Ocean acidification leads to altered micromechanical properties of the mineralized cuticle in juvenile red and blue king crabs

William D. Coffey[a], Jessica A. Nardone[a], Aparna Yarram[a], W. Christopher Long[b], Katherine M. Swiney[b], Robert J. Foy[b], and Gary H. Dickinson[a*]

[a]Department of Biology, The College of New Jersey, 2000 Pennington Rd., Ewing, NJ 08628, USA

[b]NOAA, National Marine Fisheries Service, Alaska Fisheries Science Center, Resource Assessment and Conservation Engineering Division, Kodiak Laboratory, 301 Research Ct., Kodiak, AK 99615, USA

*Corresponding author: Gary H. Dickinson, dickinga@tcnj.edu

Abstract:

Ocean acidification (OA) adversely affects a broad range of marine calcifying organisms. Crustaceans, however, exhibit mixed responses to OA, with growth or survival negatively affected in some species, but unaffected or positively affected in others. In crustaceans, the mineralized cuticle resists mechanical loads, provides protection from the environment, and enables mobility, but little is known about how OA or interactions between OA and temperature affect its structure or function. Here, the effects of OA on the mechanics, structure, and composition of the cuticle in two Alaska king crab species was assessed. Juvenile blue king crabs

(*Paralithodes platypus*) were exposed for a year to three pH levels, 8.1 (ambient), 7.8 and 7.5. Juvenile red king crabs (*Paralithodes camtschaticus*) were exposed for ~ 6 months to two pH levels, 8.0 and 7.8, at three temperatures: ambient, ambient +2°C, and ambient +4°C. Cuticle microhardness (a measure of resistance to permanent or plastic mechanical deformation), thickness, ultrastructure, and elemental composition was assessed in two body regions, the carapace and the crushing chela (claw). In both species tested, OA reduced endocuticle microhardness in the chela, but not in the carapace. There was no effect of pH or temperature on total procuticle thickness of the chela or carapace in either species. Reductions in microhardness were not driven by reduced calcium content of the shell. In fact, calcium content was significantly elevated in the carapace of blue king crabs and in the chela of red king crabs exposed to lower than ambient pH at ambient temperature, suggesting that calcium content alone is not a sufficient proxy for mechanical properties. Reduced chela microhardness, indicative of more compliant material, could compromise the utility of crushing chelae in feeding and defense.

Keywords: ocean acidification; mechanical properties; cuticle; hardness; king crab; *Paralithodes*

1. Introduction

Within the past ~200 years, atmospheric carbon dioxide (CO_2) levels have increased from ~280 μatm prior to the Industrial Revolution to over 400 μatm today (Dlugokencky and Trans, 2016; IPCC, 2001; Raven, 2005). Projections based on "business-as-usual" emission scenarios suggest a further doubling of atmospheric CO_2 from today's levels by the end of this century (Orr et al., 2005; IPCC, 2001). Absorption of increased levels of atmospheric CO_2 by the world's oceans has and continues to reduce oceanic pH levels, a process known as ocean acidification (OA). The pH of global surface waters has dropped by 0.1 pH units since the industrial revolution and is projected to drop a further 0.3 – 0.5 pH units by the year 2100 (Caldeira and Wickett, 2003; Doney et al., 2009). The decrease in pH is likely to be extreme in high latitude waters due to increased solubility of CO_2 in colder waters and local upwelling of CO_2 rich waters (Orr et al., 2005; Mathis et al., 2015). Co-occurring with this reduction in pH is an increase in sea surface temperatures. The average temperature of global sea surface waters has already increased by ~0.4°C (Roemmich et al., 2012) since the industrial revolution and is projected to increase by an additional 2-4°C by the end of the century (IPCC, 2014).

While an increase in atmospheric CO_2 and a decrease in seawater pH appears to be inevitable, the extent to which OA will affect marine organisms, particularly in the long term (many months to years), remains an area of active investigation. Meta-analyses of OA literature have highlighted a generally large and negative effect of ocean acidification on marine organisms that build a calcified shell (Kroeker et al., 2010; Kroeker et al., 2013). When all taxa were assessed together, Kroeker et al. (2013) found a significant negative effect of OA on survival, calcification, growth, development, and abundance. When taxa were analyzed separately,

however, responses varied considerably among major groups of calcifying organisms. In particular, among crustaceans, there was not a significant average effect on survival, calcification, growth or abundance (Kroeker et al., 2013). Although some crustacean species show reduced growth upon exposure to conditions that emulate OA (Long et al., 2013a; Kurihara et al., 2008), others show no effect (Carter et al., 2013; Hauton et al., 2009; Kurihara and Ishimatsu, 2008; Small et al., 2010) or even enhanced growth under OA conditions (McDonald et al., 2009; Ries et al., 2009).

Little is known about the functional responses of decapod crustaceans to OA, specifically in terms of how OA may affect the structure and mechanical properties of the mineralized exoskeleton. OA has the potential to affect both uptake of Ca^{2+} and HCO_3^- after molting and precipitation of $CaCO_3$ within the exoskeletal compartment, which requires a pH slightly above that of the hemolymph (Whiteley, 2011). The decapod exoskeleton, or cuticle, fulfills many functions including resistance to mechanical loads (e.g. those from predators and prey items), protection from the environment (including desiccation), and structural support for mobility (Chen et al., 2008; Raabe et al., 2006). Therefore, alterations in the structural or mechanical properties of the exoskeleton due to OA may significantly affect the fitness of decapod species.

The decapod exoskeleton is multilayered, consisting of an outer epicuticle, a procuticle composed of an outer exocuticle and inner endocuticle, and a thin membranous inner layer (Travis, 1963). The mineralized exo and endocuticle are composed of chitin-protein nanofibrils grouped into fibrous bundles (Chen et al., 2008; Giraud-Guille, 1984; Raabe et al., 2005; Raabe et al., 2006). These chitin-protein bundles arrange into planes, which are stacked on top of one

another, with the direction of each plane shifted slightly with respect to the last. This regular shifting of horizontal planes results in a helicoidal "twisted plywood" or "Bouligand" structure, with each 180° turn of the helix referred to as a "Bouligand layer" (Giraud-Guille, 1984; Raabe et al., 2006). Within an individual procuticle, the thickness of Bouligand layers tends to be greater in the endocuticle than in the exocuticle, resulting in denser packing of Bouligand layers in the exocuticle (Hegdahl et al., 1977; Raabe et al., 2005; Raabe et al., 2006). Amorphous calcium carbonate or nanocrystalline magnesian calcite is embedded within the chitin-protein matrix (Boßelmann et al., 2007; Dillaman et al., 2005; Roer and Dillaman, 1984).

The goal of this study was to assess the extent to which OA alone or in combination with increased seawater temperature affects functional properties of the mineralized cuticle in two commercially harvested Alaska crab species. It was hypothesized that microhardness of the cuticle, a measure of resistance to permanent or plastic mechanical deformation, would be reduced under low pH or elevated temperature and that those changes would be driven by altered structure or reduced mineral content. To test this hypothesis, juvenile blue king crabs (*Paralithodes platypus*) were exposed for a full year to three levels of pH, an ambient level of 8.1 and reduced levels of 7.8 and 7.5 (predicted global averages in surface waters for the years ~2100 and ~2200, respectively: Caldeira and Wickett, 2003). Juvenile red king crabs (*Paralithodes camtschaticus*) were exposed for ~ 6 months to two levels of pH, an ambient level of 8.0 and a reduced level of 7.8, at three levels of temperature, ambient, ambient +2°C and ambient +4°C. In both cases, individual crabs underwent several molts during the exposure (Long et al. *2017*; Swiney et al., *2017*). Following exposures, microhardness, thickness,

ultrastructure, and elemental content was assessed in two body regions, the carapace and crushing chela.

2. Materials and methods

The animals studied in this paper came from two distinct, though conceptually similar experiments, which examined a broad range of responses of red and blue king crabs to ocean acidification and warming; those data, including survival, growth, and morphology, have been published elsewhere (Long et al., 2017, Swiney et al., 2017). The response variables reported here were opportunistically made *post hoc*. Given the similarity of the experiments, data collected on the mineralized cuticles from these experiments were combined into this paper. Although the experiments were not identical, and therefore explicit statistical comparisons between the studies cannot be done, the comparisons between the two species are informative and thus have been combined into this manuscript.

2.1 Animal collection and experimental exposure

Juvenile blue king crabs, *Paralithodes platypus,* were reared from larvae at the Alaska Fisheries Science Center's Kodiak Laboratory seawater facility in Kodiak, Alaska, as described by Long (2016), from broodstock captured in commercial pots near St. Matthew Island in the winter of 2010. Thirty juvenile blue king crabs at the first crab stage (C1) stage were randomly assigned using a random number generator to each of three pH treatments (90 crabs total) 1) ambient (8.1), 2) 7.8, or 3) 7.5. The experiment began on June 17, 2011 and was ended on June 14, 2012 (363 days).

Juvenile red king crabs, *Paralithodes camtschaticus,* were also reared from larvae at the Kodiak Laboratory seawater facility from an ovigerous female collected in Bristol Bay, Alaska, in June 2011 and shipped live to the laboratory; because only one female was used, this likely represents a limited range of genetic and phenotypic diversity compared to the Bristol Bay population as a whole. Thirty juvenile red king crabs were randomly assigned using a random number generator to one of two levels of pH (ambient (8.0) or 7.8) at one of three levels of temperature (ambient, ambient +2ºC, and ambient +4ºC). This fully-crossed design yielded six experimental treatments each with 30 crabs for a total of 180 crabs. A pH treatment lower than pH 7.8 was not included in this study because in a previous study 100% mortality was observed for young-of-the-year red king crabs exposed to pH 7.5 waters after 95 d (Long et al., 2013a). The juvenile red king crab experiment began August 5, 2012 and was ended on February 4, 2012 (184 days).

Crabs were reared in tubs (53 (L) X 38 (W) X 23 (H) cm) that was placed randomly in the experimental area and which received flow-through water at the appropriate pH from head tanks as described below. One tub was used per treatment in each experiment. Juveniles were reared in individual inserts (one crab per insert) constructed from PVC pipe 40 mm inner diameter with 750 µm mesh attached to the bottom and the inserts were placed inside the treatment tub on top of a grid that was raised off the bottom of the tubs so that the tops of the inserts were just out of the water. These inserts were large enough to ensure that neither growth nor survival would be affected (Swiney et al., 2013). Water was delivered into each insert within each tub via a submersible pump connected to a manifold. Flow rates were checked visually each day for each insert and adjusted to ensure equal flow rates (each insert had its own flow valve). Three times a week, crabs were feed *ad libitum* a gel diet of Gelly Belly (Florida Aqua Farms, Inc., Dade City,

153 FL, USA) enhanced with Cyclop-eeze powder (Argent Laboratories, Redmond, WA, USA),
154 pollock bone powder (US Department of Agriculture, Agricultural Research Service, Kodiak,
155 AK, USA), and astaxanthin. Excess food was cleaned from each insert prior to feeding. Each
156 insert was checked daily for molts and mortalities. Exuvia and mortalities were recorded and
157 removed.
158
159 *2.2 Seawater chemistry*
160 Seawater acidification followed the methods described in Long et al. (2013b). Sand filtered
161 seawater was pumped into the Kodiak Laboratory seawater facility. A 160 l tank of pH 5.5 was
162 established by bubbling CO_2 into ambient seawater. This pH 5.5 water was then mixed with
163 ambient seawater in 160 l treatment head tanks (one per pH treatment) to the nominal pH via
164 peristaltic pumps controlled by Honeywell controllers using input from Durafet III pH probes in
165 the head tanks (Honeywell, Houston, TX, USA). The ambient head tank did not receive any pH
166 5.5 water. Waters from the treatment head tanks were then supplied to the treatment tubs. Blue
167 king crabs were kept at ambient temperatures for most of the year, but the water was chilled
168 when necessary to keep the temperature below 10°C, which is within the thermal tolerance range
169 for blue king crab (Stoner et al., 2013). For the red king crab experiment, to heat the water in the
170 +2ºC and +4ºC temperature treatments a 200W submersible heater was placed in each
171 experimental treatment tub. In the coldest months of the experiment, a 100W heater was added to
172 the warmest treatments to maintain the correct temperatures. For both experiments, pH_F (free
173 scale) and temperature were measured daily, typically around 9 am, in randomly selected inserts
174 in each experimental tub (5 per treatment per day for the blue king crab and 3 per treatment per
175 day for the red king crab) using a separate Durafet III pH probe calibrated daily with a TRIS

buffer. When the pH deviated from the target pH by more than ±0.02 pH units the Honeywell controller set points were adjusted to bring the pH back to the target value. For the red king crab experiment, heater set points in the +2ºC and +4ºC temperature treatments were changed daily (when necessary) immediately after the readings were made based upon the temperature measurements in the ambient temperature tubs to maintain target treatment temperatures. To characterize carbonate chemistry, weekly water samples from the treatment head tanks were taken during both experiments, poisoned with mercuric chloride, and sent to analytic laboratories for dissolved inorganic carbon (DIC) and total alkalinity (TA) analysis. Two laboratories were used over the course of the experiment due to laboratory availability to run the samples. At the first laboratory, DIC was determined using a CM5014 Coulometer with a CM5130 Acidification Module (UIC Inc., Joliet, IL, USA) using Certified Reference Material from the Dickson Laboratory (Scripps Institution of Oceanography, La Jolla, CA) (Dickson et al., 2007). TA was measured via open cell titration according to the procedure in Dickson et al. (2007). At the second laboratory, DIC and TA were determined using a VINDTA 3C (Marianda, Kiel, Germany) coupled to a 5012 Coulometer (UIC Inc., Joliet, IL, USA) using Certified Reference Material from the Dickson Laboratory (Scripps Institution of Oceanography, La Jolla, CA) and the procedures in Dickson and Goyet (1994). Salinity was measured at the same time as TA and DIC. Non-measured parameters of the carbonate system were calculated in R (V2.14.0, Vienna, Austria) using the seacarb package with the default constants and options from the measured pH and DIC (Lavigne and Gattuse, 2011).

2.3 Micromechanical testing

198 At the conclusion of experimental exposures, surviving juvenile crabs were frozen whole and
199 shipped on dry ice via overnight mail to The College of New Jersey (Ewing, NJ) for cuticle
200 assessments. Samples were stored at -80°C immediately upon receipt until analysis
201 (approximately 8 months for blue king crabs and 5 months for red king crabs). To prepare crab
202 samples for analysis, frozen, whole crabs were lyophilized on a freeze dryer (Yamoto, DC41-A,
203 Tokyo, Japan) for at least 24 hours. For each species, all crab samples were lyophilized on the
204 same day. Two anatomical regions were tested in this study, the carapace and the larger chela
205 (i.e. the "crushing claw"). Once samples were completely dry, crabs were dissected using forceps
206 and a scalpel, separating the whole carapace and crushing chela from the rest of the body. Any
207 tissue adhering to the carapace and any loose tissue found attached to the chelae was removed
208 with forceps.
209
210 Separated whole carapace and chela samples were embedded within epoxy resin to enable
211 grinding and polishing. Polypropylene cuvette holding trays were used as embedding molds,
212 each of which contained 12, 1.5 X 1.5 X 1 cm wells. Individual carapace and chela samples were
213 affixed to the bottom of one of the wells using super glue (Loctite Control Gel), oriented in such
214 a way that grinding would reveal a cross-section of the anterior-posterior axis (Fig. 1). All
215 reagents, supplies and equipment for grinding and polishing were purchased from Allied High
216 Tech Products, Inc. (Rancho Dominguez, CA, USA) unless otherwise stated. Each well of the
217 embedding mold was filled with epoxy (EpoxySet, #145-20000), which cured at room
218 temperature for at least 24 hours. Embedded samples were ground to the midline and polished on
219 a manual grinding/polishing machine (M-Prep 5). Samples were first passed through a grinding
220 series of 180, 320, 600 and 800 grit silicon carbide paper (#50-10010, 50-10020, 50-10030 and

50-10035) and then polished using a 1 μm diamond and then a 0.04 μm colloidal silica suspension (#90-30015 and 180-25010) against a polishing cloth (#90-500-500 or 180-10550). Samples were cleaned with Micro Organic soap (#148-10000) and checked under a metallurgical microscope (Jenco, MET-233, Portland, OR, USA) after each step of the grinding/polishing process, and were re-polished if necessary until the surface of each sample was completely even and free of scratches. No etching of samples was observed during grinding or polishing. Polished samples were stored in a desiccator until testing.

Vickers hardness of the polished cuticle samples was measured using a microindentation hardness tester (Clark Instrument MHT-1, SUN-TEC, Novi, MI, USA) at 0.10 N load and 5s dwell time. Embedded samples were clamped in a specimen holder to ensure a level surface for indentation. All indents were made within the endocuticle (Fig. 1D), normal to the cross-section, and 7-8 indentations were made per sample. For the juvenile crabs used in this study, the exocuticle (Fig. 1D) was too narrow for the microhardness testing procedure. Irregular indents, defined as those where the diagonals were not perpendicular, where sliding of the indenter tip was evident, or the shape was not quadrilateral, were excluded from the dataset. Samples with less than seven successful indents were excluded from the dataset. Immediately after each indentation, the indent was imaged on the hardness tester using a microscope camera (Moticam CMOS 2.0, Richmond, BC, Canada) and the length of the two diagonals was recorded. Vickers hardness numbers (VHN) were calculated as:

$VHN = 1.854 \times (F/d^2)$ where F is the applied load and d is the mean of the two diagonals produced by indentation. The VHN of each indent within a sample were averaged to determine

the mean VHN for the sample. Sample preparation and testing followed the same procedure for blue and red king crabs.

2.4 Assessment of cuticle thickness

Following hardness testing, embedded and polished samples were imaged on a metallurgical microscope (Jenco MET-233, Portland, OR, USA) equipped with a camera (Leica EC3, Buffalo Grove, IL, USA). Imaging enabled quantification of four thickness metrics (Fig. 1D): total thickness of the mineralized procuticle (endo plus exocuticle); exocuticle thickness; endocuticle thickness; and thickness of Bouligand structures. Measurements were made on digital images, taken at 20X magnification, using the camera software's measurement tools (Leica LAS EZ, V. 3.0, Buffalo Grove, IL, USA). Endocuticle and exocuticle thickness was measured along the same transect and total procuticle thickness was calculated as the sum of endocuticle and exocuticle thickness. Bouligand thickness was determined only in the endocuticle and was calculated for each transect by counting the number of visible lines within the endocuticle and dividing endocuticle thickness by this value. Each visible line is a 180° turn of the Bouligand structure (Raabe et al., 2006). Lines could not be resolved within the exocuticle. At least 15 measurement transects were taken on each sample, randomly distributed throughout the length of the sample. For each parameter replicate measurements within a sample were averaged to determine a mean value for the sample.

2.5 SEM imaging and elemental analysis

Embedded and polished chelae and carapace samples were subjected to scanning electron microscope (SEM) imaging followed by electron dispersive spectroscopy (EDS) elemental

analysis. Samples were imaged uncoated under low vacuum (50 Pa) at a range of magnifications (~100 – 10,000 X) in blue king crabs and at 1000 X magnification in red king crabs. Imaging was conducted in back-scattered electron mode (BSE) on a Hitachi SU-5000 field emission SEM (Hitachi America, Tarrytown, NY, USA). Elemental analysis employed an EDAX EDS detector (AMTEK Materials Analysis Division, Model: Octane Plus, Mahwah, NJ, USA). For each sample, a region of interest was selected which typically included at least one of the indentations made within the endocuticle (as described above). The region of interest was imaged at 1000 X magnification with an accelerating voltage of 15 kV and a working distance of ~ 8 μm. This resulted in 5000 – 8000 counts per second for EDS. A total of eight point spectra were taken within the region of interest, spread across the endocuticle. Point spectra were not taken directly on indentations. Replicate spectra within a sample were averaged to determine the calcium and magnesium content (weight percent) for each sample.

2.6 Statistical analysis

Statistical analysis was conducted using SPSS (V. 19, IBM Analytics, Armonk, NY, USA). Outliers were calculated for all parameters in SPSS as values greater than three times the interquartile range below or above the first or third quartile, respectively, and were removed from the dataset (at most two per sample). Blue king crab data (hardness, thickness metrics, and elemental content) were analyzed using a one-way analysis of variance followed by Tukey HSD post-hoc testing. Sample size for blue king crabs was 7 – 9 individual samples per treatment per body region for hardness, 5 – 9 per treatment per body region for thickness metrics, and 4 – 7 per treatment per body region for elemental analysis. Red king crab data (hardness, thickness metrics, and elemental content) were analyzed using a general liner model (GLM), with pH and

289 temperature as fixed factors. The GLM was followed by Tukey HSD post-hoc testing. Sample
290 size for red king crabs was 3 – 13 individual samples per treatment per body region for hardness,
291 4 – 13 per treatment per body region for thickness metrics, and 4 – 10 per treatment per body
292 region for elemental analysis. All available samples were tested in each analysis. No samples
293 were available for the treatment held at pH 7.8, ambient +4°C due to 97% mortality during
294 exposure (Swiney et al., *2017*). Chelae samples for the treatment held at pH 8.0, ambient +2°C
295 were damaged during processing and not included in analyses. Normality and equal variance
296 were tested for all data using Shapiro-Wilk and Levene tests, respectively, and data were log
297 transformed if necessary to meet normality and equal variance assumptions.
298
299 **3. Results**
300 *3.1 Seawater chemistry*
301 In the blue king crab experiment, target pHs were achieved throughout the experiment, and in the
302 red king crab experiment target temperatures and pHs were achieved (Table 1). In both
303 experiments pH and temperature did not vary among inserts within a treatment within a day;
304 most frequently they were identical and when they did differ it was within measurement
305 resolution of the probe (0.01 pH units and 0.1 ºC). For both experiments, pCO_2 increased with
306 decreasing pH, DIC increased with decreasing pH, and alkalinity did not vary with treatment.
307 Aragonite was supersaturated in the ambient treatment, but undersaturated in the pH 7.8 and 7.5
308 treatments; calcite was undersaturated only in the pH 7.5 treatment in the blue king crab
309 experiment (Table 1). For blue king crabs, seasonal variability in temperature resulted in highs of
310 around 9.5°C during the summer of 2011 and lows of around 1°C in the winter of 2012. In the
311 red king crab experiment, because the individual treatment tubs were heated, pHs were slightly

lower in the warmer treatments (Table 1). Ambient temperature for red king crabs ranged from 5º to 12ºC. Salinities were very stable and averaged (± SD) 31.3 ± 0.3 for the blue king crabs and 31.0 ± 0.4 for red king crabs.

3.2 Micromechanical testing

In blue king crabs, Vickers microhardness of the carapace endocuticle did not differ significantly among treatment groups (Table 2; Fig. 2). For the chela, however, a significant effect of seawater pH was observed (Table 2; Fig. 2). Microhardness was lower in the pH 7.5 treatment compared to the ambient pH of 8.1. At all pH levels, microhardness of the chelae was higher than that of the carapaces, although the magnitude of the difference in microhardness between these regions diminished with decreasing pH (i.e. 79% higher in the chelae at pH 8.1, 37% higher at pH 7.8, 35% higher at pH 7.5).

Red king crabs were exposed to ambient (8.0) and reduced pH (7.8) at three temperatures, ambient, ambient +2°C, and ambient +4°C. Neither pH nor temperature affected Vickers hardness of the carapaces (Table 3; Fig. 3). For the chelae, however, microhardness was lower at pH 7.8 than at ambient pH of 8.0 (Table 3; Fig. 3). Temperature did not alter chelae microhardness (Table 3). As in blue king crabs, microhardness values measured in the chelae were consistently higher than those measured in the carapace, although the difference between these regions diminished with decreased pH (i.e. at ambient temperature, 110% higher in the chelae at pH 8.0, 40% higher at pH 7.8).

3.3 Assessment of cuticle thickness

335 The effect of seawater pH was not significant for most thickness metrics assessed. In blue king
336 crab carapaces, total procuticle thickness and endocuticle thickness were not affected by
337 seawater pH (Table 2; Fig. 4). Exocuticle thickness and Bouligand thickness (measured within
338 the endocuticle) both differed among treatment groups (Table 2; Fig. 4). The exocuticle was
339 thinner at a pH of 7.5 as compared to the 7.8 treatment. Bouligand structures were thinner at a
340 pH of 7.5 as compared to the 8.1 and 7.8 treatments, indicative of a denser packing of Bouligand
341 structures at pH 7.5. For the blue king crab chelae, none of the thickness metrics assessed varied
342 among treatments (Table 2; Fig. 4).
343
344 In red king crabs, seawater pH did not affect any of the thickness metrics of the carapace or the
345 chelae (Table 3; Fig. 5). Temperature affected Bouligand thickness when measured in the
346 carapace, but pair-wise differences between individual treatment groups were not detected. There
347 was no effect of temperature on Bouligand thickness in chelae. Temperature had no effect on
348 total procuticle, exocuticle or endocuticle thickness (Table 3; Fig. 5).
349
350 *3.4 SEM imaging and elemental analysis*
351 At least under the magnifications tested here by SEM (~100 – 10,000 X), ultrastructure of the
352 crab cuticle in both species was similar among treatment groups for the carapace and chelae (Fig.
353 S1, S2, and S3). Bouligand (twisted plywood) structures were clearly visible in all samples, and
354 were composed of fibrous bundles with a diameter on the order of 100-200 nm. The endocuticle
355 was embedded with pore canals with a diameter of 400-500 nm. Pitting and/or erosion of the
356 mineralized cuticle was not observed for any of the samples.
357

Elemental analysis of the endocuticle in both species and body regions consistently identified ten elements: Ca, O, C, Si, Mg, Sr, P, Na, Cl and S. The vast majority of the endocuticle was composed of Ca, O and C; all other elements were found at less than 2% weight. For blue king crabs, the EDS analysis revealed a significant effect of pH on Ca content in the carapace, but not in the chelae (Table 2; Fig. 6). In the carapace Ca content was higher in crabs exposed to pH 7.5 and 7.8 as compared to those exposed to pH 8.1. Mg content did not differ among treatments in the carapace or the chelae (Table 2; Fig. 6). For red king crabs, there was no effect of pH or temperature on Ca or Mg content of carapace samples (Table 3; Fig. 7). In the chelae, however, both pH and temperature significantly affected Ca and Mg content (Table 3; Fig. 7). Ca content was higher, and Mg content was lower in the pH 7.8, ambient temperature treatment as compared to all other treatment groups (Fig. 7).

4. Discussion

Crustaceans exhibit a mixed response to OA, with survival or growth negatively affected in some species, but unaffected or even positively affected in others (reviewed in Whiteley, 2011; Sokolova et al., 2016). To date, only a few previous studies (e.g. Landes and Zimmer, 2012; Taylor et al., 2015) have assessed functional ramifications of OA on the mineralized cuticle in decapods, and, to the best of the authors' knowledge none have done so in juveniles. Here, the hypothesis that microhardness of the cuticle would be reduced under low pH or elevated temperature and that those changes would be driven by altered structure or reduced mineral content was tested in blue and red king crabs. Microhardness, the mechanical property assessed here, scales linearly with a material's Young's modulus and yield stress (Currey and Brear, 1990), and can be used to predict compressive strength (Chen et al., 2008). These metrics

381 determine the maximum stress (force per unit area) that a material can endure before it begins to
382 permanently deform. Hence, a biological material with a lower microhardness, and therefore a
383 lower yield stress, will be able to endure less stress before permanent deformation than one with
384 a higher microhardness. Alterations in the mechanical properties of the cuticle following OA
385 exposure may affect its functionality in terms of resistance to mechanical loads (e.g. those from
386 predators or prey items), protection from the environment including desiccation, and mobility.
387
388 In both crab species tested, microhardness was significantly reduced in the crab endocuticle after
389 long term exposure to reduced pH (and correspondingly reduced calcite saturation state) at
390 ambient temperature, supporting the current hypothesis. This response was body region specific,
391 with a significant reduction in microhardness at low pH in the chelae, but no effect of pH on
392 microhardness of the carapace. Under ambient pH and temperature, microhardness of the chelae
393 was substantially (about two times) higher than that of the carapace in both species tested. A
394 similar pattern was found in Dungeness crabs (*Cancer magister*) with chela hardness about three
395 times higher than that of the carapace (Lian and Wang, 2011), and chelae of sheep crabs
396 (*Loxorhynchus grandis*) were found to be about three times harder than walking legs (Chen et
397 al., 2008). Higher calcium content also occurs in chelae as compared to the carapace in *Cancer*
398 *pagurus* (Boßelmann et al., 2007), consistent with the elemental analysis shown here. For
399 decapods, building the mineralized cuticle in general is energetically expensive, involving
400 multiple active transport mechanisms (Roer and Dillaman, 1984). Given the higher hardness and
401 calcium content in the chelae, one can speculate that energy investment into this structure is
402 especially large. Within the context of OA, if energy budgets are already altered due to
403 regulation of hemolymph pH (Carter et al., 2013; Long et al., 2013a; Meseck et al., 2016; Small

et al., 2010), more energy intensive processes, such as building of the chelae, could be disproportionally affected. Further assessments of energy budgets during the process of cuticle formation are needed to test this hypothesis.

In red king crabs, temperature alone did not significantly affect microhardness of the chela or carapace. Microhardness of chela from crabs exposed to low pH at moderately elevated temperatures (ambient +2°C), however, was slightly higher than that of crabs held at low pH and ambient temperatures, suggesting an interactive effect of the two stressors. Interestingly, mortality of the same set of crabs exposed here showed a similar trend, with a reduction in mortality of crabs held at low pH and moderately elevated temperatures (ambient +2°C) as compared to those at low pH under ambient temperature (Swiney et al., *2017*). It is noted that, due to mortality during exposures (Swiney et al., *2017*) mechanical testing could not include all treatment groups, and hence may not fully represent the effect of temperature on cuticle hardness.

Differences in the response to acidified water between the crab species tested here are consistent with other measured responses in these two species. At ambient temperature, red king crabs appeared to be more susceptible to the effect of OA than blue king crabs, with a significant reduction in microhardness of the chela at pH 7.8, where water was still supersaturated with respect to calcite, while the effect of pH in blue king crabs was only significant at the lowest pH of 7.5 (undersaturated with respect to calcite). This again correlates with mortality and growth; red king crabs have significantly higher mortality and lower growth at pH 7.8 as compared to the ambient pH control group (Swiney et al., 2017; Long et al., 2013a), whereas blue king crabs only

suffer increased mortality and decreased growth at pH 7.5 (Long et al., 2017). The basis for these differences is not clear. The species have similar ranges (Somerton, 1985) and habitat requirements (Armstrong et al., 1987; Loher and Armstrong, 2000) and similar responses to temperature (Stoner et al., 2013). The differences observed here could be due to differences in exposure time, but as the blue king crab were exposed for a longer period of time this seems unlikely, especially as the response in microhardness mirrors the response of other variables. Regardless, blue king crab are more tolerant of reduced pH and seem to possess a level of phenotypic plasticity or variability in their response to low pH (Long et al., 2017) that red king crab do not (Long et al, 2013a). Based on these observations, one can hypothesize that whole organism condition and energy status affect the ability of the animal to utilize resources in energy intensive processes such as growth and calcification of the chelae. The underlying mechanism driving these processes is still unclear and more detailed experiments on the physiology of these crabs will be necessary to elucidate what is driving the differences between these two species.

The crab cuticle is hydrated in its natural state, and it is important to note that drying could alter its mechanical properties. For example, tensile testing of large sheep crab cuticles showed differences in elastic modulus, stress and strain to fracture, and toughness between wet and dry samples (Chen et al., 2008). The cuticle was also found to be anisotropic, meaning that mechanical properties depend on the direction of loading. Inherent limitations to the microhardness testing approach used here are that drying is a necessary step in sample preparation and that samples are tested only in the direction longitudinal to the surface (see Fig. 1), whereas a force is likely to be applied normal to the cuticle surface in the living crab.

Nevertheless, microhardness testing is routinely used in comparative mechanical assessments (see Meyers and Chen, 2014 for multiple biological examples), and is especially useful when comparisons need to be made over a fine (hundreds of microns) spatial scale or when samples are too small for tensile or compressive testing (Currey and Braer, 1990), as was the case in the current study. Given that all samples in the current study were prepared in the same manner and tested in the same direction, there is no evidence to suggest that the trends observed here among pH treatments are an artifact of drying or loading direction.

The hardness of a biological material is determined by its structure, organization and composition (Meyers and Chen, 2014). Given that the decapod endocuticle is a composite material, composed of fibrous bundles of chitin wrapped in protein, interspersed with monocrystalline calcite or amorphous calcium carbonate (Boßelmann et al., 2007; Chen et al., 2008; Raabe et al., 2005), changes in structure, organization and abundance of the protein, chitin or mineral could result in altered hardness. Differences in hardness between body regions or across the cuticle (e.g. within the exo vs. endocuticle) have been explained by differences in packing density of Bouligand layers (i.e. thickness of Bouligand layers: Lian and Wang, 2011; Raabe et al., 2005), calcium content (Boßelmann et al., 2007; Chen et al., 2008), porosity (Lian and Wang, 2011; Melnick et al., 1996), and protein-cross linking (Melnick et al., 1996).

At present, the mechanism(s) driving the reduced chelae hardness observed in blue and red king crabs held at low pH remain unclear. In blue king crabs there was no difference in calcium or magnesium content, or in Bouligand thickness of the chelae. Indeed, Ca content (at pH 7.8 and 7.5) was higher and Bouligand thickness lower (denser packing at pH 7.5 in blue king crabs) in

473 the carapace, though this was not manifest in altered hardness. Contrary to the prediction for
474 reduced hardness, in red king crabs, Ca content was actually elevated in the chelae of crabs at pH
475 (7.8), ambient temperature, with no change in Bouligand thickness. Co-occurring with this
476 increase in Ca content was a slight, but significant reduction in Mg content, which could
477 contribute to the reduction in hardness observed, given that even small amounts of Mg can
478 enhance hardness of a biological material (Kunitake et al., 2012; Kunitake et al., 2013). Other
479 factors that may contribute to observed alterations in chelae hardness include changes in
480 structure and organization beyond the range of what was assessed here (e.g. arrangement and/or
481 orientation of individual chitin-protein bundles), the ratio of $CaCO_3$ polymorphs present (i.e.
482 calcite vs. amorphous calcium carbonate), abundance of organic phases, or the composition
483 and/or cross-linking of protein.
484
485 These results have substantial implications for the interpretation of calcium content in
486 crustaceans in studies on OA. A large number of OA studies measure calcium content, but
487 relatively few also measure the mechanical properties of the corresponding structures. This study
488 demonstrated that maintenance or even increase of calcium content under OA conditions can be
489 accompanied by a decrease in the hardness of a structure. Thus, calcification or calcium content
490 do not necessarily correlate with cuticle mechanical properties. Caution should be used when
491 inferring mechanical properties from calcium content or calcification alone.
492
493 Although a number of reports have demonstrated altered mechanical properties of bivalve shells
494 resulting from OA exposure (e.g. Beniash et al., 2010; Dickinson et al., 2012; Dickinson et al.,
495 2013; Fitzer et al., 2015; Gaylord et al., 2011), to the best of the authors' knowledge this is only

the second study to assess mechanical properties of the decapod cuticle. Following a 5 month exposure of adult green crabs, *Carcinus maenas,* to moderately reduced pH (7.7) and/or elevated temperature (increased 5°C above ambient), Landes and Zimmer (2012) tested break resistance by quantifying the force needed to crush the portion of the shell directly involved in feeding ("denticle-like structures"). They found that neither OA nor temperature significantly affected break resistance in adult *C. maenas*. In additional to species-specific sensitivity to OA, a number of factors may contribute to the contrasting results of mechanical testing presented by Landes and Zimmer (2012) versus those reported here. For example, juveniles, which typically show greater sensitivity to OA (Sokolova et al., 2016), were used here whereas adults were tested by Landes and Zimmer (2012). Importantly, molting did not occur during the experimental exposure of Landes and Zimmer (2012), whereas crabs tested here molted several times (Swiney et al., 2017; Long et al., 2017). As such, Landes and Zimmer (2012) tested alteration in existing cuticle properties due to OA, whereas the current study assessed alterations in the deposition and assembly of cuticle formed during OA exposure. Lastly, the nature of the mechanical test itself provides inherently different information. Although both techniques provide valuable information, microhardness testing assesses the materials properties (resistance to permanent or plastic deformation) at a fine spatial scale (tens of microns) and resolution, independent of geometry and thickness of the specimen, whereas testing break resistance assesses critical failure of the material at a much larger spatial scale (mm to cm).

The consequence of a reduction in chelae hardness on crab fitness is yet to be determined, but a more compliant and less abrasion resistant cuticle could compromise the utility of the crushing claw in feeding and defense. Results of predator-prey interaction studies testing the effects of

519 OA on crabs and their molluscan prey are species and pH dependent. In *C. maenas*, reduced
520 mussel consumption was observed at very low pH (7.36), but not at moderately reduced pH
521 (7.84) (Appelhans et al., 2012). Likewise, neither moderately reduced pH (7.7) nor increased
522 temperature (increased 5°C above ambient) affected feeding ability in *C. maenas* (Landes and
523 Zimmer, 2012). As noted above, breaking strength of the chelae did not differ among treatment
524 groups. Dodd et al. (2015), found a significant reduction in oyster consumption by mud crabs
525 (*Panopeus herbstii*) under both moderate and extreme OA (pH of 8.04 and 7.05, respectively, as
526 compared to an ambient pH of 8.20), with almost no feeding during 48 hour feeding trials under
527 the extreme OA condition. Although the number of feeding attempts did not differ significantly
528 among treatment groups, the time spent in unsuccessful feeding attempts was lower at reduced
529 pH (i.e. the crabs were less persistent). Given that net calcification (measured as the change in
530 buoyant weight) did not differ in crabs among the three pH levels, the authors attribute the
531 reduction in feeding to neurological alterations due to OA rather than changes shell mass. It is
532 interesting to speculate on if altered mechanical properties of the cuticle, which, as shown here
533 do not necessarily correlate with calcification, could have contributed to the observed reduction
534 in successful feeding.
535
536 Unlike microhardness, total thickness of the procuticle was not significantly affected by OA or
537 temperature in the chela or carapace for crabs in this study, although a slight but significant
538 decrease in exocuticle thickness was observed in blue king crabs at the lowest pH (7.5).
539 Likewise, Landes and Zimmer (2012) found no effect of OA or temperature on thickness of the
540 *C. maenas* chela cuticle and Taylor et al. (2015) found that total carapace thickness was not
541 affected by reduced pH (7.53) in the shrimp, *Lymata californica*. Consistent with the elemental

542 analyses shown here, a significant increase in Ca content was observed in the carapace of *L.*
543 *californica* (Taylor et al., 2015). Use of bicarbonate in the mineralization process by crustaceans
544 (Cameron and Wood, 1985) may explain their ability to maintain or increase Ca content despite a
545 reduction in calcite saturation state. The changes in organic matrix and/or mineral deposition
546 associated with alterations in mineral content and microhardness appear to occur largely
547 independently of the total amount (thickness) of cuticle laid down.
548

549 **5. Conclusions**

550 Ocean acidification results in a complex and body region specific response in red and blue king
551 crabs, species that support fisheries in the eastern Bering Sea. While microhardness of the chela
552 (but not the carapace) was significantly reduced in both species at low pH, calcium content
553 actually increased significantly in the blue king crab carapace and in the red king crab chelae at
554 low pH. These changes occurred without a corresponding alteration in total thickness of the
555 mineralized procuticle. Direct assessments of how these alterations in cuticle properties affect
556 utility of the cuticle in feeding and defense will shed light on susceptibility of these crabs to
557 predicted future changes in ocean pH and temperature.
558

559 **Acknowledgments**

560 This project was partially funded by the National Oceanic and Atmospheric Administration
561 (NOAA) Ocean Acidification Program (W.C.L, K.M.S. & R.J.F). J.A.N. was supported by an
562 Office of Naval Research grant to G.H.D. (N00014-14-1-0491). W.D.C and A.Y. were supported
563 by The College of New Jersey's Mentored Undergraduate Research Experience (MUSE). The
564 findings and conclusions in the paper are those of the authors and do not necessarily represent

565 the views of the National Marine Fisheries Service, NOAA. Reference to trade names does not
566 imply endorsement by the National Marine Fisheries Service, NOAA.
567

568 **References**

569 Armstrong, D.A., Armstrong, J.L., Jenson, G., Palacios, R., Williams, G., 1987. Distribution,
570 abundance, and biology of blue king and Korean hair crabs around the Pribilof Islands, Outer
571 Continental Shelf Environmental Assessment Program: Final Reports of Principal Investigators.
572 U.S. Department of Commerce, NOAA, pp. 1-278.

573 Appelhans, Y., Thomsen, J., Pansch, C., Melzner, F., Wahl, M., 2012. Sour times: seawater
574 acidification effects on growth, feeding behaviour and acid–base status of *Asterias rubens* and
575 *Carcinus maenas*. Mar. Ecol. Prog. Ser. 459, 85-98.

576 Beniash, E., Ivanina, A., Lieb, N.S., Kurochkin, I., Sokolova, I.M., 2010. Elevated level of
577 carbon dioxide affects metabolism and shell formation in oysters *Crassostrea virginica*. Mar.
578 Ecol. Prog. Ser. 419, 95-108.

579 Boßelmann, F., Romano, P., Fabritius, H., Raabe, D., Epple, M., 2007. The composition of the
580 exoskeleton of two crustacea: The American lobster *Homarus americanus* and the edible crab
581 *Cancer pagurus*. Thermochim. Acta 463, 65-68.

582 Caldeira, K., Wickett, M.E., 2003. Anthropogenic carbon and ocean pH. Nature 425, 365-365.

583 Cameron, J.N., Wood, C.M., 1985. Apparent H+ excretion and CO_2 dynamics accompanying
584 carapace mineralization in the blue crab (*Callinectes sapidus*) following molting. J. Exp. Biol.
585 114, 181-196.

586 Carter, H.A., Ceballos-Osuna, L., Miller, N.A., Stillman, J.H., 2013. Impact of ocean
587 acidification on metabolism and energetics during early life stages of the intertidal porcelain crab
588 *Petrolisthes cinctipes*. J. Exp. Biol. 216, 1412-1422.

589 Chen, P.-Y., Lin, A.Y.-M., McKittrick, J., Meyers, M.A., 2008. Structure and mechanical
590 properties of crab exoskeletons. Acta Biomater. 4, 587-596.

591 Currey, J., K. Brear. 1990. Hardness, Young's modulus and yield stress in mammalian
592 mineralized tissues. J. Mater. Sci. Mater. Med. 1, 14-20.

593 Dickinson, G.H., Matoo, O.B., Tourek, R.T., Sokolova, I.M., Beniash, E., 2013. Environmental
594 salinity modulates the effects of elevated CO_2 levels on juvenile hard-shell clams, *Mercenaria*
595 *mercenaria*. J. Exp. Biol. 216, 2607-2618.

596 Dickinson, G.H., Ivanina, A.V., Matoo, O.B., Portner, H.O., Lannig, G., Bock, C., Beniash, E.,
597 Sokolova, I.M., 2012. Interactive effects of salinity and elevated CO_2 levels on juvenile eastern
598 oysters, *Crassostrea virginica*. J. Exp. Biol. 215, 29-43.

599 Dickson, A.G., Goyet, C., 1994. Handbook of methods for the analysis of the various parameters
600 of the carbon dioxide system in sea water, ORNL/CDIAC-74. Washington, DC: US Department
601 of Energy.

602 Dickson, A.G., Sabine, C.L., Christian, J.R., 2007. Guide to best practices for ocean CO_2
603 measurements. PICES special publication 3. Sidney, Canada: North Pacific Marine Science
604 Organization.

605 Dillaman, R., Hequembourg, S., Gay, M., 2005. Early pattern of calcification in the dorsal
606 carapace of the blue crab, *Callinectes sapidus*. J. Morphol. 263, 356-374.

607 Dlugokencky, E., Trans, P., 2016. NOAA/ESRL trends in atmospheric carbon dioxide.
608 http://www.esrl.noaa.gov/gmd/ccgg/trends/global.html.

609 Dodd, L.F., Grabowski, J.H., Piehler, M.F., Westfield, I., Ries, J.B., 2015. Ocean acidification
610 impairs crab foraging behaviour. Proc. R. Soc. B, 282, 20150333.

611 Doney, S.C., Fabry, V.J., Feely, R.A., Kleypas, J.A., 2009. Ocean Acidification: The Other CO_2
612 Problem. Annu. Rev. Mar. Sci. 1, 169-192.

613 Fitzer, S.C., Zhu, W., Tanner, K.E., Phoenix, V.R., Kamenos, N.A., Cusack, M., 2015. Ocean
614 acidification alters the material properties of *Mytilus edulis* shells. J. R. Soc. Interface 12,
615 20141227.

616 Gaylord, B., Hill, T.M., Sanford, E., Lenz, E.A., Jacobs, L.A., Sato, K.N., Russell, A.D.,
617 Hettinger, A., 2011. Functional impacts of ocean acidification in an ecologically critical
618 foundation species. J. Exp. Biol. 214, 2566-2594.

619 Giraud-Guille, M.-M., 1984. Fine structure of the chitin-protein system in the crab cuticle.
620 Tissue Cell 16, 75-92.

621 Hauton, C., Tyrrell, T., Williams, J., 2009. The subtle effects of sea water acidification on the
622 amphipod *Gammarus locusta*. Biogeosciences 6, 1479-1489.

623 Hegdahl, T., Silness, J., Gustavsen, F., 1977. The structure and mineralization of the carapace of
624 the crab (*Cancer pagurus* L.). Zool. Scr. 6, 89-99.

625 IPCC, 2001. Climate Change 2001 - IPCC Third Assessment Report. Cambridge, UK:
626 Cambridge University Press.

627 IPCC, 2014. Climate Change 2014: Synthesis Report. Fifth Assessment Report of the
628 Intergovernmental Panel on Climate Change. Geneva, Switzerland: IPCC.

629 Kroeker, K.J., Kordas, R.L., Crim, R.N., Singh, G.G., 2010. Meta-analysis reveals negative yet
630 variable effects of ocean acidification on marine organisms. Ecol. Lett. 13, 1419-1434.

631 Kroeker, K.J., Kordas, R.L., Crim, R., Hendriks, I.E., Ramajo, L., Singh, G.S., Duarte, C.M.,
632 Gattuso, J.P., 2013. Impacts of ocean acidification on marine organisms: quantifying sensitivities
633 and interaction with warming. Glob. Change Biol. 19, 1884-1896.

634 Kunitake, M.E., Baker, S.P., Estroff, L.A., 2012. The effect of magnesium substitution on the
635 hardness of synthetic and biogenic calcite. MRS Commun. 2, 113-116.

636 Kunitake, M.E., Mangano, L.M., Peloquin, J.M., Baker, S.P., Estroff, L.A., 2013. Evaluation of
637 strengthening mechanisms in calcite single crystals from mollusk shells. Acta Biomater. 9, 5353-
638 5359.

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

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

644 Landes, A., Zimmer, M., 2012. Acidification and warming affect both a calcifying predator and
645 prey, but not their interaction. Mar. Ecol. Prog. Ser. 450, 1-10.

646 Lavigne, H., Gattuse, J., 2011. seacarb: seawater carbonate chemistry with R. http://CRAN.R-
647 project.org/package=seacarb.

648 Lian, J., Wang, J., 2011. Microstructure and mechanical properties of dungeness crab
649 exoskeletons. In Mechanics of Biological Systems and Materials, Volume 2 (ed. T. Proulx), pp.
650 93-99. New York, USA: Springer.

651 Loher, T., Armstrong, D.A., 2000. Effects of habitat complexity and relative larval supply on the
652 establishment of early benthic phase red king crab (Paralithodes camtschaticus Tilesius, 1815)
653 populations in Auke Bay, Alaska. J. Exp. Mar. Biol. Ecol. 245, 83-109.

654 Long, W.C., 2016. A new quantitative model of multiple transitions between discrete stages,
655 applied to the development of crustacean larvae. Fish. Bull. US 114, 58-66.

656 Long, W.C., Swiney, K.M., Harris, C., Page, H.N., Foy, R.J., 2013a. Effects of ocean
657 acidification on juvenile red king crab (*Paralithodes camtschaticus*) and Tanner crab
658 (*Chionoecetes bairdi*) growth, condition, calcification, and survival. PLoS One 8, e60959.

659 Long, W.C., Swiney, K.M., Foy, R.J., 2013b. Effects of ocean acidification on the embryos and
660 larvae of red king crab, *Paralithodes camtschaticus*. Mar. Pollut. Bull. 69, 38-47.

661 Long, W.C., Van Sant, S.B., Swiney, K.M., Foy, R., 2017. Survival, growth, and morphology of
662 blue king crabs: Effect of ocean acidification decreases with exposure time. ICES J. Mar. Sci.
663 doi: 10.1093/icesjms/fsw197 .

664 Mathis, J.T., Cooley, S.R., Lucey, N., Colt, S., Ekstrom, J., Hurst, T., Hauri, C., Evans, W.,
665 Cross, J. N., Feely, R.A., 2015. Ocean acidification risk assessment for Alaska's fishery sector.
666 Prog. Oceanogr. 136, 71-91.

667 McDonald, M.R., McClintock, J.B., Amsler, C.D., Rittschof, D., Angus, R.A., Orihuela, B.,
668 Lutostanski, K., 2009. Effects of ocean acidification over the life history of the barnacle
669 *Amphibalanus amphitrite*. Mar. Ecol. Prog. Ser. 385, 179-187.

670 Melnick, C., Chen, Z., Mecholsky, J., 1996. Hardness and toughness of exoskeleton material in
671 the stone crab, *Menippe mercenaria*. J. Materials Res. 11, 2903-2907.

672 Meseck, S.L., Alix, J.H., Swiney, K.M., Long, W.C., Wikfors, G.H., Foy, R.J., 2016. Ocean
673 acidification affects hemocyte physiology in the Tanner crab (*Chionoecetes bairdi*). PLoS One
674 11, e0148477.

675 Meyers, M.A., Chen, P.-Y., 2014. Biological Materials Science: Biological Materials,
676 Bioinspired Materials, and Biomaterials. Cambridge, UK: Cambridge University Press.

677 Orr, J.C., Fabry, V.J., Aumont, O., Bopp, L., Doney, S.C., Feely, R.A., Gnanadesikan, A.,
678 Gruber, N., Ishida, A., Joos, F., Key, R.M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray,
679 P., Mouchet, A., Najjar, R.G., Plattner, G.-K., Rodgers, K.B., Sabine, C.L., Sarmiento, J.L.,
680 Schlitzer, R., Slater, R.D., Totterdell, I.J., Weirig, M.-F., Yamanaka, Y., Yool, A., 2005.
681 Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying
682 organisms. Nature 437, 681-686.

683 Raabe, D., Sachs, C., Romano, P., 2005. The crustacean exoskeleton as an example of a
684 structurally and mechanically graded biological nanocomposite material. Acta Mater. 53, 4281-
685 4292.

686 Raabe, D., Romano, P., Sachs, C., Fabritius, H., Al-Sawalmih, A., Yi, S.-B., Servos, G.,
687 Hartwig, H., 2006. Microstructure and crystallographic texture of the chitin–protein network in
688 the biological composite material of the exoskeleton of the lobster *Homarus americanus*.
689 Materials Sci. Eng. A-Struct. 421, 143-153.

690 Raven, J., 2005. Ocean Acidification Due to Increasing Atmospheric Carbon Dioxide. London,
691 UK: The Royal Society.

692 Ries, J.B., Cohen, A.L., McCorkle, D.C., 2009. Marine calcifiers exhibit mixed responses to
693 CO_2-induced ocean acidification. Geology 37, 1131-1134.

694 Roemmich, D., Gould, W.J., Gilson, J., 2012. 135 years of global ocean warming between the
695 Challenger expedition and the Argo Programme. Nat. Clim. Change 2, 425-428.

696 Roer, R., Dillaman, R., 1984. The structure and calcification of the crustacean cuticle. Amer.
697 Zool. 24, 893-909.

698 Small, D., Calosi, P., White, D., Spicer, J.I., Widdicombe, S., 2010. Impact of medium-term
699 exposure to CO_2 enriched seawater on the physiological functions of the velvet swimming crab
700 *Necora puber*. Aquat. Biol. 10, 11-21.

701 Sokolova, I.M., Matoo, O.B., Dickinson, G.H., Beniash, E., 2016. Physiological effects of ocean
702 acidification on animal calcifiers. In Stressors in the marine environment: physiological and

703 ecological responses and societal implications (ed. M. Solan and N. M. Whiteley), pp. 36-55.
704 Oxford, UK: Oxford University Press.

705 Somerton, D.A., 1985. The disjunct distribution of blue king crab, Paralithodes platypus, in
706 Alaska: some hypotheses. In: Melteff, B. (Ed.), Proceedings of the International King Crab
707 Symposium. University of Alaska Sea Grant Program, Anchorage, AK, USA, pp. 13-21.

708 Stoner, A.W., Copeman, L.A., Ottmar, M.L., 2013. Molting, growth, and energetics of newly-
709 settled blue king crab: Effects of temperature and comparisons with red king crab. J. Exp. Mar.
710 Biol. Ecol. 442, 10-21.

711 Swiney, K.M., Long, W.C., Persselin, S.L., 2013. The effects of holding space on juvenile red
712 king crab, *Paralithodes camtschaticus* (Tilesius, 1815), growth and survival. Aquaculture Res.
713 44, 1007-1016.

714 Swiney, K.M., Long, W.C., Foy, R., in press. Decreased pH and increased temperatures affect
715 young-of-the-year red king crab (Paralithodes camtschaticus). ICES J. Mar. Sci. doi:
716 10.1093/icesjms/fsw251.

717 Taylor, J.R., Gilleard, J.M., Allen, M.C., Deheyn, D.D., 2015. Effects of CO_2-induced pH
718 reduction on the exoskeleton structure and biophotonic properties of the shrimp *Lysmata*
719 *californica*. Sci. Rep. 5, 10608.

720 Travis, D.F., 1963. Structural features of mineralization from tissue to macromolecular levels of
721 organization in the decapod Crustacea. Ann. NY Acad. Sci. 109, 177-245.

722 Whiteley, N.M., 2011. Physiological and ecological responses of crustaceans to ocean
723 acidification. Mar. Ecol. Prog. Ser. 430, 257-271.

724

725 **Table and figure legends**

726 **Table 1**. Components of the carbonate system for the red and blue king crab experiments (means
727 ± s.d.). pH_F (free scale) was measured daily with a Durafet III probe in individual cells for a
728 sample size of 184 per treatment for red king crab and 246 per treatment for blue king crab, DIC
729 and alkalinity were measured weekly for a sample size of 26 per treatment for red king crab and
730 52 per treatment for blue king crab in seawater from the head tanks, and all other parameters
731 were calculated.

732

733 **Table 2.** ANOVA results for the effects of seawater pH on the mineralized cuticle of the blue
734 king crab, *Paralithodes platypus*. Significant p-values are shown in bold.

735

736 **Table 3.** GLM results for the effects of seawater pH, temperature and their interactions on the
737 mineralized cuticle of the red king crab, *Paralithodes camtschaticus*. Significant p-values are
738 shown in bold. Degrees of freedom are in subscript following F values. The interaction of pH
739 and temperature could not be calculated for chelae due to low sample availability.

740

741 **Fig. 1.** Preparation of juvenile crabs for microhardness testing and structural assessments. (A) A
742 juvenile blue king crab. Dotted line shows orientation of carapace cross-section. (B,C) Polished
743 cross-sections of a blue king crab carapace (B) and chela (C) under darkfield illumination.
744 Arrowheads surround a portion of the cross-section. (D) Close-up image of a polished chela
745 cross-section from a blue king crab, under brightfield illumination showing the distinction
746 between the endo-and exo-cuticle. Black diamonds are indentations made for micromechanical

747 testing. Bouligand structures are visible as lines within the cuticle, examples of which are
748 marked "BGD". Scale bars: A, 1 mm; B and C, 500 μm; D, 20 μm.
749
750 **Fig. 2.** Vickers microhardness of blue king crab, *Paralithodes platypus,* carapaces and chelae
751 (crushing claws) (mean ± s.e.m.). Juvenile crabs were exposed to one of three levels of pH for
752 one year. Hardness testing was conducted on polished cross-sections of the mineralized cuticle.
753 Groups marked with different letters are significantly different as shown by Tukey HSD post-hoc
754 analysis. *N* is indicated within each bar and represents individual crab samples.
755
756 **Fig. 3.** Vickers microhardness of red king crab, *Paralithodes camtschaticus,* carapaces and
757 chelae (crushing claws) (mean ± s.e.m.). Juvenile crabs were exposed to one of two levels of pH
758 at one of three levels of temperature (reported in degrees Celsius) for 184 days. Hardness testing
759 was conducted on polished cross-sections of the mineralized cuticle. Groups marked with
760 different letters are significantly different as shown by Tukey HSD post-hoc analysis. Carapace
761 and chelae data for the pH 8.0, ambient + 4°C treatment and chelae data for the pH 7.8, ambient
762 + 2°C treatment are not available due to mortality during exposure and sample damage. *N* is
763 indicated within each bar and represents individual crab samples.
764
765 **Fig. 4**. Cuticle thickness assessments of blue king crab, *Paralithodes platypus,* carapaces (left)
766 and chelae (crushing claws, right) (mean ± s.e.m.). Juvenile crabs were exposed to one of three
767 levels of pH for one year. Assessments were conducted on polished cross-sections of the
768 mineralized cuticle. Groups marked with different letters are significantly different as shown by

769 Tukey HSD post-hoc analysis. *N* is indicated within each bar and represents individual crab
770 samples.
771
772 **Fig. 5.** Cuticle thickness assessments of red king crab, *Paralithodes camtschaticus,* carapaces
773 and chelae (crushing claws) (mean ± s.e.m.). Juvenile crabs were exposed to one of two levels of
774 pH at one of three levels of temperature (reported in degrees Celsius) for 184 days. Assessments
775 were conducted on polished cross-sections of the mineralized cuticle. Carapace and chelae data
776 for the pH 8.0, ambient + 4°C treatment and chelae data for the pH 7.8, ambient + 2°C treatment
777 are not available due to mortality during exposure and sample damage. *N* is indicated within each
778 bar and represents individual crab samples.
779
780 **Fig. 6.** Calcium and magnesium content of blue king crab, *Paralithodes platypus,* carapaces
781 (left) and chelae (crushing claws, right) (mean ± s.e.m.). Juvenile crabs were exposed to one of
782 three levels of pH for one year. Assessments were conducted on polished cross-sections of the
783 mineralized cuticle, within the endocuticle, using electron dispersive spectroscopy (EDS).
784 Groups marked with different letters are significantly different as shown by Tukey HSD post-hoc
785 analysis. *N* is indicated within each bar and represents individual crab samples.
786
787 **Fig. 7.** Calcium and magnesium content of red king crab, *Paralithodes camtschaticus,* carapaces
788 (left) and chelae (crushing claws, right) (mean ± s.e.m.). Juvenile crabs were exposed to one of
789 two levels of pH at one of three levels of temperature (reported in degrees Celsius) for 184 days.
790 Assessments were conducted on polished cross-sections of the mineralized cuticle, within the
791 endocuticle, using electron dispersive spectroscopy (EDS). Groups marked with different letters

792 are significantly different as shown by Tukey HSD post-hoc analysis. Carapace and chelae data
793 for the pH 8.0, ambient + 4°C treatment and chelae data for the pH 7.8, ambient + 2°C treatment
794 are not available due to mortality during exposure and sample damage. *N* is indicated within each
795 bar and represents individual crab samples.

796 **Tables**

797 **Table 1:** Components of the carbonate system for the red and blue king crab experiments (means ± s.d.). pH_F (free scale) was
798 measured daily with a Durafet III probe in individual cells for a sample size of 184 per treatment for red king crab and 246 per
799 treatment for blue king crab, DIC and alkalinity were measured weekly for a sample size of 26 per treatment for red king crab and 52
800 per treatment for blue king crab in seawater from the head tanks, and all other parameters were calculated.
801

802

Treatment	pHF	Temperature °C	pCO2 µatm	HCO_3^- mmol/kg	CO_3^{-2} mmol/kg	DIC mmol/kg	Alkalinity mmol/kg	ΩAragonite	ΩCalcite
					Red king crab				
Ambient	8.00 ± 0.03	8.49 ± 1.99	491.28 ± 40.04	1.90 ± 0.07	0.09 ± 0.01	2.01 ± 0.07	2.12 ± 0.07	1.34 ± 0.13	2.13 ± 0.2
Ambient +2°C	7.97 ± 0.03	10.30 ± 1.92	538.00 ± 41.22	1.90 ± 0.07	0.09 ± 0.01	2.01 ± 0.07	2.12 ± 0.07	1.33 ± 0.12	2.11 ± 0.19
Ambient +4°C	7.94 ± 0.02	12.34 ± 2.01	587.97 ± 40.94	1.90 ± 0.07	0.09 ± 0.01	2.01 ± 0.07	2.12 ± 0.07	1.35 ± 0.12	2.12 ± 0.19
pH 7.8	7.79 ± 0.01	8.59 ± 1.98	826.03 ± 24.29	1.97 ± 0.05	0.06 ± 0.00	2.07 ± 0.05	2.11 ± 0.05	0.86 ± 0.06	1.36 ± 0.09
pH 7.8 +2°C	7.76 ± 0.01	10.47 ± 2.01	896.11 ± 26.58	1.97 ± 0.05	0.06 ± 0.00	2.07 ± 0.05	2.11 ± 0.05	0.87 ± 0.06	1.37 ± 0.1
pH 7.8 +4°C	7.75 ± 0.01	12.33 ± 1.94	955.08 ± 32.81	1.97 ± 0.05	0.06 ± 0.00	2.07 ± 0.05	2.11 ± 0.05	0.89 ± 0.07	1.4 ± 0.1
					Blue king crab				
Ambient	8.07 ± 0.07	5.13 ± 1.99	390.64 ± 54.27	1.89 ± 0.05	0.09 ± 0.01	2.00 ± 0.04	2.13 ± 0.07	1.42 ± 0.19	2.26 ± 0.30
pH 7.8	7.80 ± 0.03	5.11 ± 1.96	766.59 ± 44.95	1.98 ± 0.04	0.05 ± 0.00	2.07 ± 0.04	2.13 ± 0.08	0.78 ± 0.06	1.25 ± 0.10
pH 7.5	7.49 ± 0.03	5.18 ± 1.98	1627.00 ± 83.53	2.03 ± 0.04	0.03 ± 0.00	2.14 ± 0.04	2.13 ± 0.06	0.39 ± 0.03	0.62 ± 0.04

803

804 **Table 2.** ANOVA results for the effects of seawater pH on the mineralized cuticle of the blue
805 king crab, *Paralithodes platypus*. Significant p-values are shown in bold.

806

Parameter	df	F	p
Chela			
Hardness	**22**	**6.192**	**0.008**
Total procuticle thickness	17	0.174	0.842
Exocuticle thickness	17	0.780	0.476
Endocuticle thickness	17	0.069	0.934
Bouligand thickness	17	1.443	0.267
Calcium content	11	1.231	0.337
Magnesium content	11	0.674	0.534
Carapace			
Hardness	23	2.459	0.110
Total procuticle thickness	23	2.585	0.099
Exocuticle thickness	**23**	**3.684**	**0.043**
Endocuticle thickness	23	1.646	0.217
Bouligand thickness	**23**	**4.829**	**0.019**
Calcium content	**20**	**4.934**	**0.020**
Magnesium content	20	0.549	0.587

807

808 **Table 3.** GLM results for the effects of seawater pH, temperature and their interactions on the
809 mineralized cuticle of the red king crab, *Paralithodes camtschaticus*. Significant p-values are
810 shown in bold. Degrees of freedom are in subscript following F values. The interaction of pH
811 and temperature could not be calculated for chelae due to low sample availability.

Parameter	pH	Temperature	pH x Temperature
Chela			
Hardness	**$F_{1,26}$=10.8, p = 0.003**	$F_{2,26}$=1.88, p = 0.173	N/A
Total procuticle thickness	$F_{1,26}$=0.00, p = 0.992	$F_{2,26}$=0.24, p = 0.788	N/A
Exocuticle thickness	$F_{1,26}$=0.28, p = 0.603	$F_{2,26}$=0.56, p = 0.579	N/A
Endocuticle thickness	$F_{1,26}$=0.01, p = 0.930	$F_{2,26}$=0.13, p = 0.881	N/A
Bouligand thickness	$F_{1,25}$=2.45, p = 0.130	$F_{2,25}$=1.17, p = 0.326	N/A
Calcium content	**$F_{1,22}$=10.3, p = 0.005**	**$F_{2,22}$=8.53, p = 0.002**	N/A
Magnesium content	**$F_{1,22}$=8.22, p = 0.010**	**$F_{2,22}$=12.9, p = 0.000**	N/A
Carapace			
Hardness	$F_{1,21}$=1.21, p = 0.285	$F_{2,21}$=1.57, p = 0.232	$F_{1,21}$=0.09, p = 0.766
Total procuticle thickness	$F_{1,29}$=0.32, p = 0.577	$F_{2,29}$=0.28, p = 0.757	$F_{1,29}$=0.29, p = 0.592
Exocuticle thickness	$F_{1,27}$=1.86, p = 0.184	$F_{2,27}$=1.38, p = 0.270	$F_{1,27}$=0.27, p = 0.608
Endocuticle thickness	$F_{1,26}$=0.00, p = 0.963	$F_{2,26}$=0.20, p = 0.821	$F_{1,26}$=0.82, p = 0.641
Bouligand thickness	$F_{1,26}$=0.53, p = 0.474	**$F_{2,26}$=3.93, p = 0.032**	$F_{1,26}$=0.07, p = 0.799
Calcium content	$F_{1,28}$=1.23, p = 0.279	$F_{2,28}$=1.44, p = 0.258	$F_{1,28}$=0.82, p = 0.374
Magnesium content	$F_{1,28}$=0.13, p = 0.725	$F_{2,28}$=1.75, p = 0.197	$F_{1,28}$=0.18, p = 0.677

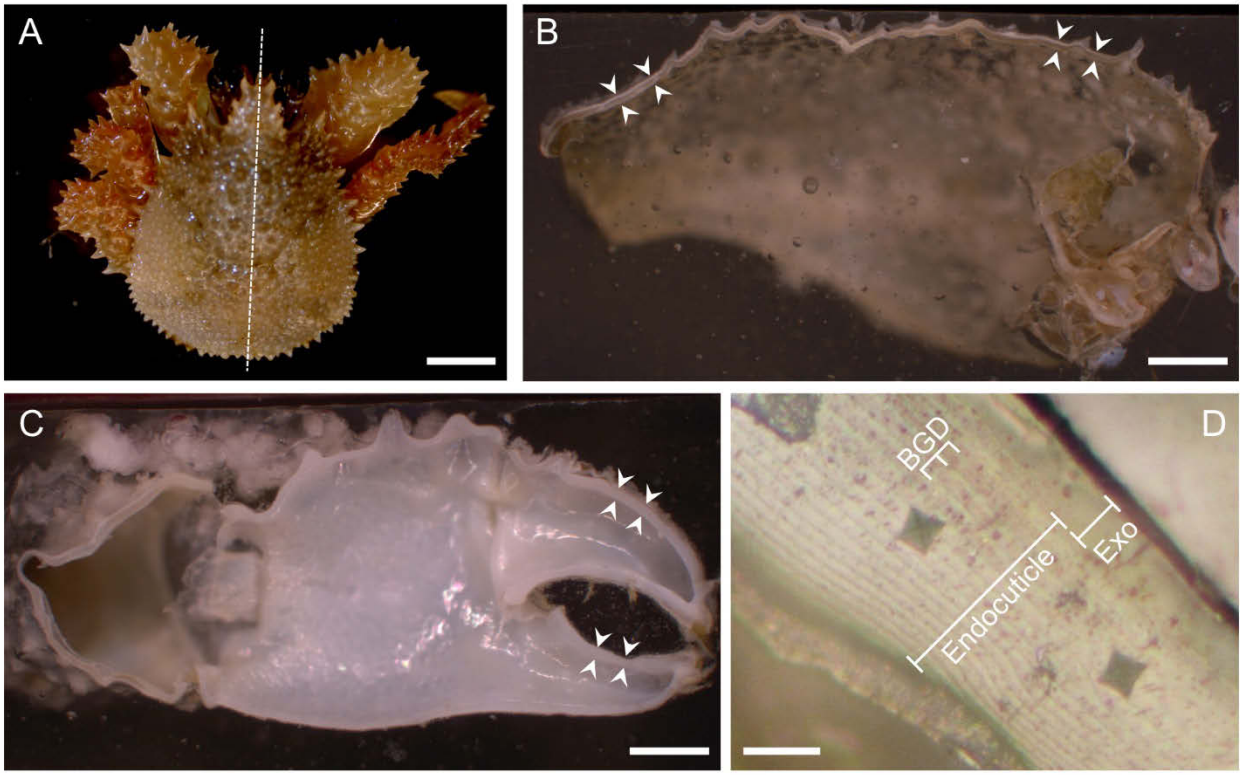


Fig. 1. Preparation of juvenile crabs for microhardness testing and structural assessments. (A) A juvenile blue king crab. Dotted line shows orientation of carapace cross-section. (B,C) Polished cross-sections of a blue king crab carapace (B) and chela (C) under darkfield illumination. Arrowheads surround the cross-section. (D) Close-up image of a polished chela cross-section from a blue king crab, under brightfield illumination showing the distinction between the endo and exo cuticle. Black diamonds are indentations made from micromechanical testing. Bouligand structures are visible as lines within the cuticle, examples of which are marked "BGD". Scale bars: A, 1 mm; B and C, 500 μm; D, 20 μm.

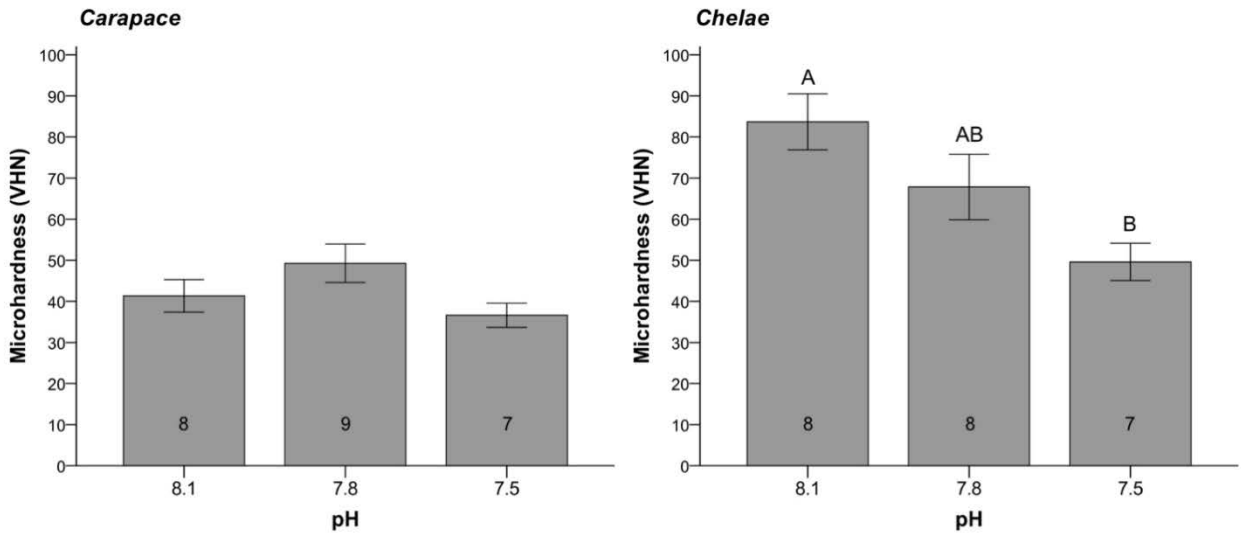


Fig. 2. Vickers microhardness of blue king crab, *Paralithodes platypus,* carapaces and chelae (crushing claws) (mean ± s.e.m.). Juvenile crabs were exposed to one of three levels of pH for one year. Hardness testing was conducted on polished cross-sections of the mineralized cuticle. Groups marked with different letters are significantly different as shown by Tukey HSD post-hoc analysis. *N* is indicated within each bar and represents individual crab samples.

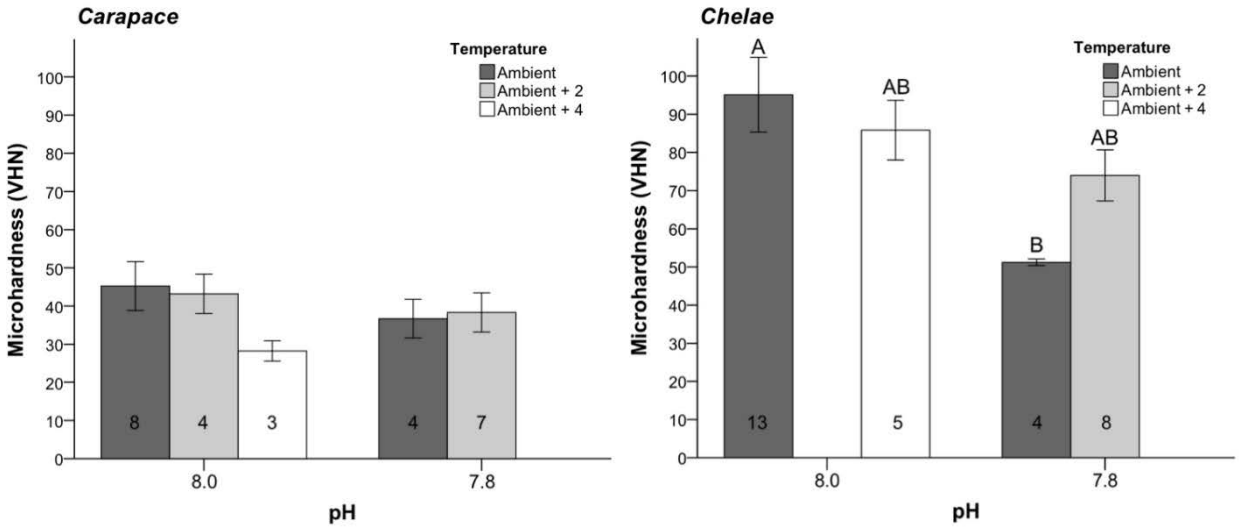


Fig. 3. Vickers microhardness of red king crab, *Paralithodes camtschaticus,* carapaces and chelae (crushing claws) (mean ± s.e.m.). Juvenile crabs were exposed to one of two levels of pH at one of three levels of temperature (reported in degrees Celsius) for 184 days. Hardness testing was conducted on polished cross-sections of the mineralized cuticle. Groups marked with different letters are significantly different as shown by Tukey HSD post-hoc analysis. Carapace and chelae data for the pH 8.0, ambient + 4°C treatment and chelae data for the pH 7.8, ambient + 2°C treatment are not available due to mortality during exposure and sample damage. *N* is indicated within each bar and represents individual crab samples.

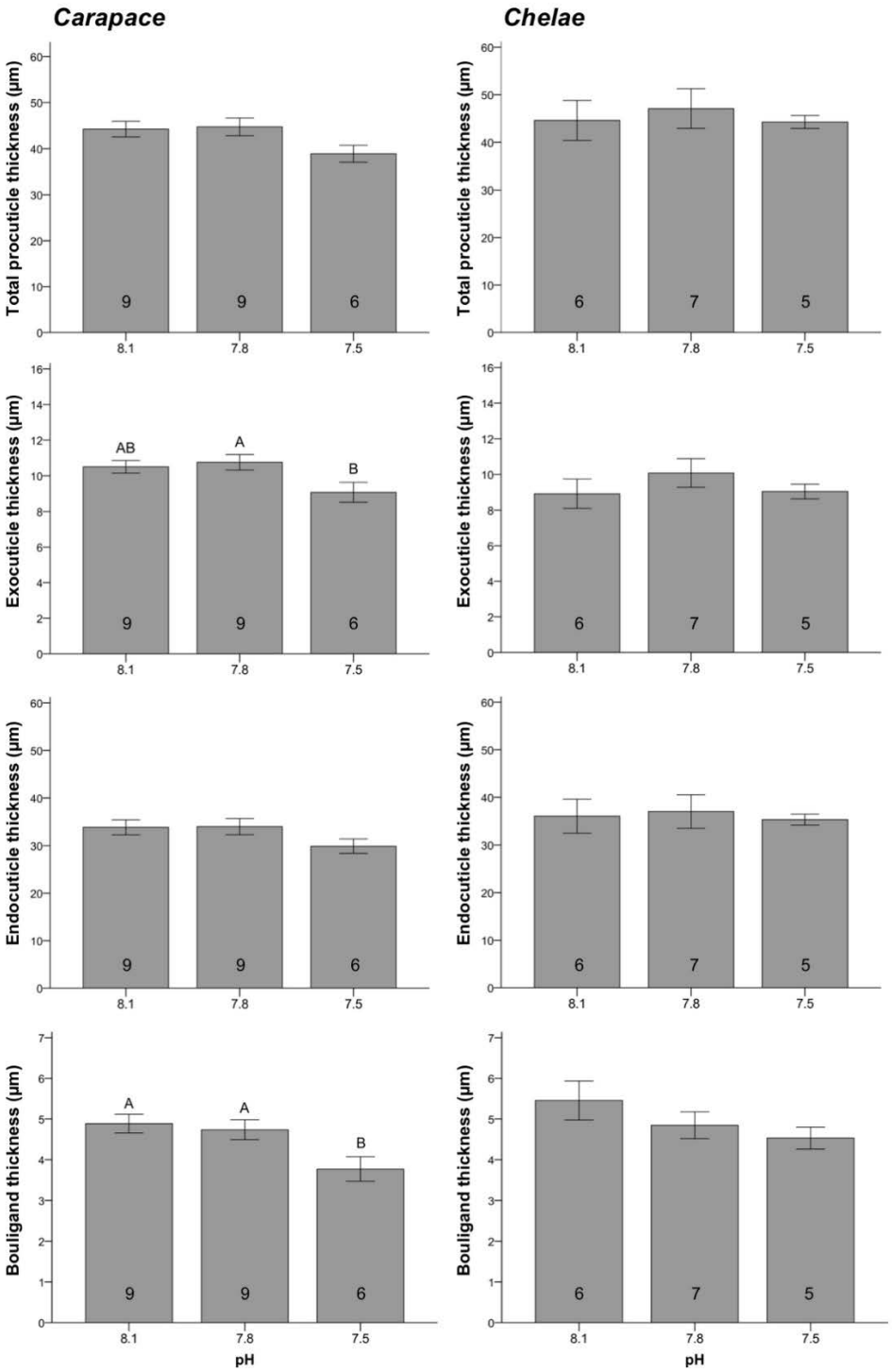
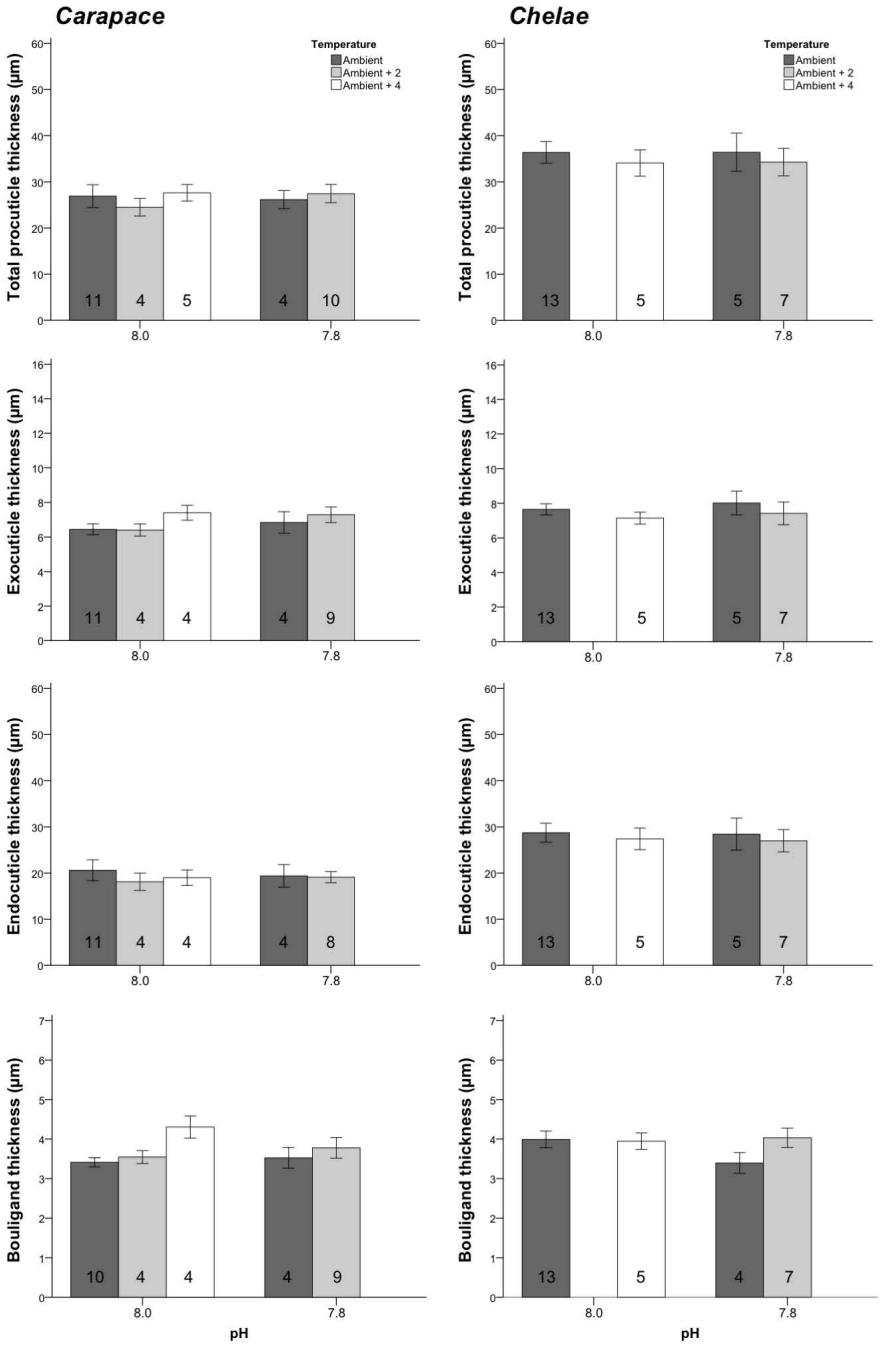


Fig. 4. Cuticle thickness assessments of blue king crab, *Paralithodes platypus,* carapaces (left) and chelae (crushing claws, right) (mean ± s.e.m.). Juvenile crabs were exposed to one of three levels of pH for one year. Assessments were conducted on polished cross-sections of the mineralized cuticle. Groups marked with different letters are significantly different as shown by Tukey HSD post-hoc analysis. *N* is indicated within each bar and represents individual crab samples



845

846 **Fig. 5.** Cuticle thickness assessments of red king crab, *Paralithodes camtschaticus,* carapaces
847 and chelae (crushing claws) (mean ± s.e.m.). Juvenile crabs were exposed to one of two levels of
848 pH at one of three levels of temperature (reported in degrees Celsius) for 184 days. Assessments
849 were conducted on polished cross-sections of the mineralized cuticle. Carapace and chelae data
850 for the pH 8.0, ambient + 4°C treatment and chelae data for the pH 7.8, ambient + 2°C treatment
851 are not available due to mortality during exposure and sample damage. *N* is indicated within each
852 bar and represents individual crab samples.

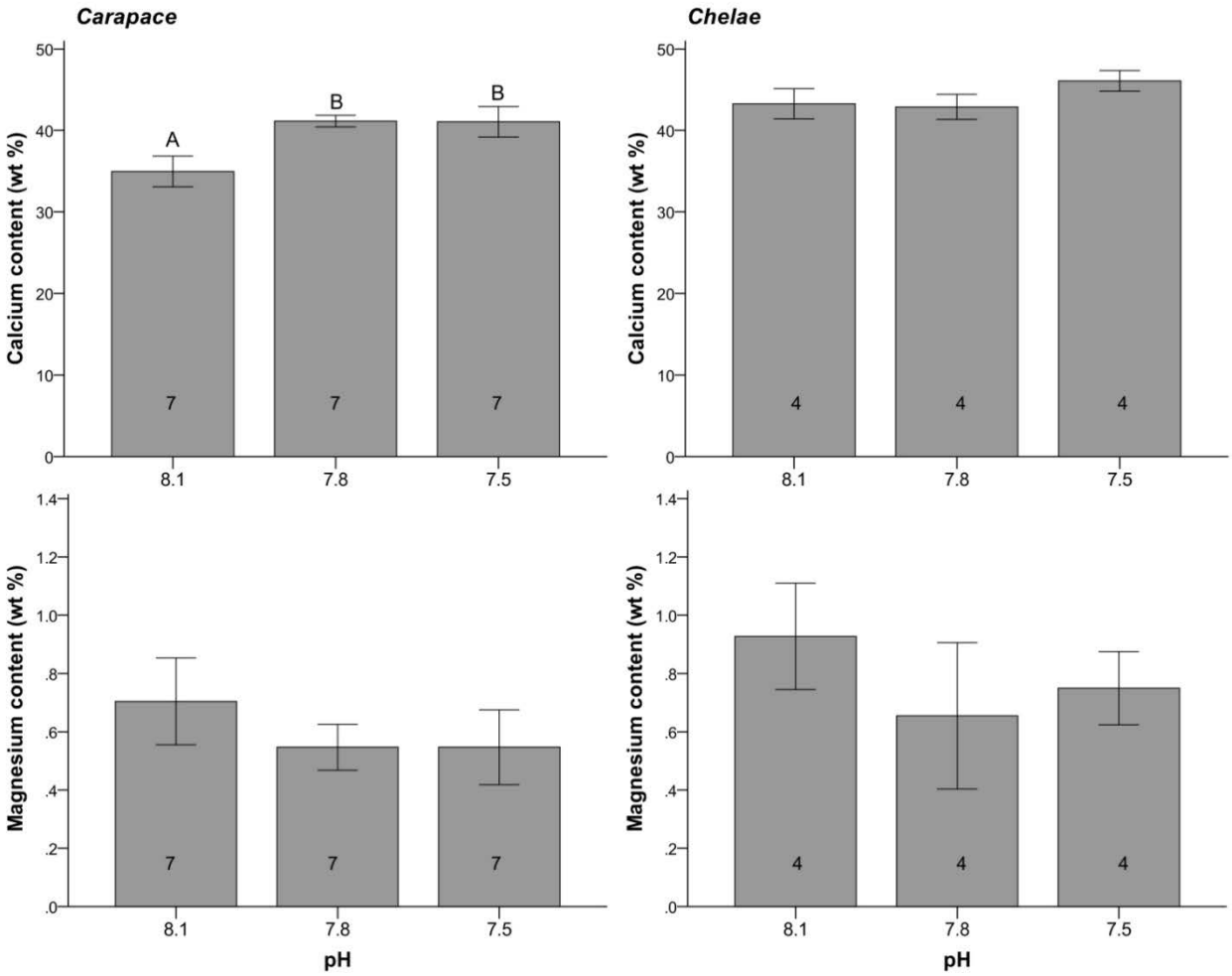


Fig. 6. Calcium and magnesium content of blue king crab, *Paralithodes platypus,* carapaces (left) and chelae (crushing claws, right) (mean ± s.e.m.). Juvenile crabs were exposed to one of three levels of pH for one year. Assessments were conducted on polished cross-sections of the mineralized cuticle, within the endocuticle, using electron dispersive spectroscopy (EDS). Groups marked with different letters are significantly different as shown by Tukey HSD post-hoc analysis. *N* is indicated within each bar and represents individual crab samples.

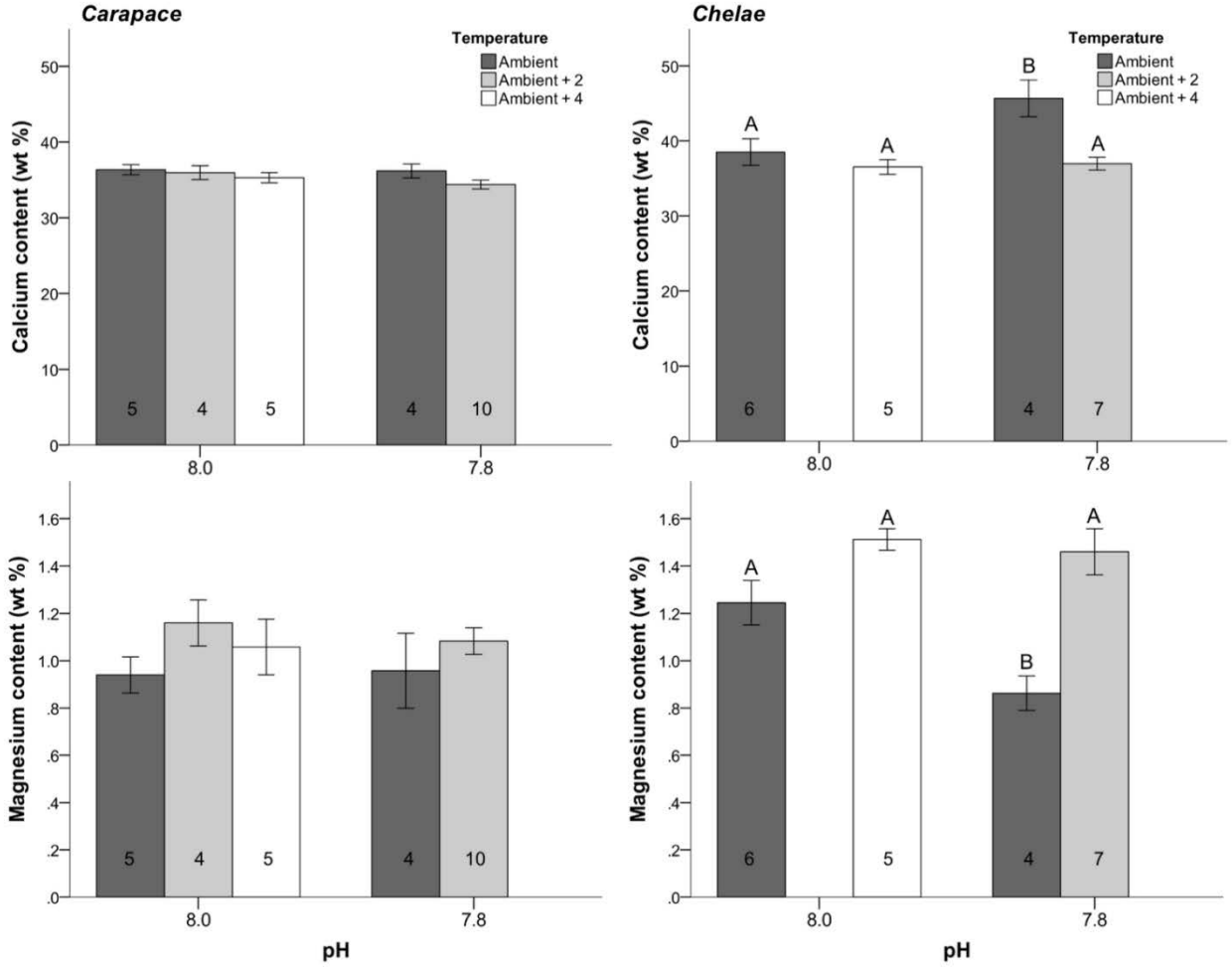


Fig. 7. Calcium and magnesium content of red king crab, *Paralithodes camtschaticus,* carapaces (left) and chelae (crushing claws, right) (mean ± s.e.m.). Juvenile crabs were exposed to one of two levels of pH at one of three levels of temperature (reported in degrees Celsius) for 184 days. Assessments were conducted on polished cross-sections of the mineralized cuticle, within the endocuticle, using electron dispersive spectroscopy (EDS). Groups marked with different letters are significantly different as shown by Tukey HSD post-hoc analysis. Carapace and chelae data for the pH 8.0, ambient + 4°C treatment and chelae data for the pH 7.8, ambient + 2°C treatment are not available due to mortality during exposure and sample damage. *N* is indicated within each bar and represents individual crab samples.