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Original Article

Effects of elevated pCO_2 on crab survival and exoskeleton composition depend on shell function and species distribution: a comparative analysis of carapace and claw mineralogy across four porcelain crab species from different habitats

Tessa M. Page[1], Samantha Worthington[2], Piero Calosi[2,3], and Jonathon H. Stillman[1,4]*

[1]*Romberg Tiburon Center and Department of Biology, San Francisco State University, 3150 Paradise Drive, Tiburon, CA 94920, USA*
[2]*Marine Biology and Ecology Research Center, Plymouth University, Drake Circus, Plymouth, England PL4 8AA, UK*
[3]*Département de Biologie, Chimie et Géographie, Université du Québec à Rimouski, 300 Allée des Ursulines, Rimouski, Québec G5L 3A1, Canada*
[4]*Department of Integrative Biology, University of California, Berkeley, Valley Life Sciences Building no. 3140, Berkeley, CA 94720-3140, USA*

**Corresponding author: tel.: (415) 338-3790; fax: (415) 435-7120; e-mail: stillmaj@sfsu.edu*

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Elevated concentration of carbon dioxide (elevated pCO_2) that cause reduced pH is known to influence calcification in many marine taxa, but how elevated pCO_2 influences cation composition of mineralized structures is less well studied. To a large extent, the degree to which elevated pCO_2 impacts mineralized structures is influenced by physiological adaptation of organisms to environments where low pH is routinely experienced. Here, we test the hypotheses that elevated pCO_2 will differently impact the relative concentrations of divalent cations (Ca^{2+}, Mg^{2+}, Sr^{2+}, and Mn^{2+}) in four closely related species of porcelain crabs distributed across intertidal zone gradients. Cation composition of carapace and claw exoskeleton was determined using inductively coupled plasma mass spectrometry following 24-day exposures to pH/pCO_2 levels of 8.0/418 and 7.4/1850 µatm during the intermoult period. Reduced pH/elevated pCO_2 caused a 13–24% decrease of carapace [Ca^{2+}] across all species, and species-specific responses in carapace and claw [Mg^{2+}], [Sr^{2+}] and [Mn^{2+}] were observed. During a 24-day exposure, reduced pH/elevated pCO_2 reduced survival probability in low-intertidal but not mid-intertidal species. Overall, the effect of reduced pH/elevated pCO_2 on exoskeleton mineral composition was muted in mid-intertidal species relative to low-intertidal species, indicating that extant adaptation to the variable intertidal zone may lessen the impact of ocean acidification (OA) on maintenance of mineralized structures. Differences in responses to reduced pH/elevated pCO_2 among closely related species adds complexity to predictive inferences regarding the effects of OA.

Keywords: acclimation, calcification, calcium, intertidal zone, magnesium, manganese, ocean acidification, pH, strontium.

Introduction

Crustacean mineralized exoskeletons provide physical and physiological protection from mechanical loads, predation and desiccation (Nagasawa, 2012). Mineralized structures in crustaceans are composed mainly of calcium carbonate, but vary in composition and mineral properties across body parts and within and across species (Boßelmann *et al.*, 2007). In addition to calcium (Ca^{2+}), exoskeletons of marine calcifying organisms include other divalent cations including magnesium (Mg^{2+}), manganese (Mn^{2+}), and strontium (Sr^{2+}) (de Lima *et al.*, 2011). Replacement of Ca^{2+} with Mg^{2+}, Sr^{2+}, and Mn^{2+} alters structural and functional properties (Baden and Eriksson, 2006; Boßelmann

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et al., 2007), and in crustaceans is associated with the processes of molting (Tao *et al.*, 2009), and hardening of the exoskeleton (Horne and Tarsitano, 2007).

Carbon dioxide (CO_2) is increasing in the atmosphere at an unprecedented rate (Pachauri and Meyer, 2014) and being absorbed by the world's oceans (Sabine *et al.*, 2004) causing a reduction in seawater pH (Meehl *et al.*, 2007), a phenomenon known as ocean acidification (OA). Following the recommendation of McElhany (2017) we have adopted the convention of referring to physiological responses as resulting from exposure to reduced pH driven by elevated concentration of CO_2 (reduced pH/elevated pCO_2), and restricting the usage of response to OA as ecological or evolutionary response to natural variation in environmental pH/pCO_2 over temporal or spatial scales.

Calcifying organisms are sensitive to reduced pH/elevated pCO_2 (Kroeker *et al.*, 2010). In particular, organisms buffer the effects of reduced environmental pH by liberating ions from calcified tissues (Wheatly *et al.*, 1991; Calosi *et al.*, 2013a; Rastrick *et al.*, 2014). Crustaceans use $CaCO_3$ in their carapace to buffer the extracellular effects of acidosis by extracting carbonate ions (CO_3^{2-}) from the carapace to bind with free protons (H^+) and form bicarbonate (HCO_3^-) (Wheatly *et al.* 1991; Michaelidis *et al.* 2005). Accordingly, reduced pH/elevated pCO_2 increases rates of mineralized exoskeleton dissolution (Bednaršek *et al.*, 2012).

Reduced pH/elevated pCO_2 is also known to cause shifts in exoskeletal cation composition in a wide range of taxa, including crustaceans (e.g. Wickins, 1984), molluscs (Ries, 2011), echinoderms (Miles *et al.*, 2007), and corals (Cohen *et al.*, 2009). For example, reduced pH/elevated pCO_2 increased intermolt exoskeleton Ca^{2+} content in adults of the prawns *Penaeus occidentalis* and *Penaeus monodon* (Wickins, 1984), decreased Ca^{2+} in spider crab larval exoskeleton (Walther *et al.*, 2011), decreased Mg^{2+} in European lobster *Homarus gammarus* larval exoskeleton, and increased Mg^{2+} in claw exoskeleton of the velvet swimming crab (Small *et al.*, 2010). Changes in exoskeleton ion composition shift skeletal strength (Chan *et al.*, 2012), so mineralization dynamics involved with acid-base regulation may cause weaker exoskeletons, increasing vulnerability to predation and other stressors (Amaral *et al.*, 2012).

Differences in physiological response to reduced pH/elevated pCO_2 have been observed among closely related species living in different pH environments: e.g. molluscs (Parker *et al.*, 2010; Maas et al. 2012), crustaceans (Lewis *et al.*, 2013), echinoderms (Byrne *et al.* 2013; Calosi *et al.* 2013a), and polychaete worms (Calosi *et al.*, 2013b). Physiological responses to reduced pH/elevated pCO_2 also vary within a species across pelagic and benthic life history stages, as has been demonstrated for the intertidal zone porcelain crab *Petrolisthes cinctipes* (Ceballos-Osuna *et al.*, 2013). Intertidal zones are pH/pCO_2 variable environments (Morris and Taylor, 1983), and organisms inhabiting intertidal regions are adapted to tolerate a dynamic environment (Magozzi and Calosi, 2015). However, intertidal zone organisms experience environmental conditions at their physiological tolerance limits (Stillman and Somero, 2000). Climate warming is predicted to increase the intensity and frequency of coastal upwelling of reduced pH/elevated pCO_2 seawater (Gruber *et al.*, 2012). If present pH variability of the intertidal zone is close to physiological tolerance limits (Stillman and Paganini, 2015), there could be detrimental impacts of OA and climate warming (Paganini *et al.* 2014).

We hypothesized that under reduced pH/elevated pCO_2 mid-intertidal zone organisms would defend exoskeleton mineralization to a greater extent than low-intertidal organisms due to their adaptation to variable environments. We tested this hypothesis in four species of porcelain crabs from different intertidal zones and oceanic regions. Porcelain crabs serve as a good model for comparative research because closely related species occupy different geographic locations and intertidal zones (Stillman and Somero, 2000). We examined exoskeleton mineralogy changes in two pairs of vertically zoned porcelain crab species. One species pair is *P. cinctipes* from the mid-intertidal zone and *Petrolisthes manimaculis* from the low-intertidal to shallow subtidal of the temperate Northeastern Pacific along the west coast of the United States (Morris *et al.*, 1980). The other species pair is *Porcellana platycheles* from the mid-intertidal zone and *Pisidia longicornis* from the low-intertidal to shallow subtidal of the English coast of the North Atlantic (Davenport, 1972). We measured survival and cation concentrations of two regions of the exoskeleton, claw and carapace, following acclimation to pH/pCO_2 values representing current and predicted levels according to the IPCC's (2013) worst case scenario (RCP8.5 Figure 12.12; Collins *et al.*, 2013).

Material and methods

Experimental design

Four confamilial species of intertidal porcelain crabs were compared between two geographic locations: Northeastern Pacific (United States) and Northeastern Atlantic (United Kingdom), and two intertidal zones: mid- and low-intertidal zone. Experiments were conducted at the Stillman Laboratory at the Romberg Tiburon Center (San Francisco, CA, USA) for the Pacific species and at the Marine Biology and Ecology Research Centre (MBERC) at Plymouth University (Plymouth, UK) for the Atlantic species, hence the methods are separately described for each location.

Animal collection

Similarly sized (carapace ~ 8–15 mm), adult male *P. cinctipes* and *P. manimaculis* were collected during low tide from the rocky intertidal zone in Pacifica, California (37°36′4″ N, 122°29′57″ W) on the 13th of January 2013. Crabs were transported to San Francisco State University Romberg Tiburon Center for Environmental Studies (Tiburon, California), transport time < 1.5 h. Seawater was collected during high tide from San Francisco Bay and sediment was allowed to settle for ≥ 1 week before use. Crabs were held in a common garden aquarium for 11 d in constant conditions ($T = 12\,^{\circ}C$, $S = 34.0$, pH 8.0) with daily water changes. Crabs were fed daily ~50 µL (~500 million cells) of Shellfish Diet 1800 (Reed Mariculture, Inc, Campbell, CA, USA, http://www.reedmariculture.com), an algal mixture of 30% *Isochrysis sp.*, 20% *Pavlova sp.*, 20% *Thalossiosira weissflogii*, and 30% *tetraselmis sp.*

Adult males (carapace ~ 7–10 mm) *P. platycheles* were collected during low tide from the rocky intertidal zone from Batten Bay, Plymouth, UK (50°21′22.91″ N, 4°7′39.03″ W) on the 29 of May 2013. Adult males (carapace ~ 2–5 mm) *P. longicornis* were collected during low tide from the rocky intertidal zone in Hannafore, Cornwall, UK (50°20′37.54″ N, 4°27′4.18″ W) on the 28 of June 2013. Crabs were transported to Plymouth University (Plymouth, UK), transport time < 2.5 h., and held together in a common garden aquarium for 11 d in constant ambient

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temperature (T = 15 °C, S = 33.5, pH 8.1) with daily water changes. Seawater was collected from the Mount Batten pier (Plymouth, UK). Sediment was allowed to settle and water was filtered before use. Crabs were fed daily following the same procedure as stated above for the US experiments.

Animal welfare

Animals in the United States and United Kingdom were kept in ambient, unfiltered seawater aquaria prior to experiment for 11 d. Daily water changes and cleanings of aquaria were done during this time, and animals were treated gently and fed daily. Upon end of experiment, animals were sacrificed in the least time possible to minimize any potential suffering.

Experimental procedure

Individuals of *P. cinctipes* and *P. manimaculis* (n = 30 *per* species) were haphazardly assigned to one of six treatments boxes (n = 10 indiv. *per* treatment box and a n = 15 indiv. *per* species *per* pH/pCO_2 treatment). Individuals were placed in separate mesh-bottomed cylinders and kept in sealed treatment boxes (three replicate boxes *per* treatment) within water tables that were maintained at a constant temperature. Each box had minimal air space and was equipped with a manifold connected to a small submersible pump supplying recirculating treatment water to each cylinder. Crabs were fed daily with ∼20 µL full concentration Shellfish Diet 1800. Complete water changes were performed daily 4 h after feeding for the duration of the 24-d experiment. Care was taken to not aerate or disturb the treatment water during changes so as to not alter the pH/pCO_2 of the seawater. Salinity and temperature were checked daily using an YSI model 30 hand-held meter (Yellow Springs Instruments, OH, USA) and dissolved oxygen (DO) was tested weekly to every other week throughout the experiment using a YSI Pro20 hand-held meter. DO was always at or above 80% air saturation. Daily checks for molts and death were performed and molts or dead crabs were removed.

P. platycheles and *P. longicornis* were haphazardly assigned to experimental conditions and boxes to achieve an n = 15 indiv. *per* species *per* treatment (n = 45 total indiv. *per* species). Crabs were placed in 120 mL perforated, plastic, screw cap bottles and kept in sealed treatment boxes (n = 5 indiv. *per* treatment box, n = 9 treatment boxes). Treatment boxes were maintained at constant temperature within sea tables. Each treatment box was supplied with continuous water flow (40 mL min^{-1}) from a header tank with constant temperature and salinity (T = 15.2 ±0.3 °C, S = 33.9 ± 0.5); this eliminated the need for water changes. Box positions were altered daily to eliminate any placement effect. Feeding and checking seawater parameters followed the same methods as stated in above paragraph for crabs in the United States.

Water chemistry manipulation

Seawater pH/pCO_2 in the United States was regulated by injection of pure CO_2 gas controlled by a pH sensor (*sensu* Widdicombe and Needham, 2007). Two treatment mixing tanks, one designated to mimic ambient pH (pH 8.0) and the other set to pH 7.4 to mimic predicted, future, ocean pH according to the IPCC's (2013) worst case scenario (RCP8.5 Figure 12.12; Collins *et al.*, 2013). Treatment water in the United Kingdom was altered using CO_2 enriched air *via* a modified and integrated version of the equilibration flow-through system used by Melatunan *et al.* (2011). Three treatment header tanks were set to target pH levels of "ambient pH" (pH 8.0) and "lowered pH" (pH 7.4). Additionally, a treatment of "extreme lowered pH" (pH 7.0) was included for the UK species, but only *P. platycheles* survived that pH level (data mainly presented in Supplementary Materials).

Seawater samples were taken in both the United States and United Kingdom to test pH/pCO_2 levels with best possible practices at each location (Dickson *et al.*, 2007). We are confident as much as possible that the methods used allowed us to obtain pH/pCO_2 levels that were comparable across geographical locations. Water samples in the United States were collected bi-weekly from treatment boxes and CO_2 mixing tanks. Seawater pH was determined spectrophotometrically (Shimadzu UV-1700 PharmaSpec, Kyoto, Japan) following a protocol modified from SOP 6b (Dickson *et al.*, 2007). In the United Kingdom, samples were collected daily from a random assortment of boxes and pH was measured immediately using a pH meter (SevenEasy, Mettler Toledo GmbH, Schwerzenbach, Switzerland) with an electrode that was calibrated daily using a three-point calibration with NBS buffers 4.01, 7.00, and 9.21 (Technical buffer solution, Mettler Toledo AG, Schwerzenbach, 1V057B, 1V103B, 1W241D). All pH measurements in the United Kingdom were then converted to total scale (Noisette *et al.*, 2014).

In the United States, seawater samples (100 mL) for total alkalinity were collected once weekly from treatment boxes and CO_2 mixing tanks, poisoned with 10 mL of saturated mercuric chloride solution in water (sat. $HgCl_2$), and stored in acid washed glass bottles. Total alkalinity was determined by titration using a Metrohm dosimat 765 pH-Meter (Metrohm, Herisau, Switzerland) following the method of Bradshaw *et al.* (1981). Water samples (100 mL) for total alkalinity in the United Kingdom were collected once weekly from treatment boxes using an identical protocol as in the United States. Total alkalinity was obtained using a fully automated Gran Alkalinity Titrator (Apollo SciTech As-alk2, Newark, DE, USA). The instrument system was equipped with a combination pH electrode (8102BNUWP, Thermo Scientific, MA, USA) and a temperature probe (Star ATC probe, Thermo Scientific) connected to a pH meter. LabVIEW (National Instruments Corporation, TX, USA) software was used to control the automated alkalinity analyzer and record total alkalinity values.

Remaining parameters of the seawater carbonate system were calculated using SeaCarb 2.4.10 (Lavigne *et al.*, 2013) in the statistical computing programme R v 3.0.1 (R Core Team, 2014) (Table 5). The following parameters were calculated: pCO_2, $[HCO^{3-}]$, $[CO_3^{2-}]$, dissolved inorganic carbon (DIC), $\Omega_{calcite}$, and $\Omega_{aragonite}$. Treatment water salinity, temperature, pH and total alkalinity were used as input parameters (seacarb, flag 8), using the total pH scale (pH_T). Seawater carbonate system calculations (i.e. TA, pCO_2, $[HCO^{3-}]$, $[CO_3^{2-}]$, DIC, $\Omega_{calcite}$, and $\Omega_{aragonite}$) from the US and UK were compared using Kruskal-Wallis rank sum tests (Supplementary Tables S8 and S9).

Determination of exoskeleton ion concentration

After each of the 24-day experiments, crabs were euthanized, and carapace and left and right chelae were sampled using Teflon coated or plastic forceps. No metal dissecting tools were used in order to eliminate possible metal contamination. Exoskeleton parts were triple rinsed with MilliQ ultra pure water, blotted dry with filter paper and stored in 1.5 mL centrifuge tubes. *P. cinctipes*

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and *P. manimaculis* exoskeleton samples were prepared in the United States and dried exoskeleton samples were transported to the United Kingdom facility for analysis.

Samples were prepared for inductively coupled plasma mass spectrometry (ICP-MS) analysis using an exoskeleton method modified from Jack *et al.* (2011). Carapace and left and right chelae were weighed to the nearest 0.1 mg with a max sample weight of 70.0 mg and digested in 2.5 ml nitric acid (75% TraceMetal^{TM} Grade, Fisher Chemical, Lougborough, UK) and sent through a microwave reaction to complete the full digestion of the exoskeletal structure (MARS 6, CEM).

Ionic concentrations (^{24}Mg, ^{44}Ca, ^{55}Mn, and ^{88}Sr) were measured using ICP-MS X-Series II (Thermo Scientific, Hemel Hempstead, UK) using a method similar to Wolf and Adams (2015). Dilutions of prepared samples were made using a 2% nitric acid solution in MilliQ water (TraceMetal Grade, Fisher Chemical). Samples were run in triplicate and all elements were analysed simultaneously. Additionally, 200-fold dilutions were made for Mg^{2+} and Ca^{2+}.

Statistical analyses

Survival was recorded and analysed after the 24-day experiment. Statistical analysis of survival probability was carried out in the *survival* package (v. 2.37-4) in R statistical program (v 3.0.1). Kruskal-Wallis rank sum tests were done to test significant differences in survival probability among species and across intertidal zone locations.

The effect of pH/pCO_2 on ions within the claw and carapace of the four porcelain crab species were analysed by linear regression model and ANCOVA. The α level to reject the null hypothesis was set to 0.05 and linear hypotheses with *p*-values below this threshold were considered to represent statistically significant effects. The intercept from each linear regression was set to test amount of ion present and the *p*-value tested the sensitivity of that ion to pH/pCO_2. For the ANCOVA, each ion was set as the dependent variable, pH/pCO_2 was set as the factor, and species was set as the covariate. Analyses were repeated with and without the interaction of pH/pCO_2 and species to test the significance of the slope and the effect of pH/pCO_2 and species on the slope of the line obtained from linear regression.

The effect of intertidal zone on the sensitivity of ions to pH/pCO_2 was compared using a two-way ANOVA testing pH/pCO_2 treatment and intertidal location as independent or combined fixed factors depending on best-fit model, which was obtained through best fit modeling *via* Akaike information criterion (AIC). Data were tested for normality using QQ normality plots. Tukey HSD post hoc comparisons were made to identify statistically significant pairwise differences between treatments and intertidal location. Values reported in the text are mean SEM.

Cation concentration ratios were obtained for $[Ca^{2+}]$ to $[Mg^{2+}]$ and $[Ca^{2+}]$ to $[Sr^{2+}]$ by using the following equation (1):

$$\frac{\left[\frac{Ca^{2+}(low)}{Mg^{2+}(low)}\right]}{\left[\frac{Ca^{2+}(avg)}{Mg^{2+}(avg)}\right]} \quad \text{(e.g. for Ca/Mg ratio)} \tag{1}$$

The effect of reduced pH/elevated pCO_2 on the ratio was found through subtracting "1.0" or "ambient" from the obtained value from equation (1) at pH 7.4. A negative response implied a decrease in $[Ca^{2+}]$ or an increase in $[Mg^{2+}]$ or $[Sr^{2+}]$ with reduced pH/elevated pCO_2. Ratios were compared by two-way ANOVA with pH/pCO_2 treatment and intertidal location as independent or combined fixed factors.

Results

Survival and condition

There was no significant effect of reduced pH/elevated pCO_2 on survival in *P. cinctipes*, *P. manimaculis*, or *P. platycheles* (Supplementary Figure S1). However, survival probability decreased with reduced pH/elevated pCO_2 in *P. longicornis* (Figure 1), as 50% mortality was observed at day 17 in pH 7.4 (Kruskal Wallace Chi-squared = 8.3, $p < 0.001$, Supplementary Table S2), and 100% mortality at day 7 in pH 7.0 (Kruskal Wallace Chi-squared = 12.3, $p < 0.0001$, Supplementary Table S2, Supplementary Figure S1). Depigmentation, through loss of the exocuticle, and autotomization were observed under reduced pH/elevated pCO_2 conditions, though were less pronounced in the mid-intertidal zone crabs *P. cinctipes* and *P. platycheles* (Figure 2).

Exoskeleton ion concentration

Differences in mineralization status of carapace & claw

Total ion content of mineralized exoskeleton under ambient pH/pCO_2 conditions (pH 8.0) varied across species and anatomy (Table 1). $[Ca^{2+}]$ ranged from 5 to 68 mol kg^{-1}, $[Mg^{2+}]$ ranged from 0.6 to 8 mol kg^{-1}, $[Sr^{2+}]$ ranged from 20 to 45 mmol kg^{-1}, and $[Mn^{2+}]$ ranged from 0.3 to 7.3 mmol kg^{-1} (Table 1). When comparing $[Mn^{2+}]$ across species, $[Mn^{2+}]$ was at least 93% higher in *P. platycheles* carapace and claw. $[Ca^{2+}]$ was found to be almost 10-fold higher in claw than carapace in all species with the exception of *P. longicornis*, in which carapace $[Ca^{2+}]$ was 15% higher than claw. $[Sr^{2+}]$ was 22 to 54% higher in carapace than claw in all four species (Table 1). $[Ca^{2+}]$ was highest in carapace and claw of *P. manimaculus* and $[Sr^{2+}]$ was highest in carapace of *P. manimaculis* ($[Ca^{2+}]$ 14 to 37% higher in carapace and 8–1246% higher in claw, $[Sr^{2+}]$ 15 to 84% higher in carapace).

Changes in skeletal mineralization status caused by exposure to low pH

We observed species-specific changes in exoskeletal cation concentrations following exposure to reduced pH/elevated pCO_2 (Figure 3). Greatest changes occurred in claw exoskeleton (Tables 2 and 3) with exception of $[Sr^{2+}]$. $[Ca^{2+}]$ decreased with decreasing pH across all species, but statistically significant decreases were only observed in the low-intertidal species, *P. manimaculis* and *P. longicornis*, where $[Ca^{2+}]$ was 16 and 67% lower, respectively (ANOVA, $p < 0.05$, Table 2 and Supplementary Table S4, Figure 3). Under reduced pH/elevated pCO_2, claw exoskeleton $[Mg^{2+}]$ decreased by 17% in *P. manimaculis*, 19% in *P. platycheles* and 28% in *P. longicornis* (ANOVA $p < 0.05$, Table 2 and Supplementary Tabler S4, Figure 3); however, did not change significantly in *P. cinctipes* (ANOVA $p > 0.05$, Table 2 and Supplementary Table S4, Figure 3). $[Mn^{2+}]$ did not significantly change in *P. manimaculis*, *P. cinctipes* and *P. longicornis* with reduced pH/elevated pCO_2 (ANOVA $p > 0.05$, Table 2 and Supplementary Table S4, Figure 3); however, it increased significantly in *P. platycheles* by 39% (ANOVA $p < 0.05$, Table 2 and Supplementary Table S4, Figure 3). $[Sr^{2+}]$ decreased significantly with reduced pH/elevated pCO_2 by 23% in *P. manimaculis*, 32% in *P. cinctipes*, and 105% in *P. longicornis* (ANOVA $p < 0.05$,

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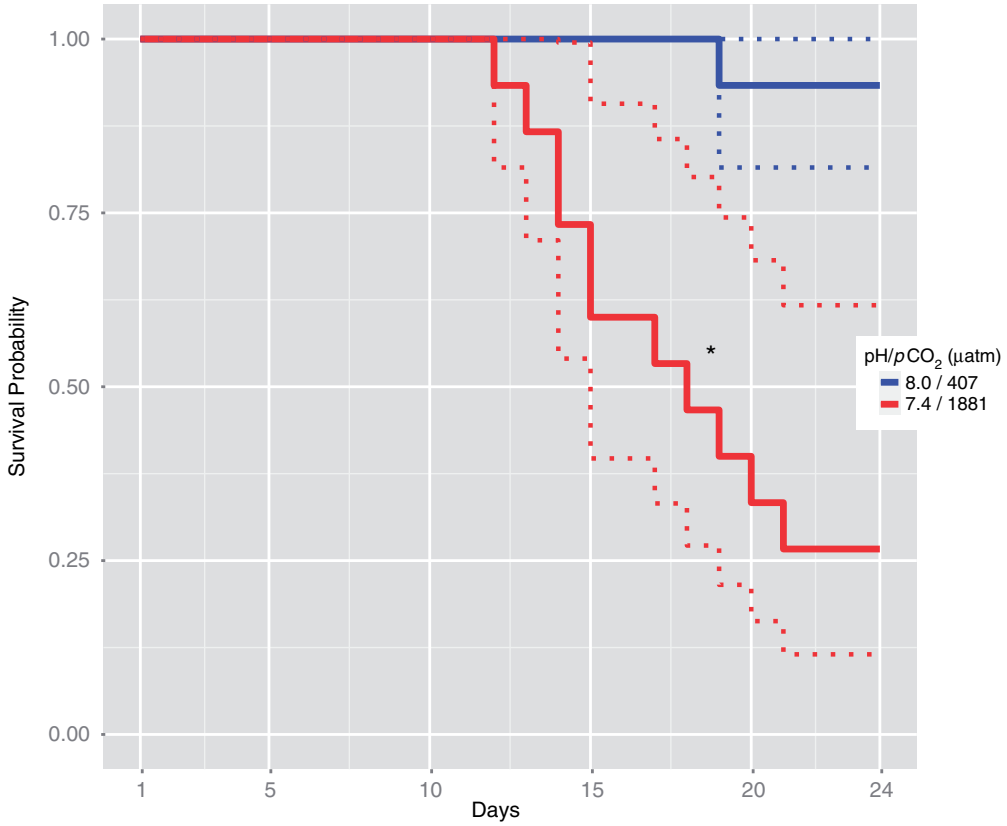

Figure 1. Survival probability of *P. longicornis* (L. 1767), the low-intertidal North Eastern Atlantic species, significantly decreased under reduced pH/elevated pCO_2. Solid lines represent mean values, and dotted lines represent 95% confidence intervals. The asterisk indicates a statistically significant difference at $p < 0.05$.

Table 2 and Supplementary Table S6, Figure 3), but did not significantly change in *P. platycheles* (ANOVA $p > 0.05$, Table 2 and Supplementary Table S4, Figure 3).

Claw $[Ca^{2+}]$:$[Mg^{2+}]$ increased with reduced pH/elevated pCO_2 by 70% in *P. manimaculis* (ANOVA $p < 0.05$, Table 4, Figures 3 and 4) and increased by 38% in *P. cinctipes* (ANOVA $p < 0.05$, Table 4, Figure 3). Claw $[Ca^{2+}]$:$[Mg^{2+}]$ was found to decrease by 15% in *P. platycheles* (ANOVA $p < 0.05$, Table 4, Figures 3 and 4) but increased in *P. longicornis* by 8% (ANOVA $p < 0.05$, Table 4, Figure 3). $[Ca^{2+}]$:$[Sr^{2+}]$ in the claw increased in the two North Eastern Pacific species by 70% for *P. manimaculis* and 38% for *P. cinctipes* (ANOVA $p < 0.05$, Table 4, Figure 4) and in *P. platycheles* by 10% (ANOVA $p < 0.05$, Table 4, Figure 3); however claw $[Ca^{2+}]$:$[Sr^{2+}]$ decreased in *P. longicornis* by 80% (ANOVA $p < 0.05$, Table 4, Figure 4).

Carapace concentration of Ca^{2+} ($[Ca^{2+}]$) decreased under reduced pH/elevated pCO_2 by 13–24% across species (ANOVA, $p < 0.05$, Table 2 and Supplementary Table S3, Figure 3). Similarly, carapace $[Mg^{2+}]$ decreased under reduced pH/elevated pCO_2 by 21% in the low-intertidal zone species *P. manimaculis*, and by 28% in mid-intertidal species *P. platycheles* (ANOVA, $p < 0.05$, Table 2 and Supplementary Table S3, Figure 3). In contrast, carapace $[Mg^{2+}]$ did not change under reduced pH/elevated pCO_2 in the mid-intertidal species *P. cinctipes* or the low-intertidal species *P. longicornis* (ANOVA, $p > 0.05$, Table 2 and Supplementary Table S3, Figure 3). Carapace $[Mn^{2+}]$ increased by 49% under reduced pH/elevated pCO_2 in *P. platycheles* (ANOVA, $p < 0.05$, Table 2 and Supplementary Table S3, Figure 3). However, carapace $[Mn^{2+}]$ was lower and did not change under reduced pH/elevated pCO_2 in the other species (ANOVA, $p > 0.05$, Table 2 and Supplementary Table S3, Figure 3). Overall, carapace $[Sr^{2+}]$ decreased across all species under reduced pH/elevated pCO_2 (ANOVA, $p > 0.05$, Table 2 and Supplementary Table S3, Figure 3), decreasing by as much as 76% in *P. manimaculis* between pH 8.0 to pH 7.4 (ANOVA, $p < 0.05$, Table 2 and Supplementary Table S3, Figure 3).

Carapace $[Ca^{2+}]$:$[Mg^{2+}]$ increased under reduced pH/elevated pCO_2 by 15–20% in *P. cinctipes* and *P. manimaculis* (ANOVA $p < 0.05$, Table 4, Figures 3 and 4), but decreased under reduced pH/elevated pCO_2 by 60% in *P. longicornis* (ANOVA $p < 0.05$, Table 4, Figures 3 and 4). Carapace $[Ca^{2+}]$:$[Mg^{2+}]$ in *P. platycheles* was not significantly affected by reduced pH/elevated pCO_2 (ANOVA $p > 0.05$, Table 4, Figures 3 and 4). Carapace $[Ca^{2+}]$:$[Sr^{2+}]$ of the two low-intertidal zone species increased under reduced pH/elevated pCO_2, by 55% in *P. manimaculis* and by 5% in *P. longicornis* (ANOVA $p < 0.05$, Table 4, Figures 3 and 4), was unchanged in the two mid-intertidal zone species, *P. cinctipes* and *P. platycheles* (ANOVA $p > 0.05$, Table 4, Figures 3 and 4).

Effect of intertidal zone location on ion concentration

Claw $[Ca^{2+}]$ and $[Mg^{2+}]$ in *P. platycheles, P. cinctipes*, and *P. manimaculis* were orders of magnitude higher than in the low-intertidal *P. longicornis* at both pH/pCO_2 levels (ANOVA

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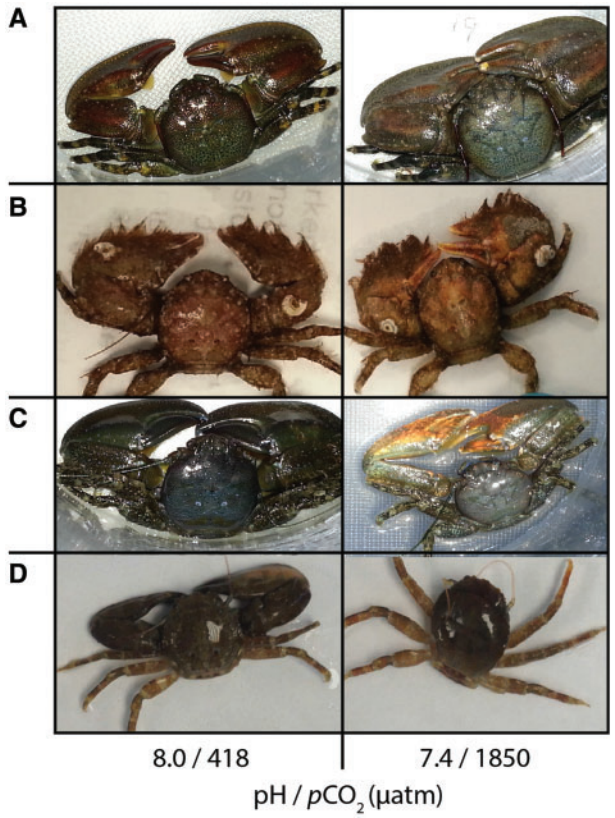

Figure 2. Coloration and condition of representative specimens of four porcelain crab species **(a)** *P. cinctipes* (mid-intertidal zone, California USA), **(b)** *P. platycheles* (id-intertidal zone, Plymouth UK), **(c)** *P. manimaculis* [lower intertidal zone, California, USA], and **(d)** *P. longicornis* (lower intertidal zone, Plymouth UK) following exposure to ambient and reduced pH/elevated pCO_2.

Table 1. Shows average concentrations of ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}) after 24 days at pH 8.0 (ambient) across four porcelain crab species, *P. cinctipes*, *P. manimaculis*, *P. platycheles*, and *P. longicornis*, in both the carapace and claw. Concentrations in mmol kg^{-1}

	Ca^{2+}	Mg^{2+}	Mn^{2+}	Sr^{2+}
Carapace				
P. cinctipes	6301	606	0.5	38.7
P. manimaculis	7191	699	0.4	44.6
P. platycheles	5244	673	7.3	24.3
P. longicornis	5804	889	1.6	30.7
Claw				
P. cinctipes	62751	6639	0.4	29.0
P. manimaculis	67787	7376	0.3	28.2
P. platycheles	55529	7720	2.7	20.0
P. longicornis	5036	768	1.4	26.8

$p < 0.05$, Supplementary Table S6, Figure 5). Neither claw $[Ca^{2+}]$:$[Mg^{2+}]$ or claw $[Ca^{2+}]$:$[Sr^{2+}]$ varied with respect to intertidal zone (ANOVA $p > 0.05$, Figure 4, Supplementary Table S7).

Carapace $[Ca^{2+}]$ of *P. platycheles* was 7% lower than *P. longicornis* at pH 8.0 (ANOVA $p < 0.05$, Table 1 and Supplementary Table S5, Figure 5) but not at pH 7.4 (ANOVA $p > 0.05$, Supplementary Table S5, Figure 5). Carapace $[Mg^{2+}]$ in the mid-intertidal *P. cinctipes* at pH 7.4 was 12% lower than in the low-intertidal *P. manimaculis*, and 35% lower at pH 8.0 (ANOVA $p < 0.05$, Supplementary Table S5, Figure 5). Carapace $[Ca^{2+}]$:$[Mg^{2+}]$ was not significantly impacted by location in the intertidal (ANOVA $p > 0.05$, Supplementary Table S7). Carapace $[Sr^{2+}]$ did not vary with respect to intertidal zonation (ANOVA $p > 0.05$, Supplementary Table S5, Figure 5); however, claw $[Ca^{2+}]$:$[Sr^{2+}]$ was higher in the mid-intertidal crabs (ANOVA $p < 0.05$, Supplementary Table S7, Figure 5).

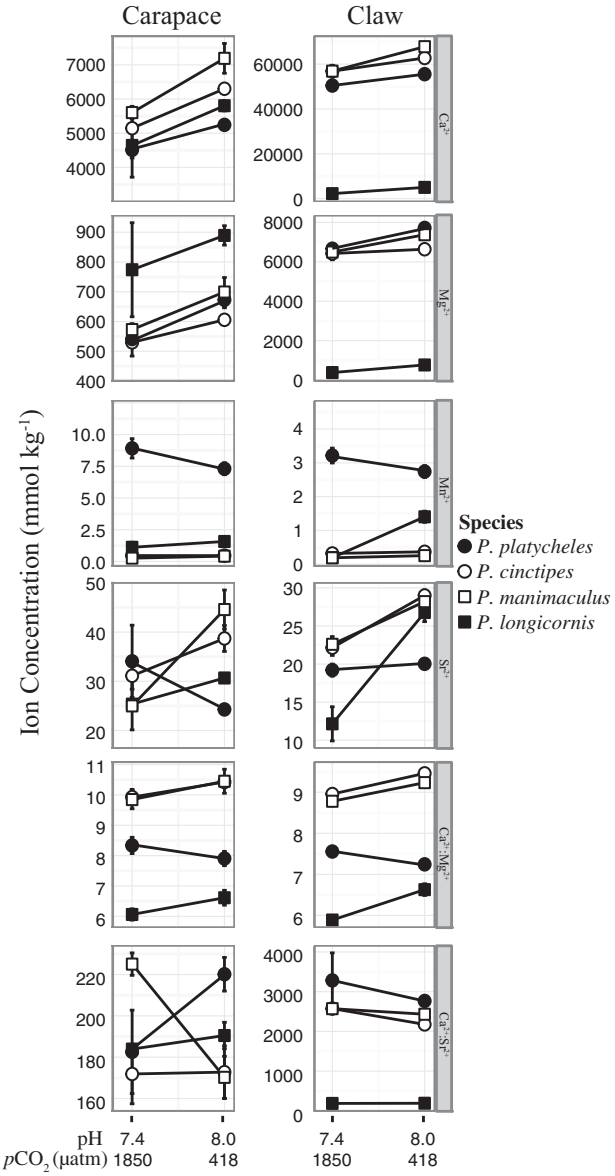

Figure 3. The response of cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}) concentrations and ratio ($[Ca^{2+}]$:$[Mg^{2+}]$ and $[Ca^{2+}]$:$[Sr^{2+}]$) within the carapace and claws of porcelain crabs was variable across species with reduced pH/high CO_2. Each point represents the mean ± SEM for $n = 4$–15 individuals *per* species *per* treatment at each pH. Circle symbols are for crabs found in the mid intertidal zone, and squares for crabs in the low zone. Black symbols are for crabs from the UK and white symbols for crabs from California. Columns are split into carapace or claw. Statistical analyses are presented in Tables 2–4.

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Table 2. Results from a linear regression model conducted on each ion and the effect of pH on these ions from pH 8.0 to pH 7.4

Species	Ion			
	Ca^{2+}	Mg^{2+}	Mn^{2+}	Sr^{2+}
Carapace				
P. cinctipes	−*	−	=	−
P. manimaculis	−*	−*	=	−*
P. platycheles	−*	−*	+*	+/−
P. longicornis	−*	−	−	−
Claw				
P. cinctipes	−	−	=	−*
P. manimaculis	−*	−*	=	−*
P. platycheles	−	−	+*	=
P. longicornis	−*	−*	−	−*

Increases in ion due to lowered pH are symbolized by '+', decreases by '−', no change by '=' and statistically significant changes by '*'. Full statistical results can be found in Supplementary Tables S5 and S6.

Table 3. Results from an ANCOVA applied to linear regression from Table 2 (see Supplementary Tables S5 & S6), testing the significance of slopes found in Figure 3

	d.f.	*F*	Sig.
Carapace			
Ca^{2+}			
pH	1	85.0	***
Species	3	13.5	***
Residuals	108		
Mg^{2+}			
pH	1	66.2	***
Species	3	16.7	***
Residuals	108		
Mn^{2+}			
pH	1	90.7	***
Species	3	95.2	***
Residuals	108		
Sr^{2+}			
pH	1	9.4	**
Species	3	0.1	n.s.
Residuals	108		
Claw			
Ca^{2+}			
pH	1	9.9	**
Species	3	272.8	***
Residuals	198		
Mg^{2+}			
pH	1	19.6	***
Species	3	209.4	***
Residuals	198		
Mn^{2+}			
pH	1	39.8	***
Species	3	123.4	***
Residuals	198		
Sr^{2+}			
pH	1	59.6	***
Species	3	17.1	***
Residuals	198		

Significance p-values: *<0.05, **<0.01, and ***<0.001.

Discussion

We found that closely related porcelain crab species (congeneric and confamilial) living in different intertidal zones vary in their physiological responses to reduced pH/elevated pCO_2. We also found that the effects of reduced pH/elevated pCO_2 on exoskeletal mineralization vary between claw and carapace. Our study suggests variation in responses of closely related species to OA may be greater than appreciated, and we conclude that accurately predicting biodiversity responses to OA requires specific taxon-focused investigations.

Survival probability under ocean acidification

Because low-intertidal zone species have not evolved to tolerate the variable conditions that characterize the high to mid intertidal zone (Stillman and Somero, 1996), low-intertidal zone species could be considered more susceptible to future changes in our environment that will be brought on by global change (Sunday *et al.*, 2011). From our results, the two low-intertidal species, *P. longicornis* and *P. manimaculus*, were the most affected by reduced pH/elevated pCO_2 with 50% mortality in *P. longicornis* occurring after half the duration of the experiment. However, the two mid-intertidal species, *P. platycheles* and *P. cinctipes*, saw zero to minimal mortality at reduced pH/elevated pCO_2. The variable effects of reduced pH/elevated pCO_2 on survival across porcelain crab species was consistent with adaptation to environmental microhabitat differences from high to low-intertidal zone. Our results support the hypothesis that mid-intertidal zone species are less susceptible to OA than subtidal, or low-intertidal, species (Byrne *et al.*, 2013; Kroeker *et al.*, 2010).

Exoskeletal mineralization of claws and carapace of porcelain crabs

Reduced carapace Ca^{2+}, Mg^{2+}, and Mn^{2+}, but elevated Sr^{2+} concentrations in *P. cinctipes, P. manimaculis*, and *P. platycheles* reflect the high elasticity of carapace relative to claw exoskeletons (Maitland, 1992; Boßelmann *et al.*, 2007). Elasticity in the carapace supports greater range of movement for gill ventilation and scaphognothite movement (Maitland, 1992), whereas claws need to be more rigid and mineralized for foraging and defense efficiency (Kropp, 1981). Porcelain crabs are generally filter feeders (Kropp, 1981), but these crabs could use their claws as defense mechanisms or have large claws to ward off other individuals from invading their territory in the intertidal zone (Rypien and Palmer, 2007). In this study, *P. longicornis* had higher carapace [Ca^{2+}], and interestingly does not use its claws for defense, using instead agility and speed to avoid predation or competition (Boßelmann *et al.*, 2007), suggesting that claw mineralization of this species may be of little consequence to fitness.

Ion-specific changes in exoskeleton mineralogy under ocean acidification

Crustaceans are known to extract ions from their carapace to resist the effects of acidification (Wheatly *et al.*, 1991). Carapace [Ca^{2+}] in all species decreased with reduced pH/elevated pCO_2, suggesting that these crabs mobilized mineral from their carapace to support their acid-base regulatory function (Wheatly *et al.*, 1991). Claws remained more mineralized than carapace under reduced pH/elevated pCO_2, except in *P. longicornis*. Decreased exoskeleton [Ca^{2+}] under reduced pH/elevated pCO_2 observed in porcelain crabs is not a general response of decapod crustacea. Exoskeleton [Ca^{2+}] was unaffected by reduced pH/elevated pCO_2 in the velvet swimming crab *Necora puber* (Small *et al.*, 2010),

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Table 4. Results of ANOVAs and Tukey HSD *post-hoc* multiple comparison analyses comparing the effects of lowered pH on the $[Ca^{2+}]$ to $[Mg^{2+}]$ ratio within the carapace

Carapace $[Ca^{2+}]$:$[Mg^{2+}]$				Carapace $[Ca^{2+}]$:$[Sr^{2+}]$			
ANOVA	d.f.	*F*	Sig.	ANOVA	d.f.	*F*	Sig.
P. cinctipes * pH	1	18.2	***	*P. cinctipes* * pH	1	0.2	n.s.
Residuals	22			Residuals	22		
P. manimaculis * pH	1	10.8	**	pH	1	76.7	***
Residuals	24			Residuals	24		
P. platycheles * pH	1	3.7	n.s.	*P. platycheles* * pH	1	0.1	n.s.
Residuals	26			Residuals	26		
P. longicornis * pH	1	223	***	*P. longicornis* * pH	1	9.6	*
Residuals	6			Residuals	6		
Claw $[Ca^{2+}]$:$[Mg^{2+}]$				**Claw $[Ca^{2+}]$:$[Sr^{2+}]$**			
ANOVA	**d.f.**	***F***	**Sig.**	**ANOVA**	**d.f.**	***F***	**Sig.**
P. cinctipes * pH	1	37.3	***	*P. cinctipes* * pH	1	159	***
Residuals	46			Residuals	46		
P. manimaculis * pH	1	34.7	***	*P. manimaculis* * pH	1	82.3	***
Residuals	40			Residuals	40		
P. platycheles * pH	1	125	***	*P. platycheles* * pH	1	4.25	*
Residuals	58			Residuals	58		
P. longicornis * pH	1	939482	***	*P. longicornis* * pH	1	36277	***
Residuals	2			Residuals	2		

Significance p-values: *<0.05, **<0.01, and ***<0.001.

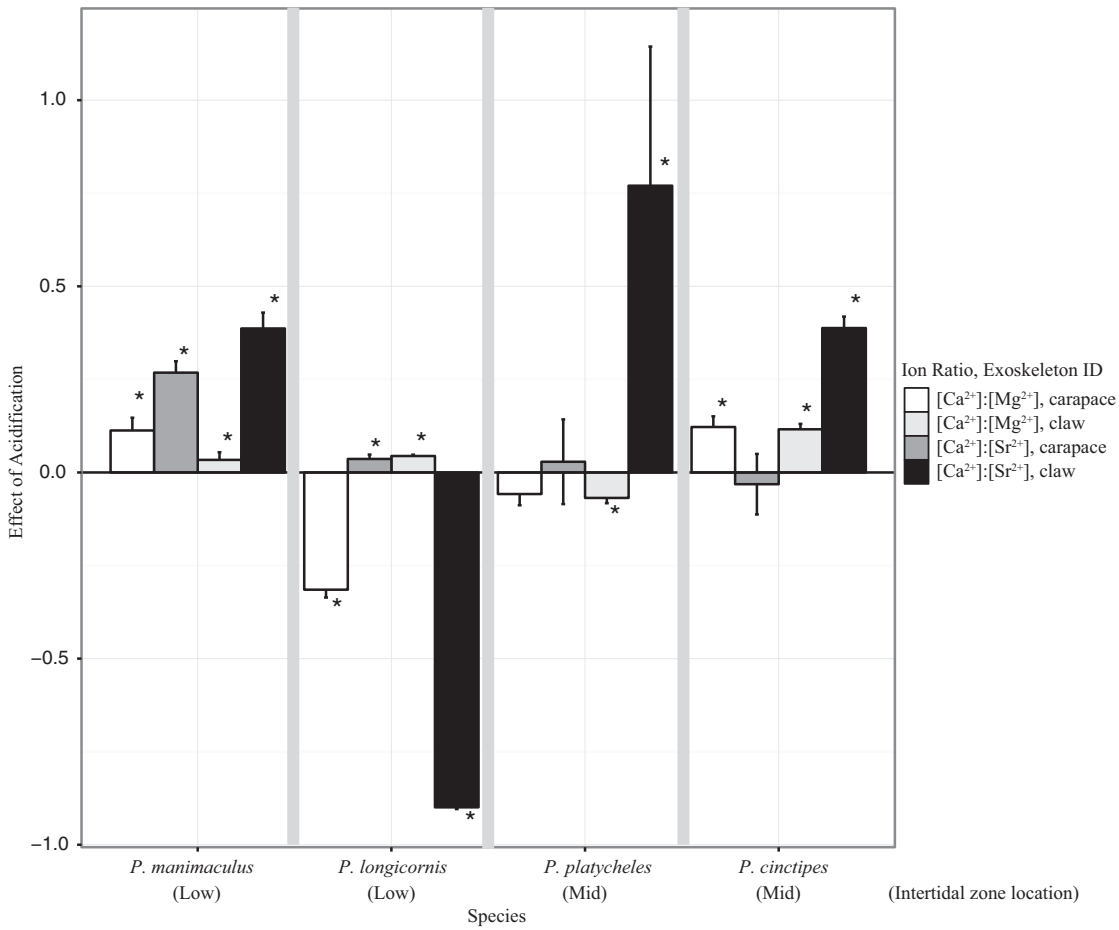

Figure 4. $[Ca^{2+}]$:$[Mg^{2+}]$ and $[Ca^{2+}]$:$[Sr^{2+}]$ in the carapace and claw of porcelain crabs responded differentially across species in response to reduced pH/elevated pCO_2. Each bar represents the mean of the ratio between average ambient $[Ca^{2+}]$:$[Mg^{2+}]$ and the treatment $[Ca^{2+}]$:$[Mg^{2+}]$ ± SEM for $n = 4$–15 individuals per species. Asterisks indicate a statistically significant difference ($p < 0.05$) in the ratio, relative to ambient conditions (~8.0 pH).

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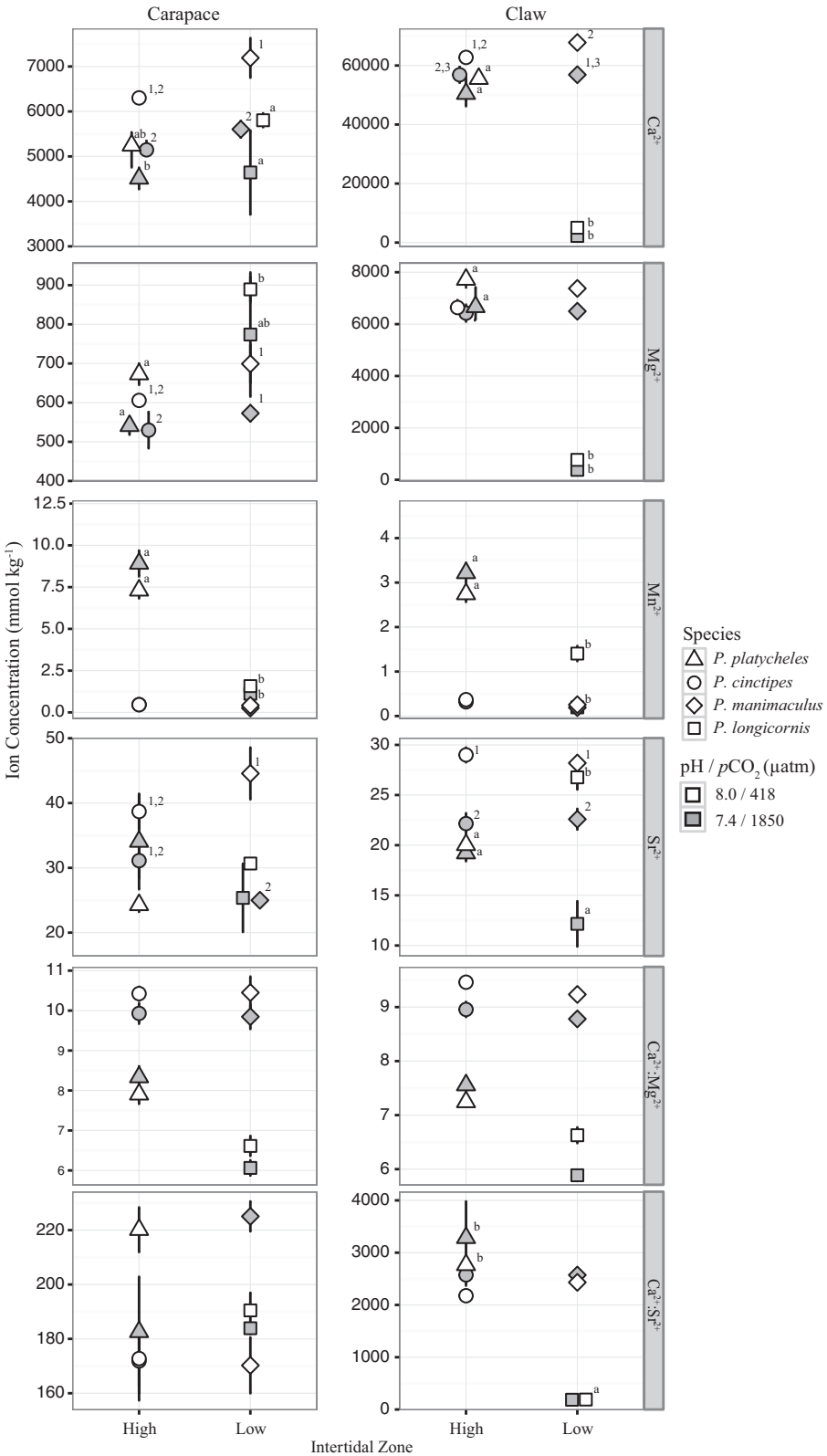

Figure 5. There was a differential response to reduced pH/elevated pCO_2 when looking across intertidal zones, high and low, and cation (Ca^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}) concentrations and ratios ([Ca^{2+}]:[Mg^{2+}] and [Ca^{2+}]:[Sr^{2+}]) within the carapace and claws of porcelain crabs. Species are divided on the x-axis by vertical distribution in the intertidal zone. Each point represents the mean ± SEM for $n = 4$–15 individuals *per* species *per* treatment at each pH. Letters above points indicate statistically significant differences ($p < 0.05$) between United Kingdom high- and low-intertidal species. Numbers above points indicate statistically significant differences ($p < 0.05$) between United States high- and low-intertidal species. ANOVA results can be found in the Supplementary Tables S5 and S6. Points were manually jittered when symbols overlapped.

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Table 5. Seawater carbonate system calculations (i.e. TA, pCO_2, $[HCO^{3-}]$, $[CO_3^{2-}]$, DIC, $\Omega_{calcite}$, and $\Omega_{aragonite}$) from the United States and United Kingdom.

Location	California USA		Plymouth, UK		
Parameter	US Ambient (pH 8.0)	US Acidified (pH 7.4)	UK Ambient (pH 8.0)	UK Acidified (pH 7.4)	UK Extreme (pH 7.0)
Salinity	34.4 ± 0.3	34.03 ± 0.09	34.1 ± 0.1	34.1 ± 0.1	34
Temperature (°C)	13.0 ± 0.3	13.0 ± 0.2	15.5 ± 0.3	15.5 ± 0.3	15.4 ± 0.1
pH_T	7.98 ± 0.01	7.41 ± 0.02	8.07 ± 0.01	7.5 ± 0.05	7.00 ± 0.02
TA (μequiv kg^{-1})	2104.4 ± 23.4	2101.3 ± 25.6	2469.7 ± 35.1	2432.8 ± 34.6	2492.4 ± 22.6
DIC*	1938.4 ± 20.3	2120.1 ± 30.8	2225.3 ± 35.4	2423.92 ± 46.7	2682.2 ± 20.2
$pCO2$ (μatm)*	430.4 ± 4.1	1816.66 ± 109.02	407.3 ± 19.8	1881.6 ± 190.5	5821.7 ± 202.1
$[HCO_3^-]$(μmol kg^{-1})*	1803.03 ± 17.80	2012.8 ± 27.8	2031.95 ± 35.1	2300.3 ± 46.3	2448.6 ± 21.3
$[CO_3^{2-}]$(μmol kg^{-1})*	118.2 ± 2.7	34.8 ± 1.4	178.3 ± 5.4	53.8 ± 8.1	17.9 ± 0.8
Ω_{ara}*	1.81 ± 0.04	0.53 ± 0.02	2.7 ± 0.1	0.83 ± 0.1	0.28 ± 0.01
Ω_{cal}*	2.83 ± 0.07	0.83 ± 0.03	4.3 ± 0.1	1.3 ± 0.2	0.01 ± 0.43

Values represent the mean ± 1 *SD*. *indicates parameters calculated using R package seacarb (version 2.4.10).

and increased under reduced pH/elevated pCO_2 in red king crabs *Paralithodes camtschaticus* (Long *et al.*, 2013).

Decreased carapace and claw $[Mg^{2+}]$, as seen under reduced pH/elevated pCO_2 across all four porcelain crab species in this study, indicates weakening of the exoskeleton (Horne and Tarsitano, 2007) and may delay rapid formation of calcified exoskeleton after a molt (Tao *et al.*, 2009), making the post-molt crabs even more vulnerable to predation. Claw, but not carapace, $[Mg^{2+}]$ has been shown to increase under reduced pH/elevated pCO_2 in *N. puber* (Small *et al.*, 2010), but the opposite was observed in porcelain crabs as these crabs likely are putting more effort into maintaining the mineralization levels within the claw than the carapace. Alternatively, it is possible that crabs are not putting more effort into maintaining mineralization in the claws, but the mineral phase in the carapace is more easily and/or quickly accessible *via* potential superficial degradation of the exoskeleton cuticle. This could ultimately result in the carapace being more soluble or easier to degrade.

Elevated $[Mn^{2+}]$ in *P. platycheles* exoskeleton could be related to the dense setae on this species. Those setae, which are typically chitinous (Goffredi *et al.*, 2008), may account for the observed higher $[Mn^{2+}]$ levels under ambient conditions, as chitin absorbs Mn^{2+} (Robinson-Lora and Brennan, 2010). Urbanization is known to increase Mn^{2+} bioavailability (Baden and Eriksson, 2006), and possibly *P. platycheles* had higher $[Mn^{2+}]$ simply because it was collected at a site near a harbor.

Intertidal zonation has previously been suggested to be an important determinant in exoskeleton ion composition (Gibbs and Bryan, 1972). That premise was not strongly supported by our data, with the only difference found between intertidal zones at one site was elevated $[Sr^{2+}]$ in *P. longicornis* (low-zone) relative to *P. platycheles* (mid-zone). Sr^{2+} may replace Ca^{2+} to defend mineralization status (de Lima *et al.*, 2011), and reduced $[Sr^{2+}]$ under OA in porcelain crabs could be detrimental. Sr^{2+} does not play a large role in biomineralized structures of crayfish (Horne *et al.*, 2009), but in mussels shell $[Sr^{2+}]$ correlated positively with growth and negatively with final size (Dodd, 1964). Whether $[Sr^{2+}]$ is correlated to size relationships and if that is important in crabs remains unknown.

Altered ion ratios under ocean acidification

Decreases in $[Ca^{2+}]$:$[Mg^{2+}]$ under reduced pH/elevated pCO_2, as was observed in the carapace of *P. longicornis* and the claw of *P. platycheles*, indicate a weaker exoskeleton prone to dissolution (Findlay *et al.*, 2010). Reduced $[Ca^{2+}]$:$[Mg^{2+}]$ under reduced pH/elevated pCO_2 was not observed across all species and exoskeletal regions, suggesting that mineralized structures are actively mobilized for acid-base regulation to counteract the effects of reduced pH/elevated pCO_2, as has been shown in urchins, bivalve molluscs and crustaceans (Calosi *et al.*, 2013a; Michaelidis *et al.*, 2005). $[Ca^{2+}]$:$[Sr^{2+}]$ could be a proxy of calcification rate (Rickaby *et al.*, 2002), and an increase in the Sr^{2+} fraction (and thus a resulting reduction of $[Ca^{2+}]$:$[Sr^{2+}]$) may aid growth, as observed in the claw of *P. longicornis* (Figure 3). *P. manimaculis*, had the greatest increase in exoskeleton $[Ca^{2+}]$:$[Sr^{2+}]$ under reduced pH/elevated pCO_2, which suggests more strongly reduced calcification under OA as compared with the other three species.

Extant levels of adaptation and sensitivity to ocean acidification

Physiological adaptation to environmental variability that characterizes coastal habitats may grant organisms some protection from the negative effects of OA (Sunday *et al.*, 2011; Engström-Öst *et al.*, 2014; Magozzi and Calosi, 2015). The largest pH-related changes in ion concentrations were observed in the low-intertidal zone species *P. manimaculis*. However, if a larger sample size of *P. longicornis* were available after the 24-day low-pH exposure (i.e. much greater starting sample size to make up for elevated mortality), we would expect a similar shift of ion concentrations in this species as well. Ion composition of claw and carapace exoskeleton, based on our data for how elevated pCO_2 induced changes in $[Ca^{2+}]$, $[Mg^{2+}]$ and $[Sr^{2+}]$, as well as organismal survival and exoskeletal dissolution, suggests that low intertidal zone species are more susceptible to OA than closely related taxa that live higher on the shore.

Conclusions

Comparisons of biomineralization responses to reduced pH/elevated pCO_2 among closely related species allows us to examine the generalizability of physiological response, how responses vary with respect to microhabitat variation, and extant levels of adaptation. Here, in four species of porcelain crabs from different intertidal zones and coastal regions we show that despite the existence of species-specific differences in responses to reduced pH/elevated pCO_2, species adapted to intertidal zone variability are in general better able to withstand the changes in pH associated with OA. Our results emphasize the importance of existing

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level of adaptation and phylogenetic considerations in informing generalized conclusions regarding physiological responses to OA.

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the article.

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