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The Physiological Response of Larval Marine Snails to Environmental Stressors

A Dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Ecology, Evolution and Marine Biology

by

Mackenzie Lane Zippay

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ABSTRACT

The Physiological Response of Larval Marine Snails to Environmental Stressors

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Embryonic and larval stages are thought to be more sensitive to environmental fluctuations than later life history stages. Therefore, examining how marine larval gastropods might be affected by climate change stressors, in this case, high temperatures and ocean acidification (OA), becomes important for predicting long-term outcomes such as recruitment success and population structure.

This study first examined the effects of temperature on larval survival of the intertidal dogwhelk, *Nucella ostrina*, over a large geographic scale. Laboratory trials concluded that veliger survival decreased abruptly within a few degrees celsius for all sites. In addition, there was a significant relationship between veliger thermal tolerance and latitude, but habitat temperature and veliger thermal tolerance were not correlated. Data further yielded that some larval populations may be living near their upper thermal limit, and as sea surface temperatures continue to rise with climate change, a couple of degrees could impact the population structure of these encapsulated developers.

A growing body of research on calcifying marine invertebrates suggests that OA can have deleterious effects on development and various physiological processes in these organisms, especially since climate change models predict oceanic pH could decrease by additional 0.2-0.3 units (from pH 8.1 to an average of 7.9-7.8) by the year 2100. In laboratory experiments designed to mimic seawater chemistry in future oceans, we examined the effects of elevated CO_2 on larvae of two marine snails, *Nucella ostrina* and *Haliotis rufescens*. Larvae were raised in culture under control and experimental CO_2 levels that span the range of current atmospheric CO_2 concentrations (385 ppm) to a "worst case" scenario (~990 ppm) predicted for the year 2100. Following development under conditions of ocean acidification, we measured larval thermal tolerance, shell integrity and shell formation. Our results showed elevated CO_2 had a subtle influence on veliger thermal tolerance for both species. In addition, shell strength in *Nucella ostrina* veligers and gene expression patterns for genes involved in shell formation in abalone larvae were not changed. These results suggest that larval forms of these species may have the capacity to withstand environmental change.

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I. Dissertation Introduction

A. STATEMENT OF THE PROBLEM

Environmental stressors, including those predicted to increase due to climate change, potentially affect the sensitive embryonic and larval stages of many marine invertebrates. The goal of my dissertation research was to examine how larvae of marine gastropods, an ecologically and in some cases commercially important taxon, are affected by climate change stressors, in this case, high temperatures and ocean acidification (OA). I explored whether the responses of these early life history stages can play a role in predicting longer-term outcomes such as recruitment success and population structure under climate change scenarios. To address this goal, I investigated the physiological responses of two marine invertebrates, the intertidal dogwhelk, *Nucella ostrina*, and the red abalone, *Haliotis rufescens*, and determined whether their larvae possess the capacity to survive any physiological challenges caused by increased temperature and elevated carbon dioxide (CO_2). In addition to temperature-induced mortality, shell integrity was assessed for *N. ostrina* veliger stage larvae, while variability in transcript levels of shell matrix genes was analyzed for abalone larvae.

Although extensive research has documented larval thermal tolerance (Andronikov 1975, Strathmann 1987, Fujisawa and Shigei 1990, Sewell and Young 1999, Anger et al. 2003, Newman et al. 2006) and the effects of hypercapnia or elevated CO_2 on marine animals (Batterton and Cameron 1978, Jouve Duhamel and

Truchot 1983, Cochran and Burnett 1996, Seibel and Walsh 2001, Kurihara et al. 2004, Pörtner et al. 2005, Spicer et al. 2007), research is sparse on how encapsulated and lecithotrophic developers will tolerate the likely scenario of both stressors concomitantly. Fewer studies still have addressed the physiological costs associated with developing under conditions of OA and thermal stress. The experiments presented in my thesis represent the first attempts to employ two alternative approaches (biophysical and molecular response) aimed at better understanding the impact of elevated CO_2 on shell formation and shell integrity in larval gastropods. Here I present the background needed to place the dissertation into the context of the field of climate change and provide justification for the experimental approaches utilized in my research. In my research I investigated larval thermal tolerance on a latitudinal scale for the intertidal dogwhelk (**Chapter II**) and physiological tolerance of CO_2-acidified seawater (**Chapter III and Chapter IV**) for encapsulated developing and lecithotrophic gastropods.

B. BACKGROUND

Four areas of investigation were central to my dissertation research: (1) the adverse effects of climate change, (2) basic gastropod larval development, (3) thermal stress effects on early life history stages, and (4) the data currently available on the effects of ocean acidification on larval development, especially shell structure and how that is measured.

1. Climate change

Marine organisms from the intertidal zone out to the continental shelf provide the US an estimated $14 trillion worth of goods and services annually (Costanza et al. 1997) with molluscs contributing 19%, or $748 million (Cooley and Dooney 2009). Coastal marine ecosystems are being threatened by climate change, a process resulting from anthropogenic activity including 'changes in atmospheric abundance of greenhouse gases (GHGs) and aerosols, land-cover and solar radiation that alter the energy balance of the climate system' (IPCC 2001). Carbon dioxide, methane and nitrous oxide are a few of the GHGs that trap some of the heat that would otherwise re-radiate to space, causing the earth to warm. According to the most recent Assessment Report from the IPCC (4^{th}, 2007), eleven of the previous twelve years (1995-2006) have ranked as the warmest years on record for global surface temperature (since 1850). Increased greenhouse gases are forecast to influence several additional abiotic variables by increasing storm frequency, intensifying upwelling, decreasing ocean pH, and increasing seawater levels and sea surface temperatures (SSTs). An organism's response to such changes can be expressed through altered physiology, morphology, and behavior at any stage of their life cycle. If the stresses are sufficiently severe, they can exceed individual tolerances and lead to local extinctions.

At the population level, climate change can impact the geographical pattern of recruitment by altering abundance and reproductive patterns. For example, as a result of climate change, species of barnacles in the northern British Isles have fewer

broods than do the same species further south in Europe (Burrows et al. 1992). Climate changes can also alter wind speeds, which can influence the strength and duration of upwelling events and ultimately impact the dispersal and recruitment (Gaylord and Gaines 2000). Alterations in oceanographic currents of the Bering Sea have already been observed, causing juvenile walleye Pollock fish, *Theragra chalcogramma* to be separated from adults resulting in increased cannibalism (Wespestad et al. 2000). Lastly, climate change is affecting the reproductive and recruitment patterns of krill (*Euphausia superba*) as a direct result of the declining area of sea ice formed near the Antarctic Peninsula (Loeb et al. 1997).

Ultraviolet radiation will also increase as a consequence of rising GHGs due to the depletion of the ozone layer (Austin et al. 1992), which could negatively affect marine larvae and algae (Peachey 2005). UV rays can impact physiological performance and impair developmental processes in marine organisms. For example, cleavage patterns were delayed in green urchin (*Strongylocentrotus droebachiensis*) embryos due to damage caused by UV radiation (Adams and Shick 1996). Some urchin species have the ability to tolerate or resist UV exposure through absorbing protective amino acids ingested from algae (Adams and Shick 1996) while some gastropod species may lay benthic egg capsules to provide protection for developing embryos (Rawlings 1996). However, as the climate continues to change it remains to be seen if these mitigation strategies will provide the safeguard needed for species' survival.

One of the major components or drivers of climate change is temperature. Global sea surface temperatures have already increased by 0.6°C during the 1990's

(IPCC 2001) and are projected to further rise by as much as 2-6°C by the year 2100 (IPCC 2007). Since many marine organisms already live close to their thermal limit (Hoegh-Guldberg 1999, Edmunds et al. 2001, Somero 2002), further temperature increases could be detrimental to their fitness and ultimately, survival. For example, intertidal turban snails and porcelain crabs are more thermally tolerant than their subtidal congeners, live closer to their upper physiological limits, and were found to be more vulnerable to climate change than the less heat tolerant species (Tomanek and Somero 1999, Stillman 2002). Such changes can have adverse effects on community structure and cause widespread shifts in biogeographical ranges of marine organisms. In an analysis of a long-term dataset, Southward and colleagues (1995) found that over a 70-year period, various fish populations shifted their distribution depending upon the temperature of the English Channel. When the channel warmed, cool-adapted fish moved into cooler waters, and warm-adapted fish swam to warmer waters. Distributional range shifts have also been documented in other marine communities including copepods (Beaugrand et al. 2002), gastropods and chitons (Zacherl et al. 2003, Rivadeneira and Fernández 2005), corals (Precht and Aronson 2004) and other fish populations (Holbrook et al. 1997, Perry et al. 2005) to name a few.

An increase in environmental temperature can play a significant role in reproductive phenology for some organisms. For example, with recent warming trends *Macoma balthica*, a north-western European clam has begun to spawn earlier in the season, while phytoplankton bloom patterns have not changed, resulting in a mismatch between larval production and food supply (Philippart et al. 2003). Due to

the pervasive influence temperature can have on species abundance and distribution, it is particularly important to investigate the affects that global warming might have on an organism's physiological response and how this could affect biodiversity and community structure in the ecosystem. Winter warming is starting to affect terrestrial species, such as the timing of pond entry for newts (*Triturus* spp.) (Pounds et al. 1999) and delays in arrival for migratory birds (Both and Visser 2001). The cumulative influence of persistent temperature changes and additional stressors due to climate change may eventually be more than a population, species or ecosystem can handle.

Another major factor of climate change is the increase in atmospheric carbon dioxide (CO_2), one of the most important GHGs. Global atmospheric CO_2 has increased markedly from the pre-industrial value of about 280 parts per million (ppm) to 379 ppm in 2005 (IPCC 2007). Due to the increase in human activities (e.g., increased burning of fossil fuels, increases in transport and industry, deforestation, residential and commercial buildings, etc.) the largest increase in annual CO_2 concentration for the last 10 years was a 1.9 ppm increase per year (IPCC 2007). Current models predict CO_2 concentrations to further increase at a rate of ~0.5% per year (Feely et al. 2008), resulting in an expected rise to 550-1020 ppm (IPCC 2007, Orr et al. 2005) by the end of 22nd century and could reach 2000 ppm by the year 2300 (Caldeira and Wickett 2003). As a result, the ocean has become a sink for atmospheric CO_2 and absorbed ~30% of emitted CO_2 in the last 200 years (Sabine et al. 2004), altering ocean chemistry. Carbon dioxide diffuses into the ocean forming carbonic acid (H_2CO_3) which quickly dissociates into hydrogen (H^+)

and bicarbonate (HCO_3^-) ions. The creation of hydrogen ions can combine with other previously free carbonate ions (CO_3^{2-}) to form more HCO_3^- according to the following formula:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow CO_3^{2-} + 2H^+$$

Therefore, the net effect of increased atmospheric CO_2 absorbed by the ocean is an increase of H_2CO_3, HCO_3^-, and H^+, and a decrease in CO_3^{2-} concentration and lower pH.

The oceans' current average pH has dropped by 0.1 units from a global average of 8.16 (CO_2 = 280 parts per million) to 8.05 (CO_2 = 370 ppm) since the early industrialization era (Ruttiman 2006). Using estimates for predicted CO_2 emissions, the pH of surface waters could fall by up to 0.4 units before the end of the century and as much 0.7 units by 2250 (Caldeira and Wickett 2003). This unprecedented high rate of CO_2 entering the oceans is referred to as 'ocean acidification' (OA) and can significantly impact marine organisms' cellular function (Seibel and Walsh 2003, Pörtner et al. 2005, Widdicombe and Spicer 2008, Pörtner 2008). Invertebrates, in particular, tend to be poor regulators of pH at the intra-and extracellular level (Pörtner et al. 2005) and require ions to be transported across cell membranes in order to maintain an acid-base balance (Fabry et al. 2008). Typically, H^+ and HCO_3^- protons are exchanged through Na^+/H^+ pumps and Cl^-/HCO_3^- transporters, respectively, to maintain internal pH (Ellington 1993, Gutknecht et al. 1995). During metabolism, when H^+ ions are buffered in the intracellular spaces, HCO_3^- is transported into the cell via Cl^- transporters to compensate for the imbalance in acidity. However, in elevated CO_2 conditions, the hydrolyzation of CO_2

causes the cellular spaces to become highly acidic and enhances the need for more HCO_3^- (Fabry et al. 2008). Bivalves and other calcifying organisms have the capacity to manage this acid-base imbalance through the dissolution of calcium carbonate ($CaCO_3$) exoskeletons, which produces enough HCO_3^- to counteract this problem (Lindinger et al. 1984, Michaelidis et al. 2005a, Fabry et al. 2008). This compensation creates a new problem however; long-term exposures to increased CO_2 could severely tax the organisms' ability to form shells or hard parts.

Calcifying organisms rely heavily on carbonate ions as they are one of the main building blocks for the calcification of exoskeletons, structures that provide stabilization of body form and protection against predators. The secretion of $CaCO_3$ occurs in many forms (Fabry et al. 2008) but aragonite and calcite are the two major forms. As atmospheric CO_2 dissolves into seawater, the concentration of CO_3^{2-} decreases, ultimately affecting the saturation state of aragonite and calcite. These decreases can have deleterious effects on marine calcifiers by weakening their shells (Kleypas et al. 2006) or skeletons, especially corals that use the more soluble form, aragonite, to build their calcareous structures (Leclercq et al. 2000, Langdon and Atkinson 2005). As seawater becomes under-saturated (saturation states <1) with $CaCO_3$, which has already been observed near the coast of California and Oregon (Feely et al. 2008), marine organisms will be more at risk to decreased calcification rates (Gazeau et al. 2007) and shell dissolution (Green et al. 2004, Orr et al. 2005).

Major changes in ecosystem structure and function, species interactions, and shifts in species' geographical ranges are a few of the consequences that are likely to result due to climate change. Not only is it important to focus our attention on adult

species and how they physiologically respond to environmental variations, it is also critical to understand how early life history stages will be affected because the larvae of many adult populations often encounter different thermal niches or environments than their adult counterparts (Dupont and Thorndyke 2009). Understanding how early life history stages may respond to such pervasive effects as increased temperature and ocean acidification can provide insight into the ramifications climate change will have on recruitment, future settlement patterns and adult population structure.

2. Gastropod larvae as experimental organisms

I evaluated the effect of climate change stressors on larvae from two different gastropod species, *Nucella ostrina* and *Haliotis rufescens*, found along the rocky shores of the Pacific coast of North America. These prosobranch marine gastropods produce two types of larvae, trochophores and veligers, which develop either as a free-swimming stage (as in the archeogastropod, *H. rufescens*; Figure 1) or within benthic egg capsules (as in the neogastropod, *N. ostrina*). The trochophore stage, uniting molluscs with the annelids and sipunculans (Willmer 1990), is shaped like a top with a posterior ciliated band known as the prototroch (e.g., Figure 1B). The prototroch can be used for feeding, locomotion or both (Strathmann 1987).

As development continues, either in the water column or inside capsules, the larva metamorphs into the veliger stage, which is characterized by the appearance of a shell (Figure 1C) and paired velar lobes. The velum bears cilia for locomotion and catching food particles. During this time, a major morphogenesis process known as

torsion occurs where the visceral mass (the mantle, gills, nephridia and anus) undergoes an 180° counter-clockwise twist bringing these organs behind the head (Underwood 1972, Pennington and Chia 1985; Figure 1D). Sensory structures, such as statocysts and larval eye spots, also develop during the veliger stage. Once the larva has completed development (Figure 1E), it becomes physiologically competent to settle and metamorphose (Figure 1F). Larvae of some species may remain in the plankton for weeks to months (Morse and Morse 1984, Pechenik 1986), while larvae of other species may never enter the plankton and hatch out as 'crawl away' juveniles (Spight 1976). With this great diversity of early developmental modes, to understand the impact of climate change on marine species we must explore how these vulnerable early life history stages are differentially impacted by environmental change.

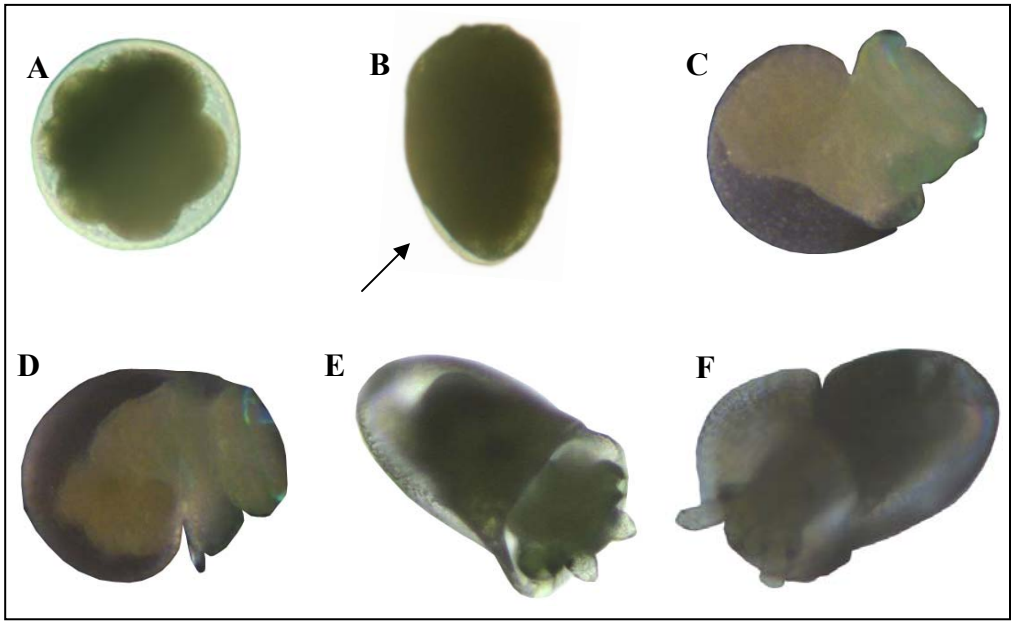

Figure 1: An example of gastropod development of the red abalone *Haliotis rufescens* raised at 15°C. At ~15 h post-fertilization (pf) the embryos have reached their 4th cleavage, 16 cell stage (A). After ~22 h pf larva hatched as trochophores (B) and the shell gland begins to form indicated by the arrow. After 36h pf the veligers are considered 'pre-torsion'(C), have a distinct larval shell and are about to undergo torsion. 'Post-torsion' veligers (D), after 86 h pf, have twisted their visceral mass 180° and formed a foot and operculum. Veligers continue to develop forming eyespots and cephalic tentacles as they prepare to settle and metamorphose (E), after ~125 h pf. A 2wk post-settled juvenile (F).

a. Nucella ostrina

Five species of *Nucella* can be found in the intertidal in the Northeastern Pacific (Morris et al. 1980, Sorte and Hofmann 2004). The biogeographical range for *Nucella ostrina* extends from Yakutat, Alaska to Point Conception, California (Collins et al. 1996), in the low to high intertidal zone (Palmer 1980, B. Menge personal communication).

All members of the genus *Nucella* have internal fertilization, and females attach to the rock several yellow, stalked capsules, in a clump. In coastal sites along the Pacific Northwest, female *N. ostrina* can produce egg capsules year-round, although they are more abundant during the winter and spring when the food supply (barnacles and mussels) is more favorable (Seavy 1977, Moran and Emlet 2001). Each capsule contains numerous eggs (~180 – 210 μm in diameter). Most of the eggs are sterile nurse eggs (unfertilized eggs or non-developing embryos) that are consumed by the developing larvae. The veliger larvae swim in the fluid-filled capsule and undergo metamorphosis while still encapsulated, eventually hatching out as 'crawl away' juveniles (Costello and Henley 1971). Typically, an average of 10-20 hatchlings leave the egg capsule 2.5-4 months after deposition (Morris et al. 1980).

Unlike juvenile and adult stages of *Nucella*, the sessile egg capsules are not able to actively avoid abiotic stressors (e.g., desiccation, wave force, solar radiation, salinity, temperature) by moving. As a result, development in the egg capsule is potentially a significant stage of vulnerability for this marine gastropod.

Encapsulation practically imprisons embryos in stressful situations that free-swimming larvae might avoid through passive dispersal or active migration (Pennington and Emlet 1986, Sulkin et al. 1983). It has been shown that encapsulating structures may not protect developing embryos from increased exposure to osmotic stress (Pechenik 1983, Woods and DeSilets 1997), desiccation stress (Spight 1976, Pechenik 1978) and oxygen deprivation (Cohen and Strathmann 1996, Lee and Strathmann 1998), likely due to the capsules high permeability of water molecules (Hawkins and Hutchinson 1988). However, capsules seem to offer some advantages in the face of abiotic stressors. For example, *Nucella emarginata* egg capsules are protective against ultraviolet radiation, allowing <5% of UV-B and <55% of UV-A to cross the capsule wall (Rawlings 1996).

While substantial literature exists on the abiotic and biotic tolerances of juveniles and hatchlings of *Nucella* spp. (e.g., salinity - Pechenik 1983; desiccation- Gosselin and Chia 1995b, Rawlings 1999; sun exposure- Moran and Emlet 2001; predation- Spight 1976, Gosselin and Rehak 2007; wave exposure- Etter 1996, Gosselin and Rehak 2007; ultraviolet radiation- Rawlings 1996), few have investigated the survival capacity of encapsulated larvae of *Nucella ostrina*. One study that comes close was done by Gosselin and Chia (1995a) where they found that *Nucella emarginata* (now *N. ostrina,* Collins et al. 1996) hatchlings (2 days old) from Barkley Sound, British Columbia, Canada could survive temperatures as high as 26°C, but severe mortality occurred at 30°C. This suggests that climate change, such as a few degrees increase in global temperature, could impact the survival of these juveniles and may cause high mortality among embryonic stages. Since

developing embryos within encapsulated structures must tolerate and survive the physical stresses of the intertidal zone and cannot seek refuge like their adult counterpart, encapsulated larvae of *Nucella ostrina* enable us to exclude behavior and thus understand the survivorship of early life history stages under adverse effects of environmental variation.

b. Haliotis rufescens

Red abalone have a distribution range spanning the west coast of North America from Sunset Bay, OR to Bahia de Tortuga, Baja California (Morris et al. 1980) and inhabit the low rocky intertidal to shallow subtidal region (20 m) (Cox 1962). Typically, the spawning season tends to be in the spring and summer (Ault 1985), but it is not well defined. Like many marine invertebrates, this snail is a broadcast spawner and releases its gametes into the water column (Shepherd 1976). After a brief embryonic period of hours, offspring hatch as free-swimming trochophores. Unlike most marine species whose larvae feed in the plankton (planktotrophic larvae), abalone produce non-feeding larvae (lecithotrophic) that rely on nutrition in an internal yolk sac to grow and develop (Thorson 1950). Because abalone larvae are positively phototactic and tend to swim upwards near the surface (Najmudeen and Victor 2004), they are exposed to the more variable environmental conditions at the ocean surface than than those planktotrophic larvae that swim at depths (e.g., polychaetes - Blake and Van Dover 2005; bivalves- Arellano and Young 2009). Abalone larvae spend between 4-12 days in the water column (Hahn 1989), depending upon the water temperature, before they

metamorphose and commit to a benthic existence (Morse 1992). Generally, settlement occurs in the presence of various species of encrusting red algae, especially crustose coralline algae (Morse et al. 1979, Barlow 1990, Moss 1999, Jackson et al. 2005).

From the late 1800s to late-1900s abalone was commercially and recreationally fished for its large, tasty foot. In 1986 the estimated catch of red abalone, alone, for central and northern California reached a peak high of 3.4 million pounds (Leet et al. 2001). In 1997, the abalone fishery was closed in California due to severe overfishing. Recovery without human intervention was doubtful considering the remaining population was <1 % of estimated historic levels (Rogers-Bennett et al. 2002). The desire to restore populations of this important marine invertebrate has led to an abundance of research that includes understanding abalone reproductive and life history cycles (Davis 1995, Babcock and Keesing 1999), diet and food supply (Shpigel et al. 1999, Bautista-Teruel et al. 2003, Garcia-Esquivel and Felbeck 2009), growth and morphology (Roberts and Lapworth 2001, Jardillier et al. 2008), shell properties (Zaremba et al. 1996, Bédouet et al. 2001, Lin et al. 2006), developing aquaculture systems (Fleming and Hone 1996, Langdon et al. 2004) and more recently, molecular physiology (Vadopalas et a. 2006, Farcy et al. 2007, Jackson et al. 2007). With the demand for abalone culturing there has been an influx of information into the key environmental factors that influence growth and survival, such as salinity (Chen and Chen 1999), light (Clarke and Creese 1998) and temperature (Steinarsson and Imsland 2003, Diaz et al. 2006, Searle et al. 2006). However, only a few of these studies have focused on larval stress tolerances

(Jarayabhand et al. 1995, Lu et al. 1999, Lu et al. 2001). Eggs, larvae and juveniles of red abalone were found to have the highest growth rate and survival between 15-18°C (Leighton 1974). Lu and colleagues (2004) found embryonic survival rates of *Haliotis diversicolor* (species of abalone from South China) were highest at 27°C with survival at 99.4%.

3. Thermal stress on early life history stages

Temperature has pervasive effects on organisms in nature (Hochachka and Somero 2002). These range from temperature effects on molecular processes to a role in defining species' biogeographical range boundaries. Early studies on embryos and larvae recognized that temperature can affect larval development by influencing the duration of the pelagic stage (Appellöf 1912, Orton 1920). For some species of asteroids (*Ophidiaster granifer*), for example, temperature can delay metamorphosis resulting in smaller juveniles and increased mortality (Yamaguchi and Lucas 1984, Wendt 1996). It has also been shown that some larvae can tolerate and develop over a broad range of temperatures. For instance, eggs of the tropical sea urchin *Echinometra lucunter* could be fertilized at 12-37 °C, but development was most successful between the temperatures of 27-34 °C (Sewell and Young 1999). Similarly, cleavage in the tropical crown-of-thorns sea star (*Acanthaster planci*) was successful over a range of 10°C (21-31 °C), while swimming gastrulae effectively developed into bipinnaria larvae over a temperature range of 13°C (18-31 °C) (Johnson and Babcock 1994). Both examples indicated that larval performance was maintained over a broad range of temperatures, implying these early life history

stages are well adapted to their tropical environment where temperatures can fluctuate from 18-27 °C (Johnson and Babcock 1994, Sewell and Young 1999).

In contrast, other marine invertebrate larvae can only survive within a narrow window of temperatures. For example, both larvae of *Haliotis diversicolor* and juveniles of *H. iris* could tolerate a 4°C window (24-28 °C and 18-22 °C, respectively) (Lu et al. 2004, Searle et al. 2006). Likewise, larvae of the sub-Antarctic crab, *Paralomis granulose*, successfully developed from +6 to +9°C (Anger et al. 2003). This narrow window of physiological performance might suggest some larval species may be more susceptible to harsh environmental conditions, such as the sub-lethal effects of temperature during low-tide (Wolcott 1973, Gosselin and Chia 1995b), and could ultimately impact the likelihood of surviving to later developmental stages. This is why it is important to understand how, and to what extent, larvae can physiologically tolerate the affects of environmental change. More detail on this issue is provided in Chapter II.

Developing larvae use various pathways and cellular level responses to compensate for the effects of thermal stress, including metabolism (Strathmann et al. 2006, Fischer et al. 2009) and oxygen consumption (Strathmann and Strathmann 1995, Cohen and Strathmann 1996, Lardies and Fernández 2002, Moran and Woods 2007, Brante et al. 2009). One of the best documented physiological responses to temperature is the heat shock response (see review by Feder and Hofmann 1999). Heat shock proteins (Hsps), or molecular chaperones, function as cellular defenses by aiding protein stabilization (Lindquist 1986, Gething and Sambrook 1992) and assist in the removal of damaged or misfolded proteins (Morimoto et al. 1997, Fink

1999) following an environmental stress. It has been shown that the expression of Hsps, either in response to short-term or long-term stress (e.g. Sanders et al. 1991, Hofmann and Somero 1995, Roberts et al. 1997, Tomanek and Somero 1999, Tomanek 2008), can dramatically contribute to the thermal tolerance of the organism (Parsell et al. 1993). Extensive research has demonstrated the importance of the heat shock response in adult marine invertebrates (e.g., bivalves-Boutet et al. 2003, Hamdoun et al. 2003, Halpin et al. 2004; gastropods-Schill et al. 2002, Tomanek and Sanford 2003, Sorte and Hofmann 2005, Martynova et al. 2007; echinoderms-Osovitz and Hofmann 2005, Holm et al. 2008) and the number of studies on marine larvae are increasing. Sconzo et al. (1986) found a positive correlation between thermal tolerance and expression of heat shock proteins in *Paracentrotus lividus* sea urchin larvae. In this study, larvae that were exposed to a higher temperature prior to a heat stress could tolerate and develop at temperatures higher than they would normally encounter, thus increasing their thermal tolerance. Others have shown that the thermal tolerance and expression of Hsps varies during development (Giudice et al. 1999, Wood et al. 1998, Chang 2005). For instance, early life stages of the oyster *Ostreola conchaphila* can survive the same lethal temperatures as that for adults (38.5-39 °C). Veliger larvae, however, induced Hsps at temperatures that were 4°C cooler than their adult counterpart (Brown et al. 2004), suggesting early life history stages maybe more vulnerable to environmental stress.

These studies linking cellular adaptations to temperature tolerance and survival highlight the mechanisms available to marine organisms to deal with current environmental fluctuations. However, several questions remain. What are the limits

of these physiological responses, and are they different for larval and adult forms? Will the expected changes to the global climate surpass these limits? Changes in environmental conditions have the potential to directly affect population connectivity, structure and biodiversity (O'Connor et al. 2007). Therefore, understanding if early life history stages have the physiological capacity to tolerate changes in temperature could provide insight into these questions.

4. Ocean acidification & larval development

Early life history stages and events including fertilization, cleavage, planktonic larvae, settlement, metamorphosis and juvenile, could all be affected by the change in ocean chemistry, especially for the benthic calcifiers (Raven et al. 2005, Pörtner and Farrell 2008; Kurihara 2008, Dupont and Thorndyke 2009). Depending on the species, different stages may be sensitive. For example, fertilization rate of two sea urchins (*Hemicentrotus pulcherrimus* and *Echinometra mathaei*) decreased with a reduction in normal pH by 1.3 units (from 8.1 to 6.8; Kurihara and Shirayama 2004). In contrast, fertilization rates of the mussel *Mytilus galloprovincialis* and the oyster *Crassostrea gigas* were unaffected by exposure to high-CO_2 seawater (pH 7.4, Kurihara et al. 2007, 2008a; pH 7.8, Havenhand and Schlegel 2009), whereas polyspermic fertilization was reported in the giant scallop *Placopecten magellanicus* at pH <7.5 (Desrosiers et al. 1996) and sperm motility was reduced in the sea urchin *Heliocidaris erythrogramma* (Havenhand et al. 2008). For some larval species, a small change in pH can affect survival. The larvae of the brittle star *Ophiothrix fragilis* experienced 100% mortality after only an 8 day

exposure to a decrease of 0.2 units of pH (Dupont et al. 2008). Similarly, embryo viability was < 94% for the intertidal gastropod *Littorina obtusata* after being raised under 6 days of acidified seawater (pH 7.6; Ellis et al. 2009). Ocean acidification can also influence development rate by increasing the time it takes to reach metamorphosis. For example, barnacle larvae (*Semibalanus balanoides*) raised under high CO_2 conditions (CO_2=922 ppm, pH =7.70) delayed their development by 19 days resulting in a 60% reduction in the number of nauplii that reached the hatching stage (Findlay et al. 2009). While development of marine shrimp embryos *Palaemon pacificus* were normal under CO_2-acidified seawater (2000μatm, pH 7.6), metamorphosed and settled juveniles were smaller than normal (Kurihara et al. 2008b). A delay in development, as mentioned above, is a disadvantage and can have profound ecological implications on the population. More discussion on this is provided in Chapters III and IV.

a. Calcification

One physiological mechanism that has received a great deal of attention within the OA research community is the effect of OA on calcification. Calcification is a complex process that varies amongst marine calcifiers and therefore, OA will likely influence them differently. In molluscs, for example, calcification occurs in the extrapallial space at a crystallization site (Wilbur 1964) whereas in ophiuroids a very thin epithelium gives rise to a mesodermal skeleton (Hart and Podolsky 2005). One study found oyster larvae from *Crassostrea gigas* were significantly smaller in size, displayed morphological abnormalities, and that some veligers completely

lacked larval shells when raised under high-CO_2 (pH 7.4; Kurihara et al. 2007). Likewise, abnormal development and altered skeletal properties were evident in brittle star larvae (*O. fragilis*) when raised under pH 7.7; 50% of the larvae were abnormal after 3.7 days and 32% of larvae were asymmetrical after 2 days (Dupont et al. 2008).

While the studies cited above have shown that OA can have a negative impact on larval skeletons, some evidence seems to support the idea that calcification will not be affected at all, or in some cases, is even positively affected by OA. For example, in a recent study, neither shell diameter nor growth rate differed in barnacle larvae (*Amphibalanus amphitrite*) that developed at pH 7.4 as compared to those individuals raised under control conditions of pH 8.2 (McDonald et al. 2009). Although fine scale differences in skeleton structure were evident in several species of sea urchin larvae raised at low pH, the overall gross morphology was not affected (Clark et al. 2009). In adult calcifiers, the coccolithophore species *Emiliania huxleyi* increased its net calcification when cultured under CO_2 levels that are predicted to occur during the 21st century (750 ppmv; Iglesias-Rodríguez et al. 2008). In addition, Findlay and colleagues (2009) found an increase in shell height, width and thickness in the gastropod snail *Littorina littorea* under low pH conditions (6.45) compared to the control (pH 8.0). Since shell shape and size of marine snails can greatly improve their overall survival and fitness against predators (Spight 1976, Rivest 1983, Moran 1999, Fisher et al. 2009), such benefits could have ecologically important consequences. Collectively, these studies indicate that the impact of OA on calcification can vary among marine calcifier species and across different

developmental stages. It has been suggested that low pH levels or high CO_2 concentrations could interfere with growth via a direct, deleterious reduction in calcification, or an indirect effect due the metabolic cost of maintaining acid-base balance (Pörtner 2008).

b. Measuring gene regulation and shell strength

The synthesis of molluscan shells originates in the endoderm during early stages of development (Marin and Luquet 2004) and the shell continues to grow during their entire life. While the formation of the early shell is elaborate and has been described for a number of genera including the mussel *Mytilus* (Medakovic 2000), the Eastern oyster *Crassostrea virginica* (Lee 1990), the brooding oyster *Ostrea edulis* (Medakovic et al. 1997), the pearl oyster *Pinctada margaritifera* (Che et al. 2001), the freshwater snails *Lymnea* and *Biomphalaria* (Kniprath 1977, Bielefeld and Becker 1991) and the marine gastropod *Nassarius* (Hickman 2001), this section will focus on innovative ways to measure shell mineralization, and less on gross larval shell synthesis. Detailed explanations of larval shell formation in bivalves and gastropods are provided by Marin and Luquet (2004) and Wilt et al. (2003).

Indentation tests, sometimes called hardness tests, are commonly applied to measure the mechanical properties of biological materials (Hornbogen 1975). Nanoindentation characterizes hardness (GPa) and elastic modulus (GPa), or stiffness, by probing hard tissues at small length-scales (~ 1μm) (Oliver and Pharr 1992, Pharr 1998, Fischer-Cripps 2002). By performing a series of indents at a given

location on the surface of the specimen, information regarding local structure and composition can be generated. Applications of this technology to biological materials have included determination of the cuticle structure of cephalopod beaks (Clarke 1986), insect cuticular proteins (Andersen et al. 1995, Kerwin et al. 1999) and composition of polychaete (*Nereis*) jaws (Waite et al. 2004). These approaches have led to the discovery that the hardness and toughness of *Nereis* jaws are at least twice those of the stiffest and hardest engineering polymers (Broomell et al. 2007). While nanoindentation has been used to determine the mechanical properties of aragonite (Kearney et al. 2006) and the fracture resistance of mollusc shells (Bruet et al. 2005, Fleischli et al. 2008), to the best of my knowledge, this biomaterials technique has yet to be used in an ecologically relevant context. Therefore, nanoindentation provides a new avenue in which to investigate the impact of ocean acidification on shell structure by mechanically testing shell integrity of larvae that developed and synthesized their shell matrix under high-CO_2 seawater. More discussion on this is provided in Chapter III.

With the advent of molecular techniques, an increasing amount of information has become available regarding the primary structure and function of molluscan shell proteins. For example, the onset of larval shell formation is marked by the activation of regulatory genes, such as *engrailed* (*en*; Moshel et al. 1998). The *en* gene was first found in *Drosophila* to control segmentation (Kornberg 1981), but homologues have since been found in bivalves, gastropods, cephalopods, scaphopods and polyplacophores (Wray et al. 1995) and have been shown to be expressed at the borders of the embryonic shell (e.g., chitons, Jacobs et al. 2000;

gastropods, Nederbragt et al. 2002; clams, Jacobs et al. 2000). Another important gene in molluscan shell formation encodes the aragonite protein, AP24 (aragonite protein of molecular weight 24 kDa). This protein has calcite binding regions to enable construction of layers of aragonite tablets, which provide the framework for mineralization in red abalone, *Haliotis rufescens* (Michenfelder et al. 2003). More detail on this is provided in Chapter IV.

There have been over a dozen shell matrix proteins identified thus far that are expressed in gastropods and bivalves (see Marin and Luquet 2004), some of which have been found in abalone (*Haliotis* spp.). For instance, Lustrin A, a nacre protein found in the inner region of the mantle is composed of calcium carbonate (aragonite) platelets, known as 'mother-of-pearl' (Jackson et al. 1988). This aragonite layer is complex and in some species is stacked in a brick like fashion, where each brick is surrounded by a proteinaceous matrix (Wilt et al. 2003). While Lustrin A has an important structural role in the nacre framework for shell formation (Shen et al. 1997), the gene expression and regulation of this shell matrix protein, and others, has not been explored. Therefore, it remains to be seen if climate change, specifically OA, will affect the way these genes are expressed during shell formation or whether it will influence the function of their gene products. To investigate this, I chose to use a technique known as quantitative real-time polymerase chain reaction (qPCR) that detects and quantifies gene expression products through the use of a fluorescent tag that binds to the transcript of interest (Porcher et al. 1992). This technology has been available for twenty-five years and has been used for a number of applications (see review VanGuilder et al. 2008). Profiling gene expression can reveal a network

of genes that are being regulated in the context of global climate change, and can provide a 'physiological fingerprint' of how organisms are responding to climate change in their natural environment.

C. DISSERTATION ORGANIZATION

Following this introductory chapter, I have organized my dissertation into three chapters that will be presented in manuscript form. The dissertation will conclude with a discussion of the salient findings and provide an overall conclusion of my studies (**Chapter V**).

In **Chapter II**, I measured the thermal tolerance of several populations of *Nucella ostrina* veliger stage larva over a latitudinal gradient along the west coast of North America. This study addresses whether the thermal tolerance of early life history stages correlate with latitude and/or habitat temperature (in press at *Marine Biology;* Zippay and Hofmann 2009).

In **Chapter III**, I investigated the effects of ocean acidification on larval development in *Nucella ostrina*. By collecting egg capsules from the intertidal and exposing them to future CO_2-acidified seawater, I was able to determine if larval thermal tolerance was physiologically compromised and whether shell integrity might be impacted by ocean acidification (In preparation for *Invertebrate Biology*). These findings led me to pursue similar questions in larvae with different modes of development.

In **Chapter IV**, I addressed the thermal tolerance among different life-history stages of red abalone (*Haliotis rufescens*), an invertebrate with a lecithotrophic style

of development, when raised under high-CO_2 conditions in the lab. These treatments were meant to mimic OA conditions that abalone might encounter within the next 100 years. I also wanted to measure whether or not there was a molecular response in genes involved in shell formation when larva developed under conditions of OA. Thus, I quantified mRNA levels for two biomineralization genes (*engrailed* and *ap24*) in larvae that were raised under different CO_2 conditions (In preparation as a manuscript).

II. Physiological tolerances across latitudes: Thermal sensitivity of larval marine snails (*Nucella* spp.)

A. ABSTRACT

One critical step in understanding how temperature will affect biodiversity in coastal ecosystems is to gain insight into how the tolerances, and ultimately survival, of early life history stages will influence the distribution and abundance of reproductive adults. We assessed the thermal tolerance of encapsulated veliger-stage larvae of a common dogwhelk, *Nucella ostrina*, that occurs in the rocky intertidal zone on the west coast of North America. Results showed that veligers collected from northern latitudes in Washington State were less tolerant of heat stress than those from central sites in California. For all sites, we found a subtle difference between the temperatures at which veligers first began to die compared to when veligers reached 100% mortality. On a biogeographical scale, the LT_{50} temperatures, a measure of larval sensitivity to lethal temperatures, for *N. ostrina* veligers displayed a strong latitudinal trend. In contrast, no relationship was found between habitat temperature and veliger LT_{50} temperature. These findings provide a conservative measurement of the upper thermal limits of encapsulated veligers while illustrating how these early life history stages could be physiologically compromised under future climate warming scenarios.

B. INTRODUCTION

Although larval forms have been traditionally recognized as a vulnerable stage in the life history of marine organisms (Jackson and Strathmann 1981, but see Hamdoun and Epel 2007), few studies have explored the tolerances of embryos or larvae in broad ecological terms (Fernández et al. 2007). Existing research has focused on thermal tolerance differences among developmental stages (e.g., Pedersen and Tande 1992, Sewell and Young 1999, Lu et al. 2004) and temperature effects on the pelagic larval duration (PLD) and their ecological consequences (O'Connor et al. 2007). Overall, the field of ecological development (see Sultan 2007) is gaining recognition as we begin to appreciate the importance of understanding the vulnerabilities of larval life history stages in marine invertebrates. In this study, we tested the thermal tolerance of veligers of the marine dogwhelk, *Nucella ostrina*, a common inhabitant of the rocky intertidal of the northeastern Pacific. Additionally, we tested whether there were any differences in physiological tolerance that could be linked to biogeographic distribution, or variations in the environmental temperature regimes, that were found across the range of this broadly distributed intertidal invertebrate.

The dogwhelks of the Northeastern Pacific coast are an ideal system for addressing the role of physiological capacities, such as thermal tolerance, in setting species range boundaries in the rocky intertidal zone. These intertidal snails lack a planktonic larval stage and develop in egg capsules that later hatch out as 'crawl away' juveniles (Morris et al. 1980). Females lay yellow, stalk-like egg capsules that

are fixed on benthic substrates (Thorson 1950) which contain numerous developing embryos (Spight and Emlen 1976). Thus, these egg capsules are routinely exposed to elevated air temperatures during emersion and cooler seawater temperatures during immersion, making them an ideal biological indicator for environmental thermal stress. *Nucella ostrina* are commonly found in the mid-high intertidal zone within and among mussel beds and barnacles. In coastal sites along the US Pacific Northwest, female *N. ostrina* can produce egg capsules year-round, although they are more abundant during the winter and spring when food supply is more favorable (Seavy 1977, Moran and Emlet 2001). During development to trochophore and later to veliger stages, embryos feed on nurse eggs (unfertilized eggs or non-developing embryos) within the egg capsule. After 2.5–4 months of encapsulated development, individuals hatch as metamorphosed juvenile snails (Costello and Henley 1971, Morris et al. 1980). Unlike juvenile and adult stages of *Nucella*, the sessile egg capsules are not able to actively avoid abiotic stressors (e.g., desiccation, wave force, solar radiation, salinity, temperature) by moving to less stressful microhabitats. As a result, this developmental stage may represent a significant stage of vulnerability for this marine gastropod. Thus, understanding the physiological responses underlying the development of encapsulated embryos is a key step in defining environmental parameters that can contribute to setting a species range boundaries.

Previous studies have shown that survivorship of early life history stages can be significantly impacted by environmental factors and ultimately influence adult populations (barnacles, Connell 1985; polychaetes, Qian and Chia 1994; oysters, Roegner and Mann 1995; snails, Gosselin and Qian 1997, Moran and Emlet 2001).

Additionally, a few studies have looked at the effects of temperature on encapsulated development (Boon-Niermeijer and Van de Scheur 1984, Przeslawski 2005, Fernández et al. 2007). To our knowledge, however, none have addressed survivorship during encapsulated development in *Nucella*. Mortality data on *N. lamellosa* juveniles estimate that 90-99% die within the first year (Spight 1976) and only 1-2% of *N. lapillus* survive the first few months of life (Feare 1970), suggesting environmental factors have a profound impact on these populations. Other investigators have addressed potential biotic and abiotic sources of mortality (e.g., salinity - Pechenik 1983; desiccation- Gosselin and Chia 1995b, Rawlings 1999; sun exposure- Moran and Emlet 2001; predation- Spight 1976, Gosselin and Rehak 2007; wave exposure- Etter 1996, Gosselin and Rehak 2007; ultraviolet radiation- Rawlings 1996) in juvenile and encapsulated individuals. However, temperature, in particular, has not been well studied with respect to earlier stages of development. In regards to temperature tolerances of *N. ostrina*, very little is known about how thermal stress affects embryonic survivorship. Gosselin and Chia (1995b) found that *N. emarginata* hatchlings around Barkley Sound (on Vancouver Island, Canada) were tolerant of temperatures as high as 26 °C; all hatchlings survived 8h of exposure at 22 and 26 °C. Mortality among hatchlings did occur at 30°C whereas adults survived all temperature treatments. This earlier study exemplifies the dichotomy that exists between the impacts of temperature on juvenile and adult marine invertebrates.

To further explore the relationship of thermal tolerance and early life history stages, we characterized the temperature tolerance of encapsulated veligers of the

intertidal dogwhelk, *Nucella ostrina*. This is the first study to measure the temperature tolerance of encapsulated *Nucella* veligers. In addition, given that *N. ostrina* has an expansive range distribution, from Yakutat, Alaska to Pt. Conception, CA (Vermeij et al. 1990, Collins et al. 1996), we tested the thermal tolerance of veligers collected from multiple sites within the biogeographic range of *N. ostrina* in order to assess the relationship between latitudinal distribution and thermal tolerance. By identifying the upper thermal limits for these embryos we hoped to contribute to our understanding of how the physiological tolerances of larval stages maps onto the distribution of the adult population.

C. MATERIALS AND METHODS

1. Study Sites and *Nucella* collections

During the breeding season N. ostrina egg capsules were collected from seven mid- to high intertidal sites between the months of May and July 2006 along the west coast of the United States from Washington State to Central California (48 to 35° N, Cattle Point, WA (CP); Boiler Bay, OR (BB); Strawberry Hill, OR (SH); Coos Bay, OR (CB); Humboldt, CA (HUM); Bodega Cove, CA (BML); and Cambria, CA (RM), see Table 1). At one collection site (Rancho Marino Reserve in Cambria, CA) N. ostrina overlaps in distribution with N. emarginata around Point Conception (Marko 1998). However, for this study, since genotyping was not conducted on the veligers, egg capsules collected from this site were called N. ostrina resembling the 'northern form' described by Palmer et al. (1990) and later classified as N. ostrina by Marko (1998). From all sites 15-30 egg capsules were haphazardly collected from 10-25 clutches by scraping off egg capsules from the substrate and placing them into a mesh holding chamber. At the end of collection, all egg capsules from the same site were combined and transported back to the laboratory. Upon arrival, the egg capsules were placed in an ambient flow-through seawater tank set to the temperature of the location where they were collected (10-19°C) while awaiting thermal tolerance trials. No batch of egg capsules was held for more than 12 h before experimentation began.

2. Field temperature data

At each collection site, two iButtons® (Dallas Maxim Semiconductor # DS1921G) were deployed in the field next to the egg capsules to record approximate habitat temperature for a given duration (April-July, 2006). Temperature loggers, programmed to record temperature every 20 minutes, were epoxied, in duplicate, to the substrate using z-spar (Splash Zone Compound, A-788) at approximately the middle intertidal height, near the egg capsules. Tidal height was an approximation considering these dogwhelk species are commonly found at the middle intertidal height. Temperature data were not recorded for Coos Bay. After 4 months of recorded data, the loggers were collected and data were downloaded using manufacturer's software and instructions.

3. Thermal tolerance trials

Using a modified protocol from Sewell and Young (1999) the egg capsules were exposed to a range of temperatures (from 13-36.5°C, see Table 2), including some of the higher temperatures that could be experienced in the intertidal zone during emersion (as recorded by deployed iButtons®). While some of the temperatures were outside the range of ecological relevancy, it was important for this study to identify the physiological limits imposed by temperature on survival for developing veligers. The number of temperature treatments varied slightly from site to site due to the number of egg capsules available for collection. Five to eight egg capsules were placed in an unsealed 20 mL glass scintillation vial with filtered seawater (0.35μm), in triplicate at each of the 6-11 different temperatures (Table 2).

To eliminate the confounding effect of desiccation stress as a variable, egg capsules were exposed to the temperature treatments while immersed in seawater. After the 1 h incubation, egg capsules (n = 5–8) were cut open and larvae were examined under a dissecting microscope where they were scored for survivorship: larvae that appeared to have ciliary movement around their velum were considered alive and those with no movement were scored as dead. Each capsule contained between 5 and 22 larvae. Temperature exposures of 1-2h in duration are commonly used to analyze the physiological performance of larvae (Boon-Niermeijer and Van de Scheur 1984, Sewell and Young 1999, Brown et al. 2004). Although it is likely the egg capsules of these species would encounter longer exposure to these temperature stress in the intertidal, we used a conservative approach for assessing mortality in this study. It is important to note that only advanced veliger-stage larvae were used for this experiment (as described by Gallardo 1979). If the larvae did not have distinct velar lobes, appearance of shell, eyespots, formation of foot and operculum, then the whole egg capsule was discarded and not used. These thermal tolerance trials were designed to measure veliger mortality across different temperatures and the data were used to calculate the LT_{50} for that particular site. LT_{50} refers to the temperature at which 50% of the total experimental population was killed by the heat treatment (Stillman and Somero 2000, Hamdoun et al. 2003).

4. Recovery experiment

To assess if the observed LT_{50} was a true indication of larval mortality as opposed to a temporary state of torpor (i.e., a temporary state of decreased

physiological activity) induced by the acute heat stress, a second experiment was conducted on *N. ostrina* larvae from Strawberry Hill, OR. Thirty-five egg capsules were collected from the field and subjected to a lethal (34.5°C) and an acute (31.7°C) heat shock for 1 h and allowed to recover for up to 1 h at the ambient seawater temperature (11°C). *N. ostrina* veligers were exposed to 31.7°C and 34.5°C, because these temperatures represent the LT_{50} and lethal temperature, respectively, for the Strawberry Hill, OR site.

Using the same thermal tolerance protocol as above, egg capsules (n = 35) were exposed to a 1 h heat shock, in triplicate, at the two temperatures (LT_{50} = 31.7°C and lethal = 34.5°C). Immediately following the exposure, 7 egg capsules were dissected and larvae survival rates were determined. The remaining 28 egg capsules for each temperature were placed in the seawater table to recover. Every 15 minutes up to 1 h, 7 egg capsules were removed from the recovery seawater table and survival rates were determined. To get a baseline measurement of larval condition in the egg capsules with no heat shock ("control"), survival rates of larvae from 21 *N. ostrina* egg capsules were quantified prior to any heat shock exposures.

5. Statistical analyses

For the thermal tolerance assays, analyses were performed using JMP 7.0 (SAS Institute Inc.) statistical software. The LT_{50} of *Nucella* for each site was calculated using a 95% confidence interval where upper and lower limits of the 50% mortality were given. Analyses for the recovery experiment were performed using SigmaStat 3.0 statistical software. The average mortality was calculated for each

recovery time point for veligers that were heat shocked at the LT_{50} temperature of 31.7°C and those that were heat shocked at 34.5°C. All values were Box-Cox transformed to meet the assumptions of normality. The mean mortality values for each heat shock temperature were compared using one-way analyses of variance.

Table 1: Collecting sites for *Nucella ostrina*

Site of Collection	Species Collected	Coordinates
Cattle Point, WA (CP)	*Nucella ostrina*	N 48°27.001' W 122°57.803'
Boiler Bay, OR (BB)	*Nucella ostrina*	N 44°50.001' W 124°03.846'
Strawberry Hill, OR (SH)	*Nucella ostrina*	N 44°14.995 W 124°06.808
Coos Bay, OR (CB)	*Nucella ostrina*	N 43°20.968' W 124°19.808'
Humboldt, CA (HUM)	*Nucella ostrina*	N 41°03.432' W 124°08.957'
Bodega Marine Reserve Bodega Cove, CA (BML)	*Nucella ostrina*	N 38°18.250' W 123°03.540'
Rancho Marino Reserve Cambria, CA (RM)	*Nucella ostrina**	N 35°33.290' W 121°10.580'

* Records may include *N. emarginata* in the southern part of the range of *Nucella* spp.

Table 2: Temperature exposure for *N. ostrina* egg capsules at a particular site

Latitude	Sites	1 hr Incubation Temperatures (ºC)
North ↓	CP	13, 15, 17.5, 19.5, 21.5, 23.5, 25.5, 27.5, 29.5, 31.5, 33.5
	BB	13, 19.5, 25.5, 29.5, 31.5, 34.5
	SH	13, 19.5, 21.5, 25.5, 29.5, 31.5, 34.5
	CB	13, 17.5, 19.5, 21.5, 25.5, 29.5, 31.5, 33.5, 34.5
	HUM	13, 19.5, 21.5, 23.5, 25.5, 29.5, 31.5, 33.5
	BML	13, 19.5, 21.5, 25.5, 29.5, 31.5, 34.5
South	RM	13, 16.5, 19.5, 21.5, 25.5, 29.5, 31, 34

C. RESULTS

1. Thermal tolerance profiles

Veliger larvae from all sites survived temperatures from 13 to 27.5°C. As temperatures further increased, mortality rapidly increased within a narrow window of temperatures. Overall, regardless of the biogeographic collection site of the *Nucella ostrina* veligers tested in the thermal tolerance trials, the temperature at which mortality was first observed was only ~ 3- 5°C less than the temperature that resulted in complete mortality. Survivorship of *N. ostrina* veligers abruptly changed within a narrow window of about 2°C for all sites (Fig. 2). For example, veligers from Cattle Point first experienced mortality at 29.5°C with 47.5% dead; temperatures 4°C higher, at 33.5°C, caused 100% mortality. A similar pattern was seen for other sites, such as Strawberry Hill and Humboldt, where only a few degrees (5°C and 3°C, respectively) separate the initial onset of mortality and 100% mortality (Fig. 2).

These mortality data were used to calculate the LT_{50} values for each collection site. In recovery experiments checking the calculated LT_{50}, no veligers were scored as alive when heat shocked at the highest temperature of 34.5°C (Fig. 3), whereas veligers exposed to the lower temperature, 31.7°C (the LT_{50} for Strawberry Hill), demonstrated some ciliary movement over time. Using a one-way analysis of variance the differences in the mean values among the recovery time points for the LT_{50} temperature (31.7°C) were not significant different ($F = 1.998$, $DF = 4$, $n = 100$, $p = 0.1008$), indicating that our protocol during the thermal trials

was accurate and decisions to score larvae as dead were not biased by reversible inactivity of the larvae.

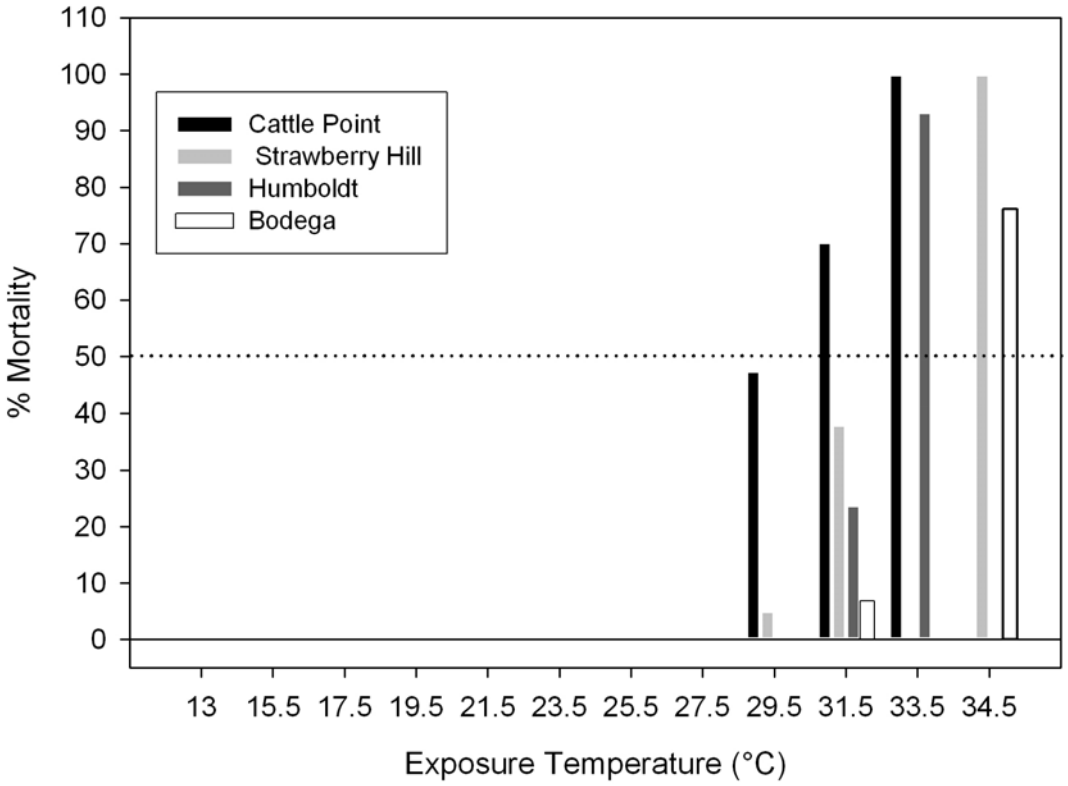

Figure 2: The thermal tolerance profile for *Nucella ostrina* veligers from 4 out of the 7 sites during May- July 2006. The *x-axis* represents exposure temperatures of egg capsules after 1hr incubation. The *y-axis* corresponds to percent mortality based upon the lack of ciliary movement around the velum after exposure. Each bar represents the number dead compared to the overall total number of larvae used (n= 5-8 egg capsules, n = 5-22 embryos per egg capsule).

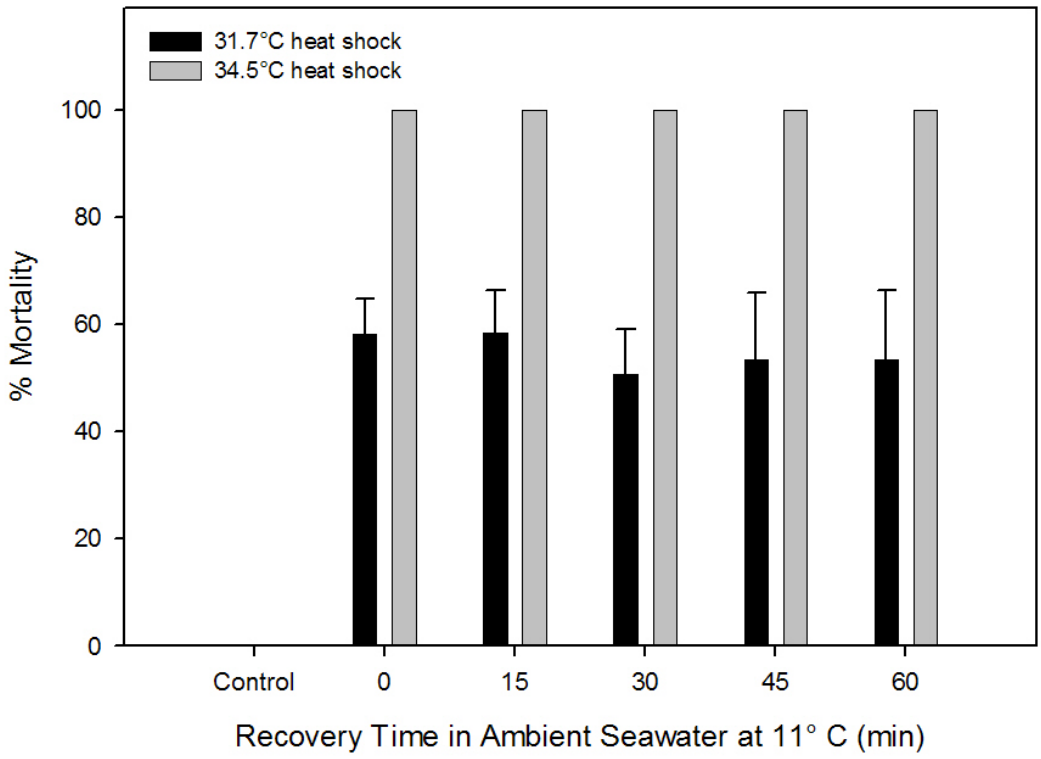

Figure 3: Recovery experiment conducted with *Nucella ostrina* veligers from Strawberry Hill, OR. The *x-axis* corresponds to the time the veligers spent recovering at 11°C post-1h heat shock. *Y-axis* is the percent mortality after time spent recovering. Each bar represents the number dead compared to the overall total number of larvae used. The dark bars correspond to veligers that were heat shocked at 31.7°C, the LT_{50} for this particular site. The light bars correspond to veligers that were exposed to their lethal temperature of 34.5°C (mean ± standard deviation). No significant difference was detected across recovery time for the LT_{50} temperatures of 31.7°C (p=0.1008) or for the lethal temperature of 34.5°C (p=1).

2. Relationship of thermal tolerance to site of collection

In the thermal tolerance trials, northern populations of veligers were more sensitive to higher temperatures when compared to the veligers collected in the southern part of the range (Fig. 4). *N. ostrina* veligers collected from the most northerly site of Cattle Point had an LT_{50} temperature of 30.1°C, 3.8°C lower than the LT_{50} for *N. ostrina* veligers from the most southern site of Rancho Marino (LT_{50} = 33.9°C, Fig. 4 and Table 3). When considered as a group, the veliger LT_{50}s showed a pattern of least to most tolerance of high temperatures in the following order: CP<BB>SH≈CB<HUM<BML≈RM (Table 3). This latitudinal trend was confirmed using linear regression (p = 0. 0011, R^2 = 0.8995).

Figure 5 shows environmental temperature data as collected by iButtons® deployed in the intertidal adjacent to the collected *Nucella* capsules for each site during April- July 2006. Although these data do not reflect the inter-capsule temperature, the iButton® data do illustrate the temperature variability experienced by the intertidal egg capsules. The mean habitat temperature had a subtle latitudinal cline; here, temperature increased as latitude decreased. However, statistically there was no correlation between the two (mean temperature vs. LT_{50}; p=0.211, Table 4). Overall, there was a great deal of variability in environmental temperature across latitude. For example, at a given site, intertidal species could experience temperatures as low as 4°C or as high as 29°C in a four month period (Humboldt, Fig. 5). Some sites (e.g., Cattle Point, Strawberry Hill, Humboldt and Bodega) had recorded maximum temperatures that were within a few degrees (~4 °C) of their

respective calculated veliger LT_{50} temperature; but no relationship was found between the two variables (maximum temperature vs. LT_{50}; $p = 0.280$, Table 4).

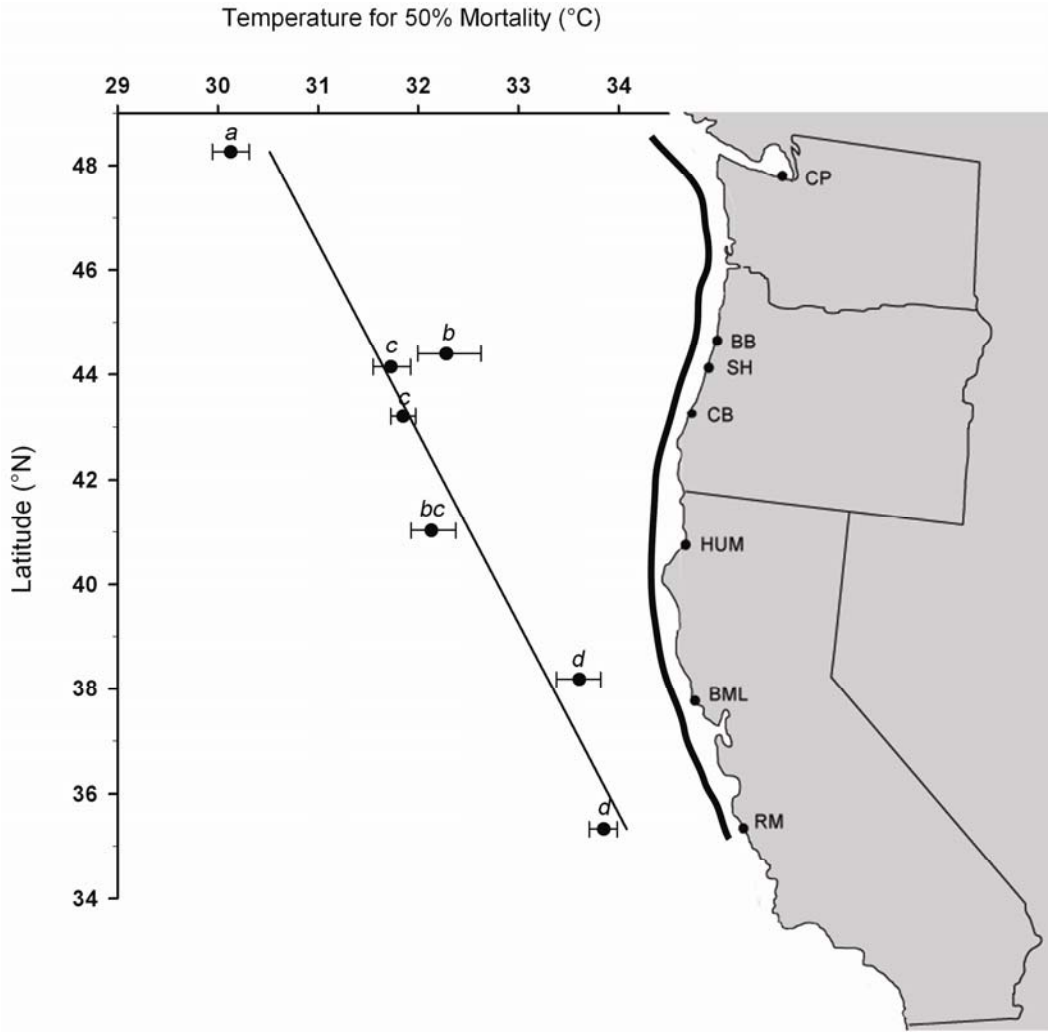

Figure 4: Latitudinal comparison of veliger thermal tolerance for *Nucella ostrina*, distribution indicated by solid black line, during May- July 2006. Black symbols on the graph represent the calculated LT_{50} for each site with error bars representing ± 95% confidence intervals. *Y-axis* represents latitude and correlates with the collection sites depicted by black dots on the map. The *x-axis* is the temperature at which 50% of the veligers died after a 1hr exposure ($p = 0.0011$, $R^2 = 0.8995$).

Table 3: Habitat temperature values for each collection site

Location	**Mean (°C)**	**Minimum (°C)**	**Maximum (°C)**	**LT_{50} (°C)**
Cattle Point	12.2	6.9	29.4	30.1
Boiler Bay	11.1	5.4	20.3	32.3
Strawberry Hill	10.8	5.1	27.2	31.7
Humboldt	12.4	5.1	28.7	32.1
Bodega	12.7	8.0	27.0	33.6
Rancho Marino	17.0	12.2	22.5	33.9

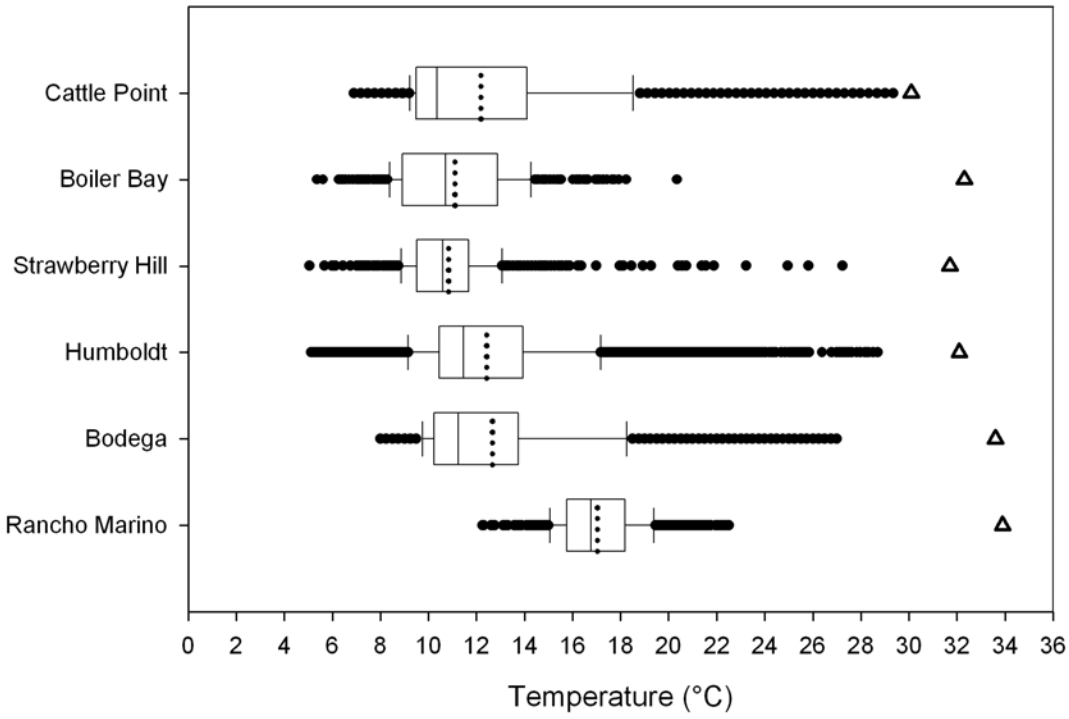

Figure 5: Habitat temperatures recorded from April-July 2006 using ibutton data loggers at each of the collection sites, except Coos Bay. The box represents 50% of the temperature data collected while the error bar to the right signifies 75% of the data and the left error bar corresponds to 25%. The dotted black line within the box equals the mean temperature. The black dots on either side of the error bars are the whiskers and show the outliers. The open triangles (Δ) indicate the veliger LT_{50} temperature for that site based on the thermal tolerance trials.

Table 4: Relationship of habitat temperature to calculated LT_{50} temperature for *Nucella ostrina* veligers across collection sites.

	slope	R^2	***p*-value**
Mean (°C)	0.972	0.356	0.211
Minimum (°C)	1.208	0.360	0.208
Maximum (°C)	-1.356	0.271	0.289

D. DISCUSSION AND CONCLUSION

In this study, we assessed the thermal tolerance of veliger larvae of *Nucella ostrina*, an intertidal dogwhelk, across its biogeographic distribution, and measured survivorship in laboratory trials across a range of ecologically relevant temperatures. There were two major findings in this study: (1) *N. ostrina* veligers displayed a sharp change in survivorship, at all sites, with mortality increasing to 100% within a few degrees, (2) there was a significant relationship between veliger thermal tolerance and latitude, but the relationship was uncorrelated with three month time series of environmental temperatures collected at each site.

1. Larval thermal tolerance

In the thermal trials, there was a rapid, linear increase in mortality occurring over a few degrees. Specifically, there was only ~3-5ºC difference between the LT_{50} temperature and the lethal temperature (100% mortality) across all the sites. Given the large-scale distribution of *Nucella ostrina* a broader range of LT_{50} temperatures might be predicted. However, our data revealed a narrow thermal window between survival and mortality where the LT_{50} temperature for *N. ostrina* veligers collected from Strawberry Hill was 31.7°C as compared to the recorded lethal temperature of 34.5°C. These slight differences in temperature indicate that there is an abrupt threshold for mortality that could have profound effects on survival where "ecological surprises" of sudden increases in temperature could precipitate mass mortality events (Harley et al. 2006). For example, despite being located in the

northern part of the range, for this study, Cattle Point is one site where veligers could experience warm habitat temperatures close to the LT_{50} for that site.

Our data on thermal tolerance are not the first in *Nucella* to illustrate a broad range of tolerance followed by a sudden drop in survivorship of the encapsulated veligers. Although the nature of the egg capsule can mitigate some of the abiotic stresses inherent to life in the intertidal (desiccation stress, osmotic shock and UV radiation) (Pechenik 1982, 1983, Hawkins and Hutchinson 1988, Rawlings 1990), there are thresholds where the protective capacity fails. For example, it has been found that late stage veligers (*N. emarginata*) can withstand up to 80% water loss from capsule chamber before suffering substantial mortality (Rawlings 1995). Capsule wall thickness helps provide protection against UV radiation. The thicker walled neogastropods, such as *N. emarginata* and *N. canaliculata*, were more resistant to UV stress and somewhat effective at protecting against UV-A radiation than the thinner walled lower intertidal species, *N. lamellosa* (Rawlings 1996, Pechenik 1999).

Similar observations of sudden shifts in survivorship in response to thermal stress have been made in early life history stages of other marine invertebrates. For example, Boon-Niermeijer et al., (1984) found that a 1°C increase (39.5-40.5°C) in heat exposure for 3-day old *Lymnaea stagnalis*, a gastropod mollusk, shifted mortality from 40% to 100%. In echinoderms, the purple sea urchin (*Strongylocentrotus purpuratus*) displayed abrupt changes in survival over just a 2°C span for both gastrulae and 4-arm larvae (Hammond and Hofmann 2009). Similarly in a non-marine invertebrate, larvae of two species of *Drosophila*, *D. melanogaster*

and *D. simulans*, had percent survivorship that shifted from 100% to near zero when exposure temperatures were increased from 37 to 39.5°C (Krebs 1999). Responses of developing embryos to temperature and the particular temperatures encountered during the normal breeding season have been most extensively investigated in echinoderms (Andronikov 1975, Strathmann 1987, Fujisawa and Shigei 1990). In a study on five tropical species of sea urchins, Rupp (1973) found that the upper thermal limits of cleavage were near 34°C whereas egg fertilization was more thermally resistant and not affected until 36°C, suggesting that larval development could cease if larvae were trapped in flat reef pools or lagoons where temperatures exceed 36°C. Similarly, Sewell and Young (1999) reported that the ability for blastulae and later staged tropical sea urchin larvae (*Echinometra lucunter*) to survive short periods (2 h) of elevated temperatures (4-5 °C higher than the 34°C upper limit for normal development), might be important if the larvae are carried to shallow waters of the Caribbean reef-flats where seawater temperatures can reach 40°C. In another experiment, increased mortality was observed in the larvae of a brooding tropical coral, *Porites astreoides*, when exposed to slight increases in temperature from 28ºC to 33ºC (Edmunds et al. 2001). Finally, in a polar species, successful larval development was achieved in a 3 ºC window, from +6 to +9°C, for the sub-antarctic crab, *Paralomis granulosa* (Anger et al. 2003).

While all the above examples help to explain the importance of understanding how temperature limitations influence early developmental stages of larvae, they also highlight the scarcity of data on the temperature impacts on non-planktonic larvae. This study contributes to our understanding of how conditions in

the intertidal might influence larval survival. In a more global context, these data also suggest that increased air and seawater temperatures due to global climate change may also increase mortality during extreme heat events (Hoegh-Guldberg 1999, Pandolfi et al. 2003).

2. Relationship of thermal tolerance and environmental temperature

While our data suggest a strong latitudinal relationship between calculated LT_{50} temperature and site, no correlation was found between LT_{50} and habitat temperature exposure for *N. ostrina* veligers even though the maximum habitat temperature for Cattle Point was only 0.7°C from its calculated LT_{50} value (Fig. 5, Table 3). *N. ostrina* veligers from other sites such as Strawberry Hill, Humboldt and Bodega experienced larger temperature differences between maximum habitat temperature and LT_{50} values (4.5, 3.4 and 6.6 °C, respectively). In general, our data show that larvae from more southerly- distributed populations were more thermally tolerant than northern populations. For *N. ostrina* veligers collected from Cattle Point the LT_{50} was 30.1°C as compared to an LT_{50} of 33.9°C of veligers collected at the more southerly location of Rancho Marino Reserve. Few other studies have addressed the relationship of biogeography and the physiological tolerances of larval forms. The only other study that we are aware of found a modest correlation between thermal tolerance and biogeography for echinoplutei larvae of *Stronglycentrus purpuratus*. Larvae spawned from Oregon adults (LT_{50} = 29.7°C) were more thermally sensitive than those larvae collected from Baja California, Mexico urchins (LT_{50} = 31°C) (Hammond and Hofmann 2009).

In contrast to the limited data on larval forms, the correlation of thermal tolerance with latitude and environment has been documented in adult forms of many marine invertebrates (see review Vernberg 1962, Stillman and Somero 2000, Somero 2005, Sorte and Hofmann 2005, Jansen et al. 2007) and in other ectotherms (e.g., *Drosophila melanogaster*-Hoffmann et al. 2002, mosquitoes-Zani et al. 2005, *Fundulus heteroclitus*-Fangue et al. 2006). Similar to our results, Urban (1994) found a correlation between LT_{50} temperature and geographical distribution for 10 bivalve species from Peru and Chile over 22° of latitude.

Overall our results provide evidence that embryos and larvae are not necessarily a hyper-vulnerable life history stage, consistent with the idea put forth by Hamdoun and Epel (2007). This observation is further supported by other studies, for example on an intertidal bivalve which found that early life stages and adults had similar thermal tolerances. The oyster, *Ostreola conchaphila*, broods its young and adults and veliger larvae survive the same lethal temperatures of 38.5-39°C (Brown et al. 2004).

In summary, the current study showed that *Nucella ostrina* veligers displayed different thermal tolerances as a function of the latitude at which they were collected. The data presented here are a conservative measurement of the upper limits of physiological thresholds of encapsulated larvae. These data are further evidence that these intertidal dogwhelks could be living near their upper thermal limits. Overall, these data suggest that larval stages of this intertidal invertebrate may not be a hyper-vulnerable life stage. However, changing thermal patterns due to climate change may significantly increase mortality during larval development and

result in population fragmentation based on the latitudinal timing of low-tides and tendency for local adaptation in this species (Sanford et al. 2006).

III. Effects of CO_2-infused seawater on a direct developer (*Nucella ostrina*)

A. INTRODUCTION

Over the last two centuries, human activities such as the burning of fossil fuels, cement manufacturing and deforestation have caused atmospheric carbon dioxide (CO_2) to increase by approximately 40% above pre-industrial levels (Doney and Schimel 2007). It is estimated that almost 50% of all anthropogenic CO_2 emitted is absorbed by the world's oceans (Sabine et al. 2004) resulting in an increase of H^+ concentration of roughly 30% (Caldeira and Wickett 2003). The production of these protons causes seawater pH to fall, creating a less alkaline, more acidic ocean, a phenomenon called ocean acidification (OA; Caldiera and Wickett 2003). Recent modeling efforts predict that OA could worsen as atmospheric levels of CO_2 continue to increase; predictions are that oceanic pH may decrease by as much as 0.4 units within 100 years and 0.7 units within 250 years (Caldeira and Wickett 2003, Steinacher et al. 2009).

Ocean acidification is predicted to lead to significant changes in water chemistry that will affect calcification and other physiological processes in many marine organisms. As pH decreases, there is a shift in inorganic carbon equilibria towards higher CO_2 and lower carbonate ion (CO_3^{2-}) concentrations (Kleypas et al. 1999). The carbonate ion is a chemical component of calcium carbonate ($CaCO_3$), and changes in its ambient concentration can alter the ability of calcifying organisms to precipitate $CaCO_3$ to form hard parts such as shells and skeletons (Fabry et al.

2008). In addition, as pH continues to decrease in the ocean, lower saturation states of $CaCO_3$ will cause a change in the saturation levels for minerals such as calcite and aragonite.

Ocean acidification is predicted to affect the biology of a variety of calcifying marine organisms (Doney et al. 2009a). Among marine invertebrates, stony corals have been noted as being most vulnerable to OA-related declines in calcification (Langdon et al. 2000, Langdon and Atkins 2005, Schneider and Erez 2006). For example, a scleractinian coral (*Oculina patagonica*) maintained under acidified seawater (pH 7.4) completely lost its skeleton as compared to those under normal conditions (pH 8.2; Fine and Tchernov 2007). Not only will coral calcification be hindered under increasing ocean acidification, but also dissolution rates could increase (Langdon et al. 2000, Yates and Halley 2006), ultimately influencing the coral reef community (Przeslawski et al. 2008). Similarly, marine invertebrates in temperate, benthic ecosystems are also expected to be affected by OA. Calcification rates of the edible mussel (*Mytilus edulis*) and Pacific oyster (*Crassostrea gigas*) declined by 25 and 10%, respectively, with increasing pCO_2 under predicted Intergovernmental Panel on Climate Change (IPCC) scenarios (Gazeau et al. 2007). In addition, shell growth and survival were reduced in the gastropod *Strombus lubuanus* when grown over a long-term exposure (6 months) at 560 ppmv CO_2 (Shirayama and Thornton 2005). Echinoderms (Kurihara et al. 2004, Shirayama and Thornton 2005, Miles et al. 2007), other molluscs (Bamber 1990, Michaelidis et al. 2005a, Berge et al. 2006) and crustaceans (Mayor et al. 2007) also have shown vulnerability to ocean acidification.

In other subtidal ecosystems, crustose coralline algae (CCA) demonstrated a lower recruitment rate and decreased percent coverage when exposed to high CO_2 (pH 7.91; Kuffner et al. 2008). The reduced abundance of CCA could greatly impact benthic communities because of its key role in these ecosystems. CCA supplies framework for some benthic invertebrates, provides settlement cues for developing larvae (Morse et al. 1988, Heyward and Negri 1999), and is a major producer of carbonate sediments (Chave 1962). Some planktonic species, such as a foraminifera (Spero et al. 1997, Bijma et al. 2002) and pteropods (Orr et al. 2005, Fabry et al. 2008), have revealed high sensitivity to high-CO_2/low pH seawater. In other cases no net change in calcification was observed for the coccolithophore *Coccolithus pelagicus* (Langer et al. 2006) and, somewhat surprisingly, net calcification rates for another coccolithophore *Emiliania huxleyi* increased when grown under increased pCO_2 (Iglesias-Rodríguez et al. 2008). Additional evidence suggests that some photosynthetic organisms (e.g., cyanobacteria and seagrasses) might also be able to tolerate the changes brought about by ocean acidification (Fu et al. 2007, Hutchins et al. 2007, Palacios and Zimmerman 2007).

While our understanding of the effects of OA on marine calcifiers is growing, most of the recent research in the field has focused on adult forms or juvenile stages of life history cycles (see Bamber 1990, Michaelidis et al. 2005b, Langdon and Atkinson 2005, Shirayama and Thornton 2005, Berge et al. 2006, Bibby et al. 2007, Kurihara et al. 2008b). Much less is known about how early developmental stages will respond (see Kurihara 2008, Dupont and Thorndyke 2009). Since mortality rates of early developmental stages can sometimes exceed

90% in their natural habitat (Gosselin and Qian 1997), it is important to understand how climate change related stress might exacerbate these natural losses.

Historically, early developmental stages have been found to be more sensitive to environmental disturbances than their adult counterparts (Thorson 1950). A recent study supported this notion by demonstrating adult copepod (*Calanus finmarchicus*) survival and egg production were not affected by CO_2-induced seawater but egg development was dramatically reduced (Mayor et al. 2007). Since it is known that the first deposition of $CaCO_3$ occurs during larval development, these stages may be highly susceptible to the potential effects of OA. A few studies have shown that early development in echinoderm larvae (fertilization, cleavage and overall development) was slower when grown under elevated CO_2 conditions (Kurihara et al. 2004, Kurihara and Shirayama 2004, Clark et al. 2009). Additionally, morphological abnormalities and larval shell synthesis were disrupted when oyster (*Crassostrea gigas*) and mussel (*Mytilus galloprovincialis*) larvae were raised in CO_2-enriched seawater (2000ppm CO_2, pH 7.4) (Kurihara et al. 2007, Kurihara et al. 2008a). Green and colleagues (2004) found mortality levels increased in juveniles of the hard clam, *Mercenaria mercenaria*, when exposed to undersaturated aragonite seawater.

The mineralogy and calcification of larval stage molluscs and echinoids is very complex, but raises the possibility of increased sensitivity to OA. While adult bivalves and other molluscs secrete aragonite, calcite or both phases of $CaCO_3$, larval veliger shells contain an amorphous form of $CaCO_3$, a transient precursor to aragonite (Weiss et al. 2002). This amorphous $CaCO_3$ precursor has been found to be

unstable and more soluble than minerals of aragonite and calcite (Beniash et al. 1997). Therefore, the process of shell deposition in early development may be exceptionally vulnerable to ocean acidification.

While it is important to note that some organisms may be capable of regulating, and therefore defending, biological processes such as calcification in response to the stress induced by OA, it is likely this will be done at a 'cost' to the animal. While Wood and colleagues (2008) found no decrease in calcium carbonate skeleton of brittle stars (*Amphiura filiformis*) exposed to lower pH treatments, they did detect distinct losses of muscle mass as pH decreased. In addition, O'Donnell et al. (2009a) demonstrated that larval urchins raised under OA conditions failed to activate the expression of defensome genes in response to mild heat stress, suggesting that developing under lower pH conditions comes at the cost of thermal tolerance. Similarly, in crabs (*Cancer pagurus*) hypercapnia (1% CO_2) caused a 5 °C decrease in the upper thermal limits of aerobic scope, thus enhancing their sensitivity to temperature and narrowing their window of thermal tolerance (Metzger et al. 2007).

Understanding these trade-offs helps to explain how an organism's physiological tolerance might be jeopardized under multiple climate factors, such as thermal stress, hypoxia and salinity, and whether marine ectotherms have the capacity to undergo thermal acclimation. Measuring calcification rates of marine organisms provides one answer to the effects of OA, but it is yet to be seen if this climate change factor can influence other physiological responses (e.g., thermal tolerance). Temperature can be one of the most predominant and influential abiotic

stressors in intertidal habitats (Stillman and Somero 2000) and has been well documented to affect multiple facets of physiological performance (e.g., development, growth, metabolism, survival). However it remains to be seen how ocean acidification could play a role in altering the thermal tolerance pattern of intertidal organisms. It has been speculated that an organism's thermal tolerance window will likely be narrowed due to enhanced CO_2 and depletion of oxygen levels, because physiological processes respond in concert to these types of stressors (Pörtner et al. 2005).

In the present study, I aimed to evaluate the effects of CO_2-acidified seawater on shell integrity and thermal tolerance of developing embryos using the intertidal gastropod *Nucella ostrina* as a study organism. This gastropod with direct development spends its entire life cycle in the intertidal region where the encapsulated embryos are routinely exposed to wide fluctuations in environmental conditions with each tidal cycle. Until recently, temperature, salinity and UV were thought to be the primary abiotic stressors impacting development of marine intertidal larvae. However an increased focus on ocean chemistry has led to the discovery that upwelled corrosive "acidified" waters from depths are shoaling to near the surface and very close to the coast, and thus may impact intertidal communities (Feely et al. 2008). This new threat has the potential to change environmental conditions for calcifying invertebrates such as *Nucella*. Using the predicted range of values for pCO_2 from the IPCC scenarios for 2100, I examined the effects of short-term CO_2-induced seawater on veliger shell integrity and tolerance of thermal stress.

B. MATERIALS AND METHODS

1. Egg capsule collection

During the breeding season of June 2008, dogwhelk egg capsules from *Nucella ostrina* were haphazardly collected at Strawberry Hill, OR, from 35-40 clutches in the mid- intertidal zone and placed into a mesh holding chamber. Each clutch contained approximately 10-15 egg capsules. The ambient seawater temperature at the time of collection was ~12 °C. After collection, egg capsules were shipped back to our laboratory at the University of California at Santa Barbara where they were placed in a flow-through seawater tank at 12.0°C for no more than two days before they were used in the CO_2 experiment.

2. Experimental set-up of CO_2 system

A CO_2 incubation system was designed to raise larvae under three different concentrations of CO_2 that were selected so that the experimental conditions spanned the Special Report Emission Scenarios (SRES) predicted by the IPCC (2007) for the year 2100. Three different CO_2 concentrations were simulated to mimic the expected CO_2 concentrations by 2100 (from IPCC); present day atmospheric CO_2 levels (~ 380 parts per million, ppm, CO_2), "most conservative" scenario (B1, ~ 570ppm CO_2) and the "business as usual" scenario (A1FI, ~ 990ppm CO_2). These gas mixing regimes resulted in seawater with the following pHs: the Control CO_2 treatment (380ppm CO_2) varied from 8.06 to 8.13 (mean 8.09 ± 0.025 SD), the Moderate CO_2 treatment (570ppm CO_2) yielded pH values from 7.99 to 8.05 (mean 8.04 ± 0.02 SD)

during the experiment, whereas that of High CO_2 treatment (990ppm CO_2) ranged from pH 7.86 to 7.94 (mean 7.90 ± 0.02 SD).

To establish these CO_2 gas mixtures, ambient air was first pulled from the atmosphere and pumped through a cylinder containing DRIERITE® desiccant and Sodasorb® that removed moisture and scrubbed carbon dioxide, respectively, from the air. The air was then passed through a 0.22µm filter to remove particulates. Next this CO_2-free air was blended with pure CO_2 using 2 mass-flow gas valve controllers (Sierra® Instruments) for each desired pCO_2 concentration. Each gas mixture was delivered to a reactor buckets at 1000mL min^{-1} (Mazzei® Injectors) and mixed with in-coming filtered seawater (0.22µm). Once CO_2 saturation was achieved in the reactor bucket, the CO_2-acidified water was delivered to the three replicate culture buckets. The CO_2 concentration of the gas delivered to each reactor bucket was continuously monitored using an infrared CO_2 analyzer (Qubit) which allowed adjustments to CO_2 delivery to be made if necessary. Temperature, salinity and pH (using a Myron L. Company, Ultrameter II^{TM}) were continuously monitored throughout the experiment (Table 5 and Figure 6).

3. Culture bucket design

The egg capsules were exposed to elevated CO_2 concentrations using a 15 l nested culture chamber design (see Appendix A). The inner bucket contained twelve 3 inch-diameter holes sealed with 64µm mesh. Each bucket pair was fitted with an external PVC side arm connecting the outer bucket (5 l) to the inner bucket (10 l) with a short pipe along one side of the inner bucket. The side-arm provided a

location to aerate the bucket with gas (200 ml min^{-1}) and generated a gently mixing current for the egg capsules within the inner bucket. The culture buckets were filled with equilibrated CO_2 seawater that was replaced at a constant rate of 1.22 l h^{-1} to provide fresh seawater without altering the pH. All culture buckets were gently mixed with a paddle and 12-v motor. Experiments did not begin until the pH of all reactor and culture buckets were stable for at least 24 h.

4. Nanoindentation

To test shell integrity, a technique known as nanoindentation (Oliver and Pharr 1992, Pharr 1998, Ebenstein and Pruitt 2006) was performed on veligers exposed to Control and High CO_2-acidified seawater. Prior to experimentation, ten egg capsules were selected, randomly dissected, and *N. ostrina* veligers were removed and placed in a 1% bleach solution (with MilliQ® purified water) for 24 h at room temperature to remove the organic material. These individuals were called "pre-CO_2" samples. Bleach was removed and the larvae were washed with 500µl of distilled (DI) water for 1-2min. The DI water was then removed and a series of ethanol rinses for 22 min were performed (500µl of 50% ethanol, 70%, 95% and 100%). A final DI rinse was applied to larvae and removed using a pipettor after 2 minutes. Larvae were then placed in the fume hood to allow for sufficient drying (2-4h) and then moved to a desiccator for storage to await nanoindentation.

For the experimental treatments, once the CO_2 system was stable, ten egg capsules were placed in the Control and High CO_2-enriched seawater for 2 weeks. After the given exposure time, the egg capsules were dissected and veligers were

carefully removed and prepared (see above preparation). Individuals were embedded within rubber molds (Ted Pella, Inc., Redding, CA) using Epofix™ cold-setting resin (EMS, Hatfield, PA), which was cured overnight at room temperature. Samples were positioned in the mold so that ring-like cross-sections of the shell would be produced during subsequent sectioning. Flat surfaces were obtained by ultra-microtomy (Leica EM UC6, Leica Mikrosysteme GmbH, Wetzlar, Germany), using a glass knife for the initial surface trimming. The final microtoming was done with a diamond histoknife using fine cutting conditions (50 nm thick sections at a speed rate of 1 mm/sec). The microtomed surfaces revealed the ring structure of the larvae shells, with typical diameter in the range 300 to 600 μm, and a shell wall thickness of 20 to 40 μm (for example see Figure 7).

To test the mechanical properties of the shell, hardness and modulus (indicative of the stiffness) were assessed by nanoindentation: the microtomed blocks were placed with double-side tape inside the chamber of a TriboIndenter™ instrumented nanoindenter (Hysitron, Minneapolis, USA), equipped with a 30 mN load force transducer and a cube-corner tip. The area function of the tip was calibrated prior to testing by indenting a fused quartz standard with known values of hardness and stiffness. Tests were conducted in load-controlled mode using the following conditions: 1 mN peak load, 0.1 mN/sec loading rate, and a holding time at peak load of 10 sec (see Appendix B). Hardness measurements quantified the resistance of the shell to probing while stiffness, or modulus, referred to the elasticity in which the probe unloaded or retracted from the sample. In a typical

sample, indents were placed along line profiles 30 to 50 μm long with 5 μm spacing between each indent, so as to encompass the outside embedding resin, the shell and the inner region. In total, three *N. ostrina* veligers from the Control and three individuals from the High CO_2 treatment were indented after the 2 week exposure. For each larvae shell sample, 5 lines of indents were accomplished for an averaged total of 25 indents. Hardness (*H*) and modulus (stiffness, *E*) were computed from indentation curves using the classical Oliver-Pharr analysis (Oliver et al. 1992, see Appendix B). Prior to statistical analysis, the data were carefully examined and indents that hit the resin/organic material as opposed to the shell walls (the difference in mechanical properties is approximately one order of magnitude) were excluded from the analysis. Additional control indents were carried out on a mature conch shell sample, to verify that measured *H* and *E* values agreed with values reported for shells of marine snails such as conch and abalone (Jackson et al. 1988, Menig et al. 2000).

5. CO_2 exposures and thermal tolerance assays

To determine if CO_2-acidified seawater affected veliger thermal tolerance, seventy egg capsules were placed into each of the nine culture buckets (three replicates for each of the three CO_2 conditions). Half were exposed for 1 week and half for 2 weeks. During the exposures no obvious developmental abnormalities or mortality of larvae was associated with any of the CO_2 treatments. After the exposures, thermal tolerance assays were performed on 35 egg capsules from each of the CO_2 culture buckets to assess larval survivorship. Five egg capsules per CO_2

treatment were placed into 5 ml of filtered seawater (0.35 μm) and incubated for 11 h at one of seven temperatures (11, 30.1, 30.8, 31.8, 33, 34 and 35 °C) in an aluminum heat block. After the 1 h thermal challenge, egg capsules were cut open and larvae were examined under a dissecting microscope to determine survival. One-two hour heat exposures are commonly used in such studies (Boon-Niermeijer and Van de Scheur 1984, Sewell and Young 1999, Brown et al. 2004) to assess the physiological performance of larvae in a given treatment. Survival was assessed based on ciliary movement around their velum. It is important to note that only advance-staged veligers (described by Gallardo 1979) were tested for this experiment; if the larvae did not have a distinct velum, eyespots, foot and operculum, the whole egg capsule was discarded and not used. Each capsule contained approximately 5-15 larvae and a minimum of 100 larvae were exposed to each temperature. These thermal tolerance assays were performed for both 1week and 2 week CO_2 exposure periods for all nine culture buckets.

6. Statistical analysis

Significance of mean shell hardness and mean stiffness between CO_2 treatments was determined using one-way analysis of variance (ANOVA). Data were Box-Cox transformed if necessary to improve normality. If significant differences were found, Tukey-Kramer HSD tests were used to conduct post-hoc comparisons among mean groups ($\alpha = 0.05$). These analyses were computed in JMP 8.0. (SAS Institute Inc.).

To evaluate the effects of CO_2 treatment on survivorship across all temperatures and exposure time (weeks), the status of individuals (alive/dead) was analyzed using likelihood ratio tests in JMP 8.0. (SAS Institute Inc.). Post-hoc comparisons using Fisher's exact test (i.e., permutation tests) were performed among main factors if significant differences were detected. False discovery rate (FDR) was run to correct for multiple comparisons and yielded expected proportion of false positives among all significant hypotheses.

Table 5: Values for the treatment buckets for the duration of the *Nucella ostrina* CO_2 experiment.

Culture Buckets	CO_2 concentration	Mean pH (±SD)	Mean Temperature (°C)	Mean salinity (‰)
Control CO_2	~380ppm	8.09 ± 0.025	11.52 (± 0.5)	33
Moderate CO_2	~ 570ppm	8.04 ± 0.02	11.33 (± 0.5)	33
High CO_2	~ 990ppm	7.90 ± 0.02	11.62 (± 0.5)	33

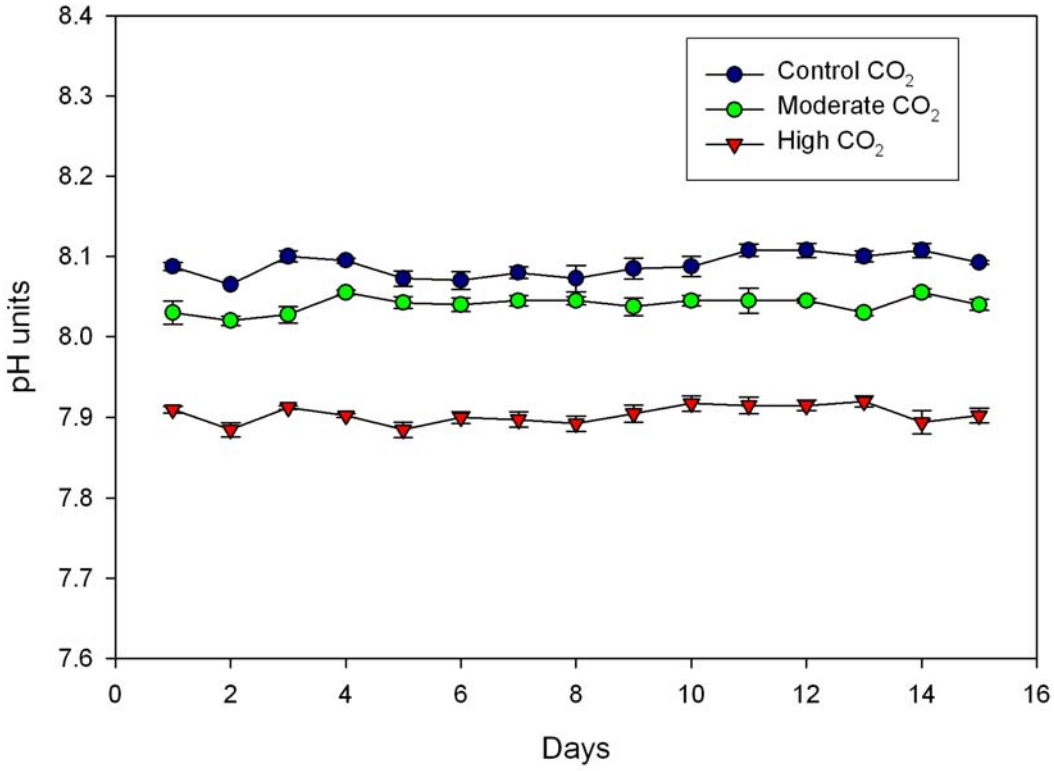

Figure 6: Measurements of the acidified seawater for the 14 day experiment with *Nucella ostrina* veligers. Data are mean pH values of the 3 replicates at each CO_2 concentration. Error bars = SD.

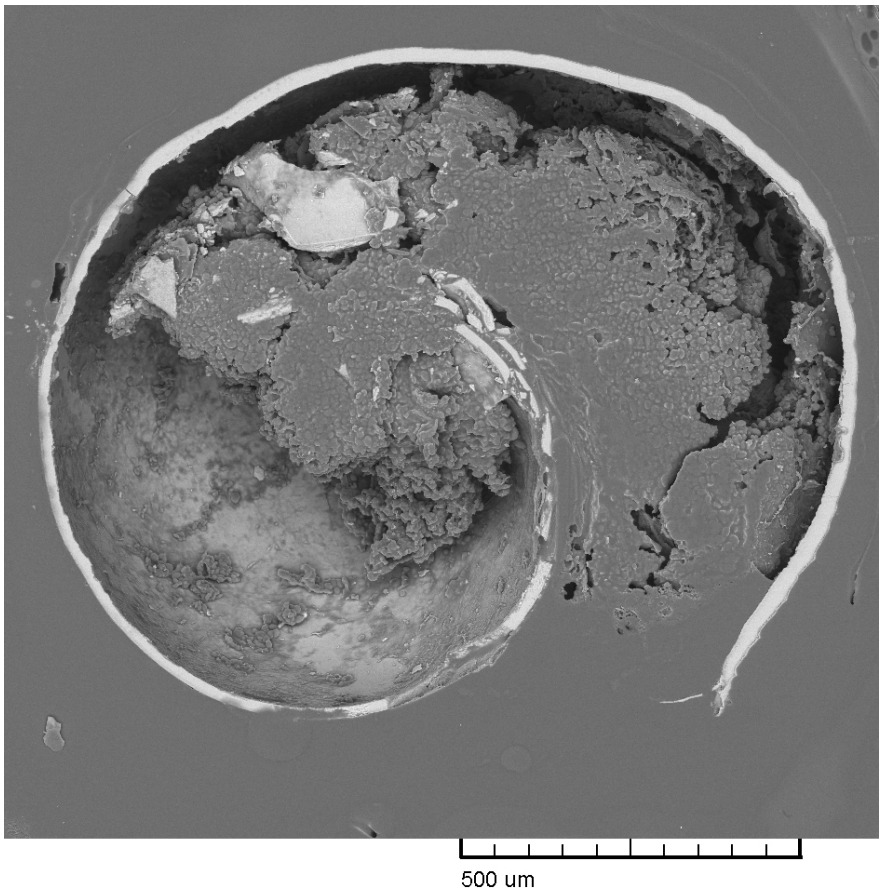

Figure 7: An example of a scanning electron microscope (SEM) image of a microtome slice of a *Nucella ostrina* veliger that was indented multiple times around the larval shell. The indented regions were done with a 1um diamond tip and cannot be seen at this magnification.

C. RESULTS

1. Shell stiffness and hardness

The shell integrity of *Nucella ostrina* veligers was measured using nano-indentation to determine if stiffness or hardness were affected by short-term exposure to CO_2-acidified seawater. Shell stiffness modulus did not differ between the Control CO_2 treatment compared to the High CO_2 treatment following a 2 week exposure (Figure 8, $p=0.9833$). However, shells of High CO_2 veligers were stiffer than were 'pre CO_2' shells (Figure 8, $p<0.0001$). Similarly, shell hardness did not significantly differ between those veligers raised under Control CO_2 and High CO_2 treatments (Figure 9, $p=0.6038$), but shells of veligers from the 'pre-CO_2' samples were more brittle than those of veligers raised under High CO_2 ($p = 0.0195$).

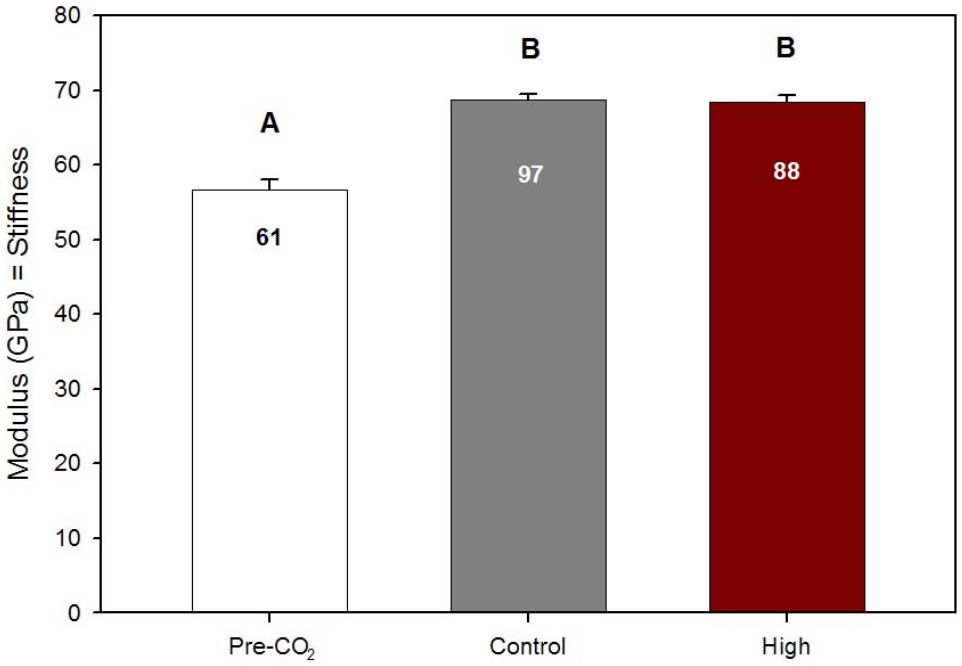

Figure 8: Shell stiffness of *Nucella ostrina* veligers after two weeks of exposure to "control" and "high" CO_2 compared to shell stiffness of veligers not exposed to CO_2 treatments. Each bar represents the mean stiffness of three individuals indented and the total number of indents is indicated by the number within the bar (Error bars = S.E.). The *x-axis* represents the two CO_2 treatments (Control = 380 ppm and High = 990 ppm) and the Pre-CO_2 were veligers sampled before the experiment began. Differences in means are signified by different letters (Tukey-Kramer, $p<0.001$).

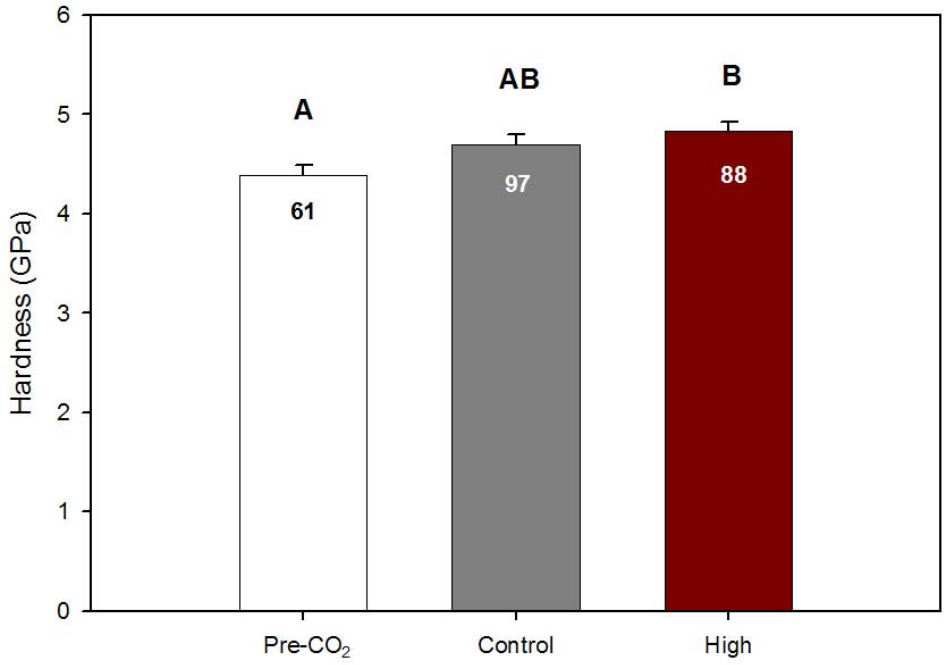

Figure 9: Shell hardness of *Nucella ostrina* veligers after two weeks of exposure to "control" and "high" CO_2 compared to shell hardness of veligers not exposed to CO_2 treatments. Each bar represents the mean hardness of three individuals indented and the total number of indents is indicated by the number within the bar graph (Error bars = S.E.). The *x-axis* represents the two CO_2 treatments (Control = 380 ppm and High = 990 ppm) and the Pre-CO_2 were veligers sampled before the experiment began. Significant differences in means are signified by different letters (Tukey-Kramer, $p<0.0195$).

2. The effect of elevated CO_2 on *N. ostrina* veliger thermal tolerance

Thermal tolerance performance curves were generated to measure veliger survival over a range of temperatures at 1°C intervals. Such performance curves illustrate the effect of a thermal challenge on survivorship of early developmental stages of *N. ostrina* in response to CO_2-driven seawater acidification.

Following a 1-week exposure to CO_2-acidified seawater, survivorship varied with temperature (Fig. 10A; likelihood ratio test; temperature, $p < 0.0001$) but CO_2 had no effect (Figure 10A; likelihood ratio test; temperature x CO_2, $p=0.4247$; CO_2, $p=1.000$). However, restriction of analysis to only those temperatures at which survivorship varied (30.8, 31.8, 33 and 34°C) suggests that at higher temperatures, CO_2 altered thermal tolerance of the veligers (likelihood ratio test; temperature x CO_2, $p=0.1856$; temperature, $p <0.0001$; CO_2, $p=0.0161$). The High CO_2-acidified seawater significantly decreased survivorship of veligers when compared to the Control CO_2 treatment ($p=0.004$). No differences in survival were detected when either Control and Moderate CO_2 treatments or when Moderate and High CO_2 treatment were analyzed (Fisher extact test; $p=0.1077$ and 0.0714, respectively).

In contrast to the differences in survival responses observed after 1 week of exposure, CO_2 treatment did not appear to affect survival of *N. ostrina* veligers after a 2 week exposure (Figure 10B). Here, temperature was the only factor that significantly affected mortality (likelihood ratio test; temperature x CO_2, $p=0.1802$; CO_2, $p=0.2850$; temperature, $p<0.0001$). However, the survival response for veligers exposed to the High CO_2 treatment underwent a transitional phase from 31.8 to 33

°C. Compared to the other CO_2 treatments, survivorship was lowest (74.4%) at 31.8 °C in the High CO_2 treatment and then switched to the highest survivorship of 70.3% at 33 °C (Figure 10B). Although this trend was not significant, it was also observed for the 34°C exposure; High CO_2 treatment had 43.9% veliger survival whereas Control and Moderate CO_2 treatments were lower (36.7% and 27.6%).

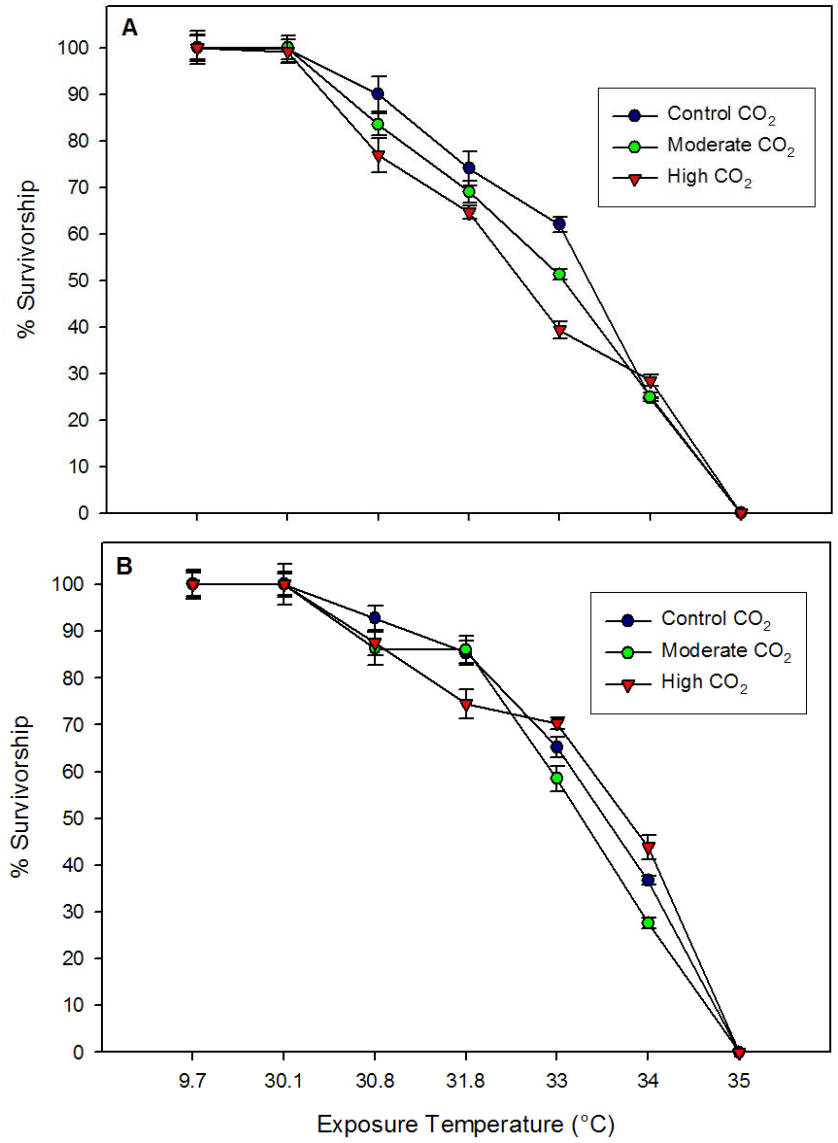

Figure 10: Thermal tolerance, presented as % survivorship of *Nucella ostrina* veligers after a (A) 1 week exposure and (B) 2 week exposure to elevated CO_2 concentrations. Four to five egg capsules were accessed for survivorship, in triplicate, at each exposure temperature (1 h in duration) and CO_2 concentration (n=100) (Error bars = ± S.D.).

D. DISCUSSION AND CONCLUSION

The majority of studies on the consequences of OA on marine calcifiers have shown that OA is generally deleterious to a number of physiological processes (Widdicombe and Spicer 2008, Fabry et al. 2008). The most commonly studied feature is calcification; notably, there is a reduction in calcification rates (Hoegh-Guldberg 2005, Kurihara et al. 2007, Clark et al. 2009, Martin and Gattuso 2009), although some have noted an increase (Wood et al. 2008, Gooding et al. 2009). Fewer studies have addressed how function or development is altered by OA stress. The research reported here adds to our understanding of the effects of OA on early-stage marine invertebrates. Specifically, I have demonstrated that the tolerance of brief bouts of thermal stress under increased acidification was unaffected in *N. ostrina* veligers. In addition, shell integrity of larvae was also unaffected by exposure to OA conditions. Overall, these results suggest that encapsulated veliger-stage *Nucella ostrina* are relatively resistant to seawater conditions that mimic OA stress in the lab, at least over the short term.

1. Shell integrity

Veliger shell strength did not change when larvae were exposed to High CO_2 treatments (pH 7.9), suggesting that developing larvae may be able to maintain shell integrity as they continue to grow in acidified seawater at least for 2 weeks. Similar results were found for the intertidal gastropod, *Littorina littorea* (Bibby et al. 2007). Adult shell thicknessdid not change between normal pH (7.97) and low pH (6.63)

when grown for 15 days under CO_2-enriched seawater. Although Bibby et al. (2007) investigated adults instead of developing larvae, both suggest that shell dissolution may not be a consequence of short-term exposures to elevated CO_2 in at least some gastropods. In contrast, differences in growth patterns were observed in the bivalve *Mytilus edulis* following 44 days of exposure to pH 6.7 when compared to control seawater of pH of 8.1 (Berge et al. 2006). These results with much lower pH than I used in my experiments suggest that, although I observed no changes in shell characteristics, shell integrity might have changed if *N. ostrina* veligers were exposed to higher concentrations of CO_2-acidified seawater and lower pH values. For example, Kurihara and colleagues (2008a) found high CO_2 of 2000 ppm (pH 7.4) interfered with larval shell synthesis in the mussel *M. galloprovincialis*. The differences in shell hardness and stiffness in the 'pre-CO_2' veligers compared to the other CO_2 treatments may have been due to the fact that these individuals were 2 weeks younger than the experimental veligers. Currently no data are available on age-specific differences in shell qualities of *Nucella* veligers.

Other physiological studies investigating the acute effects of long-term exposure of hypercapnia on bivalve calcification found significant shell dissolution to occur at pH <7.0 for *M. edulis* after 60 days (Bamber 1990) and chronic exposure (pH <7.5) in a closely related species *M. galloprovincialis* slowed down several processes, resulting in a lowered mussel metabolism and growth (Michaelidis et al. 2005b). A more recent study has shown that OA can also alter the physical characteristics of coral hard parts by significantly decreasing the internal saturation state of calcifying compartments when the saturation state of the external seawater

has decreased (Cohen et al. 2009). Cohen et al. (2009) study suggests that growth of calcifying organisms living under these environmental conditions may be inhibited due to a limited amount of energy available for proton pumping, which is required for calcification. These hypercapnia experiments provide insight into the physiological processes that might be impacted by OA.

Another possible explanation for the absence of detectable veliger shell dissolution in our experiment could be the mode of development and the protective nature of the capsule. Because the time spent in encapsulated development for *N. ostrina* embryos can be extensive, sometimes lasting 2-4 months (Morris et al. 1980), it is possible that the capsules (Pechenik 1984, Rawlings 1990) may provide protection against an environmental stressor, including the chemistry of acidified seawater. Egg capsules have been shown to protect some direct developers from osmotic shock and UV radiation (Pechenik 1982, 1983, Hawkins and Hutchinson 1988, Rawlings 1990). For example, late stage veligers (*N. ostrina*) can withstand up to 80% water loss from capsule chamber before suffering substantial mortality (Rawlings 1995). Very few OA studies with encapsulated larvae currently exist and the one other known to date found heart rates and shell morphology to be reduced in *Littorina obtusata* larvae when exposed to seawater with pH 7.6 (Ellis et al. 2009). Most other OA studies conducted on larval molluscs have focused on free-swimming planktotrophic development (Kurihara et al. 2007, Kurihara et al. 2008a). Larvae from broad-cast spawners spend their entire larval stage in the water column (e.g., mussels, oysters, clams and some gastropods) with little protection from adverse environmental perturbations. Therefore, it is possible that elevated CO_2

could be more detrimental to calcification for molluscs with free-swimming larvae as compared to those with encapsulated development. However, it is important to note that this is the first study that has used this biomaterials technique in an ecological context on an encapsulated developer and therefore we do not want to overgeneralize our results until more species have been studied.

2. Thermal tolerance

This study also examined whether a fundamental physiological characteristic for an intertidal invertebrate – tolerance of brief, high temperature stress - was compromised after a period of development at elevated CO_2. In this study, thermal tolerance as a measure of veliger sensitivity to acidified seawater only affected survival for those exposed for one week. High CO_2 treatment compromised *N. ostrina* veligers capacity to tolerate thermal exposure by as little as 10.5% and as much as 23% after one week. Few studies have examined the synergistic interaction of OA and temperature stress on developing larvae. However, O'Donnell et al. (2009a) showed a reduced expression of heat shock protein, *hsp70,* in larvae of the red sea urchin (*Stronglyocentrotus franciscanus*) when raised under elevated CO_2 conditions, suggesting that cellular mechanisms of tolerance of temperature stress may be compromised during development under OA conditions.

Studies of adult marine invertebrates have examined the interaction of OA stress and physiological tolerances. Wood and colleagues (2008) found increased calcification rates in response to OA in the brittle stars, *Amphiura filiformis*, and this was accompanied by a decrease in muscle mass. It has also been shown that

reproductive capacity can be compromised in copepods via a reduction in egg production in response to seawater pH 6.8 (Kurihara et al. 2004).

Little evidence thus far demonstrates whether organisms will have the capacity to compensate or adapt to the changes in seawater chemistry. In our experiment, veligers exposed to 2 weeks of High CO_2-acidified seawater began to show signs of compensation to thermal stress between 31.8 and 33 °C as evident from the increase in survival which continued through 34°C (Fig. 10B). These results suggest the possibility that an initial physiological response to OA could potentially be replaced by a set of different more long-term responses. It has already been documented that some organisms may have the capacity to regulate their local extracellular pH above their intracellular pH (Pörtner et al. 2004) as a means of achieving pH compensation with elevated CO_2. Species that are more tolerant will exhibit greater accumulation of bicarbonate; for example, the subtidal crab *Necora puber* exposed to extreme hypercapnia (~10,000ppmv) resulted in haemolymph bicarbonate concentrations more than four times the control levels (Spicer et al. 2007) and another crab (*Cancer magister*) fully compensated its haemolymph pH within 24 hours by accumulating more than 12mM bicarbonate (Pane and Barry 2007). A more long-term exposure of hypercapnia would need to be investigated in order to understand whether extracellular acid-base compensation could explain why High CO_2 treated *N. ostrina* veligers could tolerate thermal stress better than those veligers exposed to lower CO_2 concentrations.

Although this short-term experiment demonstrated subtle effects on thermal tolerance and none on shell integrity, chronic exposure to elevated CO_2 could result

in the accumulation of physiological changes in developing larvae. More experiments are needed to fully understand whether the compensation detected in our experiment would influence substantial downstream effects on larval development, growth and juvenile hatching. The impact of OA on an organism's response will be dependent upon its habitat, life cycle and evolutionary history (Fabry 2008, Andersson et al. 2008, Aronson et al. 2007, Dupont and Thorndyke 2009). In order for us to truly understand the impact OA has on the marine ecosystem and biodiversity, we need to focus our research priorities in understanding the processes and trade-offs that make up an organism's physiology and determine whether animals have the capacity to cope with these unprecedented changes.

IV. Impact of CO_2-acidified seawater on early life history stages of red abalone (*Haliotis rufescens*)

A. INTRODUCTION

In Chapter IV, I use larvae from the red abalone, *Haliotis rufescens*, an economically-important benthic herbivore (Gordon and Cook 2004) to investigate the physiological tolerance of marine molluscan larvae to CO_2-acidified seawater. Abalone, known for their tasty foot and treasured shell, were an exploited North American Pacific fishery with approximately 80,000 individuals taken annually prior to 1997 (Karpov et al. 2000). While adult red abalone inhabit the low intertidal to subtidal zone and have a broad distribution that spans from Oregon to Bahia de Tortuga in Baja California along the coast of North America (Morris et al. 1980), their young are free-swimming lecithotrophic larvae. Typically the larvae spend 6 to 14 days in the water column, depending upon ambient seawater temperature, at which point they metamorphose and settle (Strathmann 1987). It is during this time in the water column that the developing embryos likely are most susceptible to environmental stressors, such as ocean acidification, which could alter their development. Using predicted atmospheric CO_2 scenarios developed by the Intergovernmental Panel on Climate Change (IPCC, 2007) for the year 2100, I raised abalone larvae under different atmospheric CO_2 conditions and looked for evidence that survival was compromised in response to a thermal challenge. I also measured the expression levels and timing of expression of two genes (*engrailed* and *ap24*) involved in mollusc shell formation as larva begin to actively synthesize a $CaCO_3$

shell. Since the expression patterns of these two genes have not, to our knowledge, been documented for red abalone, I first established the expression under normal conditions and then compared this to expression under high CO_2 conditions. This study enabled us to measure the effects of CO_2-acidified seawater on survival of a thermal stress and gene regulation as a way to understand the physiological capacity of abalone larvae in a future climate change scenario.

When considering the biological consequences of ocean acidification, marine organisms that require $CaCO_3$ to build their hard-parts or skeletons could be impacted by ocean acidification. An accumulating body of research has now shown that marine organisms have variable responses to acidified seawater, but a clear pattern regarding whether resilience or tolerance has a pattern across taxa has not yet emerged. Recent work has begun to investigate the effects of elevated CO_2 on calcification in coccolithophorids (Riebesell et al. 2000, Zondervan et al. 2001), foraminifera (Spero et al. 1997, Barker and Elderfield 2002), pteropods (Feely et al 2004, Orr et al. 2005), echinoderms (Shirayama and Thornton 2005, Kurihara 2008, Clark et al. 2009, O'Donnell et al. 2009b), corals (Marubini and Thake 1999, Raven et al. 2005, Hoegh-Guldberg et al. 2007), coralline algae (Kuffner et al. 2008) and molluscs (Bibby et al. 2007, Gazeau et al. 2007, Kurihara et al. 2008a). In nearly every case, elevated CO_2 was found to negatively impact calcification, indicating that high CO_2 has the potential to impact not only individual organisms, but could change the way the entire marine ecosystems function (Hoegh-Guldberg 2005, Guinotte and Fabry 2008, Hall-Spencer et al. 2008, Przeslawski et al. 2008, Wootton et al. 2008).

Benthic invertebrates, including molluscs, can play an important role in the marine community as ecosystem engineers, as food sources for other animals (Nagarajan et al. 2006), and are major carbonate producers. Gutiérrez and colleagues (2003) found benthic marine molluscs produce 50-1000g $CaCO_3$ $m^{-2}yr^{-1}$, with oysters producing the most (90,000g $m^{-2}yr^{-1}$). Furthermore, molluscs can have huge economic importance. In 2007, molluscs harvested commercially contributed 19%, or $748 million, of the $3.8 billion US annual domestic ex-vessel revenue (Andrews et al. 2008). Estimates suggest the revenue losses for mollusc fisheries could be substantial ($0.6-2.6 billion) by the year 2060 if atmospheric CO_2 continues to rise (Cooley and Doney 2009). Thus, due to their economic importance, marine molluscs are a key group to investigate with regard to the impacts of ocean acidification.

One of the biological processes that may be impacted by OA is biomineralization, including shell calcification (Lowenstam and Weiner 1989, Simkiss and Wilbur 1989). Different species use a variety of mineral forms, including aragonite, calcite, high-magnesium calcite, amorphous $CaCO_3$ or a mixture of these phases (Fabry et al. 2008). Molluscs tend to use aragonite (Eyster 1986), a more soluble mineral of $CaCO_3$. Bivalves and gastropods generally exert low amounts of control over biomineralization, requiring passive and active ion movement along a calcification compartment isolated from ambient seawater (Weiner and Dove 2003). Therefore, these organisms depend upon sufficient CO_3^{2-} concentrations to successfully build shells. Many studies have investigated the mechanical properties and characterization of shell formation in molluscs (Kniprath 1981, Jacobs et al. 2000, Sudo et al. 1997, Weiss et al. 2000, Hattan et al. 2001, Lin

et al. 2006, etc.) but more research is needed to understand the physiological processes behind biomineralization to assess the sensitivity of these organisms to ocean acidification.

Organisms regulate a number of cellular processes during stressful events (e.g., cell cycle, protein induction, metabolism). Specific gene regulation can be one of the most rapid and sensitive responses. Using molecular tools (e.g, quantitative real-time PCR) we can quantify an organism's response to an environmental stressor, such as ocean acidification, and potentially uncover the physiological mechanism behind its impacts on calcification. Currently, there are a suite of proteins known to be required for biomineralization in molluscs (e.g., MSP1 in scallops, Sarashina and Endo 2001; MSI 60 in oysters, Sudo et al. 1997; Mucoperlin 2 in mussels, Marin et al. 2000; nacrein in turban snail, Miyamoto et al. 2003, etc). Another example is *engrailed*, a transcription factor that regulates expression of genes encoding the proteins responsible for mineralizing plates along the shell plate, shell gland and shell field in molluscs (Wilt et al. 2003). While expressed during many stages and not 'specific' to biomineralization, *engrailed* is clearly involved in regulating larval shell formation of chiton trochophores (*Lepidochitona caverna*, Jacobs et al. 2000) and scaphopods (*Antalis entails*, Wanninger and Haszprunar 2001). Another gene (*ap24*) encodes the aragonite protein 24kDa, a class of shell matrix proteins that has a calcite-binding domain and is found in the aragonite-containing nacre layer of abalone (Michenfelder et al. 2003, Wustman 2004). Assessing the expression pattern of genes responsible for shell formation under

normal atmospheric conditions compared to predicted ocean acidification conditions may be a way to assess the potential impacts of climate change.

While understanding the molecular mechanisms behind biomineralization is important to the calcification of marine organisms, it is also imperative to gain insight into the physiological consequences associated with these mechanisms. Some studies have found no net change (Langer et al. 2006) or an increase (Iglesias-Rodríguez et al. 2008) in calcification under conditions of ocean acidification, but at a 'cost'. Wood et al. (2008) found that the brittle star, *Amphiura filiformis*, increased calcification and metabolism when exposed to reduced pH, but lost muscle mass as a consequence. In another study, larvae from the red sea urchin (*Strongylocentrotus franciscanus*) raised under elevated CO_2 conditions and then subjected to a temperature shift revealed a reduced and delayed response in transcript level of *hsp70*, a gene involved in the response to acute environmental thermal stress, suggesting physiological response may be impaired at the molecular level due to CO_2 exposure (O'Donnell et al. 2009a). These results suggest that marine organisms may have the capacity to maintain protective mechanisms against ocean acidification, but could reduce an organism's ability to tolerate additional stressors (e.g., thermal tolerance, hypoxia, salinity). Climate change scenarios predict that sea surface temperatures will increase by 2-4 °C (IPCC 2007) and hypoxic 'dead zones' will likely also increase (Diaz and Rosenberg 2008), necessitating a need for making predictions for an organism's physiological tolerance to ocean acidification in a multi-stressor environment. Understanding if an organism has the ability to tolerate other stressors after being exposed to elevated CO_2 is important to investigate as

well as determining the 'costs' associated with those physiological process. In this study, I examined how development under conditions of ocean acidification might alter the ability of abalone larva to tolerate high temperature stress.

B. MATERIALS AND METHODS

1. Spawning of abalone adults

Adult red abalone (*Haliotis rufescens*) used for spawning in this study were generously provided by a local abalone aquaculture facility (The Cultured Abalone Inc., Goleta, CA). Two separate spawning events involving 4 males and 4 females during 2008 (June and September) were carried out at the abalone facility, resulting in 8 replicate cultures of larvae. Naturally, spawning tends to occur in the spring and summer, but this species has been found to spawn at any time of the year (Morris et al. 1980). Adults were maintained in unfiltered flowing seawater at approximately 15 °C at the abalone facility. Individuals of similar age class were chosen (4 yrs old; size range, females were ~12.5 cm in length and males were 8.9-10.2 cm) for spawning to eliminate age bias. At each spawning event, 1 male and 1 female were placed in individual containers with enough water to cover them. The pH of the water was first increased to 9.1 by adding 6.6 ml of 2 M Tris-(hydroxy-methylamino) methane (Tris-base, M.W. = 121.1) for each liter of water in the container. Reagent grade hydrogen peroxide (30%) was diluted to make a 6% working solution. Fifteen minutes after adding the Tris, 3ml of freshly prepared 6% hydrogen peroxide solution for every liter of water was added in the container. Water in the container was then thoroughly stirred to mix the solution. The adults were exposed to this solution for 2-2.5 h and then the water was removed. The container was rinsed thoroughly with clean seawater and adults were placed back

into container with fresh seawater. Upon the release of gametes (2-3.5 h), eggs from the each female were separately fertilized by sperm from the male spawned at the same time. Only batches of eggs with a fertilization rate of 95% were used in the experiment to ensure synchronous and normal development.

2. Experimental set-up of CO_2 system

A CO_2 incubation system was designed to raise larvae under 3 different concentrations of CO_2 that were chosen to span the Special Report Emission Scenarios (SRES) predicted by the Intergovernmental Panel on Climate Change (IPCC, 2007) for the year 2100; present day atmospheric CO_2 levels (~ 380 parts per million, ppm, CO_2), "most conservative" scenario (B1, ~ 570ppm CO_2) and the "business as usual" scenario (A1FI, ~ 990ppm CO_2). This system pulls ambient air from the atmosphere and pumps it through a DRIERITE®-containing cylinder where moisture was removed and scrubbed of carbon dioxide using soda sorb. The air was then passed through a 0.22μm filter to remove particulates. Next this CO_2-free air was blended with pure CO_2 using 2 mass-flow gas valve controllers (Sierra® Instruments) for each desired pCO_2 concentration. Each gas mixture was delivered to a reactor buckets at 1000mL min^{-1} (Mazzei® Injectors) and mixed with in-coming filtered seawater (0.22μm). Once CO_2 saturation was reached in the reactor bucket, the CO_2-acidified water was delivered to the four replicate culture buckets. We continuously monitored the CO_2 concentration of the gas delivered to each reactor bucket using an infrared CO_2 analyzer (Qubit) allowing us to make adjustments to CO_2 delivery if necessary. These gas mixing regimes resulted in seawater with the

following pHs; the Control CO_2 treatment (380ppm CO_2) varied from 8.04 to 8.07, the Moderate CO_2 treatment (570ppm CO_2) yielded pH values from 8.00 to 7.94 during the experiment, whereas that of High CO_2 treatment (990ppm CO_2) ranged from pH 7.91 to 7.84. Temperature (Omega HH81), salinity (Vista A366ATC) and pH (Myron L. Company, Ultrameter^{TM} II) were continuously monitored throughout the 6d experiment (Table 6 and Figure 11).

3. Culture bucket design

Larvae were raised in elevated CO_2 concentrations using a 15 l nested culture chamber design (see Appendix A). The inner bucket contained twelve 3 inch-diameter holes sealed with 64μm mesh. Each bucket pair was fitted with an external PVC side arm connecting the outer bucket (5 l) to the inner bucket (10 l) with a short pipe along one side of the inner bucket. The side-arm provided a location to aerate the bucket with gas (200 ml min^{-1}) and generated a gently mixing current for the egg capsules within the inner bucket. The culture buckets were filled with equilibrated CO_2 seawater that was replaced at a constant rate of 1.2 l h^{-1} to provide fresh seawater without altering the pH. All culture buckets were gently mixed with a paddle and 12-v motor. Experiments did not begin until the pH of all reactor and culture buckets were stable for at least 24 h.

4. Sampling larval cultures

Immediately upon fertilization, eggs were transported to the Marine Science Institute at the UC Santa Barbara where they were counted and divided evenly

among the three experimental CO_2 treatments for each female (24 cultures total). The experimental procedure was repeated twice with 4 parent pairs at each time resulting in 12 cultures per repeat. Fertilization batches were staggered by 1.5 h to allow us to monitor developmental timing and sample larvae based on their stage and not based on their time post-fertilization (pf). Larval cultures were raised at a constant seawater temperature of 15.15°C ± 0.5 for all CO_2 treatments. Development in each of the 12 cultures was assessed at20 h pf, 28 h pf, pre-torsion (~ 35h pf), 48 h pf, post-torsion (~ 58 h pf), 74 h pf, late veliger (~84 h pf) and pre-metamorphic (~ 120 h pf). Each of these developmental stages was chosen based on key morphological features. What is termed the 'pre-torsion' stage was identified by the formation of the prototrochical girdle and was chosen for sample collection because this is the point in development at which the larval shell begins to form. After undergoing torsion, larvae were collected at the 'post-torsion' stage which was identified by two definitive characteristics: (1) a twisting of their visceral mass inside the shell, and (2) the appearance of a defined foot. 'Late-veliger' larvae were sampled when they had distinct eye spots and an operculum. The latest stage 'pre-metamorphic' was defined by the presence of cephalic tentacles, a sign that the larvae are competent and ready to settle. Although the timing of development varied slightly (within 1.5 h) between cultures from the 4 different females, there were no visible differences in developmental timing in the CO_2 treatments as compared to controls during the course of this experiment. Because abalone larvae are lecithotrophic, larvae were not fed while conducting the 6 day experiment. Notably,

the duration of the experiment was intended to run right up to the point when the larvae were competent to settle and metamorphose.

When larvae had reached each of the desired stages (pre-torsion, post-torsion, late-veliger and pre-metamorphic), one sample of larvae was removed from each of the twelve culture chambers to measure thermal tolerance and another was taken for later analysis of gene expression using quantitative PCR. Those larvae sampled for gene expression analysis were quickly pelleted by centrifugation for 5 seconds to remove excess seawater and 1mL of TRIzol® Reagent (Invitrogen, Calrsbad, CA, USA) was added. To ensure thorough mixing, the samples were quickly vortexed and frozen at -80 °C for RNA extractions at a later time.

5. Thermal tolerance assays with CO_2 exposures

To determine if CO_2-acidified seawater affected larval abalone thermal tolerance, one sample of larvae (2700 total larvae) was removed from each of the twelve culture buckets when the desired developmental stage (pre-torsion, post-torsion, late-veliger and pre-metamorphic) was reached. The larvae were then divided equally among nine vials (each 3 ml total volume) and exposed to a range of temperatures (prior to heat shock, 15.5, 19.4, 23.6, 26.2, 29, 32, 33.8 and 35.4 °C). After a 1 h heat stress, 100 larvae from each temperature vial were pipetted onto a microscope slide for determination of survivorship. Survival was assessed based on ciliary movement around their velum. These thermal tolerance assays were performed on all male x female offspring (n=8), for all CO_2 treatments (n=3) and on all developmental stages (n=4). Our choice of acute stress temperatures was not

meant to indicate an expectation that seawater will rise to these temperatures, but was chosen to provide a gradient of stress.

6. RNA extraction and reverse transcription

Total RNA was extracted from approximately 4000 larvae from each CO_2 treatment and from each male/female pair (24 samples total). Frozen TRIzol® samples were thawed on ice and larvae were ruptured by quickly passing them 3 times through a 21 gauge needle followed by a 23 gauge and then a final pass with a 25 gauge needle. Total RNA was extracted using the guanidine isothiocyanate method outlined by Chomczynski and Sacchi (1987). Pellets were resuspended in 30µl of nuclease-free water. RNA was quantified spectrophotometrically using a ND-1000 UV/ visible spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and electrophoresed on a 1% w/v agarose gel to verify RNA integrity. RNA was stored at -80 °C. First strand cDNA was synthesized from 600ηg total RNA using oligo (dT_{18}) primer and ImProm-II™ reverse transcriptase following the manufacturer's instructions (Promega, Madison WI, USA).

7. Isolation and sequencing of candidate genes from abalone biomineralization genes

Partial sequences for *engrailed* were obtained using primers designed from conserved regions of *Haliotis asinina engrailed* (Accession No. DQ298403). The forward primer was 5'-GAC AGA GCA CGG TGG GTT AT-3' and the reverse primer was 5'- CGG CAA TCA TCA AAC TCC TT-3'. Primers were designed with

the assistance of Primer3 software (Rozen and Skaletsky 2000, http://fokker.wi.mit.edu/primer3/input-040.htm).

Polymerase chain reactions (PCRs) were carried out in a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules CA, USA) using 1.25U Taq DNA polymerase (New England Biolabs®, Ipswich MA, USA) and isolated larval cDNA. PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide and bands of appropriate size were extracted from the gels using the QIAquick® gel extraction kit (Qiagen Inc., Valencia CA, USA). The extracted PCR product was ligated into a T-vector (pGEM®-T easy; Promega), transformed into heat shock competent *Escherichia coli* (strain JM109; Promega) and colonies were grown on ampicillin LB-agar plates. Several colonies containing the ligated PCR product were selected and grown overnight in LB bacterial growth medium. Plasmids were isolated from the liquid culture using GenElute^{TM} Plasmid Miniprep kit (Sigma-Aldrich®, St. Louis MO, USA) and sequenced at the University of California Berkeley sequencing facility (http://mcb.berkeley.edu/barker/dnaseq/index.html). The sequence for *engrailed* in *H. rufescens* was deposited into GenBank (GQ245982).

8. Quantitative real-time PCR analysis of gene expression

Characterization of biomineralization genes in abalone larvae was analyzed using quantitative real-time PCR (qPCR) from the relative levels of mRNA for *engrailed*, *ap24* (Accession No. AF225915) and elongation factor 1-alpha (*EF1α*, Accession No. DQ087488) genes on an iCycler Thermal Cycler (Bio-Rad).

Gene specific primers were designed using Primer Express software (version 2.0.0; Applied Biosystems Inc., Foster City, CA, USA). Primer sequences were as follows: *engrailed* forward 5'-TTC TTC GTC TTC CTG GAT CGA-3', *engrailed* reverse 5'-CTG CTT GGG TAT ACT GCA CAA GAT-3', *ap24* forward 5'-GTC GTC GAG GAA TGT AAC ACT AAG G-3', *ap24* reverse 5'-GAG ATT TGC CGG CTG TTG A-3', *EF1α* forward 5'-GGA GGG TCA AAC CCG AGA AC-3' and *EF1α* reverse 5'-CCG ATG ATG AGT TGC TTC ACA-3'.

Quantitative real-time PCR reactions were performed with 1μL cDNA, 4ρmoles of each primer and 2X SYBR Green Master Mix (Bio-Rad) to a total volume of 20μL. All qRT-PCR reactions were run as follows: 1 cycle of 95°C for 1 min; 95°C for 20 sec and 55°C for 20 sec for 45 cycles; 1 cycle of 95°C for 1 min; 1 cycle of 55°C for 1 min. At the end of each PCR reaction, PCR products were subjected to a melt curve analysis to confirm the presence of a single amplicon.

9. Statistical analyses

Each developmental stage was analyzed separately to evaluate the effects of CO_2 treatment on survivorship across all temperatures using status of individuals (alive/dead) in a likelihood of ratio tests (JMP 8.0, SAS Institute Inc.). Post-hoc comparisons using Fisher's exact test (i.e., permutation tests) were performed among main factors if significant differences were detected. False discovery rate (FDR) was run to correct for multiple comparisons and to estimate the expected proportion of false positives among all significant hypotheses.

To quantify *engrailed*, *ap24* and *EF1α* mRNA expression, one control cDNA sample was used to develop a standard curve for all primer sets relating threshold cycle to cDNA amount and this standard curve was run on each plate. All results were expressed relative to these standard curves and mRNA values were normalized relative to *EF1α*. *EF1α* is a commonly used control gene in qPCR (Fangue et al. 2006, O'Donnell et al. 2009a) and specifically did not change in response to elevated CO_2 levels nor developmental stage (Figure 12), making it an appropriate internal control gene for this study. The mRNA expression for each biomineralization gene was analyzed using a two-way ANOVA with developmental stage and CO_2 treatments as factors. Data were transformed, when necessary, to meet assumptions of ANOVA using a Box-Cox transformation (1964). Post-hoc comparisons using Tukey-Kramer HSD tests were performed among group means if significant differences were detected.

Table 6: Recorded values for treatment buckets for the 6 day experiment

Culture Buckets	CO_2 concentration	Mean pH (± SD)	Mean Temperature (°C)	Mean salinity (‰)
Control CO_2	~380ppm	8.05 (± 0.01)	15.2 (± 0.3)	33.0
Moderate CO_2	~ 570ppm	7.97 (± 0.02)	15.2 (± 0.4)	33.0
High CO_2	~ 990ppm	7.87 (± 0.01)	15.1 (± 0.3)	33.0

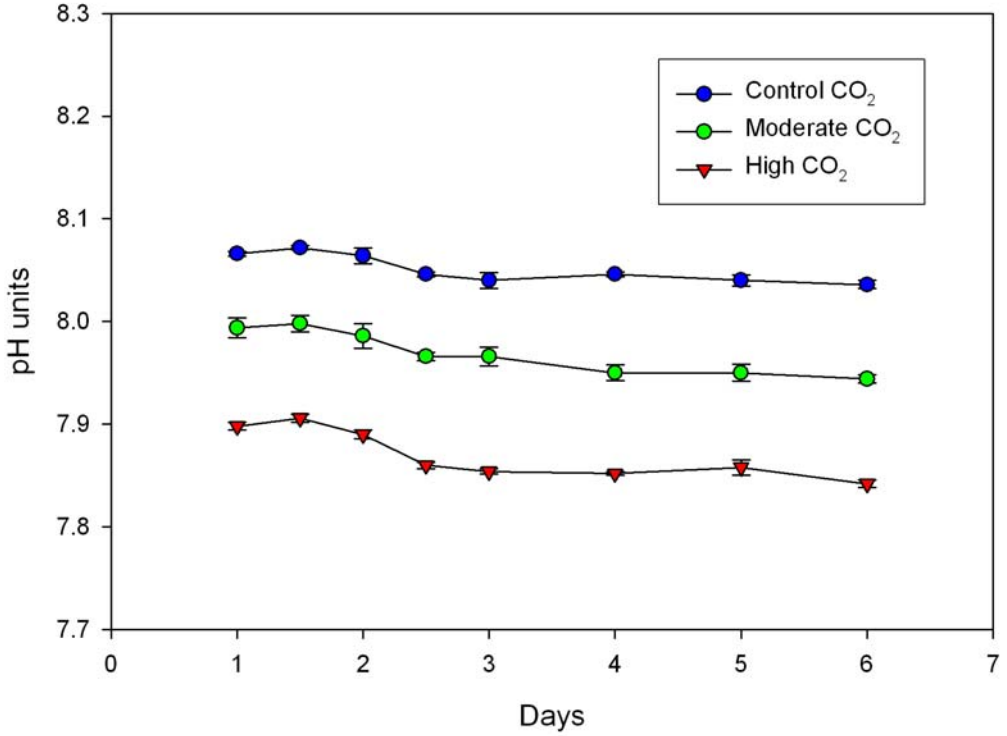

Figure 11: Measurements of the CO_2-acidified seawater for the 6 day experiment with abalone larvae. Mean pH values are plotted for the three treatments (culture and reactor buckets). Error bars = SD.

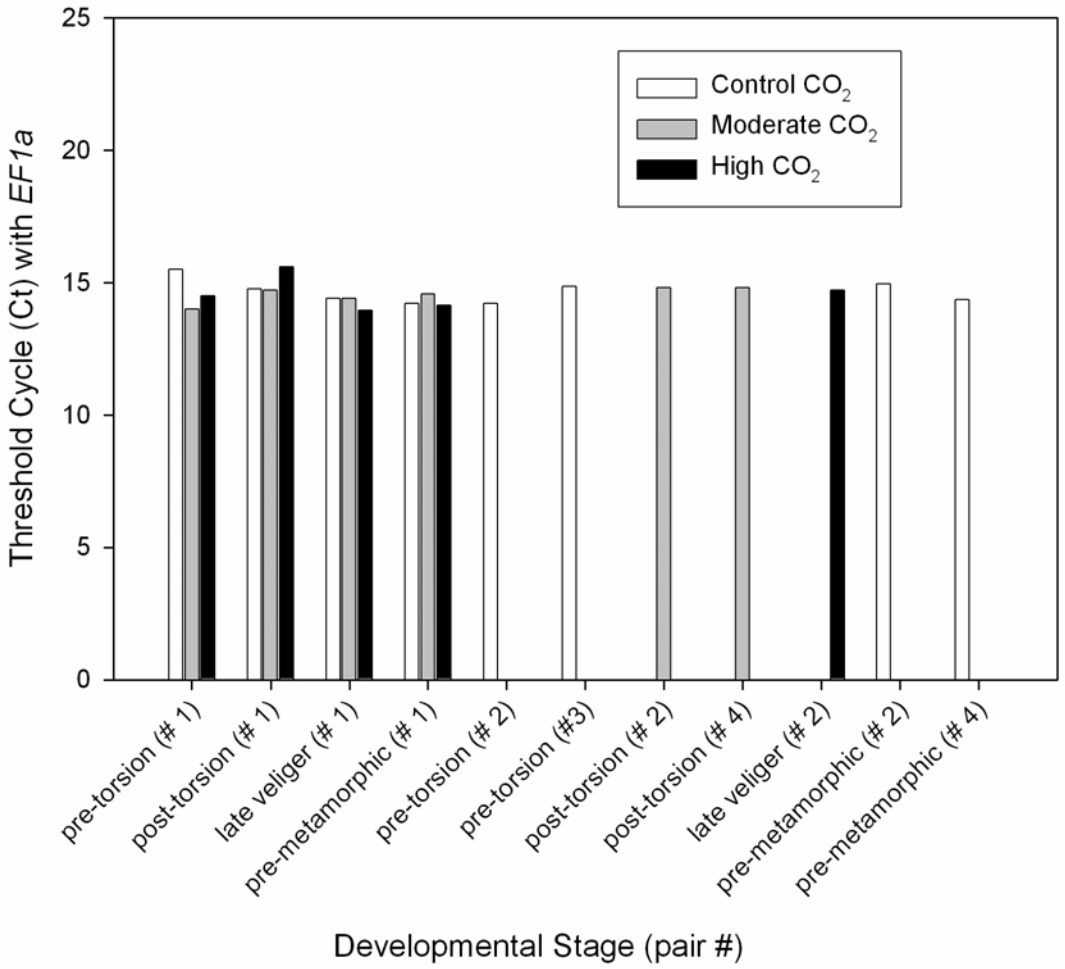

Figure 12: Threshold cycle for reference gene, *EF1α*, with mRNA from abalone larvae. The *x-axis* illustrates different developmental stages and larvae from a single set of male/female pair. Each bar represents larvae from that particular CO_2 treatment and stage.

C. RESULTS

1. Larval thermal tolerance after development in CO_2-acidified seawater

Thermal tolerance performance curves were generated to measure abalone survival over a range of temperatures. Such performance curves enabled us to examine the effect of a thermal challenge on survivorship of early developmental stages of *H. rufescens* in response to CO_2-driven seawater acidification. For all stages, larval survivorship was less influenced by CO_2-acidified seawater and more affected by temperature exposure (Figure 13 A-D). Across all temperatures tested, survivorship of pre-torsion larvae (~ 35 h pf), the youngest stage, varied with both temperature and CO_2 (Figure 13A; likelihood ratio test: temperature x CO_2, $p<0.001$). Veliger survivorship was as high as 93% for those raised under Control and Moderate CO_2 treatments at 29 °C but was only 78% for those raised under High CO_2. Analyzing just those exposure temperatures at which survivorship varied (19.4, 23.6, 26.2, 29 and 32 °C), temperature and CO_2 treatment influenced survival with a significant interaction (temperature x CO_2, $p <0.001$; temperature, $p <0.0001$; CO_2, $p=0.0267$). Multiple comparisons revealed that survivorship differed by at least 11% for exposure temperatures of 29 °C ($p<0.0001$) and 32 °C ($p<0.0001$). Further analyses demonstrated that the High CO_2 treatment significantly affected survivorship at 29 °C ($p=0.0011$), but not at 32 °C ($p=0.1867$). Thus, the High CO_2 treatment caused a significant reduction of survival by 18% when compared to the Control CO_2 treatment ($p=0.0062$) and a reduction of 13% when compared to the Moderate CO_2 treatment ($p=0.0183$, Figure 13A). Survival decreased abruptly

between 32 to 33.8 °C, but temperature ($p<0.001$), not the CO_2 treatment ($p=0.8826$) was the driver of this result.

The post-torsion larvae (~58 h pf) exhibited a similar performance curve. However, the onset of mortality was more gradual for this stage of development than was observed for the younger, pre-torsion stage (Figure 13B). Analysis of only those exposure temperatures where survivorship (19.4, 23.6, 26.2, 29 and 32°C) differed indicates that post-torsion larvae were affected only by temperature and not CO_2 treatment (likelihood ratio test: temperature x CO_2, $p=0.1949$; temperature, $p<0.0001$; CO_2, $p=0.2691$). The percentage of survival decreased as temperature increased with a pattern of 23.6>26.2>29>32 °C (Figure 13B). Hence, at the highest temperature where survival differences were calculated (32°C), survivorship fell below 65% while at 19.4°C the percentage of surviving individuals was more than 97% across all CO_2 treatments.

Survival of late veliger abalone larvae (~84 h pf) raised under CO_2-acidified seawater was affected by the interaction of exposure temperature and CO_2 treatment (Figure 13C; likelihood ratio test: temperature x CO_2, $p = 0.0020$; temperature, $p<0.0001$; CO_2, $p=0.0019$). The temperature at which the veligers were exposed to influenced survival in the High CO_2 treatment. Exposure temperatures of 23.6, 26.2, 29, and 32 °C significantly reduced late veliger survivorship by 87.5% to 17% for all CO_2 treatments ($p<0.001$). Multiple comparisons indicate that the High CO_2 treatment significantly decreased survival compared to the Control and Moderate CO_2 treatments for 3 of the 4 temperatures (Table 7). Thus, both exposure

temperature and CO_2 treatment can affect veliger survival, but High CO_2 affects survival only at certain elevated temperatures (23.6, 26.2 and 32 °C).

Temperature significantly reduced survivorship of pre-metamorphic (~120 h pf) abalone larvae raised under CO_2-acidified seawater, but CO_2 treatment did not alter this pattern of thermal tolerance (Figure 13D; likelihood ratio test: temperature x CO_2, $p = 0.0913$; temperature, $p < 0.0001$; CO_2, $p = 0.3041$). The highest exposure temperature with survivors (32 °C) had 56% survivorship compared to 23.6°C where survival was greater than 87% (Figure 13D). Thus, temperature had a significant effect on survivorship at these exposure temperatures, regardless of CO_2 treatment. Direct comparisons across developmental stages were not analyzed because temperature, and not CO_2 treatment, appeared to be the main abiotic driver in regulating survivorship for this larval stage.

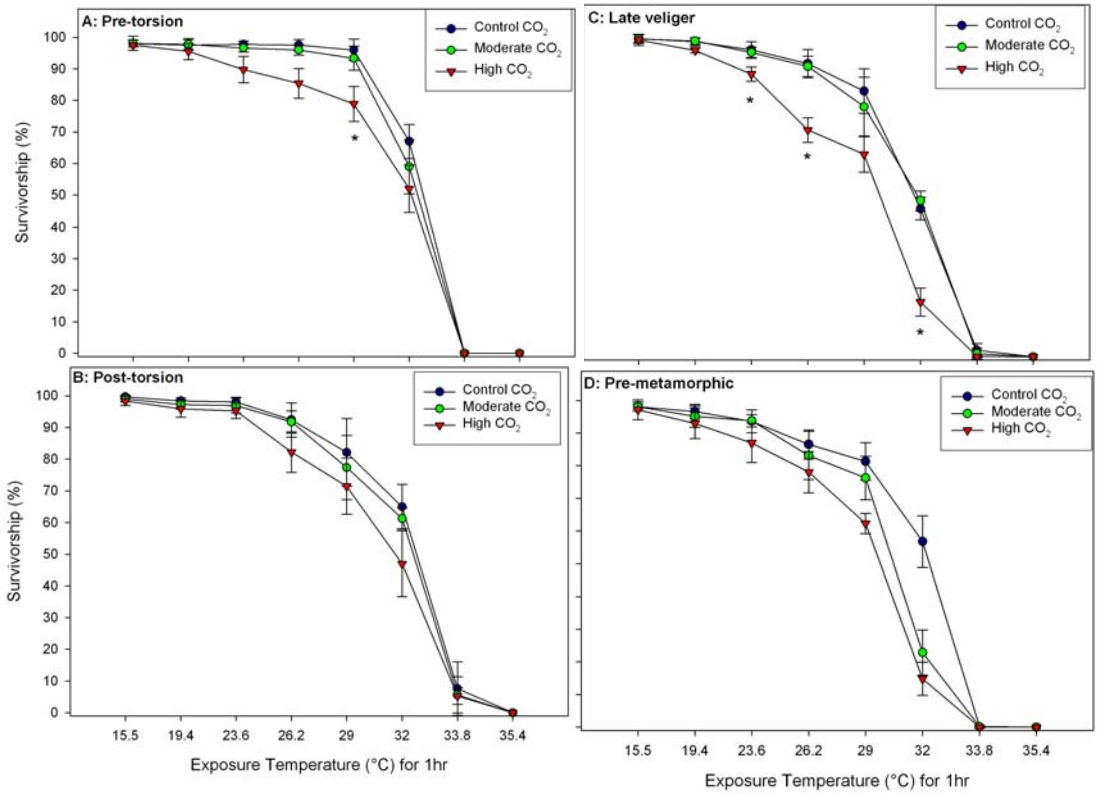

Figure 13: Thermal tolerance, presented as percent survivorship, of *Haliotis rufescens* larval stages (A) pre-torsion (~35 h pf), (B) post-torsion (~58 h pf), (C) late veliger (~84 h pf) and (D) pre-metamorphic (~120 h pf) raised under elevated CO_2 concentrations. Larvae from eight females were assessed for survivorship at each exposure temperature and CO_2 treatment (n=100 per female). Error bars = standard deviation and asterisk (*) indicates significant pairwise differences in survival for the High CO_2 treatment at a given temperature.

Table 7: Multiple comparisons using Fisher's exact test indicated survival differences between CO_2 treatments for specific exposure temperatures for late veliger stage abalone.

Exposure Temperature	CO_2 treatment comparison	p-value
23.6 °C	Control vs Moderate	0.5796
	Moderate vs High	<0.0001
	Control vs High	<0.0001
26.2 °C	Control vs Moderate	0.6122
	Moderate vs High	<0.0001
	Control vs High	<0.0001
29 °C	Control vs Moderate	0.1550
	Moderate vs High	0.1836
	Control vs High	0.0816
32 °C	Control vs Moderate	0.3673
	Moderate vs High	<0.0001
	Control vs High	<0.0001

2. Expression of Biomineralization Genes

In order to assess a molecular response of early life history stages of *H. rufescens* to CO_2-acidified seawater, mRNA levels of two biomineralization genes, *ap24* and *engrailed*, were measured. While transcript for *ap24* was found across all developmental stages, pre-torsion and post-torsion stages showed significantly higher *ap24* expression as compared to other, older stages (Figure 14A; 2-way ANOVA, $F=62.138$, $p<0.0001$). Abalone in the youngest stage had approximately 15-fold higher levels of *ap24* mRNA as compared to abalone in the post-torsion stage; the levels of *ap24* mRNA were 30-fold higher when compared to the later stages (late veliger and pre-metamorphic, Figure 14A). In contrast to the decrease of *ap24* expression as development progressed, all larval stages exhibited similar expression profiles to one another for the *engrailed* gene (Figure 14B). The oldest, pre-metamorphic, larvae appeared to have the lowest level of *engrailed* mRNA as compared to the other stages. However, this difference was not statistically significant (two-way ANOVA: stage; $F=2.715$, $p=0.0537$).

Over the course of the 6 day experiment, the expression pattern of neither gene responded to CO_2 treatment (*ap24*, Figure 14A, two-way ANOVA: $F=0.5824$, $p=0.5621$; *engrailed*, Figure 14B, two-way ANOVA: $F=0.1813$, $p=0.8347$). In addition, there was no significant interaction between stage and CO_2 treatment (*ap24*, 2-way ANOVA, $F=0.3964$, $p=0.8782$; *engrailed*, 2-way ANOVA, $F=0.7342$, $p=0.6243$).

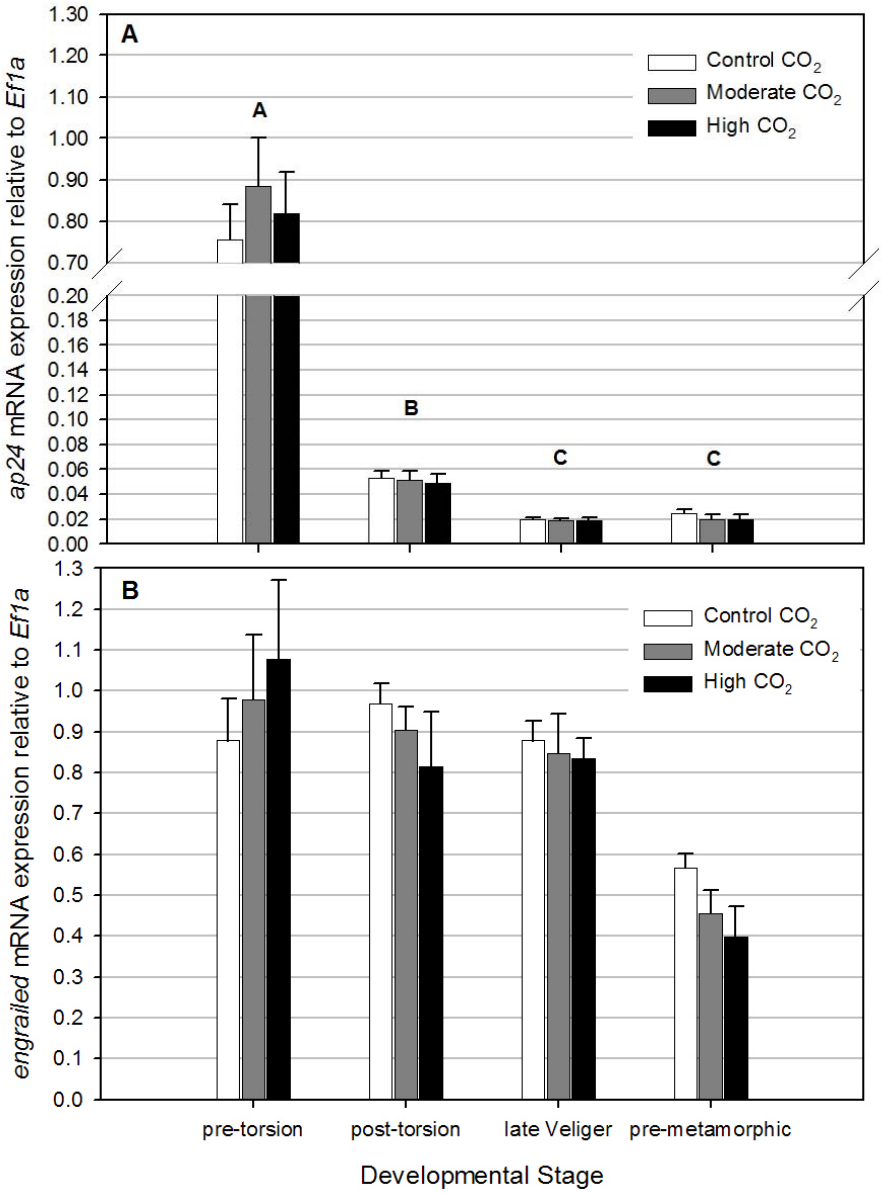

Figure 14: Relative levels of mRNA expression of (A) *ap24* and (B) *engrailed* for four developmental stages of *Haliotis rufescens* raised under three CO_2 treatments. Error bars represent standard error of the mean; larvae from 8 male/females pairs were examined. CO_2 treatment had no effect on gene expression at any developmental stage. Different letters correspond to the differences in gene expression among developmental stage only.

D. DISCUSSION AND CONCLUSION

The overall goal of this study was to determine if CO_2-acidified seawater would influence the ability of abalone larvae to tolerate thermal stress, and further, how this might affect their ability to regulate genes involved in biomineralization. The study had two major outcomes: (1) development under CO_2-acidified seawater influenced larval survivorship following a brief thermal stress, and (2) the expression levels of two biomineralization genes were not affected by CO_2 treatment for any of the larval stages, although there were stage specific differences in expression patterns.

In these studies, I found that the thermal tolerance capacity of red abalone larvae was altered by development under CO_2-acidified seawater. At the pre-torsion stage, survival of larvae developing under High CO_2 treatment was significantly lower at 29 °C, but was not altered at any other exposure temperature. A similar trend was seen for the late-veliger stage where survival decreased significantly under High CO_2 for specific temperatures (23.6, 26.2 and 32 °C). The difference in survivorship across development suggests that specific larval stages maybe more sensitive than others under elevated CO_2, and within a given stage, the effect CO_2 has on survival is not consistent. The pre-torsion stage could be more vulnerable to elevated CO_2 because these larvae are about to embark on a major developmental transition and undergo torsion, therefore making them more at risk to environmental perturbations. The same could be true for late veliger stage larvae; this stage may be more susceptible to elevated CO_2 because they are getting ready to metamorphose

and settle. Similar conclusions were found in a study with *Mytilus galloprovincialis* mussel larvae that were raised under CO_2-seawater at pH 7.43 (Kurihara et al. 2008a). This investigation found exposure to elevated CO_2 did not affect development until the trochophore stage where 70% of the CO_2-seawater larvae remained at this stage, suggesting this stage may be more sensitive to elevated CO_2 (Kurihara et al. 2008a).

These experiments also showed developing *H. rufescens* larvae can physiological tolerate short-term acute thermal stress well above ambient environmental temperatures even when exposed to other stressors (e.g., CO_2-acidified seawater). For all developmental stages, regardless of CO_2 treatment, at least 62% of the abalone larvae survived a thermal stress of 29 °C and 70% survived temperatures that were ~ 3 °C cooler. Thus, abalone larvae might have the physiological capacity to withstand brief contact with warm waters which could be encountered with sea surface temperatures predicted to rise (2 -4 °C, IPCC 2007). However, how larvae will tolerate the simultaneous effect of ocean acidification and temperature remains open. If temperatures increase beyond an organisms' thermal adaptive range it is likely early developmental mechanisms (e.g. fertilization, cleavage and gastrulation) could be impaired, never allowing larvae to reach the calcifying stages.

This study also examined expression changes in two genes associated with calcification during development under conditions of ocean acidification, *ap24* and *engrailed*. Levels of mRNA transcript for two genes involved in the regulation of biomineralization did not change in response to abalone larvae raised under

Moderate and High CO_2 treatments (pH differences of 0.8 and 0.18, respectively, when compared to the Control CO_2 treatment). The only differences observed were across developmental stages for the *ap24* gene. The peak expression of *ap24* in the earliest larval stage, pre-torsion, could be due to the fact that larvae are beginning to synthesize their shell during this time and allocation of mRNA transcript increased as a result. It is important to note that CO_2 did not appear to hinder larval shell synthesis. Although not quantified, no abnormalities in shell formation were observed between CO_2 treatments or developmental stages.

No difference in gene expression for *engrailed* was found for CO_2 treatment or developmental stage. It may not be too surprising that *engrailed* mRNA levels held steady over developmental time, since this gene product is expressed in many cells and likely has multiple functions during larval stages. For instance, expression of *engrailed* was observed around the border of a developing larval shell of a marine clam embryo (*Transennella tantilla*, Jacobs et al. 2000) whereas in a trochophore scaphopod larvae it was expressed in cells secreting shell located close to the edge of the mantle (Wanninger and Haszprunar 2001). These expression data are consistent with other results that have shown differential changes in biomineralization genes for developing purple sea urchin larvae (*Strongylocentrotus purpuratus*, Wilt 1999, Livingston et al. 2006, Oliveri et al. 2008). However, contrary to our results, a recent study found down-regulation of a suite of genes involved in spicule formation and skeletogenesis for urchin larvae (*S. purpuratus*) raised under high CO_2-acidified seawater (Todgham and Hofmann 2009). While the regulatory networks, and those genes involved, are different between sea urchins and gastropods, important

considerations need to be made when making predictions about taxa or phylum responses to ocean acidification. Each organism likely will have a varying degree of sensitivity and capacity as they attempt to physiologically cope with elevated CO_2. Therefore, since little is known about the gene regulatory pathway for shell formation in abalone, we cannot definitively say that ocean acidification will not jeopardize the expression of biomineralization genes in abalone.

Without precise measurements and an understanding of mechanical properties we cannot be certain if the CO_2 treatments had an effect on larval shell formation, even though the two genes in this study appeared to have 'normal' expression. In other words, there may be other genes or gene products that would be better biomarkers for identifying changes in shell formation under stressful environments. With the application of genome- wide approaches beginning to be developed for tropical abalone larvae (see Williams et al. 2009), we may soon have a better understanding of how ocean acidification can influence biomineralization by identifying important gene regulatory networks. Nonetheless, this was the first attempt at investigating the mRNA expression of *ap24* and *engrailed* in red abalone larvae and while the result has left us with more questions, it provides another avenue by which to pursue information regarding larval shell formation.

Within the experimental parameters of this study, we have demonstrated that red abalone larvae appear to have two windows in development - pre-torsion and late veliger stages - that were sensitive to ocean acidification; however, it is not yet known what cost this will have on organisms as they attempt to maintain such processes (e.g., calcifying shells) through development. It is possible that other

physiological mechanisms could be compromised or threatened and likely these responses might be species-specific (Fabry et al. 2008, Doney et al. 2009b). For example, a reduction in growth and metabolic depression was evident in the marine mussel, *Mytilus galloprovincialis*, when they were incubated in hypercapnia water of pH 7.3 (Michaelidis et al. 2005b). Although typical hypercapnia experiments tend to compare CO_2 concentrations higher than those predicted by IPCC scenarios (pCO_2 = 10,000 ppm in Pörtner et al. 1998), these comparative studies provide fundamental information associated with the mechanisms of acid-base regulation (Cameron and Iwama 1987, Miles et al. 2007), ion transport (Gibbs and Somero 1990), and metabolic suppression (Langenbuch and Pörtner 2004). All of which could potentially translate into great tolerance of chronic ocean acidification.

While subtle effects of larval thermal tolerance were influenced by short-term exposure of CO_2-acidified seawater in our study, chronic exposure to elevated CO_2 could have detrimental effects on larval survival, growth, calcification and settlement. And it is these early developmental stages that are often most sensitive to environmental fluctuations (Thorson 1950). This was the first study, to our knowledge, that used a molecular approach to test whether ocean acidification could affect biomineralization in gastropods. It will be important for us, as scientists, to be innovative in our approach to understanding how ocean acidification, and other climate change factors, could play a role shaping ecosystems by determining an organisms' physiological capacity to tolerate such changes.

V. General Discussion and Conclusions

The overall objective of this dissertation was to examine the physiological tolerance of marine larval snails in response to climate change factors. Within this context, I chose to focus on the early life history stages of an intertidal gastropod, *Nucella ostrina*, and the subtidal red abalone, *Haliotis rufescens*. By assessing thermal tolerance (Chapters II, IV), shell strength (Chapter III), and changes in gene expression (Chapter IV), I attempted to highlight the physiological responses of larvae to determine whether they are vulnerable to environmental variation. To accomplish this, I integrated approaches from the fields of organismal and molecular biology, as well as adapted innovative new technologies from materials science to develop a new method to assess the impacts of ocean acidification on marine calcifiers. All three approaches provided important insight into the response of gastropods to environmental variation. Below, I discuss some of the results presented in earlier chapters in the larger context of what this may mean for the direction of future climate change studies as we continue to work towards an understanding of species responses to changing habitats.

Thermal tolerance limits and range boundaries have been well studied for many marine organisms (e.g., Stillman and Somero 2000, McMahon 2001, Zacherl et al. 2003, Rivadeneira and Fernández 2005). However, little is known about the thermal tolerance of intertidal encapsulated larvae (Fernández et al. 2006, Fernández et al. 2007). In an attempt to contribute to this body of knowledge, I investigated the thermal tolerance of *Nucella ostrina* larvae across latitude (Chapter II). While the

basic question regarding larval thermal tolerance and latitude was answered by showing a clear relationship between the two, the larvae also displayed a robust capacity to cope with temperatures beyond those of ecological relevance for this species. Similarly, non-encapsulating planktotrophic larvae exposed to similar temperature ranges, have displayed high survival rates at elevated temperatures beyond their average habitat temperatures (e.g., Rupp 1973, Sewell and Young 1999). This suggests that the capsules may not provide any additional protection against the impact of temperature alone, and therefore, our current understanding of larval thermal tolerance limits and the processes setting these limits may be generalized across different developmental strategies. Furthermore, this information may help to reduce some uncertainty in predicting whether early life history stages of multiple species will be vulnerable to temperature changes in the future.

While our current knowledge of temperature effects on organismal responses has been well studied, the emerging concern with climate change and its threat on the marine ecosystem due to carbon dioxide-driven ocean acidification is relatively new. Earlier studies attempted to predict OA impacts on marine calcifiers, but some have fallen short in addressing the biological response of these organisms and their potential to mitigate the chemical changes. With our increased knowledge of the diversity of biological processes responsible for shell deposition, matrix secretion and carbonate ion sequestration, the potential for species-specific responses are becoming widely apparent. For instance, larval shell synthesis was hindered for the mussel *Mytilus galloprovincialis* when raised under high CO_2 (2000ppm, pH 7.4; Kurihara et al. 2008a) whereas positive responses were observed in the gastropod

snail *Littorina littorea* as shell height, width and thickness increased when exposed to low pH conditions (Findlay et al. 2009). Using the biomaterials approach of nanoindentation to examine the physical characteristics of a specimen, I found no significant change in the integrity of shell strength for *N. ostrina* larvae reared under CO_2 acidified seawater conditions (Chapter III).

Overall, my studies support the idea that responses to OA are likely to be species-specific. More importantly, taken together, these studies indicate that physical measurements alone (e.g., calcification, shell strength, shell dissolution) will not provide us with enough information to make predictions regarding ecosystem wide responses to OA. To the best of my knowledge, only one other study has investigated the effects of OA on encapsulated forms. Subtle decreases in *Littorina obstusata* viability, longer developmental timing, and decreased larval heart rate were observed (Ellis et al. 2009). As highlighted in Table 8, a large number of studies addressing OA impacts have been concentrated in the Echinodermata and Mollusca. My studies provide critical insight into a class of marine organisms from which little information has been previously collected, marine gastropods. In particular, my study focusing on *Nucella* raises the possibility that organisms utilizing encapsulated development as an early life history strategy may avoid the initial developmental complications that plague echinoderms and mollusks (see Table 8 for summary of OA impacts in other phyla). In addition, my study on free-swimming developers with short pelagic larval duration, such as *Haliotis*, suggest that the extent of exposure to these corrosive waters may also play a significant role in the impact felt by calcifying larvae.

One alternative approach in measuring the physical impacts of OA on marine organisms is the assessment of physiological response at the molecular level. While carbonate ions may not drive calcification directly, the regulation of internal pH on carbonate speciation within calcifying compartments will depend on an organism's ability to balance acid-base equilibrium and result in changes in calcification. Therefore, these internal cellular processes could change the regulatory patterns of genes responsible in shell formation. Early attempts to utilize this molecular approach to identify species responses have also been met with varying results. For instance, genes responsible for skeletogenesis in larvae of the purple sea urchin, *Stronglyocentrotus purpuratus*, were shown to decrease in transcript abundance when raised under High CO_2 conditions (1020 ppm), which potentially indicate a passive physiological response to changes in available carbonate ion resources (Todgham and Hofmann 2009; O'Donnell et al. 2009b). In this study, I found no significant change in transcript levels of *engrailed* and *ap24* in response to elevated exposure of carbon dioxide for developing red abalone larvae (*Haliotis rufescens*, Chapter IV). It is possible that these are not the key regulatory players involved in the biomineralization pathway and therefore may not show significant changes alternatively, other molecular mechanisms (e.g., translation, protein function) could be affected later on in development. These data together suggest that marine calcifiers will likely respond to elevated CO_2 differently, with some species at specific stages having the capacity to regulate pathways that might enable them to synthesize larval skeletons in future elevated CO_2 scenarios. The use of gene expression as a molecular tool may be able to provide global answers for an

organisms' physiological response to ocean acidification given a few specific assumptions. First, we need to achieve a deep understanding of the cellular mechanisms responsible for processes such as biomineralization and skeletogenesis in order to identify key regulatory pathways common across taxa. Second, we need to understand whether the physiological cost for these potentially adaptive responses are conserved and how these costs may impact survival.

Within the parameters of these experiments, the second assumption may prove to be difficult to assess given the effect CO_2 can have on thermal tolerance survival for larval snail species studied in this thesis. While temperature tolerance was affected by CO_2-acidified seawater in specific developmental stages of abalone (Chapter IV), the thermal tolerance of *Nucella ostrina* veligers was moderately impacted during the 7 day exposure to High CO_2 (Chapter III). However, as exposure time increased to 14 days, veligers began to increase survival by coping with higher temperatures under High CO_2. This suggests that the larvae may have the capacity to withstand these environmental changes, but at an unknown physiological 'cost' to the organism. From results of the nanoindentation, it appears as though veliger shell strength was not compromised as a result of tolerating High CO_2 however; other biological or cellular processes (e.g., metabolism, respiration, acid-base regulation, etc.) may be jeopardized to such changes in ocean chemistry. For instance, support for this argument can be seen in the brittle star *Amphiura filiformis*. While calcification increased under reduced pH, muscle mass significantly decreased for *A. filiformis* (Wood et al. 2008). These results suggest that not only is the capacity to respond to OA species-specific, but the performance trade-off for

similar organismal responses may also vary widely. Therefore, making generalized predictions regarding organismal responses to OA across entire ecosystems may prove to be more challenging than those predictions made for temperature impacts.

In order to comprehensively understand the effects of OA on marine organisms, future research needs to examine more species including some that might be considered the "winners" of OA, to continue to investigate different life history stages and, equally important, to explore species' responses to multiple environmental interactions. While single stress experiments have utility and can provide a great deal of information, it is now important to continually move forward in our thinking and design studies that focus on integrating both temperature and OA, simultaneously. By manipulations that mimic future climate change scenarios, scientists can determine if pathways other than calcification are being impacted and whether an organism has the physiological capacity to adapt to future ocean conditions.

To conclude, the findings of Chapter II are not the first to illustrate a broad range of thermal tolerance in *Nucella* spp., but these data are the first to demonstrate a heightened tolerance to thermal stress in encapsulated veligers (*Nucella ostrina*) across a latitudinal gradient. It was evident that some larval populations maybe living at their thermal limits, suggesting any future increase to global temperatures may impact these populations by possibly causing increased mortality during extreme heat events. This intertidal invertebrate species may not be a hyper-vulnerable life stage now, but it remains to be seen if they will have the capacity to cope or adapt to the changing thermal patterns predicted.

The comparisons made in Chapters III and IV are part of what makes my study unique. This was the first time that quantitative physical properties of shell strength and quantitative gene regulation were used to evaluate the effects of climate change scenarios in larval marine snails. While I did not detect a large effect of CO_2 on thermal tolerance, gene expression or shell strength, I was able to make general conclusions about the patterns I observed. However, since OA has recently become an emerging concern for marine organisms, I hope the information in this thesis can help to contribute to the body of knowledge on climate change by providing a baseline assessment of how larval marine snails may, or may not, be impacted by climate change. Moreover, I hope this field will be further aided by the innovative approaches used and enable new opportunities for testing complex questions about species responses to environmental change.

Table 8: Effects of CO_2-driven OA on early developmental stages of marine calcifiers.

Phylum/Class, Species	CO_2 (ppm)/pH tested	Effect	Reference
Anthozoa			
Acropora tenuis	1000/ 7.6	Reduced growth of polyp size	Kurihara 2008
Crustacea			
Acartia erythraea	+2000-+10000/ 7.3-6.82	Hatching rate & nauplius survival decreased at +10000ppm	Kurihara et al. 2004
A. tsuensis	2380/ 7.4	No effect on survival, body size or development	Kurihara & Ishimatsu 2008
Amphibalanus amphitrite	7.4	No developmental abnormalities; no effect on cyprid size, attachment or metamorphosis	McDonald et al. 2009
Calanus finmarchicus	8000/ 6.95	Hatching success decreased	Mayor et al. 2007
Homarus gammarus	1200/ 8.1	Reduced carapace mass & calcification; no effect on survival, zoeal progression or carapace length	Arnold et al. 2009
Palaemon pacificus	2000/ 7.6	No effect on larval growth; decreased settling size	Kurihara 2008
Semibalanus balanoides	922/ 7.7	Delayed development; reduction in nauplii hatching	Findlay et al. 2009
Mollusca-Bivalvia			
Crassostrea gigas	2000/ 7.4	No effect on fertilization, reduced larval growth, inhibition of shell synthesis	Kurihara et al. 2007, 2008
	7.8	No effect on sperm motility or fertilization	Havenhand & Schlegel 2009

Table 8: *continued*

Phylum/Class, Species	CO_2 (ppm)/pH tested	Effect	Reference
Mytilus galloprovincialis	2000/ 7.4	Shell malformation, reduced larval size	Kurihara et al. 2008a
Placopecten magellanicus	< 7.5	High polyspermy, reduced 1st cleavage	Desrosiers et al. 1996
Saccostrea glomerata	750- 1000	Decreased fertilization, abnormal development, reduced size	Parker et al. 2009
Mollusca-Gastropoda			
Haliotis rufescens	570 & 990/ 7.97 & 7.87	Little influence on larval thermal tolerance, no change in expression of *en* and *ap24* genes	Chapter IV
Littorina obtusata	1100, 7.6	Decreased egg viability, longer development, decreased larval heart rate, altered hatchling shell morphology	Ellis et al. 2009
Nucella ostrina	570 & 990/ 8.04 & 7.9	No effect on veliger shell hardness or stiffness, decreased veliger thermal tolerance after 1 wk only	Chapter III
Echinodermata-Echinoidea			
Echinometra mathaei	860-10,360/ 7.8-6.8	Decreased fertilization, reduced larval size, skeletal malformation	Kurihara & Shirayama 2004, Kurihara et al. 2004
Evechinus chloroticus	7.8-6.0	<6.5 survival decreased, 7.7 reduced larval size and calcification, no effect on gross morphology	Clark et al. 2008
Heliocidaris erythrogramma	1000/ 7.7	Reduction in sperm speed and motility, decreased developmental success	Havenhand et al. 2008
	7.9-7.6	No effect on fertilization or development	Byrne et al. 2009

Table 8: *continued*

Phylum/Class, Species	CO_2 (ppm)/pH tested	Effect	Reference
Hemicentrotus pulcherrimus	860-10,360/ 7.8-6.8	Decreased fertilization, reduced larval size, skeletal malformation	Kurihara & Shirayama 2004, Kurihara et al. 2004
Lytechinus pictus	540 & 970/ 7.87 & 7.78	Reduced larval skeleton, decreased gene expression in metabolism and biomineralization	O'Donnell et al. 2009a
Pseudechinus huttoni	7.8-6.0	<7.0 survival decreased, no effect on larval size, 7.7 reduced calcification, no effect on gross morphology	Clark et al. 2008
Sterechinus neumayeri	7.8-6.0	<6.5 survival decreased, 7.6 reduced larval size, no effect on calcification or gross morphology	Clark et al. 2008
Strongylocentrotus franciscanus	540 & 970/ 7.98 & 7.87	Decreased gene expression of *hsp70*	O'Donnell et al. 2009
S. purpuratus	540 & 1020/ 7.96 & 7.88	Decreased gene expression in biomineralization, cellular stress response, metabolism & apoptosis	Todgham & Hofmann 2009
Tripneustes gratilla	7.8-6.0	<7.0 survival decreased, 7.8 reduced larval size and calcification, no effect on gross morphology	Clark et al. 2008
Echinodermata-Asteroidea			
Ophiothrix fragilis	7.9 & 7.7	Decreased survival, larval size, abnormal development and skeletogenesis	Dupont et al. 2008

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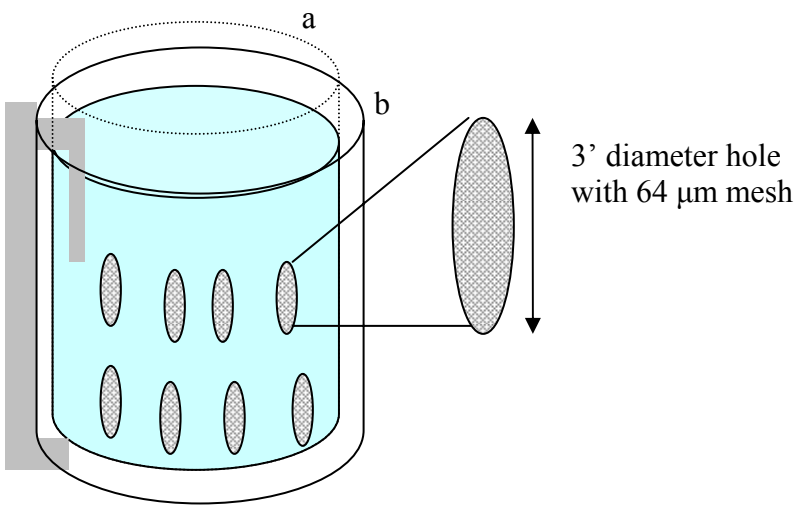
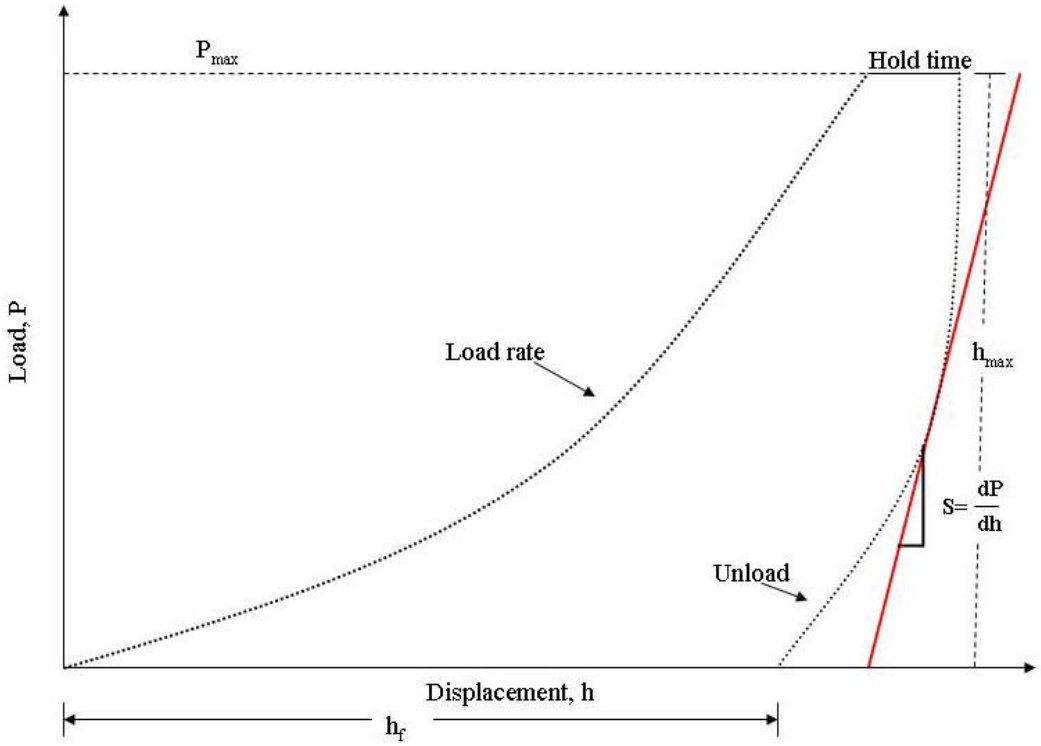
Appendix A: Nested bucket design for culturing larvae



A nested bucket design for culturing larvae (15 liters). The inner bucket (*a*) has twelve 3 inch holes with 64 μm mesh. Each bucket pair is fitted with a PVC side-arm (gray) connecting the outer bucket (*b*, 5 liters) to the inner bucket (10 liters) with a short pipe along one side. This side-arm provided a location where gas was aerated and generated a gently mixing current within the inner bucket.

Appendix B: Nanoindentation: Measures Mechanical Properties of Materials



By monitoring the load and displacement during the loading and unloading, such properties as hardness and modulus (*stiffness*) are calculated from the curve. The method is based on the notion that at some load rate (time) the sample is indented with the diamond tip and held for a duration of time (i.e., hold time). The indenter tip is then unloaded (where *S* is the slope of unloading stiffness) leaving a final depth (*hf*, impression after final unloading). The peak load (*Pmax*, maximum load) is determined by the material conforming to the shape of the indenter tip to some depth. For more of a description see Ebenstein and Pruitt 2006. Once the contact area is determined from the load displacement data the hardness (*H*) and modulus (*Er*, or stiffness) can be determined directly from the classical Oliver-Pharr analysis (Oliver et al. 1992) using the relationships:

$Er = S/2(\pi/A)$ and $H = Pmax/A$ (*A*, is the projected contact area between the indenter tip and the sample maximum load)