The Response of Molting *Homarus Americanus* to Ocean Acidification Projections

Erin Lyn McLean
*University of Rhode Island*, erinlynmclean@gmail.com

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THE RESPONSE OF MOLTING *HOMARUS AMERICANUS*

TO OCEAN ACIDIFICATION PROJECTIONS

BY

ERIN LYN MCLEAN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
BIOLOGICAL SCIENCES

UNIVERSITY OF RHODE ISLAND
2016
MASTER OF SCIENCE

IN

BIOLOGICAL SCIENCES THESIS

OF

ERIN LYN MCLEAN

Approved:

Thesis Committee:

Major Professor    Brad Seibel

Kathleen Castro

Gabriele Kass-Simon

Nasser H. Zawia
DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

2016
ABSTRACT

Marine calcifiers, especially those in their larval and juvenile stages, are thought to be most vulnerable to ocean acidification (OA) due to the effects of carbon dioxide (CO₂) on growth and shell mineralogy. However, recent evidence is contradictory. We monitored molting activity, length and weight in early benthic phase (EBP, Wahle 1992) American lobster *Homarus americanus* (Milne-Edwards 1837) under elevated CO₂ conditions (500 µatm, 1100-1300 µatm, and 2000-2700 µatm) to determine how OA affects growth at this life stage. Molted shells were analyzed for magnesium (Mg²⁺) and calcium (Ca²⁺) content by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). Exposure to higher CO₂ partial pressures over a 90-120 day period affected intermolt period length and caused decreased length and weight growth increments. The higher concentrations of CO₂ also changed the magnesium to calcium (Mg:Ca) ratio present in the mineralized shell. Shells from lobsters in the medium and high CO₂ treatments had an overall higher Mg:Ca than lobsters in the control CO₂ treatment, which can have consequences for shell dissolution in higher CO₂ conditions. Lobsters in the medium and high CO₂ treatments were also more susceptible to shell disease compared to those in the low CO₂ treatments. Taken together, these results suggest juvenile EBP lobsters may remain smaller for a longer period of time, which could make them more vulnerable to disease and/or shell dissolution in a high CO₂ ocean.
ACKNOWLEDGEMENTS

This work was funded by an American Conservation Wildlife Foundation grant to ELM, a Quebec-Labrador Foundation grant to ELM, and National Science Foundation grants ANT-1246349 and EF-1316113 to BAS. A University of Rhode Island teaching and research assistantship supported me through three years in graduate school. This work would not have been possible without the collaboration of the lobster hatcheries and their managers: David Mills at the Mount Desert Island Oceanarium and John Roy at the Sound School of Aquaculture.

My deepest gratitude to my major professor and advisor, Dr. Brad Seibel, who encouraged me, mentored me, and gave me the opportunity to participate in a wide variety of research activities. Thank you to my committee members, Dr. Gabriele Kass-Simon and Dr. Kathleen Castro, who also advised me and helped to shape this manuscript. Special appreciation is necessary for Dr. Katie Kelley, who allowed access to and taught me to use her equipment, and also reduced shell mineralogy data to a useable form. Additionally, Dr. Candace Oviatt also allowed access to equipment.

Thank you to my academic family: Matthew Birk, Abigail Bockus, Tracy Shaw, Gordon Ober, and everyone else who has been there for me to commiserate with, toss ideas back and forth to, and celebrate successes with. I am truly indebted to my loyal and hardworking team of undergraduate researchers (A. Kelly, G. Simmons, S. Tytlar, T. Schwemmer, R. Krulee, M. Francoeur, L. Salisbury, A. Myers, R. Holmes, and D. Bedortha), who came into lab to collect data, including on holidays and in the snow.
This work also could not have been completed without the love and support of my family, friends, and partner. Thank you for putting up with all the ups and downs that come with undertaking this major accomplishment. I could not have achieved this milestone without your help.
PREFACE

This thesis is written in manuscript form. I intend to submit this work to the scientific journal Marine Ecology Progress Series. The thesis is being prepared for publication.

The following authors are involved in the publication of this manuscript:

Erin L. McLean\textsuperscript{1}, Brad A. Seibel\textsuperscript{2}, Natallia Katenka\textsuperscript{3}

\textsuperscript{1} University of Rhode Island, College of the Environment and Life Sciences, 120 Flagg Road, Kingston RI 02881

\textsuperscript{2} College of Marine Science, University of South Florida, 830 1\textsuperscript{st} Street SE, St. Petersburg, FL 33701

\textsuperscript{3} University of Rhode Island, Department of Computer Science and Statistics, 9 Greenhouse Road, Suite 2, Kingston RI 02881
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MANUSCRIPT INTRODUCTION

“The response of molting Homarus americanus to ocean acidification projections”

by

Erin L. McLean*, Brad A. Seibel2, Natallia Katenka3

Will be submitted to the journal Marine Ecology Progress Series

1 University of Rhode Island, College of the Environment and Life Sciences, 120 Flagg Road, Kingston RI 02881. Email: erinmclean@uri.edu

2 College of Marine Science, University of South Florida, 830 1st Street SE, St. Petersburg, FL 33701

3 University of Rhode Island, Department of Computer Science and Statistics, 9 Greenhouse Road, Suite 2, Kingston RI 02881

Keywords: climate change; ocean acidification; growth; juvenile lobster; shell composition; calcite; aragonite; Mg:Ca ratio
INTRODUCTION

Since the Industrial Revolution, the ocean has acidified by 0.1 pH units and is expected to acidify another 0.35 by the year 2100 (Feely et al., 2004; Stocker et al., 2013: (“business as usual scenario”, RCP 8.5). The decrease in pH resulting from increased dissolved carbon dioxide (“ocean acidification”, abbreviated OA) has been shown to decrease growth, increase mortality, decrease calcification, and increase disease prevalence in many phyla, including marine invertebrates (for review: Hofmann et al., 2010).

Calcification is thought to be the physiological process most affected by elevated carbon dioxide (CO$_2$). The chemical equilibrium of seawater shifts under elevated CO$_2$ such that more of the hydrated CO$_2$ will remain as bicarbonate instead of dissociating all the way to the more bioavailable form, carbonate (Equation 1, Zeebe et al., 2012).

\[
(1) \quad \text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \rightleftharpoons \text{CO}_3^{2-} + 2\text{H}^+
\]

Under those conditions, the saturation state of calcium carbonates ($\Omega$) decreases because the concentration of carbonate ions ($\text{CO}_3^{2-}$) decreases while the concentration of calcium ions and the solubility product ($\text{K}_{sp}$, the concentration of ions that are present in a saturated solution of an ionic compound) stay the same (Equation 2, Zeebe et al., 2012).

\[
(2) \quad \text{saturation state (} \Omega \text{)} = \frac{[\text{Ca}^{2+}][\text{CO}_3^{2-}]}{\text{K}_{sp}}
\]

The mechanisms by which marine organisms produce CaCO$_3$ are diverse, but many rely on increasing pH at the calcification site in order to facilitate CaCO$_3$ precipitation (Cameron & Wood 1985, McConnaughey & Whelan 1997). The
increased pH favors the dissociation of HCO$_3^-$ into CO$_3^{2-}$, which organisms then can use to build their shells (Ries et al. 2009). Increasing the pH comes at a cost, though, and calcifiers must use energy to power mechanisms, like pumping protons through protein channels or extruding hydroxyl groups, to increase the pH of the calcifying space (McConnaughey & Whelan 1997). Several marine calcifiers representing many different phyla have shown reduced net calcification or even dissolution when presented with lower saturation states and/or elevated CO$_2$ (Kleypas & Langdon 2006, Gazeau et al. 2007, Sinutok et al. 2014, Byrne et al. 2014), supporting the link between reduced Ω and reduced calcification. It is possible to show that reduced calcification is due to an increased demand for energy required to operate those pH-increasing mechanisms.

There is presumably a higher energetic cost associated with calcification under OA conditions than under normocapnic conditions. Some calcifiers have responded to decreased Ω with upregulation, therefore increasing calcification processes rather than decreasing them. However, that upregulation comes at a metabolic cost; calcifiers deplete energy reserves in the process, which could impact routine activity (Wood et al. 2008, Vidal-Dupiol et al. 2013). Other studies have shown that decreased pH increases the energetic cost of shell production by causing an upregulation in cell transport mechanisms (Kaniewska et al. 2012).

Each polymorph of calcium carbonate used in shell building has a unique solubility product, $K_{sp}$, leading to different Ω values (Figure 1 and Equation 2; Ries et al. 2011). For example, aragonite, another common calcium carbonate, has a lower Ω than calcite, meaning it is more soluble. Most studies specific to
crustaceans have measured concentrations of magnesium (\(\text{Mg}^{2+}\)) and calcium (\(\text{Ca}^{2+}\)) in the mineralized shell because decapods produce predominantly high-magnesium calcite, which has a lower \(\Omega\) than calcite and is similar to aragonite (Boßelmann et al. 2007, Ries et al. 2011). A higher Mg:Ca ratio in a shell indicates that the \(\Omega\) required for dissolution is reached at a lower \(\text{CO}_2\) concentration (Figure 1, Ries et al. 2011).

Alterations to shell calcification or mineralogy as a result of decreasing \(\Omega\) and increasing \(\text{CO}_2\) may compromise any of the three major functions of the shell: defense from predators, feeding, and disease resistance (Kunkel et al. 2012). Decapod crustaceans were thought to resilient to the effects of increased \(\text{CO}_2\) because they regularly relocate exoskeletal \(\text{Ca}^{2+}\) and \(\text{CO}_3^{2-}\). \(\text{Ca}^{2+}\) is transferred to the gastrolith, a calcium storage organ, and \(\text{CO}_3^{2-}\) is transported to the hemolymph to add buffering capacity prior to remineralization (Roer & Dillaman 1984, Morris et al. 1986). Additionally, the lobster cuticle has a less mineralized biogenic covering (the epicuticle) to help protect it from dissolution (Aiken 1973).

Studies investigating crustacean calcification in terms of \(\text{Mg}^{2+}\) and \(\text{Ca}^{2+}\) content have shown conflicting results. Juveniles of the prawn *Paneaus monodon* increased the \(\text{Ca}^{2+}\) content in their shells in response to a decreased pH, but \(\text{Mg}^{2+}\) showed no response (Wickins 1984). The increased \(\text{Ca}^{2+}\) content was assumed to be a response to the longer intermolt period experienced as a result of reduced pH. On the other hand, elevated \(\text{CO}_2\) reduced \(\text{Ca}^{2+}\) and \(\text{Mg}^{2+}\) content in the carapace of final larval stage (Zoea IV) of *Homarus gammarus*, possibly as a result of OA-induced reduction in net calcification (Arnold et al. 2009).
Calcification and growth are directly linked. If more energy is being allocated to shell construction under OA conditions, there is less available for other processes, including growth, and such processes may be suppressed to compensate. Studies have demonstrated that growth, survival, and developmental rate of crustaceans have been hindered by OA conditions (barnacle, Findlay et al., 2009; shrimp, Kurihara, 2008; larval crab, Walther et al., 2010), but few studies have focused specifically on lobster growth and calcification response to OA. Of those that have, the responses are inconsistent – some have seen no response, others detrimental effects, still others advantageous effects. One study subjected larvae of *Homarus gammarus* to acidified seawater (1200 µatm CO₂) for 28 days, but this did not have an affect on overall carapace length or time in each larval stage (Arnold et al. 2009). Another study held juvenile *H. gammarus* for 35 days in 450 µatm, 1200 µatm, and 9000 µatm and reported a significant growth rate decrease in the 9000 µatm treatment and significantly lower total Ca²⁺ content (Small et al. 2016). Juvenile *H. americanus* were found to increase calcification under future OA conditions in Ries et al. (2009), but the lobsters were held at 25°C, exactly at their upper thermal limit (Lawton and Lavalli, 1995).

Hypercalcification due to temperature stress has been observed before. Adult lobsters developed aragonite deposits in their gills, leading to mortality events, as a result of high (~23°C) bottom temperatures (Dove et al. 2004).

The primary objective of this study was to investigate juvenile lobster growth response and molted shell mineralogy alterations in long-term exposure (90-120 days) to CO₂. Juveniles specifically were chosen because they molt
frequently, are robust in laboratory conditions, and are the first life stage of the American lobster to calcify fully (Anger 2001). Growth and shell mineralogy in lobsters have important ecological and economic consequences in terms of food web and fisheries dynamics.

METHODS

Animal collection and maintenance

Juvenile early benthic phase lobsters were obtained from the Maine Lobster Hatchery at the Mount Desert Island Oceanarium (Bar Harbor, ME) in 2014 and 2015 and the Sound School of Aquaculture (New Haven, CT) in 2015 only. The ME lobsters (starting n = 73 in 2014, n = 102 in 2015) were 4 months post hatch, raised at 17 ± 2°C and pH 7.7 ± 0.4 and the CT lobsters (starting n = 71) were 6 months post hatch, raised at 18 ± 2°C and pH 7.6 ± 0.2. Specimens were randomly distributed into the treatment tanks and kept in treatment for 90-120 days at the University of Rhode Island’s Graduate School of Oceanography in Narragansett, RI.

In 2014, each tank of <34 lobsters was fed 4.2 g of frozen Artemia naupili, thawed in water from the tank, that was randomly distributed to each individual. In 2015, the lobsters were offered 0.5 g wet weight of a gel-based diet (Mazuri Gel Diet for Crustaceans) every three days containing protein (57%), fats (14%), and essential nutrients like calcium, phosphorus, sodium bicarbonate, and fiber (29%). Food intake was monitored to ensure that growth rates and shell composition were not artifacts of feeding. Sex was not determined because at this life stage, growth rates are equal for both males and females (Hughes & Matthiessen 1962). To avoid
cannibalism and allow for consistent measurement, each individual was housed in a separate PVC pipe enclosure within the tank. The environment in the individual enclosures was identical to the homogenous tank environment, and the enclosures were moved around in the tank each day after measurement to avoid placement bias.

**Seawater Quality**

Filtered seawater (~10 µm) in the flow-through CO$_2$ regulation system came directly from Narragansett Bay from pipes fixed 1 m above the bottom (2 – 3 m below surface). This seawater had a salinity of 28-35 ppt and was heated to 17°C, with both parameters close to the optimal range for the summer molting period of this species (Hughes & Matthiessen 1962, Comeau & Savoie 2001). Temperature was measured daily using a Radiometric Thermocouple (Fisher Scientific). Specific gravity was measured in milliSiemens (mS) by a temperature-compensated conductivity meter (Pinpoint Salinity Monitor, precision and accuracy ± 1%) calibrated to a 53 mS standard. pH was measured daily using the OA Best Practices standard operating procedure (SOP) 6b, in which a spectrophotometer (OceanOptics) and pH-adjusted m-cresol purple dye were used to determine the pH of the seawater sample on the total scale (Dickson 2007). pH was also taken from a glass electrode connected to a pH meter (Orion perpHect 310). Total alkalinity was calculated from conductivity measurements using a linear regression model based on salinity, created specifically for Narragansett Bay (Turner 2014). Measured parameters are shown in Table 1.
CO₂ Treatments

Three different partial pressures of CO₂ (pCO₂) were tested: 500 µatm, the low level representing current conditions; 1100-1300 µatm, a medium level that reduced Mg:Ca ratios in Arnold et al. 2009; and 2000-2700 µatm, a pCO₂ level that gave results of increased calcification in lobsters in Ries et al. 2009. To manipulate treatment pCO₂, small amounts of pure CO₂ gas entered the tank via a peristaltic pump hose fed directly into the inflow of an aquarium pump following the modified methods of Jokiel et al. (2014) (Figure 2). The CO₂ immediately saturated in the seawater due to the movement of the impeller, which broke up the gas bubbles, creating a larger surface area for the gas to diffuse into the seawater. The CO₂ decreased experimental pH by 0.319-0.343 units for the 1100-1300 µatm treatment and 0.55-0.624 units for the 2000-2700 µatm treatment (Table 1). pCO₂ and the saturation states of calcite (ΩCa) and aragonite (ΩAr) were calculated using the measured parameters and the Excel macro CO₂Sys using the constants K1 and K2 from Mehrbach et al., 1973 that were refit by Dickson and Millero (Dickson & Millero 1987, Lewis & Wallace 1998). Calculated parameters are shown in Table 2.

Because all lobsters from each population were held in the same tank environment, the individual lobsters are not technically considered to be true statistical replicates, but are instead biological replicates. To allow for such “pseudoreplication” considerations in both the ME15 and CT populations, lobsters were placed into two different sets of tanks. These two sets were found to be not significantly different in all measured environmental conditions – pH, temperature,
and conductivity – by student’s t tests (t < 1.96) (Table 3). Therefore, all lobsters in each treatment level and population for 2015 are considered together.

The design for this experiment allowed for the natural variability of the estuarine environment to be preserved. Because most juvenile lobsters are found in inshore waters to a maximum depth of 50 m, they experience fluctuations of pH brought about by rain events, eutrophication, or other disturbances (Chang et al. 2010). The pH measurements recorded here reflect the natural variability seen in bay environments (Figure 3). When the spectrophotometric method was unavailable, pH for pCO₂ calculation was determined by the regression equation relating the glass electrode pH to the spectrophotometric pH (Figure 4).

Three different groups of lobsters were tested for their response to elevated CO₂: ME lobsters from 2014 (ME14), ME lobsters from 2015 (ME15), and CT lobsters from 2015 (CT). Each treatment population consisted of siblings from the same egg clutch. Total length, carapace length, and weight were measured daily with digital calipers and a balance. Observations on the visual condition of the lobster, such as the softness of the shell, presence of shell disease or exuvia, or missing chelae, were also recorded. In some cases, the lobster consumed the exuvia before it could be collected. In those cases, weight and length measurements were used to determine whether the lobster molted on a given day: a change in length of over 3 mm or in weight of over 0.1 g indicated a molt. Those two criteria were consistent with length and weight changes in observed molts.

Length of intermolt period was determined by the number of days between two observed molt events. Average length and weight percent increases per molt
were calculated for each lobster by taking the average length/weight before the first molt and the average length/weight after the final molt and normalizing the total percent increase to the number of molts (Equation 3).

\begin{equation}
\text{(3) Average \% increase} = \frac{\text{final length or weight}}{\text{initial length or weight}} \times \frac{1}{\text{number of molts}} \times 100\%
\end{equation}

Laser Ablation Inductively Coupled Plasma Mass Spectrometry

Molts were collected from the lobsters as soon as possible. The full molt was removed from the enclosure and the carapace was excised. Mineral analysis was done on the carapace only because the carapace represented the flattest section on the lobster molt, and a flat surface was necessary for the laser ablation to work optimally. The rest of the shed exoskeleton (chelae and telson) was returned to the enclosure with the lobster for ingestion and reabsorption of Ca\(^{2+}\). The carapace was then frozen until analysis.

The mineral content of the molts was measured according to the protocol developed for laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) (Kelley et al. 2003). Each molt was thawed and dried when ready for analysis. A small piece of the molted carapace (~2mm by ~2 mm) was affixed to a glass microscope slide using superglue (Krazy Glue). Molt pieces were chosen to ensure maximum flatness for optimal laser function. Once samples were prepared, they were analyzed in triplicate by LA-ICP-MS to determine Mg:Ca. The laser ablated the material from the shell, creating an 80 \(\mu\)m wide by 120 \(\mu\)m deep cylindrical hole for each replicate. The ablated material then became airborne in a closed, helium-filled vacuum chamber and was channeled to the ICP-MS, where the machine counted the number of atoms of each element. Samples were
compared to geological standards MACS 1 (synthetic carbonate, USGS), NSB-88a (natural dolomite limestone, NBS), and JCP-1 (natural coral calcium carbonate).

**Shell Mineral Composition**

LasyBoy VisualBasic code developed by Joel Sparks was used to determine Mg:Ca in all analyzed molt pieces. Count rate of each element was plotted over time on a log scale and suitable background and signal areas were identified and averaged. Background data was subtracted from the signal and the data were normalized to the concentration of Ca$^{2+}$ in order to remove sample-to-sample variability. Calibration curves were constructed using accepted Mg:Ca ratios in that standards to find the calibration factor with which experimental ratio values could be computed for each sample. Outliers, defined as replicates that caused a relative deviation above 10% between replicates or those that had disturbed signals, were removed. Samples that had that large deviation were often compromised, usually because the laser ablated material from the sample that was contaminated with the glue. Mean Mg:Ca ratios in each level were plotted for both populations.

**Statistical Analysis**

Statistical analysis was done using three major types of hypothesis tests: analysis of variance tests (ANOVA), Kruskal-Wallis tests, and a generalized logistic mixed effect model. ANOVA tests to determine differences between three CO$_2$ group means were performed on data that was normally distributed and had a large (n > 30) sample size (Fisher 1925, McDonald 2014). Kruskal-Wallis tests, a non-parametric test to determine differences between three CO$_2$ group means,
were performed on data that was not normally distributed and had a sample size too low (n < 30) to satisfy the central limit theorem (Kruskal & Wallis 1952, McDonald 2014). A generalized logistic mixed effect model was created to model the prevalence of shell disease in both populations and understand which factors were significantly affecting infection of lobsters in all three tested CO₂ groups (Fitzmaurice et al. 2011).

To test the equality of group means for three levels of CO₂ (low, medium, and high), Analysis of Variance (ANOVA) was applied to the set of continuous variables (pH, pCO₂, Temperature, and Conductivity). These variables were confirmed to follow a normal distribution according to a Shapiro-Wilk test (α < 0.05), McDonald 2014) and have equal variances among three CO₂ levels according to Levene’s test (α < 0.05). Since ANOVA could not be used to examine the equality of population means for variables that were not normally distributed ((α < 0.05); length of intermolt, length and weight growth increments, and Mg:Ca ratios), Kruskal-Wallis non-parametric tests (Kruskal & Wallis 1952) were applied instead. In those cases for which the Kruskal-Wallis test determined significance (α < 0.05), a post hoc multiple comparisons test designed for Kruskal-Wallis tests was applied to determine which levels were significantly different from each other (Siegel & Castellan Jr. 1988, Gastwirth 2013).

Shell Disease Analysis

To test if the shell disease prevalence was significantly different among treatments in both 2014 (n = 73) and 2015 (n = 71), we created a logistic mixed effect model using maximum likelihood estimation (Fitzmaurice et al. 2011).
A generalized logistic mixed effect model is ideal for this data because it estimates the effects of a number of covariates (Time in treatment (time), CO$_2$ level (level), the interaction of time and level, and number of days since last molt (days)) as well as a random individual lobster effect on the shell disease binary response variable. The model also provides confidence intervals and p values ($\alpha < 0.05$) for the corresponding fixed effect coefficients. The model estimates the mean of the shell disease response ($\pi$), which is the probability that a lobster in that population for a given set of parameters will contract shell disease. The logit function transforms the mean non-linearly, which allows the model to correlate a binomial variable with continuous variables (Fitzmaurice et al. 2011). The fixed effect coefficients from this model are in table 9, and the visualization of this model is in figure 11.

To further analyze the results of the generalized logistic mixed effect models, we ran a type II Wald Chi Square test. This test analyzes the nested parameters of the model (days, time, CO$_2$, and group) and checks to see if the change in deviance provided by the model parameter causes a significant difference ($\alpha < 0.05$, Moore 1977). Results from the type II Wald Chi Square test are found in table 9.

All statistical analyses were performed using the R software programming language with the following CRAN packages: lme4 (Bates et al. 2012), car (Fox and Weisberg 2011), lawstat (Gastwirth et al. 2013), pgirmess (Giraudoux 2013), plotrix (Lemon 2006), nmle (Pinheiro et al. 2013), foreign and stats (R Core Team 2012), lattice (Sarkar 2008), and ggplot2 (Wickham 2009).
RESULTS

Environmental Conditions

Measured environmental conditions for 2014 and 2015 are listed in Table 1 and calculated parameters are listed in Table 2. Summary tables listing descriptive statistics and results of ANOVA testing are listed for pH and pCO$_2$ in Table 4, and for temperature and conductivity in Table 5. In both 2014 and 2015, the three treatment levels corresponding to low, medium, and high pCO$_2$ were found to be statistically different from each other (ANOVA, $p < 0.001$) for difference in both pH and pCO$_2$ (Table 4). Neither temperature nor conductivity was statistically different (ANOVA, $p > 0.5$) (Table 5).

Mortality

Mortality was not severe for the CT lobsters (starting total length ~23 mm, weight ~350 mg, Figure 5A and B and Table 6). Mortality followed a consistent, gradual slope with no large mortality event (Figure 6A). The ME14 population of lobsters (starting total length ~15 mm, weight ~130 mg, Figures 5C and D and Table 6) also were robust and withstood routine handling well. The population suffered a steady mortality rate initially, but it leveled off after approximately 30 days in treatment for the low and medium CO$_2$ treatments (Figure 6B). In both CT and ME14, there was a slightly steeper mortality curve for the high CO$_2$ treatment, but early mortality in both populations was more likely due to laboratory and handling stress.

In contrast to the other two populations, the 2015 ME population of lobsters (starting total length ~11 mm, weight ~40 mg, Figures 5E and F and Table
6) suffered from a high mortality rate at the beginning of the study (Figure 6C). 14 out of 34 lobsters in each treatment (42% of total population) died during the acclimation period that lasted the first 7 days, so the trial period began with only ~60% of lobsters in each treatment still alive. The high mortality in all three CO$_2$ treatments was likely the result of their low weight at the beginning of the trial. The lobsters were not robust enough for routine laboratory handling. As a result, the data from this population were excluded from further analysis.

**Length of Intermolt Period**

Our hypothesis that the length of the intermolt period would increase with increasing CO$_2$ was not supported. Only the ME14 population underwent sufficient molts for statistical analysis of the intermolt period (Figure 7). The response to CO$_2$ displayed in the first molt was significantly different from the response displayed in the second and third molt periods (Kruskal Wallis test, $p < 0.00001$, Table 7), so the intermolt periods were analyzed separately. The first intermolt period was significantly longer in the high CO$_2$ treatment than in either of the other treatments (Kruskal-Wallis test, $p < 0.001$, Table 7). However, in the 2$^{nd}$ and 3$^{rd}$ intermolt periods, the high CO$_2$ treatment was significantly shorter than either of the other treatments (Kruskal-Wallis test, $p = 0.001$). The low and medium CO$_2$ levels were not significantly different from each other in any intermolt period (Kruskal-Wallis test, $p > 0.05$).

The CT population molted only twice in the 90 day trial period, so only one intermolt period was observed. There was no difference in intermolt period length among the three CO$_2$ treatments (Kruskal-Wallis test, $p = 0.56$, Table 7).
Length and Weight Growth Increments

Both the CT (n = 54) and ME14 (n = 59) populations showed a decrease in growth increments as CO₂ increased (Figure 8 and Table 8). Both populations showed the same trend in percent length and weight increases per molt. The mean length percent increase with each molt was significantly larger in the low CO₂ treatment compared to the high CO₂ treatment for both populations (Kruskal-Wallis Test, p < 0.05, Table 8). The mean weight percent increase with each molt was only significantly larger in the low CO₂ treatment in the ME14 population (Kruskal-Wallis Test, p < 0.05, Table 8). The CT population showed no significant response to CO₂ in terms of weight percent increase per molt (Kruskal-Wallis test, P = 0.33, Table 8).

Observations of Shell Disease

Lobsters in all three tanks in both the 2014 and 2015 seasons were afflicted with shell disease during the treatment time. Lobsters became infected with shell disease during the intermolt period and it was identified as rust colored spots on the carapace, telson, or chelae. When a lobster successfully completed a molt, the exuvia still had shell disease but it was not present on the new shell (Figure 9). In general, lobsters in the CO₂ treatments experienced shell disease at a far higher frequency than those in the control tank (Figure 10). The highest percentage of lobsters afflicted with shell disease at any one time was 29% in the 1300 µatm tank and 25% in the 2700 µatm tank (Figure 10A).

In 2015, the first onset of shell disease in the CT population was observed in the 1200 µatm treatment after just 3 days in full CO₂ (Figure 10B). A steady
increase in all lobsters infected happened after 70 days in treatment, but there was still a higher percentage of lobsters infected in the highest CO₂ treatment; in the final 22 days of the experiment, the percentage infected did not drop below 70%. The other two tanks never had the percentage of infected lobsters approach that level (Figure 10B).

Shell Disease Model

The equations for each generalized logistic mixed effect model list the coefficients for the fixed effects of time in treatment (time; up to 120 days in 2014 and 90 days in 2015), CO₂ level (level; low, medium, and high), the interaction of time and CO₂ level, and the number of days since last molt (days; up to 48 days in 2014 and 70 days in 2015). The reference for each model is the log odds of shell disease in a low CO₂ treatment lobster at the start of the treatment time (time = 0) with 0 days since the last molt. The units of each coefficient are the natural logarithm (ln) of the probability (π) (the log odds) of the lobster getting shell disease (a “success”) under given conditions.

2015:

\[
\ln \frac{\pi}{1-\pi} = -9.581 + 0.08 \text{ time} + 3.567 \text{ Med level} - 7.58 \text{ High level} + 0.92 \text{ days} \\
-0.052 \text{ Med level} \times \text{ time} + 0.149 \text{ High} \times \text{ time}
\]

2014:

\[
\ln \frac{\pi}{1-\pi} = -147.60 + 1.13 \text{ time} + 134.6 \text{ Med} + 132.5 \text{ High level} + 0.11 \text{ days} \\
-1.15 \text{ Med level} \times \text{ time} - 1.11 \text{ High level} \times \text{ time}
\]
Graphs of the predicted probabilities are shown in figure 11 and can be compared to the observed probabilities shown in figure 10. Significance of coefficient values and analysis of deviance values (\( \alpha < 0.05 \)) are found in table 9.

For the 2015 CT population of lobsters (n = 71), the amount of time (up to 90 days) a lobster spent in CO\(_2\), the days since the previous molt, and the interaction between time in treatment and the three CO\(_2\) levels were all highly significant components determining shell disease presence or absence (Wald II Analysis of Deviance, \( p < 0.0001 \)), but the three CO\(_2\) levels were not significantly different from each other (\( p = 0.47 \)). For the ME 2014 population of lobsters (n = 73), the days since the previous molt and the interaction between time in treatment and the three CO\(_2\) levels were both highly significant to determine shell disease presence or absence (Wald II Analysis of Deviance, \( p < 0.0001 \)), but the amount of time spent in CO\(_2\) (up to 120 days) and the three CO\(_2\) levels were not significantly different from each other (\( p = 0.84; p = 0.85 \)).

**Shell Mineralogy**

The shell composition, specifically the Mg:Ca ratio, for the CO\(_2\) treatments are shown in Figure 11. The molts from the CT population showed no significant response to CO\(_2\) levels (n = 24; Kruskal-Wallis test, \( p = 0.057 \), Table 9). In the ME14 population, the average Mg:Ca in the high CO\(_2\) treatment tank was significantly higher overall than the average Mg:Ca in the low and medium CO\(_2\) tanks (n = 74; Kruskal-Wallis test, \( p = 0.0001 \), Table 9). The mean Mg:Ca ratio (0.14) in each low treatment agrees with other published values of Mg:Ca in *Homarus americanus* (Boßelmann et al. 2007b).
DISCUSSION

Length increment was decreased significantly in both populations (Kruskal-Wallis test, p < 0.05, Table 8) in high CO₂, and weight increment was decreased significantly in the ME14 population (Kruskal-Wallis test, p < 0.01, Table 8). Molar Mg:Ca increased significantly in molted shells in the ME14 high CO₂ treatment (Kruskal-Wallis test, p = 0.0001, Table 9). Additionally, lobsters experienced a higher percentage of shell disease in higher CO₂ treatments (Figure 10).

Mortality and Reduced Growth in Elevated CO₂ Conditions

Because mortality was highest at the beginning of the trial for all studied populations, mortality is likely attributable to laboratory and handling stress rather than elevated CO₂. Previous studies have not reported mass mortalities with CO₂ levels similar to those tested here (500-2700 µatm CO₂). Mortalities were reported in 9000 µatm CO₂ in *H. gammarus*, which the authors did attribute to high CO₂ rather than laboratory and handling stress (Small et al. 2016). Crustaceans have been considered to be tolerant to high CO₂ conditions because they are able to increase hemolymph bicarbonate concentrations to buffer extracellular pH in response to acidosis (Cameron 1978, Henry et al., 1981).

Our observations of significantly reduced growth (length in CT and ME14, weight in ME14 only) in high CO₂ agree with other studies of crustaceans (shrimp, Kurihara, 2008; prawns, Wickins, 1984), including lobsters (Small et al., 2016). The lowered growth in high CO₂ observed by Small et al (2016) was attributed to metabolic depression as shown by a reduced oxygen consumption rate and a
lowered feeding rate. The present study observed no accumulation of food in the high CO₂ treatment enclosures that would suggest a lowered feeding rate in those lobsters.

Another explanation for the reduced growth increments is that the lobsters were calcium limited. Previous studies in crustaceans have determined that Ca²⁺ is required for growth (prawn, Kanazawa et al. 1984; shrimp, Davis & Lawrence 1993). If these lobsters were calcium limited as a result of OA conditions (discussed below), it may have triggered a metabolic suppression response that decreased growth increments (Figure 8, Table 8) as well as increased Mg:Ca ratios in the molted shells (Figure 11, Table 9).

Metabolic depression is an adaptive strategy for short-term adverse conditions like hypoxia (reduced oxygen conditions), temperature stress, or hypercapnia (elevated CO₂ conditions). Exposure to short term high CO₂ conditions causes metabolic depression in many different marine invertebrates (Burnett 1997, Pörtner et al. 1998, Lannig et al. 2010). While metabolic suppression may be an appropriate strategy if the exposure to CO₂ is short-lived, for chronic conditions it is likely unsustainable. As juveniles, lobsters are highly vulnerable to predation, and any reduction in growth rate caused by increased calcification or protein synthesis cost under high CO₂ will prolong this vulnerable stage of life. However, molting more frequently as seen in the ME14 population could allow those smaller lobsters to “catch up,” but that would require additional calcification events. More long-term studies in this area are needed.
The ME14 lobsters were observed over 120 days and molted 3 or more times in that period, resulting in three observed intermolt periods. For the first, the intermolt period increased only in the highest $CO_2$ treatment, which was the expected result (Kruskal-Wallis test, $p < 0.001$, Table 7). The lobsters may have been stressed by the adverse conditions and depressed metabolism initially as an response to higher $CO_2$. Metabolic suppression exhibited through fewer molts has been shown in other crustaceans exposed to hypercapnia (prawns, Wickins 1984; shrimp, Kurihara et al. 2008). Possibly, that stress response has evolved to avoid molt death syndrome (MDS), a term used for all molt-related death events. MDS is common in adverse conditions like hypercapnia (Shields et al. 2006), and when exposed to 9000 µatm, death in $H.\ gammarus$ due to MDS increased significantly (Small et al. 2016).

In contrast, in intermolt periods 2 and 3, the intermolt period for the high $CO_2$ treatment was significantly shorter than that for the medium $CO_2$ treatment (Kruskal-Wallis test, $p = 0.001$). That result implies that growth could be accelerated in higher $CO_2$ once the lobster is past that initial metabolic suppression seen in the first intermolt period.

However, the significantly reduced growth increment (length and weight, Kruskal-Wallis test, $p < 0.001$, Table 8) coupled with the shorter intermolt period suggests that the lobsters in high $CO_2$ tank are facing a physiological tradeoff: to molt more, or to grow more with each molt. Continued exposure to hypercapnia may have resulted in a compensatory strategy consisting of more frequent molting (Table 7) and smaller growth increments (Figure 8). However, that compensation
may not be sustainable in the long term due to considerable predation vulnerability post molt.

Because the CT population began the trial at a larger size, they molted only twice over the 90-day treatment, providing only a single intermolt period. No significant difference was observed in the intermolt period among CO$_2$ treatments (Kruskal-Wallis test, p = 0.56).

A similar stress reaction of more frequent molts but a lower growth increment has been seen in other crustaceans. A study on hermit crabs found that those under shell stress molted at the same rate as those not under shell stress, but those under stress grew less with each molt (Fotheringham 1976). Another study found that Indian white prawns will molt the same number of times at suboptimal pH conditions, but will not grow as much overall (Vijayan & Diwan 1995).

Taken together, the reduced growth increments and the days in intermolt results show that the growth trajectories of juvenile lobsters are altered by higher partial pressures of CO$_2$. If not compensated, growth may be reduced overall and may impact the species on the population level. Further study specifically on metabolic rates and acid-base balance under high CO$_2$ is needed to understand the effect that CO$_2$ has on early benthic phase and other life stages of lobsters.

*Implications for Shell Disease*

Shell disease was present in all tanks and all treatments, even in those that had been previously cleaned, so the disease was not transmitted solely between individuals. It is more likely that the causative agent was present in the water from Narragansett Bay. The vector of infection is still poorly understood, but previous
studies have shown that placing two diseased lobsters in the same water did not end in infection for both lobsters (Quinn et al. 2012), so it is unlikely that lobsters caught the disease from sharing the same water. It is more likely that either the lobsters were experiencing stress and their immune responses were diminished or that the causative agent was directly affected by high CO$_2$.

All populations of lobsters were affected by shell disease, but purely observational data shows that there seemed to be a higher percentage of lobsters affected in the treatment tanks than in the control conditions (Figure 10). Shell disease appeared on the lobsters as rust colored lesions on the shell, consistent with previous shell disease observations on adults (Castro et al. 2012). In many cases, the lobster would exhibit diseased spots on its carapace and telson until it molted. The diseased shell was discarded and the newly molted shell was pristine (Figure 9). Softer-shelled lobsters are more vulnerable to disease (Cawthorn 1997), so increasing the time of the post-molt period when the lobsters harden their shells could enable the proliferation of shell disease. Normally lobsters complete the calcification process 1-2 days post-molt (Aiken 1973), but in some cases in the high CO$_2$ treatments, lobsters remained “soft” well after that period of time. Those same lobsters were often the ones later infected with shell disease (E. McLean, personal observation).

The results from the Wald II Chi Square tests for both the 2014 and the 2015 models (Table 9) indicate that the interaction of time in treatment and the CO$_2$ level the lobster experienced was significant ($\alpha < 0.05$), which shows that as time in treatment increased, the probability of getting shell disease was
significantly different for each CO₂ level (Wald II Analysis of Deviance, p < 0.0001). Therefore, each set of lobsters in the three levels of CO₂ treatment contracted shell disease differently. These probabilities are visualized in Figure 11A and B. Coupled with the observational data in Figure 10, these results suggest that generally, lobsters in the medium and high CO₂ treatments are contracting shell disease more often than lobsters in the low CO₂ treatment. Large decreases in the probabilities of shell disease for each treatment are a result of molting events. Because a molt will rid the lobster of shell disease at least temporarily, a few lobsters molting within days of each other will decrease the overall averaged probability computed by the generalized logistic mixed effect model.

When the probabilities are plotted over the number of days since the previous molt (Figure 11C and D), the results show that lobsters that had longer intermolt periods had a higher chance of having shell disease. This finding agrees with previous results that observed a higher prevalence of shell disease on lobsters that had been in intermolt for longer periods of time (Laufer et al. 2005, Glenn & Pugh 2006). Interestingly, the graph also shows that the lobsters in the medium and high CO₂ treatments were more likely to develop shell disease earlier after a molt than the control lobsters. That finding would support the idea of interrupted calcification in the higher CO₂ treatments leading to a higher prevalence of disease.

One previous study investigated the immune response of a closely related lobster, *Nephrops norvegicus*, and found that a major component of the lobster immune response, prophenoloxidase (PPO), was suppressed after 4 months of
exposure to lowered pH (7.5-7.7) and higher pCO₂ water (1000-1700 µatm/kg) (Hernroth et al. 2012). PPO is normally activated when the thin calcite layer below the less mineralized biogenic epicuticle (the exocuticle) is completely breached. While not a direct investigation of shell disease, that study (Hernroth et al., 2012) suggests that lobsters are more susceptible to disease in OA conditions, a possibility supported by the present results. Possibly, lobsters that had breached calcite layers would activate PPO under control conditions, which likely kept shell disease in those tanks to a low occurrence, but those in CO₂ were unable to do so as efficiently and so were affected more by shell disease.

If the immune suppression is not responsible for the increase in shell disease in higher pCO₂ tanks, the higher pCO₂ could be directly responsible for the growth of the causative agent of shell disease. Testing disease prevalence in CO₂ along with other stressors such as temperature or salinity may be a viable avenue to understand what environmental conditions favor shell disease development.

Changes in Shell Mineral Composition

Shell mineral composition of the two study populations responded differently to high CO₂. In the CT population, there was no significant difference among the three treatments (Kruskal-Wallis test, p = 0.057, Table 9). This result is likely due to the fundamental differences caused by population: the lobsters from CT were larger and older, were reared in slightly different hatchery conditions, and are known to have a thicker shell than lobsters caught in the Gulf of Maine (Homerding 2009). Additionally, because the population was larger and older to begin with, there were fewer molts for the CT population (CT, n = 24; ME14, n =
and as such, the statistical power was decreased. As a result, the response seen in the ME14 population is considered to be more robust.

In the ME14 population, the lobsters in control and medium CO$_2$ conditions maintained a significantly lower Mg:Ca than the high CO$_2$ treatment lobsters (Kruskal-Wallis test, $p < 0.05$). A shell with a lower Mg:Ca means that the shell is less vulnerable to dissolution in more corrosive waters (Figure 1).

Surprisingly, the high treatment molts are likely to be more vulnerable to dissolution in more acidic waters. It is possible that there was a confounding effect with the diet the lobsters were fed. In 2014, they were fed Brine Shrimp Plus Blend (Ocean Nutrition), which was not enriched with calcium like the gel diet the CT lobsters were fed in 2015. However, because all treatments in each year were fed the same food, that likely would not cause differences in Mg:Ca among CO$_2$ levels in the same populations. Another possible confounding effect is that the high CO$_2$ treatment lobsters may have eaten less of the offered food. The mesh at the bottom of the enclosure was wide enough to allow for some food to drift out. That could mean that, despite the fact that we were monitoring food intake, we could have overestimated how much each lobster was eating. The mesh was not large enough to allow chelae/telson pieces to drift out, though, so each lobster was consuming their shed exoskeleton post molt. Calcium for hardening of the shell comes from mostly from the surrounding seawater and the consumption of the shed exuvia (Middlemiss et al. 2016) though, so diet may have had a small impact but likely not enough to cause the differences seen here.
In approximately one third of all molt events, the exuvia were consumed by the lobster before they could be collected for Mg:Ca determination. This has the potential to be a confounding factor in these Mg:Ca ratios – a lobster able to consume the full exuvium may have access to more Ca$^{2+}$ than one consuming the chelae and telson alone. To test that idea, we excluded shell analysis results from lobsters that had consumed the entire molt prior to the analyzed molt. Excluding that data did not change the significance or overall pattern of the data.

Seawater chemistry conditions are likely altering the lobster’s ability to uptake calcium ions for remineralization, which has been seen in other studies on crustaceans. Freshwater crayfish exposed to low pH (5.2) reduced hemolymph Ca$^{2+}$ uptake in acidic conditions, resulting in a 40% total body (including shell) decrease in calcium (Wheatly et al. 1996). More relevant to our study animal, juveniles of red king crab and tanner crabs decreased shell Ca$^{2+}$ content in acidified water (Spicer et al. 2007). The species most similar to *H. americanus*, *H. gammarus*, also decreased shell Ca$^{2+}$ content with little to no change in shell Mg$^{2+}$ content in higher pCO$_2$ conditions (Arnold et al. 2009). The results seen here agree with those findings. The treatment molts had a higher Mg:Ca, which could be due to either a direct reduction in calcium uptake, or a reduction in total calcification. Because the present study, Spicer et al. (2007), and Arnold et al. (2009) all measured shell calcium content, separating the effect of reduced uptake of Ca$^{2+}$ ions (as seen in Wheatly et al. 1996) from a reduction in calcification is difficult.

Lobsters may experience a dual hardship with regard to shell formation under OA conditions. The lower pH of the surrounding seawater may inhibit
calcium uptake, increasing the Mg:Ca. That increase in Mg:Ca will then make the shell more thermodynamically prone to dissolution in higher pCO$_2$ waters (Figure 1). Additionally, the reduction in total body calcium will have consequences for subsequent molts. There will be less total calcium available in the molted shell for the lobster to consume post molt, and there will be less calcium that could potentially be resorbed into the calcium storage organ, the gastrolith. While the gastrolith is a minor source of Ca$^{2+}$ (Wheatly 1999), the cascade of Ca$^{2+}$ reduction in each subsequent molt may inhibit development in a high CO$_2$ ocean.

**CONCLUSIONS AND BROADER IMPACTS**

Juvenile *Homarus americanus* subjected to elevated CO$_2$ conditions exhibit decreased growth and altered shell mineralogy. Both length and weight growth increments were reduced in sample populations over a 90-120 day period. The shell Mg:Ca ratio increased in the medium and high, CO$_2$ treatments relative to control. Additionally, lobsters experienced a higher percentage of shell disease in higher CO$_2$ treatments.

These results are particularly important in light of the lobster’s economic importance in Maine and southeastern Canada. Although lobster mass landings are low compared to total fisheries landings (1% in Maine, 5% in Nova Scotia), they represent a much larger proportion of income from fisheries (10% and 34%, respectively; Driscoll et al., 2015). If lobster populations grow more slowly to adult size in response to ocean acidification, as this study shows, then the population may see a bottleneck of development more severe than the one they already face as juveniles of the species. Staying smaller longer is a dangerous
position for any juvenile, as it increases the probability of predation and reduces the number surviving to either reproductive status or fishery recruitment. More research over a longer period of time is needed to confirm the effect of reduced growth and to see if lobsters will be able to compensate by molting more frequently.
Table 1: Measured parameters of seawater chemistry shown as means ± standard deviations in all treatments in 2014 and 2015. Units are: meter pH – mol/kg of H$_2$O; spectrophotometric (spec) pH – mol/kg of seawater; conductivity – mS; temperature - °C.

|                | Measured Parameters |          |          |          |
|----------------|---------------------|----------|----------|----------|
|                | Meter pH            | Spec pH  | Conductivity | Temperature |
| 2014 Low       | 8.02 ± 0.09         | 45.14 ± 2.35 | 16.70 ± 0.86 |
| 2014 Medium    | 7.68 ± 0.08         | 45.11 ± 2.29 | 16.72 ± 0.94 |
| 2014 High      | 7.40 ± 0.17         | 45.12 ± 2.26 | 16.72 ± 0.97 |
| 2015 Low       | 8.19 ± 0.13         | 7.92 ± 0.06  | 47.20 ± 1.59  | 16.44 ± 0.58 |
| 2015 Medium    | 7.87 ± 0.11         | 7.64 ± 0.08  | 47.22 ± 1.52  | 16.38 ± 0.62 |
| 2015 High      | 7.64 ± 0.15         | 7.40 ± 0.14  | 47.30 ± 1.67  | 16.31 ± 1.28 |
Table 2: Calculated parameters of seawater chemistry shown as means ± standard deviations in all treatments in 2014 and 2015. Units are: salinity – ppt; alkalinity – umol/kg of seawater; pCO$_2$ – µatm; ΩCa and ΩAr – unitless.

|       | Calculated Parameters |
|-------|-----------------------|
|       | Salinity | Alkalinity | pCO$_2$ | ΩCa | ΩAr |
| 2014  |          |            |         |     |     |
| Low   | 29.90 ± 1.71 | 2056.06 ± 108.35 | 567.77 ± 134.49 | 2.50 ± 0.52 | 1.60 ± 0.33 |
| Medium| 29.88 ± 1.69 | 2054.78 ± 106.83 | 1329.81 ± 290.49 | 1.19 ± 0.30 | 0.77 ± 0.18 |
| High  | 29.88 ± 1.66 | 2055.21 ± 105.49 | 2714.82 ± 807.85 | 0.70 ± 0.47 | 0.45 ± 0.30 |
| 2015  |          |            |         |     |     |
| Low   | 30.89 ± 0.77 | 2110.19 ± 150.35 | 533.19 ± 88.70 | 2.71 ± 0.35 | 1.73 ± 0.22 |
| Medium| 30.90 ± 0.73 | 2111.26 ± 155.31 | 1067.11 ± 227.16 | 1.54 ± 0.29 | 0.98 ± 0.18 |
| High  | 30.96 ± 0.81 | 2127.34 ± 78.36 | 2072.60 ± 830.24 | 0.93 ± 0.30 | 0.59 ± 0.19 |
Table 3: T tests between individual tank replicates in 2015; non-significance determined by tabulated t values (t < 1.96). Units are: conductivity – mS; temperature - °C; spectrophotometric (spec) pH – mol/kg of seawater.

|                     | Control   | Medium CO₂ | High CO₂ |
|---------------------|-----------|------------|----------|
|                     | Tank 1    | Tank 4     | Tank 2   | Tank 5   | Tank 3   | Tank 6   |
| Mean Conductivity   | 47.3982   | 47.0003    | 47.4194  | 47.0244  | 47.5050  | 47.0845  |
| t value             | 1.8582    | 1.9267     | 1.8754   |          |          |          |
| Mean Temperature    | 16.3919   | 16.4935    | 16.3155  | 16.4454  | 16.1550  | 16.4676  |
| t value             | 1.3096    | 1.5413     | 1.8231   |          |          |          |
| Mean spec pH        | 7.9283    | 7.9134     | 7.6449   | 7.6358   | 7.3850   | 7.4093   |
| t value             | 1.8273    | 0.8033     | 1.1947   |          |          |          |
Table 4: Analysis of Variance (ANOVA) tables for pCO$_2$ and pH values in both 2014 and 2015. Abbreviations used: Sum of squares (SS), degrees of freedom (df), mean squares (MS), F statistic (F), probability (p), critical F value (F crit). Units are: pH in 2014 – mol/kg of H$_2$O; pH in 2015 - mol/kg of seawater; pCO$_2$ – µatm.

### pH 2014

#### Summary

| Groups     | Sample size | Sum  | Mean    | Variance |
|------------|-------------|------|---------|----------|
| Low pH     | 115         | 922.36 | 8.02052 | 0.00816  |
| Medium pH  | 113         | 867.84 | 7.68    | 0.00632  |
| High pH    | 113         | 835.82 | 7.39664 | 0.02733  |

#### ANOVA

| Source of Variation | SS   | df | MS   | F     | p-level | F crit |
|---------------------|------|----|------|-------|---------|--------|
| Between Groups      | 22.25861 | 2 | 11.1293 | 800.58575 | 0.002244 |
| Within Groups       | 4.69869   | 338 | 0.0139 |        |         |        |
| Total               | 26.9573 <800.58575> | 340 <0.002244> | <0.002244> | <0.002244> | <0.002244> | <0.002244> |

### pCO2 2014

#### Summary

| Groups     | Sample size | Sum  | Mean    | Variance |
|------------|-------------|------|---------|----------|
| Low pCO2   | 115         | 65,293.82672 | 567.77241 | 18,088.45762 |
| Medium pCO2| 113         | 149,194.06799 | 1,320.30149 | 65,773.41024 |
| High pCO2  | 113         | 306,774.71594 | 2,714.8205 | 652,616.38323 |

#### ANOVA

| Source of Variation | SS   | df | MS   | F     | p-level | F crit |
|---------------------|------|----|------|-------|---------|--------|
| Between Groups      | 270,074,139.85729 | 2 | 135,037,069.92864 | 553.09703 | 0.002244 |
| Within Groups       | 82,521,741.03756 | 338 | 244,147.16283 |         |         |         |
| Total               | 352,595,880.89485 | 340 | <553.09703> | <0.002244> | <0.002244> | <0.002244> |

### pH 2015

#### Summary

| Groups     | Sample size | Sum  | Mean    | Variance |
|------------|-------------|------|---------|----------|
| Low pH     | 219         | 1,735.6114 | 7.92517 | 0.00435  |
| Medium pH  | 201         | 1,528.3362 | 7.60366 | 0.01239  |
| High pH    | 201         | 1,486.78708 | 7.39695 | 0.02081  |

#### ANOVA

| Source of Variation | SS   | df | MS   | F     | p-level | F crit |
|---------------------|------|----|------|-------|---------|--------|
| Between Groups      | 29,8843 | 2 | 14.94215 | 1,217.03499 | 0.0103  |
| Within Groups       | 7,5875 | 618 | 0.01228 |         |         |         |
| Total               | 37,4719 | 620 | <1,217.03499> | <0.0103> | <0.0103> | <0.0103> |

### pCO2 2015

#### Summary

| Groups     | Sample size | Sum  | Mean    | Variance |
|------------|-------------|------|---------|----------|
| Low pCO2   | 219         | 117,156.30911 | 534.96032 | 8,332.99878 |
| Medium pCO2| 201         | 214,785.88224 | 1,068.58648 | 56,917.65515 |
| High pCO2  | 201         | 416,999.32776 | 2,074.62352 | 672,761.44502 |

#### ANOVA

| Source of Variation | SS   | df | MS   | F     | p-level | F crit |
|---------------------|------|----|------|-------|---------|--------|
| Between Groups      | 254,065,690.89891 | 2 | 127,032,845.44945 | 531,33683 | 0.0103  |
| Within Groups       | 147,752,413.76813 | 618 | 239,081.57568 |         |         |         |
| Total               | 401,818,104.66703 | 620 | <531,33683> | <0.0103> | <0.0103> | <0.0103> |
Table 5: Analysis of Variance (ANOVA) tables for temperature and conductivity values in both 2014 and 2015. Abbreviations used: Sum of squares (SS), degrees of freedom (df), mean squares (MS), F statistic (F), probability (p), critical F value (F crit). Units are: temperature - °C, conductivity – mS.

### Temperature 2015

| Groups  | Sample size | Sum       | Mean      | Variance  |
|---------|-------------|-----------|-----------|-----------|
| Low     | 219         | 3,600.8   | 16.44201  | 0.3307    |
| Medium  | 219         | 3,587.5   | 16.38128  | 0.38832   |
| High    | 219         | 3,588.3   | 16.38493  | 0.40147   |

**ANOVA**

Source of Variation | SS      | df | MS     | F         | p-level | F crit |
--------------------|---------|----|--------|-----------|---------|--------|
Between Groups      | 0.50804 | 2  | 0.25402| 0.68011   | 0.50692 | 3.0095 |
Within Groups       | 244.26703 | 654 | 0.3735 |
Total               | 244.77507 | 656 |        |

### Conductivity 2015

| Groups  | Sample size | Sum       | Mean      | Variance  |
|---------|-------------|-----------|-----------|-----------|
| Low     | 219         | 10,337.23 | 47.20196  | 2.53845   |
| Medium  | 219         | 10,199.93 | 47.2219   | 2.2994    |
| High    | 219         | 10,358.18 | 47.29763  | 2.78261   |

**ANOVA**

Source of Variation | SS      | df | MS     | F         | p-level | F crit |
--------------------|---------|----|--------|-----------|---------|--------|
Between Groups      | 1.11464 | 2  | 0.55732| 0.21931   | 0.80313 | 3.00956|
Within Groups       | 1,654.36334 | 651 | 2.54126|
Total               | 1,655.47798 | 653 |        |

### Temperature 2014

| Groups  | Sample size | Sum       | Mean      | Variance  |
|---------|-------------|-----------|-----------|-----------|
| Low     | 115         | 1,920.0   | 16.69565  | 0.7427    |
| Medium  | 115         | 1,922.3   | 16.71565  | 0.88063   |
| High    | 115         | 1,922.5   | 16.71739  | 0.9404    |

**ANOVA**

Source of Variation | SS      | df | MS     | F         | p-level | F crit |
--------------------|---------|----|--------|-----------|---------|--------|
Between Groups      | 0.03357 | 2  | 0.01678| 0.01964   | 0.98055 | 3.02213|
Within Groups       | 292.26487 | 342 | 0.85458|
Total               | 292.29843 | 344 |        |

### Conductivity 2014

| Groups  | Sample size | Sum       | Mean      | Variance  |
|---------|-------------|-----------|-----------|-----------|
| Low     | 89          | 4,017.6   | 45.14157  | 5.50842   |
| Medium  | 89          | 4,014.35  | 45.10506  | 5.25159   |
| High    | 89          | 4,015.85  | 45.12191  | 5.11324   |

**ANOVA**

Source of Variation | SS      | df | MS     | F         | p-level | F crit |
--------------------|---------|----|--------|-----------|---------|--------|
Between Groups      | 0.05946 | 2  | 0.02973| 0.00562   | 0.9944  | 3.02998|
Within Groups       | 1,396.84618 | 264 | 5.29108|
Total               | 1,396.90564 | 266 |        |
Table 6 – Summary table for Figure 5, showing the mean ± standard deviation in length and weight at each molt event in all three populations. Units are: length – mm, weight – mg.

| CO₂ Level | n  | Change in Length and Weight Over Time |
|-----------|----|-------------------------------------|
|           |    | CT Population                       |
|           |    | Before Length | Before Weight | Length After Molt 1 | Weight After Molt 1 | Length After Molt 2 | Weight After Molt 2 |
| Low       | 17 | 22.68 ± 1.76 | 350.12 ± 78.04 | 24.81 ± 2.37 | 469.38 ± 141.50 | 28.26 ± 2.80 | 695.55 ± 278.76 |
| Medium    | 24 | 22.49 ± 1.28 | 333.25 ± 59.48 | 24.14 ± 1.53 | 428.13 ± 85.17 | 26.52 ± 2.51 | 565.75 ± 162.29 |
| High      | 13 | 23.17 ± 1.76 | 377.46 ± 102.46 | 24.65 ± 1.89 | 446.10 ± 121.92 | 26.96 ± 2.32 | 619.14 ± 220.71 |
|           |    | ME 14 Population                        |
|           |    | Before Length | Before Weight | Length After Molt 1 | Weight After Molt 1 | Length After Molt 2 | Weight After Molt 2 |
| Low       | 18 | 14.79 ± 1.68 | 135.67 ± 24.31 | 18.24 ± 1.12 | 180.87 ± 30.76 | 21.36 ± 1.31 | 290.08 ± 52.94 |
| Medium    | 17 | 15.01 ± 0.79 | 131.65 ± 18.75 | 17.98 ± 0.55 | 173.06 ± 22.97 | 20.93 ± 1.01 | 265.00 ± 40.33 |
| High      | 21 | 15.59 ± 0.98 | 137.48 ± 18.74 | 17.49 ± 2.93 | 183.70 ± 55.16 | 21.80 ± 1.75 | 282.68 ± 82.65 |
|           |    | ME 15 Population                        |
|           |    | Before Length | Before Weight | Length After Molt 1 | Weight After Molt 1 | Length After Molt 2 | Weight After Molt 2 |
| Low       | 13 | 11.62 ± 1.19 | 40.00 ± 18.81  | 13.08 ± 1.89  | 64.08 ± 33.93  | 16.80 ± 1.37  | 138.43 ± 34.95 |
| Medium    | 7  | 11.66 ± 1.21 | 41.00 ± 18.43  | 13.20 ± 1.64  | 61.00 ± 20.17  | 15.48 ± 1.28  | 101.50 ± 28.99 |
| High      | 11 | 11.88 ± 1.05 | 47.64 ± 20.21  | 13.16 ± 1.41  | 62.55 ± 24.45  | 15.32 ± 2.46  | 110.80 ± 53.81 |
Table 7: Summary table for Figure 7 showing the means ± standard deviations of the number of days spent in intermolt in the CT and ME14 populations, as well as the chi square ($X^2$), degrees of freedom (df), and probability (p) values from the Kruskal-Wallis tests.

| CO$_2$ Level | n  | Intermolt Period 1 | n  | Intermolt Period 2 | n  | Intermolt Period 3 | Among CO$_2$ Levels |
|--------------|----|--------------------|----|--------------------|----|--------------------|-------------------|
| Low          | 11 | 40.82 ± 12.53      |    |                    |    |                    | X$^2$ 1.146       |
| Medium       | 12 | 42.92 ± 7.25       |    |                    |    |                    | df 2              |
| High         | 6  | 39.83 ± 6.31       |    |                    |    |                    | p 0.564           |

| CO$_2$ Level | n  | Intermolt Period 1 | n  | Intermolt Period 2 | n  | Intermolt Period 3 | Among CO$_2$ Levels IMP 1 | Among CO$_2$ Levels IMP 2 & 3 |
|--------------|----|--------------------|----|--------------------|----|--------------------|---------------------------|-----------------------------|
| Low          | 17 | 25.35 ± 3.12       | 17 | 35.06 ± 4.08       | 13 | 34.69 ± 5.61       | X$^2$ 28.849              | 15.599                      |
| Medium       | 16 | 27.25 ± 5.01       | 16 | 37.25 ± 5.40       | 10 | 37.80 ± 3.36       | df 2                      | 2                           |
| High         | 12 | 35.25 ± 7.29       | 12 | 31.25 ± 6.11       | 5  | 30.40 ± 5.41       | p 5.43E-07                | 0.0004                     |

CT Population

ME14 Population
Table 8: Summary table for Figure 8 showing the means ± standard error of the percent increases in length and weight per molt in both the CT and ME14 populations, as well as the chi square ($X^2$), degrees of freedom (df), and probability (p) values from the Kruskal-Wallis tests.

| Population | CO₂ Treatment | n   | % Increase in Length per Molt | n   | % Increase in Weight per Molt | Kruskal-Wallis Results |
|------------|---------------|-----|-------------------------------|-----|------------------------------|------------------------|
|            |               |     |                               |     |                              | Length | Weight |
| CT         | Low           | 17  | 10.24 ± 1.09                  | 17  | 35.74 ± 5.62                 | X2     | 6.9    | 2.18   |
|            | Medium        | 24  | 8.50 ± 1.02                   | 24  | 30.43 ± 3.25                 | df     | 2      | 2      |
|            | High          | 13  | 5.92 ± 1.00                   | 13  | 22.19 ± 10.06                | p      | 0.032  | 0.336  |
| ME14       | Low           | 19  | 22.86 ± 1.07                  | 16  | 90.67 ± 5.68                 | X2     | 19.93  | 16.59  |
|            | Medium        | 18  | 19.38 ± 1.03                  | 19  | 71.41 ± 4.68                 | df     | 2      | 2      |
|            | High          | 21  | 16.17 ± 0.70                  | 21  | 51.60 ± 5.64                 | p      | 0.000004 | 0.0025 |
Table 9: Coefficients and Analysis of Deviance results from the generalized logistic mixed effect models from 2014 and 2015 (2014, \( n = 73 \); 2015, \( n = 71 \)). One asterisk represents significance to \( p < 0.05 \), three asterisks represent significance to \( p < 0.001 \).

| 2014 - ME14 Population | Analysis of Deviance Table (Type II Wald chisquare tests) |
|------------------------|----------------------------------------------------------|
| Fixed effects:         | Estimate | Standard Error | z Value | p Value | Significance | Chi Sq | df | p Value | Significance |
| (Intercept)            | -147.60  | 61.64          | -2.395  | 0.0166  | *            |        |    |         |             |
| time                   | 1.13     | 0.53           | 2.118   | 0.0342  | *            |        |    |         |             |
| Medium CO₂             | 134.60   | 61.77          | 2.179   | 0.0294  | *            |        |    |         |             |
| High CO₂               | 132.50   | 61.79          | 2.145   | 0.032   | *            |        |    |         |             |
| Days                   | 0.11     | 0.01           | 10.778  | <2E-16  | ***           |        |    |         |             |
| time:Medium CO₂        | -1.15    | 0.53           | -2.143  | 0.0321  | *            |        |    |         |             |
| time:High CO₂          | -1.11    | 0.53           | -2.079  | 0.0376  | *            |        |    |         |             |

| 2015 - CT Population | Analysis of Deviance Table (Type II Wald chisquare tests) |
|----------------------|----------------------------------------------------------|
| Fixed effects:       | Estimate | Standard Error | z Value | p Value | Significance | Chi Sq | df | p Value | Significance |
| (Intercept)          | -9.580565| 1.272605       | -7.528  | 5.14E-14| ***          |        |    |         |             |
| time                 | 0.079573 | 0.008703       | 9.143   | 2.00E-16| ***          |        |    |         |             |
| Medium CO₂           | 3.567312 | 1.612781       | 2.212   | 0.02697 | *            |        |    |         |             |
| High CO₂             | -7.57916 | 2.414262       | -3.139  | 0.00169 | **           |        |    |         |             |
| Days                 | 0.092488 | 0.007811       | 11.887  | 2.00E-16| ***          |        |    |         |             |
| time:Medium CO₂      | -0.0151975| 0.010987       | -4.731  | 2.24E-06| ***          |        |    |         |             |
| time:High CO₂        | 0.149379 | 0.024348       | 6.135   | 8.51E-10| ***          |        |    |         |             |
Table 10: Summary table for Figure 11 showing the means ± standard deviations of the Mg:Ca ratios in both the CT and ME14 populations, as well as the chi square ($X^2$), degrees of freedom (df), and probability (p) values from the Kruskal-Wallis tests.

| CO₂ Level | n  | Mg:Ca Ratio | Among CO₂ Levels |
|-----------|----|-------------|------------------|
| Low       | 7  | 0.1499 ± 0.030 | $X^2$ 5.727 |
| Medium    | 10 | 0.1876 ± 0.045 | df 2 |
| High      | 7  | 0.1477 ± 0.046 | p 0.057 |

| CO₂ Level | n  | Mg:Ca Ratio | Among CO₂ Levels |
|-----------|----|-------------|------------------|
| Low       | 30 | 0.1441 ± 0.026 | $X^2$ 18.161 |
| Medium    | 18 | 0.1561 ± 0.026 | df 2 |
| High      | 27 | 0.1904 ± 0.051 | p 0.0001 |
FIGURES

Figure 1: Solubility of calcite polymorphs common in marine calcifiers. The calcite described herein has a Mg:Ca ratio range of 0.096-0.026. The vertical colored lines show the average partial pressure of CO2 represent treatments used in this study. Low CO$_2$ treatments are shown in blue, medium CO$_2$ treatments in red, and high CO$_2$ treatments in green. Figure modified from Ries et al, 2011.
Figure 2: The CO₂ delivery system used to regulate pH and pCO₂. The peristaltic pump, shown in (A), was set to a specific RPM and slowly metered pure CO₂ gas into an aquarium pump (close up in B) for immediate solvation of gas.
Figure 3: pH variability in the 2014 and 2015 trials, measured using the NBS scale (2014, A) and the total scale (2015, B).
Figure 4: Meter determined pH as related to spectrophotometrically determined pH. The correlation is robust with an $R^2$ value of 0.691, and the linear regression equation is: $\text{Spec pH} = (0.8343 \times \text{meter pH}) + 1.0858$. 
Figure 5: Mean length and weight trajectories over time in all studied populations (CT, A and B; ME14, C and D; ME15, E and F) shown with standard error bars.
Figure 6: Mortality of all three tested populations as a percentage (CT, A; ME14, B; ME15, C) of the original number of original lobsters over time in treatment. Tables embedded within show starting and ending numbers.
Figure 7: Length of intermolt period(s) for the (A) CT (n = 29) and (B) ME14 (n = 36) populations in the low, medium, and high CO$_2$ treatments. Values are mean ± SE. Asterisks indicate significant statistical differences determined by Kruskal-Wallis tests (p < 0.05).
Figure 8: Average percent increases in length (A) and weight (B) for the CT (n = 54, average number of molts = 2) and ME14 (n = 59, average number of molts = 3) lobster populations in the low, medium, and high CO₂ treatments. Values are mean ± SE. Asterisks indicate significant statistical differences determined by Kruskal-Wallis Tests (p < 0.05).
Figure 9: Recently molted lobsters with shell disease spots on the exuvia (indicated by arrows) but no shell disease spots on the newly formed shell.
Figure 10: Observed presence of shell disease for both populations (ME14, A; CT, B) over the number of days spent in CO₂ treatment.

A  
**Shell Disease Prevalence over Time, ME14**

B  
**Shell Disease Prevalence over Time, CT 2015**
Figure 11: Predicted probability of each lobster population (ME14, n = 73; CT, n = 71) over the number of days in CO₂ (ME14, A; CT, B) and over the number of days since the previous molt (ME14, C; CT, D). Predicted probability values come from the generalized logistic mixed effect models (for equations, see equations 4 and 5; for coefficients, significance, and analysis of deviance, see table 9).
Figure 12: Change in Mg:Ca ratio of molted shells in each of the two populations (CT, n = 24; ME14, n = 75). Asterisks indicate significance from a multiple comparisons test after Kruskal-Wallis test (P<0.05).

Mg:Ca Means by Population

![Bar chart showing Mg:Ca means by population with significance markers.](chart_image)
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