

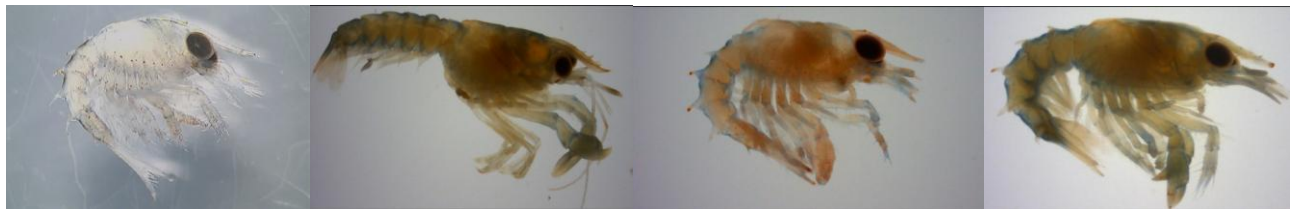
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# Physiological and developmental impacts of acidified seawater on larvae of the American lobster (*Homarus americanus*)

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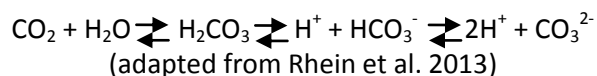
Left to right: *Homarus americanus* larvae at development stages I, II, III, and IV.

## **Abstract**

Increasing anthropogenic CO<sub>2</sub> emissions entering our oceans are increasing water acidity and might reduce the ability of lobsters to precipitate their CaCO<sub>3</sub> cuticles. Larval lobsters are thought to be more susceptible to CO<sub>2</sub>-induced acidification than adults due in part to a relatively high molting frequency. We raised *Homarus americanus* larvae in separate systems at low, mid, and high pH levels to determine whether CO<sub>2</sub>-induced acidity impacts their growth, cuticle mineralization, and development rate throughout their four larval stages. We found that neither carapace length or dry carapace mass significantly differed among pH treatments, but dry body mass of stage III and IV larvae was significantly lower at low and mid pH relative to that of larvae at high pH ( $p < 0.001$ ). Percent calcium of larval carapaces was generally lower at decreased pH and significantly so for stage II larvae ( $p < 0.05$ ), whereas the same trend of percent phosphorous among pH treatments was not significant. Larvae in low and mid pH treatments also had delayed development to each larval stage ( $p < 0.001$ ). Trends of decreased body mass, reduced calcium, and reduced phosphorous at low pH suggest a thinner and weaker cuticle, and along with delayed development, may be the result of reallocation of energy away from growth and toward acid-base balance. These physiological changes may threaten fished *H. americanus* stocks by increasing larval susceptibility to predation, disease, and time until maturity is reached.

## Introduction

Increasing acidity of marine waters is a major concern among the many environmental issues associated with global climate change. Global mean surface seawater pH, currently at about 8.1 units, has decrease by 0.1 units since pre-industrial times and is predicted to fall another 0.2-0.4 units by 2100 (Rhein et al. 2013). Because the dissolution of CO<sub>2</sub> into seawater lowers the availability of carbonate ions, marine calcifying organisms that must harness carbonate to produce CaCO<sub>3</sub> shells or exoskeletons are potentially subject to reduced fitness due to dissolution of their CaCO<sub>3</sub> components and other physiological changes associated with acidity. Species that do not use carbonate in calcification still may be impacted by stress-related effects of acidosis or hypercapnia. Ocean acidification occurs when anthropogenic CO<sub>2</sub> emissions enter the atmosphere and later dissolve into surface waters. CO<sub>2</sub> subsequently reacts with water through the following system:



In this reaction system, CO<sub>2</sub> and water combine to form carbonic acid, which dissociates into bicarbonate and carbonate ions. When this occurs, H<sup>+</sup> is released and thus pH is decreased. Since these reactions are bidirectional, as more CO<sub>2</sub> goes through this system, more carbonate is converted back into bicarbonate, decreasing the availability of carbonate ions. Below a pH of 6, bicarbonate becomes much more limited and hydrogen carbonate predominates.

The extent to which acidified water impacts marine animals depends on other physical properties of water, such as temperature. CO<sub>2</sub> is more soluble in colder waters, so calcifying organisms living at high latitudes and other cold-water regions may be more vulnerable to acidification. The vulnerability of a CaCO<sub>3</sub> producing species to ocean acidification also depends on the forms of CaCO<sub>3</sub> that compose its shell or exoskeleton. For example, pteropods have a thin, largely aragonite-based shell. Because aragonite is more soluble than pure calcite (Ries 2011), pteropods are considered highly susceptible to shell dissolution as aragonite becomes undersaturated due to sea-ice melt (Lischka and Riebesell 2012). Comparatively, crustaceans might be more resilient to shell dissolution due to a thicker exoskeleton comprised primarily of CaCO<sub>3</sub> forms less soluble than aragonite, such as magnesium-calcite. As the magnesium to calcium ratio of calcite increases though, so does its solubility (Ries 2011). Notably, it is thought that crustaceans primarily use CO<sub>2</sub> or bicarbonate (rather than carbonate) to form CaCO<sub>3</sub>, suggesting that reduced carbonate availability may not directly influence crustacean CaCO<sub>3</sub> production (Arnold et al. 2009).

Uncertainty exists regarding the effects ocean acidification may have on the physiology and ecology of many crustacean taxa. Further, despite the numerous studies investigating effects of CO<sub>2</sub>-induced acidification on animal physiology, most have focused on adults and few have focused on juvenile and larval stages. Our study focused on how CO<sub>2</sub>-induced acidification of water impacts the growth, development, and carapace mineralization of larvae of the American lobster, *Homarus americanus*. This species was selected as our study organism because it is a commercially important species in the Gulf of Maine and there is scant evidence for how it may be impacted by acidification. To date, only three published studies have investigated the effects of low pH on *H. americanus*: one on larvae (Keppel et al. 2012), and two on juveniles (Ries 2011; Ries et al. 2009). A master's thesis by Hall (2012) also investigated how low pH affects larval development of *H. americanus*. Additionally, a couple of studies have focused on larvae and juveniles of the closely related European lobster, *Homarus gammarus* (Agnalt et al. 2013; Arnold et al. 2009). For pelagic crustacean larvae such as lobsters, factors that may increase sensitivity to a low pH environment (and other environmental changes associated with climate change) include high frequency of molting relative to adults (Keppel et al. 2012), and use of amorphous calcite in part of their cuticles (exoskeletons), which is a relatively soluble carbonate form (Vezina and Hoegh-Guldberg 2008). Further, since lobster larvae have no known endogenous mechanism to delay hatching, changes in climate that mismatch the timing of blooms of their planktonic prey, if the time of lobster hatching remains fixed, could decrease larval survival rates (Green et al. 2014). Thus, focus on larval stages of lobsters is of critical importance in determining lobster susceptibility to climate change.

*H. americanus* is found along the Western Atlantic coast as far south as North Carolina and as far north as Labrador, Canada. However, lobster fisheries of southern populations, notably in Long Island Sound, have declined whereas northern fisheries in Maine are growing (Thunberg 2007). One possible cause for the collapse of southern populations in recent years may be an increase in the number of days water temperature exceeded 20 °C, leading to increased temperature stress and occurrence of shell disease (Hall 2012). Thus, we may be already observing influences of climate change on *H. americanus* populations.

Larvae of *H. americanus* progress through four pelagic larval stages during the course of 2-3 weeks (when in water at 20 °C, and longer at lower temperatures), molting to each subsequent stage before settling on the substrate to adopt a benthic lifestyle. Pre-larvae stay attached to the mother when hatched, but release into the water column when they become stage I larvae (Hall 2012). Larvae

progress to stage II and stage III, and after molting to stage IV they start searching for a desirable site to settle on the benthos. Once settled, they molt to the first juvenile stage.

As noted by Hall (2012), Stage I larvae can be identified by their long forked telsons, lack of uropods, and lack of abdominal pleopods. Stage II larvae possess abdominal pleopod buds that lack setae. Stage III larvae have setae-bearing pleopod buds and small uropods on the telson. Stage IV larvae have more obvious morphological changes and resemble a miniature version of the adult, bearing long antennae and antennules, larger uropods, a straighter (versus c-shaped) body, and large chelipeds (claws). Additionally, larvae at each stage are visibly larger than those at previous stages. In addition to these normal stages, the occurrence of intermediate stages in *H. americanus* has been observed as a result of eyestalk ablation (Charmantier and Aiken 1987; Charmantier et al. 1988) and exposure to crude oil (Wells and Sprague 1976). As a result of eyestalk ablation, several intermediate stages were characterized: stage IVa, which is morphologically intermediate between normal stages III and IV; stage IV', a post-larval stage that retains some larval morphological features but loses these in the following molt; and stage V', which occurs when a stage IVa lobster retains some of its larval features when it molts to the first juvenile stage (Charmantier and Aiken 1987). Stage IVa larvae share some characteristics with stage III larvae, including a bent body shape, short antennal flagellum, and long lateral spines, but the relative length of their uropods, telson shape, and other characteristics are intermediate between stages III and IV. Stage IV' lobsters are similar to stage IV larvae in carapace length and general body shape, but possess long setae-bearing exopodites on their pereopods and long abdominal dorsal spines. Stage V' larvae retain larval features such as dorsal abdominal spines, long postereolateral telson spines, and long setae-bearing exopodites on the pereopods (Charmantier and Aiken 1987; Charmantier et al. 1988). Although possible explanations for the presence of intermediate lobster stages include pollutants in water, malnutrition, and unfavorable salinity or temperature (Charmantier et al. 1988), no correlation between acidic water and the presence of intermediate stages of *H. americanus* has been found. Hall (2012) identified one lobster that was potentially at stage IV', though he suggested this stage was likely due to stress effects associated with a low pH environment rather than a direct effect of the low pH itself. The delay of molting to the first juvenile stage by molting to stage IV' beforehand may represent a higher energy requirement for harnessing minerals and precipitating the cuticle while in a stressful environment.

To understand how lobster cuticles may respond to low pH, the formation and structure of the cuticle must be considered. The following information about the cuticle is known from adult lobsters. The general structure of the cuticle is commonly described as a "twisted plywood" pattern. The cuticle

consists of one uncalcified innermost layer called the membranous layer and three calcified layers which, listed externally to internally, are the epicuticle, exocuticle, and endocuticle (Luquet 2012). The cuticle is composed of several  $\text{CaCO}_3$  polymorphs that vary spatially in concentration and lead to differential solubilities of these layers. Of the polymorphs known to exist in crustaceans, pure calcite is the least soluble, whereas the solubility of magnesium-containing calcite is higher and increases with the amount of magnesium present (Ries 2011). The *H. americanus* cuticle contains other compounds and trace elements, most notably phosphorous, however at least 95% is composed of  $\text{CaCO}_3$  and much is in the form of high-magnesium calcite (Kunkel et al. 2012; Ries 2011). The epicuticle lacks chitin and instead is composed of various lipids, proteins, and calcium salts (Hall 2012; Kunkel 2013). The exocuticle, also called the calcite layer, contains  $\text{CaCO}_3$  and carbonate apatite (CAp), and phosphate is deposited at the bottom of this layer (Kunkel 2013). Below this calcite layer, the endocuticle is composed of amorphous  $\text{CaCO}_3$  (ACC), which is more soluble in acid relative to other  $\text{CaCO}_3$  polymorphs (Kunkel 2013). Within the cuticle are many canals lined with CAp at their openings, each of which corresponds to a cuticular gland or sensory neurite and is used for transport of molecules secreted through the cuticle (Kunkel et al. 2012; Kunkel 2013). Relative levels of calcium and phosphorous differ throughout the cuticle. For example, in cuticular canals, the calcium to phosphorous (Ca:P) ratio in the outermost part of the canal wall is 1.76 and this ratio increases up to 7 in the innermost part of the canal (Kunkel 2013). Kunkel (2013) found that transecting through the cuticle layers (but not any cuticular canals), relative abundance of calcium is high in the exocuticle, dips in the transition to the endocuticle, and increases again in the endocuticle. Magnesium is relatively lower in abundance but follows the same general pattern as calcium. Phosphorous is low in abundance throughout this transect, with a small dip in abundance in the transition between the exocuticle and endocuticle. However, phosphorous is abundant in the canal walls and trabeculae. Overall, only the outer part of the *H. americanus* exocuticle has magnesium-calcite, and the rest of the exocuticle and the endocuticle are primarily calcified with ACC, with some CAp in trabeculae and canals (Luquet 2012; Kunkel 2013). Also notably, the dorsal carapace contains more calcite than other parts of the lobster exoskeleton (Al-Sawalmih et al. 2008).

*H. americanus* maintains a high pH layer external and adjacent to the epicuticle. The lobster solubilizes the  $\text{CaCO}_3$  from its own cuticle which, using a proton from water, is converted to bicarbonate and a hydroxyl group is released, increasing alkalinity near the cuticle surface (Kunkel et al. 2012). It is not certain however, that larval lobsters share this ability with juveniles and adults. The layer is referred to as unstirred because due to rapid ion diffusion across the cuticle, bulk water flow around the lobster

does not mix up this layer (Kunkel et al. 2012). Further, certain  $\text{CaCO}_3$  polymorphs likely have important roles in maintaining cuticle hardness and thickness, as well as protecting the lobster from microbial invasions. Magnesium is present in low levels at the calcite surface, suggesting that dissolution of  $\text{MgCO}_3$  may aid in maintaining an alkaline unstirred layer (Kunkel et al. 2012). Magnesium is also used to harden the cuticle, so it probably does not contribute much to cuticle hardness at the calcite surface. The dissolution of  $\text{MgCO}_3$ , which is more soluble than pure calcite, may be used to increase unstirred layer pH just after ecdysis (molting) has occurred, when the cuticle is softer than usual. Early post-molt, cross-linked CAp might also serve to increase cuticle hardness, and ACC might serve as an intermediate form for calcite layer maintenance at this time (Kunkel 2013). ACC is thought to act as a reservoir of  $\text{CaCO}_3$  to harden the cuticle quickly after molting or in response to injury, since ACC requires less energy to dissolve (Kunkel 2013). Further, ACC may aid calcium mobilization, helping decalcify the old cuticle during the pre-molt phase, and may contribute to flexibility of cuticle shape (Luquet 2013). Thus, even the relatively more soluble  $\text{CaCO}_3$  polymorphs of the *H. americanus* cuticle likely play important roles in maintaining a strong cuticle. Also, high density of the cuticle prevents the passage of many organic polymers, and the high pH of the unstirred layer (which depends on the dissolution of ACC or calcite) probably inhibits the mobility and metabolism of bacteria (Kunkel 2013). Thus, proper structure and function of the cuticle is likely essential to blocking the invasion of pathogens in addition to the mechanical protections it provides.

As mentioned above, larval *H. americanus* must molt to reach each subsequent development stage. Because these larval molts occur in much greater frequency than adult molts, larvae may depend even more than adults on rapid post-molt cuticle and tissue growth for protection against microbes and predators. In adults, in addition to calcite, the canals and their interface with the calcite layer are replaced with each molt. Cuticular organules are then moved away so that the new cuticle, which is considered to be mineralized from its epidermal side, can expand and new organules are formed to be placed back into the now larger cuticle (Kunkel et al. 2012). Thus, early post-molt growth seems mostly cuticle-based, whereas more tissue growth and cuticle hardening may occur afterwards. Kunkel et al. (2012) suggest that low pH during a molt may necessitate the allocation of more energy to obtain carbonate from bicarbonate, which could in turn allow more bacteria to enter through areas of the cuticle that are thinner and thus more acid vulnerable. Hence, residing in a low pH environment may result in an even more critical need for a lobster, particularly a larval one, to calcify early and quickly after molting.

Our objective in this study was to determine if rearing *H. americanus* larvae in acidified water alters their growth, carapace mineralization, and development rate, and whether effects are specific to larval age or stage. We used morphometrics to study larval growth, including measurements of carapace length, body mass, and carapace mass, and analyzed percentages of calcium and phosphorous in carapaces. We hypothesized that larvae raised in low pH water would have delayed development to subsequent stages and reduced body mass, carapace mass, and carapace length (compared to those in high pH water) due to reduced ability to mineralize or maintain the cuticle. We further hypothesized that the carapaces of older larvae (stages III and IV) raised in low pH water would have reduced calcium content due to a decrease in  $\text{CaCO}_3$  production and because these later larval stages, especially stage IV larvae, which are thought to be more calcified than younger stages, at least under normal conditions (T.J. Bowden, personal communication, April 9, 2015). We also hypothesized that phosphorous, used in CAp formation of the carapace, would not differ among pH treatments because its presence may not be directly affected, if at all, by redistribution of carbon species associated with  $\text{CO}_2$ -induced acidity. Lastly, we looked for the presence of intermediate stages to determine if they were more likely to occur in larvae kept in acidified conditions. Our results are compared with those of other studies that have investigated development of *H. americanus*, *H. gammarus*, and other crustaceans in acidified water.

## **Methods**

### **Rearing System**

All larvae were the offspring of two females (hence they were of mixed parentage) from the Downeast Institute for Applied Marine Research and Education in Beals, ME. The total number of larvae obtained was estimated using the following equation:

$$y = 0.0824 + 0.00633x$$

where  $y$  = mass and  $x$  = number of larvae (Beal 2011, as cited by Hall 2012). About 12000 larvae were obtained in total. Larvae were transported in layered paper towels soaked in cold seawater (at about  $5^\circ\text{C}$ , though the temperature was not measured), which prevented cannibalism. Larvae were raised in three separate circulation systems. Each system contained four, 64 L (17 gal) cylindrical plastic holding tanks, a header tank, and a sump tank, with a combined capacity of about 644 L (170 gal). Water passed from the sump to the header through a Bio-Bead filter, and through a chiller which maintained temperature at  $18\text{-}20^\circ\text{C}$ . Flow into the holding tanks was counterclockwise around the standpipe, which was covered with mesh so larvae were not lost through the outflow. Airstones at the bottom of each



holding tank created turbulence and inhibited cannibalism. Water was an artificial seawater mix (Crystal Sea, Marine Enterprises, MD, USA) and maintained at a salinity of 33-35 PSU. The seawater mix was made in a 454 L (120 gal) holding tank and was used to supply the core circulation system as needed. Larvae were placed in holding tanks with a target density of 1 larva per 50 mL, so there were approximately 20 larvae per L, 1320 larvae per holding tank, and 3960 larvae per system. Larvae were exposed to a light cycle of 12 h of light followed by 12 h of darkness. Lobster larvae were fed three times per day with live *Artemia*, frozen mysis shrimp, and frozen bloodworms. For the first week of the experiment, daily water changes of about 95 L were performed, and water changes were performed every other day thereafter. Water quality (including ammonia, nitrite, and nitrate levels) was tested twice on day 4 and twice on day 10 of the experiment. Due to time constraints, neither the biological filters, nor the rest of the systems, were stabilized upon the start of the experiment, having only run a few days before the lobster rearing period began.

The control system was intended to be maintained at a pH of 8.15. The header tanks in the other two systems were gassed with carbon dioxide using a simple gassing system connected to a pH probe, solenoid, and control center. One experimental system was to be maintained at a pH of 7.9 and the other at 7.6. If pH was too high in a given system, the control center switched on the solenoid, allowing more carbon dioxide to enter the system, and vice versa if pH became too low. High-precision Durafet pH probes were used to measure pH. The efficacy and stability of these probes were questionable however, despite the calibration of the probes using NIST compatible buffer standards. One probe consistently read 0.1 pH unit off from the others, and by 16 days into the experiment the probes ceased to work. Due to the malfunction of these probes, we ignored the probe readings and instead trusted the pH measurements from subsequent water analysis (see below). Temperature, salinity, and pH for all systems were recorded every day of the experiment except on days 13 and 14.

### **Water Analysis**

Water samples were collected from the first holding tank in flow about every three days throughout the experiment and mercuric chloride was immediately added to these samples in order to kill off organic matter. Analysis of water quality, including pH, total alkalinity, and dissolved inorganic carbon (DIC), was conducted by Dr. Joe Salisbury at the University of New Hampshire's Ocean Processes Analysis Lab. These analyses were performed according to equipment and testing methods outlined in the "Guide to Best Practices for Ocean Acidification Research and Data Reporting" (Riebesell et al. 2010). From this analysis, the pH for each system was found to be 0.2-0.3 units lower than our target pH, so the

treatments of target pH 7.6, 7.9, and 8.15 are henceforth referred to as "low pH", "mid pH" and "high pH", respectively.

### **Collection**

Lobster larvae were collected throughout the rearing experiment, between July 22nd and August 11th, 2013. For morphological measurements, about 10 larvae from each of the three systems were haphazardly sampled every day ( $n_{\text{Total}}=560$ ) and were placed in a fridge at 4 °C in 1.5 mL microcentrifuge tubes containing about 1 mL of 10% formal saline buffered with PBS buffer (pH 7.2). For calcium and phosphorous analysis of carapaces, at least 10 additional larvae from each system were collected each day and snap frozen at -80 °C. Out of these samples, 15 larvae from every other day (except 54 larvae on day 17 and 10 larvae on day 18), were selected for carapace mass, calcium, and phosphorous measurements ( $n_{\text{Total}}=176$ ). Although larvae in our study were from two adult females, random assignment of the larvae to pH treatments and random sampling of larvae likely allowed independence of a brood factor.

### **Morphometrics**

Using a dissecting microscope and integrated camera (Labomed "Luxeo 4D", Labo America Inc., CA, USA), all larvae were photographed and their developmental stages were identified. Carapace lengths were measured using Pixel Pro (Labo America Inc., CA, USA) and measured from the posterior end of the carapace to the posterior edge of the eye socket. Larval wet body masses were measured and dry body masses were measured after placing larvae in an oven at 60 °C for about 30 minutes. Wet masses were well correlated with dry masses ( $R^2 = 0.8442$ , data not shown), though only dry masses are presented here under the assumption that they are more accurate than wet masses. Under observation with the microscope and using photographs, samples were examined for the presence of intermediate stages. We expected that most or all intermediate stages observed, if any, would be characteristic of stage IVa previously described by Charmantier and Aiken (1987).

### **Carapace Analysis**

Using fine forceps, carapaces were isolated from larvae under a dissecting microscope. Care was taken to remove as much underlying tissue and haemolymph from carapaces as possible. Carapaces were air-dried under a clean hood and weighed on a five decimal place micro balance. These samples were then digested at room temperature in plastic vials by first adding 100  $\mu\text{L}$  of  $\text{HNO}_3$  (except for the

first 12 samples digested, which were 17-day old stage IV larvae dissolved in 15  $\mu\text{L}$   $\text{HNO}_3$ ; these were determined to be fully digested before analysis), then an equal volume of Milli-Q water a couple days later, and bringing the solution to a final volume of 2 mL with Milli-Q water once carapaces were fully digested. Prior to analysis, each sample was diluted 1:100 using 25  $\mu\text{L}$  of sample and adding 25  $\mu\text{L}$  of indium (internal standard, 200 ppb stock), 25  $\mu\text{L}$  of  $\text{HNO}_3$ , and 2.5 mL of Milli-Q water. Calcium and phosphorous concentrations of carapaces were determined by inductively coupled plasma (ICP) mass spectrometry conducted by Mike Handley at the Sawyer Environmental Research Laboratory at the University of Maine, Orono. The ICP instrument used was an "Element 2" (Thermo Fisher Scientific, Waltham, MA, USA). Specific isotopes tested for calcium and phosphorous were  $^{44}\text{Ca}$  and  $^{31}\text{P}$ , respectively. The ICP instrument was set to medium mass resolution of  $4000 \text{ m } \Delta\text{m}^{-1}$ , which is determined by the following equation:

$$R = m / (|m_1 - m_2|)$$

where R is the resolution, m is the nominal mass, m1 is the mass of one isotope or species and m2 is the mass of a separate isotope or species. The ICP instrument took 25 replicate measurements for each sample, with every set of five measurements being averaged, followed by an overall average of these five averages. Calcium and phosphorous concentrations were measured by the ICP in  $\mu\text{g L}^{-1}$ . These values were multiplied by the dilution factor (100x), converted to  $\text{mg kg}^{-1}$  using the carapace masses, then converted to percentages (by dividing by 10,000). We also calculated the ratios of percent calcium to percent phosphorous of these samples.

### Statistics

All statistics were performed in R 3.1.0 (R Core Team, 2014). Within each larval stage, two-way, between-groups ANOVA tests were run to determine if length, dry body mass, dry carapace mass, percent calcium, and percent phosphorous of carapaces vary with days post hatching (DPH) and pH treatment. Dry body and carapace mass, calcium, and phosphorous data were log-transformed for ANOVA tests in order attain normal or closer to normal distribution. Normality was assessed with the Shapiro-Wilk test. The homogeneity of variances assumption for all ANOVA tests was tested with Levene's test from the car package (Fox and Weisberg 2011). Tukey's tests were performed using the multcomp package (Hothorn et al. 2008). For carapace length and dry body mass data, a separate Tukey's HSD test for each stage was used to find specific significant differences between combined DPH and pH treatment factors. For carapace mass, percent calcium, and percent phosphorous variables, individual Tukey's tests were performed for each stage with pH as the only factor. Due to lower sample

size for carapace mass, calcium, and phosphorous data (relative to body mass and carapace length data) and the fact that for many days of the experiment only one or a few samples were measured for a given pH treatment, carapace data on larvae from different days were pooled. To determine if pH treatment caused a delay in molting to subsequent larval stages, a Cochran-Mantel-Haenszel test was performed to compare proportions of samples (only out of those used for morphometrics) at different stages among pH treatments, controlling for DPH (i.e. a test for conditional independence).

## **Results**

### **Water Parameters**

Temperature of water flowing through the high pH rearing system fluctuated modestly over the rearing period, with an average of 19.03 °C and standard deviation of  $\pm 0.56$  °C (Fig. 1). Throughout the experiment, temperature of the other two systems were checked and confirmed to be within one degree of the high pH system (data not shown). The pH levels in the three systems were not constant, likely a result of the experimenters not having the time to allow the systems to stabilize prior to the placement of larvae into the tanks. The pH of each system fluctuated with a range of 0.2-0.3 units from 3 to 16 DPH (Fig. 2). Average pH levels were about 7.86, 7.42, and 7.31 for high, mid, and low pH treatments, respectively. Total alkalinity and DIC levels decreased from 3 DPH until at least 13 DPH, and were less variable between 9 and 16 DPH, suggesting that the systems were not stabilized until about 9 DPH (Fig. 2). Table 1 summarizes means and standard errors of temperature, pH, alkalinity, and DIC for these water samples. Lower standard error values for alkalinity and DIC measurements from 9 DPH onwards indicate that the systems were approaching equilibration at this time.

### **ANOVA Test Assumptions**

Carapace length data were not normally distributed, violating the normality assumption of the ANOVA tests for datasets of each larval stage, however the homogeneity of variance assumption was met for each stage (Table 2). Dry body mass data was log-transformed for use in ANOVA tests, as this produced a closer to normal distribution. Shapiro-Wilk tests for normality indicated that dry mass data for stage III were normally distributed and data for other stages were not normally distributed. Further, dry body mass data for stage III and IV violated the homogeneity of variance assumption (this assumption was met for stage I and II samples). Both the normality and homogeneity of variances assumptions of ANOVA were met for carapace mass and calcium data for each larval stage (Table 3).

Phosphorous data for each larval stage were normally distributed and data for all stages except stage IV met the homogeneity of variance assumption.

### **Morphometrics**

One carapace length outlier from the stage III data was removed from analysis. This length value was closer to most stage IV lengths than to stage III lengths and was likely due to a data entry error, but the value intended to be recorded could not be inferred. As expected, carapace lengths within a given larval stage were far more similar to each other than lengths among stages. Larvae at each stage had carapaces about 0.7 to 1.0 mm longer than individuals from the previous stage (Fig. 3A, 3B). There was no clear or consistent pattern of carapace length among pH treatments within a given stage and day. For groups (of one stage at one day and in one pH treatment) with sample sizes of  $n > 2$ , stage I and II larvae had very similar carapace lengths among the three pH treatments. Stage III larvae had somewhat lower mean carapace lengths for low and mid pH treatments (or the mid pH treatment only) compared to high pH, on some days (10, 11, 12 and 17 DPH for stage III; 14, 15, and 17 DPH for stage IV). ANOVA tests revealed DPH as a significant factor for stage II and IV larvae (Table 2). The pH treatment was not a significant factor for larvae at any development stage, and there were no significant interactions between DPH and pH factors. Tukey's HSD post-hoc tests did not reveal any significant differences between carapace length means among pH groups for larvae at a given day and stage.

Two outliers were found for dry body mass data, one in stage I and one in stage III, and these were kept in the analysis because there was no indication they were inaccurate measurements relative to other mass values for these stages. The stage I outlier had a dry body mass of 0.4 mg and was in the high pH treatment. The only other stage I samples with a mass this low were in the low pH treatment, so this individual larva may have been malnourished, representing natural variation among the control samples. Alternatively, this low mass may have been a result of unnoticed loss of part of the sample, as dry larvae are fragile and can break apart easily, though this is less likely since considerable effort was taken to handle the samples with care. The stage III outlier was in the low pH treatment and although this individual had a carapace length and morphology indicative of stage III, it had both wet and dry masses much higher than those of other stage III larvae collected on the same day. Thus, some unknown factor, such as increased cuticle thickness or food consumption, may have contributed to a heavier than usual larva in this case. Differences in mean dry body mass among pH treatments for stage I and II larvae had no clear pattern and those on a given day were not significantly different, though mean body mass in the low pH treatment was considerably lower than in the high pH treatment at 5 and 6 DPH for stage

II larvae (Fig. 4A, 4B). Most notably, body mass was lower in low or mid pH treatments than in the high pH treatment at 11 through 13 DPH and at 17 DPH for stage III larvae, and at 15 and 17 DPH for stage IV larvae. ANOVA tests for dry body mass indicated DPH as a significant factor for all stages, as expected, and pH treatment was a significant factor for stage III and IV larvae (Table 2). There was a significant interaction between DPH and pH factors for stage III larvae. A plot of this interaction effect (not shown) was disordinal and thus the interaction effect was difficult to interpret. Tukey's HSD tests resolved significant differences between dry body mass means of the high and low pH groups for stage III larvae at 11 and 12 DPH, and a significant difference between the high and mid pH groups for stage IV at 17 DPH.

### **Intermediate Stages**

Through observation of larvae under the microscope and of photographed larvae, we did not identify any larvae to be at intermediate stages.

### **Delayed Development**

There was a distinct delay in molting of larvae to subsequent stages, in both mid pH and low pH treatments, compared to the high pH treatment (Fig. 5). For example, at 4 DPH the majority of larvae sampled from the high pH treatment had molted to stage II, but the majority of larvae from the mid pH treatment were still at stage I, and even more were at stage I in the low pH treatment. A similar pattern was seen in the transitions to stage III and stage IV, at least between the low and high pH treatments. Peculiarly, there were no stage IV larvae sampled at 16 DPH even though new stage IV larvae were present at 15 and 17 DPH (possible explanations for this are discussed below). A Cochran-Mantel-Haenszel test indicated that, controlling for DPH, the proportions of larval stages significantly differed among pH treatments ( $p < 0.001$ ). This test assumes no three-way interaction between these three factors. The structure of our dataset, and perhaps the presence of structural zeros, impaired the functionality of the test for this assumption.

### **Carapace Analysis**

Two outliers were found for carapace data: one stage II outlier with a very large percent calcium value and one stage IV outlier with very small percent calcium and phosphorous values. The stage II outlier was kept in the dataset because there was no procedural error noted to indicate the value was incorrect, even though the dry mass value of the carapace, 0.06 mg, was much lower than that of most

of the stage II carapaces. The stage IV outlier, however, was removed from the dataset because the carapace was not fully dry when weighing, thus the dry mass was overestimated and the calcium and phosphorous values were underestimated. ANOVA tests for dry carapace mass indicated DPH as a significant factor in stage II and III data ( $p < 0.01$  and  $p < 0.001$ , respectively), and pH was not a significant factor for any stage (Table 3). For all stages, there was a trend of slightly higher carapace mass in the low pH treatment, though no differences between pH treatments within a stage were significant (Fig. 6). There was no clear trend of mean percent calcium among pH treatments for stage I larvae, although there was a trend of slightly lower calcium with lower pH for stage III larvae (Fig. 7A). For stage II and IV, larvae in the low pH treatment had the lowest percent calcium out of the three pH treatments. ANOVA tests revealed DPH as a significant factor for all stages except stage II ( $p < 0.05$  for stage II and III;  $p < 0.01$  for stage IV), and pH as a significant factor for stage II ( $p < 0.05$ ). Significant differences between percent calcium means were resolved by the Tukey's HSD tests for stage II larvae, between low and mid pH treatments and between low and high pH treatments (Fig. 7A). One comparison between low and high pH treatments for stage IV larvae approached significance ( $p = 0.071$ , not shown). For stages I through III, a consistent trend of lower percent phosphorous at lower pH treatments was found, and no clear trend was seen for stage IV larvae (Fig. 7B). ANOVA tests indicated that for phosphorous, DPH was a significant factor for stage IV lobsters and pH was not a significant factor for any stage. The pH factor approached significance for stage II larvae ( $p = 0.072$ ). For no larval stage did Tukey's HSD tests resolve significant differences in percent phosphorous among pH treatments. Different trends of mean Ca:P ratio among pH treatments were observed for each larval stage (Fig 7C). For stage I larvae, Ca:P ratios were higher in the low and mid pH treatments than in the high pH treatment, and for stage III larvae the reverse trend occurred. For stage II and IV larvae, Ca:P ratio was highest in the mid pH treatment. ANOVA tests indicated that DPH was a significant factor for Ca:P ratios for stages II through IV, and for all stages Ca:P ratios of carapaces did not significantly differ with pH treatment (Table 3). Tukey's tests indicated that the difference in mean Ca:P ratio between the low and mid pH treatments for stage II larvae was significant ( $p < 0.05$ ) and that the differences between low and mid pH treatments and between low and high pH treatments for stage IV larvae approached significance ( $p = 0.62$  and  $p = 0.93$ , respectively). It should be noted that although there were no statistically significant outliers of Ca:P ratio, one stage II, mid pH sample had a ratio of about 49.8, which was much higher than all other ratios by a value of at least 20. With this sample removed the Tukey's test did not resolve any significant differences in Ca:P ratios among pH treatments for stage II larvae. We recognize that although carapace mass, calcium, and phosphorous data were pooled by DPH, DPH turned out to be a significant factor for these variables in

some groups. However, there were no significant interactions between DPH and pH, so our Tukey's tests results are not confounded by pooling the data in this way.

## **Discussion**

### **Water Analyses**

Because water temperature remained relatively constant over the course of the experiment, it is unlikely that any observed differences in development rate or other measured variables among pH treatments were confounded by temperature effects. For all systems, pH varied over time, and there are several possible reasons for this. First, owing to the fact that we only had one opportunity to obtain the larvae and place them in the system, we had to begin the rearing experiment a couple weeks after the systems were set up, thus the systems may not have equilibrated by the start of the experiment. Also, the high level of aeration in the rearing tanks (which was necessary to prevent cannibalism) likely led to considerable CO<sub>2</sub> offgassing. Our high pH treatment was much closer to our target pH of 7.9 for the mid pH treatment than the target pH of 8.15 for our control, thus this control group may reflect future, not current conditions of acidity for wild lobster larvae. Although total alkalinity in the high pH treatment on 3 and 6 DPH exceeded that of the mid pH treatment, pH and DIC profiles for mid and low pH treatments did not cross. Thus, despite measured pH being lower than our target pH for each treatment, we believe the profiles of pH, total alkalinity, and DIC over time are sufficiently far apart so that each system can be considered a distinct pH treatment.

### **Morphometrics**

Although the normality assumption was not met by the carapace length the dry body mass data for some stages, ANOVA tests tend to be fairly robust to deviations from normality. However, carapace length data deviated from a normal distribution to a much greater extent than dry body mass data, as indicated by Shapiro-Wilk tests (data not shown), thus the likelihood for type I or type II error in our data is greater for carapace length data, but likely minimal for body mass data. The homogeneity assumption of the ANOVA was only violated by stage III and stage IV body mass data, but because significant p-values of factors for these stages were so low, there is little concern over type I error resulting from body mass data.

Carapace length of larvae did not significantly differ with pH for any larval stage, suggesting that the overall size of *H. americanus* larvae is not significantly affected by an acidic environment. However, carapace length is only one of many possible variables that may indicate relative body size, such as



carapace width or total body length, so the possibility of pH affecting overall larval size cannot yet be ruled out.

Dry body mass significantly differed among pH treatments for stage III and IV larvae, with a trend of lower mass in lower pH treatments on most DPH. The occurrence of significant differences only in the later stages may indicate chronic effects of prolonged exposure to acidic conditions. Subtly reduced calcium at these stages in low and mid pH treatments, although not significant, suggests that reduced carapace mineralization may account for a portion of the reduced dry mass. However, insignificant differences of carapace mass among pH treatments for all stages indicate suggest that lower dry body mass is due to reduced body tissue growth and not reduced carapace growth.

Our results regarding how carapace length and body mass are affected by acidity are both consistent with some studies and contrasting with other studies on *H. americanus*, the European lobster *H. gammarus*, and other crustacean species. In contrast with our results, Hall (2012) found no significant differences in weight, carapace lengths, or total length of *H. americanus* larvae and juveniles between control (pH ~8.2) and experimental (pH ~7.9) treatments, except of 90 day old lobsters, for which larval weights and total lengths were significantly less in the low pH treatment. The author recognized that a larger sample size and a more accurate measuring scale (length was measured to the nearest 0.5 mm) would likely resolve more significant differences in these variables. Also inconsistent with our findings, Keppel et al. (2012) reported that carapace lengths of *H. americanus* larvae were significantly lower in acidified seawater (pH 7.7) than in control water (pH 8.1) for stages II, III, and IV. However, the sample size for this experiment was very small, with only three stage IV individuals in the acidified treatment measured and 6 individuals per treatment measured at each of the other larval stages. Thus, by chance, natural variances in carapace length observed in our study (which led to an insignificant effect of pH) may have been missed in their study. Further, although reporting that pH was steady throughout the experiment, their study did not report any total alkalinity or DIC values, so the difference in carbon chemistry between their two treatments is unclear. As a standard guideline, at least two measurable carbon parameters (such as pH, DIC, total alkalinity, and fugacity of CO<sub>2</sub>) are required for a "complete description of the system" (Dickson et al. 2007).

Agnalt et al. (2013) reported no consistent or clear effect of pCO<sub>2</sub> treatment on carapace length or dry body mass of *H. gammarus* larvae, both at temperatures of 18 °C (optimal for larval hatching and growth) and 10 °C (less optimal). Consistent with our findings for *H. americanus*, Arnold et al. (2009) found that high pCO<sub>2</sub> did not significantly affect carapace length when expressed against time or development stage, but carapace mass of stage IV *H. gammarus* larvae was significantly reduced at high

pCO<sub>2</sub> (1200 ppm) versus the control of about 315 ppm CO<sub>2</sub>. They suggested that stage IV might be the "most critical period for production of viable post-larvae" and that the low carapace mass under high pCO<sub>2</sub> at this stage may have been a result of lower CaCO<sub>3</sub> production. However, they concluded that the physiological changes they observed were likely effects of acidosis or hypercapnia impairing the lobsters' ability to maintain homeostasis, rather than a direct effect of reduced carbonate availability. This is a reasonable explanation for our results as well, and will be discussed later. Long et al. (2013) studied two commercially important crab species and reported that at their third and fifth molts, red king crab (*Paralithodes camtschaticus*) larvae at pH 7.8 were significantly smaller than control larvae (pH 8) and had a condition index (dry mass divided by the cube of carapace length) 25% lower than that of control larvae by the end of the 200-day long experiment. They found that after their second molt, tanner crab (*Chionecetes bairdi*) larvae at pH 7.5 were smaller than larvae at pH 7.8 and in the control, though the condition index (dry mass divided by the cube of carapace width, in this case) did not significantly differ among pH treatments for this species. Whereas reduced size appears to be an adverse effect of an acidified environment on *P. camtschaticus*, it is unclear if body size and mass of wild *H. gammarus* larvae and body size of *H. americanus* larvae are likely to be reduced by ocean acidification, as lab studies conducted thus far on a given lobster species have conflicting results. These discrepancies may be a result of methodological differences, such as in controlled environmental conditions. For example, in the Agnalt et al. (2013) study, ambient pCO<sub>2</sub> treatment did not remain stable and had a pH close to that of the medium pCO<sub>2</sub>, which may have prevented clear relationships between pCO<sub>2</sub> and *H. gammarus* size and mass, whereas the Arnold et al. (2009) study had a greater difference in pCO<sub>2</sub> between the control and experimental treatment, perhaps giving more opportunity for significant differences in *H. gammarus* mass to be observed.

Within a larval stage, ANOVA tests indicated carapace length only changed significantly over time for stage II and IV larvae. However, DPH was a significant factor for all stages in ANOVA tests on dry body mass, and this mass increased over time for larvae at a given stage. This may suggest differential timing of energy put into post-molt cuticle growth (at least in terms of carapace length) versus body tissue growth. The post-molt period is separated into stages referred to as A, B, and C. Stage A is just after molting, and the carapace is soft at this stage, starts to harden in stage B, and hardens further with the formation of all cuticle layers in stage C (Comeau and Savoie 2001). In adult lobsters, after each molt, cuticular organules are relocated so that the cuticle can expand, and new organules are formed and placed in the larger cuticle (Kunkel et al. 2012). In the adult blue crab *Callinectes sapidus*, most tissue growth occurs during the intermolt stage and the crab is heavier at the end of the intermolt

(Cameron 1985a). These findings support the notion that early post-molt lobsters quickly expand their new carapace to a set size and later grow in mass as the carapace hardens and tissue growth occurs. If that is the case, our observed trends of carapace length and body mass over time would indicate that larval post-molt carapace expansion is early and rapid, and that carapace size remains more or less fixed for the rest of the intermolt stage whereas body mass increases more gradually over time.

### **Intermediate Stages**

We found no evidence of intermediate stages in our larvae. It therefore seems that at least up until stage IV, larvae in low pH do not delay maturation by molting to extra, intermediate stages. Hall (2012) reported one *H. americanus* individual at reduced pH that was likely at stage IV', and suggested that this may have been a consequence of stress of low pH rather than of low pH directly. With only one potential intermediate found, it seems unlikely that the frequency of intermediate stages of *H. americanus* larvae is increased at low pH. No intermediate stages have been found in *H. gammarus* subjected to low pH either (Agnalt et al. 2013). While undoubtedly a result of eyestalk ablation, and possibly of some natural stressors, there is so far no evidence of low pH inducing intermediate stages in lobsters. Presence of intermediate stages appears to be a natural phenomenon as well, as they have been found in control, eyestalk-bearing *H. americanus* individuals (Charmantier et al. 1988).

### **Delayed Development**

Controlling for DPH, the percentages of larvae at different stages on a given day significantly differed among the three pH treatments, indicating that on average, larvae in the mid and especially the low pH treatments took longer to molt to each successive stage than the larvae in the high pH treatment. Perhaps under greater acidic or hypercapnic stress, larvae need more time to harness enough energy to complete their next molt, and more energy might be re-allocated away from growth and toward maintaining homeostasis, including maintenance of the unstirred high-pH layer external and adjacent to the cuticle. This suggests that wild larvae exposed to high pCO<sub>2</sub> and low pH may have longer pelagic larval phases. A longer larval phase would likely lead to a longer time until lobsters reach sexual maturity and increase their chances of being predated. Both these effects could lower the abundance of adult lobsters within the legally catchable size range and may put a strain on the lobster fishery. In the context of global climate change, increasing sea-surface temperature could balance this effect by increasing development rate, though these potential effects on wild populations are speculative at this

point. Additionally, a change in seasonal timing or duration of the *H. americanus* larval phase may decouple this phase with the timing of peak abundance of the larvae's prey.

There are two possibilities as to why no stage IV larvae in our dataset were at 16 DPH whereas they are present at 15 and 17 DPH. First, experimenters may have waited to collect stage IV larvae until 17 DPH, although generally stage IV larvae were collected every day and placed in separate containers because they become cannibalistic at this stage. More likely, perhaps simply by chance no larvae at 16 DPH were been observed to have molted, thus none were collected that day.

Other studies on *H. americanus*, *H. gammarus*, and additional crustacean species have also investigated development rates of larvae and post-larvae raised in CO<sub>2</sub>-induced acidic water. Keppel et al. (2012) reported that *H. americanus* larvae took about two days longer at low pH (pH 7.7) than in control conditions (pH 8.1) to molt from stage II to stage III and from stage III to stage IV, similar to our observed delay in molting. Agnalt et al. (2013) found no significant effect of pCO<sub>2</sub> on *H. gammarus* larval development rate at 10 °C and at 18 °C. Bechmann et al. (2011) discovered larvae of the northern shrimp *Pandalus borealis* at pH 7.6 (versus a control of pH 8.1) had a significant delay in development of at least two days, from stage I to II and from stage II to III. This difference between the two treatments became greater by five weeks post-hatch. Embryos of the intertidal amphipod *Echinogammarus marinus* had significantly longer development time when exposed to a high pCO<sub>2</sub> of 1900 ppm (versus a 380 ppm control) when raised at a salinity of 22 PSU but not when raised at 35 PSU (Egilsdottir et al. 2009). Growth rates of post-larval *Semibalanus balanoides* from cyprid to early juvenile stages were significantly lower at pH 7.3 than at pH 8.1 (Findlay et al. 2010). Larvae of the red king crab *P. camtschaticus* grew slower at pH 7.8 than at pH 8 in terms of carapace length and wet mass (crabs in the control were 11% longer and 61% heavier, on average, after 200 days of exposure), and larvae of the tanner crab *C. bairdi* had consistently longer intermolt periods and smaller growth increments at pH 7.5, with control crabs having carapaces 38% wider and wet masses 118% higher than crabs in the pH 7.5 treatment (Long et al. 2013). Though to what degree is species-dependent, a prolonged larval period in a low pH environment appears to be a common theme among many crustacean species, including *H. americanus*.

### Calcium and Phosphorous of Carapaces

Percent calcium of carapaces differed significantly with DPH for all stages except stage II, and differed significantly with pH treatment for stage II larvae. Percent phosphorous significantly differed with DPH for stage IV larvae and approached significance ( $p < 0.1$ ) among pH treatments for stage II. Low

sample sizes may be responsible for the lack of consistency in trends and lack of significance in some instances. For example, the small sample size ( $n=4$ ) of the low pH group for stage IV larvae may have prevented a significant difference between the low pH and other pH treatments for calcium, and may have caused the mean percent phosphorous for stage IV larvae at low pH to be considerably higher than the "true" mean, breaking the trend of lower phosphorous seen at low pH at the earlier stages. These insignificant differences may also indicate a high natural variability of calcium and phosphorous among individuals at a given stage, or may be the result of limited accuracy of the micro balance used.

It is interesting that only stage II had significant or approaching significant differences in calcium and phosphorous among pH treatments. This may be due to natural variability, measurement error, or limited sample size, as mentioned above. Conversely, some differential physiology or morphology of stage II larvae may have caused these greater differences. For example, stage II larvae may not be able to compensate as well against acidity-related stresses that may reduce calcium and phosphorous, whereas stage III larvae may be able to compensate by increasing calcium and phosphorous uptake. Stage II larvae may be more sensitive in general to stressors that could impede the molting process, as evidenced by a "critical point" (the end of the molt stage  $D_1$ ) at this stage before which eyestalk ablation results in 100% intermediate stages, mostly stage IVa (Charmantier and Aiken 1987). Also, though all larvae were fed the same diet, individuals might still preferentially select among different food quantities and types provided in order to maintain appropriate calcium and phosphorous levels. Indeed, lobsters exhibit purposeful feeding behavior in order to meet their required diet and maintain a precise calcium and phosphorous ratio (R. Bayer, personal communication, April 13, 2015).

Stage I and III larvae in our study had similar Ca:P ratios among pH treatments and ratios were between these two stages. The trend of higher Ca:P ratio in the mid pH treatment for stage IV larvae was not significant and the same trend was significant for the stage II larvae. The aforementioned high Ca:P ratio of 49.8 for a stage II larva in the mid pH treatment could indicate an abnormal diet for this individual, with excessive calcium relative to phosphorous. This individual might be considered an outlier, and with it removed from the dataset, no significant differences were observed with the Tukey's test. Thus this significant difference was likely an artifact, and our data suggest that Ca:P ratios are not significantly influenced by pH.

Here we compare our results of calcium and phosphorous of carapaces with those of other crustacean studies. Hall (2012) did not find any significant difference in percent calcium of *H. americanus* carapaces between control (pH 8.15) and acidified (pH 7.9) larvae, except at 9500 °C/h, at which average calcium of larvae of the low pH group was only 40% that of the control group. Hall (2012)

also found no significant differences in carapace magnesium concentration between pH treatments. The lack of significant differences in this study may be a result of the experimental groups being at considerably higher pH values than those of our study and thus, significantly reduced calcium and phosphorus may only occur below pH 7.9. Also, lobsters seem to calcify at point sources and mineralization in carapaces not spatially constant; this study used x-ray microanalysis at point locations to study carapace composition, so points of more variable mineral composition may have been missed (T.J. Bowden, personal communication, May 4, 2015). Ries et al. (2009) reported that after 60 days of high pCO<sub>2</sub> exposure, juvenile *H. americanus* had higher net calcification (determined from changes in buoyant weight) under higher pCO<sub>2</sub>. They suggested that this implied resistance of cuticles to dissolution may be due to protection of the cuticle from the environment by the external organic layer, and that the lobsters could be tolerant to high pCO<sub>2</sub> if they are able to use proton regulation to convert bicarbonate to carbonate or use bicarbonate directly for calcification. Similarly, Ries (2011) reported that *H. americanus* individuals in high pCO<sub>2</sub> treatments maintain over 97% of their cuticle using the same CaCO<sub>3</sub> polymorph used in the control. However, the lobsters in both these experiments were kept at 25 °C, which is near the temperature threshold for *H. americanus*. Although Ries et al. (2009) claim that lobsters in their southern range experience this temperature, wild lobsters are probably rarely present at temperatures this high, as evidenced by increased incidence of shell disease and northward shift of *H. americanus*, both correlated with rises in seawater temperature above 20 °C (Hall 2012). Therefore, increased calcification they observed may signify temperature-induced stress and pCO<sub>2</sub> effects may be confounded by a temperature that is not very environmentally relevant. The authors defend the use of this temperature in their supplemental material by stating that deleterious effects of temperature are not present because varied effects resulted for both temperate and tropical species studied (species of numerous taxa were studied). However, temperature effects may still be present in their study because temperature responses are specific to species, not simply climate.

Arnold et al. (2009) studied carapace composition of *H. gammarus* larvae and found significant reductions in carapace calcium and magnesium content at high pCO<sub>2</sub>. Specifically, when expressed as percent of carapace mass, calcium was lower at high pCO<sub>2</sub> for stage IV larvae, and magnesium was lower at high pCO<sub>2</sub> for stage III larvae. When expressed as a concentration per carapace surface area, the same was observed for calcium, and magnesium concentration was instead significantly lower at high pCO<sub>2</sub> for stage IV larvae. These results are more in line with our initial expectations for our study, suggesting that carapaces of larvae at later stages are more sensitive to a low pH environment. Also, consistent with our results they found that when expressed as a percentage of carapace weight, calcium was relatively

constant with larval stage (except it was lower for stage I larvae in our study). Expressed as a concentration (amount per surface area) though, calcium increased in successive larval stages (stage IV having the most calcium), suggesting that in *H. gammarus* calcium becomes more important, such as in mineralization of inorganic cuticle components, at later stages. It may be informative to measure calcium content per carapace surface area in a future study to see if the same may be said for *H. americanus*.

Carapace mineralization under low pH has also been studied in other decapods. Long et al. (2013) found no significant differences in calcium or magnesium (expressed as percent dry mass) among pH treatments for juveniles of the red king crab *P. camtschaticus*. In the tanner crab *C. bairdi*, they found no significant differences with respect to magnesium, but found significantly higher calcium in control crabs than at pH 7.8 and pH 7.5. Walther et al. (2011) reported that calcium content of spider crab (*Hyas araneusi*) larvae was higher at later larval stages. For a southern population of this species (the southern North Sea, 54 °N), calcium did not significantly differ among pCO<sub>2</sub> treatments for any larvae stage at 15 °C, but was lower at higher pCO<sub>2</sub>, at least in older stages, at 9 and 3 °C. For a northern population (Svalbard, North Atlantic, 79 °N), calcium was significantly lower at high pCO<sub>2</sub> for stage M3 at 15 °C, for M3, M7, and M14 stages at 9 °C, and for M7 and M14 stages at 3 °C. As expected due to the fact that CO<sub>2</sub> is more soluble in colder water, these crabs seem to be more sensitive to high pCO<sub>2</sub> at colder temperatures. This study demonstrated the importance of studying interactive effects of pCO<sub>2</sub> and temperature and studying populations of a species that occupy different regions. For instance, hypercapnic conditions could reduce an animal's thermal tolerance or vice versa. How lobsters raised at different water temperatures and from different populations may differentially respond to low pH is worth investigating in future studies, and will make research more relevant to changes in climate that wild lobster populations may be subjected to. For example, one could subject lobsters from different populations to low pH at a range of seasonal temperatures they typically experience over an average year, and at an average temperature predicted for the end of this century. Aside from this, potential delays in post-molt calcification, although not examined in our study, are also of concern and may increase an animals' vulnerability to predation.

#### **Potential Mechanisms behind Results and Broader Implications:**

There are many potential biological mechanisms that may be causing reduced calcium and phosphorous of larval carapaces in the lower pH treatments, along with reduced body mass and delayed development. Some of these explanations involve chemical changes in the low pH seawater directly

influencing these variables, whereas others involve stress responses to a hypercapnic and acidic environment. It is very possible that more than one of these mechanisms may be true. Each mechanism will be explained and the more likely mechanisms will be noted as such.

Lobster larvae, particularly stage IV larvae, which we expect to be more calcified than larvae at earlier stages, may have lower  $\text{CaCO}_3$  production at low pH due to a reduced availability of bicarbonate or other carbon species, leading to lower dry mass and lower percent calcium. This explanation seems the least likely due to the fact that relative concentration of bicarbonate in seawater is expected to be much greater than that of carbonate within the pH range we raised larvae in, and that lobsters and crustaceans in general are thought to use primarily bicarbonate, not carbonate, to calcify their exoskeletons (Arnold et al. 2009; Whiteley 2011). That said, higher external  $\text{H}^+$  concentration reportedly decreases uptake of bicarbonate across the gills (Whiteley 2011). Alternatively, lower body mass and lower percent calcium may be due to passive dissolution by the environment of  $\text{CaCO}_3$  forms of the cuticle. However, Kunkel et al. (2012) state that it is unlikely that small pH changes will inhibit cuticle production due to the lobsters' buffering ability (e.g. maintenance of the high pH unstirred layer), however to our knowledge no research has investigated whether this ability is present in *H. americanus* larvae. Dissolution of calcified gastroliths in lobster cardiac stomachs might also occur, contributing to decreases in mass (R. Bayer, personal communication, April 13, 2015), though it is not known if larvae possess gastroliths as adults do .

Lower phosphorous may be associated with reduced production (driven by reduced calcium) of compounds in the carapace that contain both calcium and phosphorous, such as CAP, which lines canals of organules and likely plays an important role in carapace rigidity (Kunkel 2013). This could explain the trend of reduced phosphorous at low pH for stages I through IV. Lower percent phosphorous in addition to lower calcium at low pH may not be so surprising, considering lobsters are known to usually maintain a consistent Ca:P ratio in their diet (R. Bayer, personal communication, April 13, 2015). Our results indicate that stage I and III larvae maintain relatively consistent Ca:P ratios from about 6 to 7, on average, among pH treatments and between these two stages. It could be important for lobsters to maintain a consistent carapace Ca:P ratio, which may be related to maintenance of proper structure of canals and organules in the carapace. It is unclear from our data whether *H. americanus* larvae naturally maintain a constant carapace Ca:P ratio or whether this is impacted by pH. If maintaining a constant ratio is as important in carapaces as it seems in diets, this may explain the trend of reduced phosphorous along with reduced calcium at low pH, at least for stage I and stage III larvae.



Another possible explanation behind our observed trends is that lobsters actively dissolve their inorganic carapace components in an effort to maintain a high pH unstirred layer, leading to lower calcium and phosphorous content. Such a process may ease formation of a new carapace for the next molt and help protect against pathogen invasion. Again however, the pH buffering abilities of larvae relative to those known in adults are uncertain, and maintained carapace length and mass at low pH suggest that carapace dissolution, if occurring, is compensated for. We did not examine carapace thickness or pH of the unstirred layer, but if these are not as well maintained in low pH conditions, pathogens would more likely be able to invade, increasing the lobster's susceptibility to diseases such as shell disease.

We propose that the most likely explanation behind our results is that various effects of acidosis and hypercapnia hinder lobsters' ability to maintain homeostasis so that more energy is allocated towards maintaining essential physiological processes, such as acid-base regulation, and less towards growth. This energy reallocation could manifest in reduced calcium and phosphorous in carapaces, reduced body mass, and a delayed development rate. As mentioned previously, under stress of high CO<sub>2</sub> and low pH, less energy may be put towards formation of the carapace and molting may be delayed so larvae can harness enough energy to complete their next molt. There are numerous ways in which lobster larvae may suffer physiological stress in acidic conditions, and ways in which they might buffer against these stresses, although there is scant evidence for their buffering abilities. For example, in addition to active dissolution of the carapace, more magnesium might be used in place of calcium at low pH to form MgCO<sub>3</sub> instead of CaCO<sub>3</sub>, and this could explain the observed maintenance of carapace length and mass in larvae at low pH. According to Kunkel (2013), this is a proposed method for increasing the pH of the unstirred layer shortly after molting, before the cuticle has become rigid. In future studies, measurement of magnesium along with calcium and phosphorous in carapaces may suggest if this hypothesis is supported by the larvae.

Additionally, larval respiration may be adversely impacted by low pH. *H.americanus* larvae breathe in oxygen through diffusion until they reach stage IV, at which the gills are developed and they breathe via advective respiration (T.J. Bowden, personal communication, April 9, 2015). The larvae also ingest much water (R. Bayer, personal communication, April 13, 2015), such as for hydraulic inflation during shedding of an old cuticle (Kunkel 2013). Such processes could lower pH of lobster haemolymph, which would decrease the oxygen affinity of respiratory pigments and thus decrease oxygen delivery to tissues (Whiteley 2011). There is potential for low haemolymph pH to be buffered by larvae, however. For example, pH buffering could occur via electroneutral ion exchange across gills, with the majority

(93%) of buffering bicarbonate likely taken up from seawater, and some perhaps being obtained from ACC (Whiteley 2011). *H. gammarus* is known to buffer well against acidosis resulting from prolonged emersion. During aerial exposure, the drop in haemolymph pH of adult *H. gammarus* (due primarily to respiratory acidosis) is well buffered by an increase of bicarbonate concentration in the haemolymph (Taylor and Whiteley 1989). This occurs along with an increase in concentration of  $\text{Ca}^{2+}$  in haemolymph, suggesting dissolution of  $\text{CaCO}_3$  in order to increase alkalinity. After 14 h of aerial exposure and after resubmergence, these lobsters maintain high haemocyanin oxygen-affinity, which may be due to increases in both  $\text{Ca}^{2+}$  and lactate ions. Use of bicarbonate to increase haemolymph pH has also been demonstrated in the crabs *Necora puber* and *Cancer magister* and the prawns *Palaemon elegans* and *P. serratus*, though chronic exposure to high  $\text{pCO}_2$  has revealed a threshold bicarbonate concentration which may represent a balance between acid-base regulation and iono-regulation (Whiteley 2011). The adult blue crab *Callinectes sapidus* appears to buffer against low blood pH during hypercapnia primarily by increasing bicarbonate uptake across the gills; buffering by the dissolution of inorganic carapace components may contribute less to overall pH compensation as it is estimated to be responsible for only about 7.5% of  $\text{H}^+$  disposal (Cameron 1985b). It would be interesting to determine if *H. americanus* larvae share these buffering methods. Additionally, in the spiny lobster *Panulirus interruptus*, two antibodies were shown to increase the oxygen affinity of hemocyanin whereas a third antibody maintains hemocyanin in a low affinity state (Pertone et al. 1997). This suggests that the *H. americanus* immune system could also play a role in maintaining the oxygen binding affinity of haemolymph at a low pH.

The stress of low pH haemolymph may induce higher respiration rate in order to obtain more oxygen, though chronically it may reduce respiratory ability and limit larval growth and survival. It is predicted that because more active, swimming animals tend to have higher respiratory rates and hemocyanin levels than sedentary animals, they are able to compensate better for haemolymph acidosis (Whiteley 2011). Thus, depending on their respiratory rates relative to the benthic juveniles and adults, pelagic larvae may be better suited to deal with this specific stress. Future research should investigate how respiratory rates of *H. americanus* larvae change through ontogeny and how they may be altered in low pH.

Reduced phosphorous and calcium in carapaces at low pH, particularly in stage II larvae, suggest that larvae may not have strong pH buffering abilities through mechanisms previously described, but maintained carapace mass suggests that lobsters might be using other elements to maintain their cuticle size and mass. Even if acid-base buffering of the larvae is strong, most buffering methods are likely energetically expensive and thus may limit larval growth and survival in the long term. Regarding

carapace mineralization, more energy may be required to obtain carbonate from bicarbonate at a low pH (Kunkel et al. 2012), and although magnesium and ACC might be produced or reserved in order to harden the cuticle when exposed to low pH, there is potential for environmental mineral resources to become limited. Studies focusing on how *H. americanus* larvae buffer pH could provide important links to how an acidified environment results in the morphological and physiological changes we have observed.

Another important consideration in future studies of *H. americanus* larvae is that responses to pH may be brood specific. For example, heart rates of porcelain crab (*Petrolisthes cinctipes*) embryos at low pH were about 37% slower than those of embryos at ambient pH, but among broods the decrease in heart rate ranged from 20 to 60% (Ceballos-Osuna et al. 2013). We did not examine brood-specific effects in our study, but potential variability in larvae vulnerability to low pH among broods is worth investigation. Other variables worth investigating in future ocean acidification studies on *H. americanus* include survival, heart rate, respiration rate, hatching success, and cuticle thickness. Also, the combined effects of temperature, salinity, and other relevant environmental factors should be tested simultaneously with low pH.

## **Conclusion**

This study reveals how the physiology and development of larval *H. americanus* are impacted by CO<sub>2</sub>-induced water acidification. When raised in low pH, these larvae have delayed development to each larval stage, reduced body mass at stages III and IV, and reduced percent calcium and phosphorous in their carapaces, especially at stage II. Slowed growth of larvae may increase their chances of being preyed on and time until they reach sexual maturity, both of which could potentially put strains on the lobster fishery. Reduced body growth and carapace mineralization observed in larvae at low pH suggest weaker or thinner cuticles in these larvae, which may increase susceptibility of larvae to predation and disease. Future research should focus on the potential pH buffering abilities of *H. americanus* larvae against respiratory and metabolic acidosis. Further, these lab-based studies give incentive for more research to predict and observe the susceptibility of wild lobsters to ocean acidification. To accomplish this, knowledge of environmental CO<sub>2</sub> and acidification data, brood and population-specific responses, and other potential ecological factors that may increase lobsters' susceptibility or resilience to ocean acidification are essential.

## **Acknowledgements**

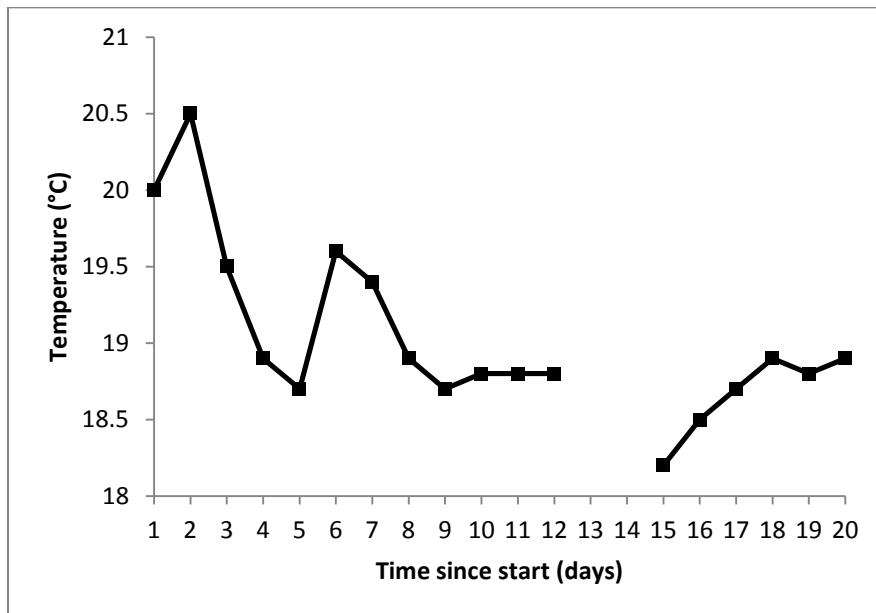
I extend many thanks to Josh Hall and Tim Bowden for conceiving this project, designing the initial experiment, raising larvae, and along with other members of the Bowden Lab, collecting and fixing the larvae. I also thank Brian Beal and staff at the Downeast Institute for providing the lobsters used in our project. I also thank Joe Salisbury for conducting water quality analysis on the rearing tank water samples and Mike Handley for his invaluable advisement and assistance with carapace digestion and ICP procedures. I further thank Wesley Cowperthwaite for his help with measuring wet weights and removing of larval carapaces, and Alexander Hauer and Nicholas Fagnoli for their assistance with carapace removal. I also thank Ivan Fernandez and Cheryl Spencer for their permission and instruction for use of their micro balance, and Martin Stokes for allowing use of his oven and balance for obtaining dry weights of larvae. Most of all, I thank Tim Bowden for his encouragement and dedicated advisement during this project.

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## Figures



*Figure 1.* Temperature profile for larval rearing experiment. Measurements were taken from water in the sump for the high pH (control) system. No temperature data were collected at days 13 and 15. Credit: Tim Bowden.

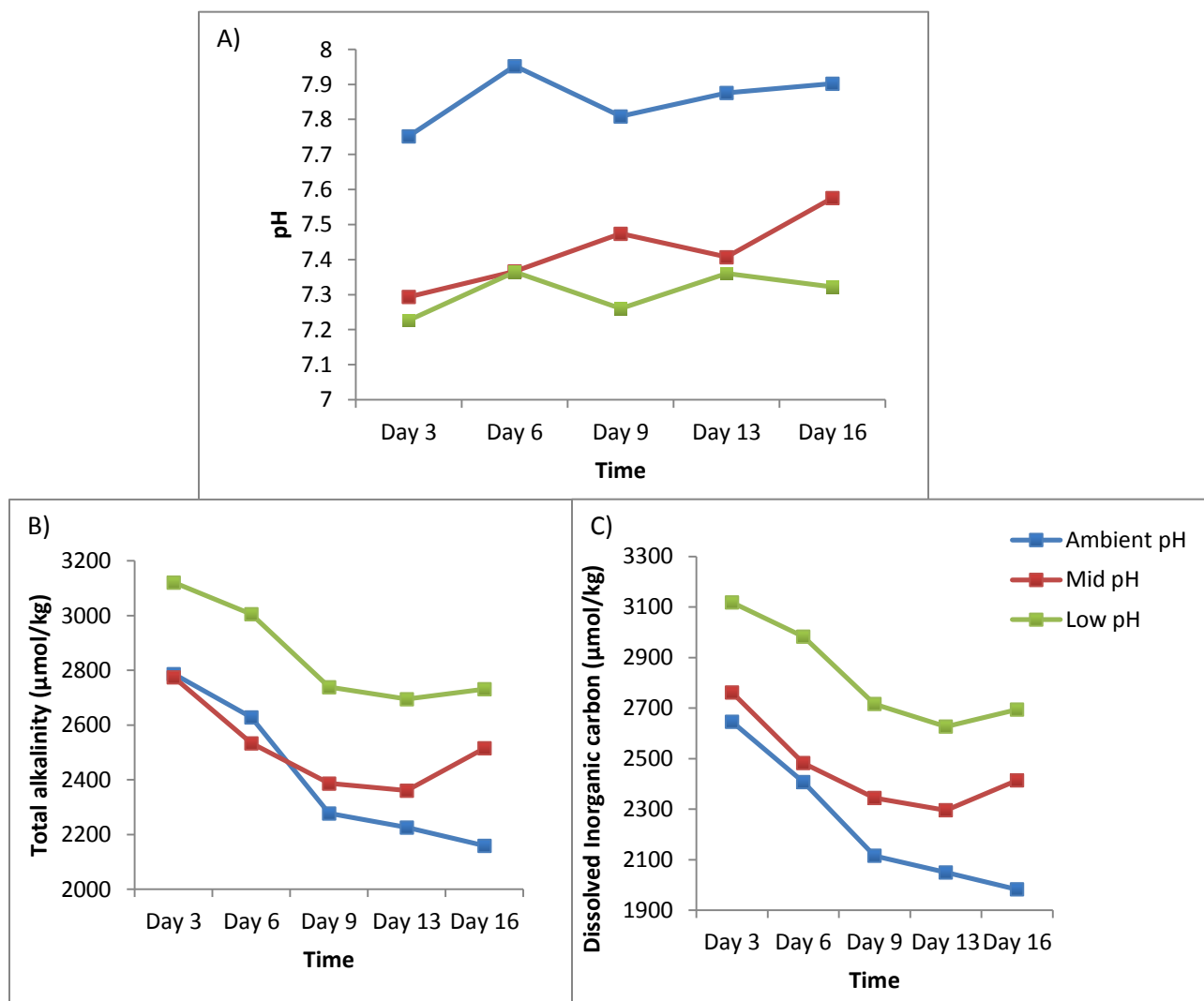


Figure 2. pH (A), total alkalinity (B), and dissolved inorganic carbon (C) for each system, at five sample points spanning the lobster rearing period. From water samples collected from our rearing systems, these values were calculated by Joe Salisbury at the University of New Hampshire's Ocean Processes Analysis Lab.



| System  | Temperature (°C) | pH          | Total alkalinity, all days (μmol/kg) | Alkalinity, Day 9, 13 & 16 (μmol/kg) | DIC, all days (μmol/kg) | DIC, Day 9, 13 & 16 (μmol/kg) |
|---------|------------------|-------------|--------------------------------------|--------------------------------------|-------------------------|-------------------------------|
| High pH | 19.03 ± 0.56     | 7.86 ± 0.08 | 2415.53 ± 275.69                     | 2220.77 ± 59.24                      | 2239.82 ± 278.67        | 2048.93 ± 66.48               |
| Mid pH  | -                | 7.42 ± 0.11 | 2514.32 ± 164.54                     | 2421.00 ± 83.12                      | 2459.38 ± 183.06        | 2351.07 ± 59.37               |
| Low pH  | -                | 7.31 ± 0.06 | 2858.49 ± 192.27                     | 2721.82 ± 83.12                      | 2827.35 ± 211.61        | 2678.76 ± 46.54               |

Table 1. Mean values ± standard deviation of temperature, pH, total alkalinity, and DIC measurements of 5 water samples collected on days 3, 6, 9, 13, and 16 of the larval rearing period, for each treatment system.

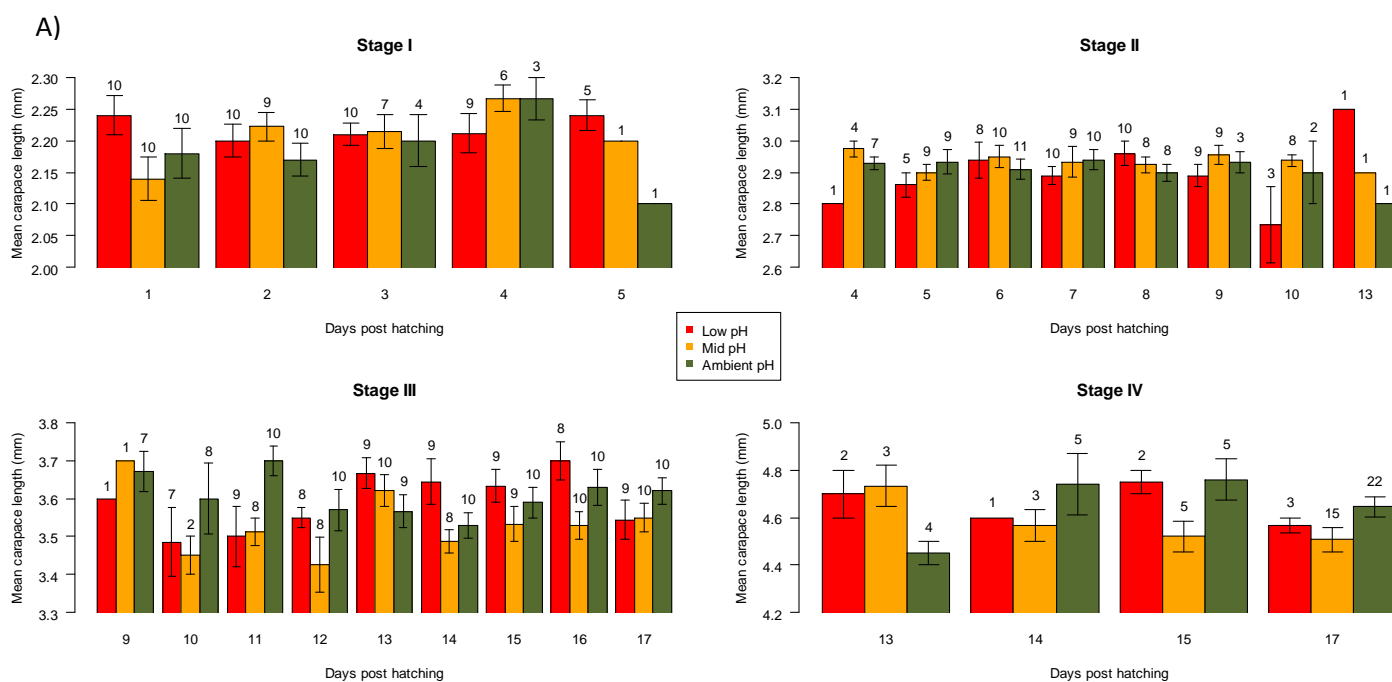
| Carapace Length          |          |                  |              | Log(Dry body mass)       |          |  |              |
|--------------------------|----------|------------------|--------------|--------------------------|----------|--|--------------|
| Stage                    | Factor   | F-value          | Pr (>F)      | Stage                    | Factor   | F-value  | Pr (>F)      |
| 1                        | DPH      | 1.03             | 0.405        | 1                        | DPH      | 2.69   | 0.0254 *     |
|                          | pH       | 1.49             | 0.226        |                          | pH       | 1.27   | 0.262        |
|                          | DPH : pH | 1.39             | 0.244        |                          | DPH : pH | 1.00   | 0.412        |
| 2                        | DPH      | 3.39             | 4.02e-05 *** | 2                        | DPH      | 5.30   | 9.38e-09 *** |
|                          | pH       | 0.45             | 0.505        |                          | pH       | 2.46   | 0.119        |
|                          | DPH : pH | 1.53             | 0.119        |                          | DPH : pH | 1.34   | 0.201        |
| 3                        | DPH      | 1.09             | 0.368        | 3                        | DPH      | 3.93   | 8.12e-06 *** |
|                          | pH       | 0.420            | 0.518        |                          | pH       | 28.2   | 2.48e-07 *** |
|                          | DPH : pH | 1.64             | 0.0808       |                          | DPH : pH | 2.71   | 0.00186 **   |
| 4                        | DPH      | 2.62             | 0.0217 *     | 4                        | DPH      | 6.67   | 6.78e-06 *** |
|                          | pH       | 1.09             | 0.299        |                          | pH       | 18.4   | 4.47e-05 *** |
|                          | DPH : pH | 1.28             | 0.280        |                          | DPH : pH | 1.58   | 0.174        |
| ANOVA Assumptions        |          |                  |              | ANOVA Assumptions        |          |  |              |
| Assumption               |          | Met?             |              | Assumption               |          | Met?   |              |
| Normality                |          | No (all stages)  |              | Normality                |          | Yes (stage III only);<br>No (all other stages) |              |
| Homogeneity of variances |          | Yes (all stages) |              | Homogeneity of variances |          | Yes (Stage I and II);<br>No (Stage III and IV) |              |

Table 2. Output from two-way, between-groups ANOVA tests using carapace length or log-transformed dry body mass as the response variable and days post hatching (DPH) and pH treatment as factors. Significant values are indicated by '\*' ( $p < 0.5$ ), '\*\*' ( $p < 0.01$ ), and '\*\*\*' ( $p < 0.001$ ). A summary ANOVA assumptions met or not met is also shown.

| Log(% Calcium)           |          |                  |            | Log(% Phosphorous)       |          |  |            |
|--------------------------|----------|------------------|------------|--------------------------|----------|--|------------|
| Stage                    | Factor   | F-value          | Pr (>F)    | Stage                    | Factor   | F-value                                  | Pr (>F)    |
| 1                        | DPH      | 4.81             | 0.0409 *   | 1                        | DPH      | 2.34                                     | 0.143      |
|                          | pH       | 0.494            | 0.491      |                          | pH       | 2.43                                     | 0.136      |
|                          | DPH : pH | 0.0130           | 0.912      |                          | DPH : pH | 0.373                                    | 0.548      |
| 2                        | DPH      | 0.300            | 0.588      | 2                        | DPH      | 1.40                                     | 0.247      |
|                          | pH       | 7.03             | 0.0127 *   |                          | pH       | 3.49                                     | 0.0715     |
|                          | DPH : pH | 1.61             | 0.214      |                          | DPH : pH | 0.331                                    | 0.569      |
| 3                        | DPH      | 5.69             | 0.0200 *   | 3                        | DPH      | 2.71                                     | 0.104      |
|                          | pH       | 1.17             | 0.284      |                          | pH       | 1.72                                     | 0.195      |
|                          | DPH : pH | 0.00500          | 0.941      |                          | DPH : pH | 0.920                                    | 0.341      |
| 4                        | DPH      | 9.10             | 0.00566 ** | 4                        | DPH      | 10.4                                     | 0.00345 ** |
|                          | pH       | 1.19             | 0.286      |                          | pH       | 0.945                                    | 0.340      |
|                          | DPH : pH | 0.0440           | 0.835      |                          | DPH : pH | 0.265                                    | 0.611      |
| <b>ANOVA Assumptions</b> |          |                  |            | <b>ANOVA Assumptions</b> |          |  |            |
| Assumption               |          | Met?             |            | Assumption               |          | Met?                                     |            |
| Normality                |          | Yes (all stages) |            | Normality                |          | Yes (all stages)                         |            |
| Homogeneity of variances |          | Yes (all stages) |            | Homogeneity of variances |          | No (stage IV);<br>Yes (all other stages) |            |

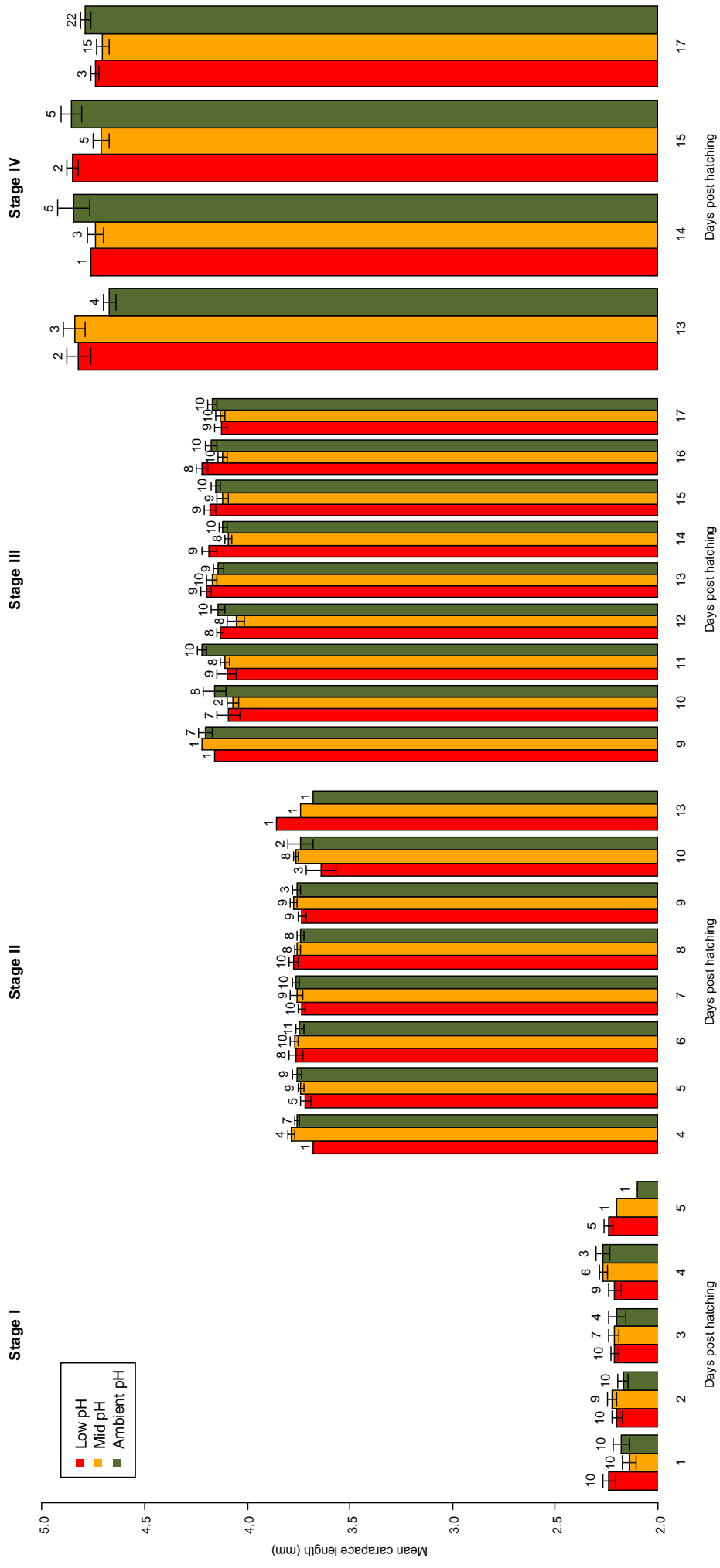
| Log(Dry carapace mass)   |         |                  |       | Log(Calcium:Phosphorous ratio) |         |                  |       |
|--------------------------|---------|------------------|-------|--------------------------------|---------|------------------|-------|
| Factor                   | F-value | Pr (>F)          | Stage | Factor                         | F-value | Pr (>F)          | Stage |
| DPH                      | 0.120   | 0.733            | 1     | DPH                            | 0.448   | 0.511            | 1     |
| pH                       | 1.52    | 0.233            |       | pH                             | 1.69    | 0.210            |       |
| DPH : pH                 | 0.519   | 0.480            |       | DPH : pH                       | 1.02    | 0.325            |       |
| DPH                      | 12.9    | 0.00114 **       | 2     | DPH                            | 4.21    | 0.0491 *         | 2     |
| pH                       | 1.31    | 0.262            |       | pH                             | 0.603   | 0.444            |       |
| DPH : pH                 | 0.0290  | 0.865            |       | DPH : pH                       | 0.574   | 0.454            |       |
| DPH                      | 21.3    | 1.88e-05 ***     | 3     | DPH                            | 31.7    | 3.93e-07 ***     | 3     |
| pH                       | 0.00800 | 0.929            |       | pH                             | 0.116   | 0.734            |       |
| DPH : pH                 | 0.162   | 0.689            |       | DPH : pH                       | 2.11    | 0.151            |       |
| DPH                      | 0.00400 | 0.953            | 4     | DPH                            | 49.7    | 1.39e-07 ***     | 4     |
| pH                       | 1.40    | 0.247            |       | pH                             | 0.00    | 0.984            |       |
| DPH : pH                 | 0.159   | 0.693            |       | DPH : pH                       | 0.609   | 0.442            |       |
| <b>ANOVA Assumptions</b> |         |                  |       | <b>ANOVA Assumptions</b>       |         |                  |       |
| Assumption               |         | Met?             |       | Assumption                     |         | Met?             |       |
| Normality                |         | Yes (all stages) |       | Normality                      |         | Yes (all stages) |       |
| Homogeneity of variances |         | Yes (all stages) |       | Homogeneity of variances       |         | Yes (all stages) |       |

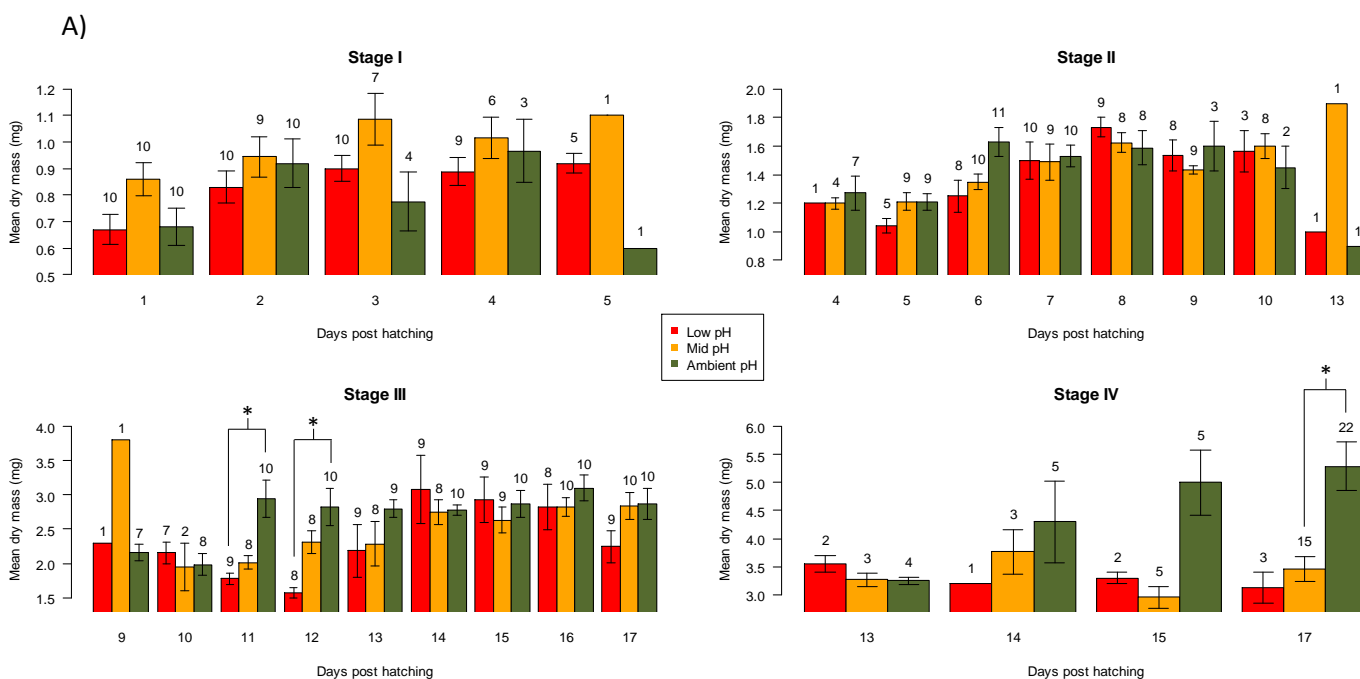
Table 3. Output from two-way, between-groups ANOVA tests using log-transformed percent calcium, percent phosphorous, dry mass, or Ca:P ratio of carapaces as the response variable and days post hatching (DPH) and pH treatment as factors. Significant values are indicated by '\*' ( $p < 0.5$ ), '\*\*' ( $p < 0.01$ ), and '\*\*\*' ( $p < 0.001$ ). A summary ANOVA assumptions met or not met is also shown.



*Figure 3.* Mean carapace lengths of lobster larvae in different pH treatments at different days post hatching and development stages. Only days in which all pH treatment groups were represented are shown. Numbers above bars indicate sample sizes and error bars represent  $\pm$  standard error of the mean. (A) Arranged to show better resolution on the y-axis, (B) arranged to show differences in lengths among stages.

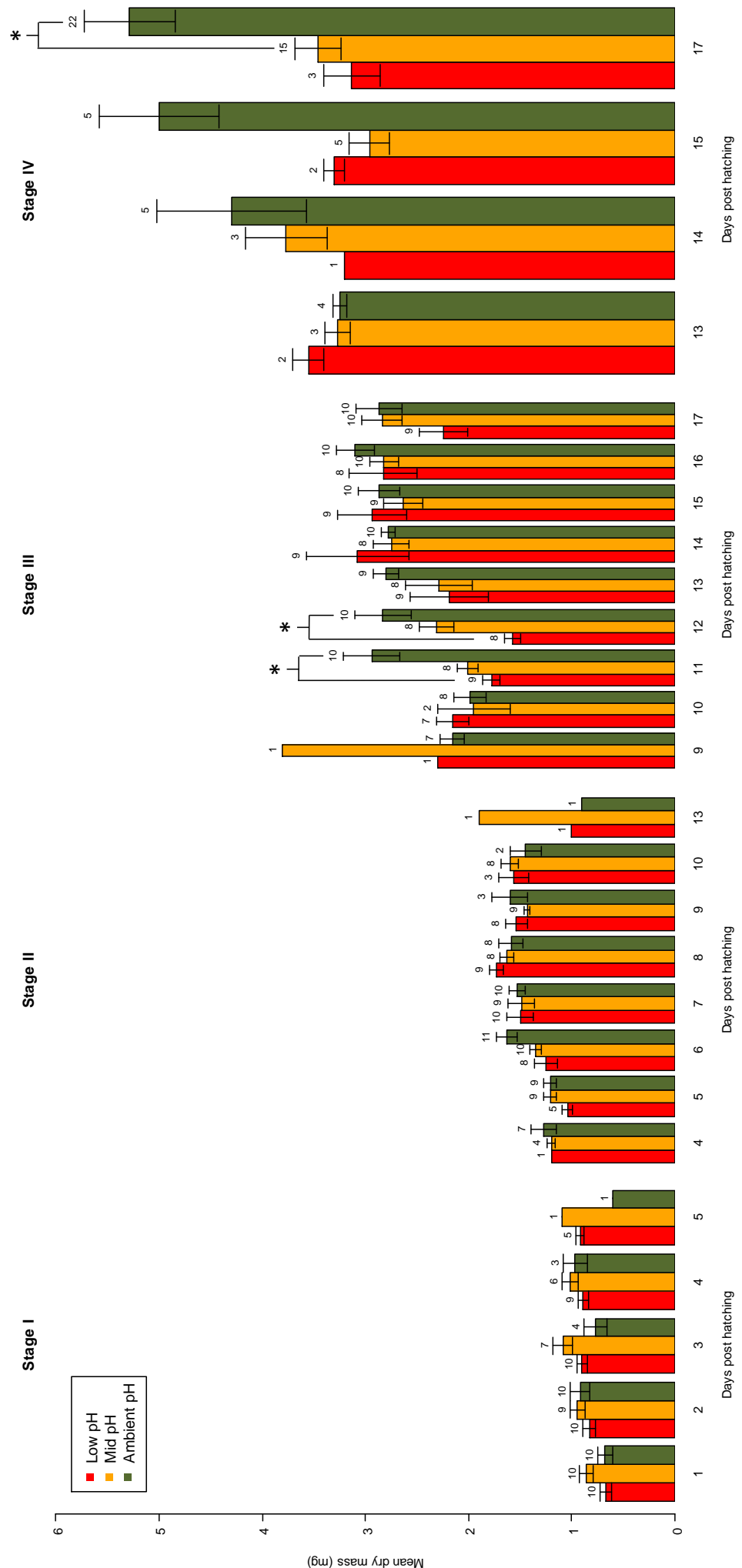
B)

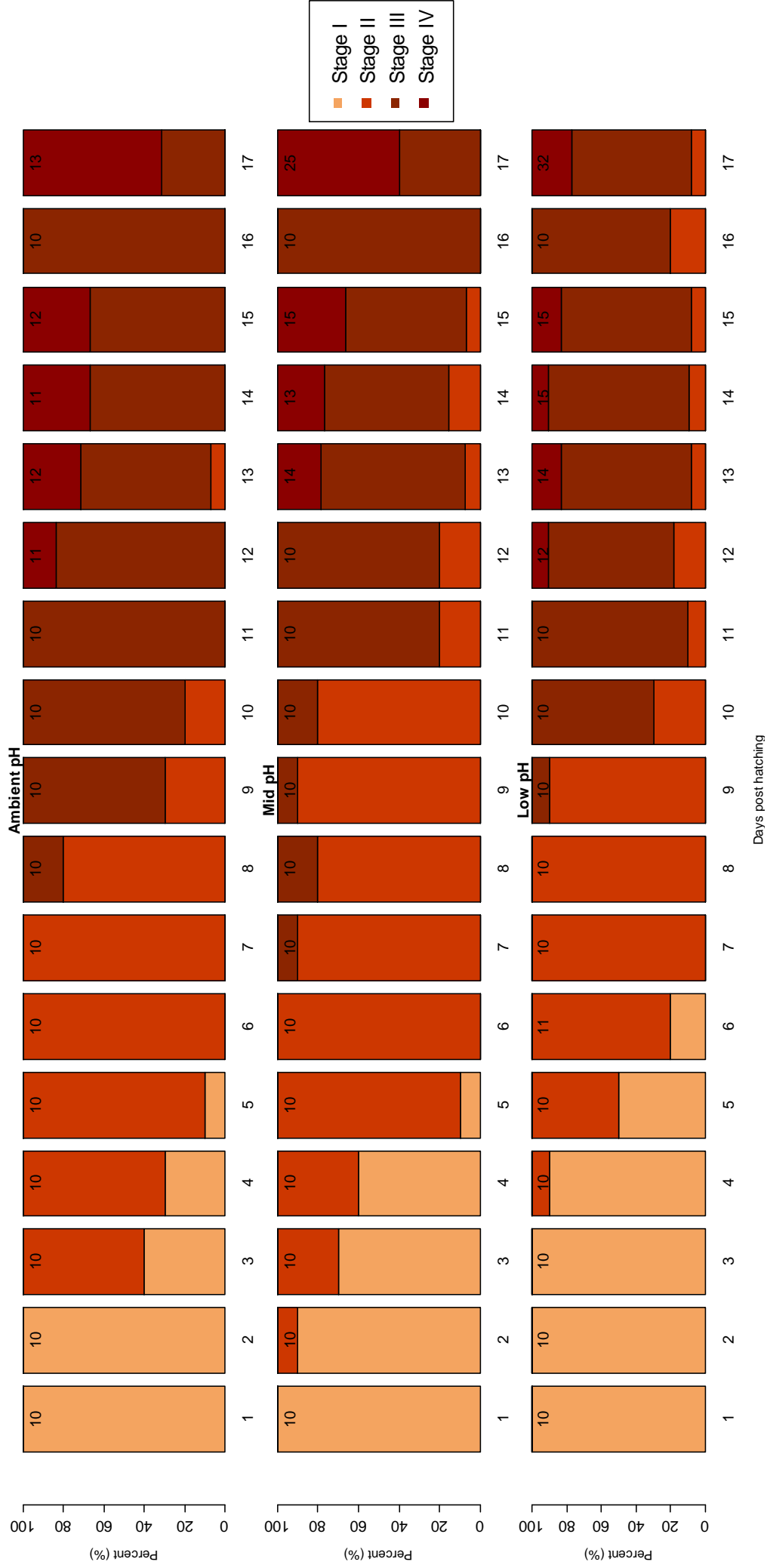




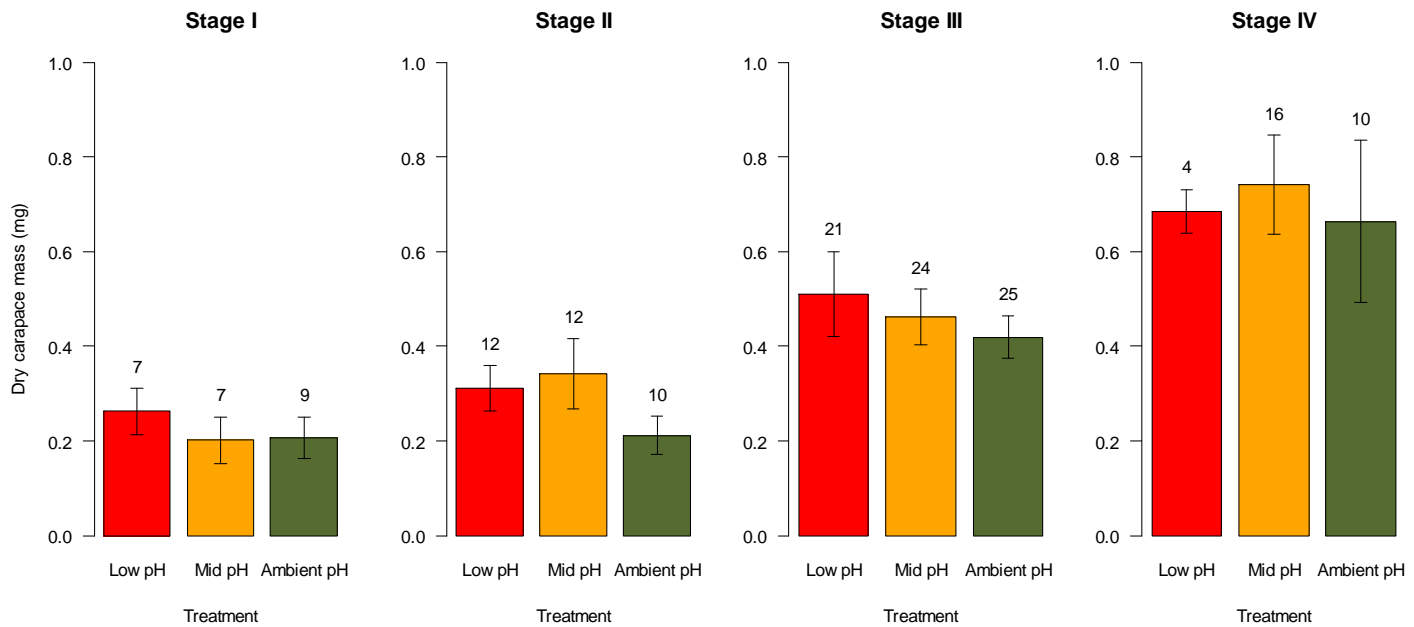
*Figure 4.* Mean dry body masses of lobster larvae in different pH treatments at different days post hatching and development stages. Only days in which all pH treatment groups were represented are shown. Numbers above bars indicate sample sizes and error bars represent  $\pm$  standard error of the mean. Asterisks indicate significant differences between two pH treatment groups on the same day, found using Tukey's HSD test. (A) Arranged to show better resolution on the y-axis. (B) Arranged to show differences in lengths among stages.

B)



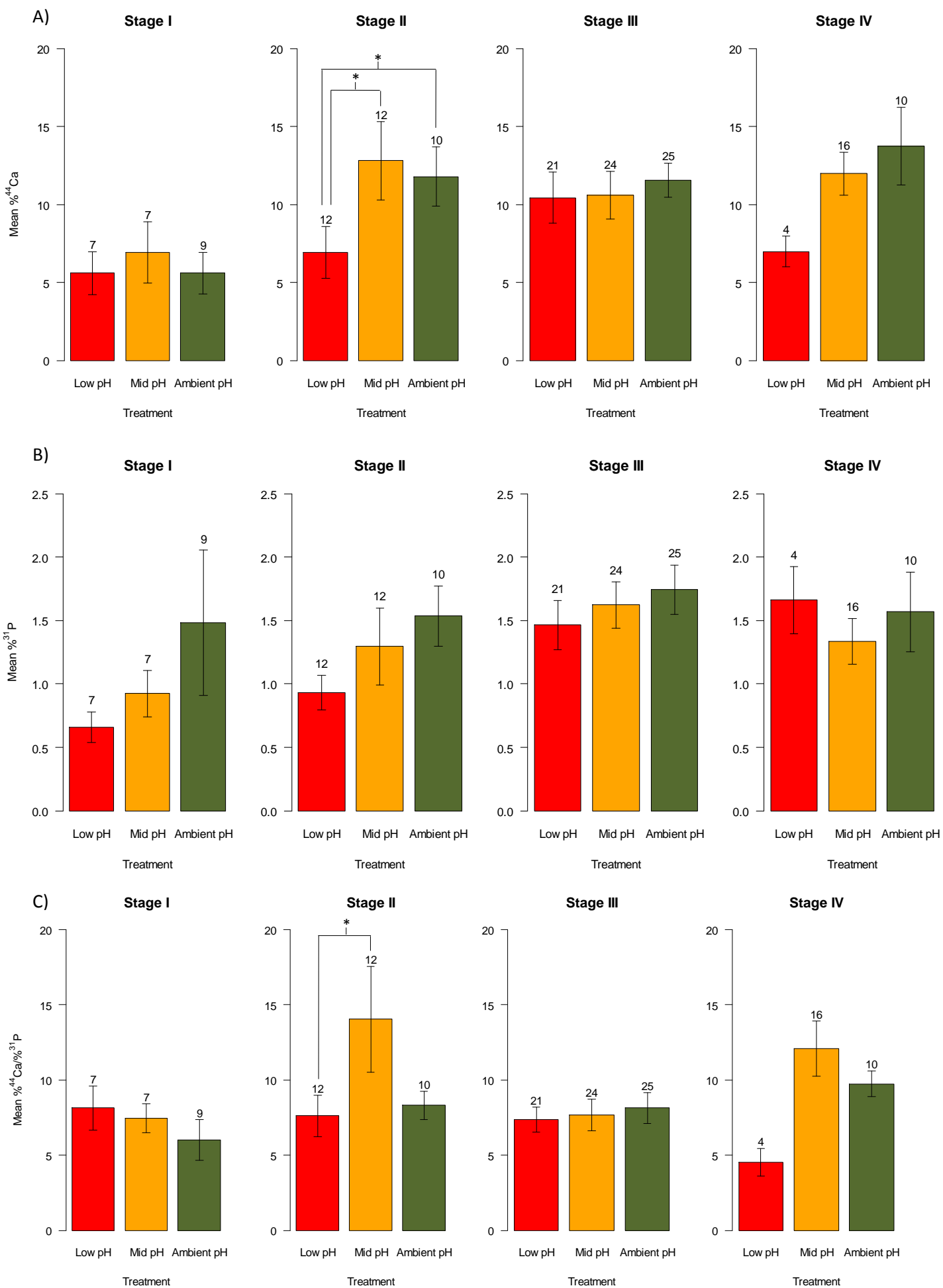


*Figure 5.* Percentages of lobster larvae at different stages at each day of the experiment, for each pH treatment. At least 10 larvae were haphazardly sampled for each pH treatment on each day, and percentages are out of all samples collected on a given day and from a given pH treatment. Only larvae used for morphometrics (not carapace analysis) are included here. Numbers near the top of each bar indicate sample sizes for that bar.



*Figure 6.* Mean dry masses of lobster larvae carapaces at different stages and pH treatments. Data from different days post hatching are pooled. Numbers above bars indicate sample sizes and error bars represent  $\pm$  standard error of the mean. Tukey's HSD tests did not resolve any significant differences among pH treatments within a stage.





*Figure 7.* Mean percentages of calcium (A) and phosphorous (B) isotopes and the ratio of calcium to phosphorous (C) of lobster larvae carapaces at different stages and pH treatments. Data from different DPH are pooled. Numbers above bars indicate sample sizes and error bars represent  $\pm$  standard error of the mean. Asterisks indicate significant differences between two pH treatment groups in the same stage, found using Tukey's HSD test. This test did not resolve any significant differences for the phosphorous data.