

Effect of pH on Larval Metamorphosis in the Marine Snail, *Ilyanassa obsoleta*

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Abstract: Elevated levels of atmospheric CO₂ 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 CO₂ release anticipate a further drop in ocean pH levels by 0.14 to 0.4 units by the end of this century. Marine organisms with calcium carbonate shells or exoskeletons are expected to undergo deleterious effects, including shell or exoskeletal dissolution or calcification with increased metabolic costs. While a number of studies have documented potential effects of ocean acidity on adult calcareous organisms, less work has focused on embryonic or developmental difficulties. Changes in ocean biochemistry can affect metabolic processes beyond calcification, and our investigations into the development of the local intertidal mudsnail, *Ilyanassa obsoleta*, 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 acidic conditions on larval *I. obsoleta*, we raised larvae in seawater adjusted to lower than normal pH levels with hydrochloric acid (HCl), measured larval growth in 15 randomly selected individuals from each culture every 5 days, and conducted experiments on larval metamorphosis in physiologically competent larvae. Although we occasionally saw fully or partially decalcified animals in our cultures, we found no important trend in growth patterns of larvae over the pH range (7.7 – 8.2) we studied. In contrast, and in support of our hypothesis, results from a number of experiments demonstrated that metamorphosis was significantly easier to elicit from larvae grown in acidic conditions. Our investigations are far from a definitive exploration of the effects of ocean acidity on the development of marine molluscs, but our data suggest that key developmental processes in marine invertebrates may be influenced in unexpected ways by continued climatic changes.

Four undergraduate laboratory assistants made significant contributions to this research project and were involved in all aspects of it, including the rearing of many larval cultures, the design and conduct of experiments, and the collection and analysis of data. A manuscript based on our findings will be submitted to the *Journal of the North Carolina Academy of Sciences*.

Introduction: Since the beginning of industrialization, not quite 250 years 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 CO₂ emissions are significantly curtailed (Caldeira and Wickett, 2003; Meehl et al., 2007; Nicholls et al., 2007; Fabry et al., 2008). 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). Most studies have focused 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 (Weiss et al., 2002; Michaelidis et al., 2005; Orr et al., 2005; Gazeau et al., 2007; Fabry et al., 2008), but much less work concerns early developmental events. Larvae of many shelled molluscs are lightly calcified compared to their adult counterparts and thus may be highly susceptible to changes in saturation levels of calcium carbonate (Green et al., 2004; Kurihara et al., 2007). Changes in seawater biochemistry that lead to general metabolic suppression and decreased growth rates (Sidorov and Polyana, 2003; Fabry et al., 2008) can also change the ciliary-based swimming behaviors of planktonic organisms (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 local marine organisms, we recorded growth rates of cultured planktonic larvae of our model organism, the intertidal mud snail *Ilyanassa obsoleta*. 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, 5HT) as a neurotransmitter that is active in the pathway that promotes metamorphosis and in the discovery that the gaseous neurotransmitter nitric oxide (NO) represses metamorphosis in unstimulated competent larvae (Couper and Leise, 1996; Froggett and Leise, 1999; Thavaradhara and Leise, 2001; Leise et al., 2004; Hens et al., 2006). Because of our knowledge of some of the pharmacology involved in the metamorphic process in *I. obsoleta*, we are in a position to study the effects of environmental perturbations on this important developmental program. Relatively few investigations have focused on potential effects of declining ocean pH levels on metamorphosis, so we used 5HT 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 *I. obsoleta*.

Methods: Larval culture techniques and methods for our pharmacological experiments are routine (Gharbiah et al., 2008), but I briefly describe them here. My students and I maintain 4 aquaria in the laboratory, each of which houses a population of about 100 adult *Ilyanassa obsoleta*, at about 26°C. We also maintain 2 aquaria in an environmental chamber at 7°C, each of which houses several hundred animals. The low temperature keeps these animals from being

reproductive, but snails will lay egg capsules upon a return to the warmer laboratory temperature. We use the chilled animals to replenish our laboratory populations as they cease to lay egg capsules during the late spring and summer. Laboratory snails are fed raw fish fillet 3 times a week. Snails in the cold are fed once every two weeks. Water in the cold aquaria is changed as necessary to maintain healthy animals for as long as possible. All adult snails are obtained from intertidal mud flats at the Center for Marine Science at UNC Wilmington during low tides from January through March. Egg capsules are collected from the walls of aquaria with a razor blade and turkey baster and are kept at 22°C in the laboratory. Egg capsules are rinsed in 70% ethanol and fresh 0.2 µm filtered artificial seawater (FIO) daily. Larvae are either put into culture within 24 hours of hatching or are discarded. Larval cultures are maintained in airlift system (Miller and Hadfield, 1986; Gharbiah et al., 2008). Each culture is started with 800-850 larvae at a density of about 1.2 larvae/ml. Cultured larvae are fed daily with two unicellular alga that are 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 antibiotics (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, until the target pH was achieved. This gave larvae some time to acclimate to lower pH levels. Growth of larvae in each culture was ascertained every 5 days by measuring the maximum shell length of 15 randomly chosen larvae at 40X magnification under a dissecting microscope.

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 550µm or greater. Larval responses to two pharmacological reagents, serotonin at 30 and 60 µM (5HT and 5HT, respectively) and 0.2mM 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 mls 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 hours 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, 1995). Untransformed means +/- s.e.m. 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 did not seem to be correlated with culture acidity. Because of the low numbers of such larvae, we did not quantify them, and assumed that any significant difference in physiology would lead to impaired growth over time. Surprisingly, growth of larvae at all pH levels appeared to be 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 real 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 obvious differences in growth rates between larvae cultures at different levels of acidity.

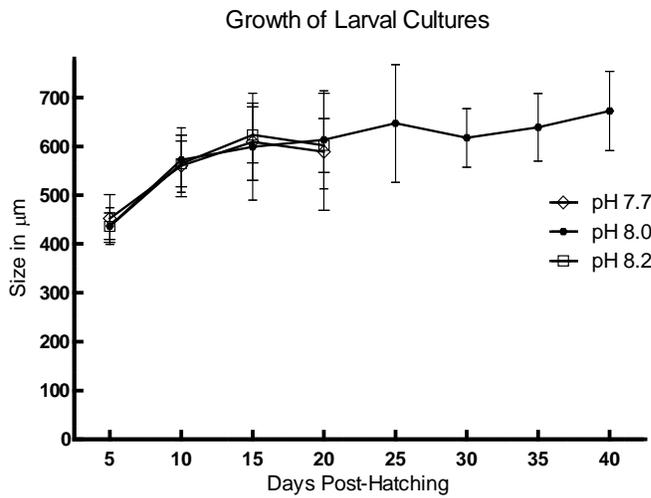


Figure 1. Growth rates of larvae in culture at 3 levels of acidity appear to be similar. Number of cultures and length of culture life varied. Most cultures were used for experiments between 15 and 20 days; a few were kept for longer times. Mean sizes \pm s.e. at pH 7.7 derived from 10 cultures for 10 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.

The classical neurotransmitter serotonin (5HT) is a known inducer of metamorphosis in this species (Levantine and Bonar, 1986; Couper and Leise, 1996) while the gaseous molecule nitric oxide (NO) inhibits this process. Typical results with these reagents are shown in Fig. 2 at

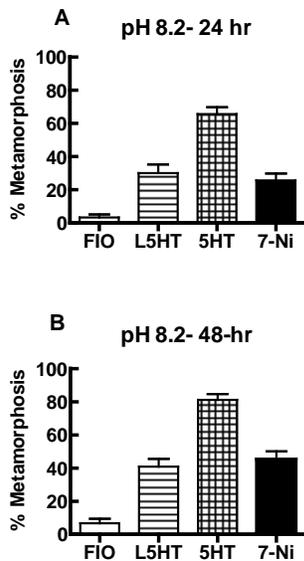


Figure 2. At pH 8.2, 60 μM 5HT typically elicits over 80% of competent larvae to metamorphose by 48 hr. Larvae are generally less responsive to 30 μM serotonin (L5HT) or to the nitric oxide synthase inhibitor, 7-Ni.

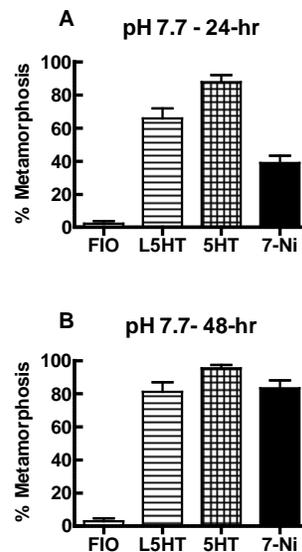


Figure 3. In a relatively acidic environment (pH 7.7), larvae metamorphose in greater numbers (c.f. Fig. 2) to all 3 treatments. Larval metamorphosis to each treatment at 3 levels of acidity is compared in Figure 4.

pH 8.2. The low concentration of serotonin (L5HT) usually yields 30-40% metamorphosis after 2 days and 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 (7NI), an inhibitor of nitric oxide synthase (NOS), and generally lower than results with 60 μ M 5HT (cf. Figs. 2 and 3).

Because we repeatedly observed higher levels of metamorphosis with both reagents at lower pH (Fig. 3) we merged the results of a few experiments in which levels of metamorphosis were similar to test whether our observations were correct. We determined that at more acidic conditions, both concentrations of 5HT induced significantly more larvae to metamorphosis (Fig. 4A-D) and that larval responses to 7-NI also declined as acidity decreased (Fig. 4E,F).

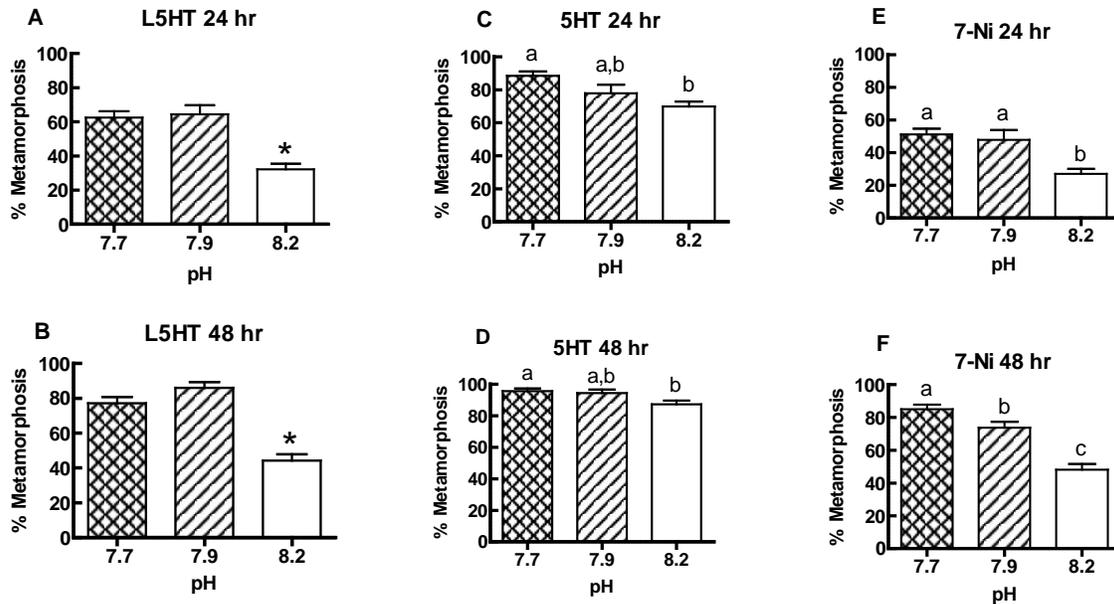


Figure 4. More larvae metamorphose in response to all treatments, 30 μ M serotonin (L5HT) (A,B), 60 μ M 5HT (C,D) and the nitric oxide synthase inhibitor, 7-Ni (E,F) under acidic conditions. Bars are means \pm s.e. for all treatments at each pH indicated. Two experiments were pooled at pH7.7 and pH8.2, yielding 36 replicates (180 animals) for all 3 treatments. One experiment is displayed at pH7.9, representing 18 replicates (90 animals) per treatment, A,B. * indicates significant differences between numbers of larvae metamorphosing at pH8.2 compared to both pH7.7 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.

Discussion: Our results indicate that the shells of planktonic larvae of the mud snail *Ilyanassa obsoleta* may not be sensitive to increasing ocean acidity for many years. We also found no difference in larval growth rates for at least 3 weeks over the range of pH levels (7.7-8.2) we studied. In contrast, as we had hypothesized, acidic environments elicited significantly more larval metamorphosis than did normal, alkaline situations (Fig. 4). Most of our experiments were conducted with larvae with shell lengths above 600 μ m, so we do not know whether an acidic environment will also make larvae metamorphose at smaller sizes. If this occurs, as we suspect, it could put juveniles in their juvenile habitat before they are physiologically ready to acclimate to benthic situations and perhaps make them more susceptible to predation. Precocious metamorphosis at any size could also put them in a juvenile

environment before sufficient diatom communities are established and available to act as the food source for these newly metamorphosed individuals (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 continue to display great adaptability to altered habitats and may remain a successful inhabitant of future soft sediment communities.

Definitive results require further studies in which the CO₂ content of seawater is altered by direct changes in gaseous CO₂ levels. This would certainly provide a better mimic of future environmental alterations. Our current data suggest that future increases in ocean acidity may have limited or subtle actions on larvae of this widespread marine snail.

Impacts and Benefits: While this research project was driven by a desire to improve our understanding of the potential impact of global warming on marine organisms, any immediate impact of this project is relatively low. However, our data do provide further support for the idea that global warming and continued anthropogenic production of excess atmospheric CO₂ can have unanticipated effects on marine communities. As mentioned, further research in this area would be required to confirm our results. Because of the labor-intensive nature of this work, and the limited space for rearing cultures that we have at UNCG, available funding did not allow us to directly manipulate partial pressures of CO₂. Nonetheless, our results strongly suggest that *I. obsoleta* may remain relatively unaffected by decline oceanic pH levels for some time to come.

Extension of Results: To disseminate our finding, we are currently crafting a manuscript to be submitted to the *Journal of the North Carolina Academy of Sciences*. In addition, as the PI updates her website in 2010, these results will be described in her listing of laboratory research projects.

Students: Four undergraduates laboratory assistants, Joshua Long, Jeremy Washburn, Nishant Shah, and Brandi Evenson, all enrolled at UNCG, made important contributions to experimental design and set up, the recording of data and its analysis. These students raised 50 larval cultures which allowed us to attempt 16 experiments (not all were completed, depending upon health of larvae and experimental conditions). Nishant Shah and Brandi Evenson received financial support from this Sea Grant minigrant.

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