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Effects of ocean acidification over the life history of the barnacle *Amphibalanus amphitrite*

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ABSTRACT: Increased levels of atmospheric CO_2 are anticipated to cause decreased seawater pH. Despite the fact that calcified marine invertebrates are particularly susceptible to acidification, barnacles have received little attention. We examined larval condition, cyprid size, cyprid attachment and metamorphosis, juvenile to adult growth, shell calcium carbonate content, and shell resistance to dislodgement and penetration in the barnacle *Amphibalanus amphitrite* reared from nauplii in either ambient pH 8.2 seawater or under CO_2-driven acidification of seawater down to a pH of 7.4. There were no effects of reduced pH on larval condition, cyprid size, cyprid attachment and metamorphosis, juvenile to adult growth, or egg production. Nonetheless, barnacles exposed to pH 7.4 seawater displayed a trend of larger basal shell diameters during growth, suggestive of compensatory calcification. Furthermore, greater force was required to cause shell breakage of adults raised at pH 7.4, indicating that the lower, active growth regions of the wall shells had become more heavily calcified. Ash contents (predominately calcium carbonate) of basal shell plates confirmed that increased calcification had occurred in shells of individuals reared at pH 7.4. Despite enhanced calcification, penetrometry revealed that the central shell wall plates required significantly less force to penetrate than those of individuals raised at pH 8.2. Thus, dissolution rapidly weakens wall shells as they grow. The ramifications of our observations at the population level are important, as barnacles with weakened wall shells are more vulnerable to predators.

KEY WORDS: Ocean acidification · Barnacle · Life history · Larval ecology · Intertidal ecology · Calcification

INTRODUCTION

The current decline in the pH of the world's oceans (ocean acidification) is an important consequence of surface water interchange with increasing concentrations of anthropogenically generated atmospheric CO_2 (Takahashi et al. 1997, Sabine et al. 2004, Caldeira & Wickett 2005, Guinotte & Fabry 2008, Riebesell 2008). Models predict that by the year 2100, mean seawater pH levels could decrease from current ambient levels of about 8.1 to 7.7 (IPCC 2007), and by 2300 to levels as low as 7.3 (Caldeira & Wickett 2003). Given these predicted declines in pH, global ocean waters will experience shifts in carbonate saturation horizons (Feely et al. 2004, Orr et al. 2005, Fabry et al. 2008). As such, the combination of lowered seawater pH and the reduced saturation states of calcium carbonate and its polymorphs aragonite and calcite are likely to have negative effects on a variety of processes in marine invertebrates both related and unrelated to calcification.

The impacts of ocean acidification on benthic marine invertebrates has been the focus of a number of recent studies (e.g. oysters, Kurihara et al. 2007; mussels, Beesley et al. 2008; sea urchins, Kurihara & Shirayama 2004; brittle stars, Wood et al. 2008, Dupont et al. 2008; hard corals, Fine & Tchernov 2007). These experimen-

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tal laboratory studies have revealed that exposure to CO_2-induced acidified seawater disrupts a variety of calcification-related and unrelated biological processes across a range of life history stages that span from fertilization (Havenhand et al. 2008) and larval development and growth (reviewed by Kurihara 2008) to aspects of juvenile and adult growth, muscle function, acid-base balance, immune response, and shell calcification (e.g. Kurihara & Shirayama 2004, Kurihara et al. 2007, Miles et al. 2007, Beesley et al. 2008, Bibby et al. 2008, Wood et al. 2008). For example, very early embryos of the oyster *Crassostrea gigas* exposed to a seawater pH of 7.4 suffer both delayed development and delayed larval shell formation (Kurihara et al. 2007). Development in the sea urchins *Hemicentrotus pulcherrimus* and *Echinometra mathaei* exposed to seawater pH ranging from 6.8 to 7.4 is characterized by decreased fertilization, reduced developmental speed, and smaller larval size (Kurihara & Shirayama 2004). Embryos and larvae of the brittle star *Ophiothrix fragilis* suffer complete mortality within 8 d even at a moderately reduced pH of 7.9 (Dupont et al. 2008). Regenerating arms in adult brittle stars *Amphiura filiformis* exposed to pH 6.8 exhibit significant muscle wastage (Wood et al. 2008), and growth of juvenile mussels *Mytilus edulis* is reduced at pH 6.7 (Berge et al. 2006) while adults become immunocompromised (Bibby et al. 2008).

While studies such as those cited above collectively report the effects of ocean acidification on a variety of discrete life history stages of marine invertebrates, they are primarily short-term studies. Indeed, to our knowledge, no studies to date have examined the impacts of chronic exposure to CO_2-driven ocean acidification across all life history stages with the exception of the brief 9 d life cycle of the copepod *Acartia tsuensis* (Kurihara & Ishimatsu 2008). This gap in our knowledge is emphasized in a recent paper by Dupont et al. (2008) indicating the importance of future studies examining the collective effects of ocean acidification across all life stages of given species.

Accordingly, here we examined the effects of CO_2-induced ocean acidification over the course of all major life history stages, from larval release of the nauplius to reproductive adult, of the common intertidal barnacle *Amphibalanus amphitrite*. Barnacles are important members of shallow marine ecosystems, and others have considered it important to understand the effects of ocean acidification on them. For example, one modeling study (Wootton et al. 2008) and another experimental technique study (Findlay et al. 2008) included barnacles in their analyses. To our knowledge, ours is only the second experimental study to investigate the effects of ocean acidification on barnacles (see Findlay et al. 2009), an ecologically important group of benthic calcified marine invertebrates.

We selected the barnacle *Amphibalanus* (=*Balanus*) *amphitrite* (Pitombo 2004), as it is a dominant fouling organism (Rittschof et al. 1984, Rittschof 2001) and is of considerable ecological importance (Henry 1959). Moreover, its growth and development from naupliar to adult stages have been well studied (Costlow & Bookhout 1958, Pechenik et al. 1993, Anil et al. 1995, Anil & Kurian 1996, Thompson et al. 1998). As a model for antifouling research (Rittschof et al. 1984, 1992, Rittschof 2001, Hung et al. 2008), standardized techniques for its culture are well established (Rittschof et al. 1984, 1992, Anil et al. 1995, Qian et al. 2003). We examined the effects of CO_2-induced seawater acidification in this common barnacle on aspects of larval condition, cyprid size, cyprid attachment and metamorphosis, juvenile to adult growth, the onset of reproductive maturity (egg production), and aspects of adult shell integrity including the forces required to break and to penetrate shells, and the levels of ash (predominately calcium carbonate) in basal shell plates.

We selected an experimental pH level of 7.4 in part because of its direct comparative value with pH levels tested in numerous previous studies of ocean acidification on calcified marine invertebrates (e.g. 23 of 30 experimental studies cited in Table 1 of Kurihara 2008), and also because it represents a pH level that is estimated to occur in the world's oceans under current models of anthropogenic CO_2 emissions (pH 7.4 is predicted to occur by approximately 2250; Caldeira & Wickett 2003). Although acclimation or microevolutionary adaptation might be expected to occur among marine invertebrates as they encounter seas with declining pH, a period of several hundred years is arguably a relatively brief period on traditional evolutionary time scales. Moreover, recently it was discovered that marine invertebrates such as barnacles, living in coastal regions along the continental shelf of western North America (Canada to Mexico), are increasingly exposed to seasonally upwelled, corrosive 'acidified' seawater (pH 7.6 to 7.7; Feely et al. 2008). Although much of this acidification is due to natural respiration processes at intermediate depths below the photic zone, the continued uptake of anthropogenic CO_2 is increasing the areal extent of exposure of marine organisms to waters that are undersaturated with respect to polymorphs of calcium carbonate (Feely et al. 2008).

MATERIALS AND METHODS

Larval collection and culture. Naupliar larvae were collected from living adult *Amphibalanus amphitrite* removed by mechanical force from wooden pilings of the pier at Duke Marine Laboratory along Beaufort Inlet,

North Carolina, USA (34° 43.0′ N; 76° 40.3′ W) in August 2008. Adult barnacles were placed in a 19 l bucket of seawater and broken apart using a hammer. The bucket containing the barnacles was subsequently placed in a dark room, and a fiber optic light was positioned against one side of the bucket to attract swimming naupliar larvae. Every 30 min for 3 h, the nauplii that had concentrated at the light source were pipetted into a 1 l glass mason jar containing aged and filtered natural seawater. Larval cultures were established by first evenly suspending the larvae in the mason jar with gentle stirring, then conducting a count of larvae in a seawater sub-sample of known volume under a dissecting microscope, and finally pipetting the appropriate volume of seawater with suspended larvae in the mason jar to transfer approximately 800 larvae into each of ten 1 l glass beakers filled with natural seawater at 35 ppt. The seawater was filtered first to remove particles >1 µm and aged with aeration for at least 1 wk at a temperature of 25 ± 1°C. Culture beakers were then placed into a walk-in temperature-controlled room held at 28°C and under a 12:12 h light:dark cycle. The chemical characteristics of the seawater were: total alkalinity (determined by titration): 2078 µmol kg^{-1} (experimental), 2278 µmol kg^{-1} (control); and saturation state (Ω): Ω_{Ar} 0.58 (experimental), Ω_{Ar} 3.27 (control), Ω_{Ca} 0.89 (experimental), Ω_{Ca} 4.98 (control). The Ω values were calculated using the Microsoft Excel spreadsheet 'co2sys.xls' v.14 (Pelletier et al., available at www.ecy.wa.gov/programs/eap/models.html) using the National Institute of Standards and Technology (NIST) pH scale.

Seawater acidification. Five of the 10 beakers were established as control beakers and were aerated continuously with atmospheric air driven by an aquarium pump. These beakers maintained a pH of 8.2 (NBS pH scale). The remaining 5 experimental beakers received a continuous mixture of air from the aquarium pump and the United States Pharmacopeia (USP) grade CO_2 from a cylinder (Airgas). The gases were mixed using a gas proportioning rotameter (Omega Engineering) to achieve a seawater pH 7.4 (NIST pH scale). The control and experimental pH levels were checked twice daily to ensure stability throughout the experiment. With this high frequency of pH checks, we were able to sustain the targeted pH values with only a small range of variation over the course of the study (pH drift ranges = 7.35 to 7.45, 8.15 to 8.25). The pH was measured with an ACCUMET BASIC Model AB15 pH meter and ACCUTU pH probe (Cat. #13-620-183) calibrated with Fisherbrand pH 4, 7, and 10 buffers (NBS standards).

Larval condition, cyprid size, attachment, and metamorphosis. Culture beakers containing naupliar larvae were maintained on a daily diet of the diatom *Skeletonema costatum* (UTEX LB 2308) (Rittschof et al. 1984). Once the naupliar larvae had developed into cyprid larvae, the culture beakers were monitored approximately every 4 h to check for cyprid attachment and metamorphosis. Both nauplii and cyprids were sub-sampled from each of the beakers on Days 2, 3, 4, and 5 post-naupliar release for later observations of potential aberrancies and, in the case of cyprids, for size measurements of carapaces. Cyprids were individually photographed under a stereo dissecting microscope (LEICA MZ6) in pixels using Adobe Photoshop v. 5.0. These measurements were then converted to millimeters based on the pixel:mm ratio (1 pixel = 0.00151 mm) determined from a photograph of a glass microscope calibration slide taken at the same magnification as the cyprid photograph. Once cyprid attachment and metamorphosis had begun in the majority of beakers (Day 5 post-naupliar release from adults), in order to obtain a measure of the proportions of hatched nauplii that had survived to attachment and metamorphosed into juvenile 'pinhead' barnacles, the numbers of settled cyprids were recorded for each beaker every 4 to 6 h for 46 h (Days 5 to 7 post-naupliar release from adults). To ensure that no juvenile 'pinhead' barnacles were counted more than once, locations of attached individuals were marked and numbered with an indelible ink pen on the outside of the glass culture beakers. Juvenile barnacles were maintained under the above culture conditions with daily addition for the first week of 50 ml of approximately 2×10^6 cells ml^{-1} of *S. costatum,* centrifuged from the growth medium and resuspended in the correct pH seawater. After the first week, barnacles were fed a 50:50 mixture of *S. costatum* and *Dunellia tertiolecta* until Day 26, when they were switched to a diet of brine shrimp (*Artemia* sp.).

Juvenile rearing and egg production. On Day 26, when the early juveniles were ready to be switched to a monospecific diet of 24-h-old brine shrimp (Holm et al. 2005), juvenile barnacles in beakers were shipped overnight to the University of Alabama at Birmingham in 100% humidity in an insulated container. Immediately upon arrival, the original pH conditions in artificial seawater were resumed (see 'Seawater acidification' above). Shipment of live *Amphibalanus amphitrite* over this period of time is a well-established method of transfer (Pechenik et al. 1993). Artificial seawater (Instant Ocean®, Spectrum Brands) pre-adjusted to the appropriate pH was changed daily and had a calcium level of 400 mg l^{-1} water. Salinity was monitored daily using a refractometer, and seawater total alkalinity was measured every other day by titration with sulfuric acid. The chemical characteristics of the artificial seawater were as follows: total alkalinity (determined by titration): 2497 µmol kg^{-1} (experimental), 2617 µmol kg^{-1} (control), Ω_{Ar} 0.71 (experimental), Ω_{Ar} 3.65 (control), Ω_{Ca} 1.08 (experimental), Ω_{Ca} 5.55 (control).

Juvenile barnacle cultures were held at 36 ppt and 25°C under 12:12 h light:dark conditions and maintained on a standardized diet of 24-h-old live *Artemia* sp. Both seawater pH and salinity were monitored daily. Moreover, to ensure high water quality, water changes were made each day using artificial seawater that had been pre-adjusted to the appropriate pH. In order to prevent overcrowding during growth, and to standardize for density-dependent growth, the settled juvenile barnacles were randomly culled down to 100 ind. beaker^{-1}.

Growth of juvenile barnacles was determined by measuring the longest dimension of the diameter of the basal shells of all individuals in each beaker weekly over a period of 8 wk. Measurements were made to the nearest 0.01 mm using Vernier calipers (Mitutoyo), and growth of each individual was followed by assigning it a unique number on the outside surface of the culture beaker below its basal plate. The onset of egg production was determined in each individual barnacle by daily visual examination for the presence of distinct yellow eggs that were clearly evident through the basal plate. The onset of egg production was observed at Week 9 post-cyprid attachment and documented though the Week 11 post-cyprid attachment.

Shell integrity and basal plate ash weight. At the end of the 8 wk growth experiment, most barnacles had become reproductive and had grown to adult size. Individuals were then subjected to several measures of shell integrity. In an experiment using a penetrometer (pin = 3 mm diameter), we measured the amount of force necessary to penetrate the central portion of shell wall plates from 25 individuals in the 7.4 and 8.2 pH treatments. Shell wall plates used in these measurements were from adult individuals of similar size (basal shell diameters ranged from 10.7 to 12.5 mm).

Upon completion of the growth and shell penetrometry experiments, the beakers containing the remaining barnacles were shipped overnight back to Duke Marine Laboratory where we immediately measured the shear forces necessary to break adult barnacles off the glass beakers in which they had settled (see Holm et al. 2005 for description of technique). The normal American Society for Testing and Materials (ASTM) procedure (ASTM 1997) was not followed because 100% of the barnacles had settled and grown to ≥5 mm basal diameter. Barnacles of this size break rather than dislodge when reared on a glass surface. Briefly, 10 barnacles in each beaker were selected and numbered on the outside of the beaker, and the basal diameter was measured as per the ASTM procedure (ASTM 1997) using Vernier calipers. Using a hand-held mechanical force gauge (Shimpo MF-5lb, #93953-10, Cole-Parmer), force was then applied at the base of the wall plate parallel to the substrate at a rate of approximately 4.5 N s^{-1}, until each barnacle broke.

Using a diamond scribe, we then marked the base plates of each barnacle used in the breakage experiment and combusted the beakers with the barnacles (500°C for at least 4 h). Combustion eliminated the glue that held the basal plates to the glass. The intact calcium carbonate base plates for each barnacle from the breakage experiment were removed and weighed to determine the relative amount of ash (calcified material) in the basal shell plates of individuals raised in the acidified pH 7.4 and ambient pH 8.2 treatments.

Statistical analyses. ***Cyprid size:*** Cyprid length and width measurements were log transformed [(x' = log (x + 1)] prior to means comparisons. At each time period, means were compared between pH treatments using a nested analysis of variance (ANOVA) with the factors being treatments and beakers within treatments. For the between-treatments test of significance, the beakers within treatments MS was used as the denominator in forming the *F*-ratio. For these comparisons, least squares adjusted mean lengths and widths are reported.

Nauplii surviving to attachment and metamorphosis: Since each beaker was initiated with approximately 800 nauplii, it was possible to determine the number of larvae that had not settled at any time period by subtraction from the number that had settled. The cumulative proportions of hatched nauplii that had survived to attachment and metamorphosis in each beaker over the course of the experiment were compared between the 2 treatments using a repeated measures ANOVA.

Juvenile to adult growth: Growth rates of juvenile barnacles were compared using an analysis of covariance (ANCOVA), where mean basal diameter (mm) for each beaker was the dependent variable and time (weeks) was the covariate. Growth rates were compared over the first 6 wk of the experiment, where the growth trajectories were approximately linear. Differences in the slopes of the regression lines between treatments were tested by the significance of the treatment × time interaction effect in the ANCOVA model.

Egg production: The mean proportion of individuals with and without eggs was compared between the 2 pH treatments at each time period using a *t*-test with the means for each beaker as the replicates.

Adult adhesion strength: When the beaker means were used as replicates, the force required to shear a barnacle from its substrate was not significantly correlated with basal plate area, so this variable was not used in the analysis. Mean shear forces (kg) were compared between treatments using a *t*-test.

Basal plate ash weight: Basal plate ash (calcium carbonate) weights were positively correlated with basal areas. Adjusted mean basal plate ash weights (mg) were compared between treatments using ANCOVA where weight was the dependent variable and basal

area (mm^2) was the covariate. The model was first run including the treatment × basal area interaction effect. When it proved insignificant, indicating homogeneity of slope, the model was re-run without the interaction effect to test the significance of the difference in adjusted ash weights between the treatments.

Wall shell penetrometry: Mean force (N) required to penetrate the shells was compared between treatments using a *t*-test. Since we hypothesized that the pH 7.4 shells would be weaker than the control shells, the test was done 1-tailed.

RESULTS

Larval condition and cyprid size. There was no evidence from microscopic observations of any instances of conspicuous morphological aberrancies in nauplii or cyprids raised in either the pH 7.4 or ambient pH 8.2 seawater treatments. Moreover, there were no significant differences ($p > 0.05$) between pH treatments in the mean length or width of the larval carapaces of cyprids at either 92 or 120 h post-naupliar release (Fig. 1).

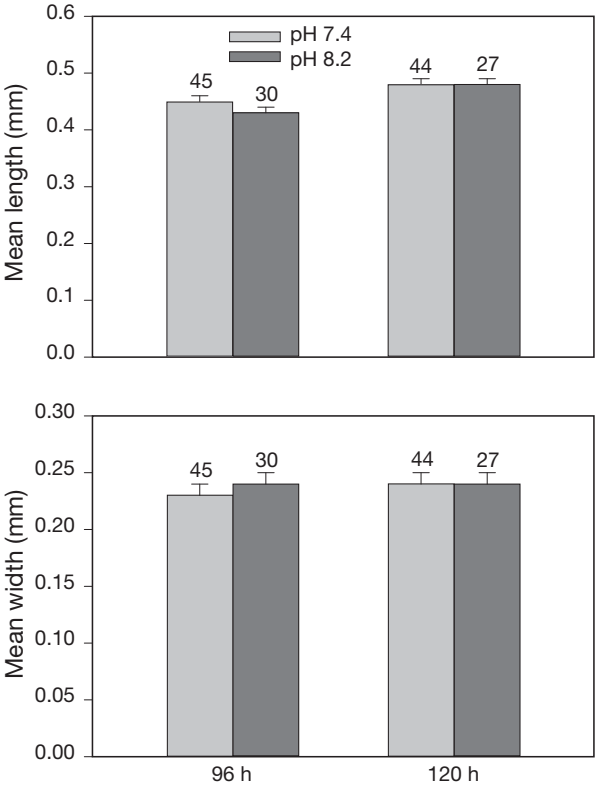

Fig. 1. *Amphibalanus amphitrite.* Mean (+1SE) lengths and widths of cyprids at 96 and 120 h post hatch. Sample sizes are above the bars

Nauplii surviving to attachment and metamorphosis. Cumulative percent hatched nauplii surviving to attachment and metamorphosis into juvenile 'pinhead' barnacles during the initial 46 h settlement period for larvae reared in pH 7.4 and pH 8.2 seawater are presented in Fig. 2. Prior to attachment, 1 of the 5 larval cultures in the ambient seawater treatment (pH 8.2) suffered mortality, most likely due to bacterial contamination. This is not unusual when culturing larvae of barnacles (D. Rittschof pers. obs.), and all remaining 9 beakers retained normal, healthy larvae throughout the experiment. Loss of 1 larval culture did not negatively affect our experimental design, as there remained ample numbers of replication within treatments for statistical evaluations. Cyprids in 5 of the 9 beakers had initiated attachment and metamorphosis at the time of our first measurement; 3 more did so at the 4 h measurement, and larvae in the 1 remaining beaker had initiated settlement at the 8 h measurement. There was no pattern of cyprid attachment and metamorphosis in the beakers initiating a few hours earlier or later as a function of pH treatment. A large degree of variability in hatched nauplii surviving to attachment and metamorphosis was seen between beakers in both treatments (Fig. 2). Be-

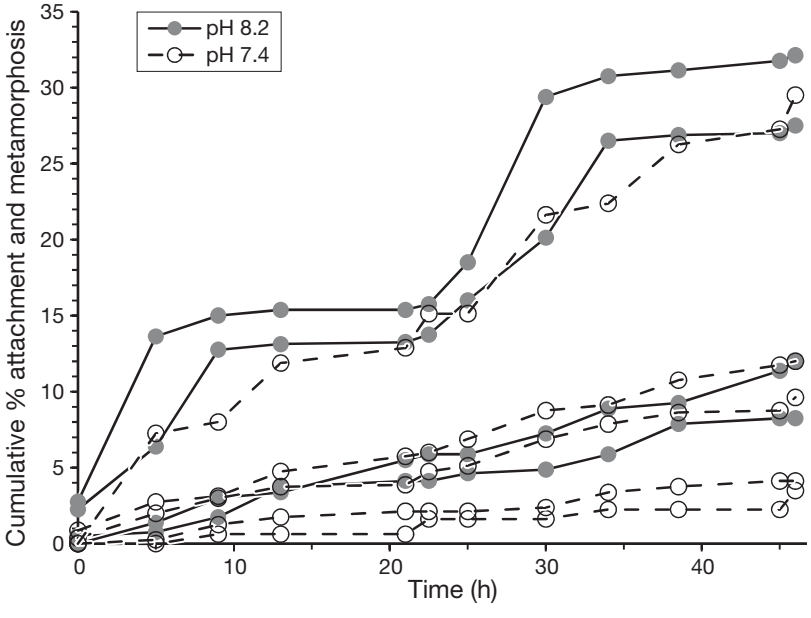

Fig. 2. *Amphibalanus amphitrite.* Cumulative percent cyprid metamorphosis and juvenile settlement of barnacles reared in pH 7.4 and pH 8.2 seawater over a 46 d period beginning 5 d post-naupliar release. The pH 7.4 treatment consisted of 5 beakers, each seeded with 800 nauplii. The pH 8.2 treatment consisted of 4 beakers, each seeded with 800 nauplii. The time series for each beaker is drawn with a line

cause of this, the difference in the proportions of nauplii surviving to attachment and metamorphosis between treatments was not significant ($F_{1,7} = 1.50$, p = 0.26). The slopes of the curves also did not differ significantly between treatments ($F_{1,7} = 1.21$, p = 0.32).

Juvenile to adult growth. Juvenile barnacles (approximately 4.5 mm basal diameter) culled randomly to densities of 100 ind. beaker^{-1} grew rapidly to adult size (approximately 8.5 mm basal diameter) in both pH 7.4 and pH 8.2 under identical nutritional conditions (Fig. 3). For the first 6 wk, growth was approximately linear (Fig. 3). At every measurement point throughout the growth experiment, basal shell diameters of barnacles were slightly, but not significantly, larger in barnacles raised at pH 7.4 than those raised at pH 8.2 ($F_{1,50}$ = 2.72, p = 0.11; Fig. 3). In addition, a comparison of growth rates (slopes) indicated that there was no significant difference in the slopes of the growth curves between the 2 pH seawater treatments ($F_{1,50}$ = 0.25, p = 0.62). Thus, there was no difference in growth rates of individuals reared in either pH.

Egg production. The mean percent of individuals with eggs at Weeks 9, 10, and 11 post-cyprid attachment are presented in Fig. 4. At no time were there any significant differences between the 2 pH treatments in the proportion of individuals producing eggs.

Adult adhesion strength. The force required to shear the barnacles from their substrate was not significantly correlated with basal plate area (p = 0.08) The mean shear force (kg) required to break shells of individuals raised at pH 7.4 was significantly greater than that for those raised at pH 8.2 (t = 2.59, 7 df, p = 0.04). Adjusted mean ± 1SE shear force values (kg) were 2.20 ± 0.06 and 1.79 ± 0.17 for barnacles raised in pH 7.4 and pH 8.2 seawater, respectively.

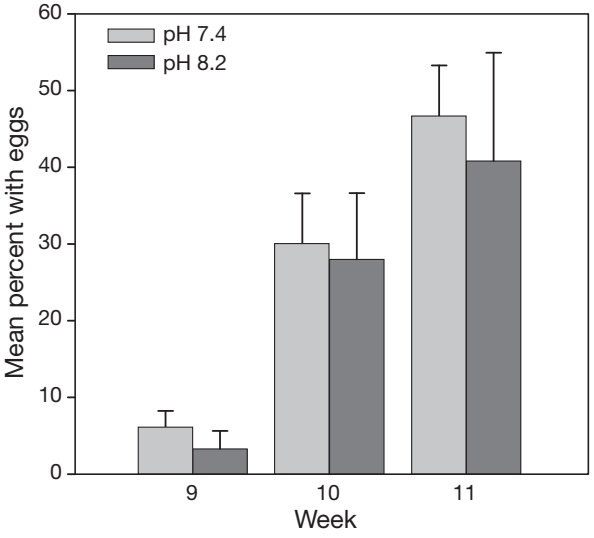

Fig. 4. *Amphibalanus amphitrite.* Mean percent (±SE) of barnacles with eggs at 9, 10, and 11 wk post settlement. Means were based on beakers as the unit of replication (n = 5 for pH 7.4 and n = 4 for pH 8.2)

Basal plate ash weight. The amount of calcium carbonate (mg ash) in basal plates was positively correlated with basal plate area in the pH 8.2 treatment (r = 0.418, p = 0.013), but not in the pH 7.4 treatment (r = 0.207, p = 0.256; Fig. 5). However, when both treatments were combined, the positive correlation was highly significant (r = 0.380, p = 0.001; Fig. 5), so ANCOVA using basal plate area as a covariate was used to compare adjusted mean ash weights between treatment groups. Mean adjusted calcium carbonate (mg ash) levels were significantly higher in the basal shells of individuals raised at pH 7.4 than that of those raised at pH 8.2 ($F_{1,64}$ = 6.452, p = 0.014; Fig. 5).

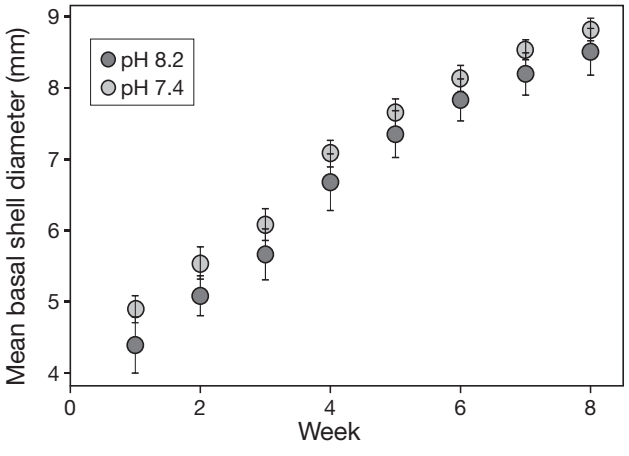

Fig. 3. *Amphibalanus amphitrite.* Mean ± 1SE basal diameter of barnacles reared in pH 7.4 and pH 8.2 seawater. Individuals were measured weekly from juvenile to adult over an 8 wk period. Means were based on beakers as the unit of replication (n = 5 for pH 7.4 and n = 4 for pH 8.2)

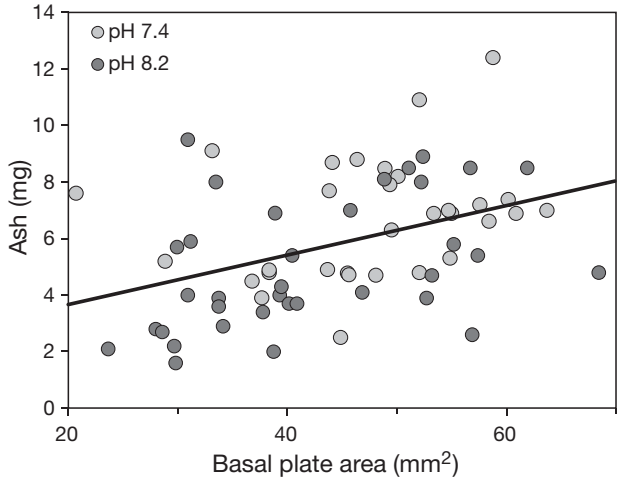

Fig. 5. *Amphibalanus amphitrite.* Ash content as a function of basal diameter for adult basal shells of barnacles reared in pH 7.4 and pH 8.2 seawater (n = 31 for pH 7.4 and n = 35 for pH 8.2). Line equation is Ash = 0.087×Area + 1.926

Adjusted mean ± 1SE ash values (mg) were 6.708 ± 0.409 and 5.247 ± 0.391 for basal plates of individuals raised in pH 7.4 and pH 8.2 seawater, respectively.

Wall shell penetrometry. Over the range of wall shell sizes used in this experiment, the force (N) necessary to penetrate the central portion of the wall shell was not correlated with shell thickness ($r = -0.031$, $p = 0.884$ for pH 7.4 and $r = 0.093$, $p = 0.659$ for pH 8.2). The mean ± 1SE force (N) was 4.750 ± 0.387 and 6.031 ± 0.385 ($n = 25$ for each treatment) for wall shells of individuals reared in pH 7.4 and pH 8.2 seawater, respectively. The mean force necessary to penetrate shells was significantly less for wall shells of individuals reared in pH 7.4 than in pH 8.2 seawater ($p = 0.024$, *t*-test).

DISCUSSION

The present study indicates that chronic exposure to elevated CO_2 seawater (pH 7.4) significantly affects some, but not all, aspects of the discrete life phases of the common intertidal barnacle *Amphibalanus amphitrite*. Early life phases including larval condition, cyprid size, proportions of hatched nauplii surviving to attachment and metamorphosis, and juvenile to adult growth were not affected by lowered pH. Nonetheless, despite compensatory calcification occurring in active growth zones of shells, ultimately this compensatory response was insufficient to prevent significant weakening of wall shells at reduced pH. Thus, importantly, our findings demonstrate that should anthropogenic CO_2 production continue at current levels, the later phases of barnacle life history will be vulnerable to oceanic pH levels that are projected to occur by 2250 (Caldeira & Wickett 2003, Riebesell 2008). Moreover, in coastal regions, the problem may be more urgent, as seasonal upwelling currently exposes marine invertebrates to corrosive acidified seawater as low as pH 7.6. This is increasing in areal extent due to ongoing anthropogenic CO_2 emissions (Feely et al. 2008). If current levels of CO_2 emissions are reduced, then it is possible that the ramifications implied by our results may not be as severe. However, it should be noted that several recent studies have found that even relatively small, and thus more near-term, decreases in seawater pH can have strong negative effects on biological processes such as fertilization in some marine invertebrates (e.g. Havenhand et al. 2008). Similar studies are needed to evaluate the effect of reductions in seawater pH on fertilization success in barnacles.

We examined hundreds of individual nauplii and cyprids with light microscopy and saw no evidence of an elevated rate of developmental abnormalities in those raised at pH 7.4. Moreover, the carapace dimensions (width and length) of larval cyprids at both 92 and 120 h post-naupliar release were similar regardless of whether they were raised in pH 7.4 or in ambient pH 8.2 seawater. This lack of effect of reduced pH on cyprid sizes, while somewhat unusual for calcified marine invertebrates examined to date (Kurihara 2008), has been found to be the case in other crustaceans. These include the nauplii and copepodites of *Acartia tsuensis* (pH 7.4; Kurihara & Ishimatsu 2008), and hatched embryos of the shrimp *Palaemon pacificus* (pH 7.6; Kurihara et al. 2008). In contrast, strong effects of ocean acidification on larvae have been observed in representative echinoderms and mollusks. For instance, echinoplutei of the sea urchin *Echinometra mathaei* become smaller and produce smaller arm sizes in CO_2-enhanced seawater (pH 7.8; Kurihara & Shirayama 2004, Kurihara et al. 2004, Kurihara 2008), while ophioplutei larvae of the brittlestar *Ophiothrix fragilis* exhibit morphological abnormalities, delayed development, and mortality, even at only a modest reduction in pH (7.9; Dupont et al. 2008). Moreover, veligers of both the oyster *Crassostrea gigas* and the mussel *Mytilus galloprovincialis* are negatively affected at pH 7.4, displaying smaller size, morphological abnormalities, and a complete lack or partial development of larval shells (Kurihara et al. 2007).

Despite the lack of effect of ocean acidification on sizes of developing cyprids or proportions of hatched nauplii that survived to attachment and metamorphosis, several studies have noted that, while ocean acidification may not affect larval development, there can be subsequent negative effects on post-settled juveniles. For example, recently settled juvenile polyps of the coral *Acropora tenuis* raised at pH 7.6 displayed malformations including aberrancies in the endoskeleton (Kurihara 2008). Among crustaceans, metamorphosing and settling juveniles of the shrimp *Palaemon pacificus* raised at pH 7.6 are smaller than those raised in ambient pH seawater (Kurihara 2008, Kurihara et al. 2008). Deleterious effects on larval settlement and juvenile recruitment success can have significant downstream effects on the population dynamics of adult marine invertebrates (Hunt & Scheibling 1997). It should be noted that the length of naupliar and cyprid development periods in other ecologically important barnacles can be considerably longer than that of *Amphibalanus amphitrite.* For example, larval development in the acorn barnacle *Semibalanus balanoides* is up to 3 to 8.5 times longer than that in *A. amphitrite* (Barnes & Barnes 1958), and is at least 10 times longer in the gooseneck barnacle *Pollicipes polymerus* (Lewis 1975). Thus, it is possible that with an increased length of exposure to reduced seawater pH there may be negative effects of ocean acidification on larval settlement and juvenile recruitment. Should reduced pH delay

larval settlement, there are known negative consequences in barnacles including reduced rates of juvenile growth (Pechenik et al. 1993).

A comparison of the slopes of growth curves in *Amphibalanus amphitrite* indicated no effect of reduced pH on growth rates, as individuals in both pH treatments grew rapidly at the same rate from juvenile to adult size. Nonetheless, sizes of barnacles (basal shell diameter) as they grew from juvenile to adult were consistently, but not significantly ($p = 0.11$), larger for barnacles exposed to ocean acidification conditions (pH 7.4) throughout the growth period. This trend suggests that there may be enhanced calcium carbonate production in the shells of juveniles as they grow into adult sizes at pH 7.4. Unequivocal evidence for acidification-induced compensatory calcification comes from 2 additional factors observed in the present study. First, shear forces required to break adult barnacles free from the substrate were significantly greater for barnacles raised at pH 7.4 than at pH 8.2. When lateral shear force was applied to individuals, they did not pull free intact from the substrate but rather suffered shell breakage, leaving their basal shell plates behind, attached to the substrate. Thus, shear force measurements provide a measure of the robustness of shells, which, in turn, provides an indirect measure of the degree of calcification. The second factor that provides evidence for compensatory calcification is that basal shell ash levels, a measure of calcium carbonate content, were significantly greater in individuals raised under conditions of ocean acidification. As the anatomical positioning of the basal shell plates renders them unexposed to the outer environment, this provides definitive evidence that calcification is enhanced under reduced seawater pH.

To the best of our knowledge, ours is only the second experimental study to demonstrate compensatory calcification in response to ocean acidification in a crustacean. Findlay et al. (2009) detected no significant differences in the Ca^{2+} concentration of the shells of live individuals of the barnacles *Semibalanus balanoides* and *Elminius modestus* exposed to seawater at pH 7.7 over 40 d when compared to those held at pH 8.0 for a similar time period. They concluded that this lack of a decline in shell Ca^{2+} concentration at the lower pH was indicative of compensatory calcification. They did not examine aspects of shell integrity to see if shells of individuals at the lower seawater pH were weakened as found in the present study (see also McClintock et al. in press). Compensatory responses have been detected in other groups of calcified marine invertebrates. In a study using the brittlestar *Amphiura filiformis* as a model calcification organism, Wood et al. (2008) demonstrated that the calcium carbonate content of regenerating arms was significantly elevated when adult brittlestars were maintained in pH 6.8, 7.3, or 7.7 seawater. Despite this compensatory calcification response, brittlestars exposed to reduced pH seawater suffered severe, and likely fatal, arm muscle wastage (Wood et al. 2008). We also discovered a significant problem that is likely to compromise compensatory calcification in *Amphibalanus amphitrite* when exposed to pH 7.4 seawater. Here, despite enhanced calcification in active shell-growth regions, wall shells, which grow from the bottom up, suffer significant dissolution as they grow upwards. Thus, by the time the wall shells have attained adult sizes, the central region of the shell has been weakened, as evidenced by our detection of a significant reduction in the force required to penetrate shells. This weakening is likely to have population-level effects, as it renders barnacles exposed to ocean acidification more susceptible to crushing and drilling predators (Connell 1961, 1985, Buschbaum 2002).

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LITERATURE CITED

Anil AC, Kurian J (1996) Influence of food concentration, temperature, and salinity on the larval development of *Balanus amphitrite.* Mar Biol 127:115–124

Anil AC, Chiba K, Kurokura H (1995) Influence of temperature and salinity on larval development of *Balanus amphitrite*: implications in fouling ecology. Mar Ecol Prog Ser 118:159–166

ASTM (American Society for Testing and Materials) (1997) Standard test method for measurement of barnacle adhesion strength in shear. Designation D5618-94. ASTM International, West Conshohocken, PA

Barnes H, Barnes M (1958) The rate of development of *Balanus balanoides* (L.) larvae. Limnol Oceanogr 3:29–32

Beesley A, Lowe DM, Pascoe CK, Widdicombe S (2008) Effects of CO_2-induced seawater acidification on the health of *Mytilus edulis.* Clim Res 37:215–225

Berge JA, Bjerkeng B, Pettersen O, Schanning MT, Oxnevad S (2006) Effects of increased sea water concentrations of CO_2 on growth of the bivalve *Mytilus edulis* L. Chemosphere 62:681–687

Bibby R, Widdicombe S, Parry H, Spicer JI, Pipe R (2008) Impact of ocean acidification on the immune response of the blue mussel *Mytilus edulis.* Aquat Biol 2:67–74

Buschbaum C (2002) Predation on barnacles on intertidal and subtidal mussel beds in the Wadden Sea. Helgol Mar Res 56:37–43

Caldeira K, Wickett ME (2003) Anthropogenic carbon and ocean pH. Nature 425:365–368

Caldeira K, Wickett ME (2005) Ocean model prediction of chemistry changes from carbon dioxide emission to the atmosphere and ocean. Geophys Res Lett 34:C09S04, doi:10.1029/2004JC002671

Connell JH (1961) Effects of competition and predation by *Thais lapillus*, and other factors on natural populations of the barnacle *Balanus balanoides.* Ecol Monogr 31:61–104

Connell JH (1985) The consequence of variation in initial vs. post-settlement mortality in rocky intertidal communities. J Exp Mar Biol Ecol 93:11–45

Costlow JD, Bookhout CG (1958) Larval development of *Balanus amphitrite* var. *denticulata* Broch reared in the laboratory. Biol Bull (Woods Hole) 114:284–295

Dupont S, Havenhand J, Thorndyke W, Peck L, Thorndyke M (2008) Near-future level of CO_2-driven ocean acidification radically affects larval survival and development in the brittlestar *Ophiothrix fragilis.* Mar Ecol Prog Ser 373:285–294

Fabry VJ, Seible BA, Feely RA, Orr JC (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. ICES J Mar Sci 65:414–432

Feely RA, Sabine CL, Lee K, Berelson W, Kleypas J, Fabry VJ, Millero FJ (2004) Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. Science 305:362–366

Feely RA, Sabine CL, Hernandez-Ayon JM, Ianson D, Hales B (2008) Evidence of upwelling of corrosive 'acidified' water onto the continental shelf. Science 320:1490–1492

Findlay HS, Kendall MA, Spicer JI, Turley C, Widdicombe S (2008) Novel microcosm system for investigating the effects of elevated carbon dioxide and temperature on intertidal organisms. Aquat Biol 3:51–62

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

Fine M, Tchernov D (2007) Scleractinian coral species survive and recover from decalcification. Science 315:1811–1813

Guinotte JM, Fabry VJ (2008) Ocean acidification and its potential effects on marine ecosystems. Ann NY Acad Sci 1134:320–342

Havenhand J, Buttler F, Thorndyke M, Williamson J (2008) Near-future levels of ocean acidification reduce fertilization success in a sea urchin. Curr Biol 18:R651–R652

Henry DP (1959) The distribution of the *amphitrite* series of *Balanus* in North American waters. In: Ray DL (ed) Marine boring and fouling organisms. University of Washington Press, Seattle, WA, p 190–211

Holm E, Oriheula B, Kavanagh CJ, Rittschof D (2005) Variation among families for characteristics of the adhesive plaque in the barnacle *Balanus amphitrite.* Biofouling 21:121–126

Hung OS, Thiyagarajan V, Qian PY (2008) Preferential attachment of barnacle larvae to natural multi-species biofilms: Does surface wettability matter? J Exp Mar Biol Ecol 361: 36–41

Hunt HL, Scheibling RE (1997) Role of early post-settlement mortality in recruitment of benthic marine invertebrates. Mar Ecol Prog Ser 155:269–301

IPCC (Intergovernmental Panel on Climate Change) (2007) Climate change 2007: the physical science basis. In: Solomon S, Qin D, Manning M, Chen Z and others (eds) Contribution of Working Group 1 to the 4th assessment report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge

Kurihara H (2008) Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates. Mar Ecol Prog Ser 373:275–284

Kurihara H, Ishimatsu A (2008) Effects of high CO_2 seawater on the copepod (*Acartia tsuensis*) through all life stages and subsequent generations. Mar Pollut Bull 56:1086–1090

Kurihara H, Shirayama Y (2004) Effects of increased atmospheric CO_2 on sea urchin early development. Mar Ecol Prog Ser 274:161–169

Kurihara H, Shimode S, Shirayama Y (2004) Sub-lethal effects of elevated concentration of CO_2 on planktonic copepods and sea urchins. J Oceanogr 60:743–750

Kurihara H, Kato S, Ishimatsu A (2007) Effects of increased seawater pCO_2 on early development of the oyster *Crassostrea gigas.* Aquat Biol 1:91–98

Kurihara H, Matsui M, Furukawa H, Hayashi M, Ishimatsu A (2008) Long-term effects of predicted future seawater CO_2 conditions on the survival and growth of the marine shrimp *Palaemon pacificus.* J Exp Mar Biol Ecol 367:41–46

Lewis CA (1975) Development of the gooseneck barnacle *Pollicipes polymerus* (Cirripedia: Lepadomorpha): fertilization through settlement. Mar Biol 32:141–153

McClintock JB, Angus RA, McDonald MR, Amlser CD, Catledge SA, Vohra YK (in press) Rapid dissolution of shells of weakly calcified Antarctic benthic macroorganisms indicates high vulnerability to ocean acidification. Antarct Sci

Miles H, Widdicombe S, Spicer JI, Hall-Spencer J (2007) Effects of anthropogenic seawater acidification on acid-base balance in the sea urchin *Psammechinus miliaris.* Mar Pollut Bull 54:89–96

Orr JC, Fabry VJ, Aumont O, Bopp L and others (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437:681–686

Pechenik JA, Rittschof D, Schmidt AR (1993) Influence of delayed metamorphosis on survival and growth of juvenile barnacles *Balanus amphitrite.* Mar Biol 115:287–294

Pitombo FB (2004) Phylogenetic analysis of the Balinidae (Cirripediaa, Balanomorpha). Zool Scr 33:261–276

Qian PY, Thiyagarajan V, Lau SCK, Cheung SCK (2003) Relationship between bacterial community profile in biofilm and attachment of the acorn barnacle *Balanus amphitrite.* Aquat Microb Ecol 33:225–237

Riebesell U (2008) Climate change: acid test for marine biodiversity. Nature 454:46–47

Rittschof D (2001) Natural product antifoulants and coatings development. In: McClintock JB, Baker BJ (eds) Marine chemical ecology. CRC Press, Boca Raton, FL, p 543–566

Rittschof D, Branscomb ES, Costlow JD (1984) Settlement and behavior in relation to flow and surface in larval barnacles, *Balanus amphitrite* Darwin. J Exp Mar Biol Ecol 82: 131–146

Rittschof D, Clare AS, Gerhart DJ, Avelin MS, Bonaventura J (1992) Barnacle in vitro assays for biologically active substances: toxicity and settlement assays using mass cultured *Balanus amphitrite* Darwin. Biofouling 6:115–122

Sabine CL, Feely RA, Gruber N, Key RM and others (2004) The oceanic sink for anthropogenic CO_2. Science 305:367–371

Takahashi T, Feely RA, Weiss RF, Wanninkhof RH, Chipman DW, Sutherland SC (1997) Global air-sea flux of CO_2: an estimate based on measurements of sea-air pCO_2 difference. Proc Natl Acad Sci USA 94:8992–8299

Thompson RC, Norton TA, Hawkins SJ (1998) The influence of epilithic microbial films on the settlement of *Semibalanus balanoides* cyprids—a comparison between laboratory and field experiments. Hydrobiologia 375-376:203–216

Wood HL, Spicer JI, Widdicombe S (2008) Ocean acidification may increase calcification rates, but at a cost. Proc R Soc Lond B Biol Sci 275:1767–1773

Wootton JT, Pfister CA, Forester JD (2008) Dynamic patterns and ecological impacts of declining ocean pH in a high-resolution multi-year dataset. Proc Natl Acad Sci USA 105: 18848–18853

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