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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
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IN
BIOLOGICAL SCIENCES

UNIVERSITY OF RHODE ISLAND

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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 μ atm, 1100-1300 μ atm, and 2000-2700 μ atm) 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.

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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*¹, Brad A. Seibel², Natallia Katenka³

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

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

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MANUSCRIPT INTRODUCTION

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

by

Erin L. McLean^{*1}, 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 (CO₂). The chemical equilibrium of seawater shifts under elevated CO₂ such that more of the hydrated CO₂ will remain as bicarbonate instead of dissociating all the way to the more bioavailable form, carbonate (Equation 1, Zeebe et al., 2012).

Under those conditions, the saturation state of calcium carbonates (Ω) decreases because the concentration of carbonate ions (CO₃²⁻) decreases while the concentration of calcium ions and the solubility product (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).

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

The mechanisms by which marine organisms produce CaCO₃ are diverse, but many rely on increasing pH at the calcification site in order to facilitate CaCO₃ 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 (Mg^{2+}) and calcium (Ca^{2+}) in the mineralized shell because decapods produce predominantly high-magnesium calcite, which has a lower Ω 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 Ω required for dissolution is reached at a lower CO_2 concentration (Figure 1, Ries et al. 2011).

Alterations to shell calcification or mineralogy as a result of decreasing Ω and increasing 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 CO_2 because they regularly relocate exoskeletal Ca^{2+} and CO_3^{2-} . Ca^{2+} is transferred to the gastrolith, a calcium storage organ, and 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 Mg^{2+} and Ca^{2+} content have shown conflicting results. Juveniles of the prawn *Penaeus monodon* increased the Ca^{2+} content in their shells in response to a decreased pH, but Mg^{2+} showed no response (Wickins 1984). The increased 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 CO_2 reduced Ca^{2+} and 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 $\mu\text{atm CO}_2$) for 28 days, but this did not have an effect 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 Ca^{2+} 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 CO_2 . 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 $17 \pm 2^\circ\text{C}$ and $\text{pH } 7.7 \pm 0.4$ and the CT lobsters (starting $n = 71$) were 6 months post hatch, raised at $18 \pm 2^\circ\text{C}$ and $\text{pH } 7.6 \pm 0.2$. 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 ($\sim 10 \mu\text{m}$) 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 $\pm 1\%$) 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 μ atm, the low level representing current conditions; 1100-1300 μ atm, a medium level that reduced Mg:Ca ratios in Arnold et al. 2009; and 2000-2700 μ atm, 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 μ atm treatment and 0.55-0.624 units for the 2000-2700 μ atm treatment (Table 1). pCO₂ and the saturation states of calcite (Ω Ca) and aragonite (Ω Ar) 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 $p\text{CO}_2$ 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 CO_2 : 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).

$$(3) \text{ Average \% increase} = \frac{\left(\frac{\text{final length or weight}}{\text{initial length or weight}}\right)^{-1}}{\text{number of molts}} * 100\%$$

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 (chela 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 μm wide by 120 μm 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 ($\alpha < 0.05$), McDonald 2014) and have equal variances among three CO₂ levels according to Levene's test ($\alpha < 0.05$). Since ANOVA could not be used to examine the equality of population means for variables that were not normally distributed ($\alpha < 0.05$); 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 ($\alpha < 0.05$), 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), CO₂ 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 (π), 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, CO₂, 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 pCO₂ 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 pCO₂ were found to be statistically different from each other (ANOVA, $p < 0.001$) for difference in both pH and pCO₂ (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 CO₂ treatments (Figure 6B). In both CT and ME14, there was a slightly steeper mortality curve for the high CO₂ 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 CO₂ 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 CO₂ was not supported. Only the ME14 population underwent sufficient molts for statistical analysis of the intermolt period (Figure 7). The response to CO₂ 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 CO₂ treatment than in either of the other treatments (Kruskal-Wallis test, $p < 0.001$, Table 7). However, in the 2nd and 3rd intermolt periods, the high CO₂ treatment was significantly shorter than either of the other treatments (Kruskal-Wallis test, $p = 0.001$). The low and medium CO₂ 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 CO₂ 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:

$$(4) \ln \frac{\pi}{1-\pi} = -9.581 + 0.08 \text{ time} + 3.567 \text{ Med level} - 7.58 \text{ High level} + 0.92 \text{ days} \\ -0.052 \text{ Med level} * \text{time} + 0.149 \text{ High} * \text{time}$$

2014:

$$(5) \ln \frac{\pi}{1-\pi} = -147.60 + 1.13 \text{ time} + 134.6 \text{ Med} + 132.5 \text{ High level} + 0.11 \text{ days} \\ -1.15 \text{ Med level} * \text{time} - 1.11 \text{ High level} * \text{time}$$

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 CO₂, the days since the previous molt, and the interaction between time in treatment and the three CO₂ levels were all highly significant components determining shell disease presence or absence (Wald II Analysis of Deviance, $p < 0.0001$), but the three CO₂ 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 CO₂ 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 CO₂ (up to 120 days) and the three CO₂ 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 CO₂ treatments are shown in Figure 11. The molts from the CT population showed no significant response to CO₂ levels ($n = 24$; Kruskal-Wallis test, $p = 0.057$, Table 9). In the ME14 population, the average Mg:Ca in the high CO₂ treatment tank was significantly higher overall than the average Mg:Ca in the low and medium CO₂ 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 $\mu\text{atm CO}_2$). Mortalities were reported in 9000 $\mu\text{atm CO}_2$ 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 CO₂ 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 a response to higher CO₂. 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 CO₂ treatment was significantly *shorter* than that for the medium CO₂ treatment (Kruskal-Wallis test, $p = 0.001$). That result implies that growth could be accelerated in higher CO₂ 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 CO₂ 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 CO₂ 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 CO₂. 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 CO₂ is needed to understand the effect that CO₂ 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 CO₂.

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 CO₂ 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 CO₂ 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 μ atm/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, $n =$

74) 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 CO₂ conditions maintained a significantly lower Mg:Ca than the high CO₂ 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 CO₂ levels in the same populations. Another possible confounding effect is that the high CO₂ 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 pCO₂ 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 Ca²⁺ (Wheatly 1999), the cascade of Ca²⁺ reduction in each subsequent molt may inhibit development in a high CO₂ ocean.

CONCLUSIONS AND BROADER IMPACTS

Juvenile *Homarus americanus* subjected to elevated CO₂ 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, CO₂ treatments relative to control. Additionally, lobsters experienced a higher percentage of shell disease in higher CO₂ 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.

TABLES

Table 1: Measured parameters of seawater chemistry shown as means $\pm$ standard deviations in all treatments in 2014 and 2015. Units are: meter pH – mol/kg of H₂O; spectrophotometric (spec) pH – mol/kg of seawater; conductivity – mS; temperature - °C.

	Measured Parameters			
	Meter pH	Spec pH	Conductivity	Temperature
2014 Low	8.02 $\pm$ 0.09		45.14 $\pm$ 2.35	16.70 $\pm$ 0.86
2014 Medium	7.68 $\pm$ 0.08		45.11 $\pm$ 2.29	16.72 $\pm$ 0.94
2014 High	7.40 $\pm$ 0.17		45.12 $\pm$ 2.26	16.72 $\pm$ 0.97
2015 Low	8.19 $\pm$ 0.13	7.92 $\pm$ 0.06	47.20 $\pm$ 1.59	16.44 $\pm$ 0.58
2015 Medium	7.87 $\pm$ 0.11	7.64 $\pm$ 0.08	47.22 $\pm$ 1.52	16.38 $\pm$ 0.62
2015 High	7.64 $\pm$ 0.15	7.40 $\pm$ 0.14	47.30 $\pm$ 1.67	16.31 $\pm$ 1.28

Table 2: Calculated parameters of seawater chemistry shown as means $\pm$ standard deviations in all treatments in 2014 and 2015. Units are: salinity – ppt; alkalinity – $\mu\text{mol/kg}$ 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
2014 Medium	29.88 ± 1.69	2054.78 ± 106.83	1329.81 ± 290.49	1.19 ± 0.30	0.77 ± 0.18
2014 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
2015 Medium	30.90 ± 0.73	2111.26 ± 155.31	1067.11 ± 227.16	1.54 ± 0.29	0.98 ± 0.18
2015 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 CO ₂		High CO ₂	
	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 pCO₂ 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 H₂O; pH in 2015 - mol/kg of seawater; pCO₂ – μ atm.

pH 2014						
Summary						
<i>Groups</i>	<i>Sample size</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>		
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						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p-level</i>	<i>F crit</i>
Between Groups	22.25861	2	11.1293	800.58575	0.	3.02244
Within Groups	4.69869	338	0.0139			
Total	26.9573	340				
pCO2 2014						
Summary						
<i>Groups</i>	<i>Sample size</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>		
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						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p-level</i>	<i>F crit</i>
Between Groups	270,074,139.85729	2	135,037,069.92864	553.09703	0.	3.02244
Within Groups	82,521,741.03756	338	244,147.16283			
Total	352,595,880.89485	340				
pH 2015						
Summary						
<i>Groups</i>	<i>Sample size</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>		
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						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p-level</i>	<i>F crit</i>
Between Groups	29.8843	2	14.94215	1,217.03499	0.	3.0103
Within Groups	7.5875	618	0.01228			
Total	37.47179	620				
pCO2 2015						
Summary						
<i>Groups</i>	<i>Sample size</i>	<i>Sum</i>	<i>Mean</i>	<i>Variance</i>		
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						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p-level</i>	<i>F crit</i>
Between Groups	254,065,690.89891	2	127,032,845.44945	531.33683	0.	3.0103
Within Groups	147,752,413.76813	618	239,081.57568			
Total	401,818,104.66703	620				

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.	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 $\pm$ standard deviation in length and weight at each molt event in all three populations. Units are: length – mm, weight – mg.

Change in Length and Weight Over Time															
CT Population															
CO ₂ Level	n	Before Length	Before Weight	n	Length After Molt 1	Weight After Molt 1	n	Length After Molt 2	Weight After Molt 2						
Low	17	22.68 ± 1.76	350.12 ± 78.04	17	24.81 ± 2.37	469.38 ± 141.50	11	28.26 ± 2.80	695.55 ± 278.76						
Medium	24	22.49 ± 1.28	333.25 ± 59.48	24	24.14 ± 1.53	428.13 ± 85.17	12	26.52 ± 2.51	565.75 ± 162.29						
High	13	23.17 ± 1.76	377.46 ± 102.46	13	24.65 ± 1.89	446.10 ± 121.92	6	26.96 ± 2.32	619.14 ± 220.71						
ME 14 Population															
CO ₂ Level	n	Before Length	Before Weight	n	Length After Molt 1	Weight After Molt 1	n	Length After Molt 2	Weight After Molt 2	n	Length After Molt 3	Weight After Molt 3	n	Length After Molt 4	Weight After Molt 4
Low	18	14.79 ± 1.68	135.67 ± 24.31	19	18.24 ± 1.12	180.87 ± 30.76	18	21.36 ± 1.31	290.08 ± 52.94	16	24.81 ± 1.43	465.75 ± 81.30	12	27.59 ± 1.68	635.00 ± 106.69
Medium	17	15.01 ± 0.79	131.65 ± 18.75	18	17.98 ± 0.55	173.06 ± 22.97	15	20.93 ± 1.01	265.00 ± 40.33	15	23.86 ± 1.40	407.93 ± 71.16	10	26.55 ± 1.51	556.00 ± 90.79
High	21	15.59 ± 0.98	137.48 ± 18.74	21	17.49 ± 2.93	183.70 ± 35.16	13	21.30 ± 1.75	282.68 ± 82.65	12	24.18 ± 2.18	408.88 ± 113.19	5	24.95 ± 2.52	460.4 ± 156.40
ME 15 Population															
CO ₂ Level	n	Before Length	Before Weight	n	Length After Molt 1	Weight After Molt 1	n	Length After Molt 2	Weight After Molt 2	n	Length After Molt 3	Weight After Molt 3	n	Length After Molt 4	Weight After Molt 4
Low	13	11.62 ± 1.19	40.00 ± 18.81	13	13.08 ± 1.89	64.08 ± 33.93	7	16.80 ± 1.37	138.43 ± 34.95	6	18.58 ± 1.62	188.00 ± 55.08	3	19.93 ± 2.42	221.67 ± 94.12
Medium	7	11.66 ± 1.21	41.00 ± 14.13	7	13.20 ± 1.64	61.00 ± 20.17	2	15.48 ± 1.28	101.50 ± 28.99	1	17.47	145.00	0		
High	11	11.88 ± 1.05	47.64 ± 20.21	11	13.16 ± 1.41	62.55 ± 24.45	5	15.32 ± 2.46	110.80 ± 53.81	4	18.12 ± 2.50	171.75 ± 77.02	2	20.31 ± 0.95	240.50 ± 3.54

Table 7: Summary table for Figure 7 showing the means $\pm$ 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.

Mean Days in Intermolt $\pm$ standard deviation							Kruskal-Wallis Results			
CT Population										
CO ₂ Level	n	Intermolt Period 1						Among CO ₂ Levels		
Low	11	40.82 $\pm$ 12.53					X ²	1.146		
Medium	12	42.92 $\pm$ 7.25					df	2		
High	6	39.83 $\pm$ 6.31					p	0.564		
ME14 Population										
CO ₂ Level	n	Intermolt Period 1	n	Intermolt Period 2	n	Intermolt Period 3		Among Intermolt Periods	Among CO ₂ Levels IMP 1	Among CO ₂ Levels IMP 2 & 3
Low	17	25.35 $\pm$ 3.12	17	35.06 $\pm$ 4.08	13	34.69 $\pm$ 5.61	X ²	28.849	15.599	13.063
Medium	16	27.25 $\pm$ 5.01	16	37.25 $\pm$ 5.40	10	37.80 $\pm$ 3.36	df	2	2	2
High	12	35.25 $\pm$ 7.29	12	31.25 $\pm$ 6.11	5	30.40 $\pm$ 5.41	p	5.43E-07	0.0004	0.001

Table 8: Summary table for Figure 8 showing the means $\pm$ 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.

Percent Increases in Length and Weight per Molt						Kruskal-Wallis Results		
Population	CO ₂ Treatment	n	% Increase in Length per Molt	n	% Increase in Weight per Molt		Length	Weight
CT	Low	17	10.24 $\pm$ 1.09	17	35.74 $\pm$ 5.62	X ²	6.9	2.18
	Medium	24	8.50 $\pm$ 1.02	24	30.43 $\pm$ 3.25	df	2	2
	High	13	5.92 $\pm$ 1.00	13	22.19 $\pm$ 10.06	p	0.032	0.336
ME14	Low	19	22.86 $\pm$ 1.07	16	90.67 $\pm$ 5.68	X ²	19.93	16.59
	Medium	18	19.38 $\pm$ 1.03	19	71.41 $\pm$ 4.68	df	2	2
	High	21	16.17 $\pm$ 0.70	21	51.60 $\pm$ 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										
Fixed effects:						Analysis of Deviance Table (Type II Wald chisquare tests)				
	Estimate	Standard Error	z Value	p Value	Significance		Chi Sq	df	p Value	Significance
(Intercept)	-147.60	61.64	-2.395	0.0166	*	time	0.0414	1	0.8388	
time	1.13	0.53	2.118	0.0342	*	CO ₂	0.3222	2	0.8512	
Medium CO ₂	134.60	61.77	2.179	0.0294	*	Days	116.1578	1	2.20E-16	***
High CO ₂	132.50	61.79	2.145	0.032	*	time:CO ₂	35.7007	2	1.77E-08	***
Days	0.11	0.01	10.778	<2E-16	***					
time:Medium CO ₂	-1.15	0.53	-2.143	0.0321	*					
time:High CO ₂	-1.11	0.53	-2.079	0.0376	*					
2015 - CT Population										
Fixed effects:						Analysis of Deviance Table (Type II Wald chisquare tests)				
	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	126.4799	1	<2E-16	***
time	0.079573	0.008703	9.143	2.00E-16	***	CO ₂	1.4996	2	0.4725	
Medium CO ₂	3.567312	1.612781	2.212	0.02697	*	Days	141.3001	1	<2E-16	***
High CO ₂	-7.57916	2.414262	-3.139	0.00169	**	time:CO ₂	82.1105	2	<2E-16	***
Days	0.092488	0.007781	11.887	2.00E-16	***					
time:Medium CO ₂	-0.051975	0.010987	-4.731	2.24E-06	***					
time:High CO ₂	0.149379	0.024348	6.135	8.51E-10	***					

Table 10: Summary table for Figure 11 showing the means $\pm$ 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.

CT Population			Kruskal Wallis Results	
CO ₂ Level	n	Mg:Ca Ratio		Among CO ₂ Levels
Low	7	0.1499 $\pm$ 0.030	X^2	5.727
Medium	10	0.1876 $\pm$ 0.045	df	2
High	7	0.1477 $\pm$ 0.046	p	0.057
ME14 Population			Kruskal Wallis Results	
CO ₂ Level	n	Mg:Ca Ratio		Among CO ₂ Levels
Low	30	0.1441 $\pm$ 0.026	X^2	18.161
Medium	18	0.1561 $\pm$ 0.026	df	2
High	27	0.1904 $\pm$ 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 CO₂ treatments are shown in blue, medium CO₂ treatments in red, and high CO₂ 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: Spec pH=(0.8343*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 CO₂ treatments. Values are mean $\pm$ 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 $\pm$ 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.

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 ($P < 0.05$).

LITERATURE CITED

- Aiken DE (1973) Proecdysis, Setal Development, and Molt Prediction in American Lobster (*Homarus americanus*). J Fish Res Board Canada 30:1337–1344
- Anger K (2001) The biology of decapod crustacean larvae. Crustac Issues 14:417
- Arnold KE, Findlay HS, Spicer JJ, Daniels CL, Boothroyd D (2009) Effect of CO₂-related acidification on aspects of the larval development of the European lobster, *Homarus gammarus* (L.). Biogeosciences Discuss 6:3087–3107
- Bates D, Maechler M, Bolker B (2012) lme4: Linear mixed-effects models using Eigen and R classes. R package version 0.999999-0. <http://CRAN.R-project.org/package=lme4>
- Boßelmann F, Romano P, Fabritius H, Raabe D, Epple M (2007a) The composition of the exoskeletons of large arthropods: example of two crustaceans: The American lobster *Homarus americanus* and the edible crab *Cancer pagurus*. Thermochim Acta 463:65–68
- Boßelmann F, Romano P, Fabritius H, Raabe D, Epple M (2007b) The composition of the exoskeleton of two crustacea: The American lobster *Homarus americanus* and the edible crab *Cancer pagurus*. Thermochim Acta 463:65–68
- Burnett LE (1997) The challenges of living in hypoxic and hypercapnic aquatic environments. Amer Zool 37:633–640
- Byrne M, Smith AM, West S, Collard M, Dubois P, Graba-Landry A, Dworjanyn SA (2014) Warming influences Mg²⁺ content, while warming and acidification influence calcification and test strength a sea urchin. Environ Sci Technol 48:12620–12627

- Cameron JN, Wood CM (1985) Apparent H⁺ excretion and CO₂ dynamics accompanying carapace mineralization in the blue crab (*Callinectes sapidus*) following moulting. J Exp Biol 114:181–196
- Castro KM, Cobb JS, Gomez-Chiarri M, Tlusty M (2012) Epizootic shell disease in American lobsters *Homarus americanus* in southern New England: Past, present and future. Dis Aquat Organ 100:149–158
- Cawthorn RJ (1997) Overview of “Bumper Car” Disease - Impact on the North American Lobster Fishery. Int J Parasitol 27:167–172
- Chang J-H, Chen Y, Holland D, Grabowski J (2010) Estimating spatial distribution of American lobster *Homarus americanus* using habitat variables. Mar Ecol Prog Ser 420:145–156
- Comeau M, Savoie F (2001) Growth increment and molt frequency of the American lobster (*Homarus americanus*) in the southwestern Gulf of St Lawrence. J Crustac Biol 21:923–936
- Davis DA, Lawrence AL (1993) Response of *Penaeus vannamei* to Dietary Calcium, Phosphorus and Calcium:Phosphorus Ratio. J World Aquac Soc 24:504–515
- Dickson AG (2007) Guide to Best Practices for Ocean CO₂ Measurements (AG Dickson, CL Sabine, and JR Christian, Eds.). North Pacific Marine Science Organization
- Dickson AG, Millero FJ (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Res Part A, Oceanogr Res Pap 34:1733–1743
- Dove ADM, LoBue C, Bowser P, Powell M (2004) Excretory calcinosis: a new fatal

disease of wild American lobsters *Homarus americanus*. Dis Aquat Organ
58:215–21

Driscoll J, Boyd C, Tyedmers P (2015) Life cycle assessment of the Maine and
southwest Nova Scotia lobster industries. Fish Res 172:385–400

Feely RA, Sabine CL, Lee K, Berelson W, Kleypas JA, Fabry VJ, Millero FJ (2004)
Impact of anthropogenic CO₂ on the CaCO₃ system in the oceans. Science (80)
305:362–6

Findlay HS, Wood HL, Kendall MA, Spicer JJ, Twitchett RJ, Widdicombe S (2009)
Calcification, a physiological process to be considered in the context of the
whole organism. Biogeosciences Discuss 6:2267–2284

Fitzmaurice GM, Laird NM, Ware JH (2011) Applied Longitudinal Analysis, Second.
Wiley

Fotheringham N (1976) Effects of shell stress on the growth of hermit crabs. J Exp
Mar Bio Ecol 23:299–305

Fox J, Weisberg S (2011). An {R} Companion to Applied Regression, Second
Edition. Thousand Oaks CA: Sage. URL:
<http://socserv.socsci.mcmaster.ca/jfox/Books/Companion>

Gastwirth JL, Gel YR, Hui WLW, Lyubchich V, Miao W, Noguchi K (2013).
lawstat: An R package for biostatistics, public policy, and law. R package
version 2.4. <http://CRAN.R-project.org/package=lawstat>

Gazeau F, Quiblier C, Jansen JM, Gattuso J-P, Middelburg JJ, Heip CHR (2007)
Impact of elevated CO₂ on shellfish calcification. Geophys Res Lett 34:L07603

- Giraudeau P (2013) pgirmess: Data analysis in ecology. R package version 1.5.8.
<http://CRAN.R-project.org/package=pgirmess>
- Glenn RP, Pugh TL (2006) Epizootic Shell Disease in American Lobster *Homarus americanus* in Massachusetts Coastal Waters: Interactions of Temperature, Maturity, and Intermolt Duration. J Crustac Biol 26:639–645
- Hernroth B, Sköld HN, Wiklander K, Jutfelt F, Baden S (2012) Simulated climate change causes immune suppression and protein damage in the crustacean *Nephrops norvegicus*. Fish Shellfish Immunol 33:1095–101
- Hofmann GE, Barry JP, Edmunds PJ, Gates RD, Hutchins D a., Klinger T, Sewell M a. (2010) The Effect of Ocean Acidification on Calcifying Organisms in Marine Ecosystems: An Organism-to-Ecosystem Perspective. Annu Rev Ecol Evol Syst 41:127–147
- Homerding MR (2009) Characterization of cuticular and hemolymph associated defense parameters in shell diseased lobsters. Stony Brook University
- Hughes JT, Matthiessen GC (1962) Observations on the Biology of the American Lobster, *Homarus americanus*. Limnol Oceanogr 7:414–421
- Kanazawa A, Teshima S-I, Sasaki M (1984) Requirements of the juvenile prawn for calcium, phosphorus, magnesium, potassium, copper, manganese, and iron Mem Fac Fish Kagoshima Univ 33:63–71
- Kaniewska P, Campbell PR, Kline DI, Rodriguez-Lanetty M, Miller DJ, Dove S, Hoegh-Guldberg O (2012) Major cellular and physiological impacts of ocean acidification on a reef building coral. PLoS One 7:e34659
- Kelley KA, Plank T, Ludden J, Staudigel H (2003) Composition of altered oceanic

- crust at ODP Sites 801 and 1149. *Geochemistry, Geophys Geosystems* 4
- Kleypas JA, Langdon CJ (2006) Coral Reefs and Changing Seawater Carbonate Chemistry. *Coast Estuar Stud* 61:73–110
- Kruskal WH, Wallis WA (1952) Use of Ranks in One-Criterion Variance Analysis. *J Am Stat Assoc* 47:583–621
- Kunkel JG, Nagel W, Jercinovic MJ (2012) Mineral Fine Structure of the American Lobster Cuticle. *J Shellfish Res* 31:515–526
- Kurihara H (2008) Effects of CO₂-driven ocean acidification on the early developmental stages of invertebrates. *Mar Ecol Prog Ser* 373:275–284
- Lannig G, Eilers S, Portner HO, Sokolova IM, Bock C (2010) Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas* - Changes in metabolic pathways and thermal response. *Mar Drugs* 8:2318–2339
- Lawton P, Lavalli K (1995) Postlarval, juvenile, adolescent, and adult ecology. In: Factor JR (ed) *Biology of the lobster Homarus americanus*. Academic, San Diego, pp 47–88
- Laufer H, Demir N, Biggers WJ (2005) Response of the American Lobster to the Stress of Shell Disease. *J Shellfish Res* 24:757-760
- Lemon, J (2006) Plotrix: a package in the red light district of R. *R-News*, 6(4): 8-12.
- Lewis E, Wallace D (1998) Program developed for CO₂ system calculations. *Ornl/Cdiac-105*:1–21
- McConnaughey TA, Whelan JF (1997) Calcification generates protons for nutrient and bicarbonate uptake. *Earth-Science Rev* 42:95–117
- McDonald JH (2009) *Handbook of Biological Statistics*. Sparky House Publ: 291

- Middlemiss KL, Urbina MA, Wilson RW (2016) Effects of seawater alkalinity on calcium and acid–base regulation in juvenile European lobster (*Homarus gammarus*) during a moult cycle. *Comp Biochem Physiol Part A Mol Integr Physiol* 193:22–28
- Moore DS (1977) Generalized Inverses, Wald’s Method, and the Construction of Chi-Squared Tests of Fit. *J Am Stat Assoc* 72:131–137
- Morris S, Tyler-Jones R, Bridges CR, Taylor EW (1986) The regulation of haemocyanin oxygen affinity during emersion of the crayfish *Austropotamobius pallipes*. II. An investigation of in vivo changes in oxygen affinity. *J Exp Biol* 121:327–337
- Pinheiro J, Bates D, DebRoy S, Sarkar D and the R Development Core Team (2013) nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-108
- Pörtner H-O, Reipschlag A, Heisler N (1998) Acid-base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level. *J Exp Biol* 201:43–55
- Quinn RA, Metzler A, Smolowitz RM, Tlusty M, Chistoserdov AY (2012) Exposures of *Homarus americanus* shell to three bacteria isolated from naturally occurring epizootic shell disease lesions. *J Shellfish Res* 31:485–493
- R Core Team (2012) foreign: Read Data Stored by Minitab, S, SAS, SPSS, Stata, Systat, dBase, R package version 0.8-50. <http://CRAN.R-project.org/package=foreign>

- R Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org/>
- Ries JB, Cohen AL, McCorkle DC (2009) Marine calcifiers exhibit mixed responses to CO₂-induced ocean acidification. *Geology* 37:1131–1134
- Roer R, Dillaman R (1984) The Structure and Calcification of the Crustacean Cuticle. *Am Zool* 24:893–909
- Sarkar, D (2008) Lattice: Multivariate Data Visualization with R. Springer, New York. ISBN 978-0-387-75968-5
- Shields JD, Stephens FJ, Jones B (2006) Pathogens, parasites and other symbionts. In: Phillips BF (ed) *Lobsters: biology, management, aquaculture and fisheries*. Blackwell Publishing, Oxford.
- Siegel S, Castellan Jr. NJ (1988) Non-Parametric Statistics for the behavioural Sciences. McGraw Hill Int 2nd Edition: 213–214
- Sinutok S, Hill R, Kühl M, Doblin MA, Ralph PJ (2014) Ocean acidification and warming alter photosynthesis and calcification of the symbiont-bearing foraminifera *Marginopora vertebralis*. *Mar Biol* 161:2143–2154
- Small DP, Calosi P, Boothroyd D, Widdicombe S, Spicer JJ (2016) The sensitivity of the early benthic juvenile stage of the European lobster *Homarus gammarus* (L.) to elevated pCO₂ and temperature. *Mar Biol* 163:53
- Spicer JJ, Raffo A, Widdicombe S (2007) Influence of CO₂-related seawater acidification on extracellular acid-base balance in the velvet swimming crab *Necora puber*. *Mar Biol* 151:1117–1125

- Stocker, TF, Qin D, Plattner GK, Alexander LA, Allen SK, Bindoff NL, Bréon FM, Church JA, Cubasch U, Emori S, Forster P, Friedlingstein P, Gillett N, Gregory JM, Hartmann DL, Jansen E, Kirtman B, Knutti R, Krishna Kumar K, Lemke P, Marotzke J, Masson-Delmotte J, Meehl GA, Mokhov II, Piao S, Ramaswamy V, Randall D, Rhein M, Rojas M, Sabine CL, Shindell D, Talley LD, Vaughan DG and Xie SP (2013) Technical Summary. In: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Stocker TF, Qin D, Plattner GK, Tignor M, Allen SK, Boschung J, Nauels A, Xia Y, Bex V and Midgley PM (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.
- Turner, C (2015) Verification of pH Fluctuations in Narragansett Bay. Open Access Master's Theses. Paper 526: <http://digitalcommons.uri.edu/theses/526>
- Vidal-Dupiol J, Zoccola D, Tambutté E, Grunau C, Cosseau C, Smith KM, Freitag M, Dheilly NM, Allemand D, Tambutté S (2013) Genes Related to Ion-Transport and Energy Production Are Upregulated in Response to CO₂-Driven pH Decrease in Corals: New Insights from Transcriptome Analysis. PLoS One 8
- Vijayan K, Diwan A (1995) Influence of temperature, salinity, pH and light on molting and growth in the Indian white prawn *Penaeus indicus* (Crustacea: Decapoda: Penaeidae) under laboratory conditions. Asian Fish Sci 8:63–72
- Walther K, Anger K, Pörtner H-O (2010) Effects of ocean acidification and warming on the larval development of the spider crab *Hyas araneus* from different latitudes (54° vs. 79°N). Mar Ecol Prog Ser 417:159–170

- Wheatly MG (1999) Calcium Homeostasis in Crustacea: The Evolving Role of Branchial, Renal, Digestive, and Hypodermal Epithelia. *J Exp Zool* 283:620–640
- Wheatly MG, Souza SCR de, Hart MK (1996) Related Changes in Hemolymph Acid-Base Status, Electrolytes, and Ecdysone in Intermolt Crayfish (*Procambarus clarkii*) at 23°C during Extracellular Acidosis Induced by Exposure to Air, Hyperoxia, or Acid. *J Crustac Biol* 16:267–277
- Wickham, H (2009) *ggplot2: elegant graphics for data analysis*. Springer New York.
- Wickins JF (1984) The Effect of Hypercapnic Sea Water on Growth and Mineralization in Panaeid Prawns. *Aquaculture*:37–48
- Wood HL, Spicer JI, Widdicombe S (2008) Ocean acidification may increase calcification rates, but at a cost. *Proc R Soc Biol Sci* 275:1767–73