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# ACIDIC SEAWATER CAN PROMOTE LARVAL METAMORPHOSIS IN THE MARINE MUD SNAIL, *NASSARIUS OBSOLETUS*

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*Abstract:* Elevated levels of atmospheric $CO_2$ have had the unintended effect of acidifying the world's oceans by at least 0.1 pH unit since the beginning of the industrial revolution. Current models of the effects of continued $CO_2$ release anticipate a further increase in ocean acidity by 0.14 to 0.4 pH units by the end of this century. Numerous marine organisms with calcareous shells or exoskeletons are expected to undergo deleterious effects, including shell or exoskeletal dissolution or calcification with increased metabolic costs. While many studies have documented potential effects of ocean acidity on adult calcareous organisms, less work has focused on embryonic or developmental changes. Alterations in ocean biochemistry can affect metabolic processes beyond calcification, and our investigations into the development of the local intertidal mudsnail, *Nassarius obsoletus*, led us to hypothesize that rising ocean acidity would increase incidents of larval shell decalcification, decrease larval growth rates, and induce precocious metamorphosis. As an initial study of potential effects of future acidic conditions on larval *N. obsoletus*, we raised larvae in seawater adjusted to lower than normal pH levels with hydrochloric acid (HCl), measured larval growth every 5 days in 15 randomly selected individuals from each culture, and conducted experiments on larval metamorphosis in physiologically competent larvae. We found no major trend in growth patterns of larvae from pH 8.2 to 7.7. However, results from a number of pharmacological experiments demonstrated that larvae grown in acidic conditions metamorphose more readily than controls. Our investigations are far from a definitive exploration of the effects of oceanic acidity on the development of this marine mollusc, but our data suggest that key developmental processes in marine invertebrates may be influenced in unexpected ways by continued climatic changes.

*Key Words:* Development; gastropod; mollusc; ocean acidification.

## INTRODUCTION

Since the beginning of industrialization, not quite 250 yrs ago, ocean acidity has dropped a tenth of a pH unit. Shallow coastal waters are particularly vulnerable to acidification, and further decreases in pH over the coming century are projected to range from 0.14 to 0.4 pH units unless global atmospheric $CO_2$ emissions are significantly curtailed (Bijma et al. 2013; Caldeira and Wickett 2003; Doney et al. 2009; Fabry et al. 2008; Hofmann and Schellnhuber 2009; Kelly et al. 2011; Meehl et al. 2007; Nicholls et al. 2007). This rapid decline in ocean pH lowers the saturation state for calcite and aragonite, with potentially negative effects on the stability of organisms with calcareous skeletons or shells (Guinotte and Fabry 2008; Zeebe et al. 2008). Early studies concentrated on the threat to coral reefs, but many shell-bearing molluscs that may be equally endangered are important ecosystem members with considerable economic value. Effects of seawater acidity on calcification rates and metabolism have been studied for some adult molluscs (Fabry et al. 2008; Gazeau et al. 2007; Michaelidis et al. 2005; Orr et al. 2005; Weiss et al. 2002) and more recent work has begun to focus on early developmental events (Green et al. 2004; Ross et al. 2011; Talmage and Gobler 2009; Talmage and Gobler 2010). Larvae of many shelled molluscs are lightly calcified compared to their adult counterparts and appear to be highly susceptible to changes in saturation levels of calcium carbonate (Green et al. 2004; Kurihara et al. 2007; Ross et al. 2011; Talmage and Gobler 2009; Talmage and Gobler 2010). Changes in seawater biochemistry that lead to general metabolic suppression and decreased

growth rates (Fabry et al. 2008; Sidorov and Polyanina 2003) can also change the ciliary-based swimming behaviors of planktonic organisms (Chan et al. 2011; Woodward and Willows 2006). Reduced growth or survival in planktonic or early juvenile stages can significantly alter annual recruitment patterns, which in turn can lead to ecosystem-wide transformations in species composition and numbers (Hall-Spencer et al. 2008). To begin to determine if low oceanic pH levels would have detrimental effects on metamorphosis in local marine molluscs, we recorded growth rates of cultured planktonic larvae of our model organism, the intertidal mud snail *Nassarius obsoletus*. We also looked for evidence of shell decalcification as an indicator of metabolic stress. We found surprisingly little evidence of shell dissolution and no link between larval growth rates and an acidic environment.

Metamorphosis is the developmental phenomenon that links planktonic larvae with their adult community and the successful completion of this event is vital for recruitment of the next generation. Previous investigations in my laboratory have resulted in the confirmation of serotonin (5-hydroxytryptamine, 5-HT) as a neurotransmitter that is active in the pathway that promotes metamorphosis. We also discovered that the gaseous neurotransmitter nitric oxide (NO) represses metamorphosis in unstimulated competent larvae (Couper and Leise 1996; Froggett and Leise 1999; Hens et al. 2006; Leise and Cahoon 2012; Leise et al. 2004; Lin and Leise 1996; Thavaradhara and Leise 2001). We used our knowledge about the pharmacological regulation of metamorphosis in *N. obsoletus* to study the potential effects of environmental perturbations on this important developmental program. Relatively few investigations have focused on potential effects of declining ocean pH levels on metamorphosis (Crim et al. 2011; Ginger et al. 2013; Green et al. 2004; Ross et al. 2011; Talmage and Gobler 2009; Talmage and Gobler 2010), so we used 5-HT and a reagent that inhibits the formation of NO, 7-nitroindazole (7-NI), to explore how an acidic environment might influence this developmental process. Results from a number of experiments support our hypothesis but are somewhat counter-intuitive; low pH levels increase the ease with which metamorphosis can be elicited from larval *N. obsoletus*.

## MATERIALS AND METHODS

Larval culture techniques and methods for our pharmacological experiments are routine (Gharbiah et al. 2008), but we describe them here briefly. Approximately 100 adult *Nassarius obsoletus* (Say, 1822, synonymous with *Ilyanassa obsoleta*) were housed in each of four aquaria in the laboratory at about 26°C. Two aquaria were maintained in an environmental chamber at 7°C, each of which held several hundred animals. The low temperature kept these animals from being reproductive, but the snails laid egg capsules upon being moved to the warmer laboratory temperature. Chilled animals were used to replenish our laboratory populations as they ceased to lay egg capules during the late spring and summer. Laboratory snails were fed raw fish fillet three times a week. Snails in the cold were fed once every two weeks. Water in the cold aquaria was changed as necessary to maintain animal vitality. All adult snails were obtained from intertidal mud flats at the CREST Research Park at UNC Wilmington during low tides from January through March. Egg capsules were removed from the walls of laboratory aquaria with a razor blade and turkey baster and kept at ambient temperature (22°C) and lighting conditions. Egg capsules were rinsed in 70% ethanol and fresh 0.2 μm filtered artificial seawater (FIO) daily to minimize bacterial contamination. Larvae were either put into culture within 24 hr of hatching or discarded. Larval cultures were maintained in airlift systems (Gharbiah et al. 2008; Miller and Hadfield 1986). Each culture was started with 800–850 larvae at a starting density of about 1.3 larvae/ml. Cultured larvae were fed daily with two unicellular algae that were also maintained in the laboratory, *Dunaliella tertiolecta* and *Isochrysis galbana.* Larvae are reared in a 1:1 mixture of natural and artificial seawater with penicillin and streptomycin (Miller and Hadfield 1986), all filtered to 0.2 μm.

The pH of each culture was tested and adjusted daily. Low pH cultures were adjusted downward in increments of about 0.05 pH units daily from pH 8.1 until the target pH was achieved. This gave larvae time to acclimate to greater acidity. Growth of larvae in each culture was ascertained every five days by measuring the maximum shell length of 15 randomly chosen larvae under a dissecting microscope at 40× magnification.

Experiments on the impacts of pH on metamorphic induction were conducted on physiologically competent larvae obtained from cultures at least 15 days past hatching in which the average shell length was 550 μm or greater. Larval responses to two pharmacological reagents, serotonin at 30 and 60 μM (L5-HT and 5-HT, respectively) and 0.2 mM 7-nitroindazole (7-NI), an inhibitor of nitric oxide synthase, were tested in untreated, 24 well plastic Falcon tissue culture plates. Five larvae were pipetted into each well, FIO was removed and 2 ml of a control or experimental solution was added. Experiments were conducted in FIO adjusted to the pH at which larvae were cultured. Numbers of larvae and juveniles were counted at 24 and 48 hr after induction (Couper and Leise 1996; Froggett and Leise 1999; Gifondorwa and Leise 2006). For statistical analysis, data were treated with an arcsine transformation and tested for significance in a one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test (Sokal and Rohlf

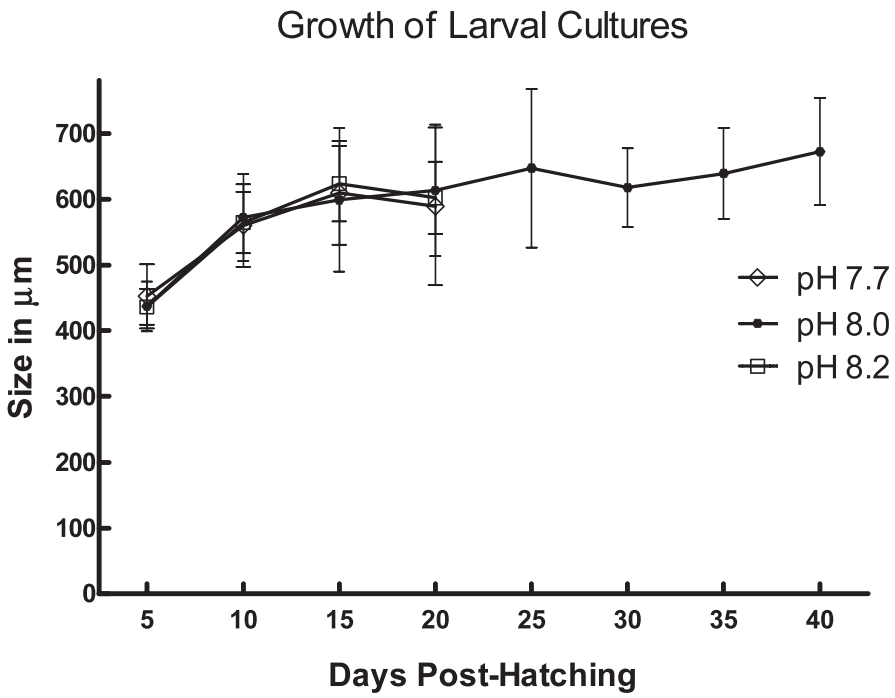


FIG. 1. Growth rates of larvae in culture at 3 levels of acidity are similar. Number of cultures and length of culture life varied. Most cultures were used for experiments between 15 and 20 days; a few were kept for longer times. Mean sizes ±SE at pH 7.7 derived from 10 cultures for 10 days, 8 for 15 days, with 3 remaining at 20 days. Mean sizes at pH 8.0 derived from 3 cultures for 25 days, 2 cultures thereafter. Mean size at pH 8.2 derived from 14 cultures for 10 days, 11 cultures at 15 days, but 2 cultures at 20 days. Apparent declines in larval sizes with age arise because cultures with larger animals were used for experiments and thus removed from the later samples.

1995). Untransformed means ±SEM are presented graphically. Arcsine transformations were conducted in Excel (Microsoft Office 2007) and analyses and graphs were prepared in GraphPad Prism 5.02.

## RESULTS

A few larvae (~1%) in each culture were found to partially or completely lack shells, but these occurrences were not correlated with culture acidity (data not shown). These results were not quantified because of low numbers of such larvae. Any significant difference in physiology should lead to impaired growth over time. Surprisingly, growth of larvae at all pH levels was similar (Fig. 1). Number of cultures and length of cultured life varied because of experimental use. Most larvae were used for experimentation after 15 or 20 days, although larvae in a few cultures were allowed to age further. A number of cultures were lost because of temperature fluctuations in the culture room during the late winter months. Heating and cooling issues in that room were subsequently resolved. Individual cultures showed no decline in size with age although average sizes appear to fluctuate because larger cultures were used for experiments and thus removed from the sample population (Fig. 1). We detected no differences in growth rates between larvae cultures at different levels of acidity.

The classical neurotransmitter, serotonin (5-HT), induces metamorphosis in this species (Couper and Leise 1996; Levantine and Bonar 1986) while the gaseous molecule nitric oxide (NO) inhibits this developmental event. Typical results with these reagents are shown in Figure 2 at pH 8.2. L5-HT typically elicits 30–40% metamorphosis after two days of exposure. Larval responses to this concentration are more dependent upon larval health than they are at the higher concentration. Percentages of metamorphosed larvae are more variable after treatment with 7-nitroindazole (7-NI), an inhibitor of nitric oxide synthase (NOS), and generally lower than results with 60 µM 5HT (cf. Figs. 2 and 3).

Because we repeatedly observed higher levels of metamorphosis with both reagents at lower pH levels (Fig. 3) we merged the results of multiple experiments to determine whether our observations were statistically significant. We found that at more acidic conditions, both concentrations of 5-HT induced significantly more larvae to metamorphosis than did solutions at pH 8.2 (Fig. 4A–D) and that larval responses to 7-NI also (as later) declined as acidity decreased (Fig. 4E, F).

## DISCUSSION

We found no difference in larval growth rates for at least three weeks over the range of pH levels (7.7–8.2) we

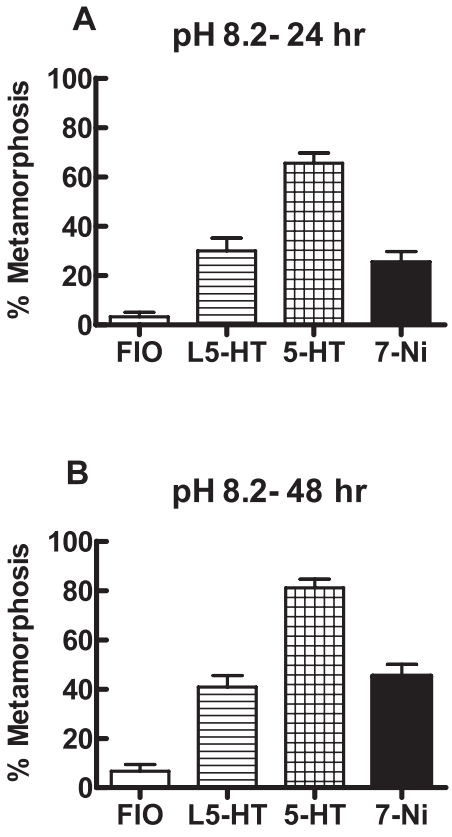


FIG. 2. Serotonin elicits significant levels of metamorphosis at a normal pH. At pH 8.2, 60 μM serotonin (5-HT) typically elicits over 80% of competent larvae to metamorphose by 48 hr. Larvae are generally less responsive to 30 μM serotonin (L5-HT) or to the nitric oxide synthase inhibitor, 7-Ni.

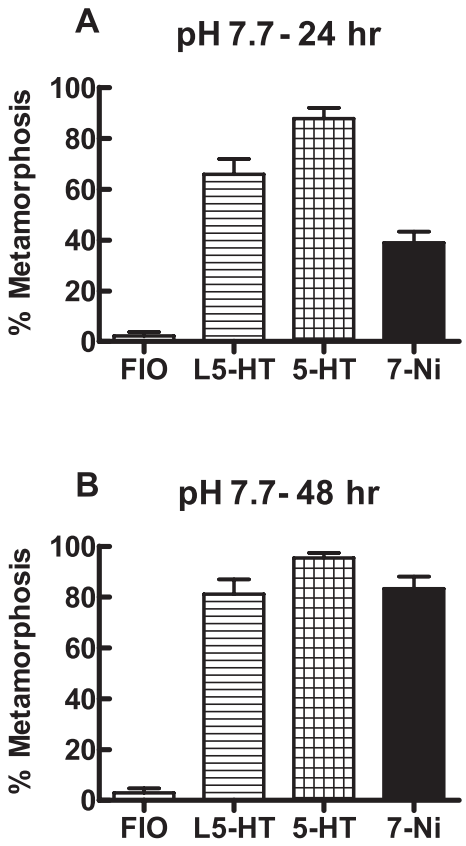


FIG. 3. Larvae are more responsive to metamorphic inducers at low pH levels. In a relatively acidic environment (pH 7.7), larvae metamorphose in greater numbers (cf. Fig. 2) to all 3 treatments. Larval metamorphosis to each treatment at 3 levels of acidity is compared in Figure 4.

studied, and only rarely saw fully or partially decalcified animals in our cultures. Thus, we suggest that the planktonic larvae of the mud snail *Nassarius obsoletus* may be insensitive to increasing ocean acidity for many years. However, experiments with other molluscan larvae have demonstrated that culture conditions and the stages of embryos or larvae used can greatly influence results obtained about larval metabolism, shell deposition, survival or metamorphosis (Ross et al. 2011). For example, Ginger et al. (2013) found that larval survival and growth rates of *Crassostrea gigas* were unaffected by seawater acidity, but as we did, these researchers used hatched larvae for experimentation and did not subject embryos or young larvae to abnormal conditions. Other studies on *C. gigas*, the oyster *Ostrea lurida*, and the clam *Macoma balthica,* in which larvae were raised from fertilized eggs or used early in the larval phase demonstrated impaired fertilization, larval growth, and shell deposition (Barros et al. 2013; Hettinger et al. 2012; Van Colen et al. 2012). While we suspect that more cosmopolitan and invasive species, like *N. obsoletus*, may have a selective advantage over their more localized relatives (Crim et al. 2011; Ginger et al. 2013; Kozloff 1983), experimental conditions that incorporate all developmental stages and that directly manipulate seawater chemistry with gaseous carbon dioxide ($CO_2$) should elicit more reliable findings.

Incubation in a low pH (6.0) solution for several hours is a method commonly used to decalcify small marine invertebrates (Cavanaugh 1956). We have used this procedure successfully on our gastropod larvae in several sets of experiments (Couper and Leise 1996; Froggett and Leise 1999; Gifondorwa and Leise 2006), but have noted, anecdotally, that this procedure can induce up to 10 or 15% of larvae to metamorphose. These unpublished observations led us to hypothesize that an acidic environment would induce precocious metamorphosis in *N. obsoletus*. As we suspected, pharmacological induction in acidified seawater elicited significantly more larval metamorphosis than did induction in normal, more alkaline seawater (Figs. 2–4). Most of our experiments were conducted on larvae with shell lengths above 600 μm, so we do not know whether an acidic environment would make smaller larvae metamorphose. If this occurs, juveniles could recruit to their benthic habitats before they are physiologically ready to endure intertidal situations,

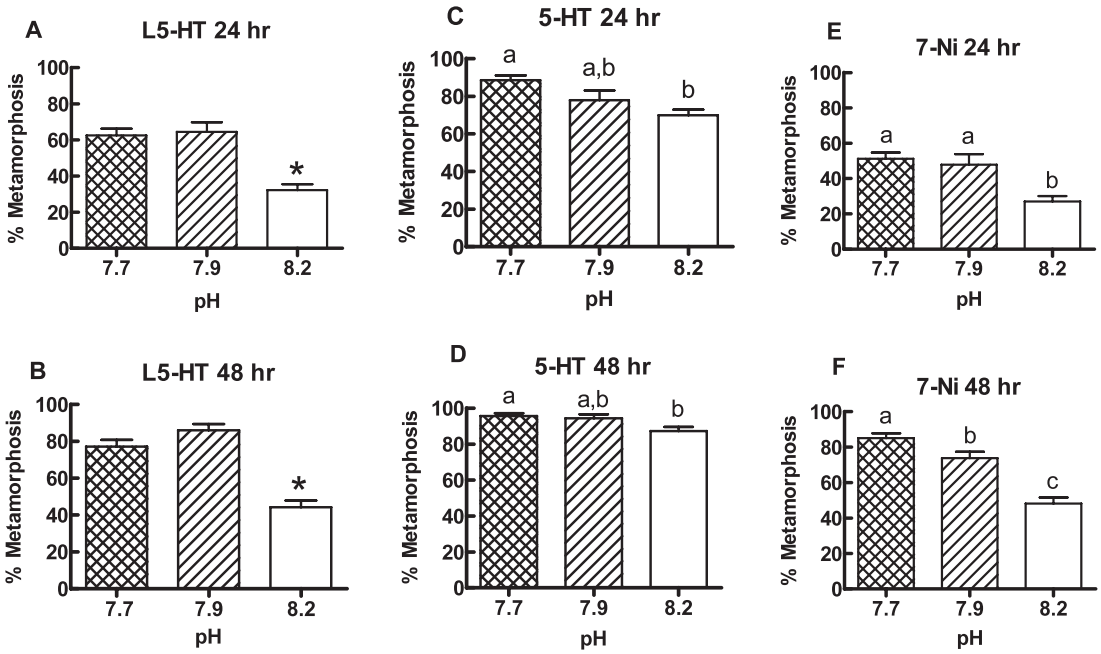


FIG. 4. A comparison of 3 metamorphic inducers at 3 pH levels. More larvae metamorphose in response to all treatments, 30 μM serotonin (L5-HT) (A,B), 60 μM 5-HT (C,D) and the nitric oxide synthase inhibitor, 7-Ni (E,F) under more acidic conditions. Bars are means ±SE for all treatments at each pH indicated. Two experiments were pooled at pH 7.7 and pH 8.2, yielding 36 replicates (180 animals) for all 3 treatments. One experiment is displayed at pH 7.9, representing 18 replicates (90 animals) per treatment, (A,B). An asterisk (*) indicates significant differences between numbers of larvae metamorphosing at pH 8.2 compared to both pH 7.7 and 7.9 at 24 and 48 hr by Tukey's Multiple Comparison Test, $p < 0.001$. Results of ANOVA were $F = 28.67$ and $p < 0.001$. C,D,E,F. Bars with different letters have significantly different means as determined by Tukey's post-hoc test at $p < 0.05$ or better.

perhaps making them more susceptible to predation or desiccation stresses. Precocious metamorphosis at any size could also place juveniles in the benthic environment before sufficient seasonal diatom communities become established and available to serve as a food source for newly metamorphosed individuals (Haines and Montague 1979; Hughes and Sherr 1983; Leise and Cahoon 2012; Leise et al. 2009). However, our metamorphic results, coupled with an apparent lack of any pH effect on growth, suggests that this widespread species may be able to adapt to altered habitats and remain a successful inhabitant of future soft sediment communities.

Oceanic acidification and a direct increase in oceanic temperatures are only two of several results of global warming. Increased absorption of atmospheric $CO_2$ also leads to oceanic deoxygenation, increased stratification of surface waters and a concomitant change in nutrient availability (Bijma et al. 2013; Hofmann and Schellnhuber 2009). Additional anthropogenic stressors, including pollution, eutrophication and surface run-off can also potentiate changes at the species and ecosystem levels (Bijma et al. 2013). Many studies of the effects of global warming on the development of marine invertebrates provide data based on permutations of one or only a few environmental transformations as researchers attempt to understand potential changes in the face of multiple and complex future trends (Barros et al. 2013; Chan et al. 2011; Crim et al. 2011; Deschaseaux et al. 2010; Hettinger et al. 2012; Landes and Zimmer 2012; Pansch et al. 2012; Ross et al. 2011; Suwa et al. 2013). Although we investigated only the effects of seawater acidity on larval development and metamorphosis, our data support the idea that global warming and continued anthropogenic production of excess atmospheric $CO_2$ may have unanticipated effects on developmental stages and events in the life histories of coastal marine invertebrates.

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