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Decreased growth and increased shell disease in early benthic phase *Homarus americanus* in response to elevated CO_2

Erin L. McLean[1,*], Natallia V. Katenka[2], Brad A. Seibel[3]

[1]College of the Environment and Life Sciences, University of Rhode Island, Kingston, RI 02881, USA
[2]Department of Computer Science and Statistics, University of Rhode Island, Kingston, RI 02881, USA
[3]College of Marine Science, University of South Florida, St. Petersburg, FL 33701, USA

ABSTRACT: Marine calcifiers, especially those in larval and juvenile stages, are thought to be most vulnerable to ocean acidification (OA) due to the effects of carbon dioxide (CO_2) on growth and calcification. However, recent evidence in lobsters is contradictory. We monitored molting activity, length, and weight in early benthic phase *Homarus americanus* (Milne-Edwards 1837) over 90 to 120 d under 3 targeted CO_2 partial pressures (pCO_2; 400, 1000, and 2000 µatm) to determine how elevated CO_2 affects growth at this life stage. Lobsters exposed to higher pCO_2 over that 90 to 120 d period exhibited altered intermolt period length and decreased growth increments (length and weight). Lobsters in the elevated CO_2 treatments were also more susceptible to shell disease. These results suggest juvenile lobsters may remain smaller, and thus more susceptible to predation, for a longer period of time and may be more susceptible to disease in a high CO_2 ocean.

KEY WORDS: Ocean acidification · Juvenile lobster · Shell disease · Growth increments

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INTRODUCTION

Due to increasing dissolved anthropogenic carbon dioxide (CO_2), ocean pH has decreased by 0.1 units and may drop by 0.35 units by the year 2100 (Feely et al. 2004, IPCC 2013; 'business as usual climate change scenario', RCP 8.5). This 'ocean acidification' (OA) has been shown to decrease calcification and/or growth while increasing disease prevalence and/or mortality in many animals, including arthropods (for review see Hofmann et al. 2010).

Organisms that produce calcium carbonate shells are thought to be most affected by OA because those shells may be more vulnerable to dissolution given a reduced saturation state. Several marine calcifiers, representing many different clades, have exhibited reduced net calcification or increased shell dissolution when presented with lower carbonate saturation states (Kleypas & Langdon 2006, Gazeau et al. 2007, Byrne et al. 2014, Sinutok et al. 2014). Decapod crustaceans are thought to be among the groups more resilient to the effects of increased CO_2 because they regularly relocate exoskeletal calcium (Ca^{2+}) and carbonate (CO_3^2) ions during molting (Roer & Dillaman 1984, Morris et al. 1986) and the lobster cuticle has a biogenic covering that protects it to some extent from dissolution (Aiken 1973). Additionally, crustaceans are able to take up bicarbonate (HCO_3^-) from the surrounding seawater for conversion to CO_3^2 (Cameron & Wood 1985).

Despite these reasons for resilience, studies have demonstrated that growth, survival, and developmental rate of many crustaceans are hindered by hypercapnic conditions (barnacle: pH 7.7, 1000 µatm pCO_2, Findlay et al. 2009; shrimp: pH 7.64, 1900 µatm pCO_2, Kurihara 2008; larval crab: pH 7.3, 3000 µatm

*Corresponding author: erinmclean@uri.edu

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pCO_2, Walther et al. 2010). Other commercially important crustaceans have been similarly investigated. Long et al. (2013) observed that when juvenile blue crabs are exposed to lower pH conditions, the crabs in the lowest pH treatment (pH 7.5, 1600 µatm pCO_2) experienced a significant increase in mortality and a decrease in growth.

There are fewer studies specifically on lobsters. In the short term (24 h), the spiny lobster *Jasus lalandi* has been shown to fully compensate the decrease in blood pH observed during the first 100 min of exposure to elevated pCO_2 (1842 µatm, pH 7.39) (Knapp 2015). In the medium term (11 d), *Homarus gammarus* larvae experienced reduced growth, increased oxidative stress, and increased DNA damage resulting from water with pH 7.85 and pCO_2 of 710 µatm (Rato et al. 2017).

However, results from long-term studies with lobsters are conflicting. Elevated CO_2 (1200 ppm, pH 8.1) caused a reduction in Ca^{2+} and Mg^{2+} content in the carapace of final larval stage (Zoea IV) of *H. gammarus*, possibly as a result of reduced net calcification (Arnold et al. 2009). Juvenile *H. gammarus* exposed for 35 d to a pCO_2 of 9000 µatm (pH 6.9) showed a significant decrease in growth rate and significantly lower total Ca^{2+} content, but no effect at lower pCO_2 (1100 µatm, pH 7.7; Small et al. 2016). Similarly, Arnold et al. (2009) found no effect of 1200 µatm pCO_2 on growth in larvae of *H. gammarus* after 28 d exposure. In addition to short-term effects, Knapp (2015) investigated chronic hypercapnia, and found that 28 wk of exposure to pH 7.4 (2065 µatm) decreased growth in the spiny lobster compared to normocapnia (pH 8, 355 µatm).

Surprisingly, another study found that juvenile *H. americanus* increased growth during 60 d exposure to pH 7.31 and 2800 ppm CO_2 (Ries et al. 2009). However, in that study, the lobsters were held at 25°C, which is believed to be their upper thermal limit and likely beyond their acclimatization temperature (Lawton & Lavalli 1995). Temperature stress is known to lead to aragonite deposits in the gills of lobsters, which has led to mortality events (Dove et al. 2004).

Under projected climate scenarios, disease susceptibility may also increase. The ability to fight off disease in crustaceans, measured primarily by phagocyte activity, has been shown to decrease at increased temperature and in acidic conditions in the laboratory (Steenbergen et al. 1978, Mikulski et al. 2000, Cheng et al. 2003, Burgents et al. 2005, Hernroth et al. 2012). Lobster shell disease serves to weaken the shell's protective ability (Gomez-Chiarri & Cobb 2012) and the expansion of the disease has correlated well with increasing temperature (Tlusty & Metzler 2012), but no work has been done yet to investigate whether hypercapnia plays a specific role in the proliferation of lobster shell disease.

The primary objective of this study was to investigate growth in early benthic phase lobsters during long-term exposure (90 to 120 d) to elevated CO_2. Early benthic phase lobsters were chosen specifically because they molt frequently, typically survive well in laboratory conditions, and are the first fully calcified life stage of the American lobster (Anger 2001). Growth and shell disease in lobsters have important ecological and economic consequences in terms of food web and fisheries dynamics.

MATERIALS AND METHODS

Animal collection and maintenance

Three groups of early benthic stage lobsters, hereafter referred to as 'populations', were obtained from 2 hatchery centers, the Maine Lobster Hatchery (Bar Harbor, ME, USA) and The Sound School of Aquaculture (New Haven, CT, USA). Populations from Maine were obtained in both 2014 (ME14, starting n = 73) and 2015 (ME15, starting n = 102), while Connecticut lobsters (CT, starting n = 71) were obtained only in 2015. The ME lobsters were 4 mo post hatch and raised at 17 ± 2°C and pH 7.7 ± 0.4, and the CT lobsters were 6 mo post hatch and raised at 18 ± 2°C and pH 7.6 ± 0.2. These are the conditions that the hatcheries have found to result in hatching and rearing success. All individuals within each population consisted of siblings from the same egg clutch collected from an egg-bearing wild female lobster. Specimens were randomly distributed into the treatment tanks (n = 34 each, 51 l capacity) and maintained for 90 to 120 d in filtered seawater (~10 µm) pumped directly from Narragansett Bay, RI, USA. The incoming seawater salinity ranged from 28 to 35 ppt (lower salinities were due to rain events in Narragansett Bay) and was heated to a target temperature of 17°C, which mimics the known seawater conditions for the summer molting period of this species (Hughes & Matthiessen 1962, Comeau & Savoie 2001). Experiments were conducted at the University of Rhode Island's Graduate School of Oceanography Marine Science Research Facility.

In 2014, each tank of approximately 20 lobsters was fed 4.2 g of frozen *Artemia naupili* each day. In 2015, lobsters were fed every 3 d on a gel-based diet (Mazuri Gel Diet for Crustaceans) containing protein

(57%), fats (14%), and the essential nutrients (29%) calcium, phosphorus, sodium bicarbonate, and fiber. While food intake was not directly measured, the lobsters were checked each day and any excess remaining food was noted. There was leftover food in the lobster enclosure in very few instances (less than 6% of days). Sex was not determined, but at this life stage, growth rates of males and females are believed to be similar (Hughes & Matthiessen 1962). To avoid cannibalism, each lobster was enclosed individually in a 4-inch (~10 cm) diameter cylinder with a mesh size of 2 mm^2. The enclosures were moved randomly in the tank after measurement to avoid placement bias.

Seawater quality

Temperature was measured daily using a radiometric thermocouple (Fisher Scientific). Conductivity was measured in milliSiemens (mS) by a temperature-compensated conductivity meter (Pinpoint Salinity Monitor) calibrated to a 53 mS standard. The pH was measured using the OA Best Practices standard operating procedure (SOP) 6b, in which a spectrophotometer (Ocean Optics USB2000) and pH-adjusted m-cresol purple dye were used to determine the pH of the seawater sample on the total scale (Dickson 2007; see Table 1). Total alkalinity was calculated from conductivity measurements using a linear regression model (alkalinity = 63.414 × salinity + 160.28) relating salinity to alkalinity created specifically from measurements in Narragansett Bay over a 14 mo span from February 2010 to April 2011 (Turner 2014). We are confident in this model because the total alkalinity and salinity measurements were extremely similar to the same parameters recorded by the US Geological Survey and the National Water Information Service in the same region, and that the correlation between salinity and alkalinity was strong at all 3 sampling sites in the study regardless of season (Turner 2014).

CO_2 treatments

Three different partial pressures of CO_2 (pCO_2) were targeted: 400 µatm, the low level representing current atmospheric conditions; 1000 µatm, a medium level that is expected by the year 2100 under business as usual scenarios; and 2000 µatm, a high treatment chosen to constrain the boundaries of performance (Barry et al. 2010). The calculated pCO_2 levels achieved varied slightly from the targets due to the method of CO_2 injection used. Small amounts of pure CO_2 gas were injected into the tank via a peristaltic pump fed directly into the inflow of an aquarium pump (Petco, King 160 Powerhead) following methods modified from Jokiel et al. (2014). The CO_2 dissolved immediately in the flowing seawater due to the movement of the impeller, which broke up the gas bubbles and thus created a larger surface area for the gas to diffuse into the seawater (Jokiel et al. 2014). The seawater pCO_2 was controlled by the rate of the peristaltic pump delivery of CO_2 gas. pCO_2 and the saturation state of calcite (Ω Ca) were calculated using the measured parameters and the Excel macro CO_2Sys using the constants from Lueker et al. (2000).

The flow-through system outlined above provided a consistent decrease in pH relative to the natural pH variability in Narragansett Bay due to freshwater input, weather events, and biological processes (Chang et al. 2010). The water flow rate in the 51 l tanks resulted in complete turnover every 3 h, therefore maintaining ammonia and oxygen levels and minimizing the presence of molting pheromones. Because the medium and high CO_2 treatments were acidifying the incoming water by approximately 0.3 and 0.55 pH units, respectively, the lobsters were experiencing the variability plus additional treatment CO_2 (Fig. 1). This is important because lobsters experience natural diel and seasonal fluctuations in pH in their benthic habitat (Price et al. 2012).

In 2014, only 1 tank was used for each treatment level (Fig. 2A). Thus, the individual lobsters are not true statistical replicates, but are instead biological replicates. To minimize the effect of such 'pseudoreplication' in 2015, both the ME15 and CT populations were placed into 2 tanks at each treatment level (Fig. 2B). These 2 tanks received the same source seawater, while pCO_2 was manipulated individually in each tank. No significant differences were found between the replicate tanks (tank set A: 1, 2, 3 and tank set B: 4, 5, 6) in any measured environmental (pH, temperature, and conductivity) or growth (percent increase in length, percent increase in weight, and length of intermolt) parameters by matched paired *t*-tests ($p > 0.05$; Rubin 1973). Therefore, CT lobsters in 2015 (tank set A and tank set B) were combined for analysis to create the aggregate population 'CT'. Light levels were not specifically controlled but were identical in all treatments and approximated diel cycles. Light is not known to affect growth in lobsters (Lawton & Lavalli 1995).

Total length, carapace length, and wet weight were measured daily with digital calipers (Husky, precision

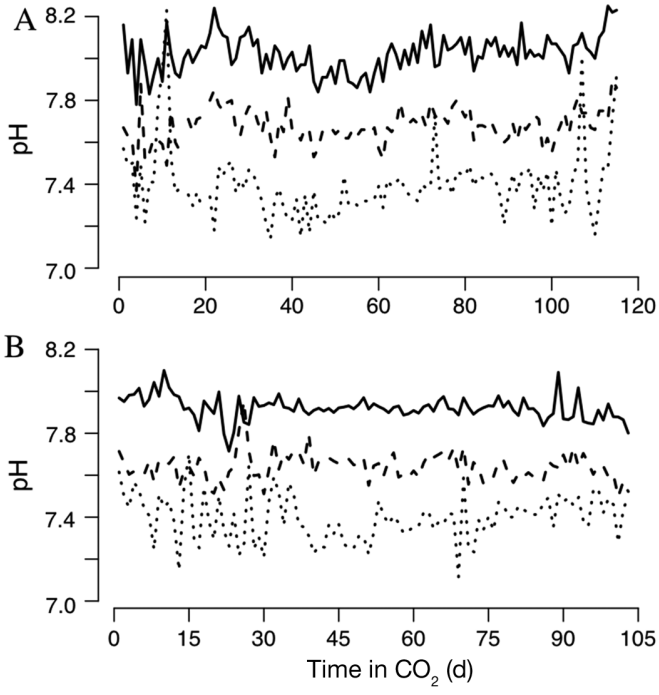

Fig. 1. pH variability in (A) 2014 and (B) 2015, showing low (solid lines), medium (dashed lines), and high (dotted lines) CO_2 levels

±0.01 mm) and a balance (Ohaus Scout Pro, precision ±0.01 g). Qualitative observations on the softness of the shell (indicated by wrinkling in the carapace), presence of shell disease, exuvia (cast off molt), or missing chelae, were also recorded. Shell disease was identified as rust colored spots on the carapace, telson, or chelae (Castro et al. 2012). In some cases, the lobster consumed the exuvia before it could be collected. In those cases, a change in length of over 3 mm or in weight of over 0.1 g was used to indicate a molting event. Those 2 criteria were consistent with length and weight changes in observed molts.

Length of intermolt period was determined by the number of days between 2 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:

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

The accepted method for calculating growth in lobsters is the difference between pre- and post-molt carapace length (Hepper 1967). However, lobsters continue to increase in size post-molt (Travis 1954); thus length and weight were averaged over the entire intermolt period (time in between molts).

Statistical analysis

Parametric analysis of variance (ANOVA) tests were used to determine differences between the 3 CO_2 treatments when the data were normally distributed and had a large (n > 30) sample size (Fisher 1925, McDonald 2014). The variables pH and pCO_2 had unequal variances according to Levene's test for homogeneity of variance ($\alpha < 0.05$), so ANOVA was run with the Welch modification for unequal variances (Welch 1951). Kruskal-Wallis non-parametric tests were run to when the data were not normally distributed and had a sample size too low (n < 30) to

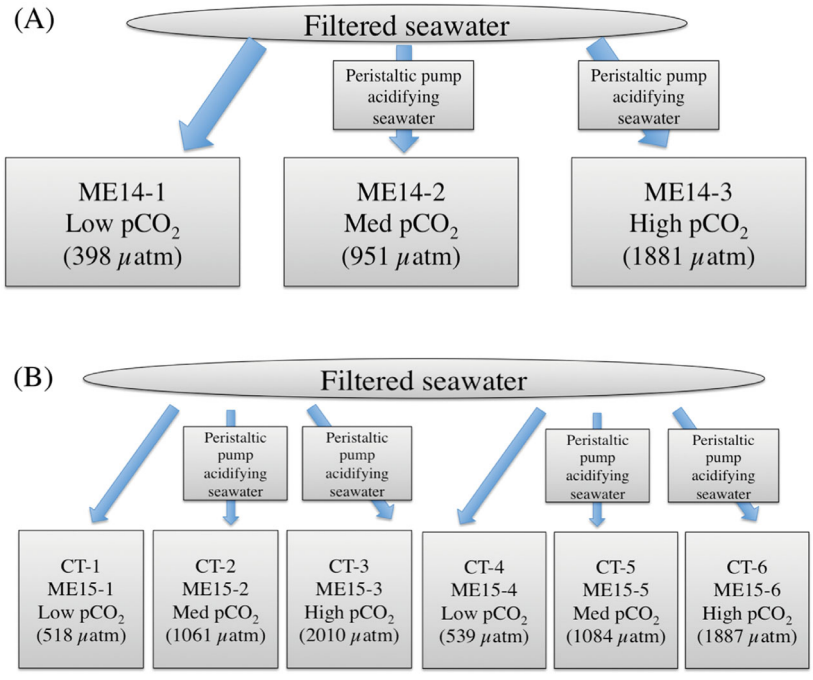

Fig. 2. Schematic of the experimental tank set up in (A) 2014 with the ME14 population (ME14, starting n = 73) and (B) 2015 with the ME15 population (ME15, starting n = 102), and CT population (CT, starting n = 71). These tanks were set up in the A-2 randomized block design (Havenhand et al. 2010, Cornwall & Hurd 2016). In 2015, the pCO_2 levels in the replicate low, medium, and high CO_2 tanks were not significantly different from each other (*t*-tests, $t < 1.96$)

satisfy the central limit theorem (Kruskal & Wallis 1952). 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 1988, Gastwith et al. 2013)

Mortality models were created using Kaplan Meier (KM) estimates of the time to death in each lobster (Singer & Willet 2003). KM survival estimates were created for all lobsters regardless of population, but log rank tests showed that the populations were highly significantly different ($p < 0.00001$, log rank test). Therefore, KM estimates were created for each population and compared. Once KM estimates were created, semi-parametric log rank tests were performed to assess significance among the 3 CO_2 levels within a population. Additionally, Cox Proportional Hazard Models were constructed for each population to relate the covariates of terminal length, terminal weight, and shell disease presence to mortality (Singer & Willet 2003).

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 3 tested CO_2 groups (Fitzmaurice et al. 2011). The model estimates the mean of the shell disease response, the probability that a lobster in that population will contract shell disease under a given set of parameters. To further analyze the results and significance of the model, we ran a Type II Wald chi-square test.

RESULTS

Environmental conditions

Seawater parameters measured directly were pH, conductivity, and temperature (Table 1). In 2014, the mean (±SE) pH was 8.02 ± 0.09 for the low treatment, 7.68 ± 0.08 for the medium treatment, and 7.40 ± 0.17 for the high treatment. The mean conductivity and temperature were statistically the same in all treatments: 45.8 ± 2.4 mS (ANOVA, $p = 0.99$) and 16.7 ± 0.9°C (ANOVA, $p = 0.98$). In 2015, the mean pH was 7.92 ± 0.06 for the low treatment, 7.64 ± 0.08 for the medium treatment, and 7.40 ± 0.14 for the high treatment. The mean conductivity and temperature were statistically the same in all treatments: 47.0 ± 1 mS (ANOVA, $p = 0.80$) and 16.4 ± 0.4°C (ANOVA, $p = 0.51$).

Seawater chemistry parameters calculated from the measured conditions were pCO_2, total alkalinity, salinity, and Ω Ca (Table 1). Alkalinity was obtained using salinity as a proxy according to the model described by Turner (2014). In 2014, the mean pCO_2 values were 398.4 ± 100.12 µatm for the low treatment, 950.89 ± 332.32 µatm for the medium treatment, and 1880.89 ± 610.80 µatm for the high treatment. Mean alkalinity for all 3 treatments in 2014 was 2055 ± 106 µmol kg^{-1} seawater and mean salinity was 29.9 ± 1.7 ppt. In 2015, the mean pCO_2 values were 527.17 ± 83.14 µatm for the low treatment, 1069.48 ± 104.41 µatm for the medium treatment, and 1938.20 ± 596.27 µatm for the high treatment. Mean alkalinity for all 3 treatments in 2015 was 2110 ± 150 µmol kg^{-1} seawater and mean salinity was 30.9 ± 0.8 ppt. In both 2014 and 2015, pH levels in the 3 treatment levels were found to be statistically different from each other (Welch ANOVA, $p < 0.0001$).

Mortality

Mortality was not significantly different among the CO_2 treatments for any of the 3 populations ($p > 0.21$, log rank test). The CT and ME14 populations suffered a steady low mortality rate initially, but that rate leveled off after approximately 30 d in treatment (Fig. 3A,B). In contrast, the ME15 population suffered a high mortality rate at the beginning of the study

Table 1. Measured and calculated parameters of seawater chemistry shown as means ± standard deviations in all treatments (low, medium, and high CO_2) in 2014 and 2015. The saturation state of calcite (Ω Ca) is unitless

	Measured parameters			Calculated parameters			
	pH (mol kg^{-1} seawater)	Conductivity (millisiemens)	Temperature (°C)	Salinity (ppt)	Alkalinity (µmol kg^{-1} seawater)	pCO_2 (µatm)	Ω Ca
2014 Low	8.02 ± 0.09	45.86 ± 2.46	16.70 ± 0.86	29.90 ± 1.71	2056.06 ± 108.35	398.40 ± 100.12	3.15 ± 0.52
2014 Med	7.68 ± 0.08	45.83 ± 2.43	16.72 ± 0.94	29.88 ± 1.69	2054.78 ± 106.83	950.89 ± 332.32	1.57 ± 0.30
2014 High	7.40 ± 0.17	45.84 ± 2.45	16.72 ± 0.97	29.88 ± 1.66	2055.21 ± 105.49	1880.89 ± 610.80	0.86 ± 0.47
2015 Low	7.92 ± 0.06	47.13 ± 1.11	16.43 ± 0.42	30.89 ± 0.77	2110.19 ± 150.35	527.17 ± 83.14	2.66 ± 0.35
2015 Med	7.64 ± 0.08	47.08 ± 1.08	16.33 ± 0.43	30.90 ± 0.73	2111.26 ± 155.31	1069.48 ± 104.41	1.48 ± 0.29
2015 High	7.40 ± 0.14	46.95 ± 1.31	16.45 ± 0.45	30.96 ± 0.81	2127.34 ± 78.36	1938.20 ± 596.27	0.89 ± 0.30

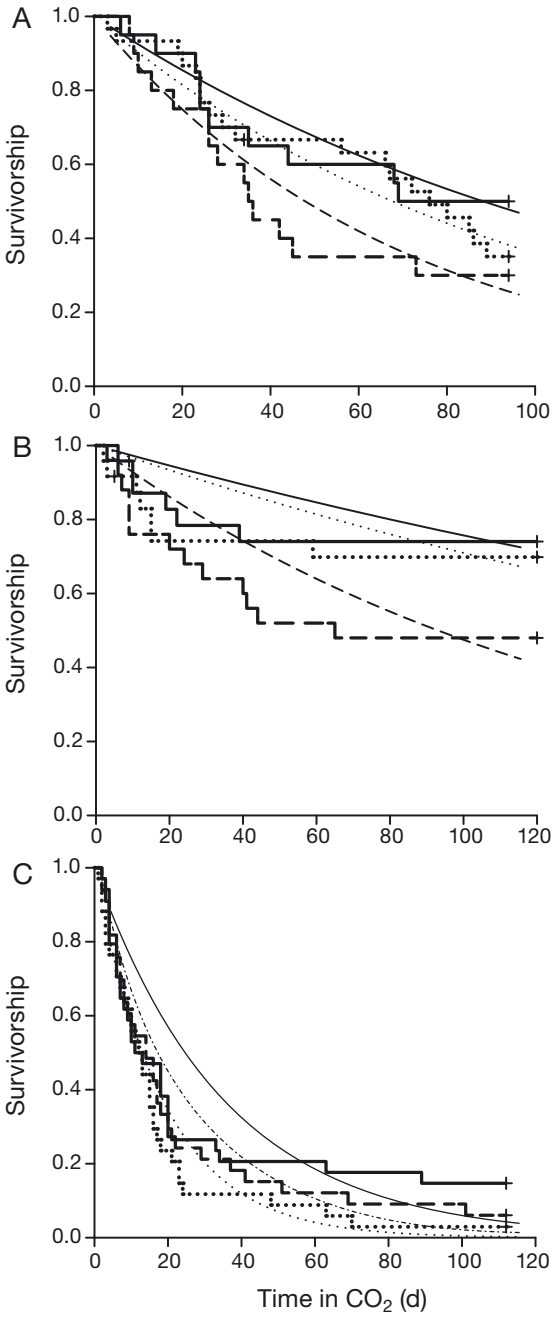

Fig. 3. Kaplan Meier (KM) estimates of mortality for all 3 tested lobster *Homarus americanus* populations as a percentage over time in treatment. (A) CT, (B) ME14, (C) ME15. Low (solid lines), medium (dotted lines), and high (dashed lines) CO_2 levels are shown. Bold lines represent the KM estimates, and thinner lines represent the hazard function

(Fig. 3C) with 14 of 34 lobsters in each treatment (42% of the total population) dying during the 7 d acclimation period. The high mortality of this population in all 3 CO_2 treatments was likely the result of their low weight at the beginning of the trial (~40 mg in the ME15 population vs. ~130 mg in the ME14 population; Fig. 4). As a result, this ME15 population was excluded from further analysis. Average lengths and weights of CT and ME14 lobsters at each molt stage can be found in Fig. 4.

Length of intermolt period

Only the ME14 population underwent sufficient molts for statistical analysis of the average length of the intermolt period using Kruskal-Wallis tests (Fig. 5). The response to CO_2 of the first intermolt period was significantly different from that in the second and third intermolt periods ($p < 0.00001$). For the first intermolt period, the low CO_2 lobsters spent 25.35 ± 3.12 d in intermolt, significantly fewer than the highest CO_2 treatment lobsters, which spent 35.25 ± 7.29 d in intermolt ($p < 0.001$, Fig. 5). For the second intermolt period, the medium CO_2 lobsters spent significantly more days in intermolt (35.06 ± 4.08) compared to the highest CO_2 treatment lobsters, which spent 31.25 ± 6.12 d in intermolt ($p = 0.001$). For the third, the medium CO_2 lobsters again spent significantly more days in intermolt (34.69 ± 5.62) compared to the highest CO_2 treatment lobsters, which spent 30.40 ± 5.41 d in intermolt ($p = 0.001$). The low and medium CO_2 levels were not significantly different from each other in any intermolt period ($p > 0.05$).

The CT population molted only twice in the 90 d trial period, so only 1 intermolt period was observed and averaged. The low CO_2 lobsters spent 40.82 ± 12.53 d in intermolt compared to the highest CO_2 treatment lobsters, which spent 39.83 ± 6.31 d in intermolt (Fig. 5). There was no difference in intermolt period length among the 3 CO_2 treatments (Kruskal-Wallis test, $p = 0.56$).

Growth increments (length and weight)

In the CT (n = 54) population, the mean length percent increase with each molt in low CO_2 was significantly higher (10.24 ± 1.09%) than in the highest CO2 treatment (5.92 ± 1.00%, $p < 0.05$, Fig. 6A). The mean weight percent increase was not significantly different between the lowest and highest CO2 treatments: 35.74 ± 5.62% in low CO_2 and 22.19 ± 10.06% in high CO_2 ($p > 0.05$, Fig. 6B). In the ME14 (n = 59) population, the mean length percent increase with each molt in low CO_2 was significantly higher than in the high CO_2 treatment: 22.86 ± 1.07% and 16.17 ± 0.70%, respectively ($p < 0.05$, Fig. 6A). The mean

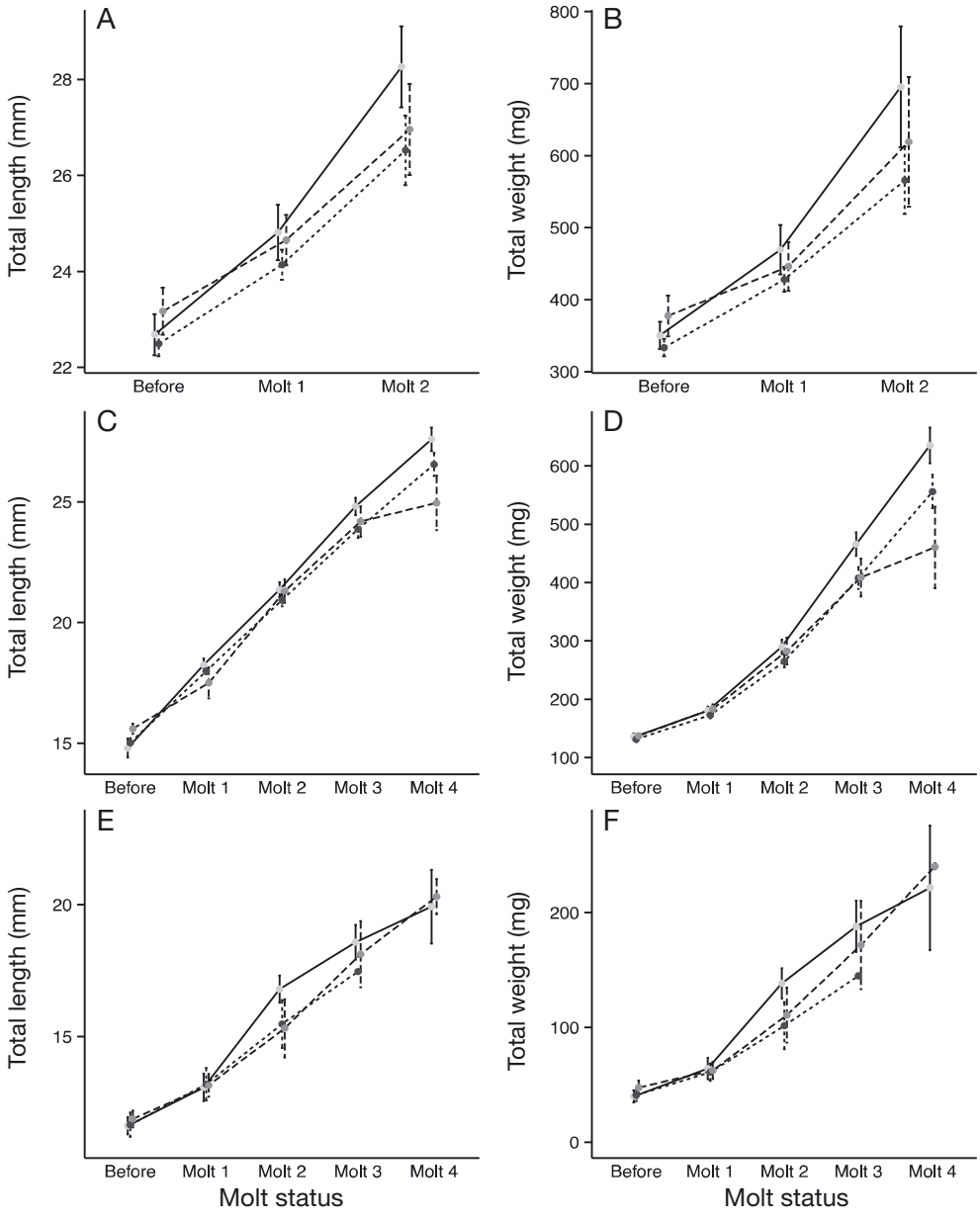

Fig. 4. Mean length and weight trajectories over time in all studied lobster *Homarus americanus* populations with standard error bars. (A,B) CT population (CT, starting n = 71); (C,D) ME14 population (ME14, starting n = 73); (E,F) ME15 population (ME15, starting n = 102). 'Before' indicates the starting length and weight the day before the experimental period began. Low (solid lines), medium (dashed lines), and high (dotted lines) CO_2 levels are shown

weight percent increase was also significantly higher in the lowest CO_2 treatment than in the highest CO_2 treatment: 90.67 ± 5.68% and 51.60 ± 5.64%, respectively ($p > 0.05$, Fig. 6B).

Observations of shell disease

Some lobsters in all treatments in both 2014 and 2015 were afflicted with shell disease, which was identified as rust colored spots on the carapace, telson, or chelae (Castro et al. 2012). When an infected lobster successfully completed a molt, the disease was not visually apparent on the new shell. Lobsters in the high and medium CO_2 treatments experienced shell disease at a higher frequency than those in the low CO_2 treatments. The highest percentage of lobsters afflicted with shell disease at any one time in 2014 was 29% in the medium CO_2 tank and 25% in the high CO_2 tank (Fig. 7A). The highest percentage

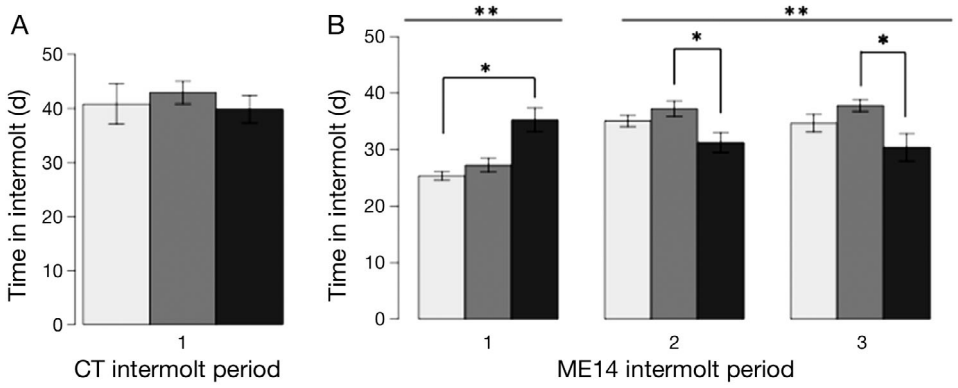

Fig. 5. Length of intermolt period(s) for the (A) CT and (B) ME14 lobster *Homarus americanus* populations in the 3 CO_2 treatments. Due to high mortality and insufficient numbers of molts, the ME15 population intermolt period was not analyzed. Low (light grey), medium (medium grey), and high (dark grey) CO_2 levels are shown. Values are mean ± standard error. Asterisks indicate significant statistical differences (Kruskal-Wallis test, *p < 0.05, **p < 0.01)

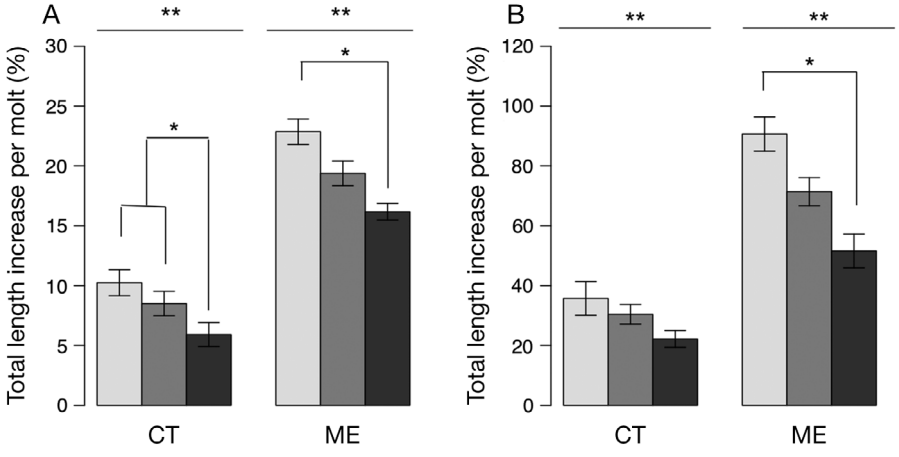

Fig. 6. Mean percent increases in (A) length and (B) weight for the CT (mean number of molts: 2) and ME14 (mean number of molts: 3) lobster *Homarus americanus* populations in the 3 treatments with standard error bars. Due to high mortality and insufficient numbers of molts, the ME15 population percent increases in length and weight were not analyzed. Low (light grey), medium (medium grey), and high (dark grey) CO_2 levels are shown. Asterisks indicate significant statistical differences (Kruskal-Wallis test, *p < 0.05, **p < 0.01)

of lobsters afflicted with shell disease at any one time in 2015 was 60% in the low and medium CO_2 tanks and 100% in the high CO_2 tank (Fig. 7B).

Graphs of the model-derived probabilities of a lobster in any population contracting shell disease are shown in Fig. 8, the generalized logistic mixed effect model coefficients and results of the Type II Wald chi-square test of significance are given in Table 2, and actual observations are shown in Fig. 7.

For the CT population of lobsters (n = 71), the amount of time (up to 90 d) a lobster spent in CO_2, the days since the previous molt, and the interaction between time in treatment and the 3 CO_2 levels were all highly significant determinants of shell disease prevalence ($p < 0.0001$, Table 2). For the ME14 population of lobsters (n = 73), the days since the previous molt and the interaction term—time in treatment interacting with CO_2 level—were both highly significant determinants of shell disease presence or absence ($p < 0.0001$), but the amount of time spent in CO_2 (up to 120 d) and the single factor of the CO_2 level were not significantly different from each other ($p = 0.84$, $p = 0.85$, Table 2).

DISCUSSION

Mortality and reduced growth in elevated CO_2 conditions

We found no significant mortality attributable to the CO_2 levels tested here (Fig. 3), although another

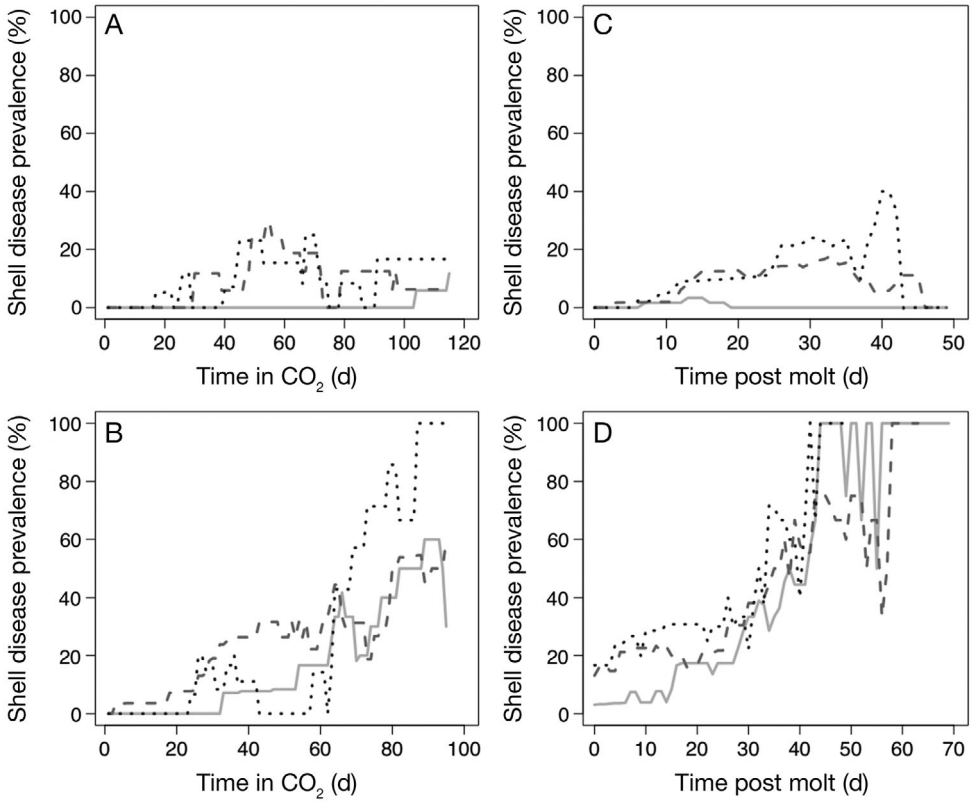

Fig. 7. Observed presence of shell disease in lobsters *Homarus americanus* over (A,B) the number of days spent in treatment and (C,D) the number of days since the previous molt. (A,C) ME14, and (B,D) CT populations. Due to high mortality, the shell disease prevalence in the ME15 population was not analyzed. Low (solid lines), medium (dashed lines), and high (dotted lines) CO_2 levels are shown

study has reported a decrease in survival in *Homarus gammarus* subjected to elevated CO_2 treatments over a 28 d term (Small et al. 2016). We did observe significant reductions in growth in high CO_2 in agreement with other studies of crustaceans (shrimp, Kurihara 2008; prawns, Wickins 1984; juvenile blue crabs, Long et al. 2013), including lobsters (Small et al. 2016, Rato et al. 2017). There are at least 3 possible explanations for this reduced growth: metabolic suppression in response to adverse conditions, reduced calcification due to a direct or indirect effect of elevated pCO_2, or increased cost of ion transport leading to reduced energy for growth.

Metabolic depression is a possible response to elevated CO_2 in some species. Pteropod mollusks reduced metabolism under 800 ppm CO_2, but only when sufficiently fed to support routine metabolism (Seibel et al. 2012). *Amphiura filiformis*, an echinoderm, reduced metabolism by 63% relative to control and *Littorina littorea*, a mollusk, reduced metabolism by 25% (Bibby et al. 2007, Wood et al. 2008). However, metabolic suppression is typically a short-term response to adverse environmental conditions (Seibel 2011) and is not likely sustainable in the long term—plus, it is not clear whether lobsters are capable of this adaptive response.

The reduced growth in lobsters exposed to high CO_2 observed by Small et al. (2016) was attributed to metabolic depression as shown by a reduced oxygen consumption rate and a lowered feeding rate. Similarly, the reduced growth seen by Rato et al. (2017) was associated with significantly lower superoxide dismutase activity (an indication of oxidative stress).

Findlay (2011) observed that the percentage of Ca^{2+} ions in the shells of 5 calcifying organisms decreased when only their shells were exposed to the lower pH conditions (pH 8.0 to 6.5), but the percentage of Ca^{2+} ions remained constant or even increased when the live animals were exposed to the same conditions. Calcification is a process that needs to be considered on a whole-body scale, with other processes such as metabolism closely linked.

The body size of the lobsters may have been reduced due to a change in energy demand caused by the increase in pCO_2 and the costs of acid–base regulation rather than a decrease in calcification.

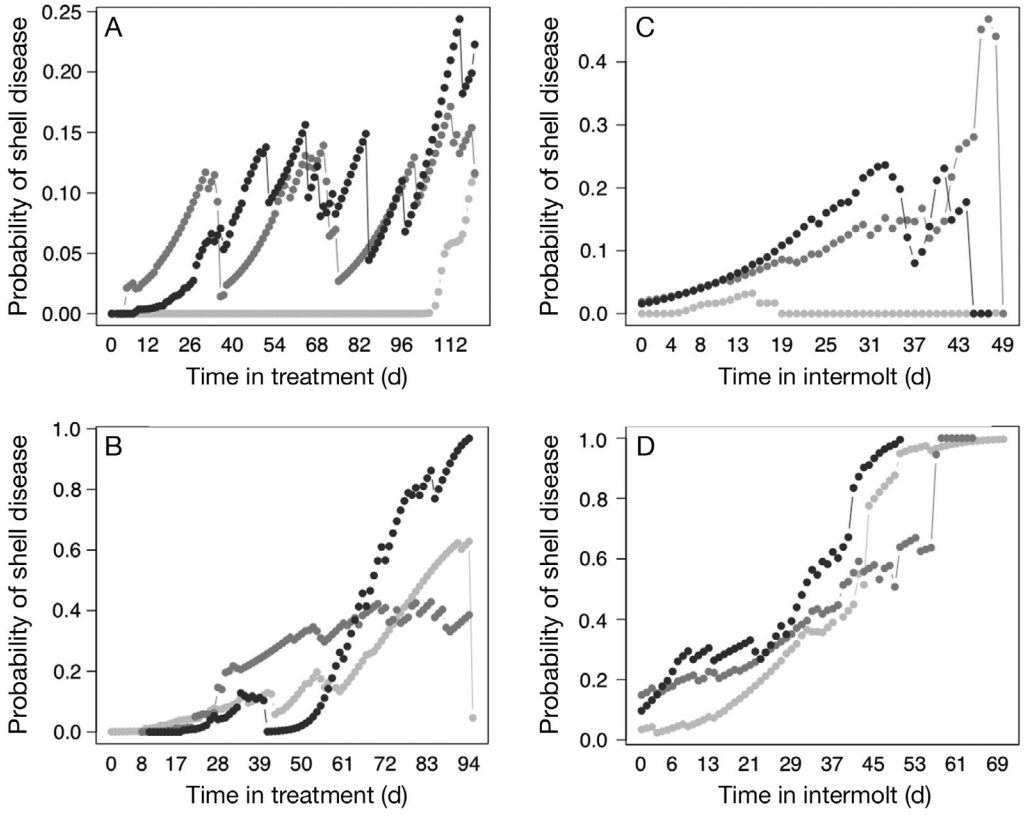

Fig. 8. Predicted probability of shell disease in lobsters *Homarus americanus* over the number of days in CO_2 in (A) 2014 and (B) 2015, and over the number of days since the previous molt in (C) 2014 and (D) 2015. (A,C) ME14, and (B,D) CT populations. Due to high mortality, the shell disease probability in the ME15 population was not modeled. Predicted probability values come from the generalized logistic mixed effect models (for equations and coefficients, significance, and analysis of deviance, see the Supplement at www.int-res.com/articles/suppl/m596p113_supp.pdf). Low (light grey), medium (medium grey), and high (dark grey) CO_2 levels are shown

Table 2. Coefficients and analysis of deviance results from the generalized logistic mixed effect models from 2014 and 2015 (2014, n = 73; 2015, n = 71). *p < 0.05, ***p < 0.001

Fixed effects:	Estimate	SE	*z*-value	p-value	Significance	Analysis of deviance (Type II Wald chi-square tests)	χ^2	df	p-value	Significance
2014 - ME14 population										
(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	2.20×10^{-16}	***
High CO_2	13250	61.79	2.145	0.032	*	Time:CO_2	35.7007	2	1.77E-08	***
Days	0. 11	0.0l	10.778	$<2 \times 10^{-16}$	***					
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										
(Intercept)	9580565	1.272605	−7528	5.14×10^{-14}	***	Time	126.4799	1	$<2 \times 10^{-16}$	***
Time	0.079573	0.008703	9.143	2.00×10^{-16}	***	CO_2	1.4996	2	0.4725	
Medium CO_2	3567312	1.612781	2.212	0.02697	*	Days	141.3001	1	$<2 \times 10^{-16}$	***
High CO_2	−757916	2.414262	−3.139	0.00169	**	Time:CO_2	82.1105	2	$<2 \times 10^{-16}$	***
Days	0.092488	0.007781	11.887	2.00×10^{-16}	***					
Time:Medium CO_2	−0.051975	0.010987 -	4.731	2.24×10^{-6}	***					
Time:High CO_2	0.149379	0.024348	6.135	851×10^{-10}	***					

There are few concrete measurements of the cost an animal incurs in regulating ions, possibly because there are a number of processes likely acting in concert that they can use to do so (for review see Heuer & Grosell 2014). Plus, the additional cost may only be transitory; once a new steady state is reached, the cost should be minimal. However, some recent work has shown the cost of protein synthesis and ion transport can increase up to 50% in sea urchins; though in that study, body size was not affected (Pan et al. 2015). Other studies have shown a decrease in size when calcifiers are exposed to elevated pCO_2 conditions. In fact, 'adapting through dwarfing' has been seen before, both recently and in the fossil record (Twitchett 2007, Garilli et al. 2015). Garilli et al. (2015) observed that 2 gastropod species living in naturally occurring high-CO_2 seeps were smaller compared to similar species living in lower CO_2 environments.

The ME14 lobsters molted 3 or more times in the 120 d observation period, resulting in 3 intermolt periods. The length of the first intermolt increased only in the highest CO_2 treatment (Kruskal-Wallis test, $p < 0.001$, Fig. 5B). Initially, the lobsters may have been stressed by the adverse conditions. Metabolic suppression resulting in fewer molts has been shown in other crustaceans exposed to hypercapnia (prawns, Wickins 1984; shrimp, Kurihara 2008). In contrast, the second and third intermolt periods were significantly shorter in the high CO_2 treatment relative to the medium CO_2 treatment (Kruskal-Wallis test, $p = 0.001$, Fig. 5B). This result implies that growth could be accelerated in higher CO_2 once the lobster adjusted to the metabolic and/or acid–base disturbance. Continued exposure to hypercapnia, beyond the first molt, may have resulted in more frequent molting (Fig. 5B) and smaller growth increments (Fig. 6), thereby compensating for the initial slow growth. 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—that is, having a suboptimal shell—molted at the same rate as those not under shell stress, but grew less with each molt (Fotheringham 1976). Another study found that Indian white prawns will molt the same number of times at low pH, but will not grow as much overall (Vijayan & Diwan 1995). However, compensation via more frequent molts may not be sustainable in the long term due to post-molt vulnerability to predation.

The hatcheries raised the lobsters with a lower pH (pH 7.7 ± 0.4 in CT, pH 7.6 ± 0.2 in ME) than the control pH reported here (Table 1). This opens up the possibility that these lobsters may have been pre-acclimated to lower pH conditions. However, since the lobsters were acclimated at pH 8.0 for 1 wk prior to our study and because significant differences among treatments were observed in this controlled study, we do not think any pre-acclimation had an impact on the observed growth reductions and intermolt period differences.

Implications for shell disease

We found an increased prevalence of shell disease in high CO_2 treatments (Fig. 7). Shell disease was present in all tanks and all treatments, so the causative agent was likely present in the incoming water from Narragansett Bay. The vector of infection is still poorly understood, but previous studies have shown that placing diseased and healthy lobsters in the same water does not end in infection for all lobsters (Quinn et al. 2012), so lobsters did not necessarily catch the disease from sharing the same water. We hypothesize that either the lobsters were experiencing CO_2-related stress and their immune responses were diminished, or that the causative agent was somehow enhanced in high CO_2.

The CO_2 level did not have a statistically significant effect on any of the mortality curves ($p > 0.21$), but the presence of shell disease did have a significantly negative effect on the probability of survival in the CT population (generalized logistic mixed effect model, $p < 0.001$, Table 2). Shell disease does not usually predicate death, except in severe cases, so this finding was surprising (Castro et al. 2012).

The results from the Type II Wald chi-square tests for both the 2014 and the 2015 models indicate that the interaction between time in treatment and the CO_2 level was significant ($\alpha < 0.05$), which shows that as time in treatment increased, the probability of contracting shell disease was significantly different for each CO_2 level (Type II Wald chi-square, $p < 0.0001$, Table 2). These probabilities are visualized in Fig. 8A,B. These results suggest that, generally, lobsters in the medium and high CO_2 treatments were contracting shell disease more often than lobsters in the low CO_2 treatment. Large decreases in the probabilities of shell disease within each treatment were a result of molting events. Molting rids the lobster of the diseased shell, but not necessarily the disease agent. Thus, lobsters molting within days of each other decreased the overall averaged probability computed by the model.

Lobsters that had longer intermolt periods had a higher chance of having shell disease (Fig. 8C,D).

This finding agrees with previous studies showing a higher prevalence of shell disease in lobsters that had been in intermolt for longer periods of time (Laufer et al. 2005, Glenn & Pugh 2006). Interestingly, lobsters in the medium and high CO_2 treatments were more likely to develop shell disease earlier after a molt than the control lobsters. Softer-shelled lobsters are more vulnerable to disease (Cawthorn 1997), so increasing the duration 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 to 2 d 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. L. McLean pers. obs.).

Immune responses have been shown in other work to decrease under hypercapnic conditions. In a 32 d exposure to high CO_2 (control pH 7.8, lowest treatment pH 6.5), mussels decreased phagocytosis — the first line of immune response — over time, though other immune response indicators such as superoxide anion production and total and differential cell counts were unaffected by treatment (Bibby et al. 2008). Immune response in lobsters has been investigated as well. Prophenoloxidase (PPO), a major component of the immune response in lobsters, was suppressed in the Norway lobster *Nephrops norvegicus* after 4 mo of exposure to lowered pH (7.5 to 7.7) and higher pCO_2 water (1000 to 1700 µatm) (Hernroth et al. 2012). PPO is normally activated when the thin calcite layer below the less mineralized biogenic epicuticle is completely breached. The authors suggested that lobsters are more susceptible to disease in OA conditions, a possibility supported by the present results. Alternatively, the higher pCO_2 could be directly responsible for the growth of the causative agent of shell disease. Because we did not directly measure any immune response parameters, we cannot determine the underlying mechanism behind the increased shell disease rates in high CO_2. Testing disease prevalence in CO_2 along with other stressors such as temperature or salinity while measuring immune response parameters may be a viable avenue to understanding what environmental conditions favor shell disease development.

CONCLUSIONS AND BROADER IMPACTS

Early benthic phase *Homarus americanus* subjected to elevated CO_2 conditions exhibited decreased growth increments (length and weight) over a 90 to 120 d period, altered intermolt periods, and an increased risk of contracting shell disease. These results are particularly important in light of the lobster's economic importance in Maine and southeastern Canada. Although lobster landings by total mass are low (1% of total fisheries landings 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 suggests, then fewer juveniles may survive to adult size. Staying smaller longer is dangerous for any juvenile, as it increases the probability of predation and reduces the number surviving to either reproductive status or fishery recruitment.

Additionally, the increased prevalence of shell disease is worth noting in light of the potential economic cost. The lobster fishery depends heavily on the success of new recruits, and shell disease may inhibit those new recruits as shown here, or affect adults by causing death during molting or premature molting in egg-bearing females (Laufer et al. 2005). Previous work has linked the decline in lobster abundance from 1997 to 2004 with an outbreak of shell disease around the same time (Gibson & Wahle 2005).

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