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Tidal height and immersion time impact predator-induced morphological shell plasticity in three members of the genus Nucella

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Tidal height and immersion time impact predator-induced morphological shell plasticity in three members of the genus **Nucella**

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of the Requirements for the Degree
Master of Science

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MASTER'S THESIS

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Caitlin E. O'Brien
November 15, 2012

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A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Caitlin E. O'Brien
October, 2012

ABSTRACT

The genus *Nucella* has long been used as a model to study phenotypic plasticity. *Nucella lamellosa*, *N. canaliculata,* and *N. ostrina* respond to waterborne cues from the predatory crab *Cancer productus* by thickening their shells at the apertural lip. This type of phenotypic plasticity is referred to as predator-induced morphological defense. The degree of constitutive and plastic lip thickening differs according to each species' native height in the intertidal: *Nucella lamellosa* lives lowest in the intertidal, typically produces the thickest shells and has the strongest response to crabs, while *N. ostrina* lives highest in the intertidal, has the thinnest shells, and the most muted response to crabs. Since crabs are restricted to immersed areas to forage, the risk of crab predation is strongly affected by the amount of time a snail is immersed by the tide. Based on these corresponding patterns, it has been suggested that relative predation risk may be driving the interspecific differences in constitutive and plastic lip thickness across these three members of *Nucella*. I hypothesized that physical parameters associated with tidal height (immersion time) also affect the anti-predator response and have played a role in directing the evolution of constitutive and inducible morphological defenses in *Nucella*.

Without "replaying the tape of life," this type of hypothesis is impossible to address. However, this system provides us with a continuous gradient of natural physical stress, predation risk, and prey response with which to test this hypothesis indirectly. Gauging the proximate effects of immersion time in isolation of and in combination with predator cue provides insight into immersion time's potential to have impacted the evolution of the predator-induced thickening response. I devised an experiment which tested the predation-induced morphological response of *N. lamellosa, N. canaliculata,* and *N. ostrina* in four immersion times representing an intertidal immersion gradient. This experiment took place in an outdoor apparatus supplied with seawater from the Salish Sea. Changes in shell weight, length, body weight, and lip thickness were measured after three months of treatment. Fully crossed and reduced generalized linear mixed models were tested for all species together and independently, and the best fit for each data set was indicated by the lowest Akaike Information Criterion value.

Nucella lamellosa N. canaliculata, and No-Crab *N. ostrina* exhibited a steep gradient of increases in shell mass and length with increasing immersion time, indicating that overall shell growth was maximized in higher immersion times. GLM modeling supported these results, indicating that immersion time was a factor describing these changes. Immersion time also affected the pattern of lip thickening in all three species and apertural teeth in *N. lamellosa*. GLM modeling indicated that immersion time was a factor describing changes in lip thickness and the frequency of apertural teeth. Interestingly, each species showed the greatest predator-induced morphological responses in the immersion treatment representing its native tidal height (*N. lamellosa* at 75% and 100%, *N. canaliculata* at 50%, and *N. ostrina* at 35%). This result corresponded with my predictions in *N. lamellosa,* but conflicted with my expectations in *N. canaliculata* and *N. ostrina*. It also demonstrates that, unlike shell and body growth in *N. canaliculata* and *N. ostrina*, the predator-induced

morphological response is best-adapted to the immersion time each species typically experiences. These findings also demonstrate that future experiments must take immersion time into account in order to ensure that this variable does not compromise experimental design.

Finally, my results provide some tentative support for a hypothesized mechanism of passive thickening in *N. lamellosa*, and ambiguous evidence in the case of *N. canaliculata* and *N. ostrina*. Evidence suggests that starvation causes a reduction in somatic growth which results in a redirection of shell deposition (shell thickening). The hypothesis posits that rather than actively increasing the rate of shell deposition, crab-exposed snails co-opt this passive mechanism to thicken their shells by reducing food consumption. In my experiment, snails in low immersion time treatments experienced reduced access to food because *Nucella* feed most efficiently when immersed. Starvation-induced lip thickening and apertural teeth appeared to occur in *N. lamellosa* in these low immersion treatments, while the results had more ambiguous implications for passive thickening in *N. canaliculata* and *N. ostrina*.

Acknowledgements

This work could not have been completed without the invaluable guidance of my advisors Benjamin Miner, Ph.D., and Deborah Donovan, Ph.D, and committee member Stephen Sulkin, Ph.D. I also benefitted from the assistance of Brian Bingham, Ph.D.

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I obtained the basic supplies required for my experimental apparatus from Hardware Sales, Keller Supply, Frontier Building Supply, Irrigation Direct, Home Depot, and Lowes in Whatcom and Skagit counties. I would be remiss if I did not mention the excellent customer service of Hardware Sales, or the generous return policies of Lowes and Home Depot.

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Table of Contents

List of Figures

List of Tables

1. INTRODUCTION

1.1 Emersion in the intertidal zone

Marine organisms living in the intertidal are subject to numerous stressors not often experienced by organisms living in the subtidal. Many of these stressors are directly related to tidal emersion, and include desiccation, hypoxia/anoxia, limited food access (due to resource scarcity or an inability to hunt), drastic fluctuations between temperature and salinity extremes, and UV exposure (Menge and Branch 2001; Morgan 2001; Sousa 2001). Emersion and the length and degree of physical stressors associated with it are typically exacerbated by increasing distance from the subtidal, creating a gradient of increasingly inhospitable conditions with increasing tidal height (Chapman 2000; Menge and Branch 2001). Thus, tidal height, which determines the duration of tidal emersion, has a great influence on the amount of physiological stress experienced by an organism. This stress imposes restrictions on intertidal organisms, forcing them to allocate energy to cope with harsher conditions that could otherwise be used for growth, reproduction, defense, or movement. For instance, the oyster *Crassostrea gigas* must keep its valves shut during tidal emersion, which forces it to deplete its oxygen stores and causes a reduction in tissue pH due to the build-up of carbon dioxide (Allen and Burnett 2008). In the bivalves *Mytilus edulis* and *Mya arenaria*, the hypoxia associated with emersion caused a destabilization of the lysosome membrane (Tremblay and Pellerin-Massicotte 1997). These stressors also potentially affect the way that these organisms are able to interact with their environments, such as the distribution of individuals, competition with conspecifics, and the expression of

traits that deter predation (Menge and Branch 2001). Organisms have evolved numerous strategies to deal with the ever-changing conditions in the intertidal, and one of these mechanisms is phenotypic plasticity.

1.2 Phenotypic plasticity

Phenotypic plasticity refers to the ability of a genotype to produce a change in phenotype as a response to environmental conditions (Bradshaw 1965). Plastic responses can take any form, including changes in life history, growth, behavior, morphology, and physiology (Miner *et al.* 2005). These changes may be induced in response to abiotic factors, such as temperature, light, or wave exposure, or to biotic factors, such as competition or predation. One example of phenotypic plasticity in the intertidal involves the barnacle, *Balanus glandula,* in which the intensity of high wave velocities induces shorter feeding tentacles than those living in less wave-swept areas (Marchinko 2003). A terrestrial example of phenotypic plasticity in response to biotic factors involves *Arabidopsis thaliana,* commonly known as the mouseear cress. Many plants can sense differences in the ratio of red light to far red light and use this information to gauge the density of neighbors with whom they are competing for sunlight (reviewed in Donohue 2003). *Arabidopsis thaliana* can use this information to respond adaptively to shading from high densities of neighboring plants by accelerating the time to flower and flowering at a smaller size (Dorn *et al*. 2000).

Phenotypic plasticity has broad-reaching implications in ecology and evolution. It impacts patterns of ecological organization by affecting the magnitude and direction of organisms' interactions with their environment, including competition, facilitation, and indirect interactions such as trophic cascades (reviewed in Agrawal 2001; Miner *et al.* 2005). Phenotypic plasticity in plants, for instance, can greatly influence the range and distribution of herbivores and by extension their predators and parasites (reviewed in Agrawal 2001). Similarly, reciprocal plastic responses between predator offense and prey defense can determine the relative abundance and biomass of both groups (reviewed in Agrawal 2001). Phenotypic plasticity has also been proposed as a potential mechanism of speciation and evolution (Agrawal 2001). In *Rhagoletis* flies, for example, phenotypic plasticity may have facilitated speciation by shifting host-preference in some flies from hawthorn to apple fruits (Prokopy *et al*. 1982; Feder *et al*. 1994). Plasticity can also facilitate a species' dispersal and subsequent diversification by enabling the organism to thrive in novel habitats and conditions (Agrawal 2001). In fact, one rapidly-developing field of inquiry is whether successful invasive species are more phenotypically plastic than their less-successful counterparts (examples include Davidson *et al.* 2011; Molina-Montenegro *et al.* 2012; Perez *et al*. 2012).

1.3 Predator-induced defenses

Predator-induced defenses are a subset of phenotypic plasticity that occur when an individual detects a predator (or damaged conspecifics) and develops a phenotypic trait to deter predation by that species (Harvell 1990). Examples include the induction of more and larger protective spines on *Acacia drepanolobium* by grazing herbivores (Young *et al.* 2003), grazer-induced toxins in phytoplankton (reviewed in Van Donk *et al.* 2011), early hatching of larval treefrogs when egg predators are detected (Warkentin 2005), and increased use of refuge by prey species sensing the presence of spiders (Schmitz 2003). A well-studied example of predator-induced morphological defense occurs in the waterflea *Daphnia pulex*: individuals exposed to kairomones from fish and larvae of the insect predator *Chaoborus* during development grow pointed "helmets" and tail spines that reduce the likelihood of consumption (Woltereck 1909; Dodson 1988). Since it involves a change in *Daphnia*'s morphology, this example is referred to as a predator-induced morphological defense (Tollrian and Harvell 1998; Bourdeau and Johansson 2012).

Predator-induced defenses differ from constitutive defenses, which are protective traits that develop regardless of environmental conditions (Duffy and Hay 2001). In nature, it is thought that organisms employ constitutive defenses when the threat of predation is high or frequent (Bourdeau 2012), and employ inducible defenses when the trade-offs associated with the response are high or if predation is intermittent (Ferrari *et al.* 2010)**.** Traditionally, researchers have considered these two forms of defense separately and exclusively of one another, but in reality most defenses exist as a "mixture" of the two

(Bourdeau 2012). In many cases, these responses are triggered by water-borne kairomones, a chemical released by the predator species and sensed by the prey (Ferrari *et al.* 2010). The genus *Nucella*, discussed in the next section, exemplifies both constitutive and predator-induced morphological defenses.

1.4 The genus *Nucella*

Instances of predator-induced morphological defense exist in many species of Gastropoda. Examples include several members of the marine genera *Nucella* and *Littorina* (Palmer 1985b; Palmer 1990; Trussell 2000; Dalziel and Boulding 2005), several genera of freshwater snail (DeWitt *et al*. 2000; Krist 2002; Lakowitz *et al*. 2008), and terrestrial pulmonates (Hoverman and Relyea 2007). The genus *Nucella* has been the target of plasticity research for over 30 years, and serves as a model for the study of predator-induced morphological defense. *Nucella lamellosa, N. canaliculata,* and *N. ostrina* are three members of the genus that co-occur along much of the western coast of North America (Collins *et al.* 1996) and exhibit predator-induced morphological plasticity (Bourdeau 2011).

These three species exhibit predator-induced morphological shell plasticity in response to the red rock crab *Cancer productus,* a predator that penetrates the shell to access the soft tissue within. Exposure of *N. lamellosa* to *C. productus* elicits thicker shells, thicker shell lips and the development of apertural teeth (Palmer 1985a; Appleton and Palmer 1988; Gibbs 1993; Edgell and Neufield 2008; Bourdeau 2009, 2010a, and 2010b;

Edgell 2010). *Nucella canaliculata* and *N. ostrina* also thicken their shell and shell lip in response to *C. productus*, but to a lesser degree than *N. lamellosa* (Bourdeau 2011) and only rarely do they develop apertural teeth (Crothers 1984). Thickening of the apertural lip can make the entire shell less likely to break during an attack, especially if force is applied perpendicular to the growing margin of the aperture (Wilbur and Yonge 1964). Shell strength and resistance to breakage in gastropods increases as (approximately) the square of shell thickness (Wilbur and Yonge 1964; Vermeij and Currey 1980). Thus, slight differences in thickness lead to disproportionally greater resistance to breakage. In *N. lamellosa*, the thicker shell morphs were 15% stronger than those of control snails in mechanical strength tests (Bourdeau 2010a).

Because the threat of predation is often stochastic, many species have evolved mechanisms to ensure that anti-predator phenotypes are only produced when predators are present (Ferrari *et al.* 2010). There are several costs associated with shell thickness and thickening that would make it unfavorable to express when not needed. One potential cost is the energetic investment in synthesizing and depositing extra shell material and the subsequently increased cost of transporting the added weight (Palmer 1981). Less obvious costs are restrictions on somatic growth (Palmer 1981) and potentially reduced reproductive capacity in thick-shelled individuals (demonstrated in *N. emarginata* by Geller 1990).

The presence of *C. productus* is patchy; some beaches have dense populations of *C. productus* while on others the species is completely absent (Robles 1989). As a result of this

patchiness and the costs associated with shell thickness, *Nucella* have evolved the ability to vary the degree of lip thickness on a population-wide and an individual basis. Those from beaches with red rock crab present possess both greater constitutive and inducible shell defense than *N. lamellosa* populations from beaches where crabs are absent (Bourdeau 2012).

Cancer productus has two basic modes of attack: crushing and peeling. In the former method, the crab grasps a shell in one or both claws (Zipser and Vermeij 1978) and applies pressure until the shell fractures. In the latter, *C. productus* grasps the shell at the aperture with one or both claws and initiates a twisting motion which chips off pieces of the apertural lip (Zipser and Vermeij 1978; Palmer 1979). For larger, thicker snails, the peeling method is usually more successful than crushing (Zipser and Vermeij 1978). *Cancer productus* is another organism strongly affected by tidal immersion: it can forage in the intertidal zone during high tides, but must retreat to the subtidal or to a rocky crevice when the water recedes (Bertness 1977; Robles *et al.* 1989). Since crabs cannot hunt during tidal emersion, snails living lower in the intertidal experience a higher degree of predation risk from red rock crab than their upper-shore congeners (Table 1).

Nucella lamellosa lives in the low intertidal and subtidal zones, *N. canaliculata* in the mid intertidal, and *N. ostrina* in the high intertidal (Table 1) (Emlen 1966; Collins *et al.* 1996, Bourdeau 2011). As mentioned previously, *N. lamellosa* has much stronger constitutive and inducible responses to red rock crab, while *N. canaliculata*'s defenses are stronger than *N. ostrina*'s (Bourdeau 2011) (Table 1). These differences in the degree of defense, both

Table 1. Native tidal height, relative predation risk, and relative constitutive and inducible lip thickness in *N. lamellosa*, *N. canaliculata,* and *N. ostrina*. Native tidal height determines relative predation risk from *Cancer productus* and appears to correspond with the relative degree of constitutive and inducible lip thickness (from Bourdeau 2011).

Species	**Native tidal height**	**Relative predation risk from *C. productus***	**Relative constitutive and inducible lip thickness**
Nucella lamellosa	Subtidal, Low intertidal	High	High
N. canaliculata	Mid intertidal	Medium	Medium
N. ostrina	High intertidal	Low	Low

constitutive and inducible, appear to mirror each species' native height in the intertidal. *Nucella lamellosa* lives lowest in the intertidal, is constitutively thicker, and most morphologically plastic, while *N. ostrina* occurs highest in the intertidal, possesses the least robust shell, and is the least plastic. These correlations have led several researchers (Connell 1970; Bertness 1977; Bourdeau 2011) to suggest that the degree of shell plasticity against crushing predators is adaptively correlated with relative predation risk according to each species' height in the intertidal. While not directly testable, this hypothesis offers an explanation for the pattern of plasticity within the *Nucella* lineage.

1.5 Current study

While some researchers have hypothesized that the degree of constitutive and inducible defense is determined by the predation risk of each tidal height (Connell 1970; Bertness 1977; Bourdeau 2011), I suspected that physical restrictions in the intertidal may also have had a great deal to do with this pattern, since increasing height in the intertidal correlates with increasing physical stress. Each species has evolved in the context of a specific pattern of tidal immersion and has developed defensive strategies that are constrained by the physical restrictions that each has historically encountered. While it is impossible to rewind and replay the "tape of life" (Gould 1989) in order to identify the evolutionary pressures that led to a particular outcome, we can test this idea indirectly by taking advantage of the gradients that exist naturally in these three species and across the

intertidal. The next step in understanding the evolution of this model system is to determine if the existing predator-induced morphological responses in each species are affected by the gradient of environmental conditions across the intertidal zone. If all *Nucella* are able to achieve an increase in the inducible thickening response with increasing immersion time, it would suggest that evolving under more "favorable" conditions might have allowed stronger inducible and constitutive thickness to develop. However, if instead *Nucella* express the greatest anti-predator response at lower immersion times, it would imply that there is a more complex interplay between the physical restrictions of emersion and predator-induced morphological shell defense that requires further investigation.

I devised an experiment to determine if immersion time affects the degree of plastic response in *Nucella* by exposing the three congeners to four immersion times roughly approximating different intertidal zones and measuring the effect on the predator-induced lip thickening response. The experiment was conducted in a specially-designed outdoor apparatus plumbed with a one-way flow-through system delivering fresh seawater during the winter of 2011-2012. I predicted that *N. lamellosa, N. canaliculata,* and *N. ostrina* would show the greatest lip-thickening response and the greatest proportion of individuals with apertural teeth in high-immersion times because these conditions allow the maximum amount of time to feed (Bertness 1977; Bourdeau 2011; Price 2012), and presumably to sequester calcium from seawater (Wilbur and Yonge 1964; Vermeij 1993), and are less thermally, aerobically, and osmotically stressful than prolonged emersion (Menge and Branch 2001; Sorte and Hofmann 2005). I also predicted that increases in shell length, shell

weight, and body weight would be maximized in higher immersion times for the same reasons. Few studies have investigated how emersion stress can affect existing constitutive or predator-induced morphological responses, and thus far no work exists regarding the effects of immersion time on the predator-induced thickening response in *Nucella*.

2. METHODS

2.1 Overview

In order to determine whether differences in immersion time caused intra- and interspecies differences in the degree of plastic lip thickening response in *Nucella*, I devised an experiment in which *Nucella lamellosa, N. canaliculata,* and *N. ostrina* from the Salish Sea were exposed to four immersion time treatments in a specially-designed outdoor apparatus. This apparatus allowed me control not possible with a field experiment, but also enabled me to take advantage of natural air and seawater conditions. In effect, it excluded some of the stressors associated with the intertidal and the tidal cycle (e.g., predation, UV exposure) while including others such as temperature, desiccation, hypoxia, and reduced food access (Bertness 1977; Menge and Branch 2001; Morgan 2001; Sousa 2001; Sorte and Hofmann 2005; Bourdeau 2011; Price 2012).

Based on measurements by Bourdeau (2011), 35% immersion was chosen to represent the high intertidal zone (*N. ostrina*'s native height), while 100% represented the subtidal in my experiment. 50% and 75% immersion represented the mid- and low-intertidal zones respectively, but these percentages were selected for convenience and not based on actual measurements. In addition to immersion time, snails were subjected to either the presence or absence of predation cue. There were five replicates per species in each immersion time by predation cue treatment combination. Each replicate consisted of the mean change of three individual snails. Replicates were distributed randomly within five blocks. Changes in shell dimensions were assessed after a three month period.

2.2 Collections

Nucella lamellosa (120 individuals) were collected from Marine Park, Bellingham during October, 2011 (average shell length (SL) = 22.27 ± 3.18 mm, minimum SL = 17.25 mm, maximum SL = 33.25 mm); *Nucella canaliculata* (120) were collected from Cattle Point and Eagle Cove, San Juan Island during September 2011 (average shell length = 22.13 ± 2.23 mm, min SL = 11.55 mm, max SL = 27.75 mm); *Nucella ostrina* (120) were collected from Cattle Point and Deadman Bay, San Juan Island (average SL = 20.95 ± 1.47 mm, min SL = 17.8 mm, max SL = 24.48 mm). After collection, snails were housed in holding tanks supplied by the flow-through seawater system at Shannon Point Marine Center in Anacortes, Washington for 8-11 weeks before the initiation of the experiment. Prior to and during the experiment, snails were maintained on an *ad libitum* diet of the barnacle *Balanus glandula*. Clumps of *B. glandula* were collected periodically throughout the experiment from Marine Park in Bellingham, and distributed to snail beakers every three weeks. Approximately 40 *B. glandula* were consumed per snail during the experiment.

Cancer productus were collected from Hat Island, Skagit County by SCUBA during September and November, 2011 and housed in the flow-through seawater system at Shannon Point Marine Center in Anacortes, Washington for 11 weeks prior to the initiation of the experiment. During the experiment, there was a 42.1% mortality of crabs and additional *C. productus* were collected as needed by crab trap and by hand at low tide in Skagit and Whatcom Counties. These individuals experienced varying acclimatization times to the flow-through seawater system prior to being introduced to the experiment. In total,

34 male and 4 female *C. productus* were collected and used. The four females were replaced by males as soon as they became available (5 days after their introduction to the apparatus) in order to ensure uniformity of cue among treatments. *Cancer productus* were maintained on a diet of *Mytilus* spp., which were collected in clumps from Marine Park in Bellingham periodically. The feeding regime was *ad libitum,* although several crabs ceased eating during the course of the experiment. Approximately 1,850 *Mytilus* were consumed during the experiment.

2.3 Experimental Apparatus

An outdoor one-way flow-through seawater apparatus was constructed at Shannon Point Marine Center in Anacortes, Washington. The apparatus consisted of four 19 mm diameter PVC pipes elevated 1.75 m off the ground, fed by water pumped by an intake system from approximately 90 m off of the Shannon Point Beach. Three of these pipes were controlled by mechanical on/off timers regulating water flow and the fourth remained on constantly. Each pipe simulated water immersion at different tidal heights by delivering water for a designated portion of the day (35%, 50%, 75%, or 100%). These pipes fed into ten 6 L predator-cue bins situated on a platform 0.45 m below. Five bins housed a single *C. productus* and several *Mytilus* spp. (predation cue) and five housed only *Mytilus* spp. (no predation cue). In total, the four pipes supplied 10 bins each for a total of 40 bins, half of which held crabs. Thus there were five replicates in every immersion time by cue type

combination. The average flow rate to each beaker when water was being delivered was 0.237 L/min ± 0.231 (error represents standard deviation unless otherwise noted). To account for this variance in flow rate, bins were blocked into 5 groups and their order within each block randomized (Figure 1). Additionally, the order of species placement within each treatment unit was randomized to account for differences in flow rate along the manifold.

The mechanical timers (RainDrip® Digital Water Timer) controlling the 35%, 50%, and 75% water pipes initiated water flow twice a day (at noon and midnight) to roughly approximate the local tidal pattern. The 35% timer shut off water to its bins after 248 min, the 50% timer after 354 min, and the 75% after 536 min. In order to account for the effects of fill time and drain time in the beakers, a subset of ten beakers in the experiment were randomly selected for observation and the average fill time (237 sec ± 231 s, rounded to 240 s/4 min) and drain time (490 s ± 285, rounded to 480 s/8 min) were determined. The water shutoff schedule was designed to account for these measurements. When the timer turned off water flow to a pipe, the connected bins drained until they were approximately half full, allowing crabs to remain immersed but stopping flow to the beakers downstream.

Each predator treatment bin was plumbed via 19 mm diameter PVC pipe to three beakers (situated one meter beneath the predation cue bins). The pipe terminated in a manifold that delivered water from the pipe into the beakers via three 3.2 mm x 6.4 mm nozzles and short sections of latex tubing (6.4 mm internal diameter) which were inserted into the mesh lids of the beakers. Together, one bin, three beakers, and the plumbing between them composed a single treatment unit (Figure 2).

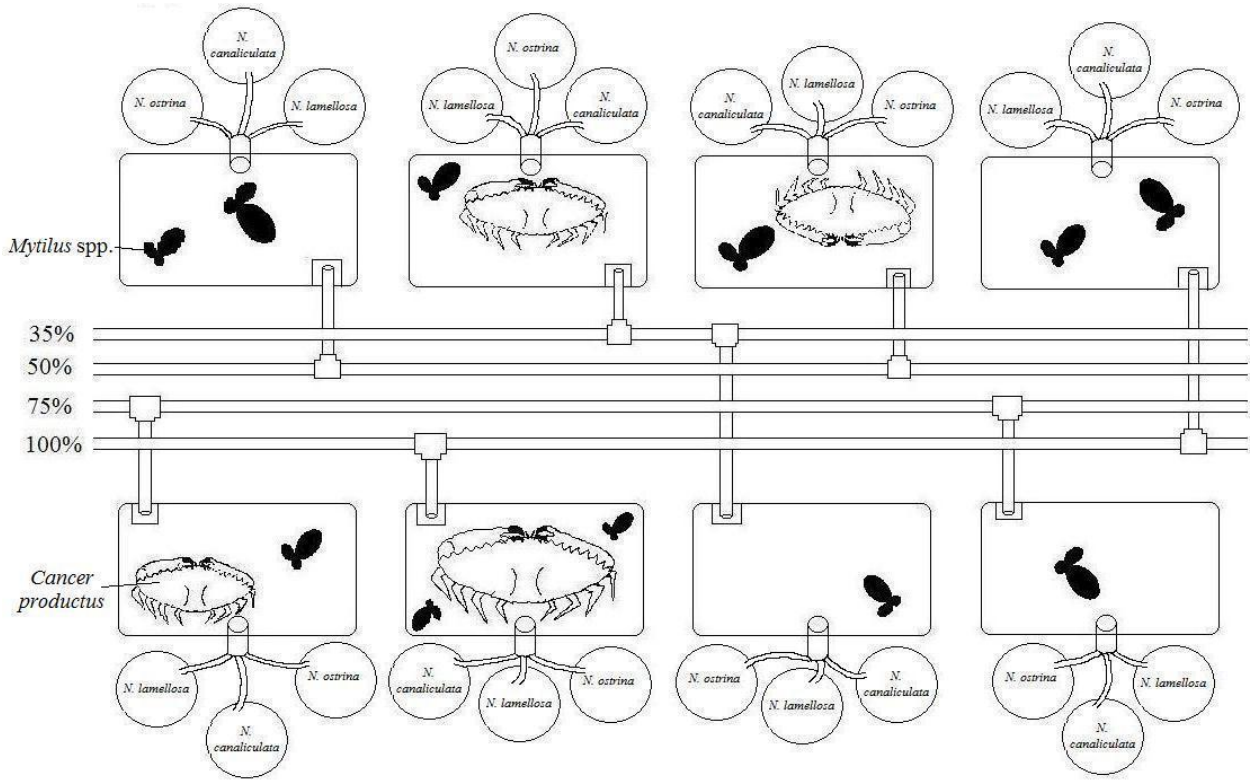

Figure 1. A single block within the experimental apparatus. Each immersion time pipe feeds two bins, one with a crab and one without. These bins then feed via a manifold into three beakers, each containing three individuals of *N. lamellosa, N. canaliculata,* or *N. ostrina*. The placement of these beakers was randomized to account for differences in flow rate along the manifold. There were five blocks in the experimental apparatus, and the order of treatment combinations independently randomized within each.

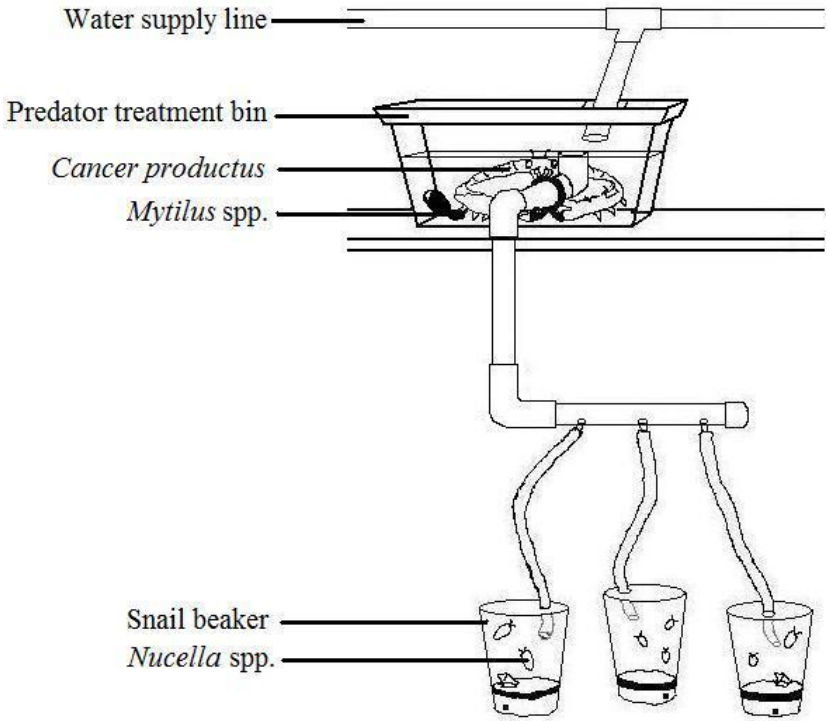

Figure 2. A single treatment unit consisting of one of four immersion times (35%, 50%, 75% or 100%) and one of two cue types (crab or No-Crab). *N. lamellosa, N. canaliculata,* and *N. ostrina* are each represented in one of the beakers. Each beaker contains three snails. The barnacle clumps which served as food for the snails and beach cobble were omitted from this diagram.

Snails were housed in 1 L plastic VWR® beakers (Figure 2). A 1.6 mm diameter hole was drilled near the bottom of each beaker to permit constant drainage of water. While water was flowing to a beaker from the predator treatment bin above, the rate of flow exceeded the amount of water that could drain through the hole, and the water overflowed through the mesh lid. When water flow from the bin above ceased, the beaker would drain completely through the hole in the bottom. An elevated mesh platform was placed in the bottom of each beaker to prevent snails or particulates from blocking the drainage hole. A small beach cobble was placed atop this platform as substrate (Figure 3). This design ensured that snails would be fully immersed when water was flowing and quickly emersed when the water was shut off.

The beakers rested in groups of three on an elevated grated platform which allowed them to drain freely. Each beaker contained three individuals of one species, and there were 120 beakers within the apparatus. 120 individuals of each species were used, resulting in 15 individuals (5 treatment units) per immersion time by ± predation cue. Individual snails were randomly assigned to their beakers and bins.

2.4 Experiment

The experiment was initiated on November 26th, 2011 by placing each animal in its pre-determined bin or beaker. The experiment was maintained for three months, terminating on February 20th, 2012. During the experiment, two snails died of unknown but

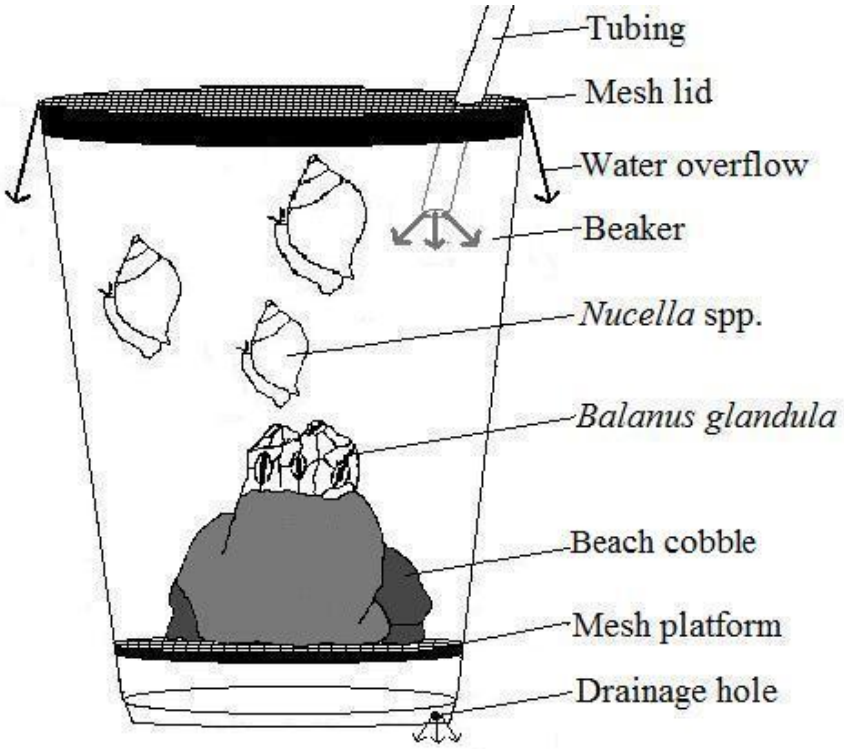

Figure 3. One of 120 beakers housing three individual snails, a small beach cobble for substrate and a clump of barnacles for food. Water from a predator treatment bin was delivered via a PVC manifold and a latex tube, and could drain freely out of the beaker through a small hole near the bottom. While water was flowing to a beaker, the rate of flow exceeded the amount of water that could drain through the hole, and the water overflowed through the mesh lid. When water flow from the bin above ceased, the beaker would drain completely through the hole in the bottom. The mesh platform prevented snails, sediment or other particulates from obstructing the drainage hole.

presumably natural causes and were excluded from data analysis, while the remaining 358 snails were frozen on Feb 27th, 2012.

Beakers containing snails were disturbed as little as possible, but removal of algae and barnacle shell fragments, and clearing of the drain hole was required every three weeks. Additionally, new clumps of barnacles were added to the beakers once every three weeks. Live *Cancer productus* were randomly assigned to treatment bins to provide predator cue. The average width of *C. productus* used in the experiment was 11.7 ± 0.31 cm. The smallest crab used was 8.1 cm and the largest 14.8 cm. Mussels were added as needed to the predation cue bins 2-3 times a week. Predation cue bins, PVC manifolds, nozzles, and tubing connecting the bins to the beakers were cleaned of detritus 2-3 times a week.

2.5 Measurements

Snails were measured between November 11 and 13, about two weeks prior to the experiment, and between February 25-27, immediately after termination of the experiment, in order to gauge changes in shell morphology. Prior to measuring, snails were labeled with numbered tags attached to the body whorl by cyanoacrylate adhesive so that each individual could be identified for iterative measurements. Shell length, lip thickness at the center and top of the aperture (subsequently averaged) were measured using Whitworth® digital calipers (Figure 4). Additionally, a subjective assessment of the presence or absence of apertural teeth was made for *N. lamellosa*. Shell and body weight were

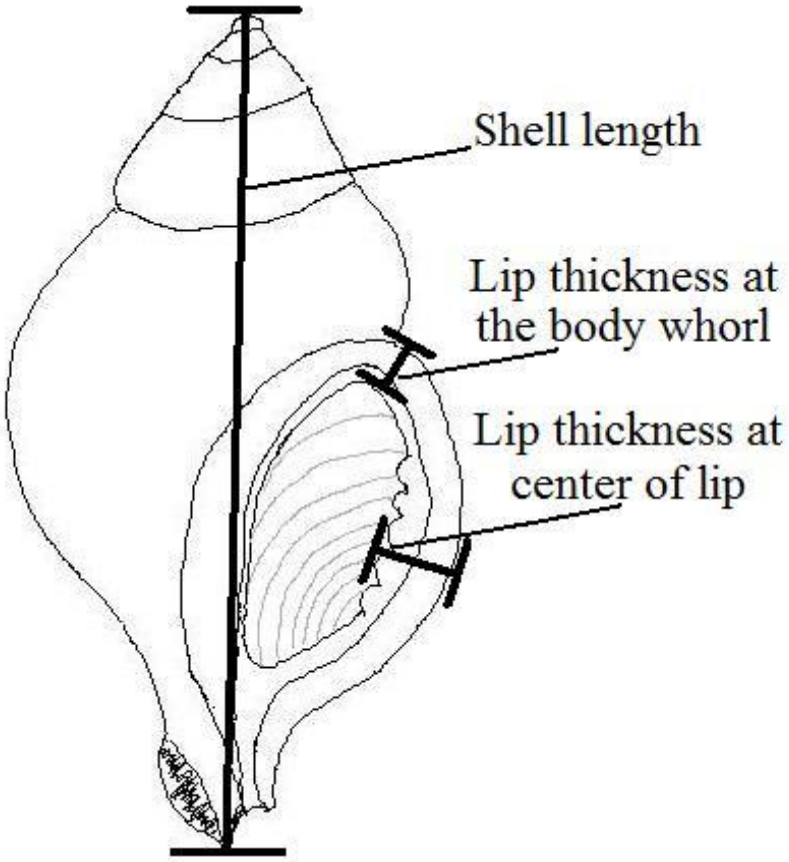

Figure 4. Shell dimensions measured with Whitworth® digital calipers included shell length and two measures of lip thickness, which were averaged.

estimated by comparing immersed and semi-dry weights corrected by experimentally-established equations, as outlined in Palmer (1982). Shell weight was calculated in *Nucella lamellosa* with the equation, 1.572 x submerged weight + 0.0162; in *N. canaliculata,* 1.558 x submerged weight + 0.0075; and in *N. ostrina*, 1.530 x submerged weight + 0.0032. Body weight was calculated by subtracting semi-dry weight from estimated shell weight.

2.6 Statistical Analyses

Changes in shell length, weight, and lip thickness were calculated for each individual snail. Analyzing differences rather than final means avoided many interactions in the statistical models. Mean change of the three (in two cases two) snails in each beaker were used for analysis, yielding 5 replicates for each of the 24 treatment groups (species*immersion time*cue). The mean treatment group changes were plotted for inter- and intraspecies comparison. For assessment of apertural teeth in *N. lamellosa*, data consisted of the number of snails in each beaker with apertural teeth at the end of the experiment.

To determine the effects of the experimental variables, generalized linear mixed models (GLMM) were fit to the data using the package “lme4” for the statistical program R. GLM models allowed the incorporation of mixed effects to account for the random variables created by the split plot design (with species nested in immersion time and cue) and the blocked arrangement of the treatment units. This reduced the number of interactions,

greatly simplifying the models. GLM modeling consists of fitting fully crossed and reduced combinations of fixed and random factors (models) to the data and assigning a relative Akaike Information Criterion (AIC) value based on goodness-of-fit. The best-fit model is judged as the one with the AIC value. The presence of a factor or interaction in the best-fit model is analogous to being associated with a significant p-value in ANOVA.

To confirm that results differed between species, shell length, shell weight, body weight, and lip thickness were analyzed with "Initial Shell Length," "Species," "Cue," and "Immersion Time" defined as fixed effects. "Treatment Unit" and "Block" were defined as random factors in order to account for the fact that beakers were nested in treatment unit. The significance of the fixed factors and their interactions were determined by comparing the fits of full models with reduced models. *Nucella lamellosa*, *N. canaliculata,* and *N. ostrina* were also analyzed individually in order to test the within-species effects of immersion time and cue on shell dimensions (including apertural teeth in *N. lamellosa*). The factor "Species" was eliminated, while "Initial Shell Length," "Cue," "Immersion Time," and "Block" were retained. Due to an apparent correspondence between native immersion time and maximal lip thickening and apertural teeth expression, these dimensions were further analyzed in all three species together and individually by recoding immersion times as "Native" or "Non-Native" and retesting GLM models. In all cases, the lowest AIC value was used to determine the model of best fit.

3. RESULTS

3.1 Overview

Changes in shell length, shell weight, body weight, and lip thickness differed by species, immersion time, and cue (sections 3.2, 3.3, and 3.4). The fact that these results differed in each species was supported by the best-fit all-species GLMMs, which showed that "Species" was a factor describing changes in all dimensions (Table 2). The inclusion of "Initial Shell Length" as a factor in all but one of the five group models suggests that the effects of "Cue" and "Immersion Time" on shell weight, body weight, and lip thickness, differed by snail size (Table 2). The interactions between "Species," "Cue," and "Immersion Time," imply that these factors influence each other's effects on shell weight, body weight, and lip thickness, and prompted further species-by-species analysis (Table 2).

3.2 Overall shell growth

Overall shell growth was represented by changes in shell length and shell weight. *Nucella lamellosa*, *N. canaliculata,* and No-Crab *N. ostrina* increased in both of these dimensions, indicating that shell growth occurred over the course of the experiment (Figures 5 and 6). Cue appeared to have some effect on shell lengthening: No-Crab *N. lamellosa* and *N. canaliculata* showed slightly larger increases in shell length and weight than Crab-exposed snails in the same immersion treatment (except in *N. canaliculata* 50%) and generalized linear mixed modeling indicated that "Cue" was a factor describing shell

Table 2. The five best-fit generalized linear mixed models describing changes in shell and body dimensions *N. lamellosa, N. canaliculata,* and *N. ostrina.* Initial Shell Length = L, Species = S, Cue = C, Immersion Time = I, N = Nativity. The best-fit model is denoted by the lowest Akaike Information Criterion (AIC) value and is listed first. Models include the main factors and interactions between factors that describe changes in snail size. The presence of a factor or interaction in the best-fit model is analogous to being associated with a significant p-value in ANOVA. "Block" and "Treatment Unit" were included as random factors in all models, but not listed in the table.

Dimension	Top 5 models	AIC values
Shell length	L + S + C + I + LxSxCxI + LxS + LxC + LxI + LxSxC + LxSxI + SxCxI + SxI + SxC + CxI	251.0266
	L + S + C + I + SxCxI + SxC + CxI +SxI	253.0166
	S + C + I + SxCxI + SxC + CxI +SxI	261.3256
	L + S + I + LxSxI + LxS + SxI + LxI	280.8741
	L + S + I + SxI	282.9779
Shell weight	L + S + C + I + SxCxI + SxC + CxI +SxI	-126.0729
	S + C + I + SxCxI + SxC + CxI +SxI	-125.5054
	L + S + C + I + LxSxCxI + LxS + LxC + LxI + LxSxC + LxSxI + SxCxI + SxI + SxC + CxI	-125.3937
	LxSxI + LxS + SxI + LxI	-116.0700
	S + I + SxI	-115.9690
Body weight	L + S + C + I + SxCxI + SxC + CxI +SxI	-370.2014
	L + S + C + I + LxSxCxI + LxS + LxC + LxI + LxSxC + LxSxI + SxCxI + SxI + SxC + CxI	-366.2237
	S + C + I + SxCxI + SxC + CxI +SxI	-345.5885
	L + S + C + I + CxI	-322.0286
	L + S + C + I + LxS + LxC + LxI + LxSxCxI + LxSxC + LxCxI + LxSxI + CxI	-320.3577
Lip thickness	L + S + C + I + SxCxI + SxC + CxI +SxI	-0.1713580
	S + C + I + SxCxI + SxC + CxI +SxI	0.2839528
	L + S + C + I + LxSxCxI + LxS + LxC + LxI + LxSxC + LxSxI + SxCxI + SxI + SxC + CxI	11.08658
	S + C + I + SxCxI + SxC + SxI	41.35416
	L + S + C + I + SxCxI + SxC + SxI	42.70742
Lip thickness, nativity	SxCxN + SxC + CxN +SxN	0.3944879
	L + S + C + N + SxCxN + SxC + CxN +SxN	1.176928
	L + S + C + N + LxSxCxN + LxS + LxC + LxN + LxSxC + LxSxN + SxCxN + SxN + SxC + CxN	17.70505
	S + C + N + CxN	46.84024
	L + S + C + N + CxN	48.82695

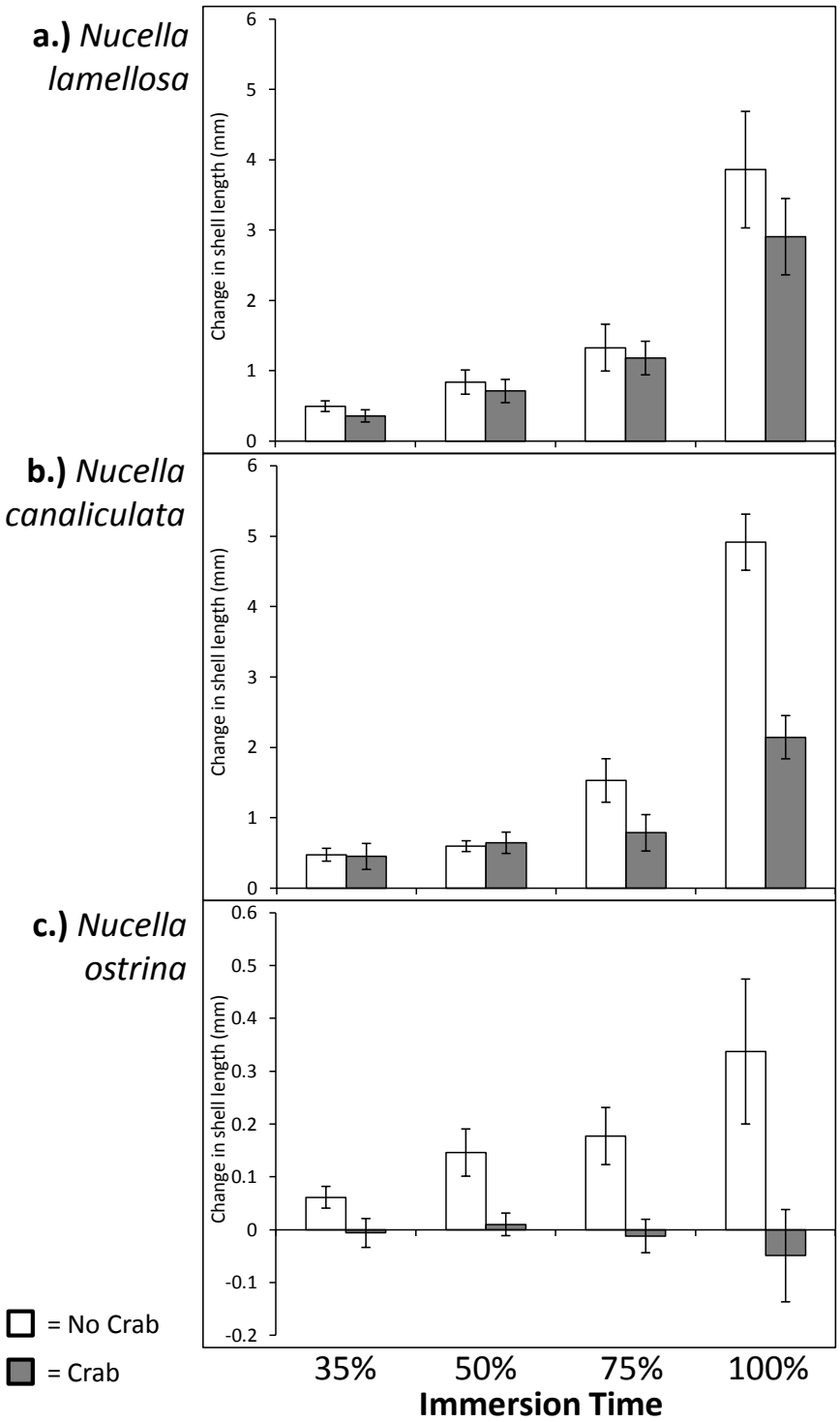

Figure 5. Changes in shell length of *Nucella* following 87 days of exposure to one of four immersion times (35%, 50%, 75%, or 100%) and either crab and mussel cue (Crab) or mussel cue only (No-Crab). Each bar represents the mean change of snails in five beakers (n=5). Error bars indicate standard error. Note that *N. ostrina* is plotted on an axis with major increments one tenth the scale of *N. lamellosa*'s and *N. canaliculata*'s.

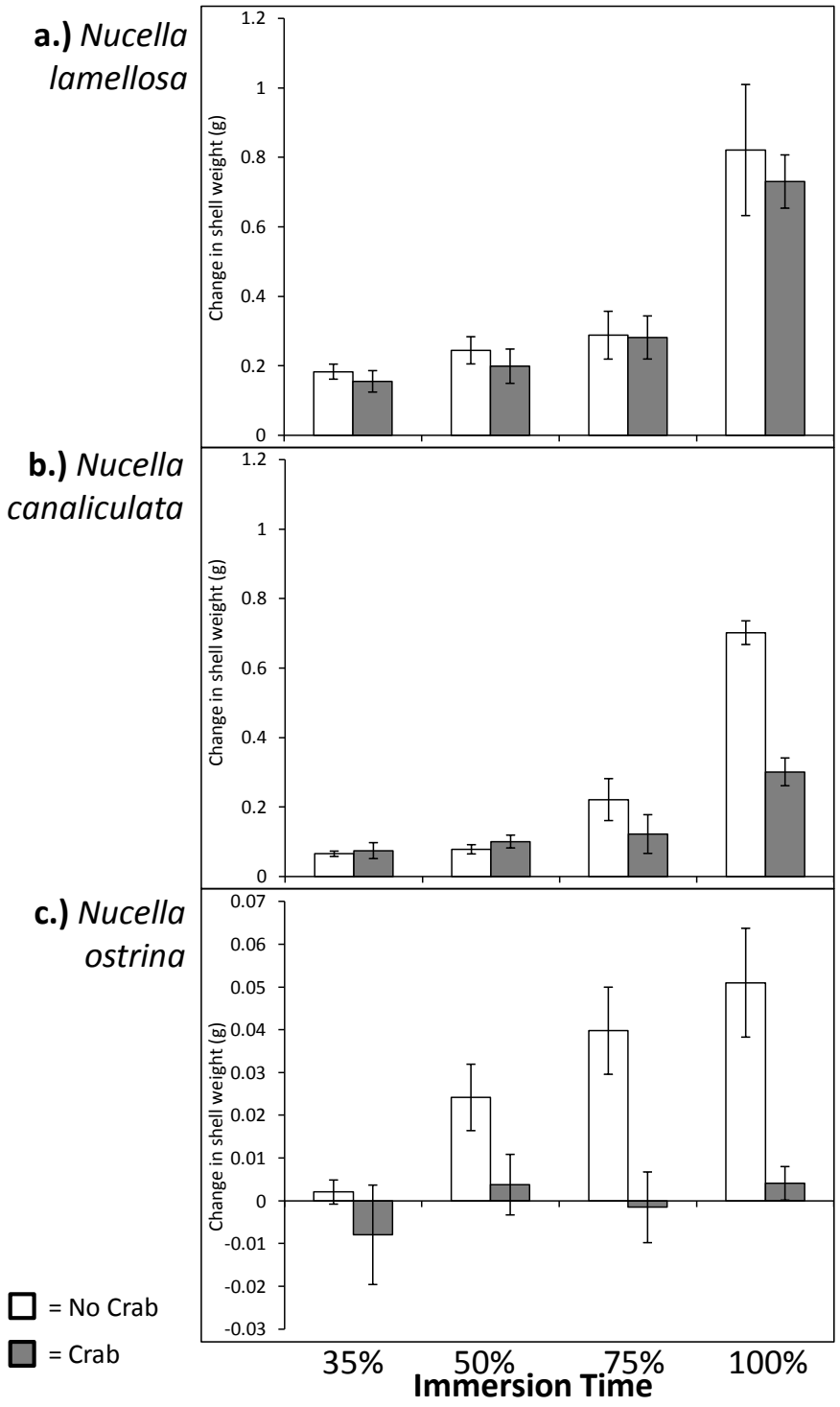

Figure 6. Changes in shell weight of *Nucella* following 87 days of exposure to one of four immersion times (35%, 50%, 75%, or 100%) and either crab and mussel cue (Crab) or mussel cue only (No-Crab). Each bar represents the mean change of snails in five beakers (n=5). Error bars indicate standard error. Note that *N. ostrina* is plotted on an axis with major increments one twentieth the scale of *N. lamellosa*'s and *N. canaliculata*'s.

growth in both these species (Tables 3 and 4). *Nucella ostrina* had a very strong response to Crab cue: Crab-exposed showed negative growth in all immersion times except for slight increases in shell length at 50% and shell weight at 50% and 100% (Figures 5 and 6). Crab-exposed *N. ostrina* showed negative shell growth in all immersion times except for slight increases in shell length at 50% and shell weight at 50% and 100% (Figures 5 and 6). Negative changes in shell length reflect erosion of the shell spire (Spight 1973), while negative changes in shell weight reflect this loss. The strong effect of cue in *N. ostrina* is reflected in the fact that "Cue" was a factor describing shell length and weight change in this species (Table 5). Predation cue appeared to suppress shell growth in all three species, although the strength of this effect was weak in *N. lamellosa* and *N. canaliculata*. GLM modeling also indicated an interaction between "Cue" and "Immersion Time" for *N. canaliculata* and *N. ostrina* (Tables 3, 4, and 5).

Immersion time had a strong effect on the pattern of overall shell growth in all three species, demonstrated by the trend of increasing gains in shell length and weight with increasing immersion time (Figures 5 and 6). The importance of immersion time is supported by the fact that the best-fit shell length and shell weight GLMMs included "Immersion Time" as a factor in all three species (Tables 3, 4, and 5). The interactions between "Immersion Time" and the other factors present in these models imply that the effects of the other fixed factors are different in each immersion time. The fact that "Initial Shell Length" is a factor describing changes in shell growth for all but shell weight in *N. canaliculata* indicates that the effects of cue and immersion time are mediated by snail size.

Table 3. The five best-fit generalized linear mixed models describing changes in shell and body dimensions of *N. lamellosa.* Initial Shell Length = L, Species = S, Cue = C, Immersion Time = I, N = Nativity. The best-fit model is denoted by the lowest Akaike Information Criterion (AIC) value and is listed first. Models include the main factors and interactions between factors that describe changes in snail size. The presence of a factor or interaction in the best-fit model is analogous to being associated with a significant p-value in ANOVA. "Block" was included as a random factor in all models, but not listed in the table.

Dimension	Top 5 models	AIC Values
Shell length	L + I + LxI	106.4999
	L + C + I + LxCxI + LxC + LxI + CxI	107.4318
	L + I + LxI	111.0915
	L + C + I	111.6828
	L + C + I + CxI	112.8836
Shell weight	L + I + LxI	-9.133772
	I	-7.932867
	L + I	-7.320556
	L + C	-6.400648
	L + C + I	-5.744526
Body weight	L + I + LxI	-109.5282
	L + I	-107.9798
	L + C + I	-107.8745
	L + C + I + LxCxI + LxC + LxI	-107.1078
	L + C + I + CxI	-105.938
Lip thickness	C + I + CxI	35.80515
	L + C + I + CxI	35.85448
	L + C + I + LxCxI + LxC + LxI + CxI	40.19557
	C + I	49.89441
	L + C + I	51.25202
Lip thickness, nativity	C + N + CxN	36.8062
	L + C + N + CxN	37.76595
	L + C + N + LxCxN + LxC + LxN + CxN	43.33042
	L + C + N	52.4643
	L + C + N + LxCxN + LxC + LxN	54.4549
Apertural Teeth	L + C + I	42.29251
	C + I	42.47349
	L + C + I + LxCxI + LxC + LxI	44.27724
	L + C + I + CxI	47.08603
	C + I + CxI	47.33334
Apertural Teeth, nativity	L + C + N	51.09833
	L + C + N + CxN	52.60127
	L + C + N + LxCxN + LxC + LxN	53.08653
	C + N	54.21911
	C + N + CxN	55.77471

Table 4. The five best-fit generalized linear mixed models describing changes in shell and body dimensions of *N. canaliculata.* Initial Shell Length = L, Species = S, Cue = C, Immersion Time = I, N = Nativity. The best-fit model is denoted by the lowest Akaike Information Criterion (AIC) value and is listed first. Models include the main factors and interactions between factors that describe changes in snail size. The presence of a factor or interaction in the best-fit model is analogous to being associated with a significant p-value in ANOVA. "Block" was included as a random factor in all models, but not listed in the table.

Dimension	Top 5 models	AIC Values
Shell length	L + C + I + CxI	98.91663
	C + I + CxI	100.0527
	L + C + I + LxCxI + LxC + LxI + CxI	102.9577
	C + I	114.9411
	L + C + I	115.5249
Shell weight	C + I + CxI	-55.2373
	L + C + I + CxI	-54.08786
	L + C + I + LxCxI + LxC + LxI + CxI	-50.3269
	C + I	-39.17781
	L + C + I	-37.42911
Body weight	L + C + I + CxI	-112.0998
	L + C + I + LxCxI + LxC + LxI + CxI	-110.6261
	C + I + CxI	-107.6756
	L + C + I	-92.33491
	C + I	-91.32865
Lip thickness	L + I + LxI	-46.9455
	L + C + I + LxCxI + LxC + LxI + CxI	-45.8962
	I	-44.59276
	L	-43.12424
	C	-42.78076
Lip thickness, nativity	L + N + LxN	-53.48464
	L + N	-47.90269
	C + N	-47.52397
	L + C + N + LxCxN + LxC + LxN + CxN	-46.21483
	L + C + N	-46.10361

Table 5. The five best-fit generalized linear mixed models describing changes in shell and body dimensions of *N. ostrina.* Initial Shell Length = L, Species = S, Cue = C, Immersion Time = I, N = Nativity. The best-fit model is denoted by the lowest Akaike Information Criterion (AIC) value and is listed first. Models include the main factors and interactions between factors that describe changes in snail size. The presence of a factor or interaction in the best-fit model is analogous to being associated with a significant p-value in ANOVA. "Block" was included as a random factor in all models, but not listed in the table.

Dimension	Top 5 models	AIC Values
Shell length	L + C + I + CxI	-37.75592
	C + I + CxI	-36.49376
	L + C + I + LxCxI + LxC + LxI + CxI	-33.74517
	L + C + I	-31.90674
	C + I	-31.57302
Shell weight	L + C + I + CxI	-197.6765
	C + I + CxI	-196.7277
	L + C + I + LxCxI + LxC + LxI + CxI	-195.3960
	L + C + I	-192.6157
	C + I	-192.2392
Body weight	C + I + CxI	-169.4711
	L + C + I + CxI	-167.8856
	C + I	-165.3911
	C	-164.5517
	L + C + I	-163.6274
Lip thickness	I	-43.6084
	L	-42.89476
	C	-42.44515
	L + I	-42.32737
	C + I	-41.75378
Lip thickness, nativity	L + N	-44.92015
	L + N + LxN	-44.68636
	C + N	-44.126
	C + N + CxN	-43.67796
	I	-43.6084

GLM modeling indicated interactions between “Initial Shell Length” and “Immersion Time” in *N. lamellosa*, and between “Cue” and “Immersion Time” in *N. canaliculata* and *N. ostrina* (Tables 3, 4, and 5).

3.3 Somatic growth

Somatic growth was represented by change in body weight. About half of the treatment groups gained body weight, indicating somatic growth over the course of the experiment, while the other half displayed decreases, indicating loss of somatic tissue (Figure 7). *Nucella lamellosa* increased body weight in all treatments except 35% No-Crab and Crab and 50% Crab. *Nucella canaliculata* increased body weight in all treatments except in 35% and 50% Crab. *Nucella ostrina* increased body weight in all treatments except 35% No-Crab and Crab and 50% Crab.

Predation cue had a clear effect on the amount of body mass gain: within each immersion treatment, each species showed a difference in the changes in body mass between No-Crab and Crab snails. In most cases, No-Crab snails showed a greater gain in body mass, or less of a decrease in body mass, than their Crab-exposed counterparts. GLM modeling supported this result in *N. canaliculata* and *N. ostrina*, including “Cue” as a factor describing body mass change (Tables 3, 4, and 5). In *N. canaliculata* and *N. ostrina*, this factor interacts with immersion time (Tables 4 and 5).

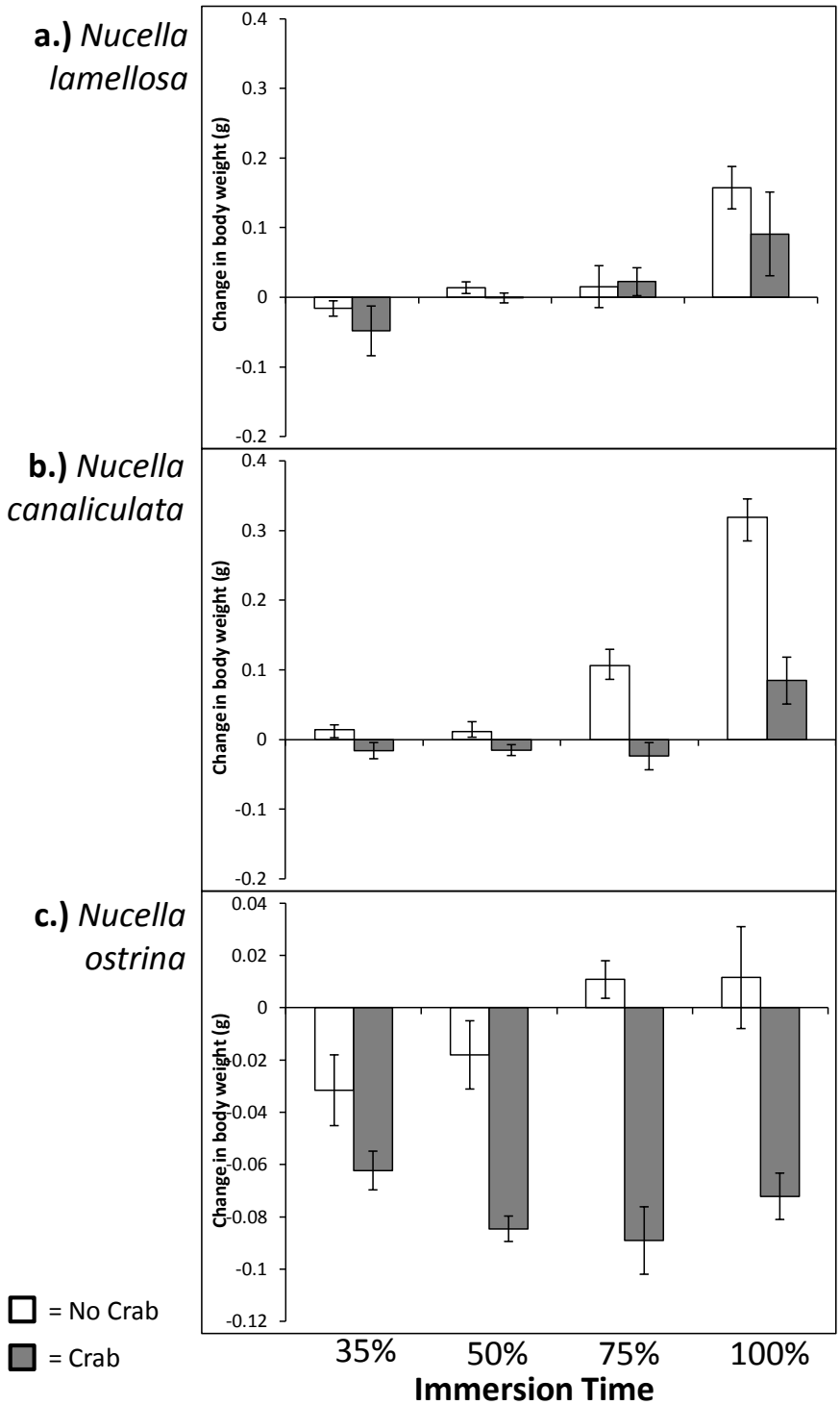

Figure 7. Changes in body weight of *Nucella* following 87 days of exposure to one of four immersion times (35%, 50%, 75%, or 100%) and either crab and mussel cue (Crab) or mussel cue only (No-Crab). Each bar represents the mean change of snails in five beakers (n=5). Error bars indicate standard error. Note that *N. ostrina* is plotted on an axis with major increments one fifth the scale of *N. lamellosa*'s and *N. canaliculata*'s.

The relative degree of body weight increase of *N. lamellosa, N. canaliculata,* and No-Crab *N. ostrina* corresponded with relative immersion time: 100% immersion time treatments showed the greatest weight increases and 35% showed decreases (Figure 7), although this pattern is not as distinct as it is for shell length and shell weight. GLM models support this pattern, including "Immersion Time" as a factor describing change in body weight (Tables 3, 4, and 5). The interactions between "Cue" and "Immersion Time" that exist in *N. canaliculata* and *N. ostrina* imply that the effects of immersion time differ by cue type and vice versa (Tables 4 and 5). "Initial Shell Length" is a factor describing changes in shell length in *N. lamellosa* and *N. canaliculata* and interacts with the other fixed factors in *N. lamellosa* (Tables 3 and 4).

3.4 Defensive responses

The defensive responses in *Nucella* were indicated by changes in lip thickness and the proportion of *N. lamellosa* exhibiting apertural teeth at the end of the experiment. Constitutive levels of lip thickness and apertural teeth expression were denoted by No-Crab treatments, while inducible responses were indicated by expression in Crab-exposed treatments. In all but two treatment groups, *Nucella* demonstrated increases in lip thickness over the course of the experiment (Figure 8) and in all but 100% *N. lamellosa*, over half of the snails in each treatment group had apertural teeth at the end of the experiment (Figure

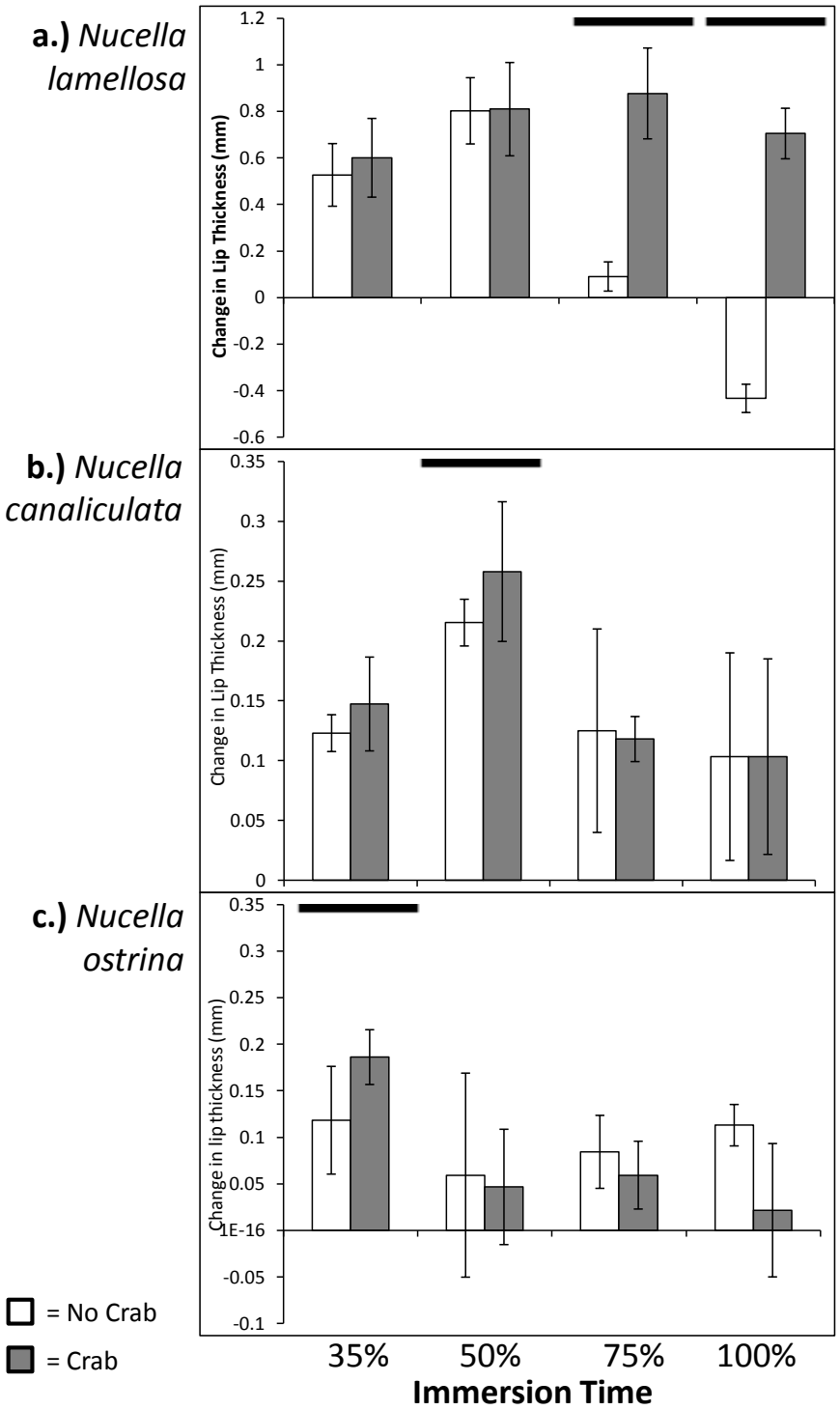

Figure 8. Changes in lip thickness of *Nucella* following 87 days of exposure to one of four immersion times (35%, 50%, 75%, or 100%) and either crab and mussel cue (Crab) or mussel cue only (No-Crab). Each bar represents the mean change of snails in five beakers (n=5). Error bars indicate standard error; horizontal bars indicate native immersion time. Note that *N. canaliculata*'s and *N. ostrina*'s are plotted on an axis with major increments one fourth the scale of *N. lamellosa*.

9). The relative degree of lip thickening between species differs from Bourdeau's (2011) findings: *N. canaliculata* actually showed a smaller difference between Crab and No-Crab at its optimal immersion time (50%) than *N. ostrina* did at its (35%) (Figure 8). The overall magnitude of thickening was still greater in *N. canaliculata* than *N. ostrina*, however (Figure 8).

In most treatment groups, *Nucella* thickened more when exposed to No-Crab cue than Crab cue. Only in 35%, 75%, and 100% *N. lamellosa*, 35% and 50% *N. canaliculata,* and 35% *N. ostrina* did Crab snails thicken more than their No-Crab counterparts (Figure 8). That the effect of cue on lip thickness varied by immersion time is demonstrated in *N. lamellosa* by the interaction between these two factors (Table 3). In contrast, apertural teeth in *N. lamellosa* showed the predicted pattern of inducible response to crab cue, in that Crab-exposed snails thickened more than their No-Crab counterparts (Figure 9) and the best-fit GLM model included "Cue," "Immersion Time," and the interaction between them as factors (Table 3). Crab cue affected lip thickening in all species and apertural teeth, but the pattern of increase varied among species.

Immersion time had a complicated effect on defensive responses in all three species. While constitutive thickness was higher at lower immersion times in all species, *Nucella lamellosa* in 35%, 50%, 75% Crab, and 100% Crab thickened to an almost equivalent degree, while at 75% No-Crab snails thickened very little and at 100%, lost lip thickness (Figure 8). The best-fit GLMM describing lip thickening in this species included "Immersion Time," suggesting that this factor had an effect on these results (Table 3). In No-Crab *N. lamellosa*,

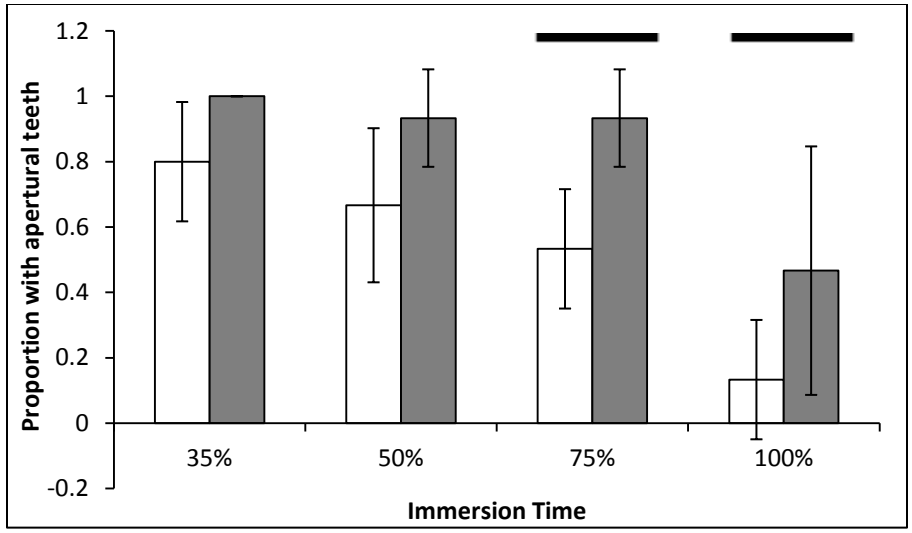

Figure 9. Frequency of *Nucella lamellosa* expressing apertural teeth following 87 days of exposure to one of four immersion times (35%, 50%, 75%, or 100%) and either crab and mussel cue (Crab) or mussel cue only (No-Crab). Each bar represents the mean change of snails in five beakers (n=5). Apertural teeth response was measured by counting the number of individuals with apertural teeth present at the end of the experiment. Error bars indicate standard error; horizontal bars indicate native immersion time.

the proportion of snails with teeth actually decreased with increasing immersion time. In 35%, 50%, and 75% Crab, nearly all snails have apertural teeth, but at 100% immersion, the proportion is closer to half (Figure 9). Immersion time was included as a fixed factor describing apertural teeth. In *N. lamellosa*, the strongest expression of constitutive shell defenses occurred in low immersion times (35% and 50%), while the greatest inducible responses occurred at 75% and 100%. *Nucella canaliculata* and *N. ostrina* showed near-equivalent thickening across immersion times. In *N. canaliculata*, the greatest constitutive and inducible lip thickness occurred at 35% and especially 50% (Figure 8). In *N. ostrina,* the greatest constitutive and inducible lip thickness occurred at 35%. GLM modeling showed that immersion time was a factor describing lip thickening in these species (Tables 4 and 5). In *N.* canaliculata, immersion time interacted with initial shell length (Table 4).

The greatest constitutive thickness in *N. canaliculata* and *N. ostrina*, and the greatest inducible response in all three species, occurred at each species' native immersion time (Figure 8): *N. lamellosa* at 75% (89.68% greater in Crab than No-Crab) and 100% (161.47% greater in Crab than No-Crab), *N. canaliculata* at 50% (16.54% greater in Crab than No-Crab; 35% *N. canaliculata* was close to this at 16.52%), and *N. ostrina* at 35% (36.44% greater in Crab than No-Crab). Additionally, the greatest difference in apertural teeth occurred at 75% (42.86% more Crab-exposed snails had apertural teeth than No-Crab) and 100% (71.43% more Crab-exposed snails had apertural teeth than No-Crab). Based on this pattern, I tested new models with "Immersion Time" recoded as "Native" or "Non-Native." The best-fit group GLM model included "Nativity" (Table 2), which justified further

species by species analysis. All individual species GLM models included “Nativity” as a factor. For changes in lip thickness of *N. lamellosa*, GLM modeling indicates interactions between “Cue” and “Nativity” (Table 3). For apertural teeth, “Initial Shell Length” and “Cue” are also factors (Table 3). In *N. canaliculata* “Nativity” interacts with “Initial Shell Length” (Table 4). In *N ostrina*, “Initial Shell Length” is also a factor (Table 5).

4. DISCUSSION

4.1 Overview

Shell length, shell weight, and body weight varied with immersion treatment, indicating that immersion time affects shell and somatic growth. Constitutive and inducible thickness and apertural teeth expression also varied among immersion treatments in each species. These findings demonstrate that immersion time can and does affect the predator-induced morphological response during the lifetime of individual *Nucella*. Interactions between "Species," "Cue," and "Immersion Time" in the best-fit models describing these changes imply that these factors influence each other's effects on *Nucella*'s growth and defense.

4.2 Immersion time affects the predator-induced morphological response in *Nucella*

Immersion time affects lip thickness and apertural teeth in individual *Nucella* regardless of the presence or absence of predation cue. This suggests that tidal height may have influenced the evolution of constitutive and plastic lip thickening in this genus through more than just relative predation risk as hypothesized by other researchers. I hypothesized that tidal height has influenced the evolution of the predator response through the physical stressors associated with emersion. Potential stressors include hypoxia, desiccation, and thermal stress (Menge and Branch 2001; Sorte and Hofmann 2005), a reduced amount of time available for snails to feed (Bertness 1977; Bourdeau 2011; Price 2012), and

presumably, a reduced amount of time to accumulate calcium from seawater (Wilbur and Yonge 1964; Vermeij 1993). Each species has evolved under a different pattern of stressors based on its native tidal height which limit its ability to respond to predators. It also impacts the ecological value of such a response, since lip thickening can affect a snail's ability to combat the stresses of tidal emersion in numerous ways. For instance, the added weight of a thickened shell probably interferes with a snail's ability to forage while emersed (Palmer 1992), and it could affect snail's ability to exchange heat with its environment (Vermeij 1993). Natural selection would dictate that *Nucella* not express this defense to a degree which would compromise an individual's ability to survive the emersion conditions at its native tidal height. Thus, where relative predation risk may dictate the necessity of lip thickness in each species as Bourdeau (2011) has hypothesized, the stressors associated with emersion may have determined each species' ability and incentive to express this response. Future experiments can address this hypothesis by raising populations of *Nucella* under a gradient of immersion times, and measuring the predator-induced morphological response of subsequent generations.

In addition to demonstrating that immersion time affects the predator-induced morphological response in *Nucella*, my results hint at a possible seasonal difference in the lip thickening rates of *N. canaliculata* and *N. ostrina*. In my experiment, the degree of predator-induced thickening (but not constitutive thickening) was slightly stronger in *N. ostrina* than in *N. canaliculata* when comparing the immersion times of optimal performance for each species (35% and 50% respectively). This particular finding is at odds

with the results of Bourdeau (2011), who found that constitutive and inducible lip thickening was greater in *N. canaliculata* than *N. ostrina*, and subsequently based his hypothesis about relative predation threat and the degree of thickening on this pattern. My experiment had many fewer replicates in each treatment group, rendering it less powerful. Potentially however, I observed a seasonal difference in the relative shell-thickening rates of these two species, as my experiment took place during the winter and Bourdeau's (2011) during the summer.

My results demonstrate that all future research with *Nucella* should control for immersion time to ensure that its effects on shell and body growth do not compromise experimental design. More importantly, careful thought should be put into the immersion regime employed during an experiment, and these considerations should be based on the type of comparisons to be made. Since previous research has demonstrated that *Nucella lamellosa* will actively avoid crab cue by crawling away from the source, even out of the water (Marko and Palmer 1991; Mach and Bourdeau 2011), researchers should also specify whether snails are free to move in and out of the water.

4.3 Native Immersion Time

A very interesting pattern emerged in the way that immersion time affected thickening: each species showed the strongest induced thickening response at the immersion treatment representing its native tidal height. *Nucella canaliculata* and *N.*

ostrina also demonstrated the greatest constitutive thickening (thickening in No-Crab treatments) at their native immersion times. Based on this finding, I recoded immersion treatments as "Native" or "Non-Native" and retested the fully crossed and reduced GLM models. The best-fit models indicated that "Nativity" was a factor describing changes in lip thickness for all species. This finding could be interpreted in a number of ways in each species.

In *N. lamellosa*, overall shell growth, body growth, and lip thickening were maximized concurrently at 100% immersion. While this does lend support to my hypothesis that higher immersion provides optimal conditions for growth and lip thickening, it could also be indicative of a simple graded response to the amount of predation cue sensed by *N. lamellosa.* Snails experiencing greater immersion also experience a greater overall volume of predation cue and may tailor thickening responses accordingly. Recent unpublished data (Tran pers. comm.) suggest that intermittent exposure to crab cue results in a reduction of the thickening response in *N. lamellosa*, but to what degree seems dependent on the pattern of exposure. In his experiment, *N. lamellosa* exposed to *C. productus* for a total of 28 days in 7 day blocks every other week for 56 days thickened significantly (27.4%) less than constant-exposure snails. However, snails exposed for 28 days every other day for 56 days had only a slight (10%) and non-significant reduction in thickening when compared to constant-exposure snails. Thus, snails experiencing less than constant exposure appear to show weak to moderate reduction in thickening response as compared to 100% exposure. In another experiment, *N. lamellosa* exposed to small *C. productus* responded significantly

less than snails exposed to larger crabs (Edgell and Neufield 2008). Presumably, larger crabs produce a higher volume of cue than smaller ones, which could be the reason for this difference in inducible defense. In my experiment, the fact that *N. lamellosa* in the 75% immersion treatment responded less than those in 100% would also tend to support to the idea that a different volumes of cue induce different degrees of defense (see next section for details regarding 35% and 50%). Future experiments could test this hypothesis by comparing the responses of crab-exposed snails subjected to partial immersion to ones experiencing full immersion but crab cue only intermittently.

Apertural teeth results in *N. lamellosa* were more ambiguous. Unlike overall shell growth and lip thickening, which were maximized in the highest immersion times, the smallest proportion of snails with teeth occurred in the No-Crab 75% and 100% treatments. This conflicted with my hypothesis that constitutive apertural teeth expression would be higher at higher immersion times due to access to greater resources and less stressful environmental conditions. However, 75% and 100% immersion did show the strongest difference between No-Crab and Crab, supporting my hypothesis that inducible apertural teeth expression would be greatest at the highest immersion times.

Lip thickening results in *N. canaliculata* and *N. ostrina* contrast with those of *N. lamellosa*: *N. canaliculata* showed the greatest constitutive and inducible thickening at 50% and *N. ostrina* at 35%. These results conflict with my hypothesis that shell secretion and growth would be maximized at higher immersion times due to more favorable conditions: Immersion time does affect thickening in *N. canaliculata* and *N. ostrina*, but not through my

hypothesized mechanism. Additionally, this result contrasts with overall shell growth (shell length and weight) and body growth in *N. canaliculata* and *N. ostrina*. This suggests that while higher immersion provided more favorable conditions for overall shell growth, the optimal conditions for inducible lip thickening occur at "native" immersion conditions. In fact, at non-native immersion times in *N. lamellosa* and *N. canaliculata*, snails show little difference in thickening between No-Crab and Crab and in 50%, 75%, and 100%. This finding is difficult to explain, because in theory, higher immersion times provide more stable thermal, aerobic, and osmotic conditions (Menge and Branch 2001; Sorte and Hofmann 2005), higher access to food (Bertness 1977; Bourdeau 2011; Price 2012), and presumably greater access to calcium to construct shell material (Wilbur and Yonge 1964; Vermeij 1993). Odder still, at 50%, 75%, and 100% No-Crab *N. ostrina* actually thickened more than Crab.

One potential reason for native immersion time being optimal may involve the balance between immersed and emersed thermal conditions: Immersion time directly controls the temperature that snails experience, and the rate of lip thickening and apertural teeth growth is influenced by environmental temperature (Wilbur and Yonge 1964; Vermeij and Currey 1980; Spight 1981; Vermeij 1983; Price 2012). Perhaps lip thickening is optimized at a small range of temperatures, a range which historically occurs most reliably at each species' native tidal height. At anything other than native immersion time, this optimal temperature range does not occur or occur often enough for substantial expression of shell defenses. This hypothesis could be tested by raising snails under different

temperature and immersion regimes and assessing the relative effects of these factors on the predator-induced morphological response.

In *N. ostrina*, the fact that No-Crab snails thickened more than Crab-exposed snails in non-native immersion times suggests that they might be employing a different anti-predator strategy in these treatments. Palmer (1985a) found evidence that thin shells are favored in *N. lamellosa* in the absence of crab partially because it allows faster growth, permitting snails to attain size refuge more quickly. Perhaps because *N. ostrina* do not have a very strong thickening response anyway, they are encouraged by the optimal shell growth conditions at higher immersion times to opt for the tactic of growing quickly in order to reach a size refuge (Palmer 1985a; Bourdeau 2011), rather than pursue a futile effort to fortify the apertural lip.

4.4 Reduced food access and passive thickening in *Nucella*

In *N. lamellosa*, thickening is thought to occur through a passive mechanism in which the rate of shell secretion remains constant but is redirected from normal shell lengthening to lip thickening (Bourdeau 2010a). When *Nucella* sense crab cues, they reduce their overall activity (Appleton and Palmer 1988; Bourdeau 2010a) and increase passive avoidance behavior (i.e., shell withdrawal) (Mach and Bourdeau 2011). In one experiment, reduced overall activity resulted in 51.9% lower food consumption, which caused 93% less somatic growth in crab-exposed *N. lamellosa* (Bourdeau 2010a). However, shell material continued

to be produced at the same rate (although the composition is different, see Bourdeau 2010a). Due to the reduction in shell growth, this newly secreted shell material was deposited perpendicular to the axis of coiling, which resulted in 88% less of an increase in shell length (Bourdeau 2010a). Bourdeau (2010a) has proposed that this mechanism has evolved to produce and regulate the predator-induced thickening response for individual *Nucella.* In support of this hypothesis, *N. lamellosa* with restricted food access thickened their shells to a similar degree as those exposed to crab effluent (Palmer 1990; Edgell and Neufield 2008; Bourdeau 2010a) and snails producing thicker shells consumed less food (Palmer 1992). Additionally, like snails exposed to crabs, starved snails with thickened shells were more likely to survive an attack by *C. productus* (Palmer 1981; Bourdeau 2010a).

In a recent review of predator-induced morphological plasticity across several genera, Bourdeau and Johansson (2012) called on researchers to investigate the passive thickening hypothesis in *Nucella* by designing experiments that subject snails to a gradient of food resources and measuring subsequent food consumption, activity, and lip thickening. My experiment may fulfill some of these requisites. It is generally believed that *Nucella* forage and feed only while immersed (e.g., Bertness 1977; Bourdeau 2011), and recent research shows that 5 hours of tidal emersion (approximately 79% immersion) depresses the rate at which *N. lamellosa* feed on barnacles (Price 2012). Whether this is true in *N. canaliculata* and *N. ostrina* is unknown, but logic would suggest that *N. ostrina,* an inhabitant of the upper intertidal, would possess the ability to eat emersed. Based on this hypothesis, the immersion time treatments in my experiment imposed a gradient of food

availability for at least *N. lamellosa*, albeit indirectly. Thus, my experiment provides the conditions called for by Bourdeau and Johansson (2012), although the scale of the project did not allow me to track snail activity or food consumption. That food availability was restricted in low immersion treatments is demonstrated by the patterns of reduced somatic growth in *N. lamellosa* and *N. canaliculata*. Interpreted in this light, my results lend support to the passive thickening hypothesis for *N. lamellosa*, but the patterns of growth in *N. canaliculata* and *N. ostrina* suggest that there may be different mechanisms operating to cause inducible thickening in these two species.

Nucella lamellosa demonstrated decreasing somatic growth and overall shell growth (length and weight) with decreasing immersion time, suggesting that emersion restricted its access to food. Mirroring the findings of Bourdeau (2010a), starvation-induced thickening appeared to occur in 35% and 50% immersion times as both No-Crab and Crab snails thickened to an almost equivalent degree, while snails in the 75% and 100% immersion times exposed to predation cue showed a much stronger thickening response than their No-Crab counterparts. In addition, Crab-exposed snails showed slightly less overall shell growth and less body growth than No-Crab snails exposed to the same immersion time (except in 75%, where the relative body weight change pattern is reversed but standard errors overlap), suggesting that they reduce feeding as a response to predation cue. Passive induction by restricted food intake might explain the fact that there is higher constitutive apertural teeth expression at 35% and 50% in *N. lamellosa*: Appleton and Palmer (1988) found that starved snails express apertural teeth to a similar degree as well-fed snails

exposed to predation cues, and in their experiment, starvation actually augmented the predator-induced morphological response. A few of the data from *N. lamellosa* in my experiment are contrary to what is predicted by the passive thickening hypothesis: there is very little thickening in 75% No-Crab despite low somatic growth, while 100% Crab had substantial somatic growth and substantial thickening where only thickening would be predicted to occur. In addition, 75% and 100% Crab showed only slightly higher degrees of thickening than 35% and 50% Crab, but much higher degrees of overall shell growth and somatic growth. In general however, these findings provide evidence in support of the hypothesis that reduced food induced passive thickening in *N. lamellosa*.

Nucella canaliculata demonstrated very little body growth in 35% and 50% immersion time, suggesting that it too suffered from reduced access to food in low immersion. Like *N. lamellosa*, Crab-exposed *N. canaliculata* demonstrated smaller body weight increases (or even decreases) and, in most immersion times, less overall shell growth than their No-Crab counterparts. In further accordance with the passive thickening hypothesis, 35% and 50% No-Crab thickened almost as much as their Crab-exposed counterparts and the greatest induced thickening responses occurred in these immersion times. However, support for the passive thickening mechanism is not as clear as it is for *N. lamellosa* in that patterns of relative overall shell growth do not correspond with what the passive thickening mechanism would predict. 35% and 50% Crab-exposed *N. canaliculata* show slightly more or equal overall shell growth than No-Crab, where reduced shell growth due to thickening would be predicted. Additionally, 75% and 100% Crab show less overall

shell growth than No-Crab where equal shell growth would be predicted. Finally, strong differences in somatic growth between Crab and No-Crab should yield strong differences in thickening, but at 75% and 100% where there is a strong difference, there was very little difference in lip thickening in *N. canaliculata*.

In all treatments except 75% and 100% No-Crab, *N. ostrina* demonstrated decreases in body weight, suggesting that food access was very restricted, especially at low immersion times. This is surprising given the fact that *N. ostrina* live in the upper intertidal, which forces them to spend a great deal of time out of water. A species native to such conditions would be expected to have the ability to eat emersed. Nevertheless, the low gains in body weight of No-Crab *N. ostrina* support the use of the immersion gradient as a proxy for food access for this species. All Crab-exposed snails lost a great deal of body weight, especially at 35% and 50%, suggesting that predation cue induced *N. ostrina* to reduce feeding. Crab-exposed snails also exhibited reduced overall shell growth. Finally, Crab-exposed snails at 35%, which showed the strongest thickening response, also demonstrated negative shell growth. These findings support the hypothesis of passive thickening for *N. ostrina*, but the preponderance of data suggest that a mechanism other than passive thickening is operating in *N. ostrina.* While my results show that thickening is associated with low shell and somatic growth, low/moderate thickening is associated with even lower body growth (but not lower shell growth) and the greatest thickening response (35% Crab) does not correspond with greatest reduction in somatic growth, as the passive thickening hypothesis would predict.

5. CONCLUSION

In *N. lamellosa*, constitutive shell length, shell weight, body weight, and lip thickening followed a pattern of increasing gains with increasing immersion time: The largest increases in shell length, shell weight, body weight, and lip thickness occurred at 100% immersion, and the smallest at 35%. Similarly, the greatest difference between No-Crab and Crab *N. lamellosa* in lip thickening and the proportion of individuals with apertural teeth (i.e., the greatest inducible response to predation cues) occurred at 100%, and the smallest at 35%. This suggests either that higher immersion provided more favorable conditions for shell growth, body growth, and the predator-induced morphological response, or that *N. lamellosa* have a graded response to the amount of crab cue that they sense. My results also provide some tentative support for a mechanism of starvation-induced passive thickening proposed by Bourdeau (2010a).

The concurrence between overall shell growth, body growth, and predator-induced morphological response in *N. lamellosa* differs from *N. canaliculata* and *N. ostrina*, in which lip thickening results contrasted with shell length, shell weight, and body weight. Like *N. lamellosa*, shell and somatic growth tended to increased with higher immersion time, but the greatest lip thickening occurred at the immersion time representing native tidal height. This suggests that while higher immersion provided more favorable conditions for shell and body growth, native immersion time provides the optimal conditions for the thickening response. The implications of these results for starvation-induced passive thickening in *N.*

canaliculata and *N. ostrina* are ambiguous, since both species demonstrated some responses in accordance with the hypothesis and some contrary to it.

My experiment demonstrated that native immersion time optimizes the thickening and apertural teeth development in *N. lamellosa,* and predator-induced thickening in *N. canaliculata* and *N. ostrina.* Thus, within the lifetime of a single individual, a plastic response can be modified by environmental constraints. This suggests that the evolution of the predator-induced morphological defense in *Nucella* has been shaped by the physical constraints of emersion. Future hypotheses concerning the evolution of phenotypic plasticity must take this variable into account, and future experiments with *Nucella* must take measures to ensure that immersion time doesn't compromise experimental design.

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