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EFFECT OF pH ON GENE EXPRESSION AND THERMAL TOLERANCE OF EARLY LIFE HISTORY STAGES OF RED ABALONE (*HALIOTIS RUFESCENS*)

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ABSTRACT A growing body of research on calcifying marine invertebrates suggests that ocean acidification will have deleterious effects on development and various physiological processes in these organisms. In laboratory experiments designed to mimic seawater chemistry in future oceans, we examined the effect of pH reduction, driven by the carbon dioxide (CO_2) acidification of seawater, on larvae of the red abalone, *Haliotis rufescens*. Following development under CO_2-acidified conditions, we measured 2 indicators of physiological response to low pH in 4 stages of larval development: (1) tolerance of acute thermal challenges and (2) quantitative real-time polymerase chain reaction-determined expression of 2 genes involved in shell formation (*engrailed* and *ap24*). The results showed that low pH (pH 7.87 vs. pH 8.05 for control treatments) had a significant effect and decreased larval thermal tolerance for some developmental stages (pretorsion and late veliger), but not for others (posttorsion and premetamorphic veligers). In contrast to the thermal tolerance data, decreased pH did not affect the expression pattern of the 2 shell formation genes in any of the abalone larval stages. The results indicate larval stages were differentially sensitive to low pH conditions and this variability may play into the resilience of individual species to withstand environmental change in the longer term.

KEY WORDS: abalone, ocean acidification, early development, pH, *Haliotis*, climate change, gene expression

INTRODUCTION

During the past 200 y, the ocean has been a sink for atmospheric carbon dioxide (CO_2) and has absorbed approximately 25–30% of emitted CO_2 (Sabine et al. 2004, Marland et al. 2006, Gruber et al. 2009, Takahashi et al. 2009). As atmospheric, anthropogenic CO_2 concentrations continue to rise in the future and additional CO_2 is absorbed, seawater chemistry will be further altered by continued reductions in surface pH (a process known as ocean acidification), elevated partial pressure of dissolved CO_2, reductions in carbonate ion (CO_3^{2-}) concentrations, and declining saturation states of calcium carbonate ($CaCO_3$) minerals (Caldeira & Wickett 2003, Raven et al. 2005, Feely et al. 2008). Recent modeling efforts predict that atmospheric CO_2 will increase from the current global average of ~385 ppm (Hofmann et al. 2009) to 450–650 ppm by 2060 (Intergovernmental Panel on Climate Change 2007, Cao & Caldeira 2008, Steinacher et al. 2009), a scenario that would cause a decrease in oceanic pH by an additional 0.2–0.3 units (to an average of 7.9–7.8) and reduce carbonate saturation states by ~25% (Doney et al. 2009).

An organism's response to these ongoing changes in ocean chemistry will depend upon its sensitivity to decreases in seawater pH, CO_3^{2-} concentrations, and resulting $CaCO_3$ saturation state. These changes in the carbonate system that occur during an ocean acidification scenario have been predicted to affect greatly those marine organisms that require $CaCO_3$ to build their hard parts or skeletons. Recent work has investigated the effects of elevated CO_2 on calcification in numerous organisms including coccolithophorids (Riebesell et al. 2000, Zondervan et al. 2001), foraminifera (Spero et al. 1997, Barker & Elderfield 2002, Moy et al. 2009), pteropods (Feely et al. 2004, Orr et al. 2005, Comeau et al. 2009), echinoderms (Shirayama & Thornton 2005, Kurihara 2008, Clark et al. 2009, Todgham & Hofmann 2009), corals (Marubini & Thake 1999, Langdon & Atkinson 2005, Schneider & Erez 2006, Hoegh-Guldberg et al. 2007, Herfort et al. 2008, Marubini et al. 2008), coralline algae (Kuffner et al. 2008) and molluscs (Bibby et al. 2007, Gazeau et al. 2007, Kurihara et al. 2008a). In most cases, elevated CO_2 was found to affect calcification negatively, an outcome that has been demonstrated for a variety of taxa (see Fabry et al. (2008) and Hofmann et al. (2010) for a review). In light of the role these marine calcifiers play as key organisms in their respective environments, future ocean acidification has the potential to impact not only individual species, but could change the way entire marine ecosystems function (Hoegh-Guldberg 2005, Guinotte & Fabry 2008, Hall-Spencer et al. 2008, Przeslawski et al. 2008, Wootton et al. 2008, Baskett et al. 2009).

Molluscs highlight this situation in many ways: They are key ecosystem engineers; they are critical members of many benthic and, in some cases, pelagic ecosystems; and they are economically important in fisheries worldwide. Molluscs are recognized as important ecosystem engineers (Gutiérrez et al. 2003), forming mussel beds (Norling & Kautsky 2007, Buschbaum et al. 2009) and oyster beds (Wells 1961, Zimmerman et al. 1989, Coen et al. 2007, Brumbaugh & Coen 2009). They are recognized as food sources for other animals (Nagarajan et al. 2006) and are major $CaCO_3$ producers (Fabry 1990, Comeau et al. 2009). Gutiérrez et al. (2003) found benthic marine molluscs produce 50–1,000 g $CaCO_3/m^2/y$, with oysters producing the most $CaCO_3$ (90,000 g/m^2/y). In addition, pteropods, a pelagic gastropod, are thought to contribute a large fraction of carbonate flux in parts of the oceans (e.g., 11–13 g/m^{-2}/y in the Southern Ocean (Honjo et al. 2000, Hunt et al. 2008)). Finally, molluscs have a relatively high economic importance. For example, in 2007, molluscs harvested commercially contributed 19%, or $748 million, of the $3.8 billion U.S. annual domestic ex-vessel revenue (Cooley & Doney 2009). 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 & Doney 2009). Thus, as a result of their central ecological and economic importance, marine molluscs are a key group to investigate with regard to the impacts of ocean acidification.

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One of the biological processes in molluscs that may be impacted by ocean acidification is biomineralization, the process that is central to forming a mineralized $CaCO_3$ shell (Lowenstam & Weiner 1989, Simkiss & Wilbur 1989). Different species contain a variety of mineral forms, including aragonite, calcite, high-magnesium calcite, amorphous $CaCO_3$, or a mixture of these phases (Lowenstam & Weiner 1989). Molluscs tend to have aragonite (Eyster 1986), a more soluble form of $CaCO_3$. Bivalves and gastropods have some regulatory control over biomineralization, with passive and active ion movement along a calcification compartment isolated from ambient seawater (Weiner & Dove 2003). Many studies have investigated the mechanical properties and characterization of shell formation in molluscs (e.g., Kniprath 1981, Sudo et al. 1997, Jacobs et al. 2000, Weiss et al. 2000, Hattan et al. 2001, Lin et al. 2006), but more research is needed to understand the core physiological mechanisms behind biomineralization to assess better the sensitivity of these organisms to ocean acidification.

One potential way to assess the impact of ocean acidification on an organism's physiology is to examine the response at the molecular and biochemical levels. Organisms regulate a number of cellular processes during stressful events (e.g., cell cycle, protein induction, metabolism), and the regulation and modulation of gene expression can be one of the most rapid and sensitive responses to environmental stress (e.g., Schulte 2001, Gracey et al. 2008, Place et al. 2008). Using molecular tools (e.g., quantitative real-time polymerase chain reaction (qPCR) or DNA microarrays (see Hofmann et al. (2008)) we can quantify an organism's response to an environmental stressor, such as ocean acidification, and potentially gain insight into the physiological mechanism driving its impact on calcification. Currently, there are a suite of proteins known to be required for biomineralization in molluscs (e.g., MSP1 in scallops (Sarashina & 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). 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). Although expressed during many stages and not specific to biomineralization, *engrailed* is involved in regulating larval shell formation of chiton trochophores (*Lepidochitona caverna* (Jacobs et al. 2000)) and scaphopods (*Antalis entails* (Wanninger & Haszprunar 2001)). Another gene (*ap24*) encodes the aragonite protein of the 24k-Da class, 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 et al. 2004) shells. Assessing the expression pattern of genes responsible for shell formation under current atmospheric conditions compared with predicted ocean acidification conditions may be a way to assess the potential impacts of climate change on biomineralization.

Although understanding the molecular mechanism that may defend or act compensatorily to preserve biomineralization under the altered seawater chemistry of ocean acidification, it is also imperative to gain insight into the physiological costs and consequences of functioning in an altered environment. Wood et al. (2008) found 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 stress, suggesting physiological response may be impaired at the molecular level as a result of CO_2 exposure (O'Donnell et al. 2009). These results suggest that marine organisms may have the capacity to maintain protective mechanisms against ocean acidification, but this could come at a significant "cost" to an organism's ability to tolerate additional stressors (e.g., thermal tolerance, hypoxia, salinity). Climate change scenarios predict sea surface temperatures to increase by 2–4°C (Intergovernmental Panel on Climate Change 2007), and hypoxic "dead zones" will likely also increase (Diaz & Rosenberg 2008, Brewer & Peltzer 2009). Thus, it is necessary to begin to understand whether an organism has the ability to tolerate abiotic stressors in a high CO_2 environment as well as to determine the "costs" associated with those physiological responses.

To investigate the physiological tolerance of marine molluscs to increased acidity, we studied the larvae from the red abalone, *Haliotis rufescens* (Swainson 1822), an economically important benthic herbivore (Gordon & Cook 2004), and assessed the consequences of developing under low pH conditions in CO_2-acidified seawater. Abalone, appreciated for their meat and shell, were an exploited North American Pacific fishery, with approximately 80,000 individuals taken annually prior to 1997 (Karpov et al. 2000). Although adult red abalone inhabit the low intertidal to subtidal zones 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 6–14 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 CO_2-acidified water to decrease pH of the seawater, we raised abalone larvae under different pH conditions and looked for evidence of survival being compromised in response to a thermal challenge. We also measured the expression levels and timing of 2 genes (*engrailed* and *ap24*) involved in mollusc shell formation as the larvae begin to synthesize a $CaCO_3$ shell. Because the expression patterns of these 2 genes have not, to our knowledge, been documented for red abalone, we first established the expression under normal conditions and then compared this with expression under conditions when pH was lowered in CO_2-acidified seawater. This study enabled us to measure the effects of increased acidity on survivorship in response to a temperature challenge and gene regulation as a way to understand the physiological capacity of abalone larvae under future climate change conditions.

MATERIALS AND METHODS

Spawning of Abalone Adults

Adult red abalone (*H. rufescens*) used for spawning in this study were provided by a local abalone aquaculture company (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. In nature, spawning tends to occur during the late spring into summer, but this species has been found to spawn at any time of the year (Morris et al. 1980). Adults were maintained in unfiltered seawater at approximately 15°C at the abalone facility. Individuals of similar age class were chosen (4 y 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 2 M Tris-(hydroxy-methylamino) methane (Tris base; molecular weight, 121.1 g/mol) for each liter of water in the container to prepare for spawning. Fifteen minutes after adding the Tris solution, 3 mL/L freshly prepared 6% hydrogen peroxide solution was added to the container. Water in the container was then thoroughly stirred to mix the solution. The adults were exposed to this solution for approximately 2–2.5 h and then the water was removed. The container was rinsed thoroughly with clean seawater and adults were placed back into a 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.

Experimental CO_2 System Used for Culturing Abalone

A CO_2 incubation system was designed to raise larvae under different, but controlled, concentrations of CO_2 (Fangue et al. 2010, in prep.). For the experiments described here, 3 different CO_2 concentrations were chosen specifically to span the emission scenarios predicted by the Intergovernmental Panel on Climate Change (2007) for the year 2100. There was a present-day atmospheric CO_2 level of ~380 ppm; an intermediate scenario, the B1 at ~570 ppm CO_2; and the less optimistic, "business as usual" scenario, the A1FI of ~990 ppm CO_2. The CO_2 system is briefly described here. This system is designed to remove moisture from ambient air as it is passed through a Drierite-containing (W.A. Hammond Drierite Co. Ltd., Xenia, OH) column and also scrubbed of CO_2 by a second material (Sodasorb HP; Amron International, Vista, CA). The air was then passed through a 0.22-μm 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, Monterey, CA) for each desired partial CO_2 concentration. Each gas mixture was delivered to a reactor bucket at 1,000 mL/min (Mazzei Injectors, Bakersfield, CA) and mixed with incoming filtered seawater (0.22 μm). After CO_2 saturation was reached in the reactor bucket, the CO_2-acidified water was delivered to the 4 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 Systems, Kingston, Ontario, Canada) allowing us to make adjustments to CO_2 delivery if necessary. We also measured certified reference standard gas mixtures (100 ppm and 1,900 ppm CO_2, ±1%) that bracket our experimental CO_2 levels to ensure that our CO_2 analyzer did not drift away from calibration during our experiments.

These CO_2 mixing regimes resulted in seawater with the following pH levels: the control pH treatment (~380 ppm CO_2) varied from 8.07–8.04, the moderate pH treatment (~570 ppm CO_2) yielded pH values from 8.00–7.94 during the experiment, whereas the lowest pH treatment (~990 ppm CO_2) ranged from pH 7.91–7.84. Temperature (HH81; Omega Engineering Inc., Stamford, CT), salinity (A366ATC; Vista Series Instruments, China), and pH (Ultrameter II; Myron L. Company, Carlsbad, CA) were continuously monitored throughout the 6-day experiment (Table 1 and Fig. 1).

Culture Bucket Design

Larvae were raised under various pH treatments using a 15-L nested culture chamber design (modified from the design described in Todgham and Hofmann (2009)). The inner bucket contained 12 7.6 cm diameter holes sealed with 64-μm 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) and generated a gentle mixing current for 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 to provide fresh seawater without altering the pH. All culture buckets were gently mixed with a paddle driven by a 12-V motor. Experiments did not begin until the pH of all reactor and culture buckets was stable for at least 24 h.

Sampling Larval Cultures

Immediately upon fertilization, eggs were transported to the seawater workroom on the campus of the University of California, Santa Barbara, where they were counted and divided evenly among the 3 experimental CO_2 treatments for each female (24 cultures total). The experimental procedure was repeated twice with 4 parent pairs each time, resulting in 12 cultures per experimental trial. Fertilization batches were staggered by 1.5 h to allow us to monitor developmental timing and to sample larvae based on their stage and not based on their time postfertilization (pf). Larval cultures were raised at a constant seawater temperature of 15.15 ± 0.5°C for all CO_2 treatments.

Developmental progression in each of the 12 cultures was monitored and samples were collected when the abalone had reached 4 distinct stages: pretorsion (~35 h pf), posttorsion (~58 h pf), late veliger (~84 h pf), and premetamorphic (~120 h pf). Each of the distinct developmental stages was chosen based on key morphological features. What we called the pretorsion stage was identified by the formation of the prototrochical girdle and was chosen for sample collection because this is the

TABLE 1.

Recorded values for treatment buckets for the 6-day experiment.

Culture Buckets	Approximate CO_2 Concentration (ppm)	Mean pH (± SD)	Mean Temperature (°C)	Mean Salinity (‰)
Control	380	8.05 (±0.01)	15.2 (± 0.3)	33.0
Moderate	570	7.97 (±0.02)	15.2 (± 0.4)	33.0
Low	990	7.87 (±0.01)	15.1 (± 0.3)	33.0

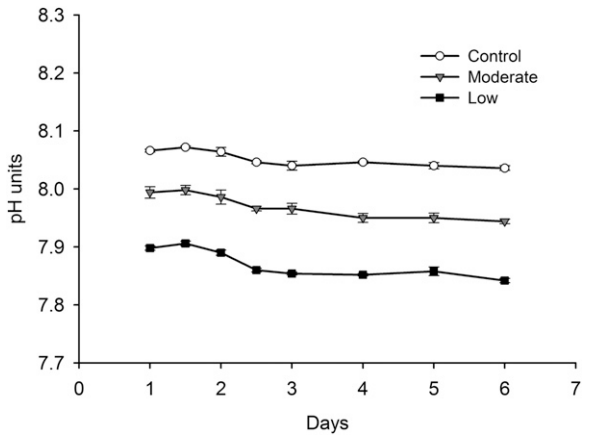

Figure 1. Measurements of the pH-acidified seawater for the 6-day experiment with red abalone larvae. Mean pH values are plotted for the 3 treatments (culture and reactor buckets). Error bars = ±SD.

point in development at which the larval shell begins to form (Fretter 1967, Bose et al. 1991). After undergoing torsion, larvae were collected at the posttorsion stage, which was identified by 2 definitive characteristics: (1) the twisting of their visceral mass inside the shell and (2) the appearance of a defined foot. Late-veliger stage larvae were sampled when they had distinct eye spots and an operculum. The latest stage, premetamorphic, 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 as an effect of pH treatment during the course of this experiment (data not shown). Because these 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. It is important to note that increased acidity did not appear to hinder larval shell synthesis. Although not quantified, no abnormalities in shell formation were observed between pH treatments or developmental stages.

When larvae had reached each of the desired stages (pretorsion, posttorsion, late veliger, and premetamorphic), 1 sample of larvae was removed from each of the 12 culture chambers to measure thermal tolerance and another was taken for RNA extraction and analysis of gene expression using qPCR. Those larvae sampled for gene expression analysis were quickly pelleted by centrifugation for 5 sec to remove excess seawater, and 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA) was added. To ensure thorough mixing, the samples were quickly vortexed and frozen at –80°C for later RNA extractions.

Thermal Tolerance Assays with Lowered pH

To determine whether lower pH affected larval abalone thermal tolerance, 1 sample of larvae (2,700 total larvae) was removed from each of the 12 culture buckets when the desired developmental stage (pretorsion, posttorsion, late veliger, and premetamorphic) was reached. The larvae were then divided equally among 9 vials (each 3 mL total volume) and exposed to a range of temperatures (prior to heat shock, 15.5°C, 19.4°C, 23.6°C, 26.2°C, 29°C, 32°C, 33.8°C, and 35.4°C). 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 temperature exposure and therefore increasing physiological stress. After a 1-h heat stress, 100 larvae from each temperature vial were visually inspected under a compound microscope for determination of survivorship. Survival was assessed based on ciliary movement around their velum. These thermal tolerance assays were performed on the offspring of male/female pairs ($n = 8$) for all pH treatments ($n = 3$) and on all developmental stages ($n = 4$).

RNA Extraction and Reverse Transcription

Total RNA was extracted from approximately 4,000 larvae from each pH treatment, each male/female pair and at each developmental stage. 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 30 µL nuclease-free water. RNA was quantified spectrophotometrically using a ND-1000 UV visible spectrophotometer (NanoDrop Technologies, Wilmington, DE) and electrophoresed on a 1% w/v agarose gel to verify RNA integrity. RNA was stored at –80°C. First-strand complementary DNA (cDNA) was synthesized from 600 ng total RNA using oligo (dT_{18}) primer and Improm-II reverse transcriptase following the manufacturer's instructions (Promega, Madison, WI).

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 & Skaletsky 2000) (v.0.4.0; Whitehead Institute for Biomedical Research; http://fokker.wi.mit.edu/primer3).

PCRs were carried out in a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) using 1.25 U Taq DNA polymerase (New England Biolabs, Ipswich, MA) and reverse-transcribed 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). 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 Plasmid Miniprep kit (Sigma-Aldrich, St. Louis, MO) and sequenced at the University of California at Berkeley sequencing facility (http://mcb.berkeley.edu/barker/dnaseq/index.html). The sequence for *engrailed* in *H. rufescens* was deposited into GenBank (GQ245982).

Quantitative Real-Time PCR Analysis of Gene Expression

Characterization of biomineralization genes in abalone larvae was analyzed using qPCR from the relative levels of messenger RNA (mRNA) for *engrailed*, *ap24* (accession no. AF225915), and elongation factor 1-alpha (*EF1*α, 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). 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′; *EF1*α forward, 5′-GGA GGG TCA AAC CCG AGA AC-3′; and *EF1*α reverse, 5′-CCG ATG ATG AGT TGC TTC ACA-3′.

qPCRs were performed with 1 μL cDNA, 4 pmoles of each primer, and 2× SYBR Green Master Mix (Bio-Rad) to a total volume of 20 μL. All qPCR 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, and 1 cycle of 55°C for 1 min. At the end of each PCR, PCR products were subjected to a melt curve analysis to confirm the presence of a single amplicon.

Statistical Analyses

Each developmental stage was analyzed separately to evaluate the effects of pH treatment on survivorship across all temperatures using status of individuals (alive/dead) in a likelihood of ratio tests (JMP 8.0, SAS Institute Inc., Cary, NC). 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 was used to correct for multiple comparisons and to estimate the expected proportion of false positives among all significant hypotheses.

To quantify *engrailed*, *ap24,* and *EF1*α mRNA expression, 1 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 *EF1*α. *EF1*α is a commonly used control gene in qPCR (Fangue et al. 2006, O'Donnell et al. 2009, Lang et al. 2009) and specifically did not change in response to decreased pH or developmental stage (data not shown), making it an appropriate internal control gene for this study. The mRNA expression for each biomineralization gene was analyzed using a 2-way analysis of variance (ANOVA), with developmental stage and pH treatments as factors. Data were transformed, when necessary, to meet assumptions of ANOVA using a Box-Cox transformation (Box & Cox 1964). Post hoc comparisons using Tukey-Kramer Honestly Significant Difference tests were performed among group means if significant differences were detected.

RESULTS

Larval Thermal Tolerance When Exposed to CO_2-Acidified Seawater

Temperature survivorship curves were generated to measure abalone survival over a range of temperatures. Such performance curves enabled us to examine the effect of lowered pH, as driven by CO_2 acidification of seawater, on survival of thermal stress during early developmental stages of *H. rufescens*. For specific stages, development at lower pH did have an influence on survivorship of the larvae in response to temperature (Fig. 2). For all stages, the response to temperature was similar, with a gradual drop off in survival as temperature increased, regardless of pH treatment. The results are described next for each of the stages.

Pretorsion larvae, the youngest stage assessed in this study, were influenced by pH and displayed a reduction in survivorship between the pH treatments. Across all temperatures tested, survivorship varied with both temperature and pH (Fig. 2A; likelihood ratio test: temperature × pH, $P < 0.001$). Analyzing just those temperatures at which survivorship varied (19.4°C, 23.6°C, 26.2°C, 29°C, and 32°C), temperature and pH treatment influenced survival with a significant interaction (likelihood ratio test: temperature × pH, $P < 0.001$; temperature, $P < 0.0001$; pH, $P = 0.0267$). Multiple comparisons revealed that survivorship differed for exposure temperatures of 29°C ($P < 0.0001$) and 32°C ($P < 0.0001$). In terms of percentages, survivorship at 29°C was as high as 93% for those raised under control pH treatment (pH 8.05), but was reduced to 78% for those raised at 29°C under low pH (pH 7.87). Further analyses demonstrated that the low acidity treatment significantly affected survivorship at 29°C ($P = 0.0011$), but not at 32°C ($P = 0.1867$). Thus, at 29°C, the low pH treatment significantly reduced survival by 18% when compared with the control pH treatment ($P = 0.0062$), and a 13% loss of survivorship occurred when compared with the moderate pH treatment ($P = 0.0183$, Fig. 2A). Although there was an abrupt increase in larval mortality between 32–33.8°C, temperature ($P < 0.001$), and not the pH treatment ($P = 0.8826$), was the influential factor.

The posttorsion 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, pretorsion stage (Fig. 2B). Analysis of only those exposure temperatures when survivorship (19.4°C, 23.6°C, 26.2°C, 29°C, and 32°C) differed indicated that posttorsion larvae were affected only by temperature and pH treatment (likelihood ratio test: temperature × pH, $P = 0.1949$; temperature, $P < 0.0001$; pH, $P = 0.2691$). The percentage of survival decreased as temperature increased with a pattern of 23.6°C > 26.2°C > 29°C >32°C (Fig. 2B). Hence, at the highest temperature when survival differences were calculated (32°C), survivorship fell below 65%, whereas at 19.4°C the percentage of surviving individuals was more than 97% across all pH treatments (Fig. 2B).

As abalone larvae continued to develop in acidified seawater, late-veliger survivorship was affected by both exposure temperature and pH (Fig. 2C; likelihood ratio test: temperature × pH, $P = 0.0020$; temperature, $P < 0.0001$; pH, $P = 0.0019$). Although veliger survivorship did not differ among pH treatments for 19.4°C ($P = 0.7426$), multiple comparisons indicated the low pH treatment did significantly reduce survival for 23.6°C, 26.2°C, and 32°C, but not 29°C (Table 2). For example, as temperature exposures increased from 23.6–32°C, veliger survivorship under the low pH treatment decreased from 87.5–17%, respectively (Fig. 2C). This was the largest mortality effect for any of the pH treatments, and for this late stage, CO_2-driven seawater did negatively impact the thermal tolerance of veliger abalone larvae.

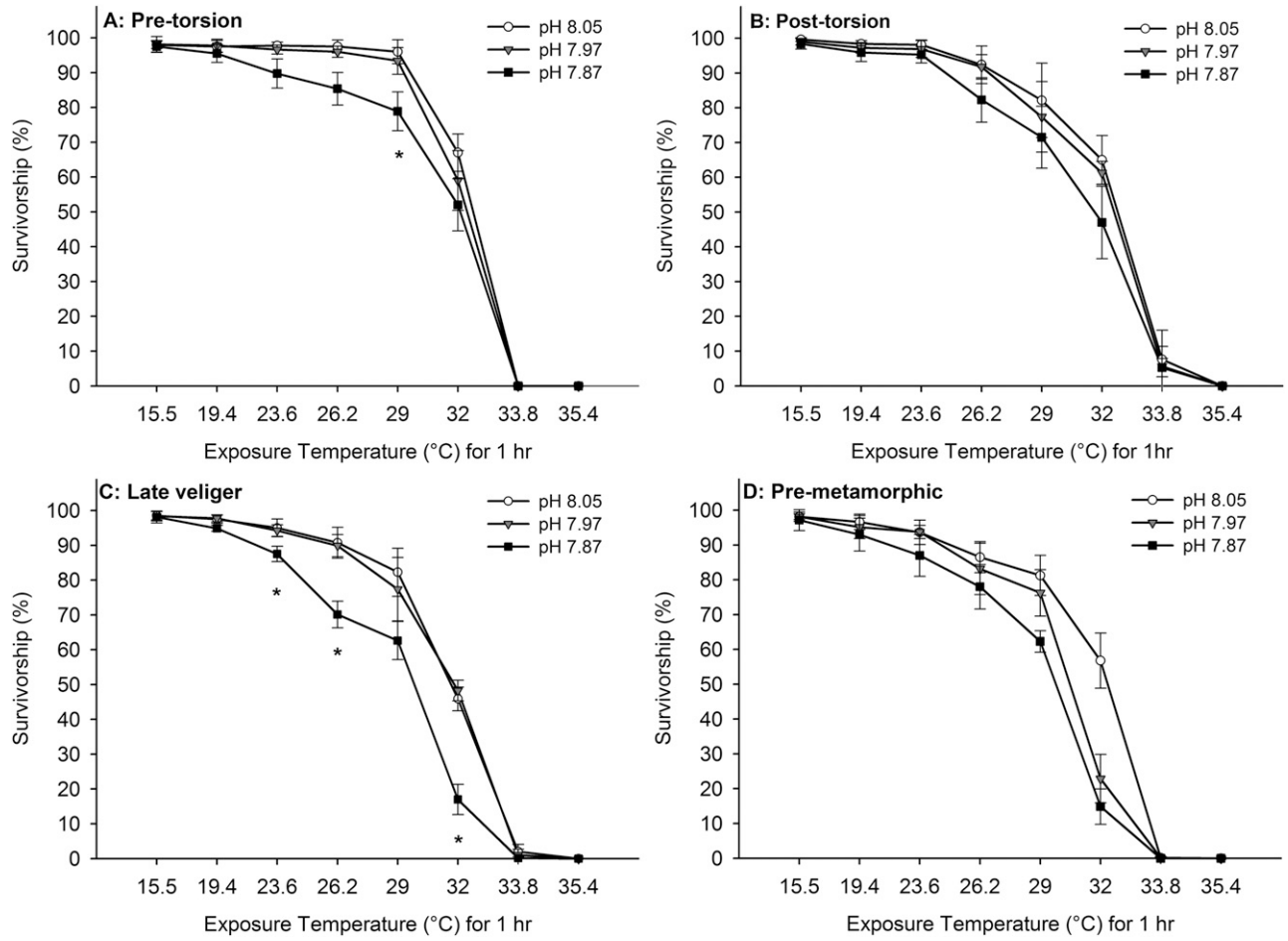

Figure 2. (A–D) Thermal tolerance, presented as percent survivorship, of *Haliotis rufescens* larval stages—pretorsion (~35 h pf; A), posttorsion (~58 h pf; B), late veliger (~84 h pf; C), and premetamorphic (~120 h pf; D)—raised under elevated CO_2 concentrations. Larvae from 8 male/female pairs were assessed for survivorship at each exposure temperature and pH treatment ($n = 100$). Error bars = SD. *Significant pairwise differences in survival for the low pH treatment at a given temperature.

Temperature significantly reduced survivorship of premetamorphic (~120 h pf) abalone larval raised under CO_2-acidified seawater, but pH treatment did not alter this pattern of thermal tolerance (Fig. 2D; likelihood ratio test: temperature × pH, $P = 0.0913$; temperature, $P < 0.0001$; pH, $P = 0.3041$). The highest exposure temperature with survivors (32°C) had 56% survivorship compared with 23.6°C, when the percentage of survival was greater than 87% (Fig. 2D). Thus, temperature had a significant effect on survivorship at these exposure temperatures, and this did not change as a function of pH treatment.

Expression of Biomineralization Genes

To assess a molecular response of early life history stages of *H. rufescens* to CO_2-acidified seawater, mRNA levels of 2 biomineralization genes, *ap24* and *engrailed*, were measured. Although transcript for *ap24* was found across all developmental stages, pretorsion and posttorsion stages showed significantly higher *ap24* expression compared with other, older stages (Fig. 3A; 2-way ANOVA, $F = 62.138$, $P < 0.0001$). Abalone in the youngest stage had approximately 15-fold higher levels of *ap24* mRNA compared with abalone in the posttorsion stage; the levels of *ap24* mRNA were 30-fold higher when compared with the later stages (late veliger and premetamorphic, Fig. 3A). 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 (Fig. 3B). The oldest, premetamorphic, larvae appeared to have the lowest level of *engrailed* mRNA compared with the other stages. However, this difference was not statistically significant (2-way ANOVA, stage; $F = 2.715$, $P = 0.0537$).

Throughout the course of the 6-day experiment, there was no differential response to pH treatment in either gene (*ap24* (Fig. 3A), 2-way ANOVA, $F = 0.5824$, $P = 0.5621$; *engrailed* (Fig. 3B), 2-way ANOVA, $F = 0.1813$, $P = 0.8347$). In addition, there was no significant interaction between stage and pH treatment (*ap24*, 2-way ANOVA, $F = 0.3964$, $P = 0.8782$; *engrailed*, 2-way ANOVA, $F = 0.7342$, $P = 0.6243$).

DISCUSSION

The overall goal of this study was to determine whether CO_2-acidified seawater would influence the ability of abalone larvae to tolerate thermal stress and, furthermore, how the expression of genes involved in biomineralization might change to provide evidence of a physiological response (e.g., a stress response or

TABLE 2.

Multiple comparisons using Fisher's exact test indicated survival differences between pH treatments for specific exposure temperatures for late-veliger stage abalone.

Exposure Temperature	pH Treatment Comparison	*P* Value
23.6°C	Control vs. moderate	0.5796
	Moderate vs. low	<0.0001
	Control vs. low	<0.0001
26.2°C	Control vs. moderate	0.6122
	Moderate vs. low	<0.0001
	Control vs. low	<0.0001
29°C	Control vs. moderate	0.1550
	Moderate vs. low	0.1836
	Control vs. low	0.0816
32°C	Control vs. moderate	0.3673
	Moderate vs. low	<0.0001
	Control vs. low	<0.0001

compensation). The study had 2 major outcomes: (1) developing in acidified seawater influenced larval survivorship in response to a brief thermal stress and (2), in contrast, the expression levels of 2 biomineralization genes were not affected by pH treatment for any of the larval stages, although expression of one of the genes varied across development.

In our thermal stress trials, the thermal tolerance of red abalone larvae was altered when the larvae developed in CO_2-acidified seawater, although different stages displayed different responses (Fig. 2). At the pretorsion stage, survival of larvae developing under low pH (pH 7.87) was significantly lower at 29°C, but was not altered at any other exposure temperature. A similar trend was seen for late-veliger-stage larvae when survival decreased significantly under low pH for specific temperatures (23.6°C, 26.2°C, and 32°C). The difference in survivorship across development suggests that some developmental stages may be more sensitive than others to pH changes. Notably, the pretorsion stage may be more vulnerable to lower pH because these larvae are about to begin a major developmental transition and undergo torsion, therefore making them more sensitive to environmental perturbations (Collin & Voltzow 1998, Mahroof et al. 2005, Gunter & Degnan 2007, Jardillier et al. 2008, Ueda & Boettcher 2009). The same could be true for late-veliger-stage larvae. Larvae at this stage may be more susceptible to low pH because they are about to metamorphose and settle. Larvae that are near settlement stage and entering metamorphosis have been shown to be influenced by ocean acidification through alterations in developmental rate (Talmage & Gobler 2009). A study with *Mytilus galloprovincialis* mussel larvae raised in CO_2-acidified seawater at pH 7.4 (2,000 ppm) found that 70% of the larvae were delayed in development at the trochophore stage and, when development continued, abnormalities in morphology were observed (Kurihara et al. 2008a). Similarly, barnacle larvae (*Semibalanus balanoides*) raised under high CO_2 conditions (CO_2 = 922 ppm, pH 7.70) experienced a developmental delay of 19 days, resulting in a 60% reduction in the number of nauplii that reached the hatching stage (Findlay et al. 2009a). Finally, metamorphosed and settled juvenile marine shrimp *Palaemon pacificus* raised in CO_2-acidified seawater (2,000 µatm, pH 7.6) were smaller in size compared with the control shrimp (Kurihara et al. 2008b).

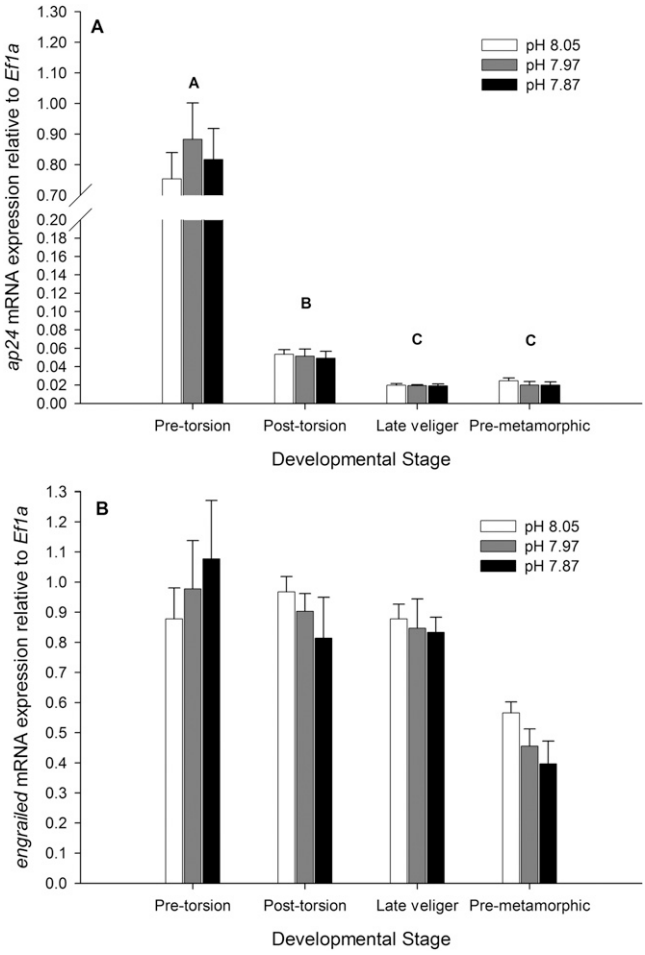

Figure 3. (A, B) Relative levels of mRNA expression of *ap24* (A) and *engrailed* (B) for 4 developmental stages of *Haliotis rufescens* raised under 3 pH treatments. Error bars represent SEM. Larvae from 8 male/female pairs were examined. Different letters correspond to significant differences in gene expression among developmental stage only, and not pH treatment.

Although the current study has shown later stages of abalone development to be vulnerable to ocean acidification, in other studies, fertilization and the earliest embryonic stages of marine calcifiers have been found to be highly sensitive to enhanced acidity. Fertilization rate of 2 sea urchins (*Hemicentrotus pulcherrimus* and *Echinometra mathaei*) decreased with a reduction in pH by 1.3 units (from 8.1–6.8 (Kurihara & Shirayama 2004)). In addition, polyspermic fertilization was reported in the giant scallop *Placopecten magellanicus* at a pH level less than 7.5 (Desrosiers et al. 1996), and sperm motility was reduced in the sea urchin *Heliocidaris erythrogramma* (Havenhand et al. 2008). In contrast, fertilization rates of the oyster *Crassostrea gigas* were unaffected by exposure to high-CO_2 seawater (pH 7.8 (Havenhand & Schlegel 2009)). In terms of early development, the first cleavage was delayed with decreasing pH in the giant scallop *P. magellanicus* (Desrosiers et al. 1996). More recently, researchers have found the success of copepod hatching has decreased with increased CO_2 levels (Mayor et al. 2007, Kurihara et al. 2004a, Kurihara et al. 2004b). In the context of ocean acidification, these types of data—fertilization and early development impacts—are unknown for abalone and should be targets of future investigation for this economically important species.

Our experiments showed, for some stages of development, that *H. rufescens* larvae can physiologically tolerate short-term acute thermal stress well above ambient environmental temperatures even when exposed to other stressors (e.g., CO_2-acidified seawater). A few studies have explored the interaction between ocean acidification and thermal stress. One such study on crustaceans found that the edible crab *Cancer pagurus* experienced a reduction in physiological performance, responding to elevated CO_2 conditions with a significant decrease in the upper thermal limit for aerobic scope (Metzger et al. 2007). In larvae of the red sea urchin (*S. franciscanus*), development at elevated CO_2 and lowered pH resulted in a decrease in the expression of thermal defense genes, suggesting that the larvae would be more vulnerable to thermal stress (O'Donnell et al. 2009). In a microcosm experiment, postlarvae growth and development of barnacle *S. balanoides* were negatively impacted by reduced pH (pH 7.7) and not affected by increased temperature (+4°C (Findlay et al. 2009b)). Whether abalone larvae will tolerate the simultaneous occurrence of ocean acidification and ocean warming in nature remains unanswered.

This study also examined expression changes in 2 genes, *ap24* and *engrailed,* associated with calcification during development under acidic conditions. Levels of mRNA transcript for *ap24* and *engrailed* did not change when abalone larvae were raised at lower pH (pH differences of 0.08 and 0.18, respectively, when compared with the control pH treatment). The only differences observed were across developmental stages for the *ap24* gene (Fig. 3A). The peak expression of *ap24* in the earliest larval stage, pretorsion, may be linked to the onset of larval shell synthesis, and allocation of mRNA transcript increased as a result. Thus far, the molecular process regulating molluscan biomineralization is poorly understood. However, Michenfelder et al. (2003) found the AP24 protein in *H. rufescens* to have oligosaccharides, an important feature associated with calcium carbonate in sea urchin spicule matrix proteins (Cho et al. 1996), indicating oligosaccharides may be involved in mineral binding functions.

No difference in gene expression for *engrailed* was found for pH treatment or developmental stage (Fig. 3B). It may not be too surprising that *engrailed* mRNA levels held steady over larval development, because this gene product is expressed in many cells and likely has multiple functions during larval development. The protein, Engrailed, is a transcription factor with known roles in controlling morphology and ectoderm differentiation, and functions to produce ectodermal boundaries to differentiate specific neurons and to pattern appendages in *Drosophila* development (Kornberg et al. 1985). In molluscs, expression of *engrailed* was observed around the border of a developing larval shell of the marine clam (*Transannella tantilla* (Jacobs et al. 2000)), and in scaphopods it was expressed at the trochophore stage in cells located close to the edge of the mantle (Wanninger & Haszprunar 2001). These expression data are consistent with others that have shown differential changes in biomineralization genes for developing purple sea urchin larvae (*S. purpuratus* (Wilt 1999, Livingston et al. 2006, Oliveri et al. 2008)). However, in contrast, a recent study found a downregulation for a suite of genes involved in spicule formation and skeletogenesis for urchin larvae (*S. purpuratus*) raised under high CO_2-acidified seawater (Todgham & Hofmann 2009). The regulatory networks, and those genes involved, are different between a sea urchin and a gastropod. Therefore, important considerations need to be made when making predictions about taxa or phylum-level responses to ocean acidification (Hendriks et al. 2009). Each organism will have a varying degree of sensitivity and capacity to cope physiologically with acidified conditions. Without precise measurements and an understanding of mechanical properties, we cannot be certain if the pH treatments had an effect on larval shell formation, even though the 2 genes in this study appeared to have "normal" expression. In other words, there may be other better biomarkers for identifying changes in shell formation under acidified environments. With the application of genomewide approaches in development 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.

In conclusion, although the effects of ocean acidification on all economically valuable species are not known, it is estimated that a 10–25% decrease in U.S. harvest of molluscs could result in a revenue loss of $75–187 million the following year (Cooley & Doney 2009). In other countries, estimates predict China, Chile, and Brazil could also suffer, with an expected decline in total catch of 6–13% by the year 2055 (CO_2 concentration, 720 ppm (Cheung et al. 2009)). The production of abalone aquaculture has become vital to this endangered species by reducing further depletion of natural populations, specifically *H. rufescens* (Rogers-Bennett et al. 2004), and providing a global production value of ~$300,000 (FAO 2007). Thus, understanding how climate change factors affect growth and survival for commercially cultivated species will be effective in yielding production and profitability. For example, 3 commercially and ecologically valuable shellfish (*Mercenaria mercenaria*, *Argopecten irradians,* and *Crassostrea virginica*) had reduced larval survival, delayed metamorphosis, and lowered growth during development under elevated CO_2 (~66 Pa and ~150 Pa CO_2 (Miller et al. 2009, Talmage & Gobler 2009)). Therefore, future efforts need to identify vulnerabilities of various aquaculture species to future environmental change (e.g., Somero 2010). Because different strains and even genotypes will display differential resilience to ocean acidification, it is vital that we begin to examine the physiological capacity of economically important species that play an important role in the welfare of human society (Easterling et al. 2007, Battisti & Naylor 2009).

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