UNIVERSITY OF CALIFORNIA, SAN DIEGO

The effects of reduced pH on decorator crab morphology, physiology and behavior

A Thesis submitted in partial satisfaction of the requirements
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in

Marine Biology

by

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The Thesis of Ashley Rankin is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

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DEDICATION

This thesis is dedicated to my family and friends who have always supported and encouraged me in the pursuit of my dream to become a marine biologist. I would also like to dedicate this thesis to my advisor Dr. Jennifer Taylor, and the rest of the Taylor lab for all of the advice, ideas and cherished memories they have given me throughout this process.

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ABSTRACT OF THE THESIS

The effects of reduced pH on decorator crab morphology, physiology and behavior

by

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Crabs in the family Majoidae camouflage by decorating their exoskeletons with organisms and debris from their environment. This form of camouflage, involving both the act of decorating and carrying of these decorations, is thought to be energetically costly, and may present a trade-off under stressful environmental conditions. The energetic cost of decoration behavior has been evinced by reduced organic content due to elevated metabolism. In the context of previous research demonstrating that many marine calcifiers experience metabolic costs under experimental ocean acidification conditions,

we hypothesized that decorator crabs exposed to reduced pH will have insufficient energy to support regulatory processes along with decoration behavior. Thus, we predicted that energy will be allocated towards growth and calcification at the expense of decoration behavior. Dwarf teardrop crabs, *Pelia tumida*, were exposed to ambient (pH=8.0, pCO_2=613 µatm) and reduced (pH=7.75, pCO_2= 894 µatm) pH conditions for five weeks. Half of the animals in each treatment were given two sponge species, *Halichondria panacea* and *Haliclona permollis*, to decorate with, whereas the remaining animals were not allowed to decorate. At the end of the experiment, all animals were analyzed for exoskeleton mineral content (Ca and Mg) using EDX and ICP-MS, organic content (a proxy for metabolism) using TGA, and decoration behavior by quantifying sponge mass and percent cover. Overall, decorator crabs showed no signs of energy limitation under reduced pH conditions. Neither growth, exoskeleton mineral content, nor organic content of crabs differed among pH or decoration treatments. In addition, both sponge mass and percent cover remained the same across pH treatments, indicating no effect of reduced pH on decoration behavior, and thus the ability to camouflage. The maintenance of physiological processes without metabolic costs in *P. tumida* exposed to reduced pH radiates from the emerging trends on the susceptibility of crustaceans to changes in ocean chemistry associated with ocean acidification.

Introduction

Marine organisms showcase a remarkable assortment of mechanisms to achieve crypsis, often through morphological adaptations such as transparency, reflectance, adaptive coloration, and mimesis. Decoration camouflage, in which organisms attach material (organisms or debris) from the environment to their bodies, is both a morphological and behavioral strategy that is used by nearly 25% of metazoan phyla (Berke et al., 2006). The prevalence of decoration camouflage suggests that it is an effective strategy to avoid predation and increase survival (Hultgren & Stachowicz, 2008). Several experiments have provided evidence that decorating can reduce predation rates on crabs by as much as 50% (Berke et al., 2006; Hultgren & Stachowicz, 2008; Stachowicz & Hay, 1999; Thanh et al., 2003). Similarly, predator avoidance by the decorated sea urchin, *Sterechinus neumayeri*, was as high as 95% in the field, whereas undecorated urchins avoided predation only 30% of the time (Amsler et al., 1999). Decorations are effective because they provide visual and tactile camouflage in certain habitats (Wicksten, 1993), but also provide chemical defenses, with the use of noxious algae (Stachowicz & Hay, 1999), and mechanical defenses, with materials that detach upon predator contact (Amsler et al., 1999). Thus, decoration camouflage provides multiple channels of predator protection for marine animals; however, these benefits do not come without costs.

Decoration behavior is considered to be energetically expensive to maintain, requiring that animals expend energy to find and manipulate decorations, as well as place and carry these decorations on their bodies (Berke et al., 2006; Berke & Woodin, 2008;

Dumont et al., 2007; Kehas et al., 2005; Wicksten, 1993). Augmenting the costs of active decorating are the costs that come with developing the morphology for decoration attachment, such as setae and adhesives (Wicksten, 1993). Berke & Woodin (2008) demonstrated in a series of starvation experiments that decorated *Oregonia gracilis* crabs had consistently greater tissue catabolism, and thus higher metabolic activity, than undecorated crabs. This suggests that carrying decorations results in greater energetic costs. Increased metabolic costs have been demonstrated in a range of other animals that carry objects as well, including hermit crabs carrying mollusk shells (Herreid & Full, 1986) and ants carrying food (Lighton et al., 1993; Moll et al., 2012). The trend for decoration behavior to become reduced or absent as animals grow larger is commonly attributed to its energetic costs and trade-offs (Berke and Woodin, 2008; Hultgren & Stachowicz, 2011; Wicksten, 1993).

Despite the energetic costs, decoration behavior is especially prominent in crabs from the family Majoidae, known as decorator crabs. These crabs collect organisms, such as algae, sponge, and hydroids, from their environment and attach them to their carapace using specialized setae (Wicksten, 1976). Decorator crabs have multiple types of setae, with the hooked setae used specifically for decorating (Berke & Woodin, 2009). To decorate, the crabs grasp and tear an object with their chelae and bring it to their mouth for processing by the mouthparts. After abrading the edges of the object, the crabs fix the decorations by impaling or trapping them on or between the setae. Decorator crabs, therefore, expend significant energy in developing the morphology and behavior underlying decoration camouflage. Indeed, many species have evolved an ontogenetic

shift to lower setal density (fewer setae) and decreased decoration rates as the animal grows larger (Berke & Woodin, 2008, 2009). Once a size refuge is reached, some species reduce or stop decoration behavior altogether. This reduction in decoration behavior is hypothesized to be due to the high energetic costs involved in decorating.

Decoration behavior may be too expensive for some animals to maintain during times of stress, when homeostatic demands may be high and energy resources limited. For example, under hypoxic stress both sea urchins and decorator crabs have been observed to discard their decorations (Riedel et al., 2014). Similarly, decoration behavior (i.e., number of animals decorated and the amount of decoration) is negatively affected in sea urchins exposed to elevated temperature (Brothers & McClintock, 2015; Zhang et al., 2017). Decoration behavior is thus sensitive to animal stress and therefore might be susceptible to other environmental stressors, such as ocean acidification (OA). OA is a major concern in climate change, with a 0.3 drop in ocean pH expected by 2100 (IPCC, 2014). Simulated OA conditions have shown that many marine animals experience sub-lethal stress leading to metabolic depression (Langdon et al., 2000), reduced growth rate (Beniash et al., 2010; Michaelidis et al., 2005), reduced energy storage ability (Langenbuch & Pörtner, 2002), and changes in calcification (Langdon et al., 2000).

For marine calcifiers, the impact of OA conditions is generally negative (Kroeker et al., 2010), particularly in respect to the calcification process, though for crustaceans the responses are varied. For some species and life stages, exoskeleton calcium content decreases under reduced pH conditions (Long et al., 2013, 2016; Swiney et al., 2015), yet increases in others (Findlay et al., 2011; Ries et al., 2009; Taylor et al., 2015), or remains

unchanged (deVries et al., 2016; Findlay et al., 2010a,b; Kroeker et al., 2010; Lowder et al, 2017; Small et al., 2010). Though crustaceans possess strong internal pH regulation that may explain the maintenance of calcification, other physiological processes may be compromised (Kurihara, 2008; Melzner et al., 2009; Metzger et al., 2007). In general, stressful conditions can increase the metabolic activity of animals as their body compensates for changes in the environment and attempts to maintain homeostasis (Koehn & Bayne, 1989). With increased energy demand to cope with a stressful environment, crustaceans may not have sufficient energy to perform all of their normal physiological processes or behaviors, as evident in some species that experience reduced growth rates under OA conditions (Berge et al., 2006; Kroeker et al., 2010) and others that experience reduced swimming performance under reduced pH and increased temperature (Dissanayake & Ishimatsu, 2011).Reduced pH could be problematic for decorator crabs, which benefit from decorating behavior that is sensitive to stress and energy availability.

Given the energetic expense of decoration behavior and the potential metabolic costs of responding to OA, we hypothesized that decorator crabs would face a trade-off between growth, calcification, and decoration behavior due to energy limitations under reduced pH conditions. We predicted that decorated crabs would have higher energetic costs than undecorated crabs, and that crabs in reduced pH would have higher energetic costs than those in ambient pH, resulting in decreased decoration behavior. We tested our hypothesis and predictions using the decorator crab, *Pelia Tumida*, also known as the dwarf teardrop crab. This species can be found along the coast of California in intertidal

and subtidal waters (Bisby et al., 2005). It lacks the ontogenetic shift in decoration behavior that is seen in other species, most likely due to its small size (adult carapace width: 10-15 mm; Berke & Woodin, 2008). Thus, this species experiences the energetic cost of decorating throughout its lifetime. Here we exposed *P. tumida* to ambient (pH = 8.0, pCO_2 = 613 μatm) and reduced pH (pH = 7.75, pCO_2 = 894 μatm) conditions for five weeks, after which growth, exoskeleton calcification, organic content, and decoration behavior were analyzed to determine the effects of reduced pH on animal physiology and behavior.

Materials and Methods

1) Animal collection and maintenance

Forty-eight decorator crabs of the species *Pelia tumida* were collected from the pier pilings of Scripps Pier, Scripps Institution of Oceanography (SIO), San Diego, CA and housed in the Hubbs Hall experimental aquarium. Crabs were held in individual tanks receiving flow-through seawater for up to 3 months until the start of the experiment. Prior to and throughout the experiment, crabs were fed an equal diet of Tilapia pieces (cut to 1cm x 3 cm in size) 3 times a week, with excess food removed after 24 hours.

2) Experimental design and maintenance

The experimental OA system consisted of two large header tanks (60.5 L) that each received filtered seawater pumped in from the SIO Pier at ambient pH_{SWS} (7.99 ± 0.03), pCO_2 (894 ± 65.9 μatm), temperature (19.3 ± 2.6 °C), and salinity (33.5 ± 0.1 PSU) (mean ± s.d. during the experimental period). One header tank was maintained at ambient pH while the second header tank was adjusted for reduced pH_{SWS} (7.75 ± 0.02). Target pH was selected based on current predictions for decreased ocean surface pH of 0.3 pH units by the year 2100 (IPCC, 2014). Reduced pH was accomplished by bubbling in 100% CO_2 into the treatment header tank, which was controlled by an Apex Lite aquarium controller (pH accuracy 0.01; Neptune Systems, Morgan Hill, CA, USA). Both pH and temperature of the header tanks were continuously monitored with the Apex controller and data logged every 20 minutes.

Each header tank supplied flow-through seawater to 24 randomly placed experimental plastic cups (32 oz.) that housed individual crabs, for a combined total of 48 individuals. A small inert rock was placed in each cup for crabs to crawl on. Crabs ranged from 6 mm to 14 mm in carapace width and were distributed semi-randomly across treatments so that each treatment had the same size range. Experimental pH was gradually adjusted over the course of 3 days in an attempt to minimize stress. Once the target pH was reached, the experiment was run for 5 weeks. Crabs were checked for molts and deaths daily, with exuviae promptly removed.

3) Water chemistry

Daily readings of pH and temperature were taken from each header tank and experimental cup using a portable probe (HQ40d, probe PHC201, accuracy 0.01 pH, 0.01°C temperature, Hach, Loveland, CO, USA). All pH probes were calibrated weekly using NBS buffer solutions. Water samples were also taken from each header tank and a subset of experimental cups at the beginning (1 sample from each pH treatment) and end (2 samples from each pH treatment) of the experiment in accordance with standard operating procedures (Dickson et al., 2007). Water samples were submitted to the Dickson laboratory at SIO for analysis of pH_{SWS}, density-based salinity, and total alkalinity (TA) (Table 1). Carbonate and aragonite saturation states, concentrations of carbonate and bicarbonate, and pCO_2 were calculated using CO_2Sys 2.01 (Table 1) (Pierrot et al., 2006). For calculations, dissociation constants of K_1 and K_2 were from Mehrback et al (1973), refit by Dickson and Millero (1987). The HSO_4 constant was from Dickson (1990), the $[B\}_T$ value was from Uppstrom (1974), and the seawater pH scale

was used. Water samples were used to calculate the average difference between the Hach pH probe readings and spectrophotometric pH values. Corrected daily measures of pH from the Hach probe were then averaged for the experimental cups in each treatment (Table 1).

4) Decoration behavior

Just prior to the start of the experiment, crabs were carefully cleaned of all decorations under a dissecting microscope using tweezers and a probe. Caution was taken to minimize damage to setae. Animals were then patted dry using Kim wipes, measured for carapace width and length using digital calipers, and weighed on a balance (RADWAG PS 3500/C/2, RADWAG, Radom, Poland).

Within each experimental treatment (ambient and reduced pH), 12 of the crabs were allowed to decorate (decorated crabs) while the other 12 crabs were not allowed to decorate (undecorated crabs). Decorations were given each week only to the decorated crabs in each treatment. *P. tumida* primarily uses sponge for decoration, but it also covers with red algae, bryozoans, and hydroids (pers. Observ.). As most animals were thoroughly covered in sponge at the time of collection, this was the decoration chosen for the experiment. Two sponge species, *Halichondria panacea* and *Haliclona permollis,* were collected from the Scripps Pier flume and held in a separate flow-through tank. Through preliminary observation, it was determined that crabs showed no preference between the two sponge species. New sponge cut to 3 cm x 3 cm x 1 cm pieces were given to each decorated crab once a week so that they could decorate *ad libitum* for 24 hours, after which time the sponge was removed. Decorator crabs typically decorate

immediately once a decoration source is provided, so this time was considered sufficient for crabs to decorate (Thanh et al., 2005; Yamaguchi et al., 2006).

Weekly photographs were taken of the dorsal carapace of each crab using an Iphone 6 camera to track decoration throughout the duration of the experiment (Figure 1). To minimize disturbance to the animals, crabs were kept in seawater while being transferred to a small container with a grid placed on the bottom for general size reference. A general assessment of crab decoration was determined from these weekly photographs by ranking as 1) partially decorated (<50% decoration cover) and 2) highly decorated (>50% decoration cover). To equalize handling stress among treatments, undecorated crabs were sham-handled each time that the decorated crabs were photographed, using the same protocol, but with no photograph taken.

At the end of the experiment, decorations were further quantified by photographing the dorsal carapace of each crab using a HD digital camera (Leica DFC290, Buffalo Grove, 206 IL, USA) attached to a stereomicroscope (Leica M165 C, Buffalo Grove, IL, USA). These images were then analyzed using ImageJ software to calculate sponge total area (Figure 2). The total area of sponge was then divided by the carapace area to give the percent cover of sponge.

Following imaging, decorations were carefully removed from each crab, dried in a fume hood for 6 days, then weighed using a digital microbalance (Sartorius 1602 MP 6, Data Weighing Systems, Inc., Elk Grove, IL, USA). Decoration mass was then corrected for animal size by dividing the mass of sponge by the final body mass of the crab.

Decoration behavior was characterized as both the mass of sponge carried and the percent cover of sponge.

Once cleaned, crabs were measured and weighed by the procedure described above. The crabs were then euthanized by being placed in a -20° freezer for 30 minutes before being dissected for analyses of exoskeleton morphology and organic content.

5) Exoskeleton calcification

Cleaned crab carapaces were bisected so that one half could be analyzed for structure and elemental content using scanning electron microscopy (SEM) equipped with an energy dispersive x-ray spectroscope (EDX) and the other half of the carapace analyzed for elemental composition using inductively coupled plasma mass spectroscopy (ICP-MS). Carapace samples were carefully cleaned using a paintbrush and a probe to remove excess debris.

For SEM, carapace samples were fractured using forceps, dried in a critical point drier (AutoSamdri 815 Series A, Tousimis, Rockville, MD, USA), secured to a double 90° SEM mount revealing the cross-section, and sputter coated with iridium. Cross-sections of carapace samples were then examined with an ultra-high resolution SEM equipped with EDX (XL30 SFEG with Sirion column, Field Emission Incorporated, Hillsboro, OR, USA). SEM imaging was done at 10 kV acceleration voltage. An overview image of the whole cuticle cross-section along with magnified images of the epicuticle, exocuticle and endocuticle layers were taken for each animal (Figure 7). Total

cuticle thickness was averaged from 5 measurements taken from each whole cuticle image.

Mineral composition of the carapace cuticle was examined using EDX by magnifying each image so that the whole cuticle filled the screen. Spectra were collected at a 20 kV acceleration voltage and a minimum of 5,000 counts per second. A semi-quantitative analysis of all elements in the cuticle cross-section was conducted on all samples. Ca and Mg, which are key elements in cuticle mineralization, along with C, O, Na, Cl, Al, P, S were found consistently in all samples, with some samples also containing small amounts of Si and K. We focused specifically on the amount of Ca and Mg in each sample, which was calculated as the weight percent (wt %) and atomic percent (at %) relative to all detected elements, excluding the iridium coating.

For elemental trace analysis using ICP-MS, cleaned carapace samples were weighed and placed in Teflon vials for digestion with 0.5 ml of concentrated Teflon-distilled (TD) nitric acid (HNO_3) on a hotplate at 120°C for >24 h. Samples were dried down and diluted by a factor of 4000 with 2 % TD HNO_3 before being transferred to pre-cleaned centrifuge tubes for analysis. Samples were doped with an indium solution at this time to monitor instrumental drift. Measurements were done using a *ThermoScientific iCAPq* c ICP-MS (Thermo Fisher Scientific GmbH, Bremen, Germany), in standard mode. Masses of Mg and Ca were sequentially measured for 30 ratios, resulting in internal precision of <2 % (2 s.d.). Elements were corrected for total mole fraction (ambient pH/decorated $n = 10$, ambient pH/undecorated $n = 8$, reduced pH/decorated $n = 10$, reduced pH/undecorated $n = 9$). Raw data were corrected off line for instrument

background and drift. Samples were bracketed by internal standards of crab carapace (n = 2), which allowed for calculations of absolute values. The standards yielded external precision of better than 1% for Mg and Ca (2 s.d.).

6) Organic content

The metabolic state of an organism can be inferred through its overall organic content (Berke & Woodin, 2008; Brey et al., 1988; Lares & Pomory, 1998). Here, we refer to organic content as the overall mass of proteins, lipids and carbohydrates. A crab with sufficient energy resources presumably has relatively higher organic content, and thus higher lipid stores, muscle, and protein, compared to animals with higher energetic costs, which require the burning of this organic content to supply energy. Therefore, we designate animals with lower organic content as having higher energetic costs and higher metabolism (Berke & Woodin, 2008). Organic content is thus considered a proxy for metabolic activity.

After removal of the carapace, all internal organs and soft tissues, including the hepatopancreas (the primary storage organ), were carefully removed using a probe and forceps to excise all tissue from the cephalothorax. Due to the small size and improbability of separating each organ, all internal tissues were massed together for analysis. To prevent degradation of tissue, samples were placed in a petri dish and stored in a -80° C freezer for up to 50 days until analysis was performed.

Organic content of each crab was attained through thermogravimetric analysis (TGA) of the tissue sample using a SDT Q600 (TA Instruments, New Castle, DE,

USA). Samples were heated in a 9 microliter alumina crucible, from room temperature to 800℃, at a rate of 10℃/min in air. Previous studies using TGA on biological samples aided in interpretation and temperature designations of our TGA curves (Gai et al., 2013; Kebelmann et al., 2013; Kristensen, 1990). Within our sample curves, water was observed to burn off at 200° C, proteins, lipids and carbohydrates burned from 200° C to 450° C, and carbonaceous materials burned from 450° C to the end of the procedure (Figure 3). The temperature of 450° C was chosen as the end point of the organic content burn off because it was consistent with temperatures in previous studies on microalgae (476° C; Gai et al., 2013), and it was invariable throughout all samples. To attain pure organic content, the region encompassing lipids, proteins and carbohydrates was extracted by subtracting the percent organic content and percent water from the total percent change in mass, and subsequently subtracting the percent water content from the percent organic content to calculate organic content alone (Figure 4). Percent water content was also calculated because studies have shown that changes in water content are inversely related to changes in organic content, so we would therefore expect an increase in water content as metabolism increases and organic content decreases (Brown & Murphy, 1991). The use of TGA ensures that no water or carbonaceous materials impacted the determination of organic content. Measuring tissue pyrolysis via TGA was chosen over other metabolic methods, such as respirometry, because of complications with using live decoration, as also noted by Berke and Woodin (2008). Caloric intake was not used due to difficulty in collecting and measuring the debris of unconsumed food.

7) Statistical analyses

All statistical analyses were performed in R Version 3.2.3 (R Core Team, 2015). All water parameters, growth, mineralization, decoration and organic content data were tested for normality using a Shapiro-Wilk test and for homogeneity using Bartlett's test. Kruskal-Wallis tests were used to compare most data across treatments due to lack of normality. Crab mass was log transformed and used to calculate percent growth. Due to the lack of molting during the experiment, growth in carapace width was not expected and therefore not presented here. Weekly decoration categorically ranked data were analyzed with a Wilcoxon rank sum test. One- way ANOVA was used to analyze differences in decorating behavior (sponge mass and percent cover) between sexes, as well as the ICP-MS Ca content; Mg content data were not normally distributed and thus a Kruskal-Wallis was used. All data are reported as mean ± s.d.

Content from the materials and methods section of this thesis is being prepared for publication in 2017. Rankin, Ashley, Seo, Kyungah, Graeve, Olivia, Taylor, Jennifer R. A. (2017). The effects of reduced pH on decorator crab morphology, physiology and behavior. The thesis author was the primary investigator and author of this paper.

Results

1) Water chemistry

Experimental pH was stable throughout the experiment for both ambient pH_{SW} (adjusted Hach pH readings: 7.99 ± 0.03) and reduced pH_{SW} (adjusted Hach probe readings: 7.75 ± 0.02 treatments. Temperature varied throughout the experiment (17.3 °C – 23.7 °C), but was not significantly different between treatments (ambient pH: 19.3 ± 2.6 °C, n = 1102; reduced pH: 19.1 ± 2.4 °C, n = 1084) (Kruskal-Wallis, df = 1, p = 0.18).

2) Survival and growth

Similar levels of mortality occurred among treatments, with 4 deaths in ambient pH/undecorated, 2 in ambient pH/decorated, 3 in reduced pH/undecorated, and 3 in the reduced pH/decorated treatment. These deaths spanned the duration of the experiment and could not be attributed to any specific factor. The experiment was ended after five weeks to prevent any further mortality. Over this time, only 2 animals molted (one from each of the undecorated treatments), thereby making linear growth (carapace width) analysis irrelevant. Intermolt growth in mass occurred with log percent growth in in mass not being significantly different between treatments (Kruskal-Wallis, df = 3, n= 32, p = 0.46) (Figure 3).

3) Decoration behavior

Neither measure of decoration behavior (sponge percent cover and mass) differed among treatments (Figure 6). Weekly measures of percent sponge cover showed no trends within (Pearson's Chi-squared test, week 1: p = 0.84; week 2: p = 1.0; week 3: p = 0.65; week 4: p = 1.0) or across treatments (Wilcoxon test, W = 32.5, p = 0.50), and the final percent cover was also not significantly different between the decorated ambient and reduced pH treatments (ambient pH/decorated: 35.3 ± 25.6%; reduced pH/decorated: 44.5 ± 23.9%) (Kruskal-Wallis, df = 1, n = 18, p = 0.87) (Table 2). The mean mass of the sponge carried by individuals at the end of the experiment was not significantly different between ambient and reduced pH treatments (ambient pH/decorated: 0.013 ± 0.006 g; reduced pH/decorated: 0.017 ± 0.008 g) (Kruskal-Wallis, df = 1, n =18, p = 0.93) (Figure 6). Further, sponge mass and percent cover did not differ between sexes (decoration mass: one-way ANOVA: P = 0.07, $F_{1,17}$ = 3.92, n = 18, percent cover: one-way ANOVA: P = 0.76, $F_{1,17}$ = 0.09, n = 18). Though the undecorated animals were not given sponge to decorate with, some crabs had small amounts of sponge on their carapace at the end of the experiment (ambient pH/undecorated: 0.0014 ± 0.0025 g (n =2), reduced pH/undecorated: 0.0031 ± 0.0035 g (n = 5)). This sponge is most likely due to regrowth of sponge that was not removed during the initial cleaning process.

4) Exoskeleton structure and composition

There were no visible differences in the structure of the carapace cuticle between treatments (Figure 7). Total cuticle thickness of the carapace did not differ between pH or decoration treatments (ambient pH/decorated: 0.014 ± 0.004 mm; ambient

pH/undecorated: 0.013 ± 0.003 mm; reduced pH/decorated: 0.012 ± 0.001 mm; reduced pH/undecorated: 0.012 ± 0.002 mm) (Kruskal-Wallis, df = 3, *n* =34, p = 0.87).

Elemental analysis of the carapace cuticle with EDX showed no significant difference in either wt% Ca (ambient pH/decorated: 39.15 ± 6.1%; ambient pH/ undecorated: 34.25 ± 3.1%; reduced pH/decorated: 35.57 ± 4.5%; reduced pH/undecorated: 34.97 ± 6.1%) (Kruskal-Wallis, df = 3, *n* = 33, p = 0.16) or wt% Mg (ambient pH/decorated: 2.53 ± 0.4%; ambient pH/ undecorated: 2.83 ± 0.5%; reduced pH/decorated: 2.98 ± 0.5%; reduced pH/undecorated: 2.70 ± 0.6%) (Kruskal-Wallis, df = 3, *n* = 34, p = 0.26) (Figure 8). ICP-MS analysis of the mean concentration of Ca in the carapace cuticle confirmed no difference among treatments (ambient pH/decorated: 6.08 ± 0.50 μmol mg^{-1}; ambient pH/ undecorated: 6.22 ± 0.37 μmol mg^{-1}; reduced pH/decorated: 6.13 ± 0.25 μmol mg^{-1}; reduced pH/undecorated: 6.49 ± 0.54 μmol mg^{-1}) (Kruskal-Wallis, df = 3, *n* = 37, p = 0.12) (Figure 9). Likewise, the mean concentration of Mg did not differ among treatments (ambient pH/decorated: 0.60 ± 0.07 μmol mg^{-1}; ambient pH/ undecorated: 0.59 ± 0.05 μmol mg^{-1}; reduced pH/decorated: 0.60 ± 0.06 μmol mg^{-1}; reduced pH/undecorated: 0.56 ± 0.05 μmol mg^{-1}) (Kruskal-Wallis, df = 3, *n* = 37, p = 0.31) (Figure 9).

5) Organic content

Percent total organic content of crab soft tissue did not differ between pH or decoration treatments (ambient pH/decorated: 12.05 ± 3.0%; ambient pH/undecorated: 9.86 ± 1.7%; reduced pH/decorated: 11.21 ± 3.0%; reduced pH/undecorated: 10.93 ±

2.7%) (Kruskal-Wallis, df = 3, n = 34, p = 0.56) (Figure 10). The percent of total water content supported this result, also showing no significant differences between pH and decoration treatments (ambient pH/decorated: 24.03 ± 4.4%; ambient pH/undecorated: 21.76 ± 3.4%; reduced pH/decorated: 23.32 ± 4.8%; reduced pH/undecorated: 22.43 ± 4.3%) (Kruskal-Wallis, df = 3, n = 34, p = 0.65).

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Discussion

Decoration behavior provides protective benefits for decorator crab species, but the energetic costs associated with this behavior may make it difficult to maintain under potentially stressful environmental conditions like ocean acidification. The present study investigated the decorator crab *Pelia tumida's* energetic availability and decorating behavior under the reduced pH conditions associated with near-term ocean acidification. Results show that mineralization (Ca and Mg content), energetic availability (percent organic content), and decorating behavior (sponge percent cover and sponge mass) were unaffected by the reduced pH conditions of this experiment, suggesting that this species of decorator crab maintains multiple biological processes while compensating for decreases in external pH.

1) Decorator crab tolerance to OA conditions

The decorator crab *P. tumida* shows tolerance to medium-term exposure to pH levels that mimic forecasted changes in ocean chemistry by the year 2100 (IPCC, 2014). Such experimental conditions are sufficient to elicit a variety of detectable responses in other crustacean species, including changes to survival, metabolism, and calcification (Kroeker et al., 2010; Kurihara et al., 2008; Long et al., 2013), but not for any of the metrics measured in this study on *P. tumida*. In comparison, another decorating majoid crab, *Hyas araneus*, showed upregulation of genes related to metabolism, acid-base balance, and stress in comparable pH conditions (pH = 7.81, pCO_2 = 991 μatm) (Harms et al., 2014), highlighting the species-specific responses so far observed in crustaceans.

The contrasting responses of *H. araneus* and *P. tumida* to reduced pH may also be related to differences in habitat and geographic distribution. *H. araneus* is an arctic species that lives subtidally, whereas *P. tumida* is a temperate species that spans subtidal and intertidal zones. Arctic species are generally expected to be less equipped to deal with ocean parameter perturbations (Fabry et al., 2008). In contrast, the coastal waters off California are thought to be naturally stressful because of changes in temperature and calcite saturation states that result from upwelling events (Feely et al., 2008; Gruber et al., 2012). The pH at the collection site for *P. tumida* can fluctuate from 7.9 to 8.13 over the course of 18 months (data from Kram, S. L., Takeshita, Y., Dickson, A., Martz, T., & Smith, J. E. Scripps Ocean Acidification Real-time (SOAR) Dataset, Scripps Institution of Oceanography). Though these crabs are adapted to live in a variable pH environment, forecasted changes in ocean pH may overextend their physiological tolerance limits, resulting in detectable responses as observed in intertidal porcelain crabs (Paganini et al., 2014) and barnacles (Findlay et al, 2010b). The experimental pH used in this study on *P. tumida* was below its natural range, yet insufficient to elicit any detectable responses, at least over the time-scale of this experiment. *P. tumida* likely has a broad physiological tolerance range similar to that of mantis shrimp, which experience no stress at pH values far below what they encounter in nature (deVries at al., 2016). The mechanisms underlying broad tolerance in crustacean species is yet to be characterized, but would be valuable for assessing species susceptibility to ocean acidification conditions.

2) Non-treatment related mortality

Significant mortality occurred during the experiment, but was not related to treatment conditions. A total of 12 crabs (25%) died over the course of the experiment. These deaths were distributed among treatments and crab body size, ruling out treatment and crab age as potential factors. No detectable changes in behavior or indication of infection, disease, or stress were observed in any of the animals and none of the deaths were associated with molting. Furthermore, water flow and food supply remained sufficient and consistent for the duration of the experiment. Preliminary work showed that *P. tumida* survived well in this experimental system for longer than 3 months, so the mortality during this experiment remains unexplained. It is possible that mortality was related to when individuals were collected, as collection took place over three months prior to the experiment. Animals collected from the same location but at different time points may have been exposed to different conditions in nature or the aquarium facility that could have affected their adjustment to the experimental conditions.

3) Exoskeleton mineralization is uncompromised

The calcification process in crustaceans, as opposed to most other marine calcifiers, is protected from changes in ocean carbon chemistry through the epicuticle barrier that separates the site of calcification from the external environment along with the acid-base regulatory system that maintains internal pH (Pörtner, 2008; Wheatly & Henry, 1992). These factors may explain why *P. tumida* in this study, like a variety of other crustacean species [barnacles (Findlay et al., 2010a), crabs (Long et al., 2013; Small et al., 2010), grass shrimp (Lowder et al., 2017)], exhibits no net change in calcification under reduced pH conditions. The carapace exoskeleton of *P. tumida* showed no changes

in calcium or magnesium content under reduced pH conditions according to either method of elemental analysis used (EDX and ICP-MS).

Germane to these results is the absence of molting during this experiment; only two crabs molted and therefore molt-related calcification did not take place. Calcification peaks following molting, when new cuticle is formed and hardened through cross-linking and deposition of calcium carbonate. It is therefore not surprising that no changes in mineral content were observed, because all animals used in this experiment were in the intermolt stage. Though minimal compared to post-molt calcification, there is still a flux of calcium across the epithelium and a net uptake of calcium in the cuticle that takes place during the intermolt stage (Dall, 1965; Roer, 1980). For intermolt mantis shrimp, *Neogonodactylus bredini,* calcium content of the cuticle continued to increase up to 6 months following molting, indicating long-term accretion (deVries et al., 2016). Our data show that intermolt calcification and maintenance processes were unaffected by reduced pH conditions on the timescale of this experiment, though this may not be the case for long-term exposure.

Like most crustacean OA studies that examine calcification, we focused only on one region of the exoskeleton. Due to the small size of *P. tumida*, we only sampled the carapace, but it is possible that other regions of the crab exoskeleton are more sensitive to reduced pH conditions. In mantis shrimp, for example, changes in Mg content occurred in the merus of the raptorial appendage, but not the carapace, under reduced pH (deVries et al., 2016). This region-specific response to reduced pH was also observed in the velvet crab, *Necora puber*, which had increased Mg in the chelae, but not the carapace (Small et

al., 2010). This variable response within an individual is not surprising given that the calcification process itself is malleable, enabling localized control of mineral content for regions that support specialized functions. Inherent variation in calcification within an individual animal requires that localized measurements of mineral content from multiple exoskeleton regions be considered if accurate descriptions of the crustacean response to environmental stressors are to be made.

3) No measurable energetic costs of coping with reduced pH

Maintaining homeostasis of physiological processes under environmental stress requires additional energy for some organisms, materializing as increased metabolism and reduced growth rates. This energetic trade-off has been observed in a variety of marine calcifiers whose strong acid-base regulation maintains calcification under reduced pH conditions, but at metabolic expense (Findlay et al., 2010b; Findlay et al., 2011; Harms et al., 2014; Long et al., 2013; Portner et al., 2000; Stumpp et al., 2011; Thomsen & Melzner, 2010; Wood et al., 2008). For instance, when exposed to moderate increases in pCO_2 [991 µatm (Harms et al., 2014) and 490 µatm, 1,100 µatm, 2,400 µatm (Schiffer et al., 2013), the decorating spider crab, *Hyas araneus,* sustained net calcification, but experienced increased metabolism and stress. We predicted a similar response in the decorator crab *P. tumida,* but it showed no indication of metabolic costs, as measured by changes in wet weight and organic content, under reduced pH conditions in this study. The current study did not, however, take into account the complexity of a species' metabolic response to reduced pH. When *H. araneus* was exposed to a high pCO_2 level

of 1,960 µatm, metabolic rates decreased rather than increased (Harms et al., 2014), revealing a disparity of outcomes specific to carbon chemistry parameters Reduced metabolic rates under increased pCO_2 conditions have been observed in other crustaceans as well, includingthe velvet swimming crab, *Necora puber* (Small et al., 2010), and the prawn, *Metapenaeus joyneri* (Dissanayake & Ishimatsu, 2011). Metabolic depression is thus another potential outcome for animals exposed to environmental stress, but it is also typically associated with reduced growth rates (Beniash et al., 2010; Michaelidis et al., 2005; Whiteley, 2011). *P. tumida* showed no signs of elevated or depressed metabolism, suggesting that the pH conditions used in this experiment either did not impose a sufficient stress to affect metabolism or that the crab's energy budget already accounts for physiological energetics under stress (Koehn & Bayne, 1989).

Metabolic rates are commonly analyzed using respirometry, but measuring oxygen consumption is problematic for these small decorating crabs due to contributions from live decoration material. Here we chose to measure organic content as a proxy for energetic availability, or metabolism. Pyrolysis of tissues has been effectively used to characterize energetic availability in studies on a variety of organisms. Using the green sea urchin *Lytechinus variegatus,* Lares & Pomory (1998) investigated how organic content from different body parts are used during periods of starvation. The organs of interest (gut, body wall and gonad) were separated and then burned in a muffle furnace to obtain the organic content (weight) of each organ. Through this method, it was revealed that the gonads provided more energy storage than either the body wall or gut when subjected to long term starvation. Organic content is a reasonable measure of energetic

availability and the best way to quantify organic content is to use the ash-free dry weight (ADFW) of the tissue relative to animal size (Brey et al., 1988). It was shown in the bivalve *Laternula elliptica* that the percent AFDW decreases as oxygen consumption (measured through respirometry) increases, suggesting that the more organic content available, the lower the metabolism (Ahn & Shim, 1998). With this reasoning, the present study used TGA to determine AFDW, and therefore metabolism indirectly, by separating water content from the organic content percentage. TGA revealed no differences in organic content for animals under experimental conditions, suggesting that *P.tumida's* energetic costs are not altered by reduced pH as hypothesized and observed for a variety of other crustacean species.

4) Decoration behavior neither costly nor compromised

Surprisingly, we did not detect an energetic cost of decorating for *P. tumida*, in contradiction to expectation and previous studies on decoration behavior energetics in decorator crabs and sea urchins (Berke et al., 2006; Berke and Woodin, 2008). The cost of decoration behavior has only been measured in a few species of decorator crabs, but it is likely inconstant given the diversity of species and the interconnection of habitat, body size, ontogeny and sexual dimorphism with the evolution of decorating in this group. The energetics required for decorating are presumably unique to the individual species. For *P. tumida*, a small species that continues decorating throughout its ontogeny, the costs may be minimal. Decorator crab species that do not undergo an ontogenetic shift in decoration behavior tend to be small (5-40 mm CW; Berke & Woodin, 2008), presenting the possibility that decorating may not be as costly for smaller crabs. Yet, *Oregonia gracilis*

demonstrates a cost of decoration for both juveniles (3-6 mm) and subadults (11-18 mm) (Berke & Woodin, 2008), refuting the idea of a size refuge from decoration costs. Species may potentially minimize decoration costs by using different types and amounts of decoration material or by reducing activity and being slow-moving (Olmstead & Denno, 1992). Indeed, small decorating crabs facilitate blending in with their background by remaining still and inactive during day light hours (Wicksten, 1993).

We hypothesized that under energetically stressful conditions, decoration behavior would be reduced in order to allocate energy to other important physiological processes, as was observed in the sea urchin *Strongylocentrotus droebachiensis* (Berke et al., 2006). *P. tumida* did not, however, change its decoration behavior (percent cover and total mass of sponge) when maintained under reduced pH conditions. Decoration behavior was likely unaffected by reduced pH conditions because there were no signs of energy limitation. To study energetic trade-offs, it is important to control and monitor energy intake (Comoglio et al., 2004; Hervant et al., 1999), which is why studies on decoration behavior energetics use starvation conditions (Berke and Woodin, 2008). We chose not to introduce starvation stress in this experiment in an attempt to explore realistic responses to reduced pH conditions. *P. tumida*, like most other decorating majoid crabs, are likely not food-limited in nature because they inhabit areas with abundant background material that they consume (Wicksten, 1993).

In this study crabs were fed consistent, measured amounts of food throughout the experiment, but quantifying unconsumed food was not feasible. We therefore have no precise measure of the amount of food consumed per individual. If decorated animals in

the reduced pH treatment had higher energetic costs, but satiated this with increased feeding, it was not detectable. Additionally, some species of decorator crabs are known to use their decorations as a food cache (Kilar & Lou, 1986). Percent sponge cover in individuals was not stable throughout the duration of the experiment, indicating that some sponge may have fallen off, deteriorated, or possibly been consumed. Thus some crabs may have consumed more food than others, providing them with higher caloric intake or different food quality. The variation in decoration material over time was the same for crabs in ambient and reduced pH treatments, dispelling the possibility that decoration food cache was used to supplement energy in response to pH stress. Overall, metabolic costs may not have been detected because the decorator crabs were not energy limited in this experiment, an important factor for some species in coping with OA conditions (Melzner et al., 2011; Pansch et al., 2014; Thomsen et al., 2013).

Content from the discussion sections of this thesis is being prepared for publication in 2017. Rankin, Ashley, Seo, Kyungah, Graeve, Olivia, Taylor, Jennifer R. A. (2017). The effects of reduced pH on decorator crab morphology, physiology and behavior. The thesis author was the primary investigator and author of this paper.

Conclusions

Crustaceans exhibit responses to experimental OA conditions that vary among species, life histories, habitats, and experimental conditions, making it opportune, yet challenging, to decipher the critical aspects driving tolerance in this diverse group of animals. The current study on *P. tumida* showed no changes in morphology (Ca and Mg content), physiology (organic content; lipids, carbohydrates and proteins) or behavior

(decoration percent cover and mass) under reduced pH conditions, adding to the growing collection of crustacean species that appear to be tolerant to experimental OA conditions. This contrasts other studies focusing on energy limitation and behavioral aspects in response to reduced pH, emphasizing the need to further crustacean research to include more species as well as a wider view of possible ecological responses.

Table 1 Water temperature and carbonate chemistry parameters [mean (s.d.)]. Measurements for pH_{sws}, salinity, and total alkalinity (TA) were measured in the Dickson laboratory (n=12). Corrected pH_{sws} and temperature taken from daily Hach probe measurements (n=2188). pCO_2, HCO_3, Ω Ca and Ω Ar were calculated using CO2SYS.

Treatment	pH_{sws}	pH_{sws} Hach	pCO_2 (µatm)	Temperature (°C)	Salinity (PSU)	TA (µmol/kgSW)	HCO_3 (µmol kg^{-1})	CO_3 (µmol kg^{-1})	Ω Ca	Ω Ar
Reduced pH	7.75 (.02)	7.94 (0.004)	894 (65.8)	19.3 (2.6)	33.5 (0.1)	2225.6 (8.2)	1915.0 (76.8)	125.8 (28.1)	3.04 (0.69)	1.97 (0.46)
Ambient pH	7.99 (.03)	8.18 (0.02)	613 (108.9)	19.1 (2.4)	33.5 (0.1)	2226.3 (9.7)	2000.3 (37.3)	91.6 (11.5)	2.21 (0.28)	1.43 (0.19)

Figure 1 *Pelia tumida* individual exhibiting decorating behavior with sponge over the course of the experiment. Decorations were ranked as 1 (>50% sponge cover) and 2 (<50% sponge cover).

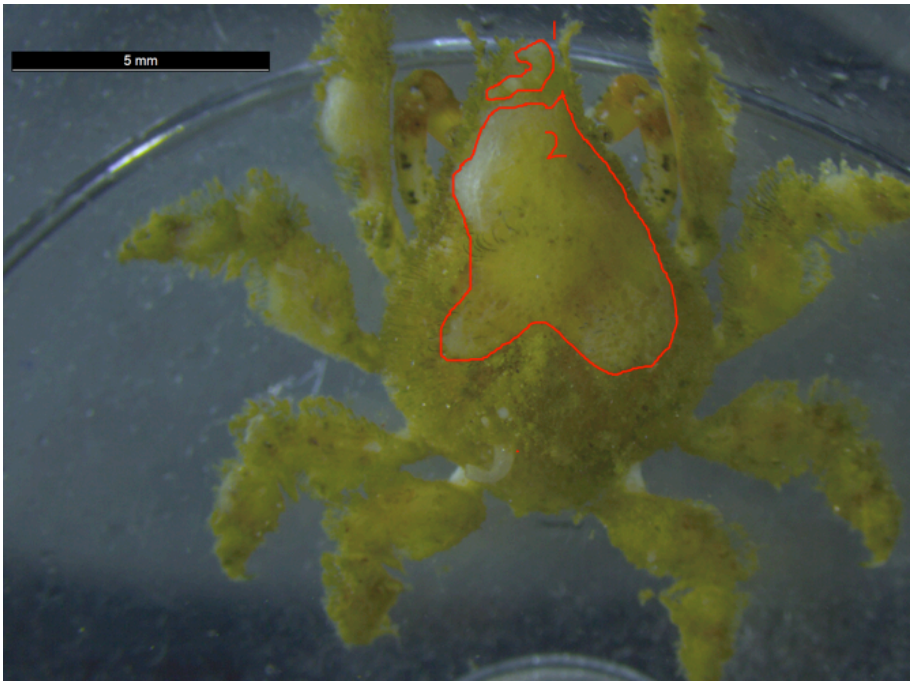

Figure 2 Decorated *Pelia tumida* under a dissecting microscope. Red outline indicates the area encompassing sponge, which was mapped through ImageJ and used to calculate the percent cover of sponge for each animal. Scale bar =5 mm.

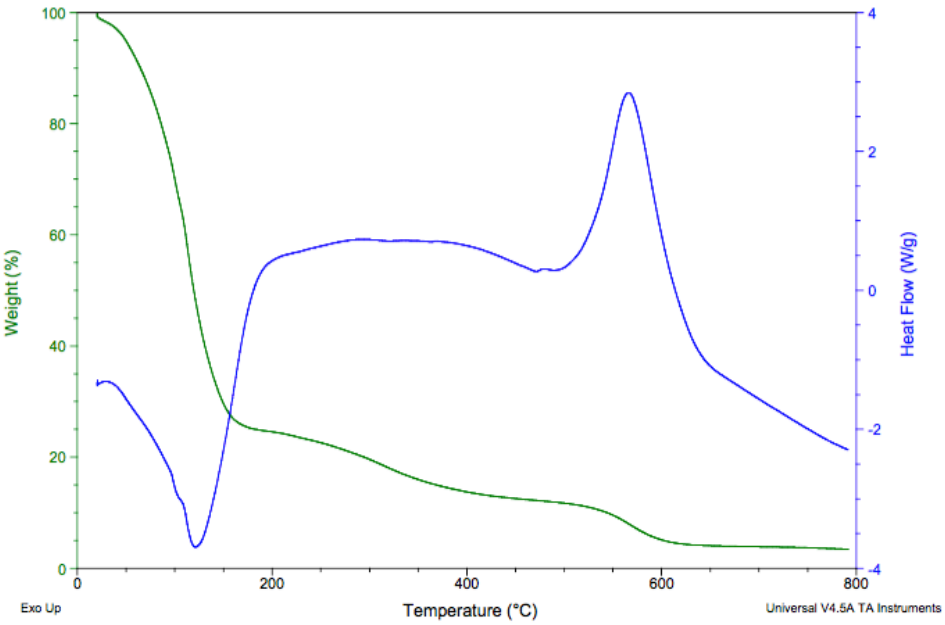

Figure 3 Example of TGA curve for *Pelia tumida* in ambient pH/ decorated treatment. The green curve represents weight percent of sample, which decreases as the tissue is burned during pyrolysis. Changes in slope indicate changes in heat flow, represented by the blue curve, indicating a change in the type of material being burned.

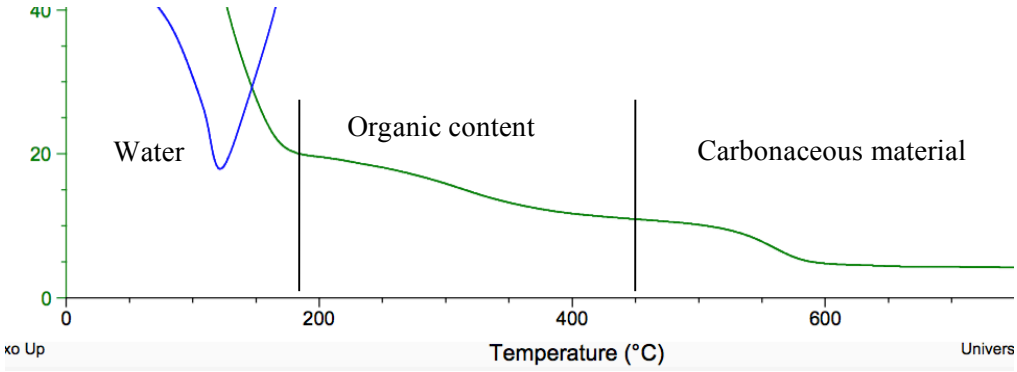

Figure 4 Representative section of TGA curve used to determine organic content. The first designation point begins where the water burn- off point ends (~200 °C), indicating the removal of all water from the tissue. The end designation point was set at 450 ° C, as determined by literature for when organic material is removed from the tissue.

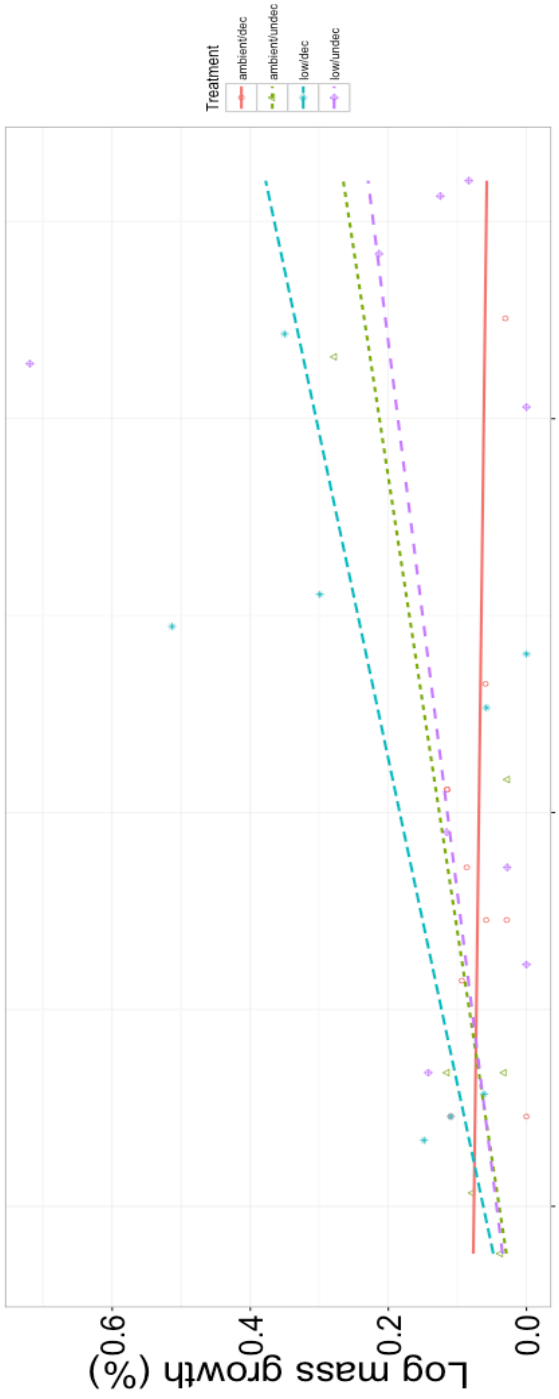

Figure 5 Crab growth. Log percent growth in mass for crabs in ambient pH/decorated, ambient pH/undecorated, reduced pH/decorated and reduced pH/undecorated treatments. Growth in mass did not differ between treatments.

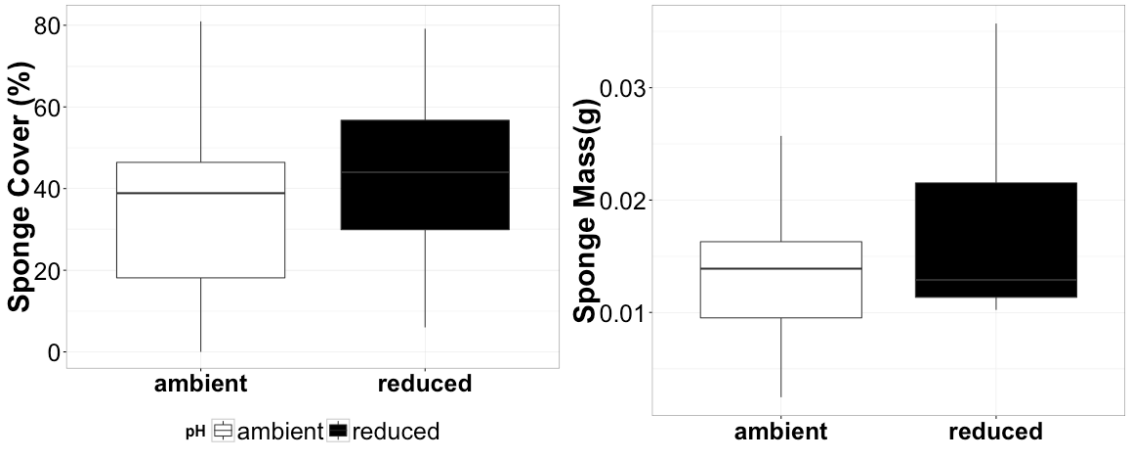

Figure 6 Sponge cover (%), calculated in ImageJ, and sponge dry weight (g) from ambient and reduced pH treatments. No significant difference was found between pH conditions.

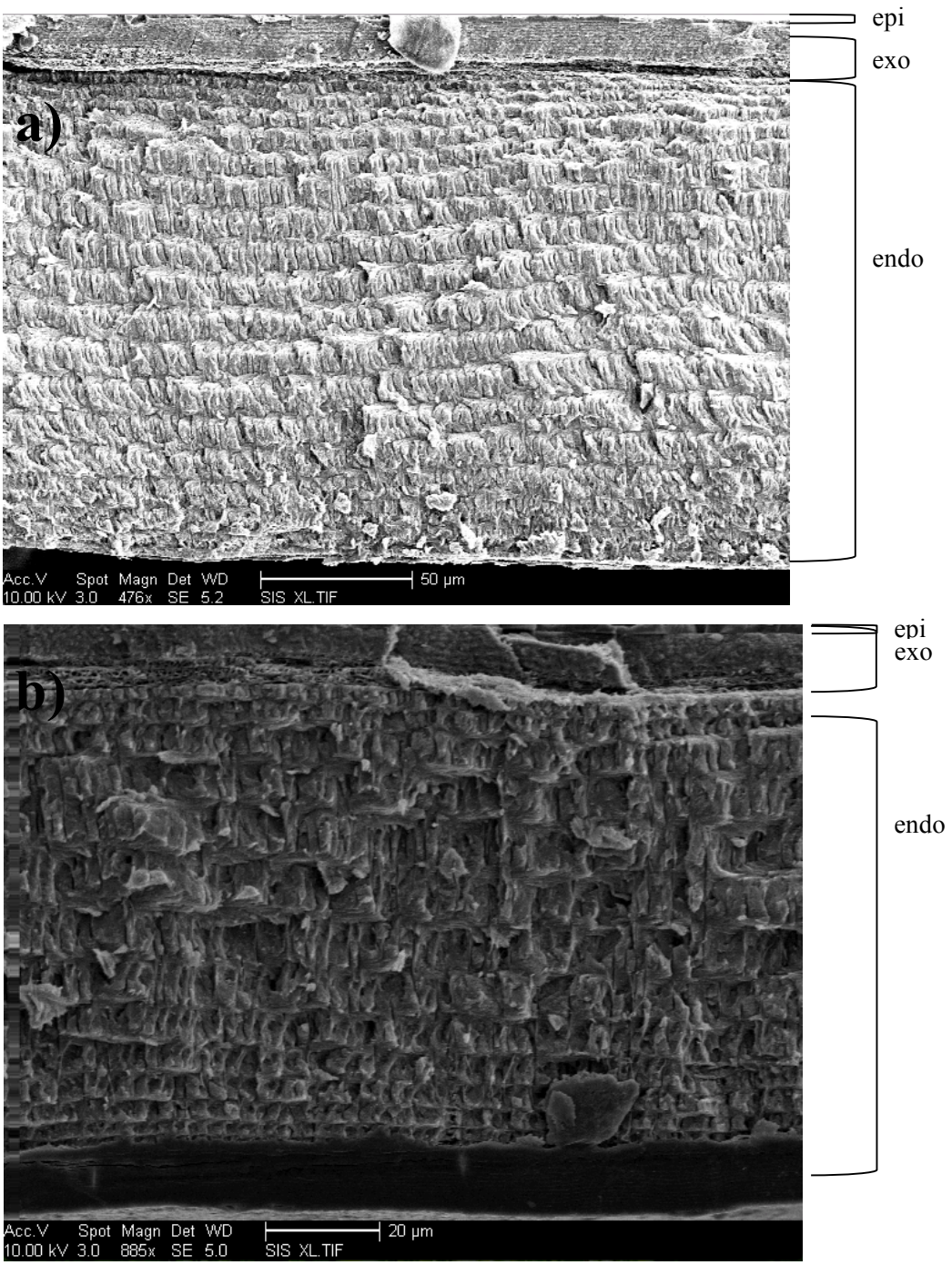

Figure 7 Scanning electron micrographs of carapace cross-section from undecorated crabs in (a) reduced pH and (b) ambient pH treatments. Cuticle layers are noted on the right, epi=epicuticle, exo= exocuticle, endo= endocuticle. Scale bar for (a) = 50 µm and (b)= 20 µm. There was no visible difference in cuticle morphology between treatments.

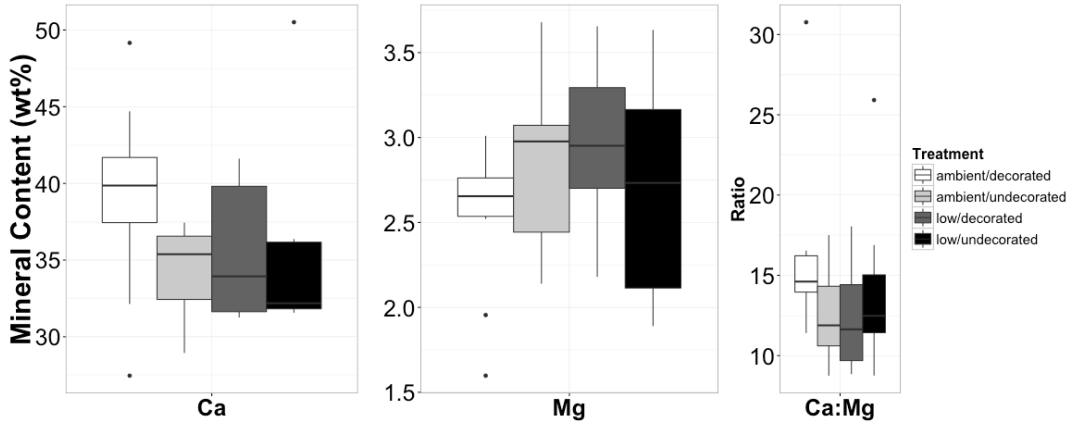

Figure 8 Mineral content, in weight percent, of the carapace cross-section as determined by EDX. (a) Ca, (b) Mg, and (c) the ratio of Ca:Mg. Neither Ca nor Mg differed between treatments.

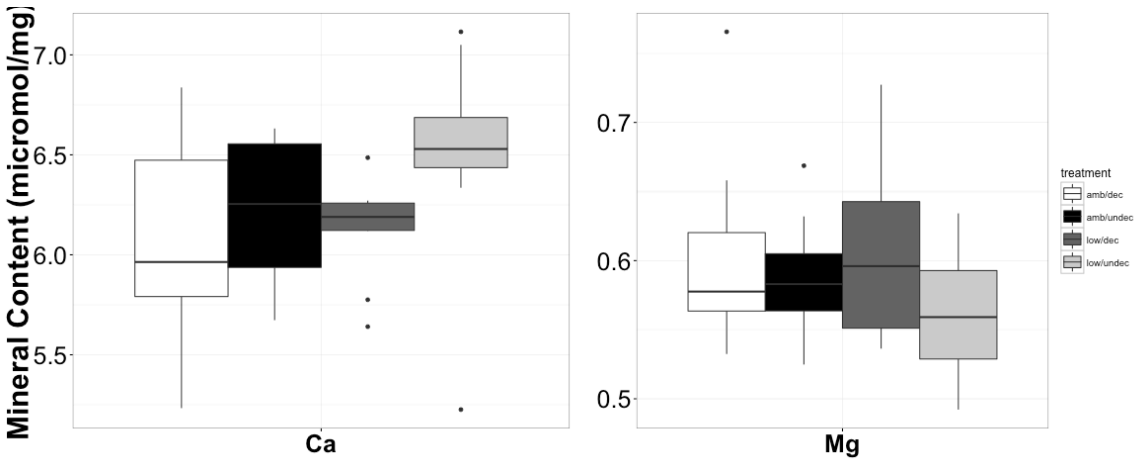

Figure 9 Mineral content, in μmol/mg, of crab carapace determined by ICP-MS. Neither (a) Ca nor (b) Mg differed between treatments.

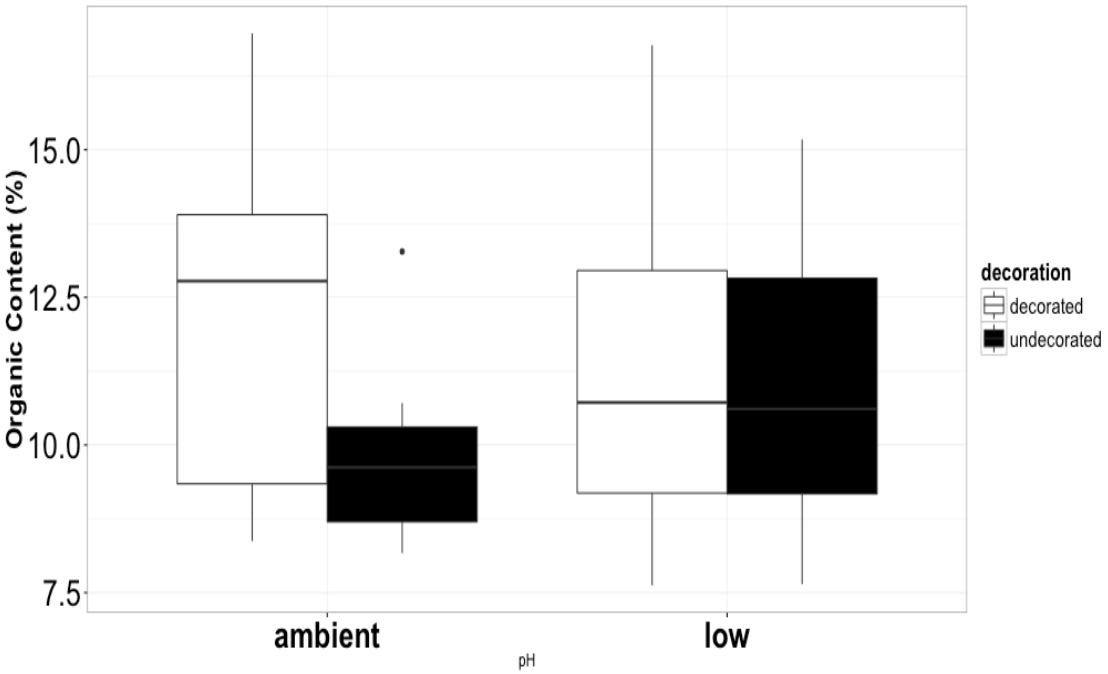

Figure 10 Total percent organic content of crabs from each treatment. Organic content includes lipids, carbohydrates and proteins and is calculated from TGA curves. There was no significant difference in crab organic content between treatments.

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