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ENVR 451 - Research in Panama

THE EFFECTS OF WATER TEMPERATURE ON REPRODUCTION IN CALYPTRAEID GASTROPODS

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Presented to Dr. Roberto Ibáñez and Dr. Rafael Samudio

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1. LOGISTICS OF INTERNSHIP

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1.2 Supervisor

We would appreciate it if McGill could send a thank-you note to our host supervisor, Dr. Rachel Collin, at the following address:

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1.3 Host Institution

Smithsonian Tropical Research Institute (STRI) Naos Marine Laboratory

The Smithsonian Tropical Research Institute (STRI) in Panama is a branch of the Smithsonian Institution located in Washington DC in the United States. STRI has been dedicated

to the study of biological diversity in the tropics since 1923. STRI has been closely linked to the Panama Canal; its oldest field station in Panama is Barro Colorado Island (BCI), a mountain that became an island when the Chagres River was dammed to flood the canal. In 1923, the Governor of the Panama Canal Zone declared BCI a biological reserve, making it the first such site in the Americas. Today, BCI is one of the world's leading research institutes.

STRI's facilities are used by over one thousand visiting scientists yearly from all over the world due to STRI's unique opportunities for long-term and short-term studies in Panama. STRI offers facilities for its fellows, visiting scientists and thirty-eight staff scientists to achieve their research goals.

In order to provide the necessary facilities, STRI has set up multiple research stations throughout Panama, one of which is the Naos Marine Laboratory, where our internship has taken place. In 1964, STRI established its first marine laboratory in an old military bunker on Naos Island along the causeway. It is a ten-minute drive from Panama City and is located at the Pacific entrance of the Panama Canal. This facility has allowed scientists to study many aspects of marine biology, including the formation of coral reefs, as well as the community, physiological, and behavioural ecology of organisms of the rocky intertidal. It also specializes in molecular work, including DNA sequencing.

1.4 Time spent on internship

Number of full days spent on project in Panama: 36 full days

Number of full days spent in the field in Panama: 1 full day

2. INTRODUCTION

2.1 Abstract

The marine environment on the Pacific coast of Panama is defined by two major aquatic seasons. The first is characterized by displacement of surface waters and upwelling of cold, nutrient-rich waters from beneath, while the second is characterized by warmer waters and lower nutrient availability. The natural variability in environmental conditions in this region is currently being heightened by anthropogenic climate change, which has already led to a global increase in sea surface temperature (SST), and which may cause more intense and unpredictable ENSO events and fluctuations in temperature in the coming years. This study seeks to understand the effect of such changes in temperature on reproduction in the calyptraeid gastropod *Crepidula marginalis*. For the purposes of this project, 240 snails were collected from Playa Chumical in Panama, and were then divided into mating pairs and split into two groups of 60 pairs, one to be kept in 24ºC water, and the other to be kept at 29ºC. Sex change and reproduction were monitored for both treatments, and data was gathered on larval size, breeding frequency, breeding success, rate of sex change, and date and size at first reproduction. The results of the various statistical analyses show that, in warmer temperatures, female *C. marginalis* initiate reproduction earlier, generate a greater number of broods in a given period of time, and produce smaller larvae. These findings suggest either that smaller larvae are better adapted to warmer temperatures, or that females must make a tradeoff between offspring quality (i.e. larger size) and quantity in different environmental conditions. They also indicate that climate change may have important implications for offspring fitness and survival, although more research is required to better understand how offspring size and fitness are related to each other and to water temperature.

2.2 Theory and Background Information

In recent years, anthropogenic climate change has begun to pose serious threats to tropical marine ecosystems. Global sea level has risen at a rate of 1.7 ± 0.5 mm/year over the last century, while global sea surface temperature (SST) has increased by 0.6ºC since 1950 (IPCC 2007). In tropical regions, SST has risen by close to 1ºC since 1900, and is currently increasing at a rate of 1-2ºC per century (Hoegh-Guldberg 1999). Furthermore, a global temperature increase of up to 2.6ºC is predicted in coastal regions by the end of the 21st century (IPCC 2007). These observed changes in water temperature have been coupled with alterations in the pattern and intensity of El Niño – Southern Oscillation (ENSO) cycles over the last several decades, which typically have a period of between two and seven years (Trenberth and Hoar 1996). Although the mechanism driving these cycles is not well-understood, they consist of a coupled atmospheric-oceanic feedback, whereby an initial positive anomaly in the sea surface temperature (SST) of the eastern equatorial Pacific weakens the transport of warm surface waters away from the coast, allowing for upwelling of cold water from beneath. This reinforces the positive SST anomaly, creating a positive feedback loop (Wang 2001). An El Niño event is therefore defined as a prolonged period of anomalously warm sea surface temperatures in the eastern equatorial Pacific, and generally lasts for between nine and twelve months, with occasional episodes lasting up to three or four years (Climate Prediction Center 2005). El Niño events are also counterbalanced by occasional prolonged periods of unusually cold water temperatures, known as La Niña episodes.

Guilderson and Schrag (1998) documented a shift in the climate of the eastern tropical Pacific beginning after the El Niño event of 1976, which they suggested was caused by an increase in SST in that region. They argue that this shift in climate has led to an augmentation of

the frequency and intensity of El Niño episodes since that time. Several other authors have made similar observations about a trend toward greater amplitude and frequency of El Niño events in the last quarter of the 20th century (McGowan et al. 1998; Trenberth and Hoar 1996). Still others have predicted a further shift in this direction during the 21st century, as a result of increasing water temperatures and rising greenhouse gas concentrations. For instance, Collins (2000) simulated ENSO cycles with greenhouse gases at four times pre-Industrial levels, and found that they had 20% greater amplitude and double the frequency of those currently observed. It has also been predicted that an intensification of SST variability will be observed if CO_2 concentrations double in the coming years (Merryfield 2006). Essentially, it appears that anthropogenic climate change may not only increase water temperatures, but may also significantly increase the amount of environmental variability, and particularly the degree of variation in water temperature, with which Pacific coastal organisms have to contend.

Changes in environmental temperature have major implications for reproduction in many species; in fact, temperature has long been regarded as one of the most important factors regulating the breeding period of many marine organisms (Orton 1920). Rising temperatures lead to an increase in the metabolic rate and energy demand of ectotherms, which often forces them to make tradeoffs in terms of energy allocation to different activities. For many organisms, such tradeoffs can result in lowered reproductive success (Nilsson et al. 2009). Shifts in temperature have been shown to influence the likelihood and the timing of reproduction, gamete viability, development time, and the length of the interval between broods for many different species. For instance, Donelson et al. (2010) found that the frequency of reproduction of *Acanthochromis polyacanthus*, a coral reef damselfish, declines with increasing temperature. They also found evidence for a reduction in the rate of spermatogenesis at elevated temperatures. Another study

of several commercial salmonid species likewise showed that elevated water temperature can reduce gamete viability and can negatively affect the development process (Pankhurst and King 2010). In a review of the effects of climate change on benthic invertebrates of tropical reefs, Przeslawski et al. (2008) suggest that some species will experience reduced fecundity in warmer waters. Of course, there is also much evidence to suggest that rising water temperatures will improve reproductive success for some species. Marine copepods have been shown to have shorter brood intervals at warmer temperatures, indicating a higher frequency of reproduction (Rhyne et al. 2009; Williams and Jones 1999). Furthermore, Przeslawski et al. (2008) clearly demonstrate that development and growth rates are positively correlated with temperature for many marine invertebrates.

The effects of changing temperature on reproduction in marine organisms are not limited to adjustments in reproductive success of the parent; variation in temperature may also have important implications for juvenile fitness and survival. Donelson et al. (2010) found that egg size of *Acanthochromis polyacanthus* was reduced at higher temperatures, while Emlet and Sadro (2006) obtained a similar result for the larvae of the barnacle *Balanus glandula*. This is significant because small egg and larval sizes often lead to decreased juvenile growth rate and lower survival rate (Donelson et al. 2008; Emlet and Sadro 2006). Rhyne et al. (2009) also suggested that marine copepods have decreased juvenile survival at elevated temperatures. In effect, the impacts of changing water temperature on marine species may be quite significant, but will most likely vary greatly among taxa. This suggests that studies of the relationship between temperature and reproduction must be conducted using a broad diversity of organisms in order to gain a full understanding of the potential consequences of climate change in marine environments.

It has been predicted that the effects of climate change will be particularly severe for tropical marine organisms, as they have evolved in a relatively stable and predictable thermal environment (Donelson et al. 2010; Tewksbury et al. 2008). However, it is certainly untrue that all tropical marine environments experience a low degree of seasonality or fluctuation in climatic conditions. For instance, the marine environment on the Pacific coast of Panama is characterized by a large degree of diel, seasonal, and interannual variability. Tides in this region are semi-diurnal, with amplitudes of up to 6 m; furthermore, water level at high tide fluctuates according to monthly lunar cycles (D'Croz and Robertson 1997). Seasonal variation in climate is largely determined by the movements of the Inter-Tropical Convergence Zone (ITCZ), which is located directly over Panama between May and December, creating a prolonged rainy season. Between January and April, when the ITCZ is located slightly to the south of Panama, the country experiences its dry season. During this time, northeast trade winds cross the isthmus from the Caribbean side, forcing Pacific coastal surface water away from the shore. This displaced water is replaced by cooler, saltier, more nutrient-rich waters that move up to the surface through a process of upwelling. Thus, the Pacific coastal marine environment is defined by two major sets of conditions that coincide with the wet and dry terrestrial seasons; during the dry season, coastal waters have a mean temperature of 24ºC as well as high nutrient availability from upwelling, while they have a mean temperature of 29ºC and are less productive during the wet season, when wind movement is less important (Collin, personal communication). Finally, interannual fluctuations in marine environmental conditions in this region are largely the product of the ENSO cycles.

Clearly, the diversity of species living in the coastal waters of the eastern Pacific, and particularly those in the intertidal zone, must be well-adapted to fluctuations in environmental

conditions. Not only must they be equipped to deal with lengthy periods of exposure to the open air, but they must also be able to cope with high variability in salinity, nutrient availability, and water temperature. For instance, it is estimated that marine environments along the Pacific coast of Panama can experience a range of temperatures from 15-31ºC (D'Croz et al. 2001). Therefore, it is difficult to predict how intertidal organisms in this region will respond to future variation in water temperature that arises as a result of climate change; analysis of the relationships between temperature and reproduction over a broad range of taxa is necessary in order to elucidate the potential effects of this variation. Gastropods of the family Calyptraeidae are one characteristic group in the rocky intertidal habitat off the Pacific coast of Panama, and their longevity, sedentary lifestyle, and high frequency of reproduction make them ideally suited to this type of study. Calyptraeid gastropods are a diverse and widespread family of filter-feeding marine snails that are generally found in rocky intertidal zones. All members of this family are protandrous sequential hermaphrodites. Within this family, the genus *Crepidula* is the best-studied group, and it is well-represented by a number of species along the coast of the tropical eastern Pacific (Collin 2003a). Two major modes of development are represented in this genus; some species are direct developers, producing juveniles that will immediately attach to the substrate, while others produce planktotrophic larvae that remain motile for some time before attaching and becoming sessile (Collin 2003b; Collin 2004). Significant intraspecific variation in egg and hatchling size has been recorded for *Crepidula* species exhibiting both developmental modes, but little is known about the causes underlying this variation, or about the environmental variables that influence offspring size (Collin, unpublished manuscript). Likewise, it has been suggested that female *Crepidula* may produce broods less frequently at higher temperatures, though this has not been studied in great detail (Collin and Salazar 2010). For these reasons, the

relationship between temperature and reproduction in snails of the genus *Crepidula* warrants some further analysis.

2.3 Research Question and Objectives

With this study, we seek to determine the effects of temperature on various characteristics of reproduction in Calyptraeid gastropods of the genus *Crepidula*. We first examine the effects of two different temperature treatments on hatchling size and then analyze the difference in reproductive success, frequency of reproduction, rate of sex change, and date and size at onset of reproduction between females maintained in each treatment. We hypothesize that females living in warmer waters will have a lower frequency of reproduction and reduced reproductive success, that they will produce smaller hatchlings, and that they will change sex at a slower rate and initiate breeding later than those living in colder waters. In a broad sense, we hope that in completing this project, we will be contributing to an understanding of the ecology and life history of marine gastropods in the tropical eastern Pacific. Although our project will be only one small piece in the larger context of the work performed in Dr. Rachel Collin's laboratory, we hope that it will provide valuable information that will lead to a more detailed picture of the challenges facing organisms in coastal marine environments. Our other major goal in completing this project is to further the understanding of the potential impacts of global climate change on reproductive success and juvenile fitness in marine invertebrates.

This project has also allowed us to pursue a number of more specific objectives. First, we have had the opportunity to spend one full day collecting animals in the field, which provided us with experience in some basic field techniques. We have also been responsible for raising a large number of marine snails in a laboratory setting, and for monitoring their reproductive status and ensuring their continued health. We have collected and analyzed data on the frequency of

reproduction and the hatchling size of these snails at different temperatures, in order to draw conclusions about the ecological implications of changing water temperature. To do so, we had to keep all of our animals in an environment of constant temperature, as well as record the hatching date of every brood and collect the offspring immediately subsequent to hatching in order to obtain size measurements. Our proposed experimental design and analysis required that we learn how to maintain organisms in a laboratory environment, that we organize a database containing all of the information we collected, and that we familiarize ourselves with some basic statistical techniques. In completing all of these elements of the project, we have gained valuable experience that may further our careers in biological research.

Although we hope that the results of this project have scientific merit in and of themselves, we also anticipate that our experiment will be used as a kind of pilot project for further research conducted in Dr. Collin's laboratory. More specifically, we have begun collecting data on the effects of abrupt shifts in temperature on brood interval and hatchling size, by switching each of our females to the second temperature treatment after they have successfully produced two broods. Knowledge of the response of these individuals to such disturbances is important, since fluctuations in SST may become more intense in the coming years (Merryfield 2006). Unfortunately, we are currently collecting only preliminary data for this part of the project, but we hope that this experiment will be continued after our departure, and that the data produced may be combined with our results to generate a more complete picture of the potential consequences of climate change.

Finally, we have also produced a short, informative video that introduces the concept of our project along with the daily routine of life in the Collin laboratory. This video is to be posted on the STRI website in order to generate more interest in marine ecological research on the part

of young scientists. We hope that this video will bring more students with an interest in marine biology to the Naos Marine Laboratories, where they too may further the research being conducted on the changing environment of the rocky intertidal zone.

3. METHODOLOGY

3.1 Study Species

The species *Crepidula marginalis* has been chosen for the purposes of this study, as it is abundant, relatively distinctive, and easy to manipulate and rear in an artificial setting. It is also among those *Crepidula* species that produce planktotrophic larvae. As for all species belonging to this family, *C. marginalis* is a protandrous sequential hermaphrodite, meaning that it begins its life as a small male and gradually becomes female as it grows (Collin 2003a). The male has a penis extending from one side of its head, which becomes progressively shorter over time, eventually disappearing altogether (Appendix 5). The female oviduct and female genital papilla appear shortly after, and are located underneath the foot on one side of the body (Appendix 6). While there is some variation in colour among individuals, this species is generally characterized by a white shell with several thin brown lines extending from the umbo to the edge of the shell. Males mate with females by climbing onto their shells and extending the penis underneath the edge of the shell to locate the female genital papilla. Females produce broods of many eggs that are contained in several capsules (Appendix 8). Upon emergence from the oviduct, the capsules are kept underneath the shell, between the neck and the propodium of the female, until hatching, at which point the larvae disperse into the surrounding water (Appendix 9).

3.2 Study Site

Most of our work was conducted at the Naos Marine Laboratory of the Smithsonian Tropical Research Institute, which is located on Isla Naos, along the causeway extending out

from the mainland just to the west of Panama City (Fig. 1). Our field work, however, was carried out at Playa Chumical, a beach situated near Veracruz, to the west of the Panama Canal. This beach was chosen for its large expanse of rocky intertidal habitat, and because it is known as a site where *Crepidula marginalis* are to be found in abundance. It is also located some distance from the city, which means that the water reaching it should be somewhat less polluted, and it is not heavily frequented by large crowds of people.

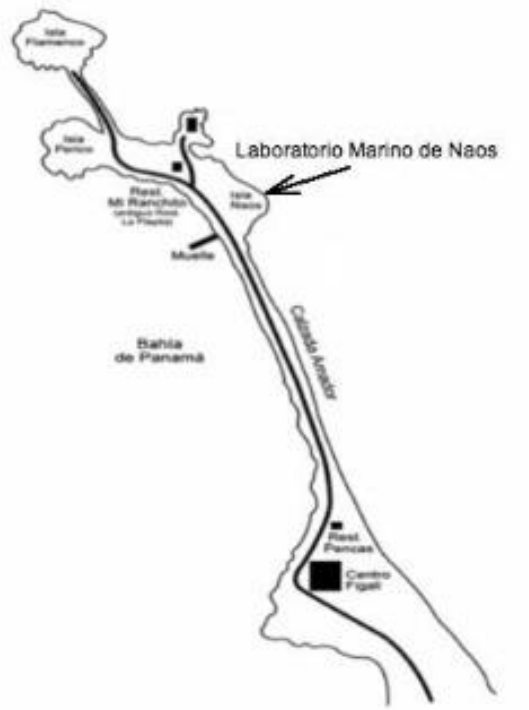

FIGURE 1. Location of Naos Marine Laboratory, Isla Naos, Panama

3.3 Field Collection

We collected marine snails of the species *Crepidula marginalis* from the rocky intertidal habitat of Playa Chumical on 31 January 2010 (Appendix 4). The lowest tide of the monthly lunar cycle occurred on this day, meaning that the greatest proportion of the rocky intertidal was exposed to the open air. The samples were also collected beginning just after the daily low tide had been reached, and for three hours after that time, to ensure access to as many individuals as

possible. *C. marginalis* tend to fasten to the underside of rocks in the intertidal, where they are better protected from the open air, and are more frequently submerged in water. In order to collect them, we overturned small- and intermediate-sized rocks and pried the *C. marginalis* off with our fingers. We gathered a total of approximately 240 individuals, so that we could have a total of 120 female samples, each with a male mate. Since these animals are protandrous sequential hermaphrodites, we selected individuals in the field according to size. We gathered around 120 of the smallest individuals to use as males, as well as another 120 individuals of an intermediate size, such that they were still male, but would become female shortly after their arrival in the laboratory. This was done to ensure that none of the individuals would have reproduced as a female prior to the start of our experiment.

Approximately one month after the beginning of our experiment, we discovered that many of our smaller males were losing their male genitalia and becoming female. At this time, we returned to Playa Chumical for a second half-day of field collection, and we gathered around 30 new males to replace those that had begun to change sex, and to have some extra in case they were needed later on in the experiment. The sex of these new males was verified using a dissecting microscope before they were added to the samples.

3.4 Laboratory Maintenance

Upon our return to the Naos Marine Laboratory, we used a dissecting microscope to verify that all individuals were still male, and we then placed one individual of each size category in a 350-mL plastic cup filled with sea water, where they generally remained for the duration of the experiment. The animals were fed daily with a solution containing 3.86×10^6 cells of the alga *Isochrysis galbana*, and the water in each cup was changed every other day. Between 31 January and 19 March, the sex of each individual was monitored weekly, until the

larger males became female. In cases where both individuals lost their male genitalia simultaneously, the two animals were placed in separate cups and a new male was added to each cup, such that an additional sample was created. Dead snails were also replaced by new individuals up until the onset of reproduction, at which point samples were discarded from the experiment if one of the individuals died. Once a male and female were present together in a cup, if they failed to produce a brood within about the first three weeks, the male would be switched with the male of another sample that had also not produced eggs. In some instances, it appears that females who consistently fail to reproduce with a given male produce eggs readily with a new partner. However, after a female laid her first brood, the male was never replaced with another, even in the case of death, so as to avoid the confounding factor that would be introduced by having multiple broods sired by various males.

3.5 Data Collection

The 120 samples were randomly divided into four groups of 30 individuals, two of which were kept in an incubator set at 29ºC, while the other two groups were kept in a second incubator at 24ºC. These temperatures approximate those that would be experienced in the natural environment during the warm and cold seasons, respectively. For the purposes of our experiment, only a single group of samples in each temperature treatment was required; however, this project is to be continued after our departure with the aim of testing the effect of an abrupt shift in temperature on hatchling size in *C. marginalis*. For this reason, the 60 samples in each incubator were divided into a control group and an experimental group. The control samples are to be maintained in their original incubator throughout the experiment, while the experimental samples are to be switched to the alternate incubator immediately after their second brood has hatched.

Reproduction was monitored by checking the underside of each female for eggs twice daily; once a female had produced a brood, she was checked twice daily until the eggs hatched into larvae. Lay and hatch date data were compiled in spreadsheets (Appendices 1 & 2). Sometimes, females would lose their broods, meaning that they would eject the unhatched eggs from underneath their shells. When this occurred, the eggs were no longer viable. Upon hatching, the larvae of successful broods were collected immediately into a solution of 70% ethanol, which was used to kill and preserve them. Individual larvae were then photographed on their side under a microscope with 100x magnification (Appendix 10). In general, we photographed between 30 and 40 larvae from each brood, although this target was not attained in a few cases where females produced very few offspring. A stage micrometer was also photographed for each brood, at the same magnification as the larvae, in order to provide a scale for larval size measurements. The Shell Convexity and Diameter plug-in of the IMAGEJ program was then used to obtain area, perimeter, and length measurements for each larva. Larvae had to be photographed lying flat on their sides in order for the IMAGEJ program to make accurate estimates of size. The shell length of each female was measured with Vernier calipers after each of her broods hatched, at the same time as the larvae were collected. Shell length was measured along the longest axis of the shell, from the umbo to the edge of the shell. Size measurements could only be gathered after the larvae had hatched, since removing the female on the lay date in order to measure her length would have disrupted the egg capsules.

3.6 Data Analysis

A database was constructed that included all the information gathered throughout the experiment, including the water temperature experienced by each female, the number of successful and unsuccessful broods produced by each female, the lay date and the hatching date

for each successful brood, the lay date and the loss date for each unsuccessful brood, the size of the female on each hatching date, and the area, perimeter, and length of each of the photographed larvae from each brood (Appendix 3). Once this data was gathered, we employed JMP 8 statistical software to analyze the effect of water temperature on hatchling size, frequency of reproduction, proportion of total broods lost, date of sex change, and date and size at first reproduction. We employed a nested ANOVA to test for significant differences in larval size between temperature treatments, with individual females and brood number nested within temperature. This was done in order to distinguish between the variation caused by the different temperatures and that produced by variation within and among females. The proportion of total broods that were lost in either treatment was compared using a Fisher's exact test, as this data consisted of a count rather than mean values, and because the sample size was relatively small. To test for differences between the frequency of reproduction in each treatment, we used t-tests to compare the mean brood duration and interval between broods at each temperature. Brood duration is defined as the number of days between the lay date and the hatch date of a particular brood of eggs, while brood interval is defined as the number of days between the hatch date and the lay date of the subsequent brood. The progression of sex change in the two groups was analyzed using a survival test, which plots the decline in proportion of the total number of individuals that had not undergone sex change over time. For the purposes of this analysis, individuals were recorded as male until they had fully lost the penis and developed a visible oviduct, at which point they were considered female. Finally, t-tests were employed once more to compare mean date and size at first reproduction between treatments.

4. RESULTS

4.1 Effect of Temperature on Larval Size

Larval size measurements were obtained from a total of 56 broods and 42 females. Of these, 36 broods and 25 females were from the warm incubator, while 20 broods and 17 females were from the cold. An average of 27 photos were taken from each brood, each of a distinct larva. A nested ANOVA was employed to test for a significant difference in larval size between treatments, using individual female and brood number as nested factors. Area was used as a measure of larval size, as a certain amount of variation in the shape of individual larvae exists, meaning that the length of the larva is not necessarily the best estimate of its size. Temperature was found to have a significant effect on larval size, with larvae on average 11,321 μm^2 larger in the cold treatment (nested ANOVA, $df = 1$, $F = 3761.07$, $p < 0.0001$) (Fig. 2). However, both individual female and brood number were also found to have significant effects on larval size, with later broods characterized by a smaller mean larval size (female: $df = 40$, $F = 67.72$, $p < 0.0001$; brood number: $df = 14$, $F = 18.84$, $p < 0.0001$).

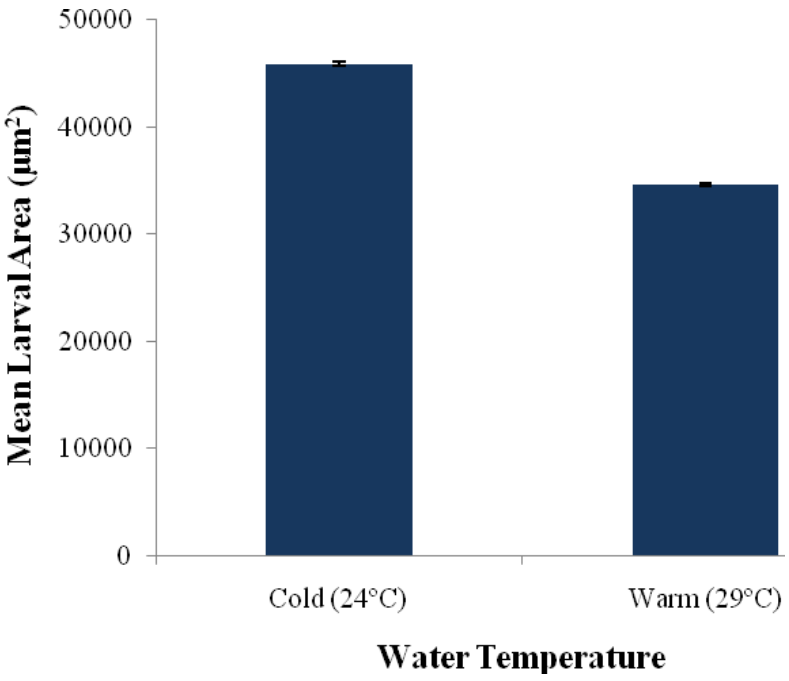

FIGURE 2. The effect of different temperature treatments on mean larval area. Larval size is significantly larger at colder temperatures (nested ANOVA, df = 1, F = 3761.07, $p < 0.0001$). Error bars represent standard error.

4.2 Effect of Temperature on Reproductive Success

Of a total of 32 broods produced in the cold incubator throughout the course of the experiment, 12 were lost, meaning that the female ejected the egg capsules out from underneath her shell before the larvae had hatched, after which the eggs could not survive. In the hot incubator, a total of 60 broods were produced, 16 of which were lost. A Fisher's exact test was used to compare the proportion of total broods that were lost between the two treatments. A smaller proportion of broods failed in the warmer temperature; however, no significant difference was detected in the ratio of lost to successful broods in the two temperatures ($p > 0.1$) (Fig. 3).

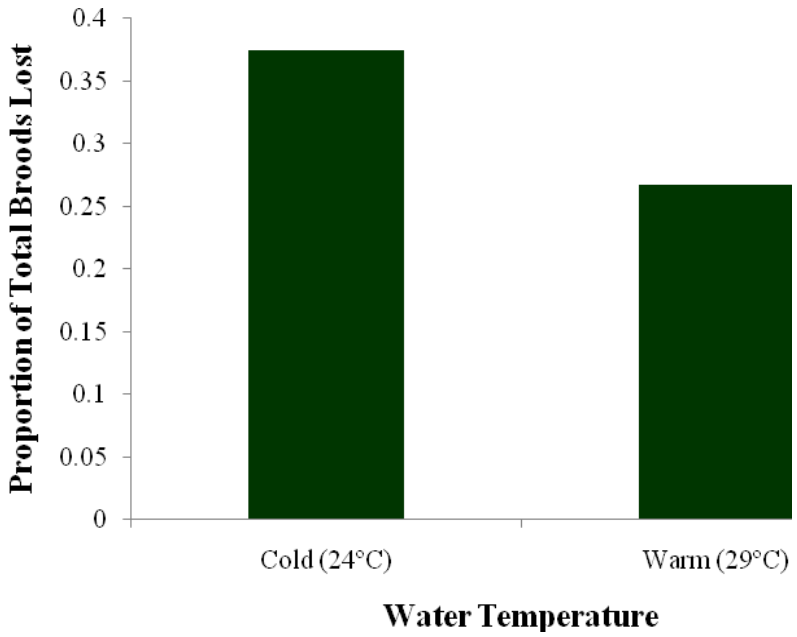

FIGURE 3. Effect of temperature on reproductive success. No significant difference between the two treatments exists (Fisher's exact test, $p > 0.1$).

4.3 Effect of Temperature on Frequency of Reproduction

Brood duration data were gathered for 21 broods from 19 females in the cold incubator, as well as 42 broods from 27 females in the hot incubator. Data on the interval between broods were collected for 10 broods from 8 females in the cold treatment, and 24 broods from 20 females in the warm treatment. Two separate two-tailed t-tests were used to compare the mean brood duration and interval between temperature treatments. A significant effect of temperature on brood duration was found, with an average duration of 8.44 (±1.14) days in the warm temperature and 10.05 (±1.68) days in the cold ($p < 0.0001$). The opposite trend was observed for the interval between broods, with an average interval of 4.00 (±2.18) days in the warm treatment and 3.27 (±1.56) days in the cold treatment. However, this result was not statistically significant ($p > 0.1$) (Fig. 4).

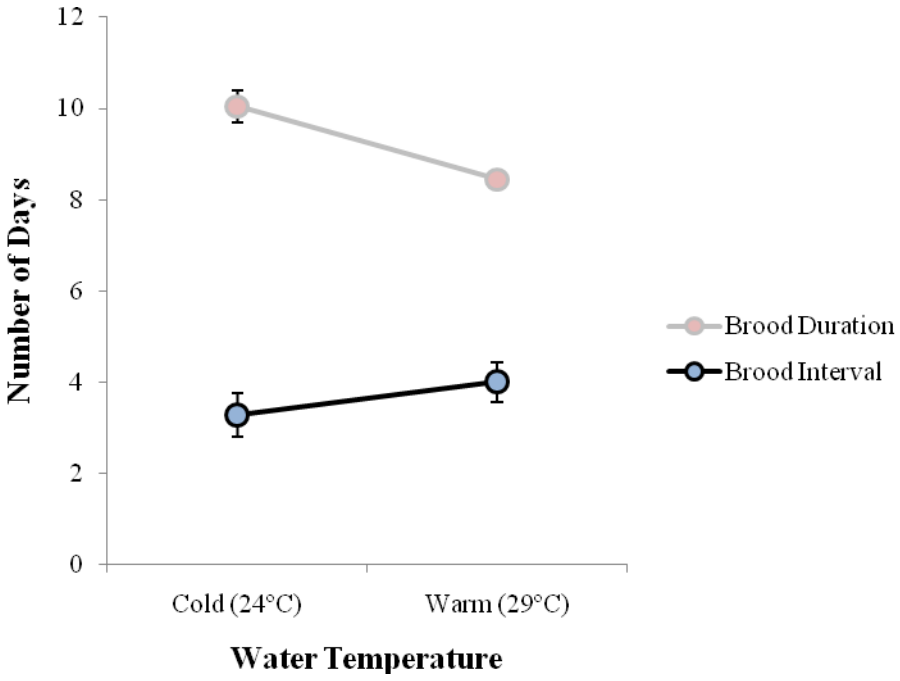

FIGURE 4. Effect of temperature on brood duration and interval between broods. Brood duration is significantly longer in the colder temperature treatment (t-test, $p < 0.0001$). Error bars represent standard error; where they are not visible, the error is smaller than the size of the marker.

4.4 Effect of Temperature on Rate of Sex Change

The sex of 125 larger males, 64 from the warm treatment and 61 from the cold, was monitored weekly in order to document the progress of the sex change process. A survival analysis, which measures the decline in the proportion of individuals that are not yet female, was employed to assess whether differences exist in the progression of sex change between the two incubators. It was found that temperature has no significant effect on the rate of sex change, meaning that individuals in both incubators became female along the same time frame ($p > 0.05$) (Fig. 5).

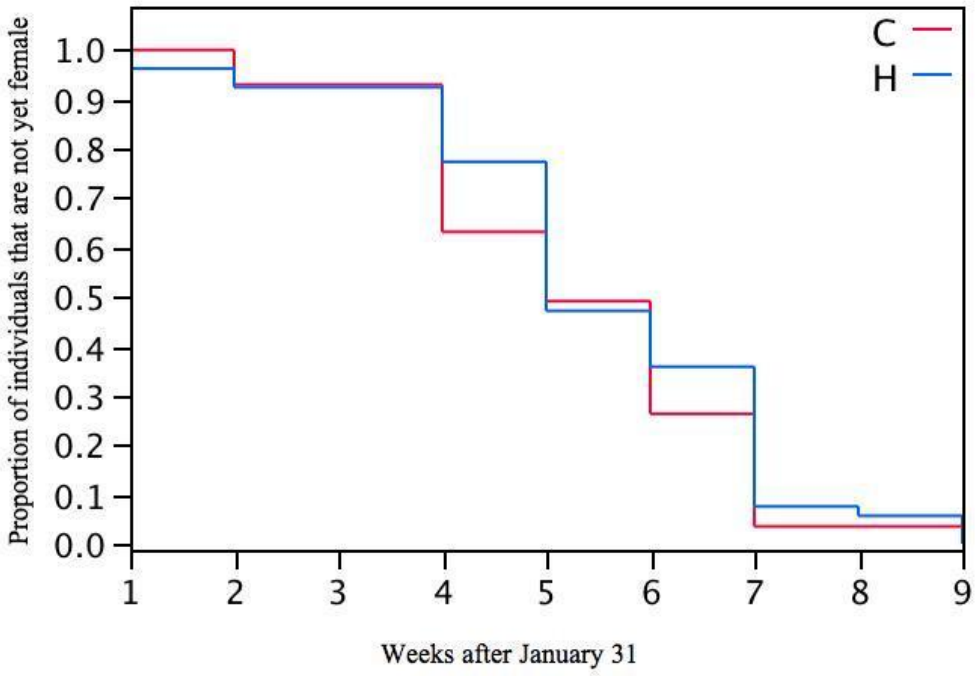

FIGURE 5. Effect of temperature on the rate of sex change from male to female. No significant difference was detected between the two curves, meaning that the progression of the transformation between the two incubators occurred in a similar manner (survival test, $p > 0.05$).

4.5 Effect of Temperature on Date and Size at First Reproduction

Data from the first episode of reproduction were collected from 28 females in the cold incubator and 40 from the hot incubator. Data from both successful and unsuccessful first broods were included. Two separate two-tailed t-tests were used to compare the mean date and female size at first reproduction between the temperature treatments. Date at first reproduction refers to the hatching date, since size measurements were taken on this date rather than the lay date. In the

warm incubator, the mean date at first reproduction was 2 April, while it occurred ten days later, on 12 April, at the colder temperature. Furthermore, the mean length of the females was 15.96 (±1.64) mm in the cold incubator and 14.30 (±1.37) mm in the warm incubator. Both the variation in date and size at first reproduction between temperature treatments are statistically significant ($p < 0.01$) (Fig. 6).

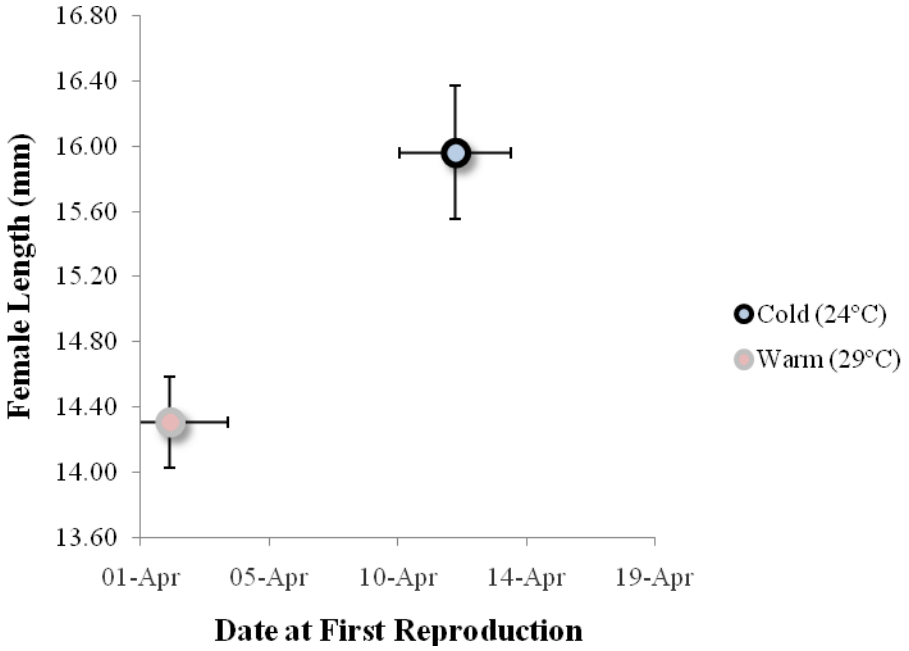

FIGURE 6. Effect of temperature on date and female size at first reproduction. Females initiated reproduction significantly later and were significantly larger at the onset of reproduction in the cold temperature treatment (t-test, $p < 0.01$). Error bars represent standard error.

5. DISCUSSION

5. 1 *Summary of Results*

The results of the various statistical analyses show that temperature has no effect on the rate of sex change in *Crepidula marginalis*. However, they also show that, in colder

temperatures, female *C. marginalis* wait for longer after sex change before initiating reproduction, and that they are larger when they first produce offspring. Likewise, females in colder temperatures maintain their broods of egg capsules for longer underneath their shell before releasing them as larvae. However, there is no significant difference in the interval of days between broods in either temperature treatment. Furthermore, no significant variation was detected in the success of reproduction between the two temperatures. These results refute the initial hypothesis, which suggested that frequency and success of reproduction would decline with increasing temperature, and that sex change and onset of reproduction would occur earlier in the cold temperature. Finally, the results demonstrate that larvae of *C. marginalis* are larger at lower temperatures, which lends support to the original hypothesis.

5. 2 Larval Size

In accordance with the results of previous studies of various marine organisms, a negative relationship exists between water temperature and larval size in *C. marginalis* (Donelson et al. 2010; Emlet and Sadro 2006). Although the reasons for this variation in size with temperature are not well-understood in this organism, there is evidence to suggest that a similar relationship between temperature and offspring size may be quite a widespread phenomenon that extends across a broad range of taxa. For instance, Collin and Salazar (2010) explain that larger offspring size at colder temperatures has been documented across several unrelated groups, including insects, fish, and marine invertebrates. This suggests that variation in offspring size may represent a common physiological adaptation that allows a particular species to flourish in a wide diversity of environmental conditions. In endotherms, for example, larger body size is favoured at lower temperatures because of the declining surface area-to-volume ratio with increasing size, which makes heat retention less energetically demanding (Meiri and Dayan 2003). A similar

rationale has been used to explain inverse relationships between temperature and size in certain ectotherms (Cruz et al. 2005), and the same trend has been documented in invertebrates (Cushman et al. 1993). Therefore, it is possible that the results obtained for *C. marginalis* are evidence of the ability of individual females to adjust offspring size in order to improve their physiological and metabolic adaptations to their natural surroundings.

However, it also seems possible that variation in larval size in *C. marginalis* may be related to the important changes in nutrient availability that these organisms experience in their natural environment along the Pacific coast of Panama. As was stated earlier, the presence of nutrients in the water increases dramatically during the dry season, between January and April, when winds force the warm surface water away from the coast, causing cold, nutrient-rich water to well up from underneath. It may be that the seasonal drop in water temperature acts as a cue that stimulates an increase in offspring size of various marine organisms, such that they are able to take advantage of the sudden increase in available nutrients. This would suggest that the change in temperature is not itself the most important variable; rather, it may simply act as a proxy for food availability, which could have more significant implications for the survival and fitness of marine organisms.

Although larval fitness was not assessed in this experiment, because of the need to kill and fix the larvae immediately after hatching in order to obtain accurate measurements of their size, various studies have demonstrated a positive relationship between offspring size and juvenile growth and survival (Donelson et al. 2008; Emlet and Sadro 2006). Therefore, although it is possible on the one hand that the different larval sizes of *C. marginalis* are perfectly adapted to the different environmental conditions in which they exist, it is also possible that certain metabolic constraints prevent offspring from attaining their ideal size at elevated temperatures.

For instance, if food is limiting at high temperatures, because of the higher metabolism attained by females at elevated temperatures and the reduced availability of nutrients (Collin and Salazar 2010), it is possible that smaller larvae may have reduced fitness and may not be perfectly adapted to their natural environment, but that they are simply the best that the females can manage in those conditions.

Although the results demonstrated a significant effect of temperature on larval size, they also showed that individual female and brood number are responsible for a substantial proportion of the variation in hatchling size. While these other sources of variation were not examined further in this study, it is interesting to note that there are significant differences within and among females, and that offspring size is the combined result of a number of different factors. In particular, it was found that larval size declines with increasing brood number. This finding counters the results obtained by Collin (unpublished manuscript), which state that brood number has no effect on egg size in two species of *Crepidula*; however, it conforms to the conclusions drawn by Ito (1997), who found that egg size declines after the onset of reproduction in the opisthobranch *Haloa japonica*, and related this decline to a diminishing investment in reproduction through time.

5.3 Reproductive success

Throughout the experiment, many of the females in both incubators lost multiple broods of eggs. However, the cause or causes of these egg losses are not really known. There was a higher proportion of broods of eggs lost during this experiment than during previous experiments on *Crepidula* executed in the Collin Laboratory (Collin, personal communication). There are many different factors that affect the reproductive success of an organism. For instance, Baeza (2007) found that brood capacity is constrained with increasing body size, causing a higher

proportion of brood losses in a marine shrimp. Furthermore, as seen in *Biomphalaria glabrata,* success in reproduction could be affected by the presence of parasites, forcing the females to compensate for future loss of reproduction by producing many eggs after the onset of infection (Minchella and Loverde 1981).

In this study, we tested the effect of water temperature on brood loss. We found that there was no significant effect of temperature on the proportion of broods lost. However, our sample size for this analysis was relatively small; therefore, perhaps no significant difference could be observed. We believe that nutrient availability may have been a limiting factor for the warm temperature treatment because it was observed that after feeding, the water was cleared within several hours. Knowing that organisms have higher metabolisms at higher temperatures, perhaps the food limitation caused nutritional stress (Collin and Salazar 2010) eliciting the female to lose her eggs. Of course, there are many other factors that could have contributed to this elevated proportion of egg loss which could not be controlled. For instance, the sea water in which the snails were kept was taken directly from the ocean, which may carry chemical cues or debris. This potential reduction in water quality could have affected the snails in an unknown way, leading to the loss of eggs.

5.4 Breeding Frequency

For the purposes of this study, the number of days between the laying and the hatching of a brood of eggs (brood duration) and the number of days between the hatching of one brood and the laying of the next (brood interval) were used as estimates of breeding frequency. Clearly, if the duration of either of these periods were to decrease, this would allow the female to produce a greater number of broods within the same span of time. The results from this study show that brood duration is significantly shorter at higher temperatures, but that brood interval is

marginally longer in the high temperature treatment. However, it is important to note that the sample size from the cold incubator for brood interval was quite small, with data from just ten broods and eight females being used. Therefore, the results from the test for differences in brood interval may not be totally reliable.

In this study, it was hypothesized that breeding frequency would be lower at higher temperatures, in part because Collin and Salazar (2010) observed a trend to this effect in an experiment involving *Crepidula ustulatulina* and *C. atrasolea*. As explained above, they suggested that food availability may have been a limiting factor at the higher temperature, as metabolism increases with temperature, meaning that the demand for nutrients should be augmented in warmer water. Nonetheless, the opposite result was obtained in this experiment; breeding frequency was found to be higher in the warm incubator. This might suggest that food was not a limiting factor in this experiment; however, the concentration of algae fed to the organisms was the same as that used by Collin and Salazar (2010). Therefore, it may be more likely that the observed increase in breeding frequency in the higher temperature treatment is another adaptation of *C. marginalis* to a change in environmental conditions.

As stated earlier, it is possible that smaller larvae are better adapted to warmer temperatures, either metabolically or as a result of the reduced nutrient availability that is characteristic of the warmer aquatic season in the marine environment of the Pacific coast of Panama. It may also be true that females do not have the energy reserves required to produce large larvae during the warm season. It is conceivable, therefore, that a reduction in brood duration at warm water temperatures may not in itself be a product of temperature change, but may simply be a side-effect of the reduction in hatchling size. In other words, if larvae that hatch earlier tend to be smaller, it may be an advantage to females to reduce their brood duration in a

warmer environment in order to maximize their own fitness or that of their offspring. However, it is unlikely that brood duration is the most important determining factor of hatchling size; according to the results of Collin and Salazar (2010), egg size in certain species of *Crepidula* is larger at lower temperatures, which indicates that the effect of temperature is manifested before the larvae even begin to develop. It seems improbable, then, that the increase in reproductive frequency with temperature is simply an artifact of the need for reduced hatchling size.

Another explanation for the observed increase in brood duration in the higher temperature treatment is that it represents a tradeoff between greater hatchling size and greater quantity of larvae. If it were true that larval fitness is positively correlated with size in *C. marginalis*, it could be that females increase their reproductive frequency in warmer water in an effort to compensate for the reduced probability of survival of each individual larva. In this way, they could be ensuring that their reproductive success remains relatively constant over a range of environmental conditions. This type of quantity-versus-quality tradeoff is well-documented in the literature, with a broad range of taxa known to sacrifice offspring size in order to produce a greater number of juveniles in certain conditions (Einum and Fleming 2000). For example, a similarly positive relationship between temperature and number of offspring, as well as a negative correlation with offspring size, has been documented for the carabid beetle *Notiophilus biguttatus* (Ernsting and Isaaks 2000).

5.5 Rate of Sex Change and Date and Size at First Reproduction

The results from this study show that the distribution of sex change dates for both temperature treatments were remarkably similar. However, they also demonstrate that the onset of reproduction took place significantly later for those individuals living in colder water. The fact that sex change occurred at the same rate in both incubators makes it unlikely that delayed

reproductive maturity is at the root of this difference in date at first reproduction. Furthermore, female size was shown to be significantly greater during initiation of reproduction in the cold treatment, which also makes it unlikely that females were growing much more slowly in cold water. It seems, therefore, that individuals are simply waiting to commence reproduction for a longer period of time after becoming female in the cold temperature treatment.

Delayed onset of reproduction at lower temperatures could be related to energetic constraints faced by the female. The production of larger offspring requires a greater investment of energy, which may not be within the capacity of a smaller, less mature individual. Direct relationships between parent size and offspring size are commonly found in the literature (Bernardo 1996; Christians 2002), although Collin (unpublished manuscript) did not find that female size contributed to intraspecific variation in egg size in two species of *Crepidula*. In this case, it could be that individuals that initiate reproduction early simply cannot achieve the hatchling sizes that they will be able to produce later in their lives. This effect of temperature on the timing of first reproduction could be another component of the tradeoff between offspring quality and quantity. Beginning to reproduce earlier in life could be another strategy to increase the overall number of hatchlings produced in a given period of time, while sacrificing the size and potential fitness of each individual hatched.

5.6 Implications of Climate Change for Reproduction

These results provide evidence for two different strategies of reproduction in *Crepidula marginalis* at different temperatures. In warmer water, females of this species will initiate reproduction earlier, produce broods more frequently, and produce smaller larvae than will females living at colder temperatures. This experiment tested the effects of two temperature treatments that were intended to simulate the natural conditions experienced by this organism

during the two distinct aquatic seasons. If these two reproductive strategies represent a tradeoff between offspring quality and quantity, it is possible that overall reproductive success of the individual female remains relatively consistent over both seasons, and that the female is well-adapted to this natural variation in temperature. However, it is difficult to predict how the effects of anthropogenic climate change may influence these adaptations in the coming years. For instance, if sea surface temperature rises significantly in this region, it is possible that overall mean hatchling size will decrease, leading to a general decline in offspring fitness. Alternatively, if smaller larvae are perfectly adapted to the warmer environment, and are not simply the product of a tradeoff between offspring size and number, this could lead to a proliferation of *C. marginalis* in this environment, which could cause important changes in nutrient concentrations and in the community structure of the intertidal zone.

Finally, it is also possible that anthropogenic climate change will disrupt the balance between water temperature, offspring size, and nutrient availability in this region. Climate change scenarios predict both an overall increase in sea surface temperature and an increase in the intensity of abrupt temperature shifts and of El Niño events, and this could have important consequences for organisms that rely on temperature as an indication of some other factor. For example, it is possible that changing temperature acts as a proxy for nutrient availability and as a cue to instigate changes in larval size in *C. marginalis*. However, it is conceivable that future fluctuations in temperature may not be tied to variation in nutrient availability. If larval size increased subsequent to a decline in temperature that was not accompanied by an increase in nutrient concentration, this could have detrimental impacts on offspring survival and fitness. On the other hand, if increases in temperature were experienced independently of declines in nutrient availability, this could actually be beneficial for the larvae. Therefore, although they are difficult

to predict, the potential implications of climate change for reproductive success in *C. marginalis* cannot be ignored.

5.7 Directions for Future Research

Although this study provided some interesting preliminary results about the effects of temperature on reproduction in calyptraeid gastropods, further studies are certainly required to gain a greater understanding of the potential implications of climate change for the rocky intertidal habitat. One major area of future research should be the relationship between offspring size and nutrient availability. Our results show that temperature has an effect on larval size even when food availability remains unchanged; however, it is still unknown whether temperature change stimulates changes in offspring size because of anticipated variation in nutrient concentrations. If so, any disruption of the link between water temperature and food availability could have serious implications for marine organisms. Greater knowledge of the role that nutrient availability plays in determining larval size would help to predict the future consequences of climate change in this region.

Another area where more work is required is the study of changes in fitness with larval size in *C. marginalis*. Although a large body of literature suggests that offspring fitness increases with increasing size, it is not known whether this is the case for this study organism. Without an understanding of the effects of size on fitness, it is impossible to know whether smaller larvae are better adapted to warmer temperatures, or whether they are symptomatic of reduced energetic resources on the part of the parent. If the latter is true, it would suggest that smaller larvae are not ideally adapted to their environments, but rather that they are the outcome of a tradeoff between larval abundance and quality. Offspring fitness and likelihood of survival must be tested for

different juvenile sizes in order to determine how the species is likely to respond to abrupt shifts in temperature and to overall warmer waters.

5.8 Limitations of Results and Difficulties Encountered

Although we obtained some significant results and our methodology was consistent throughout the experiment, there were still some constraints that limited the relevance of our results. Firstly, we tried to keep the measurement of the larva as consistent as possible by assigning each group member to one task; one person took the pictures of the larva while the other used ImageJ software to measure it. This was important because setting the scale on ImageJ requires estimating the beginning and end of the stage micrometer; this must be done in the same way each time to get consistent data. However, even though each student had her task, some pictures of the larvae may have been less focused than others causing the measurements to be slightly different. Furthermore, measuring the larvae in ImageJ requires estimating the boundaries of the larvae, consequently making our data a little less reliable than anticipated.

We also had a constraint with sample size for several variables that we tested. As previously mentioned, a small sample size for reproductive success may have limited the results obtained. Additionally, when a female lost a brood, no larvae were collected from that reproductive episode, and the subsequent brood was then assigned the brood number that should have been allocated to the lost brood. This means that successful broods are numbered relative to each other, and do not always represent the true sequence of reproduction. Furthermore, having a brood lost between broods did not allow us to get a complete data set for brood duration and interval between broods, which reduced our sample size for both variables. Lastly, due to brood losses and time constraints, we had a small sample size for number of broods for each female,

giving us less variation in our data and causing some of our data to not be completely representative of *C. marginalis'* reproduction.

Indeed, time constraints played a role in restricting not only the number of broods collected but also the original project as a whole. At the beginning of our internship we had planned to study the effects of abrupt shifts in temperature on larval size of *C. marginalis*. After collecting the snails in the field at the end of January, it was expected that the snails would have changed sex and begun reproducing by the end of February; however, the sex change took longer than predicted and the snails had only started reproducing in mid-March and reproduction was not fully underway until April. We did not have a choice but to collect males in the field because we had to ensure that the female's full reproductive life was controlled in the laboratory for our experiment. Our original methodology was the same as that used for this paper; however, after the second brood of larvae, the snails would have been switched into the opposite incubator until they produced another two broods. Nevertheless, as mentioned previously, not many females had yet produced a second brood, by the end of the experiment; therefore, the effects of shifts in temperature could not be studied. Another difficulty we had was controlling for water quality. As mentioned before, the sea water used for the snails was directly taken from the ocean, making it hard for us to know whether the quality of the water affected some of our results.

Due to these uncontrollable and unpredictable events, which are to be expected when working with live organisms because there are many variables that affect them, we needed to be adaptable and innovative. We needed to work with the data we had collected and find ways to change our question in an appropriate and advantageous manner. Additionally, we felt that we needed to make novel contributions to the ecology of intertidal marine organisms and to the study of global climate change. However, having rudimentary knowledge in statistical analysis,

this was another challenge to overcome. Fortunately, our supervisor was extremely supportive and helped us every step of the way by teaching us how to use the program JMP 8, by helping us decipher the meaning of the statistical results and by stimulating us to think critically and logically to extract the biological meaning of our data. Furthermore, replicating natural environments is nearly impossible in the laboratory, thus making conclusions about the natural environment complicated.

Lastly, we felt that we did not have as much time in the laboratory as we would have liked. Indeed, having a greater presence in the laboratory would have allowed us to follow the progression of the snails more closely, as well as the experiment as a whole.

6. CONCLUSION

The goal of our internship was to draw conclusions about the effects of changing temperature on properties of reproduction in the calyptraeid gastropod, *Crepidula marginalis*. This organism is a characteristic species of the rocky intertidal zone of the Pacific coast of Panama, which is defined by two major aquatic seasons, one with cold water and high nutrient availability, and the other with warmer water and lower nutrient concentrations. We also sought to shed light on the potential impacts of anthropogenic climate change for the survival and fitness of marine intertidal organisms in this region. The results of our study show that, in warmer water temperatures, female *C. marginalis* initiate reproduction earlier, generate broods more frequently, and produce smaller larvae. This could indicate that this species has different reproductive adaptations for different environmental conditions, and that smaller larvae are better suited to warmer temperatures, either due to reduced thermal or nutritional requirements. However, it may also be true that smaller larvae have a reduced likelihood of survival and decreased fitness, but that they are the best that can be produced in a metabolically or

nutritionally stressful environment. In this case, small larval size coupled with greater breeding frequency may result from a tradeoff that sacrifices some offspring quality for a larger number of juveniles. Heightened rates of anthropogenic climate change may disrupt the balance between water temperature, nutrient availability, and offspring size in the coming years, which could entail serious consequences for the survival and fitness of this marine organism; however, little is yet known about the most likely effect of climate change on reproduction in *C. marginalis*. Future research must seek to examine the relationship between nutrient availability and offspring size, as well as the effect of changing temperature on survival and fitness of the offspring of intertidal organisms.

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9. APPENDICES

APPENDIX 1. Sample of spreadsheet used to monitor the reproductive status of the females on a daily basis

Month: Feb 2010

C. marginalis

CC: Control Low Temp
CH: Control High Temp
EH: Exp. High Temp
EC: Exp. Low Temp

B: Brood
-: No brood
H: Hatch

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
CC 1																															
CC 2																															
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APPENDIX 2. Sample spreadsheet to record the day the female laid eggs, the date of hatching or loss of brood, female size at larval hatching and the tube number of the collected larvae.

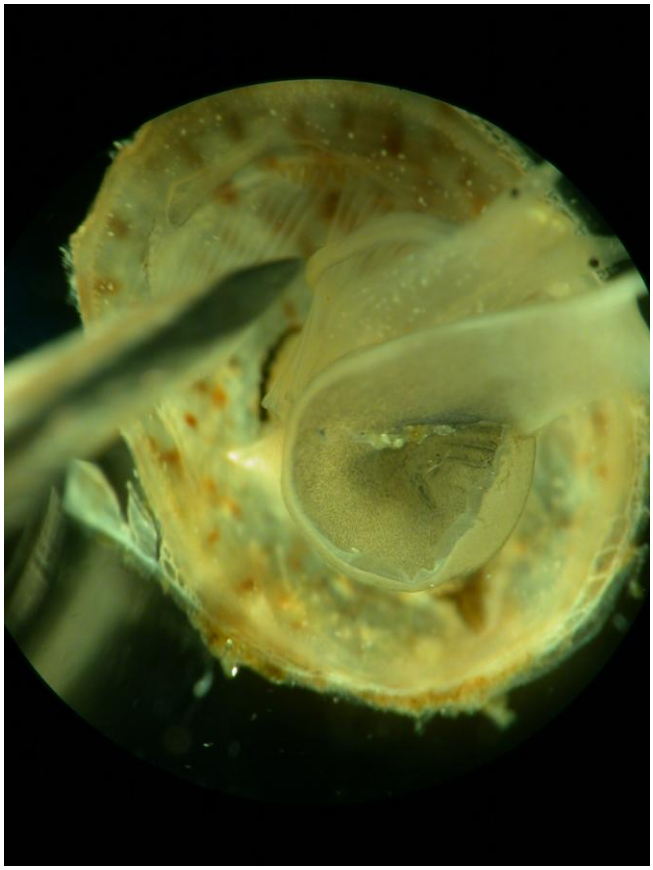
	First Brood					Second Brood				
Female	**SIZE**	**Lay Date**	**Hatch Date**	**Loss Date**	**Tube #**	**SIZE**	**Lay Date**	**Hatch Date**	**Loss Date**	**Tube #**
EH151	14.30	7-Mar-10	14-Mar-10		729	15.20	26-Mar-10	1-Apr-10		16
EH152	13.65	28-Mar-10	4-Apr-10		22*	13.80	7-Apr-10	15-Apr-10		44
EH153		9-Apr-10		9-Apr-10						
EH154	14.55	12-Apr-10	20-Apr-10		64					
EH155										
EH156	14.90	1-Apr-10	9-Apr-10		31		15-Apr-10			
EH157	15.90	20-Mar-10	29-Mar-10		11 few		4-Apr-10		9-Apr-10	
EH158										
EH159		31-Mar-10		31-Mar-10						
EH160	14.00	1-Apr-10	10-Apr-10		36		15-Apr-10			
EH161	15.25	18-Mar-10	27-Mar-10		6	15.10	31-Mar-10	9-Apr-10		29
EH162										
EH163	11.00	6-Apr-10	15-Apr-10		46		19-Apr-10			
EH164										
EH165	16.50	11-Apr-10	18-Apr-10		58					
EH166	14.30	29-Mar-10	10-Apr-10		35		15-Apr-10			
EH167										
EH168	14.15	8-Mar-10	15-Mar-10		1	14.10	20-Mar-10	29-Mar-10		9
EH169	12.90	19-Mar-10	28-Mar-10		8	13.05	1-Apr-10	9-Apr-10		28
EH170										
EH171										
EH172										
EH173		7-Apr-10		9-Apr-10			18-Apr-10			
EH174	15.40	13-Mar-10	23-Mar-10		3	15.75	26-Mar-10	4-Apr-10		21*
EH175	14.15	25-Mar-10	4-Apr-10		20*	14.15	7-Apr-10	16-Apr-10		49
EH176										
EH177	13.85	12-Mar-10	20-Mar-10		2		26-Mar-10		28-Mar-10	
EH178	16.50	24-Mar-10	1-Apr-10		17	16.50	5-Apr-10	13-Apr-10		41
EH179										
EH180	13.55	5-Apr-10	12-Apr-10		38		15-Apr-10			
EH181		13-Apr-10								

APPENDIX 3. Sample spreadsheet of compiled data for whole experiment

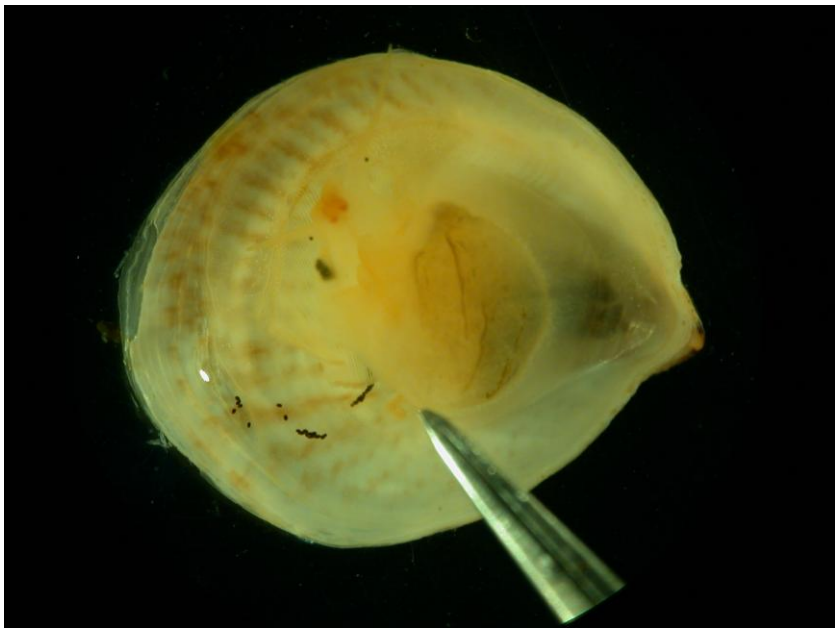
Female	T.	Female length	B. #	Date B.	H. Date	Date B.L	Next B.	B.L	B. length	Int/S	Int/L	Tube #	Area	Perim.	Feret
CH52	H	15	1	38797	38807		38810	38815	10		3	14	32639.879	773.965	268.737
CH52	H	15	1	38797	38807		38810	38815					32203.226	807.575	266.01
CH52	H	15	1	38797	38807		38810	38815					35906.55	821.752	284.669
CH52	H	15	1	38797	38807		38810	38815					37491.954	817.573	286.377
CH52	H	15	1	38797	38807		38810	38815					30679.708	788.379	259.661
CH52	H	15	1	38797	38807		38810	38815					31656.18	762.515	263.086
CH52	H	15	1	38797	38807		38810	38815					33130.268	810.36	270.711
CH52	H	15	1	38797	38807		38810	38815					32885.496	814.81	272.369
CH52	H	15	1	38797	38807		38810	38815					36229.504	828.278	286.835
CH52	H	15	1	38797	38807		38810	38815					35737.27	842.286	285.276
CH52	H	15	1	38797	38807		38810	38815					33896.717	799.36	275.909
CH52	H	15	1	38797	38807		38810	38815					37569.29	841.756	284.798
CH52	H	15	1	38797	38807		38810	38815					31765.805	784.891	267.32
CH52	H	15	1	38797	38807		38810	38815					28260.743	715.916	244.402
CH52	H	15	1	38797	38807		38810	38815					36847.353	828.053	285.759
CH52	H	15	1	38797	38807		38810	38815					33790.706	788.255	275.646
CH52	H	15	1	38797	38807		38810	38815					37243.8	869.362	294.849
CH52	H	15	1	38797	38807		38810	38815					33413.554	796.599	270.051
CH52	H	15	1	38797	38807		38810	38815					31499.277	790.798	265.817
CH52	H	15	1	38797	38807		38810	38815					29748.591	740.77	256.132
CH52	H	15	1	38797	38807		38810	38815					34822.452	795.662	277.137
CH52	H	15	1	38797	38807		38810	38815					35927.537	826.376	291.17
CH52	H	15	1	38797	38807		38810	38815					32968.061	798.316	274.713
CH52	H	15	1	38797	38807		38810	38815					33549.24	796.028	274.377
CH52	H	15	1	38797	38807		38810	38815					32138.958	779.868	272.946
CH52	H	15	1	38797	38807		38810	38815					29781.033	718.597	250.946
CH52	H	15	1	38797	38807		38810	38815					36824.905	867.722	290.955
CH55	H	15.95	1	38792	38801		38804		9	3		5	37274.54	824.744	286.475

APPENDIX 4. Maura collecting *C. marginalis* at Playa Chumical, Veracruz

APPENDIX 5. Penis of a male *Crepidula marginalis*



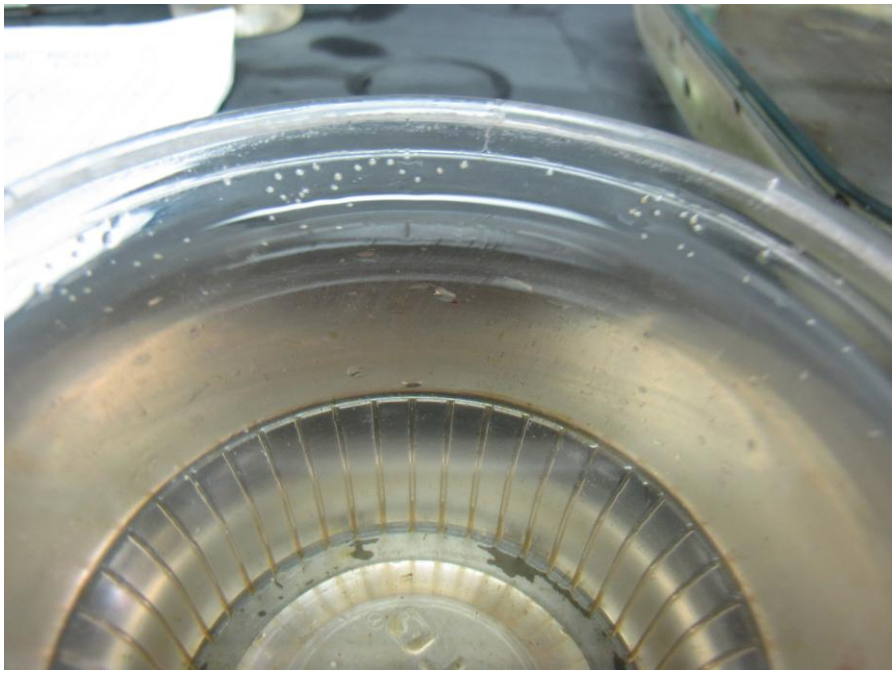
APPENDIX 6. Oviduct of a female *Crepidula marginalis*



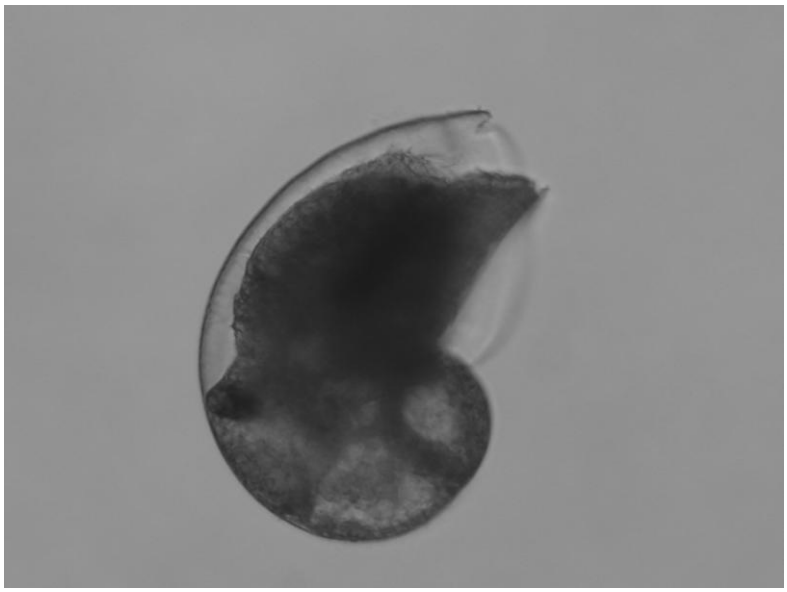
APPENDIX 7. Incubators at 29ºC and 24ºC where the snails were kept

APPENDIX 8. Female *C. marginalis* holding a brood of eggs under her shell

APPENDIX 9. Hatched larvae (white dots along the edge of the cup)



APPENDIX 10. A picture of the larvae of *C. marginlas* through the microscope



APPENDIX 11. Please see attached video on CD. Along with this report, the video represents a part of our final product.