Adult snow crab, *Chionoecetes opilio*, display body-wide exoskeletal resistance to the effects of long-term ocean acidification

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ABSTRACT

Structural and mechanical properties of the decapod exoskeleton affect foraging, defense, and locomotion. Ocean acidification (OA) poses a threat to marine biomes and their inhabitants, particularly calcifying organisms. Vulnerability of the snow crab, *Chionecetes opilio*, a commercially important, high-latitude species, to OA has not been explored. Although all oceans are experiencing acidification, abiotic factors in high-latitude areas increase the rate of acidification. We examined the effect of long-term (2-year) exposure to decreased seawater pH (7.8 and 7.5; P_{CO2} ~760 and 1550 µatm, respectively) on exoskeletal properties in post-terminal-molt female *C. opilio*. Since the effects of OA vary among body regions in decapods, exoskeletal properties (microhardness, thickness, and elemental composition) were measured in five body regions: the carapace, both claws, and both third walking legs. Overall, adult *C. opilio* exoskeletons were robust to OA in all body regions. Decreased pH had no effect on microhardness or thickness of the exoskeleton, despite a slight (~6%) reduction in calcium content in crabs held at pH 7.5. In contrast, exoskeletal properties varied dramatically among body regions regardless of pH. The exoskeleton of the claws was harder, thicker, and contained more calcium but less magnesium than that of other body regions. Exoskeleton of the legs was thinner than that of other body regions and contained significantly greater magnesium concentrations (~2.5 times higher than the claws). Maintenance of exoskeletal properties after long-term OA exposure, at least down to pH 7.5, in adult *C. opilio* suggests that wild populations may tolerate future ocean pH conditions.

INTRODUCTION

The absorption of anthropogenic CO_2 has caused oceanic pH levels to decrease by ~0.1 units since the beginning of the industrial revolution (Caldeira and Wickett, 2003; Orr et al. 2005; Doney et al. 2009; Doney et al. 2020; Leung et al. 2022). This phenomenon, known as ocean acidification (OA), is predicted to persist and cause pH in ocean surface waters to drop another ~0.3 units by 2100 and ~0.5 units by 2200 (Caldeira and Wickett 2003; Orr et al. 2005; IPCC 2014; Gattuso et al. 2015). Reduced pH of seawater, along with associated changes in carbonate chemistry, can significantly decrease survival and growth in myriad marine taxa, with calcified

algae, corals, and mollusks standing out as the most vulnerable (Kroeker et al. 2010; Kroeker et al. 2013). Although crustaceans were not initially believed to be particularly vulnerable to the effects of OA (Kroeker et al. 2010, 2013; Whittman and Pörtner 2013; Byrne and Fitzer 2019), recent studies with larval and juvenile crustaceans have demonstrated that elevated pCO_2 levels can increase mortality (Miller et al. 2016; Giltz & Taylor 2017, Long et al. 2021), reduce growth (Swiney et al. 2017; McLean et al. 2018), and alter energetics (Long et al. 2019) and behavior (Gravinese et al. 2019). In addition, at all crustacean life stages, OA has been shown to alter the formation and maintenance of the mineralized exoskeleton (Taylor et al. 2015; Meseck et al. 2016; Glandon et al. 2018; Bednaršek et al. 2020; Dickinson et al. 2021; Siegel et al. 2022), potentially limiting the defensive, predatory, and locomotive abilities of these organisms (Page et al. 2016; Coffey et al. 2017). Much of the OA research studying physiological and ecological responses of crustaceans to decreased pH has involved only short-term (~30 days) to medium-term (~ 6 month) exposure to OA; however, many crustaceans can live for a decade or longer, which makes long-term exposure experiments critically important (Whiteley 2011; Siegel et al. 2022).

There have been relatively few studies explicitly exploring the effect of OA on structural and mechanical properties of the mineralized decapod exoskeleton. The exoskeleton protects animals from both environmental (e.g., desiccation, hydrodynamic or mechanical forces) and predatory risks and, in the case of the claws (chelae) and mandibles, is critical for capturing, subduing, and consuming prey. The crab exoskeleton is multilayered, consisting of an outer epicuticle, a procuticle composed of an outer exocuticle and inner endocuticle, and a thin, uncalcified membranous inner layer (Travis 1963; Roer and Dillaman 1984). The exo- and endocuticle are formed by chitin-protein nanofibrils interlacing to create helical structures known as "Bouligand" or "twisted plywood" layers, which are embedded with nanocrystalline magnesian calcite or amorphous calcium carbonate (Bouligand 1972; Roer and Dillaman 1984; Raabe et al. 2006; Boßelmann et al. 2007). When the mechanical properties of the cuticle are compromised, vital functions such as foraging, defense against predators, and locomotion, can suffer reductions in performance efficiency (Juanes and Hartwick 1990). The cuticle provides muscle-attachment sites in many regions of the body, making the functionality of appendages contingent on its integrity (Meyers et al. 2013). Observed effects of OA include reduced microhardness (resistance

to permanent or plastic mechanical deformation) in the claws—but notably not in the carapace—of decapods; this could compromise the 'crushing' abilities of the claws, potentially diminishing defense and foraging abilities (deVries et al. 2016; Coffey et al. 2017; Dickinson et al. 2021). In order to thoroughly investigate how this complex exoskeletal structure is responding to our rapidly changing ocean, more body-region-specific analyses must be conducted on decapod species.

Although the entire ocean is absorbing atmospheric CO_2 and experiencing acidification, high-latitude regions are likely to acidify faster than lower-latitudes regions due to the higher solubility of CO_2 in colder waters (Fabry et al. 2009; Cumming et al. 2011). The Bering Sea has a set of environmental conditions that make its waters particularly vulnerable to OA (Opsahl and Benner 1997; Pilcher et al. 2019). The low temperatures, poorly buffered water, and high climate variability in this region are just some of the factors that make the Bering Sea a research priority in terms of potential biological responses to OA (Mathis et al. 2011a).

The snow crab, *Chionoecetes opilio*, is one of the many valuable commercial species that inhabit the Bering Sea. It has a distribution that spans the northern Pacific and Atlantic Oceans, and the Arctic Ocean (Jadamec et al. 1999). In the Bering Sea, snow crabs are distributed along the continental shelf and upper slope, with most individuals occurring at 50–200 m (Zacher et al. 2020). The lifespan of snow crabs is estimated at 14–16 years for males, and 11–12 years for females, making them a relatively long-lived decapod species (Adams, 1979). Both male and female snow crabs can live 3–5 years after completing their terminal molt and reaching sexual maturity (Alunno-Bruscia & Sainte-Marie 1998; Ueda et al. 2009). In Alaska, snow crabs have supported valuable fisheries, bringing in an ex-vessel revenue of $101.7 million in 2020 (Garber-Yonts and Lee, 2020; NOAA Fisheries 2021). Understanding how future ocean conditions will impact Alaskan snow crab populations is essential to protecting these stocks from possible overharvest (ADF&G 1991).

Carbonate chemistry in snow crab habitat varies both seasonally and spatially. Currently, seasonal stratification combined with benthic remineralization results in pCO_2 values dropping from late summer/early fall highs of 1600 µatm (pH about 7.5) to about 400 (pH 8.1) in the winter when storms mix surface waters down (Mathis et al. 2014). Similarly, across the Bering

Sea shelf, aragonite saturation states in the summer grade from greater than 2 (pH about 8) in shallow water at 60 m or less, to below 1 (pH about 7.8) at depths below 100 m (Mathis et al, 2011b). Projections for the greater Bearing Sea shelf show that average shelf pH is currently below 7.8 for about half the year and below 7.5 for a negligible amount of time, but this will grade to being below 7.8 for about 90% of the year and below 7.5 for 40% of the year by 2100 (Pilcher et al. 2022).

The effects of OA on exoskeletal properties have not been assessed previously in snow crabs. Previous work on a congeneric species, the southern Tanner (hereafter Tanner) crab *Chionoecetes bairdi*, however, revealed high susceptibility of the adult exoskeleton to OA (Dickinson et al. 2021). Two-year exposure to OA conditions resulted in thinning of the cuticle, internal and external dissolution, reduction in claw hardness, and alterations in mineralogy of the carapace. Hence, the goal of this study was to assess the effects of ocean acidification on exoskeletal properties of adult snow crab, *C. opilio*. Post-terminal-molt female snow crabs were held in ambient (~8.1) or reduced pH seawater (7.8 and 7.5) for a period of two years. We then evaluated microhardness and thickness of the two major structural layers of the cuticle, the endocuticle and exocuticle, within five different body regions: the carapace, left and right claws, and left and right third walking legs. Elemental composition in each body region was also assessed. These assessments are crucial because variations in mechanical, elemental, and structural properties of the exoskeleton can lead to differences in functionality.

MATERIALS AND METHODS

Overview

The work presented here is part of a broader project examining the effects of OA on snow crabs, *Chionoecetes opilio*. In brief, ovigerous snow crab were held in the laboratory for two years through two brooding cycles, and embryonic development and hatching successes were monitored. After eggs hatched in the first year, the same adult females were provided with a male to mate with and they extruded a second clutch of embryos. All females used for exoskeleton assessments brooded two clutches of eggs, one per year, for each of two years; there were no differences in reproductive output among treatments. Each year, larvae that hatched

were used in a series of experiments to determine the effects of OA on the larval phase. At the end of the second year, the adult crabs were sacrificed and samples were taken to examine the effects of OA on the exoskeleton of the females. The results of the embryonic and larval studies are presented elsewhere (Long et al., 2022a & b). Sample preparation, and mechanical, structural, and elemental testing generally followed Dickinson et al. (2021), with an expansion of the number of body regions and exoskeletal layers assessed.

Animal collection and OA exposure

Mature female snow crabs, *Chionoecetes opilio*, were collected from the Bering Sea during the eastern Bering Sea trawl survey (Daly et al. 2014) and transported to the NOAA Alaska Fisheries Science Center's Kodiak Laboratory. Upon arrival and throughout the experiment, crabs were held in flow-through, sand-filtered seawater at ambient salinity from Trident Basin (intakes 15 and 26 m) chilled to 2°C with recirculating chillers. Crabs were fed to excess twice a week on a diet of chopped squid and herring. After a brief holding period, 25 crabs were randomly assigned to each of three pH treatments: ~8.1 (ambient), 7.8, or 7.5. Two different holding systems were used during this experiment during different parts of the brooding cycle; however, in both systems the holding conditions were the same, with water acidified with the addition of CO_2, temperatures chilled to a constant 2°C, and flow through seawater at ambient salinity. During the majority of the brooding cycle, crabs were held in experimental tanks (0.6 x 1.2 x 0.6 m), one per treatment. During this period, water was acidified per Long et al. (2013a). In brief, water was acidified by mixing ambient seawater with seawater from a super-acidified tank (pH 5.5, acidified via bubbling of CO_2) in head-tanks (one per treatment). The ambient-treatment head-tank contained only ambient water with no input from the super-acidified tank. Super-acidified water was mixed into acidified head-tanks via peristaltic pumps that were regulated by Honeywell controllers and Durafet III pH probes placed inside the head tanks (see Long et al. 2013a for a diagram of this system). As embryos neared hatching, adult female crabs were moved into individual 68-L tubs. This was necessary so that the number of larvae hatched from each female could be counted (see Long et al. 2022a for details). Tubs received recirculating flow from 2000-L tanks that received flow-through water that was acidified by direct bubbling of CO_2 controlled by a Durafet III pH probe (Fig. S1). Although this design, holding crabs in a single tank for each treatment, or in individual tubs with water recirculating from a common

head tank, is technically pseudoreplication, there is no known mechanism by which the presence of other crabs might have affected the exoskeleton of each other and we ignore tank affects in all analyses. Both of the experimental setups supplied crabs with water at the same temperatures and, in acidified treatments, with water acidified with CO_2 to the same pH and using the same feedback mechanism. In addition, all crabs were transferred between the setups at the same time negating any potential bias caused by the two different sets of holding conditions.

Temperature and pH (free scale) were measured in experimental units daily using a Durafet III pH probe calibrated with TRIS buffer (Millero 1986). Water from the head tanks was sampled once per week (N = 98 per treatment) and samples were poisoned with mercuric chloride and analyzed for dissolved inorganic carbon (DIC) and total alkalinity (TA) at an analytical laboratory. DIC and TA were determined using a VINDTA 3C (Marianda, Kiel, Germany) coupled with a 5012 Coulometer (UIC Inc.) according to the procedure in DOE (1994) using Certified Reference Material from the Dickson Laboratory (Scripps Institute, San Diego, CA, USA; Dickson et al. 2007). The other components of the carbonate system were calculated in R (V3.6.1, Vienna, Austria) using the seacarb package (Lavigne and Gattuso 2012). Crabs were held in experimental conditions for two years and were monitored for mortality daily. At the end of the two-year exposure period, surviving crabs were sacrificed and cuticle samples were taken and kept frozen at -80°C. The total number of surviving crabs was 4 in the ambient treatment, 13 in the pH 7.8 treatment, and 10 in pH 7.5 treatment. Samples were transported on dry ice to The College of New Jersey (Ewing, NJ) for analysis. All samples remained frozen during transit and, upon arrival, were kept at -70°C until further use.

Table 1. Seawater chemistry parameters. pH and temperature were measured daily (N=681 per treatment). Dissolved inorganic carbon (DIC) and alkalinity were measured weekly (N=98 per treatment). Other parameters were calculated (see Materials and Methods). pH_F, pH on the free proton scale; $\Omega_{Calcite}$, calcium carbonate saturation; SW, sea water. Data are means ± SD.

	pH 8.1	**pH 7.8**	**pH 7.5**
pH_F	8.11 ± 0.08	7.80 ± 0.02	7.50 ± 0.02
Temperature (°C)	2.09 ± 0.32	1.97 ± 0.30	2.05 ± 0.31

P_{CO2} (µatm)	362.18 ± 68.33	760.98 ± 43.95	1548.29 ± 102.11
DIC (mmol kg^{-1} SW)	2.01 ± 0.04	2.09 ± 0.05	2.15 ± 0.06
HCO_3^- (mmol kg^{-1} SW)	1.90 ± 0.05	2.00 ± 0.04	2.04 ± 0.06
CO_3^{2-} (mmol kg^{-1} SW)	0.09 ± 0.02	0.05 ± 0.00	0.02 ± 0.00
Total alkalinity (µmol kg^{-1} SW)	2110 ± 20	2090 ± 20	2110 ± 20
$\Omega_{Calcite}$	2.19 ± 0.37	1.11 ± 0.06	0.57 ± 0.04

206

207 ***Sample Preparation***

208 Cuticle samples were taken from standardized locations in five body regions: the carapace, both
209 claws, and both third walking legs. From each crab and each body region, two cuticle samples
210 were cut using a water-cooled diamond band-saw (Gryphon, C-40); one of these was embedded
211 in epoxy resin and polished for micromechanical and structural assessments while the other was
212 used for elemental analyses. All segments were lyophilized for ~18 hours (Yamato, DC41-A)
213 immediately after cutting. Within the carapace, the two segments were cut immediately adjacent
214 to one another, both taken from the posterior margin. For left and right claws, the dactylus
215 (movable finger) and pollex (fixed finger) were cut from the manus; dactyli were embedded and
216 used for micromechanical and structural assessments while pollexes were used for elemental
217 analyses. Similarly, for the left and right legs, the most distal segment (the dactyl or
218 dactylopodite) was embedded and used for micromechanical and structural assessments while
219 the segment proximal to this (the propodus or propodite) was used for elemental analyses. Note
220 that a portion of the crabs were missing a claw or third walking leg at the end of the experimental
221 exposure so samples could not be taken; for consistently, other legs were not substituted for the
222 third walking leg.

223

224 Cuticle segments to be used in micromechanical and structural analyses were embedded
225 individually in epoxy resin (Allied High Tech, Epoxy Set), ground, and polished as described in
226 Coffey et al. (2017) and Dickinson et al. (2021). Samples were ground and polished on a
227 grinding/polishing machine (Allied High Tech, M-Prep 5 or Met-Prep3 PH-4). Grinding steps
228 employed a series 180, 320, 600 and 800 grit silicon carbide papers, followed by polishing with a

1 μm diamond suspension and a 0.04 μm colloidal silica suspension until the samples were completely smooth and free of scratches. Griding and polishing was used to produce a cross-section along the anterior-posterior axis of carapace samples (normal to the dorsal surface of the carapace), while grinding/polishing of claw and leg dactyl samples produced a cross-section along the longest (longitudinal) axis. Polished samples were stored in a desiccator until testing.

Micromechanical properties

Vickers microhardness testing was conducted on embedded and polished samples. Testing was conducted on a microindentation hardness tester (Mitutoyo, HM-200) following standard procedures (ASTM 2017). Indents were made at 1 g load, 5 s dwell time. Two series of indents were made: one in the endocuticle and one in the exocuticle. The two cuticle layers could be readily differentiated from one another on the hardness tester (under reflected light), as there was a dramatic difference in the thickness of Bouligand layers when moving from the endocuticle to the exocuticle (i.e., layers were more densely packed in the exocuticle). Within each layer, 10 replicate indents were made, with the first indent approximately 500 μm from the edge of the sample and each subsequent indent spaced about 200 μm apart. For leg (dactylopodite) samples, the most distal tip of the sample was avoided, as cuticle wear and damage was visible in many samples. Individual indents were measured directly on the hardness tester under a 100 X objective and Vickers microhardness values were automatically calculated. Microhardness of replicate indents within a sample and within a cuticle layer were averaged to determine the mean microhardness for each sample.

Cuticle thickness

Following microhardness testing, the same embedded samples were used to quantify four structural metrics: total thickness of the cuticle, exocuticle thickness, endocuticle thickness, and thickness of individual Bouligand layers that comprise the endocuticle. Samples were imaged under a reflected light microscope (Zeiss, AxioScope A1 with a Zeiss, AxioCam 105 color camera) using a 2.5 X objective (~100 X total magnification) and darkfield illumination. Panoramic images of the entire sample were constructed using the camera's analysis software (Zeiss, Zen v. 2.3; Fig. S2). Thickness was measured on digital images following the methods of Nardone et al. (2018) and Coffey et al. (2017). A grid was placed on each image (200 x 200 μm

for carapace samples; 500 x 500 μm for claw and leg samples) and cuticle thickness was measured using a linear line tool each time the grid crossed the sample. Total thickness and endocuticle thickness were measured separately at each point; exocuticle thickness was calculated as the difference between total and endocuticle thickness. The endo- and exocuticle layers were differentiated from one another based on the thickness of Bouligand layers (Roer and Dillaman 1984); there was a distinct shift when moving from the endocuticle to the exocuticle in Bouligand layer thickness (i.e., layers were thinner and more densely packed in the exocuticle; Fig. S2). This resulted in a clear shift in coloration under darkfield illumination. At least 10 replicate measurements were made for each parameter within each sample, with the total number of measurements dependent on the size of the sample. Replicate measurements for each metric (total thickness, exocuticle thickness, endocuticle thickness) were averaged separately to determine the mean for each sample. Thickness of the Bouligand layers that comprise the endocuticle was measured by taking three additional images of the endocuticle under a 50 X objective (~1,600 X total magnification) and brightfield illumination. The three images were spaced roughly evenly along the length of the polished sample. Within each image, three separate distance lines were drawn perpendicular to the Bouligand layers using the camera's analysis software; each line spanned 10 distinct Bouligand layers. The total length of the line was divided by 10 to determine average thickness of individual Bouligand layers. The 9 replicate measurements (3 images with 3 measurements per image) were averaged to determine the mean Bouligand thickness for each sample. A similar procedure was attempted within the exocuticle, but the density of Bouligand layers precluded accurate measurements.

Elemental composition

Elemental composition was measured at the U.S. Geological Survey's Coastal and Marine Science Center, St. Petersburg, FL. Inductively coupled plasma optical emission spectrometry (ICP-OES) was used to measure calcium, magnesium, and strontium content within the carapace, right and left claws, and right and left third walking leg. Methods followed those described in Gravinese et al. (2016) and Steffel et al. (2019). Samples were cut from each body region, as described above, and any adhering tissue was removed using a scalpel and forceps. Samples were first oxidized by sonication in a 1:1 mixture of 30% H_2O_2 and 0.1 M NaOH for 20 minutes. This was followed by sonication in Milli-Q water for 5 minutes. This oxidation procedure was

repeated before samples were removed from solution and dried overnight at 90°C. Dried samples were ground into a fine powder by mortar and pestle, and the oxidation process described above was repeated on the powdered samples. Oxidized samples were dried again at 90°C for at least 3 hours before analyses. Samples were weighed and acidified in 2% HNO_3, then measured for Ca^{2+}, Mg^{2}+, and Sr^{2+} using a PerkinElmer 7300 dual-view ICP–OES. Elemental weight-percentages were calculated for each sample by multiplying concentration by the volume of HNO_3 added prior to ICP-OES analysis, and then dividing by the total dry weight of the sample using the conversion 1 ppm = 1 mg/L (Long et al., 2013b).

Statistical analysis

The exoskeletal properties of *C. opilio* were assessed using a combination of multivariate and univariate statistical procedures. Multivariate approaches incorporated all measured variables to assess the effect of seawater pH on exoskeletal properties, as well as if these properties varied among body regions. Variables were normalized (expressed in terms of their z value) before multivariate analysis and visualized with a non-metric multidimensional scaling (nMDS) plot based on a Euclidian-distance resemblance matrix. Differences among treatments were then analyzed with a permutational analysis of variance (PERMANOVA) with treatment fully crossed with body region and crab identification number (unique to each individual crab) nested within treatment as factors. Differences in dispersion were analyzed with a permutational analysis of dispersion (PERMDISP) in order to help differentiate between effects of differences in data location and dispersion. These analyses were followed by a principal component analysis (PCA) and SIMPER analysis, which were used to identify the factors driving differences among body regions. Multivariate analyses were conducted using Primer (v. 7, Primer-E). The effect of seawater pH and body region on each individual micromechanical, structural, or elemental variables was assessed using a general linear model (GLM) for each variable, followed by Tukey HSD *post hoc* testing. Treatment pH and body region were treated as fixed factors; crab identification number was used as a blocking factor, with crab identification number nested within treatment pH. Univariate analyses were conducted in SPSS (v. 25, IBM Analytics). For nMDS, PERMANOVA, PERMDISP, and PCA data for each individual body region (i.e., carapace, left claw, right claw, left leg, right leg) was included separately within the analyses. Data from the two claws and two legs were combined for SIMPER and univariate analyses. All

datasets generated during the current study are available as a supplemental document and sample sizes for structural, mechanical and chemical analyses are included in Table S1.

RESULTS

Exoskeletal properties of snow crabs differed among body regions but not among pH treatments (PERMANOVA, Table S2). Dispersion, a measure of spread in multivariate data analogous to variance in univariate statistics, differed among body regions (pseudoF = 2.755, p = 0.033), but not pH treatments (pseudoF = 0.829, p = 0.440) or crabs (pseudoF = 0.661, p = 0.860). When both PERMDISP and PERMANOVA are significant, this indicates that either just the dispersion differs among treatments or that both dispersion and location (multivariate analog for the mean) differ; examination of an nMDS plot can help to distinguish between these two possibilities (Anderson et al. 2008). The nMDS plot showed clear differences among sampled body regions with legs, claws, and the carapace all separating from one another and having virtually no overlap; from this we conclude that the significant PERMANOVA was driven by differences in both location and dispersion (Fig. 1A). Conversely, there were no differences among pH treatments (Fig. 1B), at least under the experimental conditions and sample size tested here. Post-hoc pairwise comparisons (PERMANOVA) showed that each body region differed significantly from all other body regions ($p < 0.05$), except that the left and right legs were not significantly different from one another. Of note, and as shown in in Fig. 1A, post-hoc pairwise comparisons show a significant difference between the left and right claws ($p < 0.05$), although there is some overlap of the two in the nMDS plot (Fig. 1A).

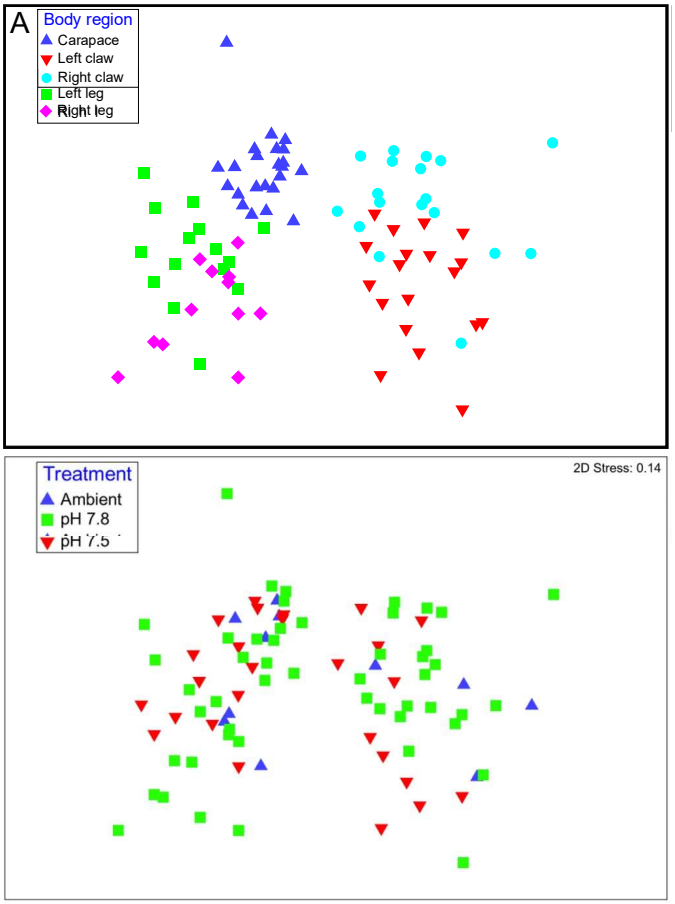

Fig. 1. Non-metric multidimensional scaling (nMDS) plots incorporating micromechanical, structural, and elemental variables. The same plot is coded by either (**A**) body region or (**B**) pH treatment. Data were normalized prior to analysis (see text for details). Stress is 0.14.

Principal component analysis (PCA) was used to visualize which factors drove the differences among body regions and SIMPER analysis was used to quantify the differences (Fig 2; Table S3). In general, the exoskeleton of claws was thicker, harder, and had higher calcium content (but lower magnesium content) than that of the carapace and legs. The carapace exoskeleton was thicker but less hard than that of the legs. Magnesium content tended to be highest in the legs.

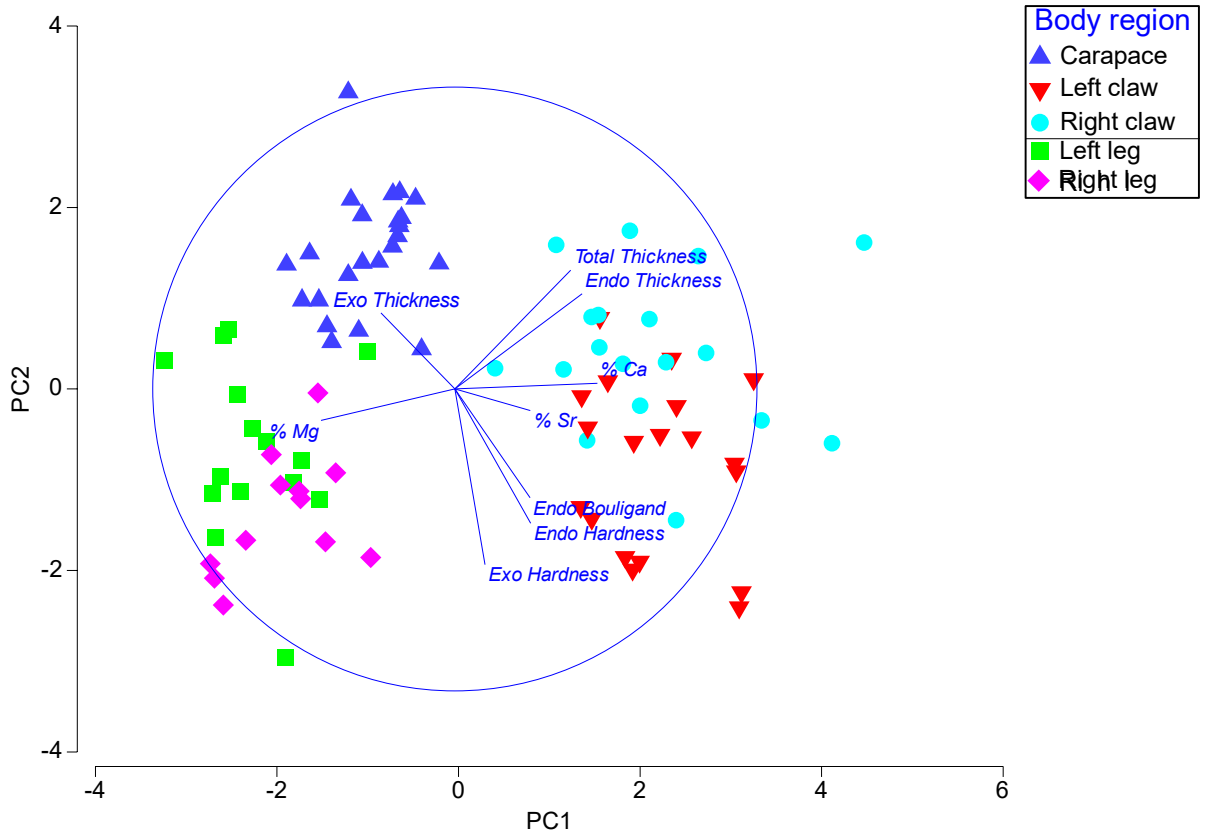

Fig. 2. Principal component plot of observations of exoskeletal properties (microhardness, elemental content, and structure) among body regions. Data were normalized prior to analysis (see text for details). Vectors indicate the loadings of the variables. PC1 and PC2 contain 46% and 20% of the overall variance, respectively.

To further assess the effects of seawater pH, body region, and their interaction, each micromechanical, structural, or elemental variable was also assessed individually. Results were generally in agreement with multivariable assessments showing a strong effect of body region, but minimal effect of seawater pH, on exoskeletal properties. Seawater pH did not affect microhardness in either the endocuticle or exocuticle (GLM: $p > 0.05$; Fig. 3 & Tables S4 & S5). Microhardness, however, varied among body regions for both cuticle layers (GLM: $p < 0.0001$). Endocuticle microhardness of the claws was 73% greater than that of the carapace and 38% greater than the legs (Tukey HSD: $p < 0.05$). Exocuticle hardness was ~60% greater in the claws and legs as compared to the carapace (Tukey HSD: $p < 0.05$) but did not differ significantly

between the claws and legs. The interaction of pH and body region was not significant for either layer.

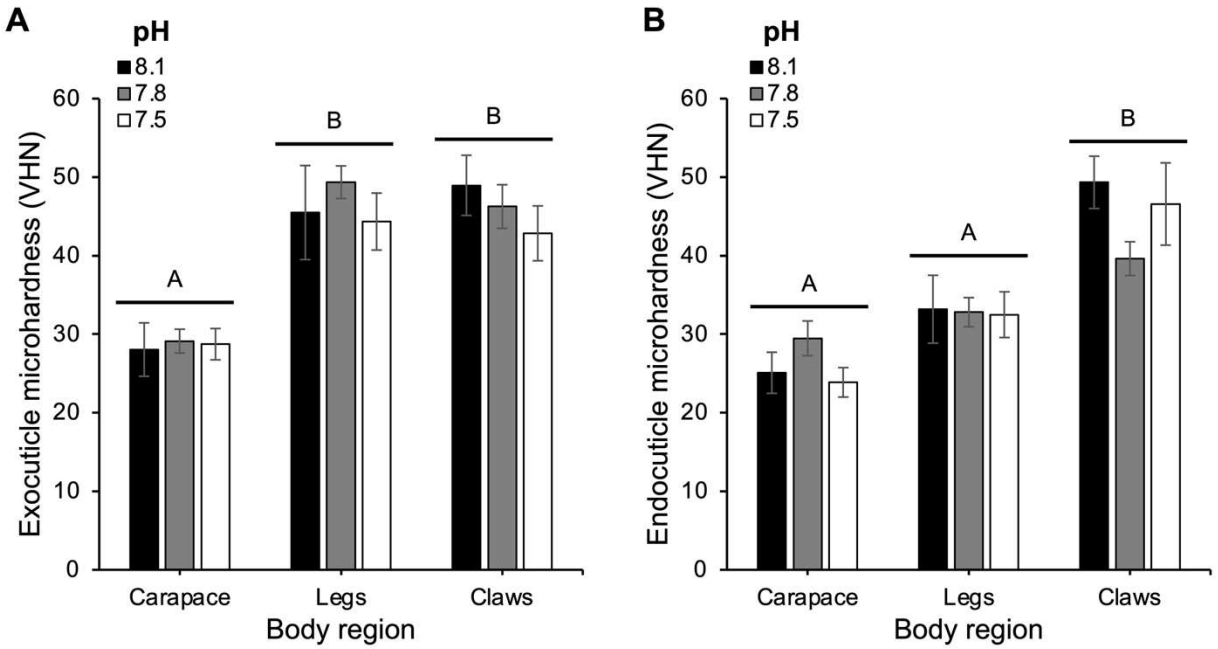

Fig. 3. Vickers microhardness tested in the *C. opilio* exocuticle (**A**) and endocuticle (**B**) after exposure to one of three pH levels for 2 years. Means ± SE are shown. Different letters represent significant pairwise differences between body regions (Tukey HSD: $p < 0.05$). pH treatments did not differ from one another. $N = 3–22$.

Treatment pH did not affect any of the structural variables assessed (GLM: $p > 0.05$; Fig. 4 & Tables S4 & S5), but the effect of body region was significant in all cases (GLM: $p < 0.0001$). For total cuticle thickness and endocuticle thickness, each body region differed from each other region (Tukey HSD: $p < 0.05$; Fig. 4A & C); total thickness was greatest in the claw, intermediate in the carapace, and lowest in the legs. Exocuticle thickness showed the opposite response, with thickness lower in the claws as compared to the carapace and legs (Tukey HSD: $p < 0.05$; Fig. 4B). Thickness of the Bouligand layers that comprise the endocuticle differed among each body region, with Bouligand layer thickness greatest in the claws and lowest in the carapace (Tukey HSD: $p < 0.05$; Fig. 4D). The interaction of pH and body region was not significant for any of the structural variables assessed.

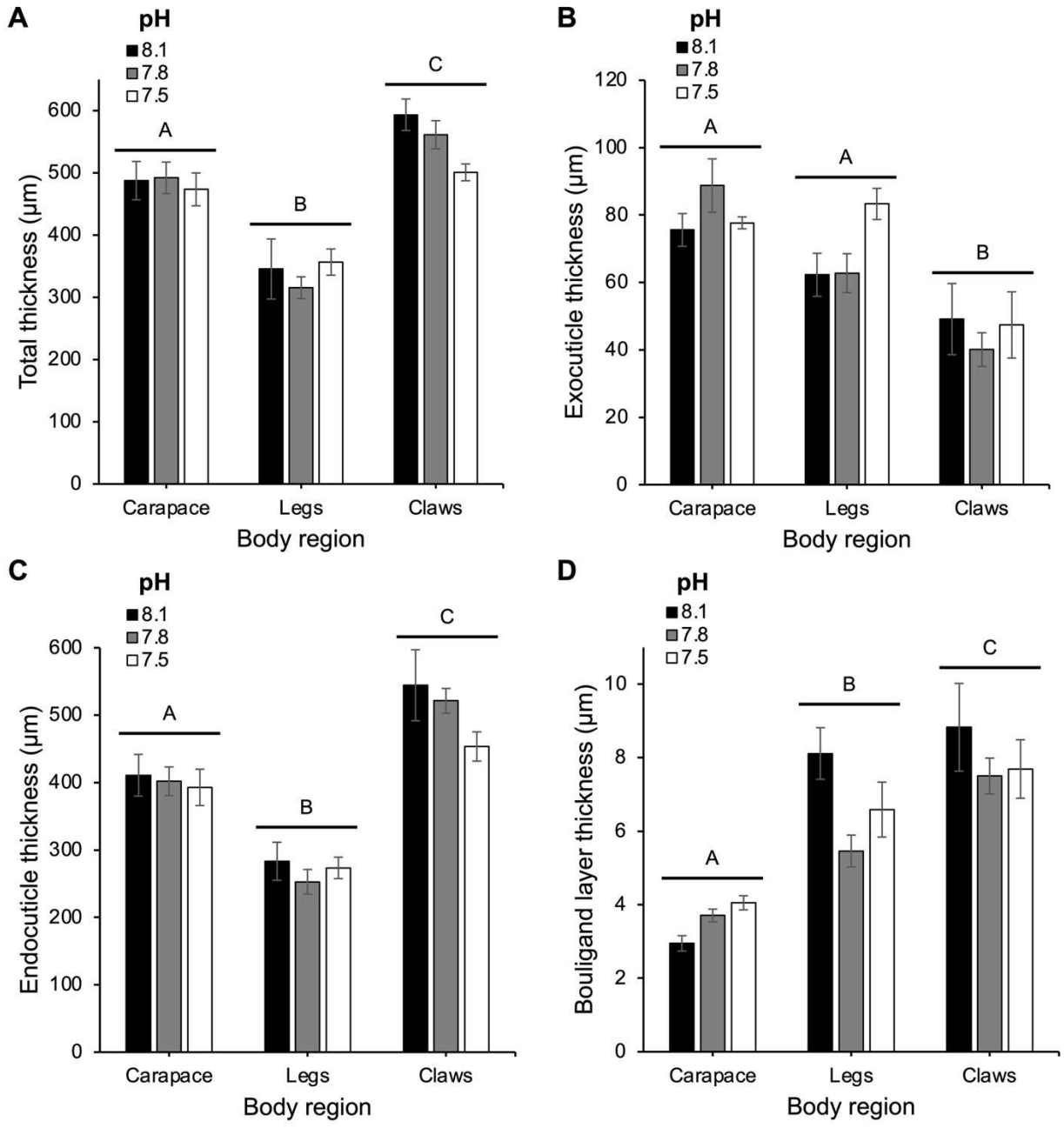

Fig. 4. Structural variables measured in the *C. opilio* cuticle after exposure to one of three pH levels for 2 years. Means ± SE are shown. Different letters represent significant pairwise differences between body regions (Tukey HSD: $p < 0.05$). pH treatments did not differ from one another. $N = 3–22$.

Unlike other measured variables, there was a slight, but significant, effect of treatment pH on calcium and magnesium content (GLM: $p < 0.05$; Fig. 5A–B & Table S4). Calcium content was

~6% greater in crabs held at pH 8.0 and 7.8 as compared to those at pH 7.5 (Tukey HSD: $p < 0.05$). Magnesium content differed between the pH 7.8 and pH 7.5 treatments, with magnesium content about 8% higher at pH 7.5 (Tukey HSD: $p < 0.05$). Overall, the effect of pH treatment on strontium content was not significant (GLM: $p = 0.075$; Fig. 5C & Tables S4 & S5). The effect of body region was significant for all elemental variables assessed (GLM: $p < 0.0001$; Fig. 5 & Table S4). Among body regions, each body region differed from each other body region for calcium and magnesium content (Tukey HSD: $p < 0.05$). Calcium content was greatest in the claws, intermediate in the carapace, and lowest in the legs with calcium content in the legs about half that of the claws. In contrast, magnesium content was greatest in the legs, intermediate in the carapace, and lowest in the claws; magnesium content was 2.5 times greater in the legs than the claws. Strontium content was greater in the claws as compared to the legs and carapace (Tukey HSD: $p < 0.05$), but did not differ between the legs and carapace. The interaction of pH and body region was not significant for calcium, magnesium, or strontium content.

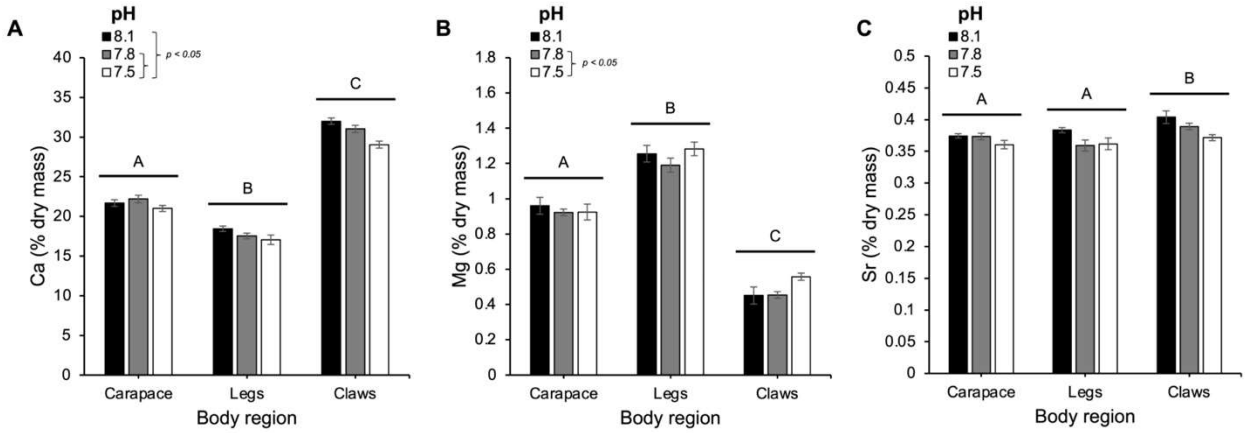

Fig. 5. Elemental content measured in the *C. opilio* cuticle after exposure to one of three pH levels for 2 years. Means ± SE are shown. Letters denote significant pairwise differences between body regions and brackets represent significant pairwise differences between pH treatments (Tukey HSD: $p < 0.05$). $N = 3–22$.

DISCUSSION

In this study, we quantified the effects of OA on adult snow crab exoskeletons in multiple body regions after a two-year exposure in order to understand how future ocean conditions might influence activities crucial to survival such as feeding, defense, and locomotion. Multivariate

analyses of all measured variables and body regions showed no effect of exposure pH on the exoskeletal properties of *C. opilio*, at least under the experimental conditions (reduced pH 7.8 and 7.5) and sample sizes tested here. Although there was a slight (~6%) decrease in exoskeletal calcium content at reduced seawater pH (7.5), microhardness and thickness were unaffected by decreased pH at any level, suggesting that this difference may have little practical consequence. On the other hand, there were substantial differences among the body regions, which highlights that the structural and mechanical properties of the decapod exoskeleton are well-adapted to the physical demands placed on those particular body regions. In contrast to other decapod species (e.g., Coffey et al. 2017; Dickinson et al. 2021), it appears that adult snow crabs are relatively resilient to the effects of reduced pH in terms of exoskeletal properties.

Since decapods use calcium carbonate, in the form of nanocrystalline magnesian calcite or amorphous calcium carbonate, to harden their exoskeletons (Roer and Dillaman 1984; Dillaman et al. 2005), it is possible that changes in seawater carbonate chemistry could affect both the formation and maintenance of their cuticles (Siegel et al. 2022). There are three primary mechanisms by which reduced pH could affect the decapod exoskeleton. First, if the calcium carbonate saturation state of seawater (Ω) drops below 1 then external (abiotic) dissolution could occur (i.e. thermodynamically, dissolution is favored; Waldbusser et al. 2016). In decapod crustaceans, the epicuticle, the predominantly organic (wax and protein) outermost layer of the cuticle (Roer and Dillaman, 1984; Fabritius et al., 2012), effectively protects the calcified cuticle layers from direct contact with seawater (Ries et al. 2009). Hence, external dissolution would be restricted to either sites where the epicuticle had been damaged or sites, such as on the denticles on the claws, where the epicuticle has been worn off by constant use (Rosen et al. 2020; Dickinson et al. 2021). Second, shifts in environmental pH can cause changes in the hemolymph pH of decapods in the short-term, and the extent to which these changes are compensated for can vary among species (Pane and Barry, 2007). If osmoregulatory functions, which are the primary means by which decapods maintain acid-base balance in their hemolymph (Melzner et al. 2009; Whitely 2011), are unable to completely compensate for the change in pH, a prolonged decrease in hemolymph pH could make it more difficult to precipitate calcium carbonate during shell formation or lead to internal dissolution of the exoskeleton. It is important to note, however, that most decapods that are able to maintain acid-base homeostasis under ocean acidification

conditions do so, at least in part, by buffering their hemolymph with bicarbonate via Cl^-/HCO_3^- exchange at the gills (Pane and Barry 2007; Whiteley 2011, Appelhans et al. 2012). This response could make precipitation of calcium carbonate more likely, and could explain why many decapods show increased calcification rates or content in response to ocean acidification (Ries et al. 2009; Long et al. 2013b; Glandon et al. 2018). Finally, ocean acidification can induce changes in the expression of genes involved in cuticle formation; red king crab adults and juveniles both exhibited an increase in the expression of such genes (Stillman et al. 2020). The findings of this study, showing that the micromechanical and structural properties of the snow crab were not altered by exposure to decreased pH levels, suggests that snow crabs may be relatively resistant to long-term exposure to reduced pH. Thus, post-terminal molt snow crabs may possess a largely in-tact epicuticle, have strong acid-base regulatory capacity, are able to alter their gene expression to maintain their cuticles, or a combination of these traits. Future experiments should examine the physiological response and gene expression patterns in snow crab to elucidate the mechanism(s) of exoskeletal growth and maintenance.

Elemental analysis of the exoskeleton revealed a slight, but significant, reduction in calcium content and increase in magnesium content in crabs exposed to pH 7.5. This shift in elemental composition of the carapace also increased the Mg^{2+}:Ca^{2+} ratio of the exoskeleton. Higher calcite Mg^{2+}:Ca^{2+} ratios correspond to higher solubility (Morse et al. 2006; Andersson et al. 2008; Chen et al. 2008) but also higher strength, as substitution of Mg^{2+} within the calcium carbonate matrix can impact fracture propagation and dislocation motion (Magdans and Gies 2004; Kunitake et al. 2012; Kunitake et al. 2013). Despite these alterations in mineral content of the cuticle, there were no changes in cuticle thickness or micromechanical properties. This suggests that either the 6% reduction in calcium content was not sufficient to cause a detectible difference in micromechanical properties of the cuticle, or that the elevated magnesium content increased the hardness of the mineral resulting in no net change in overall hardness levels. These results also highlight that calcium content alone is not a direct predictor of cuticle mechanical or structural properties in decapods. In both juvenile red and blue king crabs, elevated calcium content under OA conditions was accompanied by diminished microhardness (Coffey et al. 2017). Similarly in Tanner crabs, calcium content in the claws was unchanged despite decreased microhardness, whereas in the carapace a decrease in calcium content did not affect microhardness (Dickinson et

al. 2021). Like the snow crabs in this study, the decreased calcium content in the carapace of Tanner crabs was accompanied by an increase in magnesium content and FTIR spectroscopy showed a shift in the mineral phase of calcium carbonate from amorphous calcium carbonate to calcite (Dickinson et al. 2021). It may be, then, that the disconnect between calcium and hardness is at least partially explained by the mineral phase of calcium carbonate. These findings highlight that researchers should be cautious in making inferences regarding cuticle strength or mechanical properties in decapods based on calcium content measurements alone.

The finding that exoskeletal properties of adult snow crabs are not particularly susceptible to OA is unexpected because of the apparent vulnerability of the Tanner crab (*Chionoecetes bairdi*), the snow crab's close relative, to OA. Both the snow crab and the Tanner crab have life expectancies upwards of 10 years, live at similar depths, and endure the highly variable pH fluctuations of the Bering Sea for the duration of their relatively long lives. A similar long-term OA exposure experiment showed that adult Tanner crabs experienced 15% and 31% reductions in the total thickness in the claw and carapace, respectively, in response to exposure to pH levels of 7.5 (Dickinson et al. 2021). Reduced pH also caused decreased endocuticle hardness of adult Tanner crabs, whereas the micromechanical properties of snow crabs were unaffected by pH treatment level. Although the mechanisms driving observed differences between *Chionoecetes* species in susceptibility to OA remain unknown, it is worth noting that the species-specific differences described here mirror those reported for other life stages in these species. For example, in Tanner crab, OA exposure during oogenesis resulted in a 70% reduction in hatch success (Swiney et al. 2016). OA increased mortality and reduced growth and calcification in juvenile Tanner crabs (Long et al., 2013a), and in adults, increased hemocyte mortality and decreased intracellular pH were observed after OA exposure (Meseck et al. 2016). In contrast, hatching success, survival, and embryonic morphology were unaffected by OA in snow crabs, and both direct and carryover effects of OA on larval survival, morphology, and calcification were negligible (Long et al. 2022a & b). The findings of this study paired with previous findings support that snow crabs, although morphologically and ecologically similar to the Tanner crabs, are better equipped for survival in extreme pH conditions.

Although there was little variation in exoskeletal properties among pH treatments, exoskeletal properties varied dramatically among body regions. We found that claws were harder and thicker, and that they contained more calcium but less magnesium than the carapace and legs. The exoskeleton of the legs was thinner than other body regions but contained substantially more magnesium. These observations add to a growing body of evidence that the structural and mechanical properties of the crustacean exoskeleton vary, often dramatically, with function (e.g., Boßelmann et al. 2007; Chen et al. 2008; Politi et al. 2019; deVries et al. 2021; Inoue et al. 2021; Wang et al. 2022). Such variation in exoskeletal properties among body regions has been observed both in animals assessed directly after field-collection (e.g. Steffel et al. 2019; Rosen et al. 2020) as well as those exposed to laboratory conditions for months to years (e.g. Coffey et al. 2017; Dickinson et al. 2021; deVries et al. 2021; Lowder et al. 2022). Here, claws were found to be hard and resistant to mechanical deformation within the exo- and endocuticle, making them resistant to wear and abrasion and able to withstand high mechanical force from predatory or defensive uses. Though thin, the outer mineralized layer of the legs, the exocuticle, showed microhardness substantially higher than the inner endocuticle (consistent with Chen et al. 2008), with exocuticle microhardness comparable to that of the claws. As the most distal segment of the leg, the dactylopodite is likely to experience almost constant wear and abrasion as they are the segment of the leg that comes in contact with the sea floor; the enhanced microhardness of the leg exocuticle found here supports greater resistance to wear and abrasion. Elevated magnesium content in the legs may contribute to elevated hardness (Kunitake et al. 2012; 2013) and may also stabilize amorphous calcium carbonate (ACC) within the exoskeleton (Weiner et al. 2003; Addadi et al. 2003). Calcium content, magnesium content, and thickness of the carapace was intermediate to the legs and claws, with consistently lower hardness as compared to the claws. Although the carapace must protect the internal organs, it must also be sufficiently flexible and elastic to enable movement (Boßelmann et al. 2007). Altogether, the body region specific differences observed support highly-adaptable mineralization processes within the Crustacea (Lowenstam and Weiner 1989).

In terms of body-region-specific differences in exoskeletal properties, one surprising finding was the separation of left and right claws in multivariate analyses. The right claw was thicker but exhibited lower hardness in both the exo- and endocuticle compared with the left claw. This

exoskeletal asymmetry is unusual because, unlike crab species that display strong claw dimorphism, snow crabs appear by eye to have bilateral chelal symmetry. For example, fiddler crabs not only have evident bilateral chelal asymmetry, but the right and left claws differ in function (Pope et al. 2000; Darnell et al. 2011). The major claw of the male fiddler crab functions as an ornament and weapon in courtship contests, whereas the minor claw is used for feeding, foraging, and grooming (Crane 1966; Christy 1982). Although the male fiddler crab serves as an extreme example, chelal asymmetry as a result of handedness, or heterochely, is well-developed and immediately apparent in many decapod species (Vermeij 1977; Abby-Kalio and Warner 1989; Seed and Hughes 1997; Schenk and Wainwright 2001). Behavioral bias in claw preference for performing various activities can induce morphological asymmetries in Brachyrun crabs, resulting in species-wide heterochely (Smith and Palmer 1994). There is very little evolutionary insight into the heterochely of snow crabs, as handedness in other members of the genus *Chionoecetes*, *C. japonicus* and *C. bairdi,* has not been examined. The basis for varying chela micromechanical properties in this species may very well be attributed to functional differences between the two claws (Govid et al. 1985; Herrick 1895). Experiments assessing the snow crab's behavioral responses to predator and prey presence would be beneficial in gaining more insight on these aspects of *Chionoecetes* behavior.

CONCLUSIONS

Exoskeletal structural integrity is critical in crustacean locomotive, predatory, and defensive activities. Although decreased pH levels can cause exoskeletal dissolution in a number of crustaceans (Pansch et al. 2014; Nardone et al. 2018; Bednaršek et al. 2020; Dickinson et al. 2021), adult snow crab, *C. opilio*, display resilience to predicted changes in seawater chemistry, at least under the experimental conditions tested here. These findings suggest that snow crab populations in the eastern Bering Sea may not be drastically affected by ocean acidification, although studies with a more extreme reduction in pH (i.e., below 7.5) are necessary to fully assess their physiological tolerance. This study also revealed a dichotomy within the *Chionoecetes* genus. The susceptibility of Tanner crabs to exoskeletal dissolution was particularly high (Dickinson et al. 2021), whereas snow crabs did not experience any apparent cuticle dissolution when exposed to reduced seawater pH (down to pH 7.5). This is despite the

fact that *C. bairdi* and *C. opilio* reside in the same depths of the eastern Bering Sea and have similar life histories. Additional ecophysiological assessments of these closely related species are needed to determine the mechanisms driving the differences between these species.

Supplementary Information. The online version contains supplementary material available at:

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Author contributions. GHD, WCL, and RJF contributed to the conceptualization of the study. GHD, WCL, BVS, KES and RBA developed methodology. AM, SS, WCL, KMS, and BVS carried out experimental exposures and collected data. TA, WCL and GHD analyzed data and wrote the first draft of the manuscript. All authors commented on manuscript drafts and read and approved the final manuscript.

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Data availability. The datasets generated during and/or analyzed during the current study are available as a supplemental document.

DECLARATIONS

Conflict of interest. All authors declare that they have no conflict of interest.

Ethical approval. All applicable international, national, and/or institutional guidelines for the care and use of invertebrate animals were followed.

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