaller shells, a

shell length of 250 μm was chosen as the size at which the weights would be attached. Three sizes of poly(methyl methacrylate) beads (PMMA; density: 1.18 g/ml, ACROS Organics, NJ) were used: ~100 μm, ~140 μm, and ~180 μm in diameter. Each size bead was attached to 15 veligers and 10 of the best ones (e.g., least amount of glue, no air bubbles in the weld) were chosen from each treatment and randomly assigned to a 10 mL well in one of seven six-well plates along with 10 control veligers with no weights attached. Every other day, the veligers were moved to fresh filtered seawater and fed a tripartite algal mixture of equal numbers of cells at a combined concentration of 150 cells/mL. Since fully-grown veligers (~350 μm) are less-inclined to swim, the study was stopped when veligers had reached ~325 μm in shell length (16 days from set-up).

Attachment of beads to larval shells

When manipulating organisms of small size, it is necessary to have the proper tools. One key tool among larval biologists consists of a human eyelash affixed with tape to the blunt wooden end of cotton-tipped applicator. The tip of the eyelash was dipped in fresh egg whites and gently dragged across a clean surface to reduce the albumen remaining on the eyelash to a small amount. The wet albumen on the tip of the eyelash was then carefully lowered onto a pre-measured PMMA bead, gluing it to the bottom of the eyelash, and left to dry for at least one minute. Next, a veliger was isolated in a small drop of seawater on a slide; once the albumen had dried, a pipette was used to remove the water around the veliger. The bottommost portion of the bead was very carefully touched to the surface of a thinly-spread drop of cyanoacrylate glue and then quickly touched to the spire-side of the larval shell. The eyelash (now attached to the bead that is attached to the veliger) was then submerged in a dish of filtered seawater, instantly curing the cyanoacrylate. While the albumen dissolved in the seawater, an additional eyelash tool was used to gently free the now-weighted veliger (Figure 4.2A, 4.2B).

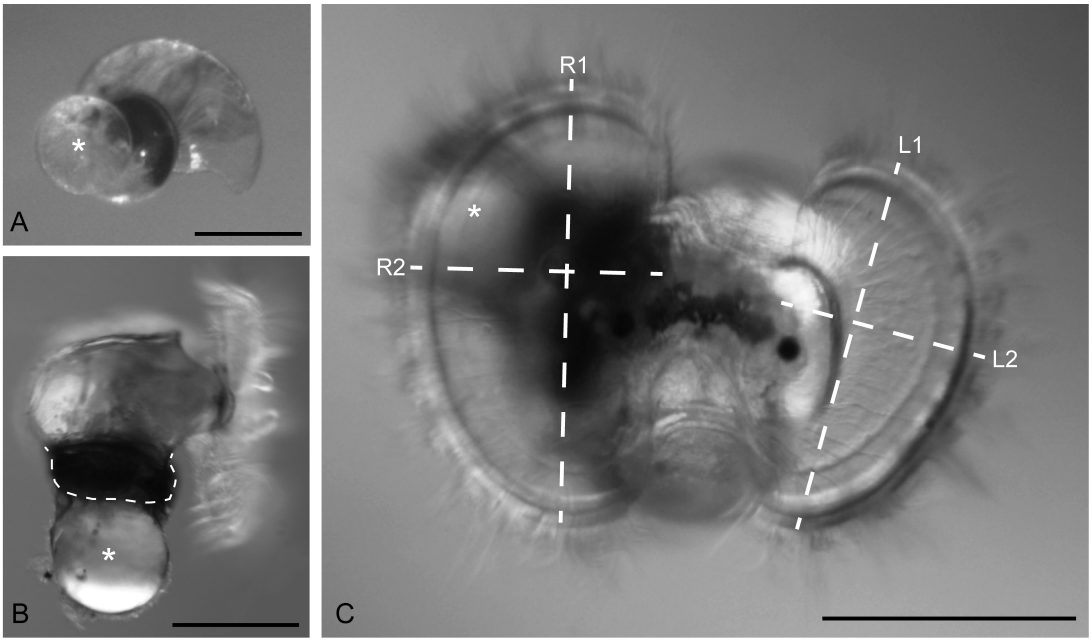


Figure 4.2. Weighted veligers and velar measurements. (A) The beads (*) were glued to the spire of the larval shell. (B) A dorsal view of a veliger swimming with its bead (*). The spire of the shell (dotted line) can be seen in reference to the bead. (C) The length and width of the right (R1, R2) and left (L1, L2) velar lobes were used to calculate the relative and total velar area. The attached bead (*) can be seen underlying the larger right velar lobe. Scale bars = 200 μm.

Velar measurements

All shell lengths were measured using an ocular micrometer on an Olympus SZH10 dissecting microscope. To measure velar dimensions, veligers were filmed swimming upwards against a coverslip under a Zeiss compound microscope. Horizontal movement was limited by confining the veliger within a square of window screen. The length (L) and width (W) of each velar lobe was measured later from video stills (Figure 4.2C) and used to calculate the area of each using the formula for estimating the area of an ellipse [(L/2)*(H/2)*π]. Total velar area was calculated by adding together the area of the R and L velar lobes.

Veliger and bead masses

Veliger mass in water (excess mass) was estimated from Stokes' equation for terminal sinking velocity (Vogel, 1994). Veligers of a range of sizes were briefly fixed in

formalin (one minute in 5% formalin in FSW) to force them into their shells and were then gently introduced to the surface of a stable water column sitting in a temperature-controlled water bath set at 14°C. Veligers were allowed to sink through two of the marked 2 cm intervals before sinking velocity was timed using a stop watch (see Figure S4.1 in Appendix).

Excess mass is the weight of an object in seawater and is the product of the excess density of an object and its volume. Excess density is the difference between the density of an object and seawater. Viscous forces dominate over inertial forces at Reynolds numbers <1 and according to Stokes' equation, at the terminal sinking velocity (U) of a sphere of radius 'a', drag forces ($6\pi a\mu U$) are equal to gravitational forces $[(4/3\pi a^3)(\rho-\rho_o)g]$ where ρ is the density of the sphere, ρ_o is the density of seawater (1023.856 kg/m^3; 32 ppt salinity, 14°C, 1 atm), μ is the dynamic viscosity of seawater (0.001235 kg/ms; 32 ppt salinity, 14°C, 1atm), and g is gravitational acceleration. Solving for U gives the equation $U = [2a^2g(\rho-\rho_o)]/9\mu$. The equation was adjusted for the drag and volume of a veliger, whose shape and volume was approximated as an oblate spheroid (Happel and Brenner, 1983) where 'b' is the equatorial radius along the axis of rotation (shell width/2) and 'a' is the polar radius [(shell length + shell height/2)/2]. For a veliger 280 μm in length, $U = [2ab(\rho-\rho_o)g]/8.46\mu$; for a veliger 325 μm in length, $U = [2ab(\rho-\rho_o)g]/8.37\mu$. Both equations were solved for the density of each size veliger (ρ), which was used to find its excess mass. The excess mass of a veliger 280 μm in length is 0.33 μg and that of a veliger 325 μm in length is 0.80 μg. The excess mass of the PMMA beads was calculated from its known density (1.18 g/mL; ACROS Organics, NJ) and the radius of each size sphere. From smallest to largest, the PMMA beads weighed 0.0769 μg, 0.2243 μg, and 0.4929 μg in water representing 23.1%, 67.4%, and 148% of the estimated weight (in water) of a veliger 280 μm in length and 9.62%, 28.1%, and 61.6% of the weight (in water) of a veliger 325 μm in length. Although the added weight

of glue likely varied from veliger to veliger, the amount of glue used per veliger was less than 20% of the bead's volume. The density of the cyanoacrylate used (1.05 g/mL; super thin insta-cure, Bob Smith Industries, Atascadero, CA) has a density higher than seawater but less dense than the beads; even assuming 20% of the bead's volume was used in glue, this would be the equivalent of adding only an additional 1/30th of the excess mass of the bead.

Veliger swimming paths

At the end of the study, the horizontal swimming path for a veliger carrying the largest diameter bead (180 μm) and for an unweighted veliger of the same size was filmed under an Olympus SZH10 dissecting microscope. The veligers were corralled within a rectangle of window screen to restrict horizontal movement. Swimming paths, average velocity, and maximum velocity were obtained using the MTrackJ plugin (Meijering *et al*., 2012) for ImageJ software; this plugin allows manual tracking of a moving particle frame by frame and calculates velocity at each point by dividing the distance traveled from the previous point by the frame interval (in this case 1/30 sec from a video frame rate of 30 fps).

Statistical methods

Linear regression statistics and equations for the relationships of shell ratio vs. velar ratio and total shell area vs. total velar area for bi-lobed and multi-lobed veligers were generated using SPSS. The regression equations for bi-lobed veligers were used to make predictions of the increases in velar ratio and total velar area with increases of shell asymmetry and total shell area for the weighted veligers of *L. scutulata*: shell area was measured for all treatment groups as described for the literature measurements with increases in shell asymmetry representing the additional area of the added weight to the right side of the shell. The data point representing *L. scutulata* fell below the regression

lines for both variables (velar ratio and total velar area). In order to make projections specific to this species, these differences (0.0344 and 67,977.267 μm^2, respectively) were used to reduce the y-intercepts of the bi-lobed regression equations so that the line passed through the measurement value for unweighted *L. scutulata*.

Two veligers died in the unweighted, 100 µm, and 140 µm groups, and there were no usable frames to measure velar area for one veliger in the 180 µm group; therefore, the number of veligers analyzed for the unweighted, 100 µm, 140 µm, and 180 µm groups were 8, 8, 8, and 9, respectively.

An ANOVA comparing the shell lengths of veligers from each of the four treatment groups (control, 100 µm, 140 µm, 180 µm) was conducted to ensure that there were no significant differences in shell size between treatment groups that could influence interpretations of velar ratio or total velar area. There were no outliers, as determined by inspection of a boxplot; the data were normally distributed, as determined by Shapiro-Wilk's test ($p \geq 0.141$); and there was homogeneity of variances, as determined by Levene's test ($p = 0.164$). An ANOVA was then conducted to compare velar ratio and total velar area between the four treatment groups. There were no outliers in the velar ratio data, as determined by inspection of a boxplot; all data were normally distributed as determined by Shapiro-Wilk's test ($p \geq 0.137$); and there was homogeneity of variances ($p = 0.708$) as determined by Levene's test. An ANOVA was run with a post-hoc one-tailed Dunnett's test to compare the control group (unweighted) with each of the three weighted groups with the expectation that the velar ratio of the control would be less than the weighted groups.

One outlier was identified in the total velar area data and an ANOVA with a post-hoc one-tailed Dunnett's was run with and without the outlier to compare the control group (unweighted) with each of the three weighted groups, with the expectation that the total velar area of the control group would be less than the weighted groups. Regardless of

inclusion or exclusion of the outlier, all data were normally distributed as determined by Shapiro-Wilk's test ($p \geq 0.083$), and variances were approximately homogeneous ($p \geq 0.03$) as determined by Levene's test.

RESULTS

A linear regression of literature measurements demonstrated that total shell area significantly predicted total velar area for bi-lobed veligers, explaining 63% of variation (adjusted $R^2 = 0.634$, $F_{1, 31} = 56.521$, $p < 0.0005$; Table 4.3, Figure 4.3A, 4.3B). For multi-lobed veligers, a linear regression demonstrated that, like bi-lobed veligers, total velar area increases with total shell area, explaining 92% of the variation (adjusted $R^2 = 0.915$, $F_{1, 36} = 400.615$, $p < 0.0005$; Table 4.3, Figure 4.3B). Multi-lobed veligers have a greater ratio of velar area to shell area (Figure 4.3C, Table 4.2).

Table 4.3. Regression equations and statistics.

	equation	*L. scutulata* adj. equation	Adj. R^2	(df); F	*p*
Bi-lobed					
Shell area vs. velar area	y = 1.702x + 41575.592	y = 1.702x – 26401.675	0.634	(1, 31); 56.521	<0.0005
Shell ratio vs. velar ratio	y = 0.209x + 0.924	y = 0.209x + 0.890	0.582	(1,47); 67.878	<0.0005
Multi-lobed					
Shell area vs. velar area	y = 8.078x – 878945.32	n/a	0.915	(1, 36); 400.615	<0.0005
Shell ratio vs. velar ratio	y = -0.014x + 1.067	n/a	0.002	(1, 52); 1.132	0.292

A linear regression demonstrated that shell ratio significantly predicted velar ratio for bi-lobed veligers, explaining 58% of variation (adjusted $R^2 = 0.582$, $F_{1, 47} = 67.878$, $p < 0.0005$; Table 4.3, Figure 4.4); as shells become more asymmetric relative to the velar midline, so does the relative size of the two velar lobes with the larger side corresponding

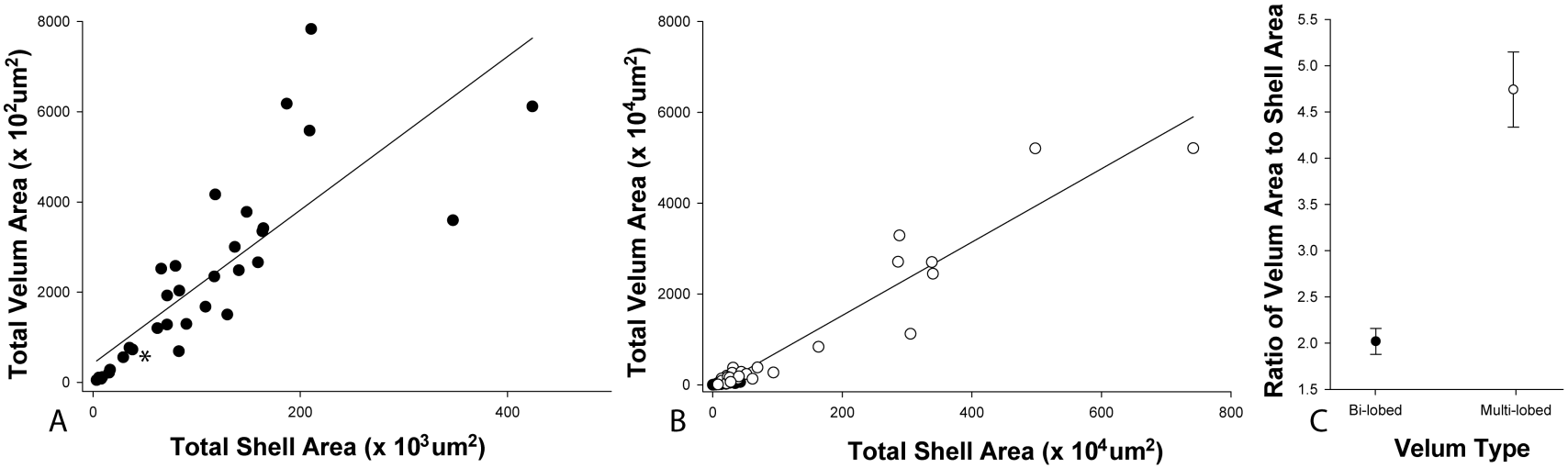


Figure 4.3. Total velar area increases with total shell area for bi-lobed (A, B; filled circles; N=33) and for multi-lobed (B; open circles; N=38) veligers. The data point representing *L. scutulata* (*) is shown relative to the bi-lobed regression line in (A). Note the difference in scale between (A) and (B) and the location of the bi-lobed data in (B) (bottom left corner). (C) Multi-lobed veligers (N=54) have a greater ratio of velar area to shell area than bi-lobed veligers (N=49). Error bars are ± 1 SE.

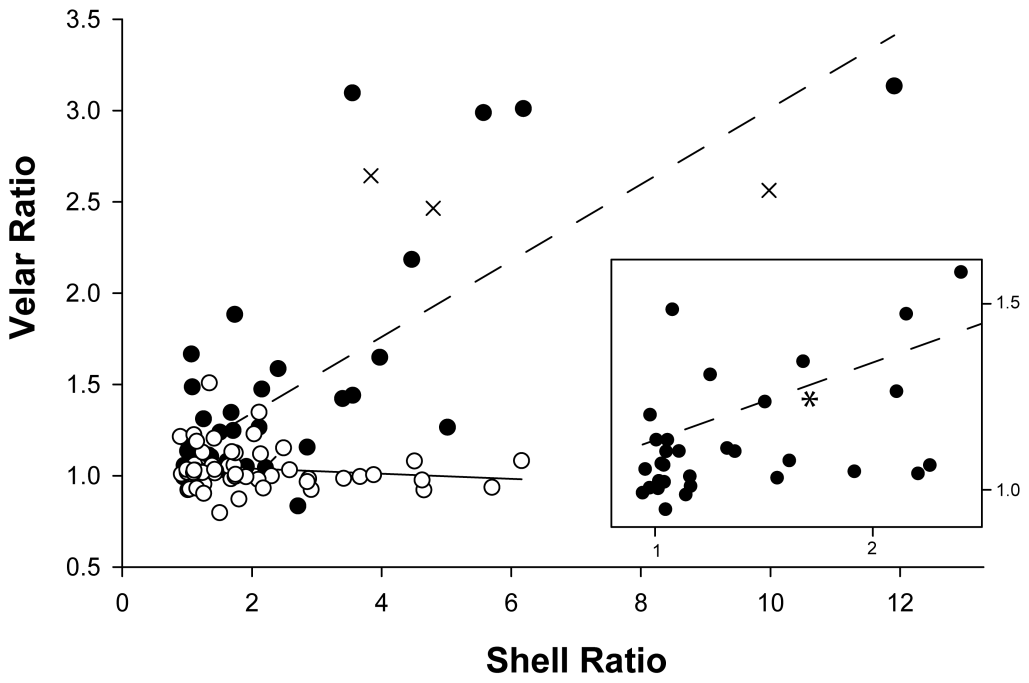


Figure 4.4. The ratio of area of the two sides of the velum vs. the ratio of area of the two sides of the shell relative to the velar midline. The asymmetry in velar area increases with asymmetry in shell area for bi-lobed veligers (filled circles; N=49) with the larger side of the velum corresponding with the larger side of the shell but not for multi-lobed veligers (open circles; N=54). Sinistrally-coiling bi-lobed veliger shells are indicated with an 'x'. The position of the data point representing *L. scutulata* (*) relative to the regression line of the bi-lobed data is shown inset.

to the side with a larger shell area. Interestingly, unlike bi-lobed veligers, shell asymmetry does not predict velar asymmetry for multi-lobed veligers (adjusted $R^2 = 0.002$, $F_{1, 52} = 1.132$, $p = 0.292$; Table 4.3, Figure 4.4): despite often large asymmetries in shell area relative to the velar midline, velar area remained approximately equally distributed.

Area increases with shell length for both the right and left velar lobes of *L. scutulata*; however, the increase in area of the right lobe is greater than that of the left lobe, resulting in a growing asymmetry between the two (Figure 4.5). Soon after hatching (~190 μm in shell length), the two lobes are approximately equal in size. By a shell length of 280 μm, the right lobe is ~27% larger than the left. Between shell lengths of 325 μm and the maximum

measured shell length (334 μm), the right lobe is 30% larger than the left, although this species can reach larval shell lengths of ~350 μm.

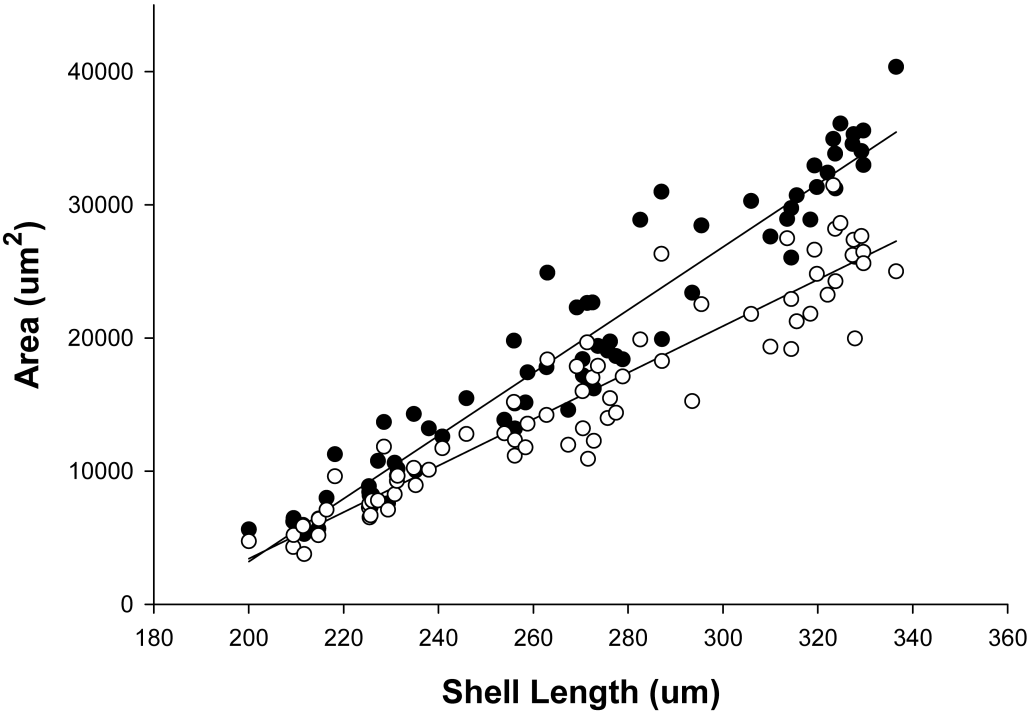


Figure 4.5. Velar asymmetry in veligers of *Littorina scutulata*. The relative areas of the left velar lobe (open circles) and right velar lobe (filled circles) gradually become more asymmetric as shell length increases.

From the start, all veligers with the smallest diameter bead (100 μm) and some with the medium diameter bead (140 μm) were observed to be capable of vertical swimming. Veligers with the largest diameter bead (180 μm) could not yet swim but instead spun in place with some horizontal movement. By four days following set-up, most of the veligers with the largest diameter bead were observed to be capable of vertical swimming although it was clearly difficult for them: in attempting to lift off the substrate, they often initially pivoted around the grounded weight, ending up velar-side down. When they successfully ascended off of the bottom, they frequently stopped swimming soon after and sank back down. When swimming, they regularly wobbled out of the relatively-straight vertical path expected of a typical unweighted veliger. Horizontal swimming paths for a veliger 325 μm

in length bearing the largest diameter bead (Figure 4.6A) vs. an unweighted veliger of the same size (Figure 4.6B) illustrate the difference in difficulty of swimming, both in duration and velocity: although both veligers were filmed over ~5 seconds of swimming, the average swimming speed was 2.6 mm/s for the unweighted veliger (with a maximum speed of 8.3 mm/s attained over a distance of 0.278 mm in 1/30 sec) and only 0.9 mm/s for the weighted veliger (with a maximum of 3.7 mm/s attained over a distance of 0.125 mm in 1/30 sec).

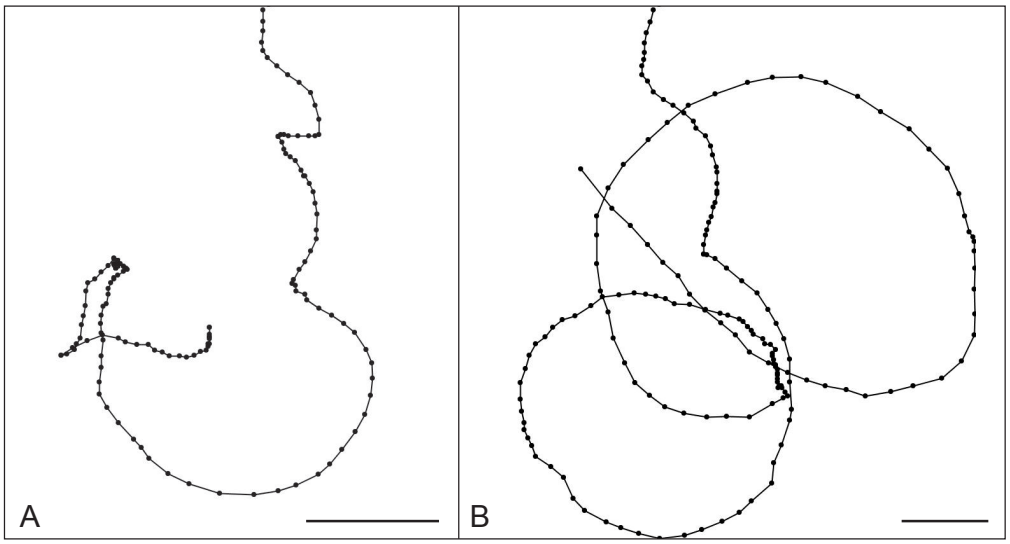


Figure 4.6. Horizontal swimming paths of an unweighted (A) and weighted veliger (B) of the same size over 5 seconds when viewed from above. The weighted veliger is carrying the largest diameter bead (180 μm). Each point represents one frame of video taken at 30 fps. The average velocities for the weighted and unweighted veliger were 0.9 and 2.6 mm/s with maximum velocities of 3.7 and 8.3 mm/s, respectively. Scale bars = 500 μm.

For veligers with experimentally weighted shells, shell lengths did not differ significantly between treatment groups ($p = 0.634$). The overall ANOVA model for the analysis of total velar area was not significant ($p = 0.110$, Welch's $p = 0.229$) with and without the outlier, respectively. Inclusion of the outlier yielded a significant difference in total velar area between unweighted veligers (58622 μm^2 ± 2150 SE) and the 100 μm group (70332 μm^2 ± 4557 SE, $p = 0.039$) but was not significantly different from the 140 μm (65003 μm^2 ± 4817 SE, $p = 0.240$) and 180 μm (59825 μm^2 ± 2280 SE, $p = 0.654$) groups

(Figure 4.7). Upon removal of the outlier, there was no longer a significant difference between the unweighted group and the 100 μm group (p = 0.085), and the difference between the control and the other two groups remained insignificant (for the 140 μm group, p = 0.378; for the 180 μm group, p = 0.784; Figure 4.7). The predicted values for the groups bearing the small, medium and large beads generated using the regression equation of bi-lobed literature measurements (see methods) were 76,766 μm^2, 98,861 μm^2, and 116,453 μm^2, respectively (Figure 4.7).

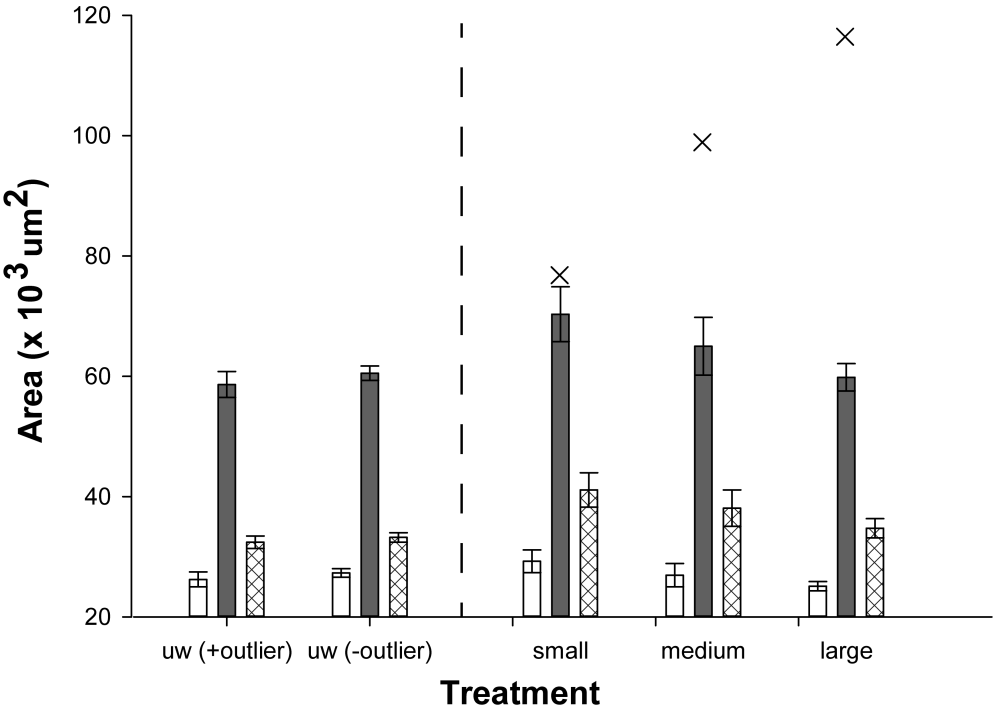


Figure 4.7. Left lobe area (open bars), total velar area (gray bars), and right lobe area (cross-hatched bars) for unweighted veligers (uw) vs. veligers bearing small (100 μm), medium (140 μm) and large (180 μm) beads. The significant difference between the unweighted and small-weight group depends on inclusion of an outlier in the unweighted data. Predicted values (X) were generated using the slope of the regression equation for total shell area vs. total velar area for bi-lobed veligers surveyed in the literature (Table 4.3); the equation was adjusted (see methods) for predictions specific to the *L. scutulata* data point (* in Figure 4.3A). Error bars are ± 1 SE.

The overall ANOVA model for the analysis of velar ratio was nearly significant (p = 0.056) and post-hoc tests showed that the velar ratio of unweighted veligers (1.25 ± 0.04

SE) was significantly smaller than both the 100 µm (1.41 ± 0.06 SE, $p = 0.026$) and 140 µm (1.41 ± 0.05 SE, $p = 0.023$) groups (Figure 4.8). The difference in velar ratios between the unweighted control group and the 180 µm group (1.38 ± 0.04) was not significant ($p = 0.057$; Figure 4.8). The predicted values for the groups bearing the small, medium and large beads generated using the regression equation of bi-lobed literature measurements (see methods) were 1.40, 1.52, and 1.73, respectively (Figure 4.8).

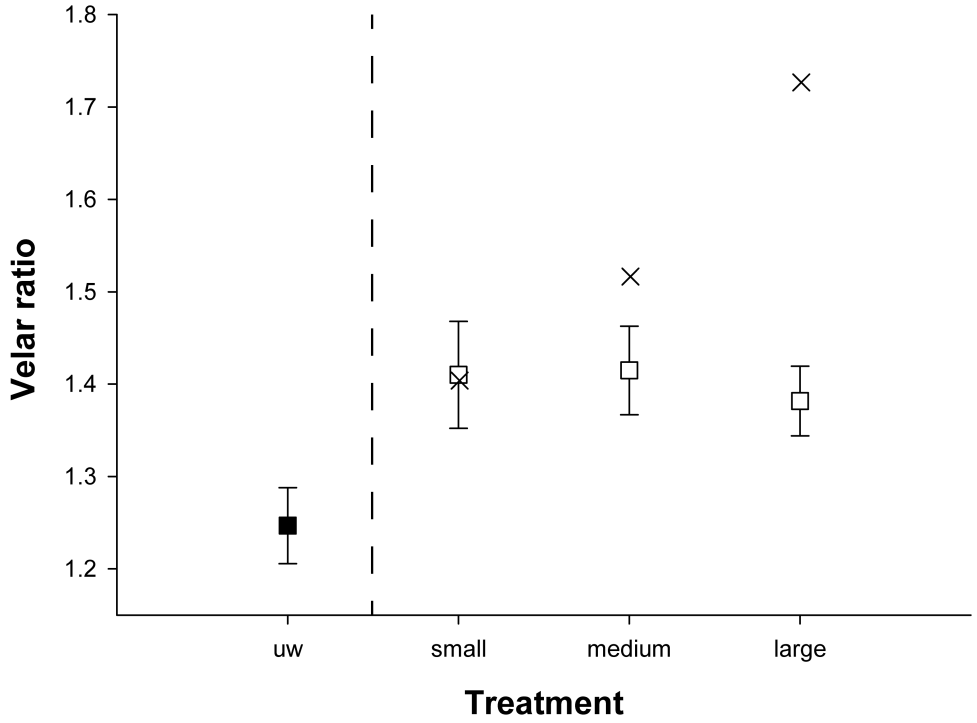


Figure 4.8. Ratio of right lobe velar area to left lobe velar area for unweighted veligers (uw) and veligers bearing small (100 µm), medium (140 µm) and large (180 µm) beads. The velar ratio of the small and medium groups was significantly different from that of unweighted veligers. Predicted values (X) were generated using the slope of the regression equation for shell ratio vs. velar ratio for bi-lobed veligers surveyed in the literature (Table 4.3); the equation was adjusted (see methods) for predictions specific to the *L. scutulata* data point (* in Figure 4.4 inset). Error bars are ± 1 SE.

DISCUSSION

Literature survey of velar asymmetry

Measurements from the literature indicate that velar asymmetry is common in several gastropod families (e.g., Cerithiopsidae, Collumbellidae, Muricidae, Raphitomidae,

Triphoridae; Table 4.1) with the spire of the shell protruding to the right or left side of the larva depending on the direction of shell coiling and corresponding with the larger side of the asymmetrical velum.

For both groups, as total shell area increases, so does total velar area (Figure 4.3A, 4.3B). For bi-lobed veligers, as weight distribution becomes more asymmetric (as indicated by shell asymmetry relative to the velar midline), the asymmetry in velar lobe area also increases, with the larger lobe co-occurring with the side of larger shell area (Figure 4.4). Interestingly, for most instances of veligers exhibiting multiple pairs of lobes, they are more or less of equal area on both sides despite a commonly large disparity in weight distribution (Figure 4.4). It is apparent from the literature measurements that multi-lobed veligers tend to be larger in shell size with correspondingly larger velar area: the largest total velar area of any bi-lobed veliger measured was ~780,000 μm^2, far less than nearly all of the total velar areas measured for multi-lobed veligers. Correspondingly, the smallest measured total shell area for a multi-lobed veliger (~82,300 μm^2) is greater than nearly half of the total shell areas measured for bi-lobed veligers and the largest total shell area for a multi-lobed veliger (~7,420,000 μm^2) is ~17X greater than the largest measured total shell area for a bi-lobed veliger.

A larger body necessitates increases in propulsive capabilities and food capture, two characteristics directly influenced by the length of the ciliated band (e.g., Strathmann and Leise, 1979; Chia *et al*., 1984; Gallager, 1988; Hansen and Ockelmann, 1991). Compared to bi-lobed veligers, those with multi-lobed vela have a greater velar area (and therefore a longer ciliated band) relative to the size of the shell (Figure 4.3C, Table 4.2) and they are able to effectively increase the velar perimeter relative to surface area through the possession of multiple extensions of velar tissue. The augmented propulsion of a longer ciliated band may decrease or negate the effects of an unbalanced weight distribution.

Dividing a larger velar area (supporting the larger shell) into multiple, often long, lobes with low elevations likely increases weight-carrying capacity (e.g., Grünbaum and Strathmann, 2003) and may also reduce vulnerability to shear relative to the surface area of a bi-lobed velum bearing the same perimeter length. In addition, studies have indicated that veligers can exert fine control over ciliary beat and velar musculature (e.g., Mackie *et al*., 1976; Arkett *et al*., 1987; Kuang and Goldberg, 2001; Braubach *et al*., 2006; Strathmann *et al*., 2014); the possession of multiple independently orienting lobes may also give the veliger finer control over directed movements. All of these characteristics suggest multi-lobed veligers with highly asymmetric shells can overcome the effects of asymmetrical weight distribution without a corresponding asymmetry in velar proportions.

The apparent changes in velar morphology with increases in shell size also correlate with the amount of time spent in the plankton. Although definitive planktonic durations for many gastropod veligers have yet to be determined, small bi-lobed vela are indicative of short larval durations while large elaborate vela are associated with long larval durations and settlement at larger sizes (e.g., Lebour, 1932, 1937; Strathmann and Strathmann, 2007).

Collectively, all figures measured from the literature suggest that veligers, either through preference or physical restriction, orient their shell axis more or less perpendicular to the cephalopedal axis and the cephalopedal axis more or less corresponds to the dorsoventral velar axis. As it would be gravitationally favorable to hold the shell axis in the same plane as the velar axis, it is interesting that this was only observed in one instance: A veliger of *Philbertia leufroyi* was depicted as holding its shell axis parallel with the central velar axis, more or less equalizing the amount of weight beneath each side of the velum despite having a conical shell with a large spire (Lebour, 1934b). Similarly, many adult snails have evolved improved shell balance by carrying their shell so its center of gravity is aligned with the cephalopedal axis. This is accomplished by tilting the axis of coiling upward

and/or regulatory detorsion, (a process which decreases the angle between the shell axis and cephalopedal axis by rotating the spire of the shell more posteriorly or anteriorly in dextral vs. sinistral coiling shells, respectively) (e.g., Linsley, 1977; Okajima and Chiba, 2011, 2012). A study by Romero *et al*. (2004) reported a change in orientation of the cephalopedal axis relative to the shell from what was typically observed in larvae to that of adults directly following metamorphosis. The reasons for the apparent difference in shell orientation between larval gastropods and adults is unclear but could reflect physical differences in larval vs. adult musculature and their limitations, or, alternatively, could be a behavioral artifact that enables a faster retreat of both the velum and cephalopedal mass into the larval shell.

Experimental manipulation

> "Why don't you stretch out on the sofa, so's you can rest your handicap bag on the pillows, honeybunch." She was referring to the forty-seven pounds of birdshot in a canvas bag, which was padlocked around George's neck...George weighed the bag with his hands. "I don't mind it," he said. "I don't notice it any more. It's just a part of me."
>
> Vonnegut (1961, p. 764-765)

I found myself in the role of the 'handicapper general', taking these balanced swimmers and curbing their perfection through the addition of weights. Just as George grew accustomed to his 'handicap bag', so too did the veligers adjust to theirs. In order to regain pre-weighted swimming abilities, I expected that the velar lobes would increase allometrically (with a larger amount of growth occurring for the lobe coinciding with the location of the added weight) as well as in total velar area in proportion to the amount of added weight.

Veligers were still capable of swimming even with a fairly large proportion of additional weight attached. For planktotrophic veligers, the velum functions both as a swimming structure and a feeding structure (e.g., Strathmann, 1987) and is often far enlarged and more complex compared to the vela of many non-feeding lecithotrophic

veligers (e.g., Fretter, 1967; Strathmann, 1978; Hickman, 1992; Moran, 1997; Hadfield *et al*., 1997; Strathmann and Grünbaum, 2006); therefore, the ability to swim even with excess weight may be due in part to the possession of an inherently larger feeding (and therefore swimming) structure. However, although the veligers can technically carry the weight, the amount by which the velum would need to grow to return to pre-weighted swimming abilities (e.g., speed, maneuverability) is unknown (e.g., see McDonald and Grünbaum, 2010).

The total velar area increased significantly only for the group with the smallest diameter bead (100 μm) and only with the individual larval outlier included (Figure 4.7). This increase of 20% compared to the total velar area of unweighted veligers falls within the range of food-induced plasticity previously observed for veligers of the same species: veligers raised in a low food environment exhibited velar areas 36% larger than veligers raised in a high food environment (data from Chapter III). An increase in total velar area for unweighted veligers proportional to that observed from the food study (+ 36%) falls within the observed range including the predicted value for the group bearing the smallest diameter bead. In order to reach the predicted increase in total velar area for veligers carrying the medium diameter bead (140 μm), there would have to be an increase of 69%. Similarly, in order to reach the predicted increase in total velar area for veligers carrying the largest diameter bead (180 μm), there would have to be an increase of nearly 100%. The prospect of reaching the predicted velar areas for veligers carrying the medium and large diameter beads likely exceeds the capabilities of veligers of *L. scutulata*, and, although both of these groups did show increases in velar area, the amounts were small and not significant (+11% and 2%, respectively).

In comparing the differences in size of individual velar lobes between veligers raised in a low vs. high food environment, both lobes are ~35% larger in a food-limited

veliger compared to a veliger from a high food environment (data from Chapter III). This indicates that when shell asymmetry is unchanged, the velar lobes increase equally in size. The ratio between the area of the two velar lobes increased when weight was added to the side of the shell with the slightly larger velar lobe, but, similar to the results for total velar area, the amount of increase appears to reach a limit and was not correlated with the amount of added weight (Figure 4.8). The predicted increase in velar ratio with an increase in shell asymmetry fell directly within the range of plasticity observed for the veliger carrying the smallest diameter bead, a change in velar proportions that was significantly different from unweighted veligers. A significant change in velar ratio was found also for veligers carrying medium diameter beads, but the increase in velar ratio fell short of the value predicted, again indicating a limitation of response despite increasingly exaggerated changes in weight distribution. Although not all changes were significant, all three groups show a disparity in individual lobe growth (Figure 4.7). For example, veligers carrying the smallest diameter bead showed an increase of 27% for right velar lobe area and only an increase of 12% for left velar lobe area, indicating that although both lobes were growing, the lobe corresponding with the weighted side grew nearly 2X as much. Only in the small weight group was there enough combined growth to achieve a significant increase in total velar area.

The reasons for the limited allometric response for the different weight groups and lack of significant increase in total velar area for the groups with intermediate and largest additional weights (relative to the control) are unclear but may be due to species-specific limitations, behavioral covariates, or inequitable exposure to the gravitational cues (the increase in weight and change in weight distribution) due to differences in swimming frequency/duration; although veligers with the largest weights were eventually capable of vertical ascent, they spent less time suspended off of the bottom and the horizontal

swimming paths indicated shorter strained durations at a much-reduced velocity (Figure 4.6).

It would be interesting to repeat this study but more closely follow velar dimensions to determine how fine-tuned the changes in area are between each lobe and for total velar growth; e.g., from the onset of the study, do both lobes continue to grow, albeit the right lobe at a faster pace than the left, is there an initial cessation in growth of the left and continued or enhanced growth of the right, and how does this balance change over the time allotted? In addition, the initial hypothesis was that weights glued to the spire side of larval shells would exaggerate the difference in size between the right and left velar lobes. A correlated hypothesis is that weights glued to the umbilicus side could either equalize a pre-existing asymmetry between velar lobes or even inverse the ratio, creating a larger left velar lobe on a larva with a dextral shell or a larger right velar lobe on a larva with a sinistral shell. I initially tried to glue weights to both the spire- and umbilicus-side of the larval shells of *L. scutulata*, but quickly realized that weights glued to the concave umbilicus side of the shell often trapped air bubbles and occluded the operculum from opening completely. This approach may be more feasible on larger veligers and/or veligers with different shell morphologies.

Growth of the velum overall and growth of its component lobes utilize the same mechanisms (e.g., cell division and/or cell stretching, see Chapter III), therefore plasticity for overall growth can likely co-occur with plasticity for allometric growth even if environmental pressure is normally lacking. Both the sensory requirements and mechanistic foundation are already in place - the only necessary addition is to be able to instruct one lobe to grow more than the other. Plasticity in response to food was observed as total velar growth for veligers of *Littorina scutulata*, with relatively equal increases of both velar lobes (Chapter III). This study demonstrates that this species is also capable of

allometric growth in response to an artificially-imposed weight, exaggerating a slight preexisting asymmetry. In combination with results obtained from measurements of veligers from the literature, this study corroborates the hypothesis that the natural occurrence of asymmetric velar lobes is to offset the unequal weight distribution of a helically-coiled larval shell.

Bridge to Chapter V

The present study examined the plastic ability of a marine larva in response to one of many environmental stimuli encountered in a pelagic environment. However, environmental variation and the necessity to respond to it does not end upon settlement and metamorphosis. The juvenile organism remains subject to many of the same stressors but with added complications that come with an intertidal lifestyle, including increased sun exposure.

CHAPTER V

SUNLIGHT AND COLORATION IN THE PURPLE URCHIN *STRONGYLOCENTROTUS PURPURATUS*

This chapter represents work done in collaboration with Laurel Hiebert and Bailey Counts: Laurel Hiebert helped plan and implement the rearing study and field-collected comparison study. Bailey Counts helped develop and implement the methods for comparing pigments levels in urchin tube feet. I performed all statistical analyses and did all of the writing.

INTRODUCTION

Strongylocentrotus purpuratus is an urchin commonly found in the mid- to lower intertidal of rocky shores exposed to moderate to strong wave action (Durham *et al*., 1980; Pearse and Mooi, 2007). This species is named for its characteristic purple coloration; however, they do not start off this way. Newly-settled *S. purpuratus* are light green while juveniles can range in color from green to purple depending on the local habitat: green juveniles can be found on the underside of rocks in intertidal boulder fields whereas equivalently-sized purple juveniles are found in adjacent pit fields on the surfaces of rocky substrate (e.g., Swan, 1952; Ebert, 1967; personal observations; Figure 5.1). Swan (1952) first noted differences in color within individuals: some purple spines often had green cores but regenerated spines were purple throughout. While investigating the growth and repair of spines of *S. purpuratus,* Ebert (1967) also noted a color difference of green vs. purple juveniles between microhabitats: green urchins found in one tidepool were completely covered with shells, shell fragments, and algae while purple urchins found in a second tidepool were uncovered. This led him to suspect that light exposure might be the cause of the apparent difference in pigment composition. An initial test demonstrated that clipped spines of both purple and green urchins equally split between a dark vs. light treatment regenerated as green in the dark treatment and purple in the light treatment (Ebert, 1967).

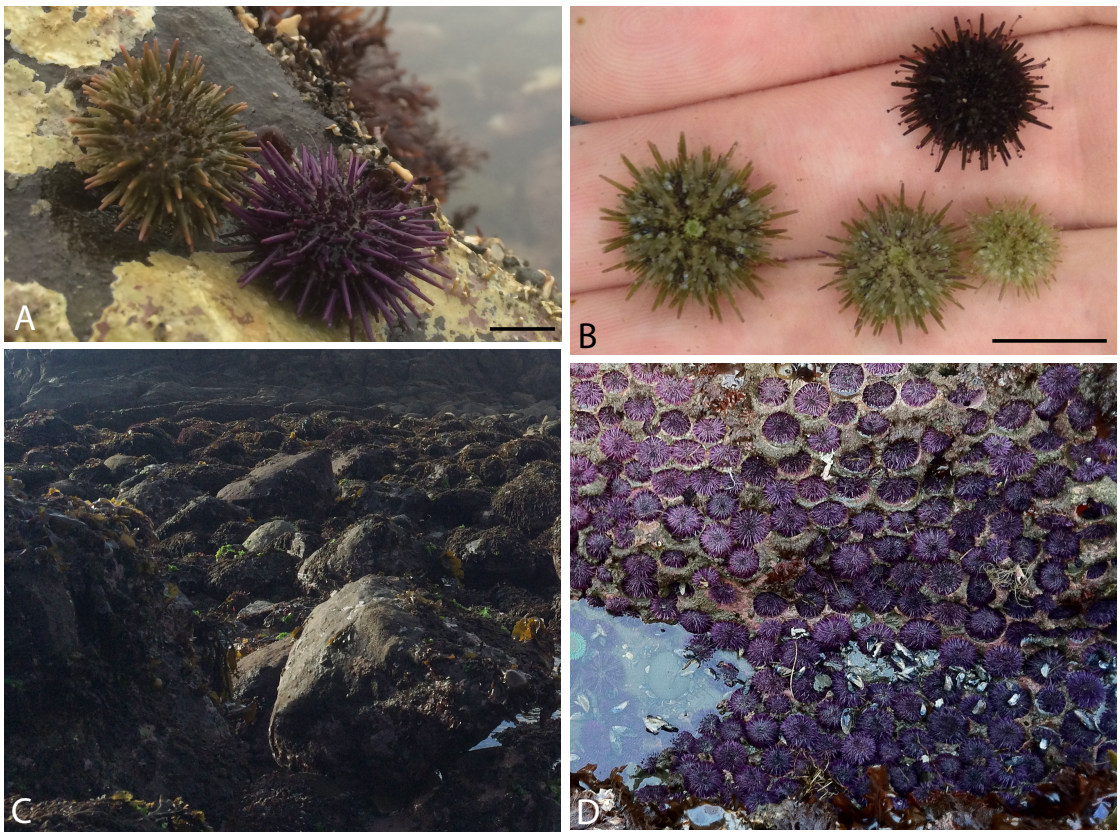


Figure 5.1. Green and purple juveniles of varying sizes (A and B) can be found in their respective habitats: on the underside of rocks in a boulder field habitat (C) or in adjacent pit fields located on the surface of rocky substrate (D). Scale bars = 10 mm.

The solar radiation reaching earth is categorized into three ranges based on wavelength frequency: ultraviolet (UVR; < 200-400 nm), photosynthetically active radiation (PAR; 400-700 nm), and infrared (>700 nm). Ultraviolet radiation is further subdivided into UVA (320-400 nm), UVB (280-320 nm), UVC (200-280 nm), and vacuum UV (< 200 nm) (e.g., Karentz, 1994). By the time it reaches earth's surface, 95% of ultraviolet radiation consists solely of UVA; the majority of UVB and all UVC and vacuum UV having been scattered or absorbed by atmospheric gases (e.g., ozone and nitrogen) (de Mora *et al*., 2000; Maverakis *et al*., 2010). Despite this, both UVA and especially the remaining UVB directly damage DNA and various proteins and also indirectly produce equally damaging reactive oxygen species (e.g., Vincent and Neale, 2000).

Color variation, both between and within species, is common in shallow water echinoids (e.g., Serafy, 1974; Marcus, 1983; Growns, 1989); however, the relationship between light exposure and urchin phenotype up to this point is mostly anecdotal. Most common in the literature is the well-documented shading behavior of urchins in which a variety of materials are held in place across the surface of the urchin by tube feet (e.g., Lees and Carter, 1972). This is most often attributed to protection from light (e.g., see Millott, 1975; Adams, 2001; Verling *et al*., 2002), although several other factors such as camouflage or protection from predators (e.g., see Millott, 1975; Amsler *et al*., 1999), protection from surge (Lees and Carter, 1972; James, 2000; Dumont *et al*., 2007), protection from desiccation/temperature (e.g., see Millott, 1975), and functions related to feeding (e.g., food collection/storage: Péquignat, 1966; Dix, 1970; Douglas, 1976) have been proposed. The studies by Adams (2001), Verling *et al.* (2002), and Dumont *et al*. (2007) showed that urchins exhibit significantly greater covering behavior in response to UVA and UVB radiation than to PAR alone. A study by Kehas *et al*. (2005) looked at the difference in the intensity of the covering response between normally pigmented wild-type *Tripneustes ventricosus* and naturally-occurring albino forms and found that the albino forms covered a significantly greater proportion of their aboral surface with provided material, implying that the lack of pigment conferred an increased susceptibility to the harmful effects of UVR. Similar results were found between the less pigmented species *Lytechinus variegatus* and a more pigmented species, *Arbacia punctulata* (Sharp and Gray, 1962). Covering intensity has also been correlated with the intensity of light as indicated by season (e.g., Moore *et al*., 1963) and time of day (Millott, 1956; Sharp and Gray, 1962).

Other studies have observed differences in color both between and within species correlating either with geography or microhabitat (e.g., Kristensen, 1964; see Anderson *et al*., 1969; Chesher, 1970; Serafy, 1974; Growns, 1989; see Kelly *et al*., 2013, etc.). For

example, urchins of *Echinometra mathaei* exhibit four distinct morphotypes whose darkness in color decreases with depth (Nishihira *et al*., 1991) and lighter colored urchins of *Heliocidaris erythrogramma* tend to be found under rocks, also exhibiting stronger covering behavior than the darker individuals found on the upper surfaces of rocks (Growns, 1989). A study by Kristensen (1964) showed that young individuals of *D. antillarum* kept in the dark were much lighter than individuals kept in ambient light, which developed the typical black dermis of this species in one to two months; this color difference is reflected in the depth range of this species, with shallower individuals being darker than ones found at depth.

Urchins, *S. purpuratus* in particular, represent a model system for the study of early development. They are also models for studies examining the effects of UVR on these processes and on the animals themselves, with the majority of work done on embryos and larvae (Lamare *et al*., 2011). Collectively, the protective strategies identified for echinoderms against UVR include behavioral avoidance, repair of damaged DNA, proteins or lipids, mitigation of oxidative stress (e.g., through the use of antioxidants), and the production of UVR-absorbing compounds (Lamare *et al*., 2011).

Of a variety of compounds known to directly and/or or indirectly provide protection from UV radiation, there are at least three known to occur in urchins: carotenoids, mycosporine-like amino acids (MAAs), and naphthoquinones.

Carotenoids are widespread lipid-based pigments known to act as protectants against the effects of UV radiation. They function as sunscreening agents, deal with UV-induced oxidative stress (e.g., as antioxidants or free radical scavengers), and help to dissipate excess energy (e.g., Britton, 1983; Edge *et al*., 1997; Mathews-Roth, 1997; Rastogi *et al*., 2010; Dahms and Lee, 2010). Exposure to UVR has been shown to stimulate enhanced production of carotenoids in cyanobacteria (e.g., Ehling-Schulz *et al*., 1997), phytoplankton

(e.g., Barlow *et al*., 2007), fungi (e.g., Libkind *et al*., 2004), plants (e.g., Merzlyak and Chivkunova, 2000), and macroalgae (e.g., Lee and Shiu, 2009); however, animals cannot produce carotenoids and must obtain them through their diet (Moeller *et al*., 2005). Most research concerning carotenoids and urchins has been focused on the gonads, where the highest concentrations are generally found (e.g., see Kelly and Symonds, 2013). A study done by Lamare and Hoffman (2004) was able to correlate higher sensitivity to UV radiation with decreased levels of carotenoids in the eggs of four species of urchins, including *S. purpuratus*. At least six different carotenoids have been isolated from *S. purpuratus* (Fox and Scheer, 1941; Griffiths, 1966; Lamare and Hoffman, 2004), although the only study not focusing on eggs/gonads only found traces of carotenoids in the skin (Fox and Scheer, 1941).

Mycosporine-like amino acids (MAAs) are small colorless compounds with strong absorption maxima occurring in both UVA and UVB wavelength ranges and are widely accepted as photoprotective compounds (e.g., Singh *et al*., 2008; Rastogi *et al.,* 2010; Dahms and Lee, 2010). Produced in response to UV radiation in many algae, phytoplankton, and cyanobacteria (e.g., Singh *et al*., 2008; Rastogi *et al.*, 2010; Rastogi and Incharoensakdi, 2014), primary and secondary consumers can selectively incorporate these compounds from their diet (e.g., Carroll and Shick, 1996; Whitehead *et al*., 2001; Adams *et al*., 2001; Gravem and Adams, 2012). Although much work has been done looking at MAAs in echinoderms, only in tropical holothurians has a high concentration been found in the epidermis (e.g., Shick *et al*., 1992; Bandaranayake and Rocher, 1999). In urchins, the largest concentrations found have been in the ovaries with only small to trace amounts in the body wall (e.g., Carroll and Shick, 1996; Karentz *et al*., 1997; McClintock and Karentz, 1999; Gravem and Adams, 2012). The explanation for this is that embryos and larvae are more vulnerable to UVR near the water's surface than the benthic adults. Even though a large

portion of UVR is reflected at the water surface or scattered/absorbed in the water column, UVB and UVA can still penetrate to depths greater than 16 m and 46 m, respectively (e.g., Tedetti and Sempéré, 2006), well within the depth range inhabited by many intertidal urchins. Urchins of *S. purpuratus* inhabit a range of 0 to 90 m but are most common in the mid to low intertidal where they are frequently in shallow-water or air-exposed for hours at time during low tides (e.g., Farmanfarmaian and Giese, 1963; Pearse and Mooi, 2007; personal observations). Although Gravem and Adams (2102) did find some association between epidermal MAA content and microhabitat-dependent sun exposure, the amounts of MAAs in the epidermis of *S. purpuratus* were relatively small compared to gonadal levels and to reported levels of MAAs in other echinoderms in general. What then, if not carotenoid or MAA pigments, is primarily protecting these urchins from the sun?

The color of echinoids is attributed primarily to the production of polyhydroxylated 1, 4-naphthoquinones, a type of quinone pigment (e.g., Goodwin, 1969; Anderson *et al*., 1969; Grossert, 1972; Needham, 1974; Fox, 1976; Britton, 1983) with the exception of some irregular urchins, whose color is largely due to the presence of carotenoids with or without naphthoquinones (Kawaguti and Yamasu, 1954; Tsushima and Matsuno, 1990). Quinones, also found in lichens, fungi, higher plants, and arthropods, are well known for their light-absorption properties, with at least one absorption maximum commonly occurring in UVR wavelengths (Spruit, 1949; Britton, 1983). The different forms of 1,4-naphthoquinones found in echinoderms have traditionally been referred to as echinochromes and spinochromes (e.g., Fox, 1976; Needham, 1974; Britton, 1983). Echinochrome A, first described by MacMunn (1885), can be found both in soft tissues and coelomic fluid (primarily within red spherule cells; e.g., Johnson, 1969; Smith *et al*., 1992; Johnstone, 2013) and calcareous parts (test and spines). Spinochromes are found only in the test and spines

in the form of calcium salts (e.g., Goodwin and Sriusukh, 1950; Goodwin, 1969; Anderson *et al*., 1969; Britton, 1983).

In the past, naphthoquinones have been suggested to function in respiration (see Tyler, 1939), in photoreception (e.g., Millot and Yoshida, 1957), as algistats (Vevers, 1963, 1966; Kittredge, 1971), or as antimicrobial agents (Johnson, 1969; Johnson and Chapman, 1970a, 1970b; Service and Wardlaw, 1984; Gerardi *et al*., 1990; Haug *et al*., 2002). Naphthoquinones are known to act as valuable antioxidants by scavenging harmful free radicals, as do carotenoids, and by chelating iron (Lebedev *et al*., 2005; Kuwahara *et al*., 2009; Zhou *et al*., 2011; Li *et al*., 2013; Pozharitskaya *et al*., 2013; Powell *et al*., 2014). A minimum of four naphthoquinone pigments including echinochrome A and spinochromes A, B and E have been found in *S. purpuratus*, all of which have been shown to have absorption maxima in the UVA/UVB range (Goodwin and Srisukh, 1950; Griffiths, 1965; Anderson *et al*., 1969; Kuwahara *et al*., 2009, 2010; Zhou *et al*., 2011; Powell *et al*., 2014). Despite the existence of persistent correlative evidence linking UVR protection and naphthoquinone pigments in urchins, a recent review of the history of UVR and echinoderms made no mention of it (Lamare *et al*., 2011). Due to their ability to absorb UVR and antioxidant properties, it is very likely that the production of naphthoquinone pigments may protect urchins against UVR exposure (e.g., Powell *et al*., 2014).

The induction of UVR-absorbing compound production or accumulation in response to UVR with a corresponding decrease in susceptibility to damage is a widespread phenomenon, occurring in cyanobacteria (e.g., Ehling-Schulz *et al.*, 1997; Rastogi and Incharoensakdi, 2014), nematodes (Baker *et al*., 2012), crustaceans (e.g., Hansson, 2000; Hansson *et al*., 2007; Hylander and Hansson, 2013), plants (e.g., Chalker-Scott, 1999; Merzlyak and Chivkunova, 2000; Zu *et al*., 2010; Ji *et al*., 2016), fish (see Leclercq *et al*., 2010), snails (Ahlgren *et al*., 2013), and humans (e.g., Friedmann and Gilchrest, 1987;

Miyamura *et al*., 2006; Costin and Hearing, 2007), to name a few. The study by Kristensen (1964) demonstrated light-induced pigment production in *Diadema antillarum* but the light was ambient and not specifically UVR, and consequent susceptibility to UVR was not determined, although the photoprotectant qualities of melanin (this urchin's primary pigment) are well established (e.g., Friedmann and Gilchrest, 1987; Kollias *et al*., 1991; Gilchrest *et al*., 1995; Costin and Hearing, 2007). Based on the variation in color of juvenile urchins of *S. purpuratus* depending on local microhabitat, we hypothesized that light, specifically ultraviolet radiation, is the link between habitat and color in juvenile urchins of this species and that the production of pigment is a phenotypically plastic protective response to light exposure.

To test the role of UVR in urchin coloration, juvenile *Strongylocentrotus purpuratus* of the green variety were collected from the field and kept under ambient solar radiation, ambient solar radiation filtered of UVR, or in the dark for ~three months. Pigment production was monitored over time via photographic assessment of a purple:green ratio and at the end of the study by a spectrophotometric measure of dermally extracted echinochrome levels with the expectation that UV radiation will produce purple urchins with a higher amount of pigment while urchins isolated from UV radiation will remain green in color (indicating less pigment). Cost of pigment production was evaluated by measuring changes in test size and spine length. In order to test the hypothesis that the production of pigment confers reduced susceptibility to damage by UVR, righting time and tube foot extension of urchins was measured following exposure to elevated UVA and UVB radiation at the end of the study. To ensure characteristics of urchins resulting from the rearing study were representative of color variants grown in the field, this assay was repeated for field-collected green and purple variants. The tube feet also exhibit differences in color and presumptive pigment levels both between and within individuals (Figure 5.2);

paler tube feet found on individuals collected from the sheltered boulder-field habitat are expected to contain less pigment than the darker tube feet found on individuals from the light-exposed urchin pits. Since urchins from the exposed pit environment are always oriented aboral-side up and appear to have lighter-colored tube feet on the more-sheltered oral side, we also hypothesized that there would be a difference in pigment levels of tube feet from the aboral vs. oral sides of purple urchins but that there would no difference in pigment levels between the aboral and oral sides of the generally sheltered green urchins.

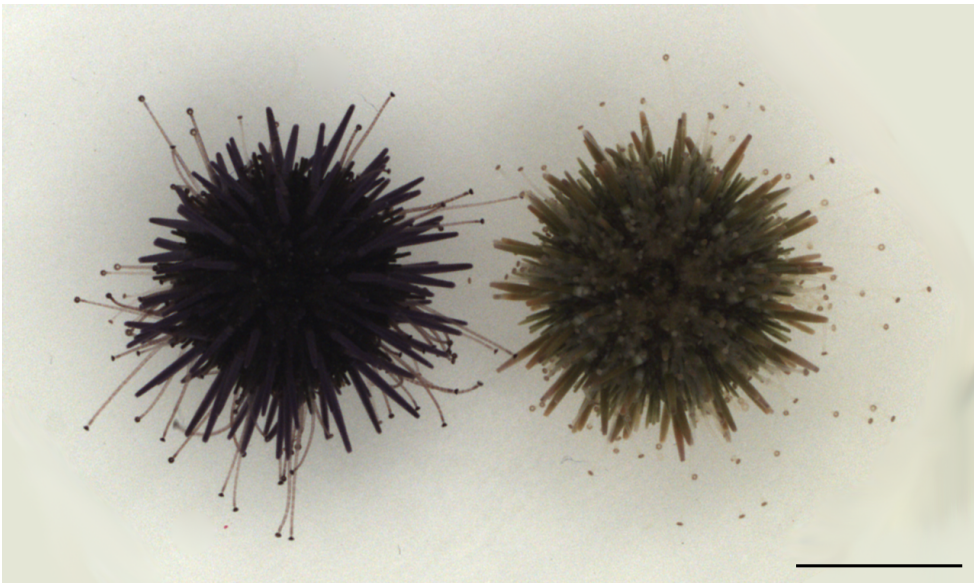


Figure 5.2. Green and purple juveniles displaying differences in overall color and in the amount of pigment located in the dark podia of purple juveniles and in the relative lack of pigment in the light podia of green juveniles. Scale bar = 10 mm.

MATERIALS AND METHODS

Rearing study set-up

Twenty-four juvenile green urchins of *Strongylocentrotus purpuratus*, ~2 cm in test diameter, were collected in late March, 2014, from the underside of large rocks in an intertidal boulder field at Cape Arago, Oregon (43°18'13.73"N, 124°24'3.09"W; Figure 5.1C). The urchins were kept in opaque containers in running seawater until the start of the study, not exceeding one week.

These urchins were randomly assigned to flow-through containers covered with different acrylic panels giving three different light treatments: dark (opaque cover - no light), -UV (OP3 cover – allows PAR but blocks 98% UVR), and +UV (OP4 cover – allows PAR and UVR). The OP3 and OP4 acrylic panels are manufactured by CYRO industries (Parsippany, NY). There were eight replicates per treatment. The urchins were individually kept in small, plastic containers with plastic-mesh bottoms and sides. The central portion of the container lid was cut-out, leaving only the locking rim of the lid, the top of which was glued to the underside of the appropriate type of acrylic panel. This container sat within a larger opaque container with an opening for a small hose providing individual water flow and a hole in the bottom where water and waste could exit (Figure 5.3A). The containers were suspended in an outdoor sea table lined with opaque black plastic. This set-up was designed primarily to completely restrict the light encountered by the urchins to that which did or did not enter from above (dependent on the acrylic panel type) and to restrict any leakage of light between adjacent treatments. Every third day the set-up was cleaned and the urchins were fed uniformly sized pieces of *Ulva* sp. Although urchins sometimes initially held the food over their aboral surface, the algae was always eaten or pulled underneath within one hour after presentation. Other than very brief exposures during set-up, cleaning, and transportation indoors for photography (< 15 seconds of outdoor conditions), and during photography (<two minutes of artificial lighting; see below), the light experienced was restricted to the experimental treatments. The study ran for 108 days, during which the urchins were photographed seven times. One urchin in the dark treatment group died after 87 days and was immediately frozen at -80°C.

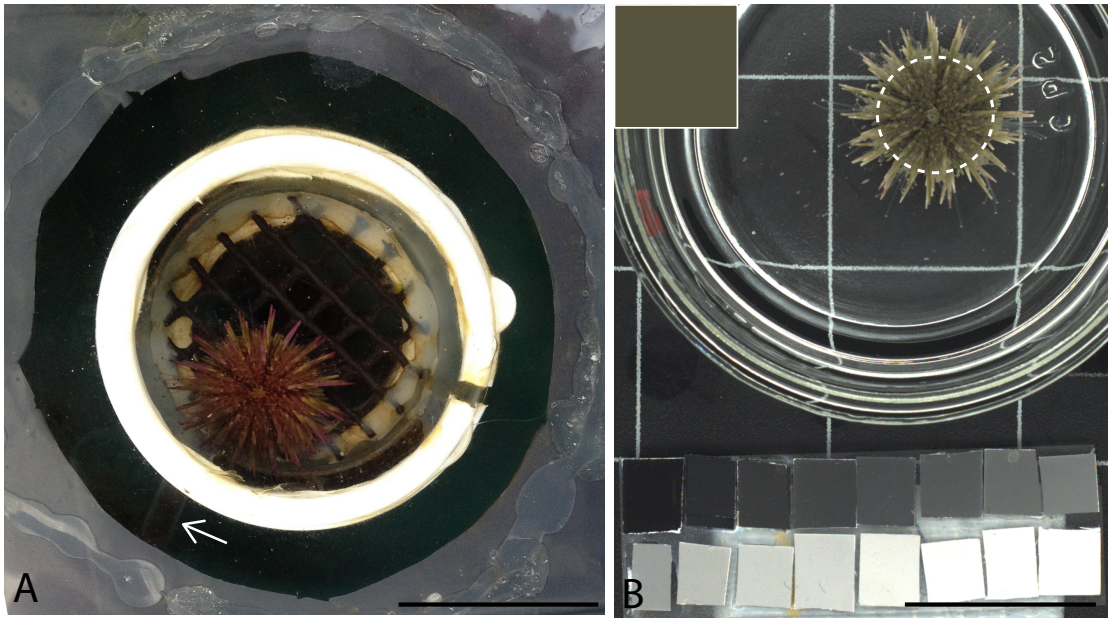


Figure 5.3. Rearing study set-up and color analysis. (A) Urchins were kept in small containers with mesh bottoms and sides and received individual water flow (white arrow). The containers sat inside larger opaque plastic pots (green). The centers of the container lids were removed and the remaining rim (white ring) was glued to the underside of the appropriate acrylic panel (Opaque, OP3, OP4). A shroud of black plastic extended from the edges of the panel to eliminate the leakage of light into/out of the containers (margins). (B) Urchin color was analyzed by photographing the urchins with a set of spectrally-flat gray standards (bottom). An averaging blur was applied and color intensity was measured for each of the gray calibration series and for an area of the urchin just inside the test margin (dashed line). The resulting color of the averaging blur for the urchin pictured is shown in the top left. The RGB intensity values and known reflectance values of the standards were then used to convert the intensity values of the urchin into RGB reflectance values. The ratio of (R +B)/G indicated whether an urchin was more green or purple in color. Scale bars = 30 mm.

Field-collected comparison

To corroborate that the characteristics of the urchins resulting from the rearing study (color, UVR susceptibility, pigment levels) were representative of naturally occurring purple and green juveniles, we sampled eight juvenile green urchins of *S. purpuratus* from the same boulder-field environment as before. Eight juvenile purple urchins of *S. purpuratus* were also collected from a nearby pit-field habitat (43°18'14.34"N, 124°24'5.25"W; Figure 5.1D), where the urchins experience more sunlight exposure. The methods used for photographing these urchins, analyzing the color ratios, assaying UVR susceptibility, and

pigment extraction and spectrophotometric measurement are all as described below for the rearing study except where noted.

Photography and color analysis

The change in color of the urchins was followed over time by initially and then periodically photographing the urchins with gray standards (Figure 5.3B). Overall methods for color analysis were modified from Tedore and Johnsen (2012). Urchins were transported indoors in beakers of seawater held within an opaque black box. Each urchin was just-submerged in filtered sea water (FSW) in a small glass finger bowl placed on a copy stand equipped with four 120V 75 watt daylight bulbs attached to the copy stand at a set height and distance from the urchin. The attachment fixture for the camera, a Grasshopper Express (digital firewire model GX-FW-28S5C; Point Grey Research Inc., Richmond, BC, Canada) with a Nikon AF Nikkor 35-70 mm f/3.3-4.5 lens, was also at a set height. Alongside the fingerbowl was a slide affixed with 16 squares from a gray series, ranging from black to white, of high-quality spectrally-flat standards (Color-aids Gray Set; Hudson Falls, NY, USA). To verify the spectral flatness of the standards, the reflectance of each was measured under an integrating sphere (ISP-REF, Ocean Optics, Dunedin, FL) connected by a fiber optic cable to a spectrometer (USB2000, Ocean Optics, Dunedin, FL, USA). To create an even and consistent light field, a cylinder of translucent white vellum was placed around the urchin and gray standards. Urchins were photographed using Astro IIDC imaging software (version 4.08.00; Aupperle Services and Contracting) with images saved in an uncompressed TIFF file format. The color balance was first established using the Astro IIDC imaging software by selecting a region of pixels within the lightest gray standard used without being overexposed and designating it as gray. This setting was saved and used for all future photographs.

For an 8-bit RGB image, each pixel contains separate intensity components for the

red, green, and blue channels expressed as integers ranging from 0-255. Once each photograph was imported into Adobe Photoshop CC (Adobe, Inc., San Jose, CA, USA), an averaging blur was applied to an equal and consistent area selected within each of the darkest nine gray series squares (those inclusive of the intensity range of the urchins); this tool calculates the average intensity of the RGB components for all inclusive pixels and applies the average values across the selected area. The average intensity of the R, G, and B channels for each gray series square was plotted against the measured reflectance values, resulting in a calibration curve for each channel that could be fitted with an exponential equation. Because the standards were spectrally flat, the average intensity values for the R, G, and B channels should be identical but separate curve-derived equations were still generated for each channel to ensure accuracy. Next, another averaging blur was applied to a circular area just inside the edge of the test of the urchin (Figure 5.3B). After the average intensity values were measured using the color picker tool, the appropriate calibration curve-derived equation was used to convert the R, G, and B intensity values of the urchin into reflectance values. To determine the difference in intensity of purple vs. green, the ratio of (R+B)/G reflectance was calculated for each urchin where an increase in this value represents an urchin becoming more purple in color.

Urchin test and spine growth

The initial and final test diameters and average spine length from the rearing study were measured from photos using ImageJ software (NIH, Bethesda, MD). Test size was measured as the shortest distance across the urchin through the center of the periproct. Average spine length was measured as the distance from the edge of the test to the tip of the three longest spines (Figure 5.4A).

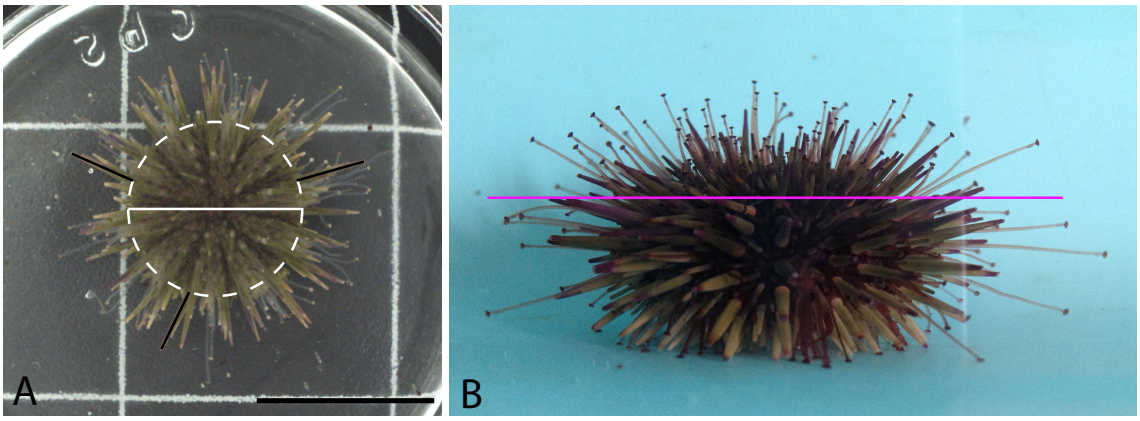


Figure 5.4. Growth and UVR susceptibility tube foot assay. (A) The change in test size was determined by measuring the shortest distance across the test through the central periproct (white line). The three longest spine lengths were measured from the edge of the test (dashed line) to the tip of the spine (black lines). (B) Prior to and following each of the UVR exposures, the urchins were photographed from the side so the number of tube feet extending from the upper third of the test + spine height (pink line) could later be counted in ImageJ. Scale bar = 20 mm.

UVR susceptibility

To determine if higher levels of pigment in the urchins resulting from the +UV and -UV treatments provided a protective benefit against UVR exposure compared to the urchins kept in the dark (and that remained green), we exposed urchins to artificial UVR. Due to limitations of time and space, the exposure study was done in four sets of six with each set containing two randomly chosen urchins from each treatment. As a positive control for the functionality of the UVR-filtering OP3 acrylic, one of the two urchins was randomly placed under either UVR-transmitting OP4 acrylic or the OP3 acrylic for the duration of the exposure experiment. Due to the death in the dark treatment group, one set had only five urchins with the single urchin from the dark treatment group placed under OP4 acrylic. This resulted in six 'conditions' of urchins (rearing treatment group / exposure group: Dark / -UV, Dark / +UV, -UV /-UV, -UV / +UV, +UV / -UV, +UV / +UV). The urchins were placed in individual glass bowls in a sea table with flowing water; the water level in the bowls was just enough for the urchins to remain submerged. The OP4 or OP3 acrylic panel was placed

over the bowl opening and was located 5 cm below the UVR source. The urchins were first exposed for one hour to UV light from two UVA-340 lamps, which closely mimic the levels and proportions of UVA and UVB light found in the natural solar spectrum at a slightly elevated level based on the distance from the bulbs; the output at the 340 nm peak is reported to be 0.83 W/m^2 at a distance of 5 cm (Q-lab Corp., West Lake, OH, USA). Immediately following exposure, righting time and tube foot extension were again assessed as described above. The urchins were then placed under a UVR gel transilluminator (Fotodyne Inc., Hartland, WI) with a peak emission of 300 nm for ½ hour; the intent of this exposure was not to mimic natural conditions but was to provide hypernatural UVB radiation to exacerbate differences in urchins with different pigment levels that may have been missed under the more natural UVA-340 UVR source. Prior to and immediately following each exposure, all urchins were photographed several times from the side so that the average number of tube feet extending from the aboral surface could later be counted (this was done using ImageJ software by counting the number of tube feet extended from the aboral surface above a line representing the aboral third of the urchin's test + spine height) (Figure 5.4B). After exposure the righting time of each urchin was then measured by placing the urchin oral-side up while submerged in FSW in a sea table and timing how long it took to flip upright (Figure 5.5). The average of three sequential flip times was used. Directly after the assays following the UVB exposure (within ½ hour), the urchins were halved with a dremel saw, the insides of the tests cleaned and rinsed, and each half was then immediately frozen and stored in a -80°C freezer until further use.

For the follow-up study comparing field-collected green (G) and purple (P) juveniles, four conditions were tested (ecotype / exposure group): P / -UV, P / +UV, G / -UV, G / +UV.

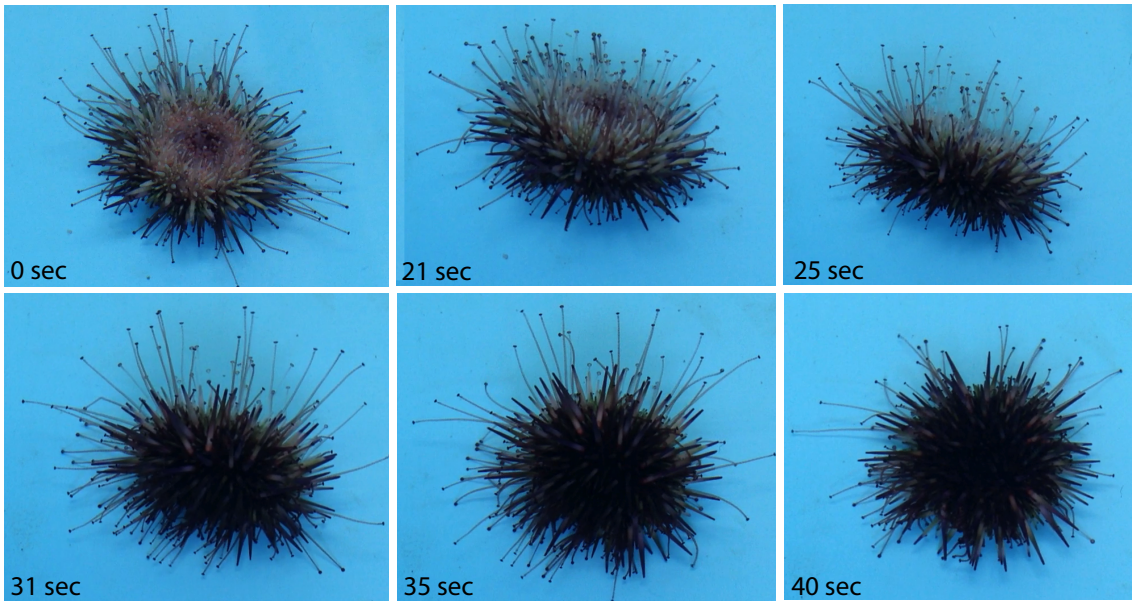


Figure 5.5. UVR susceptibility righting time assay. Prior to and following each of the UVR exposures, the time it took the urchin to right itself when placed oral-side up was measured. An example of the righting process over the course of 40 seconds is shown above.

Pigment extraction and absorbance measurements – rearing study

The method for obtaining a crude extract of dermal pigment was modeled after Bay *et al*. (1983) and Growns (1989). A pre-weighed 0.2 g piece of each frozen urchin (from a consistent location on the aboral side) was placed in 5 mL of acidified ethanol (1:3 dilution of 25% HCl in 95% EtOH). The sample was gently swirled for 20 seconds and then pushed through a 0.45 µm filter into a UV/vis cuvette. The absorption spectrum between 200-600 nm was then immediately measured using a BioSpec-mini spectrophotometer (Shimadzu, Japan).

The absorbance values analyzed (indicative of pigment amounts) were those at two of the three peaks identified by the spectrophotometer for each sample (see statistical methods below). Although a previous method compared echinochrome levels by looking at the absorption for the visible peak alone (Bay *et al*., 1981, 1983), we also included one of the two ultraviolet peaks. For all absorbance results, peak 1 is located at a λ of ~325 nm and

peak 2 at a λ of ~479 nm and will be referred to as peak 1 and peak 2 from here on forward. The locations of the peaks naturally vary slightly from sample to sample regardless of urchin treatment or condition (for example, from the rearing study, the average location of peak 1 and 2 was 322.6 nm ± 1.13 SE and 478.9 nm ± 0.18 SE, respectively).

Pigment extraction and absorbance measurements – tube feet

Fifteen urchins of each ecotype were collected from the aforementioned boulder field and pit habitats. Only urchins at least 40 mm in test diameter were collected due to the potential complications of assaying small tube feet. These larger boulder-field urchins are overall darker in color than the green juveniles but still much lighter in color than equivalently-sized urchins from the adjacent pit habitat; the tube feet, however, are often just as light as those seen on the juveniles. The urchins were brought back to the lab where they were kept in running seawater in the dark. All urchins were processed within one week beginning with one of the two smallest urchins (see below) and continuing randomly. Urchins were measured using calipers and the average size (diameter in mm ± SE) of the green and purple urchins was 48.1 ± 1.2 and 47.8 ± 1.2, respectively. Using fine scissors, 20 randomly-selected extended tube feet and suckers were cut as close to the base of the podia as possible from both the aboral and oral sides and were placed in separate pre-weighed vials. Any excess seawater was removed with a fine pipette. After being homogenized as much as possible using a plastic pestle, each vial was weighed and, when necessary, some of the homogenate was removed to adjust the weight to the lowest measured tube foot weight representing the smallest urchin (12 mg). A volume of 3 mL of acidified ethanol (1:3 dilution of 25% HCl in 95% EtOH) was added to the vial and gently swirled for one minute. The extract was then pulled into a syringe and pushed through a 0.45 μm filter into a UV/vis cuvette. The absorption spectrum between 200-600 nm was then immediately measured

and the absorbance values at the reported peaks for tube feet from the four conditions (P_Aboral, P_Oral, G_Aboral, G_Oral) were recorded.

Statistical methods

The color ratio results were analyzed with a repeated measures ANOVA in SPSS with Bonferroni-adjusted post-hoc pairwise comparisons at each date. The assumption of sphericity was violated, as determined by Mauchly's test of sphericity, $p < 0.0005$; Therefore, a Huynh-Feldt correction was applied ($\varepsilon = 0.683$). Paired t-tests were used to determine if, within each treatment group, there was a significant change in color between the beginning and end of the study. The data representing the difference between the beginning and end within each treatment group were normally distributed, as determined by Shapiro-Wilk's test ($p \geq 0.207$), and had no outliers, as determined by boxplot inspection.

The color ratio results between the field-collected green and purple juveniles of *S. purpuratus* were analyzed using an independent samples t-test. No outliers were found, as determined by boxplot inspection, and the data for both groups were normally distributed, as determined by Shapiro-Wilk's test ($p \geq 0.212$). Equal variances were not assumed as determined by Levene's test ($p = 0.021$).

A one-way ANOVA with post-hoc Tukey analysis was used to analyze the change in size of both test diameter and spine length. Data representing the difference in test diameter and spine length from the beginning of the study to the end of the study were normally distributed for both variables as determined by Shapiro-Wilk's test ($p \geq 0.059$), no outliers were found as determined by boxplot inspection, and equality of variances was determined by Levene's test ($p \geq 0.093$).

To analyze the UVR susceptibility of urchins from the rearing study, repeated measures ANOVAs in SPSS were used to compare the differences in righting time and tube foot extension between the six conditions (Dark / -UV, Dark / +UV, -UV / -UV, -UV / +UV,

+UV / -UV, +UV / +UV) before, following exposure to the UVA-340 light source, and following exposure to the intense UVB. The assumption of sphericity was violated for the righting time data, as determined by Mauchly's test of sphericity, $p < 0.0005$; therefore, a Huynh-Feldt correction was applied ($\varepsilon = 0.665$). Sphericity was not violated for the tube foot extension data ($p = 0.434$). At each measurement point, a special a-priori contrast was used to test whether the Dark / +UV urchins differed in their righting time and tube foot extension from the other groups, which were not expected to be affected.

To analyze the UVR susceptibility of the field-collected urchins, repeated measures ANOVAs were also used to compare the differences in righting time and tube foot extension between the four conditions (P / -UV, P / +UV, G / -UV, G / +UV) before, following UVA-340 exposure, and following exposure to the intense UVB. The assumption of sphericity was violated for the righting time and tube foot extension data, as determined by Mauchly's test of sphericity, $p \leq 0.008$; therefore, a Huynh-Feldt correction was applied ($\varepsilon = 0.659$ and 0.849, respectively). At each measurement point, a special a-priori contrast was used to test whether the G / +UV urchins differed in their righting time and tube foot extension from the other groups, which were not expected to be affected.

To analyze pigment levels of urchins from the rearing study: when analyzed with an ANOVA, peak locations were not statistically different from one another, regardless of treatment: For peak 1, $p = 0.375$; for peak 2, $p = 0.418$); therefore, the absorbance values at the reported peaks were analyzed with a one-way ANOVA and post-hoc Tukey analysis; one outlier was identified in the peak 1 data but did not significantly alter the model or post-analyses outcomes so it was left in. The absorbance data for each peak within each treatment was normally distributed as determined by Shapiro-Wilk's test ($p \geq 0.064$) and equality of variances was determined by Levene's test ($p \geq 0.085$).

To analyze pigment levels of field-collected urchins: when analyzed with an independent samples t-test, peak locations were not statistically different from one another, regardless of condition (for peak 1, $p = 0.756$; for peak 2, $p = 0.776$), therefore, the absorbance values at the reported peaks were analyzed with an independent samples t-test. No outliers in the data were found, as determined by boxplot inspection, the data for each peak within each condition was normally distributed as determined by Shapiro-Wilk's test ($p \geq 0.057$), and equality of variances was determined by Levene's test ($p \geq 0.403$).

To analyze pigment levels in the tube feet of green and purple ecotypes: when analyzed with a one-way ANOVA, peak locations were not statistically different from one another between the four conditions examined (P_Aboral, P_Oral, G_Aboral, G_Oral; $p \geq 0.923$). Therefore, the absorbance values at the reported peaks were analyzed using a one-way ANOVA. All data were normally distributed, as determined by Shapiro-Wilk's test ($p \geq 0.054$), and no outliers were found as determined by boxplot inspection. There was heterogeneity of variances for peak 1 data ($p < 0.0005$) but equal variances for peak 2 data ($p = 0.079$) as determined by Levene's test. Post-hoc Games-Howell and Tukey analysis were used for peak 1 and peak 2, respectively, to determine differences between groups.

RESULTS

Color analysis

There was a statistically significant interaction between treatment and time on urchin color ($F_{6.829, 68.287} = 13.044$, $p = <0.0005$), meaning that the treatments resulted in different effects on urchin color over time (Figure 5.6). At the first date photographed (April 4, 2014), urchin color was not significantly different between treatment groups ($p = 1.000$). After the first week following set-up, the urchins began to diverge in color, although not yet significantly so ($p \geq 0.096$). Two weeks following set-up, both the +UV and -UV urchins were significantly more purple than the urchins from the dark treatment (-UV: $p = 0.045$; +UV: $p =$

0.004) and did not significantly differ from each other (p = 0.797). By four weeks following set-up and continuing until the study was ended, the difference in color between the dark treatment group and the -UV and +UV groups grew ($p \leq 0.001$) and the difference between the -UV and +UV groups remained statistically insignificant ($p \geq 0.672$; Figure 5.7).

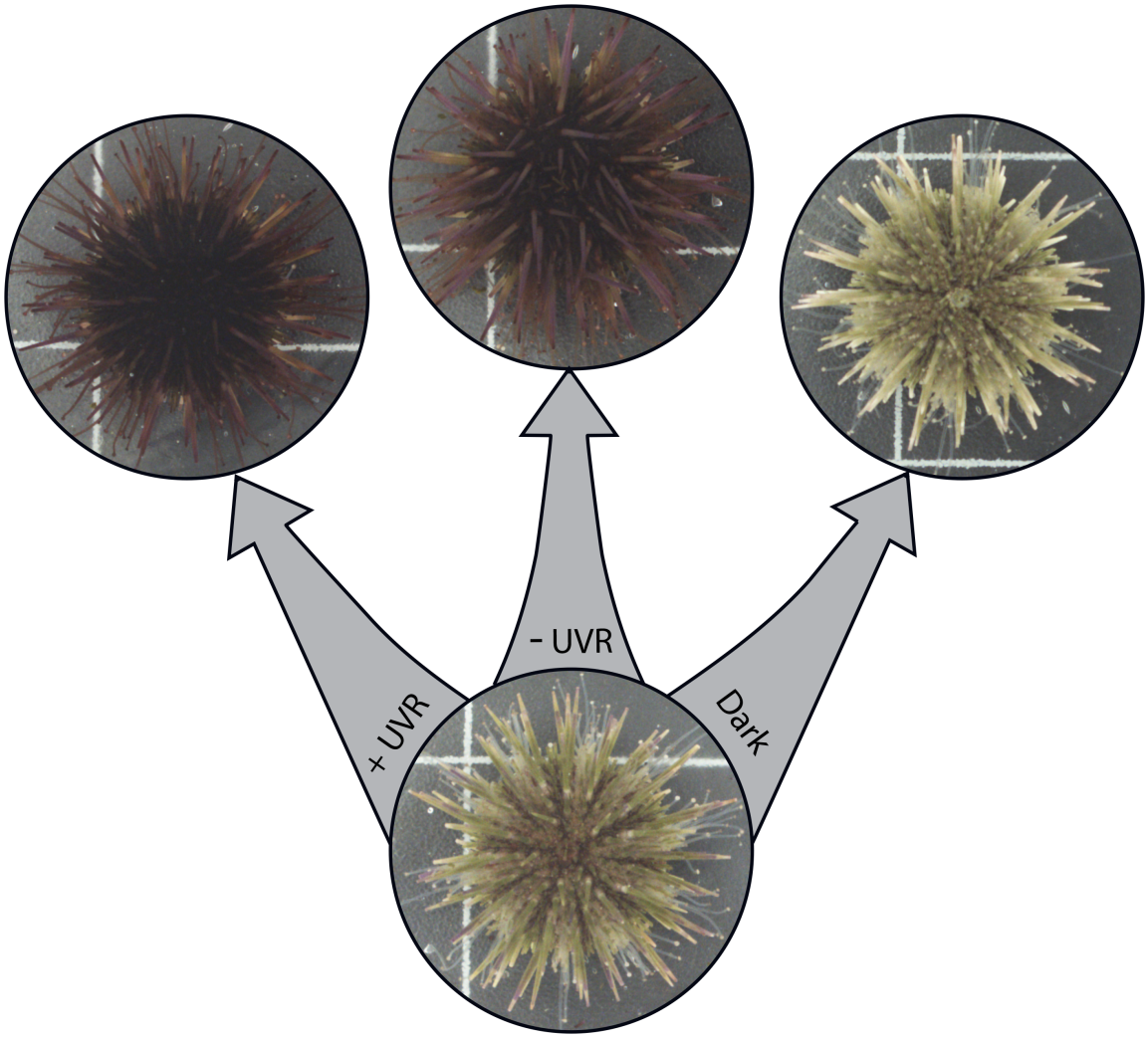


Figure 5.6. The resulting colors of urchins raised in full sunlight (+ UVR), in sunlight filtered of UVR (- UVR) and in the dark.

For the dark treatment group, the color ratio decreased slightly, from 1.937 ± 0.021 SE to 1.924 ± 0.015 SE, with a non significant change of 0.013 ± 0.013 SE (t_6 = 0.943, p = 0.382). For the -UV treatment group, the color ratio increased from 1.941 ± 0.250 SE to

2.022 ± 0.017 SE, with a significant change of 0.081 ± 0.013 SE ($t_7 = 6.014$, $p = 0.001$). For the +UV treatment group, the color ratio increased from 1.943 ± 0.009 SE to 2.045 ± 0.004 SE, with a significant change of 0.102 ± 0.007 SE ($t_7 = 15.306$, $p < 0.0005$).

For the field-collected urchins, as expected, the green individuals were less purple in color (1.944 ± 0.010 SE) than the purple ones (2.043 ± 0.005 SE), with a significant difference in color ratio of 0.099 ± 0.011 SE ($t_{9.995} = 9.095$, $p < 0.0005$; Figure 5.7).

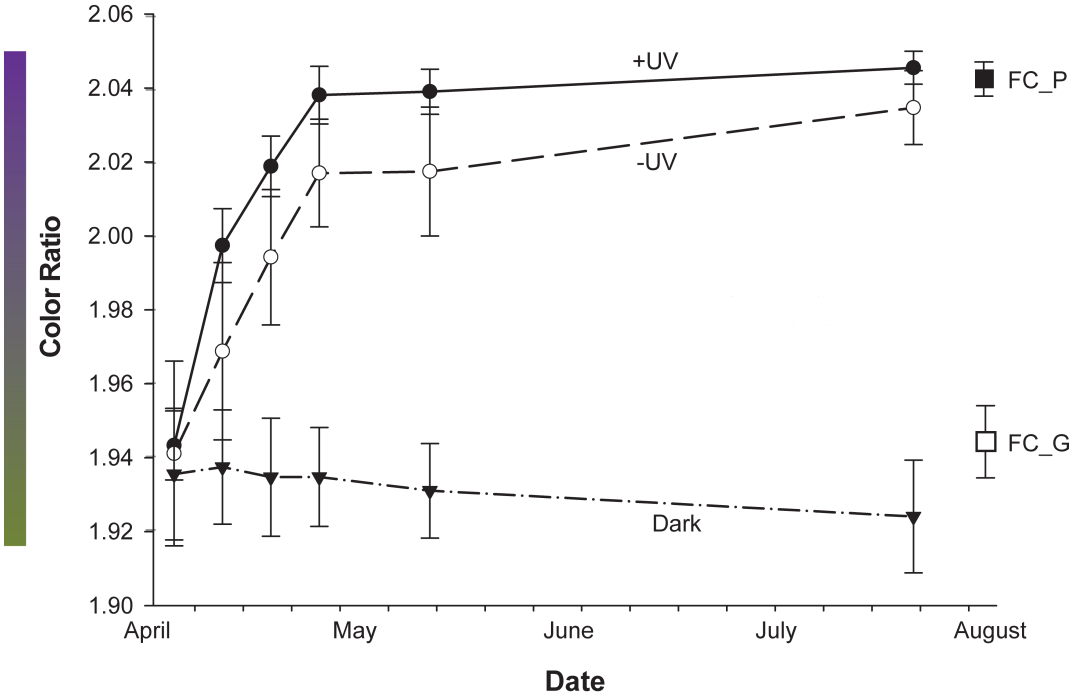


Figure 5.7. The change in color over time of urchins raised in full sunlight (+UV), in sunlight filtered of UVR (-UV), and in the dark in comparison with field-collected purple (FC_P) and green juveniles (FC_G) of equivalent sizes. The color ratio represents the relative reflectance of red+blue vs. green with higher numbers indicating an urchin becoming more purple. Error bars are ± 1 SE.

Urchin growth

The change in test diameter was significantly different between the three treatment groups ($F_{2, 20} = 13.624$, $p < 0.0005$; Figure 5.8) but spine growth was not significantly different ($F_{2, 20} = 1.970$, $p = 0.166$; Figure 5.8). Urchins kept in the dark grew significantly

more in test diameter (0.362 cm ± 0.017 SE) than the –UV urchins (0.182 cm ± 0.032 SE, p = 0.002) or the +UV urchins (0.139 cm ± 0.038 SE, $p < 0.0005$), which were not significantly different from each other (p = 0.587). Urchins kept in the dark did not significantly differ in spine growth (0.238 cm ± 0.024 SE) from the –UV urchins (0.294 cm ± 0.019 SE, p = 0.152) nor the +UV urchins (0.258 cm ± 0.018 SE, p = 0.762).

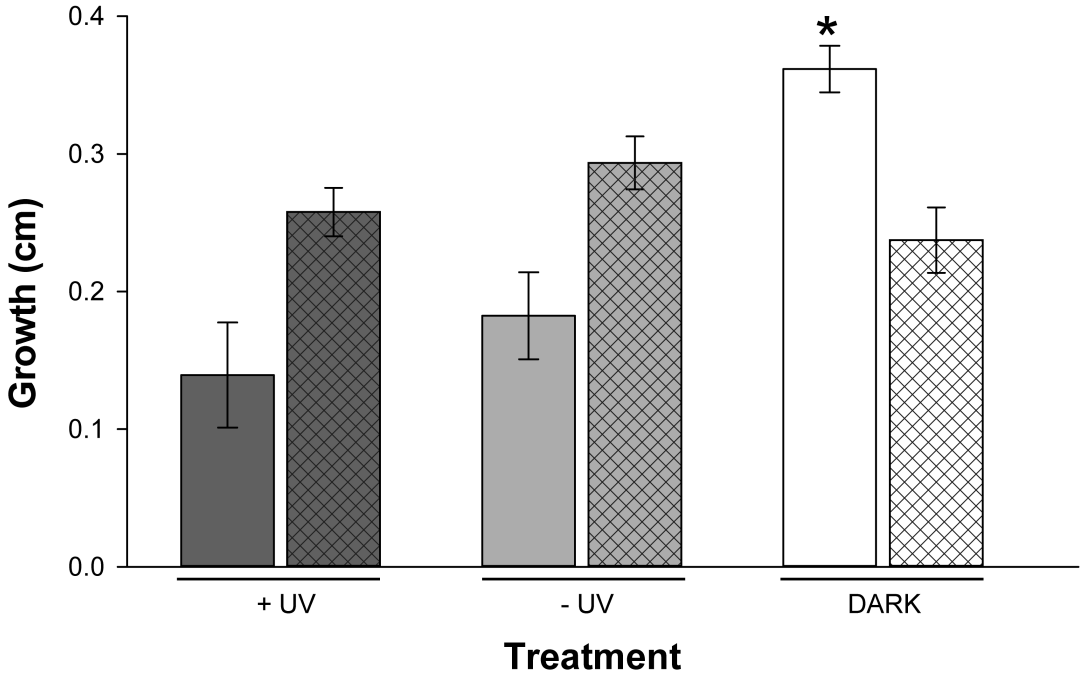


Figure 5.8. The amount of test growth (solid bars) and spine growth (cross-hatched bars) for urchins raised in full sunlight (+UV), in sunlight filtered of UVR (-UV), and in the dark. Error bars are ± 1 SE.

UVR susceptibility

For the urchins from the rearing study, there was a statistically significant interaction between condition and exposure for both assays measured (righting time: $F_{6.655, 22.626} = 18.214$, $p < 0.0005$; tube foot extension: $F_{10, 34} = 19.342$, $p < 0.0005$), meaning that the urchin conditions (Dark / -UV, Dark / +UV, -UV / -UV, -UV / +UV, +UV / -UV, +UV / +UV) resulted in different effects of UVR exposure on righting time (Figure 5.9) and the number of tube feet extended (Figure 5.10). There was no difference in initial righting time ($p \geq$

0.116) or tube foot extension ($p \geq 0.092$) between groups. Following the UVA-340 exposure, there was still no difference in righting time ($p \geq 0.065$), however, there was a significant reduction in the number of tube foot extended in the Dark / +UV urchins (40.3 ± 5.1 SE) compared to the other conditions (64.9 ± 2.9 SE; $p \leq 0.041$). Following the hypernormal UVB exposure, righting time was greatly increased (413.33 sec ± 82.19 SE) and tube foot extension was greatly reduced (6.8 ± 2.6 SE) for the Dark / +UV urchins compared to the other conditions (righting time 62.70 sec ± 3.87 SE, $p < 0.0005$; tube foot extension 65.7 ± 3.0 SE, $p < 0.0005$).

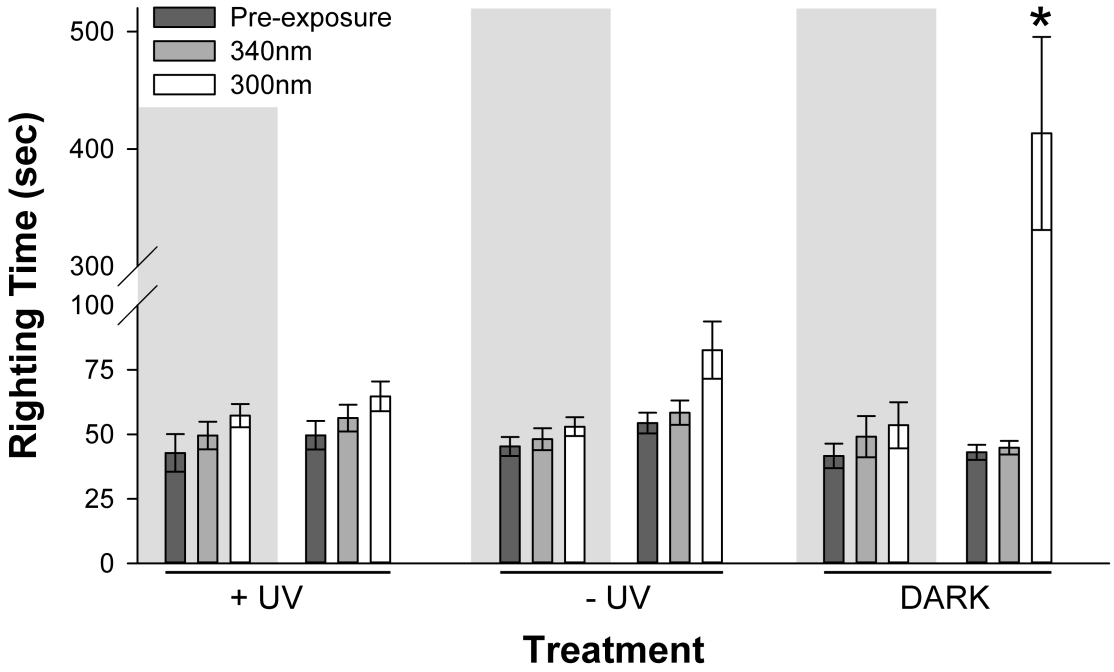


Figure 5.9. Righting time prior to (Pre-exposure) and following exposure to elevated ambient-mimicking UVR (with a peak of 340 nm) and to hypernormal UVB (with a peak of 300 nm) for urchins raised in full sunlight (+UV), in UVR-filtered sunlight (-UV), or in the dark. Half of each group was placed under the UVR-filtering acrylic during the exposures as a positive control for its filtering capacity (grayed regions); the other half was placed under UVR-transmitting acrylic. Error bars are ± 1 SE.

For the field-collected urchins, there was a statistically significant interaction between condition and exposure on both assays measured (righting time: $F_{3.956,\ 15.825}$ =

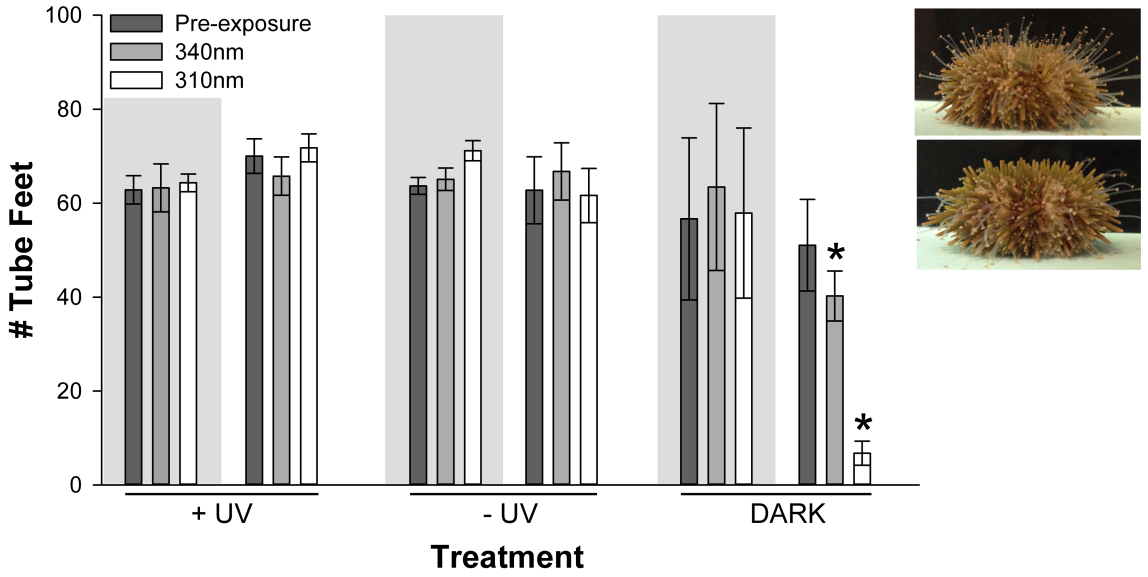


Figure 5.10. Tube foot extension prior to (Pre-exposure) and following exposure to elevated ambient-mimicking UVR (with a peak of 340 nm) and to hypernormal UVB (with a peak of 300 nm) for urchins raised in full sunlight (+UV), in UVR-filtered sunlight (-UV), or in the dark. Half of each group was placed under the UVR-filtering acrylic during the exposures as a positive control for its filtering capacity (grayed regions); the other half was placed under UVR-transmitting acrylic. An example of an urchin prior to (top) and following hypernormal UVB exposure (bottom) is shown in the upper right. Error bars are ± 1 SE.

90.229, p <0.0005; tube foot extension: $F_{5.097,\ 20.387} = 29.174$, p <0.0005), meaning that the urchin conditions (P / -UV, P / +UV, G / -UV, G / +UV) resulted in different effects of UVR exposure on righting time (Figure 5.11) and tube foot extension (Figure 5.12). There was no difference in initial righting time ($p \geq 0.126$) or tube foot extension ($p \geq 0.345$). Following the UVA-340 exposure, there was still no difference in righting time ($p \geq 0.091$), however, there was a significant reduction in the number of tube feet extended for the G / +UV urchins (60.3 ± 2.1 SE) compared to the other conditions (73.6 ± 2.1 SE; $p \leq 0.035$). Following the hypernormal UVB exposure, righting time was greatly increased (527.42 sec ± 47.68 SE) and tube foot extension was greatly reduced for the G / +UV urchins (14.1 ± 3.1 SE) compared to the other conditions (righting time 57.42 sec ± 1.96 SE, $p < 0.0005$; tube food extension 76.4 ± 2.8 SE, $p < 0.0005$).

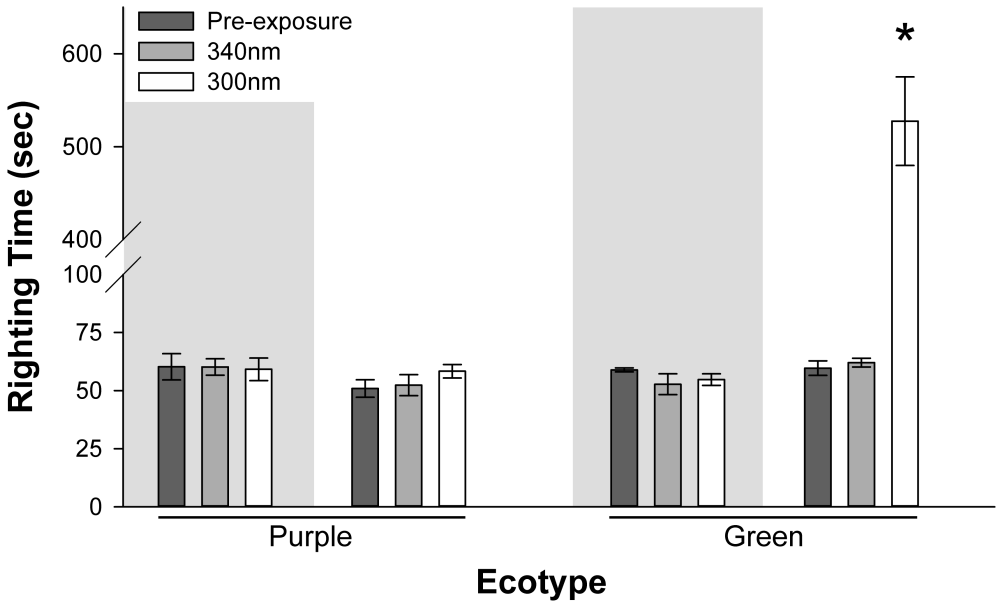


Figure 5.11. Righting time prior to (Pre-exposure) and following exposure to elevated ambient-mimicking UVR (with a peak of 340 nm) and to hypernormal UVB (with a peak of 300 nm) for field-collected purple and green juveniles. Half of each group was placed under the UVR-filtering acrylic during the exposures as a positive control for its filtering capacity (grayed regions); the other half was placed under UVR-transmitting acrylic. Error bars are ± 1 SE.

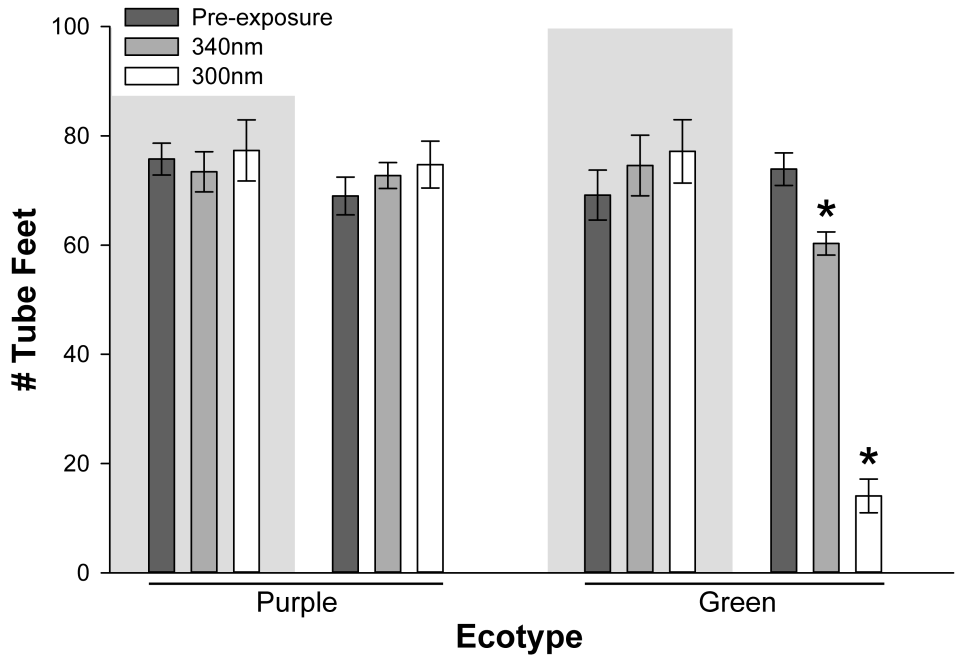


Figure 5.12. Tube foot extension prior to (Pre-exposure) and following exposure to elevated ambient-mimicking UVR (with a peak of 340 nm) and to hypernormal UVB (with a peak of 300 nm) for field-collected purple and green juveniles. Half of each group was placed under the UVR-filtering acrylic during the exposures as a positive control for its filtering capacity (grayed regions); the other half was placed under UVR-transmitting acrylic. Error bars are ± 1 SE.

Pigment levels

The absorption maxima and spectral topography for all measurements were characteristic of polyhydroxylated naphthoquinones (e.g., Spruit, 1949; Millott, 1957; Kuwahara *et al*., 2006; Powell *et al.,* 2014) with two peaks in the ultraviolet range and one occurring in the visible range (Figure 5.13). Although it is possible small amounts of spinochromes were also present, when compared with the results of the study whose methods were replicated for obtainment of the extract (Growns, 1989) and to others (e.g., Service and Wardlaw, 1984; Powell *et al.,* 2014), the absorption spectrum and maxima are nearly identical to that seen for echinochrome A.

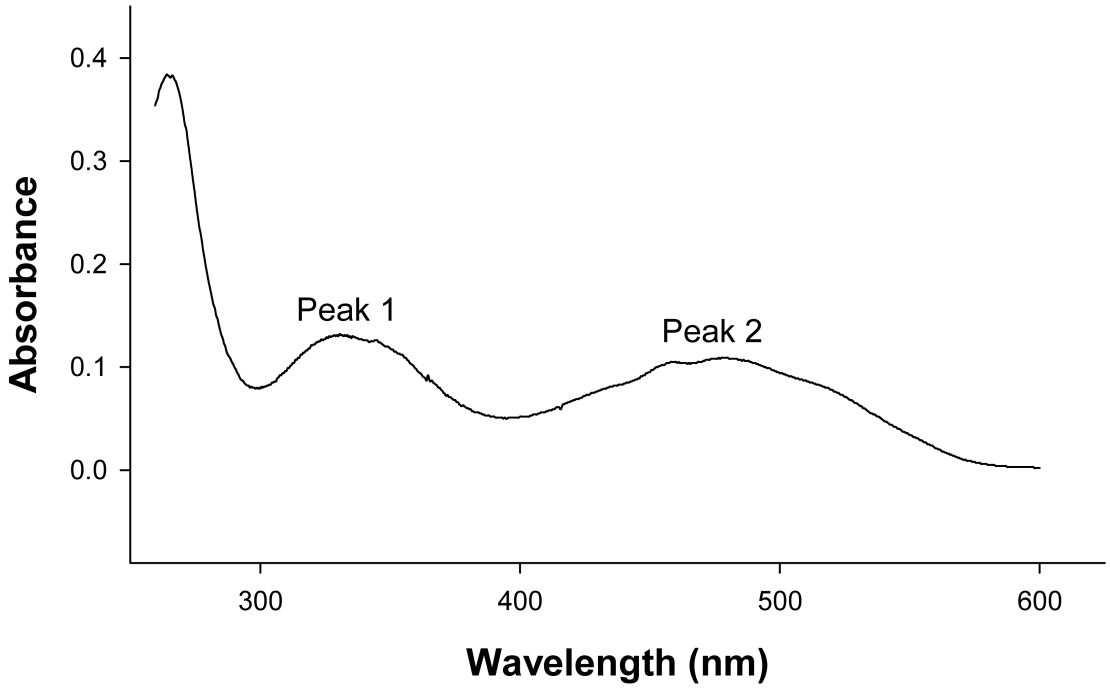


Figure 5.13. A typical absorption spectrum of dermally-extracted pigment from green to purple juveniles of *S. purpuratus* or from adult tube feet. Absorbance (indicative of pigment levels) was compared at one of the peaks in the UVR region (~325 nm; peak 1) and at the peak in the visible region (~479 nm; peak 2).

For urchins from the rearing study: the amount of echinochrome, as indicated by the absorbance values, was significantly different for both peaks between the three treatment

groups (peak 1: $F_{2, 21} = 8.207$, $p = 0.002$; peak 2: $F_{2, 21} = 15.163$, $p < 0.0005$; Figure 5.14). At both peaks, the urchins kept in the dark had significantly lower absorbance values, meaning less pigment (peak 1: 0.239 ± 0.030 SE; peak 2: 0.099 ± 0.010) than the -UV urchins (peak 1: 0.375 ± 0.026 SE, $p = 0.043$; peak 2: 0.199 ± 0.017 SE, $p = 0.001$) and +UV urchins (peak 1: 0.448 ± 0.050 SE, $p = 0.002$; peak 2: 0.210 ± 0.019 SE, $p < 0.0005$), which were not significantly different from each other (peak 1: $p = 0.360$; peak 2: $p = 0.857$).

For the field-collected urchins: as indicated by absorbance values at both peaks, the green urchins had less echinochrome pigment (peak 1: 0.296 ± 0.021 SE; peak 2: 0.128 ± 0.015 SE; Figure 5.14) than the purple urchins (peak 1: 0.611 ± 0.029 SE; peak 2: 0.288 ± 0.015 SE), with a significant difference in absorbance for peak 1 of 0.315 ± 0.036 SE ($t_{14} = 8.757$, $p < 0.0005$) and a significant difference in absorbance for peak 2 of 0.160 ± 0.213 SE ($t_{14} = 7.515$, $p < 0.0005$).

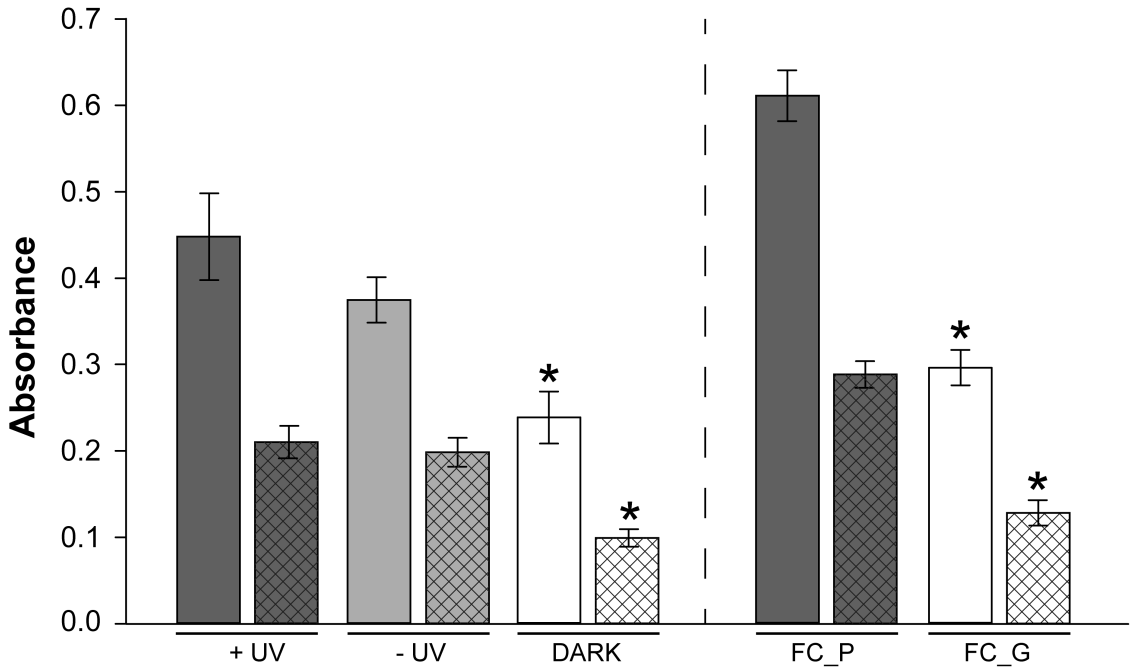


Figure 5.14. Absorbance at peak 1 (solid bars) and at peak 2 (cross-hatched bars) for crude dermal extractions from urchins raised in full sunlight (+UV), in UVR-filtered sunlight (-UV), or in the dark and for field-collected purple (FC_P) and green (FC_G) juveniles. Error bars are ± 1 SE.

There was a significant difference in echinochrome levels in the tube feet among the four conditions assayed (P_aboral, P_oral, G_aboral, G_oral) (Welch's $F_{3, 29.038} = 59.599$, $p < 0.0005$ for peak 1 and $F_{3, 56} = 59.404$, $p < 0.0005$ for peak 2; Figure 5.15). The amount of pigment in the tube feet located on the aboral side of the purple urchins from the pit habitat (P_aboral - peak 1: 0.710 ± 0.046 SE; peak 2: 0.432 ± 0.024 SE) was significantly greater than the amount of pigment from tube feet from the oral side of the purple urchins (P_oral - peak 1: 0.515 ± 0.028 SE, $p < = 0.009$; peak 2: 0.317 ± 0.018 SE, $p < 0.0005$). In the green urchins from the boulder-field, however, there was no significant difference in the amount of pigment from tube feet from the aboral (G_aboral - peak 1: 0.265 ± 0.026 SE; peak 2: 0.160 ± 0.015 SE) and oral sides (G_oral - peak 1: 0.223 ± 0.014 SE, $p = 0.506$; peak 2: 0.138 ± 0.012 SE, $p = 0.835$). As expected, when comparing the aboral and oral sides between the two ecotypes, the tube feet from the aboral side of the purple urchins contained more pigment than those from the aboral side of the green urchins (peak 1: $p < 0.0005$; peak 2: $p < 0.0005$) and the tube feet from the oral side of the purple urchins contained more pigment than those from the oral side of the green urchins (peak 1: $p < 0.0005$; peak 2: $p < 0.0005$).

DISCUSSION

The results of this study provide the first set of cohesive evidence demonstrating that the production of UVR-protective pigment in an echinoderm is an environmentally conditional response dependent on light exposure. Juvenile green urchins of *Strongylocentrotus purpuratus* kept under full solar or UVR-filtered radiation developed higher levels of echinochrome and sustained less damage from subsequent exposure to UVR than those kept in the dark. The development of echinochrome corresponded with a change in color of urchins exposed to light, reflecting overall color differences in body wall, spines and tube feet observed in the field. Similarly sized field-collected individuals of both the

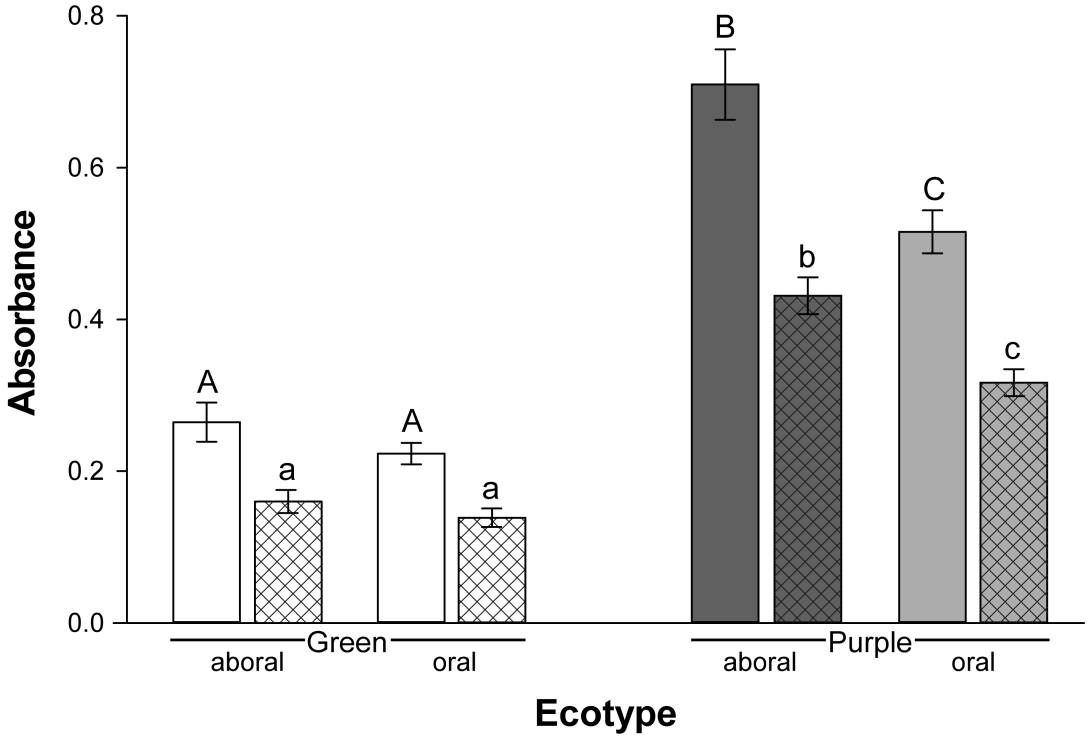


Figure 5.15. Absorbance at peak 1 (solid bars) and peak 2 (cross-hatched bars) for dermal extractions from tube feet taken from the aboral and oral sides of field-collected green and purple ecotypes. Error bars are ± 1 SE.

green and purple variety showed equivalent pigment characteristics to those resulting from the experimental study.

Color and microhabitat

The color of echinoderms is due to a combination of varying levels of dermal echinochrome and naphthoquinone salts within the calcareous spines and test.

The darkening response of light-exposed urchins was due in large part to the production of epidermal echinochrome overlying the spines and test and within the dermis of the test. Echinochrome A exists as granules within a subpopulation of cells called red spherule cells (Johnson, 1969). Colorless spherule cells also exist and although it has been proposed that these cells are colorless precursors of red spherule cells (e.g., Matranga *et al*., 2000; Johnstone, 2013), results of Johnstone (2013) supported alternative suspicions that stress-induced increases in red spherule cells (and therefore echinochrome) resulted from rapid

cell division (Matranga *et al*., 2000). In addition to increased dermal pigmentation in urchins kept in the light (likely due to division of red spherule cells), there were also assuredly color changes of the calcareous structures. Color variation of tests and spines, both between and within species, are due to varying amounts of component pigments (e.g., Goodwin and Srisukh, 1950). For example, purple and green spines or regions of spines of urchins of *Paracentrotus lividus* are due to proportionally greater amounts of spinochrome A vs. spinochrome B, respectively. Both of these pigments can be found in *S. purpuratus* and color variation in the tests and spines of purple and green urchins likely represent a similar relationship. Other possible causes of color variation may come from structural features such as naphthoquinone dimers, naphthoquinone-protein moieties, or from differences in pH (e.g., Tyler, 1939; Service and Wardlaw, 1984; see Millott, 1957; Pozharistckaya *et al*., 2013).

As a rule of thumb, only purple urchins are found in the exposed pit habitat, but both green and purple ecotypes can be found in the boulder fields with a wide variety of intermediate shades and sizes (personal observation). It is possible that some purple urchins happen to be dislodged from their pits and are washed into the boulder field habitat, but the boulder field environment introduces extra complexities for a growing recruit than those seen in typical pit habitat. Secluded microhabitats easily inhabited when smaller will sensically decrease in accessibility as the urchins grow and exposure to sunlight will likely become more frequent. Although urchins inhabiting pits are considered relatively sedentary (Yusa and Yamamoto, 1994; Grupe, 2006; Gravem and Adams, 2012), urchins in the boulder field habitat may move around more both because of size restrictions due to growth, lack of a designated refuge, lower population densities, and in order to obtain sufficient food. Field observations and the results of this study corroborate the notion that

the urchin's microhabitat influences the level of light (and probably UVR exposure) experienced.

Costs and trade-offs

Urchins kept in the dark grew more in test size than urchins kept in the light with or without UVR; this indicates that there is a trade-off to the production of pigment, a common feature of conditional traits (e.g., Tollrian and Harvell, 1999; Callahan *et al*., 2008). If no costs were incurred from pigment production, it would be expected to be a fixed phenotype independent of environmental variation (Via and Lande, 1985). In the presence of light, the fitness benefits of reduced UVR susceptibility outweigh the costs of pigment production (a consequent decrease in growth rate); likewise, unnecessary pigment production in a green juvenile living in a sheltered microhabitat would incur a fitness deficit: energy that could have been allocated to growth would be used for a trait that is of no benefit in its current environment. The lack of difference in spine growth between the light-exposed urchins vs. those kept in the dark suggests that available growth was allocated to the spines as opposed to the test. While longer spines might block UVR reaching the test by increasing the shading capacity of the spines, they also serve protective functions (e.g., against predators: Strathmann, 1981, Guidetti and Mori, 2005). Other echinoids exhibiting intraspecific color differences report the darkest phenotypes as being the smallest, although other influences of microhabitat of these variants cannot yet be ruled out (Chesher, 1970; Nishihira *et al*., 1991). Costs and/or trade-offs associated with pigment production have been identified in other organisms such as fish (e.g., Rodgers *et al*., 2013), humans (e.g., Jablonski and Chaplin, 2010), copepods (e.g., Hansson, 2000; Hansson *et al*., 2007; Gorokhova *et al*., 2013), and plants (e.g., Gwynn-Jones and Johanson, 1996)

UVR susceptibility

Considering the righting behavior of urchins is executed by use of the podia, the correspondence of tube foot extension with righting time was expected. Urchins with high numbers of extended tube feet righted themselves very quickly, usually in less than a minute. Even with much reduced and damaged aboral tube feet following UVB exposure, the green urchins were still eventually able to right themselves (usually by relying primarily on extension of podia located closer to the oral surface). In the absence of the complimentary righting time data, one could argue that the dark-raised green juveniles simply retracted their podia, as has been observed in response to UVR in other urchins (e.g., Sharp and Gray, 1962); however, most podia did not reemerge even 20 minutes following cessation of UVB and those that did were often limp, indicating damage rather than a behavioral response. Recovery after a longer period of time may have been possible (e.g., see Sharp and Gray, 1962) but urchins were scarified and frozen within one hour of exposure to later extract pigment, so this could not be determined. The significant reduction in the number of tube feet following near ambient levels of UVR, however, may be an indication of a behavioral response of the tube feet rather than inherent damage as near ambient levels of UVR did not significantly increase the righting time. Clearly the UVB exposure is not representative of ambient conditions; its purpose was to demonstrate a photoprotective function of the induced pigment. Having said that, the benefits afforded by the pigment is impressive considering the apparent lack of impact of such intense UVR on the pigmented urchins.

Environmental cue

Some behavioral studies have indicated urchins can distinguish between UVR and PAR/darkness; for example, more urchins exhibit a covering response when subjected to UVR, but not under UVR-filtered light or in the dark (e.g., Adams, 2001; Verling *et* al., 2002; Dumont *et al*., 2007), thus a similar trend was expected for the morphological response.

Urchins kept in the presence of UVR were expected to turn purple while urchins sheltered from UVR were expected to stay green. Those kept in the dark remained green and those kept in full sunlight turned purple, results that were anticipated. However, urchins grown in sunlight filtered of UVR also turned purple with color ratios, pigment levels, and UVR susceptibility that were not significantly different from urchins grown in the presence of UVR. This suggests different mechanisms may be in place for behavioral and morphological responses to light; for the latter, urchins likely use light collectively as a reliable indicator for the presence of UVR since, at least for intertidal organisms, PAR and UVR are concurrent and the ability to distinguish between the two is unnecessary. However, for urchins living at depths beyond the reach of UVR, pigmentation in response to wavelengths other than UVR is energetically wasteful. It is possible urchins rely on an overall decrease in light intensity with depth or may be responding to only a portion of the PAR spectrum. It would be particularly interesting to determine if a response in pigment production can be elicited under blue light, the wavelength capable of greatest depth penetration (Ruiz-González, 2013). It should also be noted that the UVR-filtering acrylic used in the study filters out most (98%) but not all UVR – thus a very small amount of UVR may still have triggered the production of pigment; however, considering the insignificant differences between these urchins and those kept under full sunlight, this seems unlikely.

Although there was no significant quantitative differences found between urchins kept under ambient light with UVR and urchins kept in ambient light filtered of UVR, we could consistently tell urchins from the two treatments apart: those exposed to UVR were visibly darker. This suggests that although PAR is capable of inducing preventative pigment production, the presence of UVR exaggerates the response. There may be two mechanisms in place for the induction of pigment production – a preventative response triggered by the presence of light even in the absence of UVR and a second response in reaction to direct

damage or increased levels of reactive oxygen species, cues that have been shown to trigger or enhance pigment production in some plants (Sepúlveda-Jiménez *et al*., 2004; Wang *et al.,* 2007), bacteria (e.g., Lan *et al*., 2009), and vertebrates (e.g., Gilchrest and Eller, 1999).

In addition to light, UVR-absorbing compound production or accumulation in other organisms has been demonstrated in response to other stressors such as temperature (e.g., Christie *et al*., 1994), chemical kairomones from predators (e.g., Ahlgren *et al*., 2013), or desiccation (Jiang *et al*., 2008). In urchins, studies have demonstrated significant increases in echinochrome and echinochrome-containing red spherule cells following injection with bacteria, in response to pollutant exposure, or injury (e.g., Holland *et al*., 1967; Matranga *et al*., 2000; Johnstone, 2013), indicating that light is only one of many stressors that might induce the production of this multifunctional pigment.

Functions of echinochrome

The results of the UVR-exposure study clearly indicate decreased susceptibility of purple urchins to UVR (both likely in the ability of echinochrome to absorb UVR and function as an antioxidant). Just because light is a cue inducing the production of pigment does not necessarily restrict its function solely to protection against stressors due to UVR - the pigment may provide benefits indirectly-linked to light exposure. For example, echinochrome has been implicated to act as an algistat (Vevers, 1963, 1966), a role that is only beneficial in the presence of light. Light or not, echinochrome is also often found in acutely higher concentrations at the site of injury, possibly acting as both a protective barrier and general disinfectant (e.g., Ebert, 1967; Johnson and Chapman, 1970a, 1970b; Höbaus, 1979; Coffaro and Hinegardner, 1977; Service and Wardlaw, 1984). Echinochrome is even the active substance in a cardioprotective drug called 'histochrome' which is involved in treatment following a heart attack (Lebedev *et al*., 2005). Regenerating spines of a pigment-deficient urchin of *S. franciscanus* were infected with a variety of microorganisms

compared to echinochrome-dense and infection-free injuries of normally pigmented individuals (Johnson and Chapman, 1970b); considering the only urchin to die over the course of the study belonged to those kept in the dark, this may indicate a potential limitation of lacking pigment in the way of reduced immunological defenses. This notion is contingent on the urchin requiring the presence of light to produce the pigment, even if it is for a purpose other than protection against UVR – a possible co-dependency that should be further investigated (e.g., will an injured green juvenile kept in the dark still produce echinochrome defenses?)

Echinoids and flavonoids – a common ground

One of the most well studied areas of UVR-induced protection is in plants. Like in urchins, compounds are produced by plants that directly or indirectly deal with harmful UVR by acting as sunscreens (e.g., flavonoids) that protect the underlying photosynthetic layer or by functioning as antioxidants or protective enzymes (e.g., Strid *et al*., 1994; Jansen *et al*., 1998; Barnes *et al*., 2015). Many of these compounds alter the color of the plant from green to red or purple, an opportune likeness to the color change seen in the urchins. For example, the leaves of *Cotinus coggygria* remain green or accumulate very low levels of anthocyanins when grown in the absence of or in reduced UVR but attain their characteristic purple color only under sufficient UV radiation (Oren-Shamir and Levi-Nissim, 1997). In the husk tissue of maize plants, differing only in the amounts of UVR-absorbing anthocyanin flavonoids, tissue from purple husk plants is protected from UVR-induced damage compared to tissue from green husk plants (Stapelton and Walbot, 1994).

In plants, the level of pigmentation and UVR-protectant compounds vary depending on UVR-exposure both among plants (e.g., see Barnes *et al*., 2015), between regions on the same plant (sun and shade leaves; Jaakola *et al*., 2004; Lenk and Buschmann, 2006), and between different regions on individual leaves (longitudinal gradient, Wagner *et al*., 2003;

top vs. bottom, Pietrini *et al*., 2002). For example, in barley leaves, the concentration of UVR-absorbing pigments increases from base to tip; Wagner *et al*., 2003). These patterns are mirrored in the regional differences in pigment in urchins of *S. purpuratus* and correlative degrees of sun exposure: when color variation exists along the length of a spine, the tips are always purple in color becoming green near spine bases (personal observation). Also, the sheltered oral surface of purple urchins tends to be lighter in color (indicative of less dermal pigment) than the darker aboral surface (indicative of higher amounts of dermal pigment); this pattern was confirmed by the significantly higher amount of pigment in tube feet collected from the aboral surface vs. the oral surface. The lack of difference in pigment levels of tube feet between the oral and aboral sides of boulder-field urchins was expected considering the sheltered nature of the urchin as a whole.

Future considerations

The possibility of a long-term change in an urchin's level of light exposure, such as when a purple urchin is washed into the light-sheltered boulder fields, brings up an intriguing question: can a purple urchin go back to being green? If so, would this occur only with cessation of further pigment production in combination with growth or are urchins capable of resorbing existing pigment? Reversibility of induced morphologies depends on a variety of factors such as those related to costs of maintaining the induced trait, the timing of environmental heterogeneity relative to an organism's ability to change, and developmental constraints (e.g., Relyea, 2003; Hoverman and Relyea, 2007; Orizaola *et al*., 2012). The easiest way to determine reversibility of pigment levels in adult purple urchins would be to monitor the color of the podia over time for urchins kept in the dark. Although regenerating spines in the dark are known to be green instead of purple (Ebert, 1967), color reversion in adult tests and spines is unlikely.

The methods outlined in this study could easily be applied to other organisms such as the local sand dollar *Dendraster excentricus*, the juveniles of which also exhibit green and purple color variations and possess a similar pigment profile as *S. purpuratus* (Goodwin, 1969; Fox, 1976, R. Emlet, personal communication). It would be interesting to see if subtidal species are also capable of this response and if the capability varies with depth. Although both urchins of *S. purpuratus* and *S. droebachiensis* can be found intertidally, the latter can be found much deeper (to 1,138 m; Lamb and Hanby, 2005). Although it is known for its green color, the color of the tube feet and dermal surface of urchins of *S. droebachiensis* can range from very light in color to dark purple, while the spines are almost always green. Urchins of *Strongylocentrotus pallidus* are found even deeper (to 1,600 m) and are correspondingly paler (Lamb and Hanby, 2005).

Prior to settlement as juveniles, the larvae of *S. purpuratus* and other marine invertebrates are also vulnerable to the damaging effects of UVR (e.g., Pennington and Emlet, 1986; Hovel and Morgan, 1999). Although many larvae utilize depth adjustments to escape the reach of UVR (e.g., Pennington and Emlet, 1986; Hansson *et al*., 2007; Hylander and Hansson, 2013), the usage of UVR-absorbing compounds is also a common strategy (e.g., Hansson, 2000; Miner *et al*., 2000; Hansson *et al*., 2007; Hylander and Hansson, 2013). The same UVR-transmitting, UVR-filtering, and dark treatments could be used to determine if enhanced pigment production can be induced in larval echinoids possessing echinochrome-containing cells in their ectoderm. Increased pigment may act in conjunction with behavioral responses to provide enhanced protection from the effects of UVR, although increases in pigmentation may also make zooplankton more visible to potential predators (e.g., Hansson, 2000) unless non-pigmented MAAs are used (e.g., Hylander and Hansson, 2013).

During trials of UVR exposure prior to the study, we often noticed that within a few days of UVB exposure, many spines of the green urchins fell off and the aboral surface turned a deep purple – this may be a favorable system for increasing our understanding of the involvement of naphthoquinones following UVR-induced injuries, the dispersal capabilities of urchin pigments, and the ability for them to be rapidly produced. Genes upregulated during echinochrome synthesis have been identified (e.g., Calestani *et al*., 2003) and could be an additional method used to follow light-induced pigment production on a finer scale.

CHAPTER VI

CONCLUSIONS

The studies comprising this dissertation add valuable knowledge to the understanding of phenotypic plasticity in marine invertebrates by providing novel examples of phenotypic change induced by a variety of environmental stimuli.

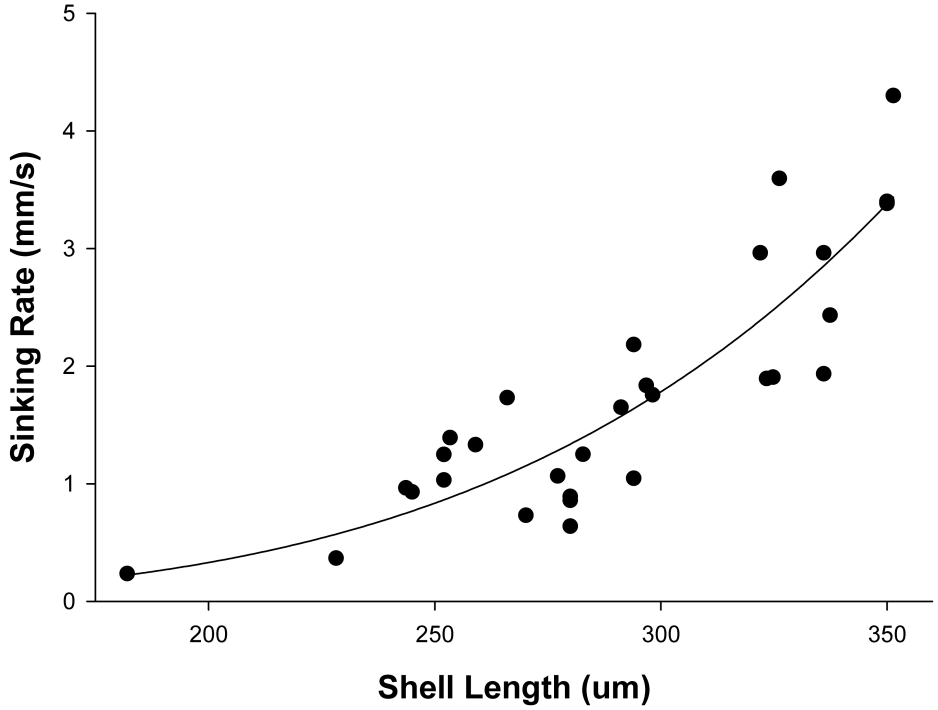
In Chapter II, I broadened an earlier report of inducible phenotypic plasticity in larval shells of veligers of *Littorina scutulata* (Vaughn, 2007) by using a different species of predatory crab zoea, measuring an additional shell response variable, and including methods to address cue specificity. When compared to controls in seawater, veligers developed rounder shells, smaller apertures, and reinforced apertural margins in response to the presence of predators. In the presence of predators consuming conspecific veliger larvae, veligers formed smaller apertures and the thickest apertural margins, but the shell shape was not significantly different from control veligers. The different responses to different treatments with planktonic predators indicate that larvae can vary shell characteristics and may indicate a trade-off reflective of cue-specific defenses indicative of risk. The induced-defenses resulted in enhanced survival of veligers when directly paired with predators.

In Chapter III, I provide the first example of an inducible offense in larvae of the gastropod family Littorinidae. Veligers raised in a food-limited environment developed larger vela and longer cilia relative to shell size, but cilia length was not significantly different relative to velar size between the two treatments. These changes reflect a trade-off in growth of larval vs. postlarval structures retained following metamorphosis (e.g., shell) depending on when food is scarce or abundant, respectively. The swimming speed of veligers raised in low vs. high food concentrations both increased over time but was faster for veligers raised in a high food environment, perhaps due to behavioral differences. Velar

proliferation was initially greater for veligers raised at a high food concentration but declined much faster than the relatively constant levels observed for food-limited veligers over a longer period of development, partially explaining the mechanism for growth of the larger velar size in food-limited veligers.

In Chapter IV, I demonstrated that velar asymmetry is a common occurrence in gastropod veligers. Measurements from literature figures indicate that while velar area increases with shell area for veligers possessing two or multiple velar lobes, asymmetry in shell area relative to the velar midline is not always reflected in velar proportions. I experimentally manipulated the weight distribution of veligers of *L. scutulata* through the attachment of different sized beads to their shells in order to test the plasticity of velar growth and velar symmetry. Results show that veligers of *L. scutulata* are able to allometrically modify growth of the two velar lobes and can enhance total growth of the velum in response to changes in total weight and weight distribution; however, the degree of plasticity is limited and does not scale with increasing amounts of weight.

The work described in Chapter V revealed that color variation in juveniles of *S. purpuratus* is due to differences in the production of pigment relative to varying levels of light exposure and that this plasticity is beneficial in protection against photodamage. Urchins kept under full or UVR-filtered sunlight developed more pigment, sustained less damage from subsequent UVR exposure, and grew less in test size than those kept in the dark. Field-collected juveniles of both purple and green ecotypes showed similar characteristics as those resulting from the rearing study. Pigment levels also reflected differences in color of tube feet between the two ecotypes and indicate regional specificity between the aboral and oral sides dependent on light exposure.



S4.1 Sinking rates of veligers of *L. scutulata* over a range of shell sizes.

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Chapter V

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