The combined effects of ocean acidification and sea surface temperature rise on larval sea hare (Aplysia dactylomela) development

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The increase in partial pressure of CO2 in the atmosphere, largely from anthropogenic sources, is affecting marine calcifying organisms. This is caused by a reduced availability of carbonate due to ocean acidification. Simultaneously, the sea surface temperature (SST) is rising due to the greenhouse effect associated with increased CO2 in the atmosphere. This study investigates the effects of decreased pH and increased temperature on the larval development of Aplysia dactylomela, an ecologically important marine gastropod that produces a calcium carbonate shell as an embryo. An ocean acidification and simulated sea surface temperature system was used to treat A. dactylomela egg masses in 4 treatments based on the Intergovernmental Panel on Climate Change (IPCC) predictions for 2100: control, heated (+3°C), acid (pH 7.6), and acid plus heat (+3°C, pH 7.6). This study found a statistically significant decline in rates of survival and amount of movement for all treatments relative to the control, with the acid plus heat treatment having the lowest rates of survival and movement. These results suggest that over the next century there may be further negative consequences for the larval development and thus species success and survival of A. dactylomela as well as other marine calcifying organisms important to coral reef ecosystems.

Introduction

Anthropogenic carbon dioxide (CO2) emissions have increased to unprecedented levels over the last several decades and are predicted to continue rising [1]. The atmospheric concentration of CO2 has risen over 100 ppm from pre-industrial levels of 280 ppm to nearly 400 ppm in 2014 [1]. Much of this steady increase in CO2 in the Earth’s atmosphere has been absorbed by the planet’s largest CO2-trap: the ocean. The additional atmospheric CO2 has led to a greater CO2 partial pressure (pCO2) in the oceans, which has reduced oceanic pH by 0.1 units since pre-industrial times [2]. The steadily decreasing oceanic pH, known as ocean acidification, has a detrimental effect throughout the oceans and especially on sensitive coral reef environments [3].

Ocean acidification reduces the concentration of calcium carbonate (CaCO3) in the ocean and thus limits the ability of coral reefs and other calcifying organisms to grow [2]. The current global oceanic average pH of approximately 8.1 allows for the super saturation of CaCO3 needed by reef building corals and other calcifying organisms; however, the Intergovernmental Panel on Climate Change (IPCC) predicts that oceanic pH will continue to decrease by as much as 0.3-0.4 units within this century [1]. These changes will affect many calcifying and CaCO3 skeleton-building organisms, both as mature invertebrates and during embryonic development. The decrease in the concentration of carbonate ion (CO3²⁻) in the ocean will weaken the ability of marine calcifying organisms to build shells or exoskeletons out of CaCO3. This under-saturation can lead to pitting, or the degradation and deformation of the exoskeletons or CaCO3 shells [2]. Species that are dependent on embryonic shell development are susceptible to population bottlenecks due to decreased mortality as larvae. This is because of their increased vulnerability to acidic conditions [4]. A bottleneck in early life stages may cascade and impact the species overall success and continued persistence [4]. While ocean acidification has been well established as a problem for calcifying corals, it is also known to affect the growth and development of other marine calcifying organisms like mollusks and other benthic invertebrates [3,5].

The long-term increase in atmospheric CO2 and other greenhouse gases has also been shown to impact global temperatures, which in turn affects the sea-surface temperature of the ocean [6]. The IPCC predicts a sea surface temperature (SST) increase of 2.4°C to 6.4°C by 2099 assuming greenhouse gas emissions continue to increase as they have over the last several decades [1]. An increase in temperature can lead to an increase in rates of metabolic reactions and the acceleration of embryonic development until a species-specific temperature threshold is reached [4]. Increases in temperature above this threshold are detrimental and can result in a dramatic increase in mortality [4]. This increase in temperature is especially concerning to tropical organisms that are adapted to a relatively small temperature range.

One such organism potentially affected by the increase in SST and ocean acidification is the spotted sea hare, Aplysia.
dactylomela, a marine gastropod found on sandy substrates in coral reefs. Sea hares, like other opisthobranch gastropods, are simultaneous hermaphrodites that reproduce via internal fertilization and have the capability of sperm storage [7]. Sea hare eggs are laid in discreet capsules within a gelatinous string that is usually attached to algae or under a hard surface at a rate of 5.9 cm min⁻¹, which is approximately 41,000 eggs min⁻¹ [8]. Because survival to hatching and through the larval planktonic stage to sexual maturity is incredibly low, sea hares lay thousands of eggs to ensure that enough sea hares will survive to reproduce [8]. The sea hares develop a CaCO₃ shell during their embryonic development, which later becomes covered in tissue as they mature [9].

The spotted sea hare serves as a useful model animal because of its ecological importance and the available previous research on its embryonic development. The spotted sea hare plays an essential role in maintaining coral reef health by helping to control the macroalgal population. Without the sea hares and other key herbivores, the macroalgal population could overrun coral reefs and there could be a transition from reef dominated to algal dominated systems. Previous studies have been conducted regarding A. dactylomela embryonic development under the predicted changing oceanic conditions. One such study explored the effects of both lower pH and increased SST on the embryonic development over their incubation time prior to hatching [10]. However, the research did not quantitatively investigate survival to hatching rates or the condition of the hatched larvae. Understanding the survival rates of the larvae and their health upon hatching is critical to understanding how these sea hares, and many other marine calcifying organisms, will survive in the changing oceans of the future. The goal of this work was to quantify the effects of ocean acidification and sea surface temperature rise would have on the health and viability of A. dactylomela during embryonic development.

METHODS

Study Site

This study was conducted on Heron Island (23° 26' 31.2" S, 151° 54' 50.4" E), 80 km east of Gladstone in Queensland, Australia. The island is located on the leeward edge of a 27 km² coral reef platform and has a deep lagoon with patches of coral. The surrounding reef is very high in diversity, supporting 72% of all the Great Barrier Reef coral species and a high proportion of endemic species [11].

Egg Mass Collection

Five sea hares (Apllysia dactylomela) were collected from the reef flat off the southern coast of Heron Island and kept in a tank with unfiltered water pumped in directly from the reef. They were fed the macroalgae Laurencia and allowed to copulate for several days. The sea hares were collected on 11 October 2014, and three egg masses were first sighted on 15 October 2014. The masses were randomly and evenly split into small semipermeable mesh containers and then allocated randomly to a treatment tank resulting in one mesh container per treatment tank (Figure 1). All of the eggs were placed in treatment tanks within 12 hours of being laid.

Experimental Design

Four conditions were used in this experiment: control, heated, acidic, and acidic plus heat. The control treatments used a flow-through system directly connected to the reef to keep the egg masses at ambient temperature and pH. The ambient temperature ranged from 22°C to 25°C, and the pH was approximately 8.1. The heated treatments simulated an increase in SST and were maintained at +3°C above ambient temperature. The temperatures in these tanks ranged from 25°C to 28°C. The acidic treatments were maintained at pH 7.6, the IPCC estimated oceanic pH for 2100, with ambient temperature. The tanks under acidic plus heated conditions were maintained at pH 7.6 as well as +3°C. There were three replicates of each treatment leading to a total of
Ocean Acidification System

The ocean acidification system used for this experiment was designed to model the increased oceanic pH values based on IPCC predicted concentration of CO$_2$ in the atmosphere (ppm). Unfiltered seawater was pumped directly off the reef flat into large sumps then into the treatment tanks, which allowed for a constant flow of fresh water to the embryos and for all non-experimental parameters (such as dissolved oxygen) to remain the same as within the natural environment. The tanks in this system used as controls received the water from the reef flat with no alteration. The water for the acidic treated tanks was pumped into a large 200 L sump where an automated CO$_2$ injection system was used to elevate the dissolved CO$_2$ concentrations to reach a pH of 7.6 by injecting CO$_2$ into a diffuser and rapidly dissolving CO$_2$ into the seawater. A pH control unit (Aquatronic, AEB Technologies, Italy) was used to monitor and maintain the desired pH level in the sump. This control unit was connected to a pH probe in the sump and controlled an electronic solenoid connected to a cylinder of CO$_2$ that would be opened if the pH rose above 7.65 and closed when the pH returned to 7.6. The water from the sump was then pumped into the treatment tanks for both the acidic and acidic plus heat treatments.

Aquarium heaters were used to heat another large 200 L sump of unfiltered seawater from the reef flat to $+3^\circ$C. This water was then pumped into the heated treatment tanks. The tanks that were both acidic and heated received the acidic water from the large pH controlled sump and had individual aquarium heaters within the tank to increase the temperature $3^\circ$C. Thermometers in the tanks allowed for temperature monitoring to ensure the temperature stayed within the desired range.

Image Analysis

A small segment (~2 cm) of each egg mass and larvae collected from within the mesh containers were viewed under an OPTIKA B-600 Biological Microscope with an attached camera. Images of each egg mass were taken through the connected computer. The images were analyzed using ImageJ Software (version 1.48) to determine the shell length. Lengths were found in pixels then converted to millimeters using a calibrated scale from the microscope software (QC Pro), which was accomplished by taking pictures of a 1 mm scale under the same microscope. The longest length of the shell, which is visible when the organism is on its side, was used as the standard for shell length measurements. At least 3 images were taken from each tank and used to get 5 shell length measurements from each tank as a representative sample.

### Data Analysis

The percent of larvae with pitted shells (Figure 3), survival rates, and degree of movement were quantified through two one-minute long observations under a microscope. Survival and movement rates were assigned a value using a five-point scale to most accurately quantify the entire egg mass from the representative sample taken (Table 1). Movement rate was based on the percent of larvae seen moving as well as the frequency and duration of movement observed. These observations were then fit to the five-point scale seen in Table 1.

![Non-Pitted and Pitted](image)

**Figure 3.** Images of *A. dactylomela* larvae taken under a compound microscope at 20 times magnification. The non-pitted sample with a visibly smooth shell was taken from the control treatment while the pitted image with noticeable deformities in the shell was taken from the acidic plus heat treatment. Both were taken on the day larvae hatched. Scale bar = 100µm.

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**Table 1.** The five-point scale used to quantify survival and movement within the sea hare larvae.

![Figure 4.](image)

**Figure 4.** Average larvae survival to hatching with standard error as observed under a microscope and categorized on a five-point scale by treatment. Significant difference (p<.05, ANOVA) are indicated by a (*).
A one-way analysis of variance (ANOVA) was used to analyze the means and variance in the data collected for survival and movement rates as well as shell-length to determine statistical significance. Due to the extreme heterogeneity of the treatments, an ANOVA could not be performed for the shell pitting data. Instead, a Cochran’s Q Test was performed instead.

RESULTS

General Observations

There was a visible reduction in the overall health of the egg masses from the control sample to the samples treated in acid (pH 7.6). The gelatinous strings in the egg masses changed color, degraded, and began falling apart even before the larvae hatched. These visible signs of degradation were present to a lesser extent in the egg masses treated with heat. When the egg masses hatched, there was observable movement of larvae in the mesh containers in the control and heat-treated samples, while in the acid and acid plus heat-treated samples, there were visible numbers of dead larvae floating on the surface. The heat-treated egg masses, including the acid plus heat-treated egg masses, hatched first. The acid and control treatments all hatched the following day. No statistical analysis was done on the difference in condition between the day the larvae hatched and the following day because no second day observations could be taken for the control samples due to the limited time available at the research station. However, it was clear that the average percent of surviving larvae and the amount of visible movement decreased dramatically, particularly in the acid plus heat-treated larvae where the average survival dropped from category 3 (40-60% survival) to category 1 (0 – 20% survival).

Survival Rates

Significantly more larvae survived to hatching in the control treatments than the experimental treatments (Figure 4, ANOVA p-value = 0.0012). The differences between the control treatments and all the other treatments were statistically significant. There was no significant difference in the amount of survival between acid and the acid plus heat treatments.

Amount of Movement

The amount of movement visible under a microscope differed significantly between the control treatment and all other treatments (Figure 5, ANOVA p-value = 0.0002). The amount of movement visible under the microscope corresponded to the amount of movement qualitatively observed within each of the mesh containers after hatching, with the highest percentage of movement seen in the control treatment. The larvae in the heat treatment had a higher rate of movement than the larvae in the acid plus heat treatment; however, this difference was not statistically significant.

Shell Length

Figure 5. Average amount of movement observed under a microscope with standard error on the day the larvae hatched as categorized on a five-point scale by treatment. Significant difference (p< .05, ANOVA) are indicated by a (*).
Shell length was significantly longer in larvae that survived to hatching in the control treatment than all other treatments (Figure 6, ANOVA p = 0.001). The largest difference in shell length was observed between the control and heat treatments.

**Percent of Larvae with Pitted Shells**

Only two of the treatments (acid and acid plus heat) had larvae that showed signs of pitted shells. A Cochrane’s Q Test found a significant difference between the treatments with pH 7.6 and the treatments at ambient pH (p-value = 0.0143). There was no significant difference between the larvae in the acid treatment versus those in acid plus heat treatment.

**DISCUSSION**

The results of this study suggest that the increased SST and decreased pH in the oceans of the future will have serious negative impacts on the health and survival of the gastropod *A. dactylomela* and other marine calcifying organisms. Not all control treated egg masses survived to hatching, which is expected and normal for egg masses [8]. However, the control treated egg masses had significantly higher survival rates to hatching than all the other treated egg masses. The control egg masses looked healthier and showed fewer signs of discoloration and deterioration. The acid plus heat treatment had the lowest survival rates, which is expected as those larvae were subjected to the most stressful conditions. However, there was no significant difference in survival rates from the acid only treatment compared to the control treated egg masses.

These findings suggest that fewer larvae may survive to hatching as the ocean warms and acidifies from climate change. A higher larval mortality could ultimately cause the population to decline as fewer organisms survive to sexual maturity and particularly poor survival rates lead to population bottlenecks [4]. Other calcifying larvae such as other gastropods or arthropods that are dependent on high CaCO₃ saturation levels to build and maintain shells or exoskeletons necessary for survival could face similar challenges in the future.

The *A. dactylomela* larvae that did survive exhibited reduced movement in all non-control treatments. The amount of movement was studied as an indicator of general health and activity level of the larvae, with higher amounts of movement potentially indicating healthier larvae. The hypothesized existence of an organism-specific threshold temperature under which metabolism increases and beyond which activity rapidly declines may explain the difference between the results of this study and previous research, which found that heat-treated embryos showed an increased amounts of movement [4, 10]. Though heat-treated egg masses hatched first, which may suggest that development occurs faster in heated conditions, there was reduced movement in all experimentally treated larvae. This reveals that even the egg masses that survive to hatching may not be healthy enough to survive the planktonic larval phase. This is supported by the observed reduction in survival and amount of movement one day after hatching in the heat-treated egg masses. The decreased swimming mobility and overall fitness of the larvae could lead to reduced larval survival rates to sexual maturity.

Although natural variation in shell length is expected, the significant difference in shell length between the experimental and control larvae suggests that larvae in the experimental treatments did not develop as fully as those in the control treatment. While the larvae in the heat treatment developed faster, as demonstrated by their earlier hatching time, they had the smallest average shell lengths of any treatment. This suggests that while higher temperatures may accelerate development rate, they may not develop to be as large. The reduced size of the shells in the acid and acid plus heat larvae suggests that the environmental stress of ocean acidification could also lead to smaller larvae due to decreases in development. Because smaller larvae may be more susceptible to predation and less able to compete for food, the smaller shells may also contribute to higher larval mortality rates.

Pits and other deformations in the CaCO₃ shells of larvae could affect sea hare survival to sexual maturity and the overall species population. The CaCO₃ shells developed as embryos are crucial for protection from predators and harmful UV, and thus damaging these shells could make the larvae more vulnerable to environmental factors [12]. The deformations were only present in egg masses in pH 7.6 treatments (acid and acid plus heat treatments), which suggests that ocean acidification may affect proper shell development and lead to deformities that may compromise the larva’s ability to survive. Studies of other calcifying larvae such as sand dollars and sea urchins similarly found a reduced calcification rate and increased deformities in larvae treated under acidic conditions [12]. The outer mantle of the sea hare protects its shell from further pitting and deformations from acidic conditions when the sea hares become mature adults. However, many other marine calcifying organisms, such as reef building corals, are affected by shell pitting throughout their life, and this study suggests that at the highly reduced pH expected in the ocean within the century, deformities and calcification difficulties would become increasingly present. This could result in population declines of many calcifying organisms, which could interrupt the food web on coral reefs as well as within other ecosystems.

Not only are oceans becoming more acidic, but they are also warming. This adds a secondary stress to marine organisms [1]. However, there was no significant difference observed between the acid and the acid plus heat treatment in this study. This could be due to limited numbers of measurements and replicates in this study. Qualitatively, the egg masses in the acid plus heat treatments appeared more discolored and deteriorated than the egg masses in the acid treatment alone, which supports the observed lower survival rate in the acid plus heat treatment. There was no significant difference observed in any metric between the acid treatment and the heat treatment, with the exception of shell pitting, which suggests that both stressors are similarly detrimental. These results demonstrate a need for further investigation on the combined effects of acidification and temperature rise on the development of sea hare larvae and other marine organisms.

This study suggests that the increasingly acid and warm waters due to climate change may threaten the future survival of *A. dactylomela*. This has broader implications for coral reef ecosystems overall as sea hares play a critical role in maintaining the balance between macroalgae populations and corals. As coral reefs struggle to calcify in waters under-saturated with carbonate due to ocean acidification, macroalgae will have greater opportunity to grow on the damaged reefs. A decrease in the population of a key herbivore like *A. dactylomela* would become a
compounding problem as there would be reduced herbivory on the macroalgae. These two factors together could contribute to a phase shift from a coral to an algal dominated reef and the loss of valuable coral reef ecosystems around the world [13]. This shift could also disrupt entire reef ecosystems, as many other organisms that are dependent on the corals for food and shelter would also come under threat due to their loss of habitat.

Further research on the impacts of acidification and sea surface temperature rise is key to understanding how sea hares will react to the changing ocean environment. Continued observations of sea hare larvae throughout the planktonic larval stage, and perhaps over the course of their entire life, will offer insight into how acidification and heat affect the survival of sea hares to sexual maturity. Such observations could help predict how the sea hare populations will be impacted in the future. Understanding the threats to key herbivores like sea hares can inform population management, which is key to preventing coral reefs from shifting to algal dominated systems in the future. Assessing the impact of ocean acidification and sea surface temperature rise on sea hares and other marine calcifying larvae will be critical in understanding and protecting sensitive coral reef environments and other ecologically important calcifying organisms that are important in the biological food web as well as the marine carbon cycle.

ACKNOWLEDGEMENTS

I would like to thank the staff at Heron Island Research Station for their help in the field and Dr. Selina Ward for designing and providing the experimental ocean acidification system. I would like to thank Dr. Kevin Arrigo for his help with the project. I would also like to thank the International Programs Office of University of Queensland and the Bing Overseas Program of Stanford University for making this research possible.

REFERENCES


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Anja is a senior majoring in environmental systems engineering with a focus on the coastal environment. Originally from Seattle, Washington, Anja’s drive to care for the marine environment started young from growing up in and around the Puget Sound. At Stanford, Anja does research in the environmental engineering department and pursues her other passion of rock climbing by both working at the Stanford climbing gym and being on the climbing team. She plans to continue her research on impacts on the ocean in graduate school pursuing a doctoral degree in environmental engineering studying plastic pollution. In her free time, Anja loves getting outside to go climbing, hiking, and exploring the world.