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Original Article

Effects of high pCO_2 on Tanner crab reproduction and early life history—Part I: long-term exposure reduces hatching success and female calcification, and alters embryonic development

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Ocean acidification, a decrease in ocean pH due to absorption of anthropogenic atmospheric CO_2, has variable effects on different species. To examine the effects of long-term exposure on Tanner crab (*Chionoecetes bairdi*) embryonic development, hatching success, and calcification, ovigerous females were reared in one of three treatments: ambient pH (~8.1), pH 7.8, and pH 7.5 for 2 years. Embryos and larvae in year 1 were from oocytes developed in the field and appear resilient to high pCO_2. Embryos and larvae in year 2 were from oocytes developed under high pCO_2 conditions. Oocyte development appears sensitive to high pCO_2, effects carryover and altered embryonic development, and reduced hatching success with on average 71% fewer viable larvae hatched in the pH 7.5 treatment than in the other treatments. Per cent calcium was reduced among females exposed to pH 7.5 waters, and their carapaces were noticeably more pliable than those in the other treatments. Softer carapaces may result in reduced defences against predators, and a reduction in the ability to feed on prey with hard parts such as shells. The results from this long-term study suggest that projected ocean pH levels within the next two centuries will likely have a pronounced impact on Tanner crab populations unless the crab are able to acclimatize or adapt to changing conditions.

Keywords: calcification, *Chionoecetes bairdi*, embryonic development, hatching success, ocean acidification, Tanner crab.

Introduction

Carbon dioxide (CO_2) is released into the atmosphere with the burning of fossil fuels, during cement production, and by other human activities (The Royal Society, 2005). Absorption of this anthropogenic CO_2 by the world's oceans has reduced global mean surface water 0.1 pH units below pre-industrial levels, an increase in pH of ~26% (Caldeira and Wickett, 2003; Intergovernmental Panel on Climate Change, 2014). This reduction in ocean's pH due to anthropogenic CO_2 is referred to as ocean acidification. In addition to reducing oceanic pH, surface waters that are currently supersaturated ($\Omega > 1$) with respect to calcium carbonate (both calcite and aragonite forms), which is needed to build and maintain calcified structures, such as shells for marine organisms, are expected to become undersaturated ($\Omega < 1$; Orr *et al.*, 2005).

High latitude waters are particularly vulnerable to ocean acidification because of a loss of sea ice, high rates of primary productivity, increased oceanic uptake of anthropogenic CO_2, and carbonate ion concentrations are naturally low in these waters compared with mid and low latitudes (Fabry *et al.*, 2009). High latitude waters are predicted to be the first to be persistently undersaturated with respect to calcium carbonate, which could occur within decades, not centuries as previously suggested (Orr *et al.*, 2005; Fabry *et al.*, 2009; Mathis *et al.*, 2011).

Ocean acidification has a variety of effects on different life stages of decapods including decreased survival (Kurihara *et al.*, 2008; Long *et al.*, 2013b), decreased growth (Kurihara *et al.*, 2008; Long *et al.*, 2013b), decreased egg production (Kurihara *et al.*, 2008), decreased calcification (Arnold *et al.*, 2009; Long *et al.*, 2013b),

increased deformities (Agnalt *et al.*, 2013), increased calcification (Long *et al.*, 2013a), increased hatch duration (Long *et al.*, 2013a), and altered haematology physiology and function (Meseck *et al.*, 2015). Carryover effects "occur when an individual's previous history and experience explains their current performance in a given situation" (O'Connor *et al.*, 2014). Carryover effects of ocean acidification between life stages can occur such as was observed in the crab *Hyas araneus*; larvae hatched and reared in acidified waters from embryos that developed in acidified waters had greater mortality and developmental delays than when just larvae were reared in acidified waters (embryos developed in ambient waters; Schiffer *et al.*, 2014).

Tanner crab (*Chionoecetes bairdi*) occur in the Pacific Ocean from Oregon to Alaska, in the Bering Sea, Aleutian Islands, and the Sea of Okhotsk (Jadamec *et al.*, 1999), and have historically supported valuable commercial fisheries in Alaskan waters. In recent years, reduced stock sizes have resulted in many fishery closures (North Pacific Fishery Management Council, 2013; Spalinger, 2013). Tanner crab are found at depths ranging from subtidal to ~440 m with mature male and female Tanner crab migrating to the deeper waters where they remain (Stevens *et al.*, 1993; Jadamec *et al.*, 1999). The depths that Tanner crab are found in are within the range of depths where the highest dissolution rates of calcium carbonate are in the North Pacific, Gulf of Alaska, and eastern Bering Sea (Fabry *et al.*, 2009; Feely *et al.*, 2012; Mathis *et al.*, 2014). Mating occurs after a female undergoes her molt to maturity, which is terminal (final), and in subsequent years she can mate while hard shelled or use stored sperm to fertilize egg clutches (Paul, 1984; Paul and Adams, 1984). Extrusion of a fertilized egg clutch occurs within 48 h of mating, brooding duration is ~12–16 months and larval hatching typically occurs in April and May (Donaldson and Adams, 1989; Swiney, 2008).

It is critical to examine the potential effects of ocean acidification, including carryover effects, on different life stages of Tanner crab. In this study, we exposed ovigerous Tanner crab to ambient pH, pH 7.8, and pH 7.5 treatments for 2 years which allowed us to observe direct and/or carryover effects of high pCO_2 during the entire reproductive cycle (oogenesis through larval hatching). The first year of the study examined effects of high pCO_2 on embryos and larvae from oocytes developed in the field under ambient conditions, whereas the second year of the study examined effects of high pCO_2 on embryos and larvae from oocytes developed under high pCO_2 conditions (during year 1). We specifically studied the effects of high pCO_2 on Tanner crab embryonic development, the mean number of viable larvae hatched, hatching success, mean hatch duration, mean brooding duration, and calcification rates of adults.

Methods

Sample collection and laboratory study

Ethical approval for this research was not required by any federal, state, or international laws because the study was conducted on invertebrates which are not covered under these laws.

The experiment was divided into 2 years based on reproductive cycles. Year 1 began 21 June 2011 when ovigerous females brooding newly extruded eggs were placed in the treatments and ended when larval hatching occurred in May 2012. Year 2 began in May 2012 after the females extruded a new clutch of eggs and ended 15 July 2013 after larval hatching occurred. Multiparous female Tanner crab were collected using crab pots and a 3-m beam trawl from Chiniak Bay, Kodiak, Alaska (57°43.25′N, 152°17.5′W) May and June 2011, and brought to the Alaska Fisheries Science Center's Kodiak Laboratory seawater facility in Kodiak, Alaska. Only females that appeared healthy, were brooding at least a three-fourth full clutch of newly extruded eggs, had at least one claw, and not missing more than three legs total were used in the experiment. At the beginning of year 1, 42 of the experimental females extruded their egg clutch in the field. An additional six females that hatched larvae in the laboratory for a related high pCO_2 experiment (Swiney *et al.*, 2016) were mated with mature males that were collected with the females in May and June 2011 and included in this study to increase the sample size to 48 females.

At the beginning of the experiment (year 1), 48 females were randomly assigned to one of three acidification treatments based on projected future changes to ocean pH: (i) ambient pH (~8.1), (ii) pH 7.8 (ca. ~2100), and (iii) pH 7.5 (ca. ~2200; Caldeira and Wickett, 2003; Intergovernmental Panel on Climate Change, 2014) for a total of 16 females per treatment. Mean female size at the beginning of year 1 was 98.7 (standard deviation, SD = 4.8) mm carapace width and female size did not vary with treatment (ANOVA, $F_{2,45} = 1.115$, $p = 0.337$). Crab died throughout the experiment, and at the end of year 1 there were 12 females in the ambient treatment, 11 females in the pH 7.8 treatment, and 12 females in the pH 7.5 treatment. After these females extruded a new clutch, they were used in year 2 of the experiment. Females are receptive to mating after larval hatching is complete (Paul, 1984) so at the end of year 1 (spring 2012) a mature male was placed in a female's tub to mate after her larvae hatched. Males were collected in the field March 2012 and held in ambient waters until they were placed with females. The females were checked daily and the day that a new clutch was extruded was recorded. It is not known if individual females mated or used stored sperm to extrude a new clutch, but all females extruded a viable clutch. Mean female size at the beginning of year 2 was 98.2 (SD = 5.2) mm carapace width and female size did not vary with treatment (ANOVA, $F_{2,32} = 2.593$, $p = 0.09$). Brooding duration for each female was defined as beginning the day of egg extrusion in 2012 (beginning of year 2) and ended when larval hatching began in 2013. At the end of year 2, there were 10 females in the ambient treatment, 6 females in the pH 7.8 treatment, and 7 females in the pH 7.5 treatment.

Each female was reared in an individual 68-l experimental tub with the treatment water flowing at 1 l min^{-1}. Water was chilled when needed and allowed to fluctuate seasonally to ensure appropriate temperatures. The mean daily temperature was 5.0 (SD = 1.5)°C for both years of the experiment, varied seasonally from a low of ~1°C in January 2012 to a high of ~9°C in August 2011, and did not vary among treatments (Kruskal–Wallis, $H = 0.761$, $p = 0.684$; Figure 1 and Table 1). Crab were fed twice weekly a diet of fish and squid in excess except they were not fed during larval hatching. Females were examined daily to ensure they were alive and pH and temperature were recorded daily for each tub (see below for more detail).

Once a month, ~20 eggs were randomly sampled from each female. The embryonic developmental stage was determined using methods described by Swiney (2008) for Tanner crab which was based on Moriyasu and Lanteigne (1998) methods for staging snow crab, *Chionoecetes opilio*, embryonic development. Un-eyed eggs were stained for 5 min with Bouin's solution to facilitate observation of the external morphology of the embryos; eyed eggs were not stained. The embryonic stages were determined under a compound microscope at ×50 magnification (Table 2). Additionally, digital images of 10 fresh eggs from each female were taken with a

digital camera attached to a compound microscope at a total magnification of ×63. Using image analysis software (Image Pro Plus Versions 6.00.260 and 7.0.1.658, Media Cybernetics, Inc., Rockville, MD, USA), egg area and diameters (maximum, minimum, and average) were measured. Once embryos were discernible, embryo areas and yolk areas and diameters (maximum, minimum, and average) were also measured. Finally, when embryos become eyed, eyespot area and diameters (maximum, minimum, and average) were measured (Figure 2).

Larval hatching occurred at the end of years 1 and 2; hatching duration and the number of viable larvae hatched was estimated for each female each year. Hatching duration was defined as the first day 50 or more larvae were hatched and ended when females began to strip their pleopods clean to remove empty egg cases and unhatched eggs (Donaldson and Adams, 1989). Hatching duration was not determined for the females that did not strip their pleopods clean. Before larval hatching, nets were placed on the outflow of each tub to retain all the larvae from each female and newly hatched larvae were collected and dried daily at 60°C until a constant weight was achieved. Average weight per larva for each female each year was calculated from 3 to 6 replicates (number varied with female) of 50 dried larvae. The average weight per larva for each female was used to calculate how many larvae each female hatched daily. If less than ~200 larvae hatched, then all the larvae were counted. On days when live larvae were needed for experiments, they could not be dried [for larval experiment details, see Swiney *et al.* (2016)], so the number of viable larvae hatched was estimated by counting the number of larvae in three to four subsamples of known volume and calculating the total number hatched for the day. For each female, total number of viable larvae hatched was estimated by adding together the number of larvae hatched daily. The number of viable larvae hatched is a little less than what the true number of larvae hatched would have been since we sampled eggs monthly for the embryonic development portion of the project; however, given that the smallest females typically release >100,000 larvae and fecundity increases with female size, the ~240 eggs removed from each crab is negligible.

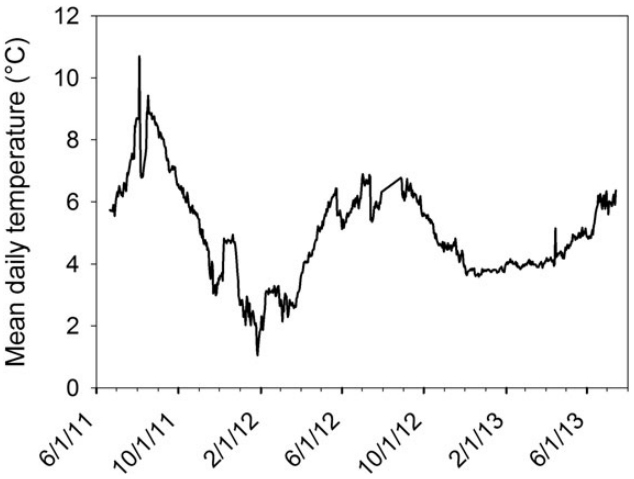

Figure 1. Mean daily temperature (°C) for the duration of the experiment.

Hatching success was defined as the per cent of viable larvae hatched divided by the calculated total number of larvae that could have hatched (number of viable larvae hatched + number of non-viable larvae hatched + number of eggs that did not hatch). The percentage of non-viable larvae hatched and the percentage of eggs that did not hatch were also calculated. Non-viable larvae that hatched were larvae that did not molt past the pre-zoea stage to the first zoeal stage. For each female, the debris from pleopod cleaning was collected, examined microscopically, and all viable larvae, non-viable larvae, and dead eggs counted in a volumetric subsample. At the end of year 2, 10% of females in the ambient treatment (1 of 10), 33% of females in the pH 7.8 treatment (2 of 6), and 86% of females in the pH 7.5 treatment (6 of 7) did not hatch their entire brood or clean their pleopods. Microscopic examination of these clutches confirmed that they were carrying dead eggs. At the end of the experiment when the females were sacrificed, the abdominal flap with dead eggs attached was removed from these females, frozen, and later processed to estimate the number of dead eggs.

At the end of the experiment (July 2013), calcium and magnesium content was analysed for 9 ambient, 5 pH 7.8 and 5 pH 7.5 treatment females from the same portion of their carapace (approximately a square centimetre of the posterior margin region) by an analytical laboratory.

Seawater acidification

Sand filtered seawater was pumped into the laboratory from 15 and 26 m depth intakes in Trident Basin, which is ~30 m from the laboratory. Only one intake line is in use at any given time and typically lines are switched when maintenance is needed on a line. Seawater was acidified using the same methods described in Long *et al.* (2013a). In short, a 170-l tank of pH 5.5 seawater was established by bubbling CO_2 into ambient seawater. This pH 5.5

Table 1. The mean and SD of water chemistry parameters in the three treatments during years 1 and 2 of the experiments.

Treatment	Temperature (°C)	Salinity (PSU)	pH_F	pCO_2 (μatm)	HCO_3^- (mmol/kg)	CO_3^{-2} (mmol/kg)	DIC (mmol/kg)	Alkalinity (mmol/kg)	$\Omega_{Aragonite}$	$\Omega_{Calcite}$
Year 1										
Ambient	5.03 (2.07)	31.26 (0.28)	8.06 (0.06)	418.6 (52.2)	1.90 (0.05)	0.09 (0.01)	2.00 (0.04)	2.12 (0.03)	1.32 (0.16)	2.11 (0.25)
pH 7.8	4.97 (2.07)	31.27 (0.030)	7.80 (0.04)	773.7 (29.2)	1.97 (0.04)	0.05 (0.00)	2.06 (0.04)	2.10 (0.03)	0.76 (0.05)	1.22 (0.08)
pH 7.5	4.93 (2.07)	31.25 (0.28)	7.50 (0.03)	1595.5 (78.9)	2.03 (0.04)	0.03 (0.00)	2.14 (0.04)	2.09 (0.02)	0.39 (0.03)	0.63 (0.05)
Year 2										
Ambient	4.97 (0.95)	31.16 (0.36)	8.10 (0.08)	373.9 (67.9)	1.89 (0.04)	0.10 (0.02)	2.01 (0.03)	2.15 (0.02)	1.52 (0.27)	2.43 (0.43)
pH 7.8	4.93 (0.95)	31.19 (0.34)	7.80 (0.02)	786.2 (31.6)	2.00 (0.03)	0.05 (0.00)	2.09 (0.03)	2.13 (0.03)	0.78 (0.04)	1.25 (0.06)
pH 7.5	4.92 (0.92)	31.14 (0.35)	7.50 (0.03)	1598.2 (50.7)	2.06 (0.03)	0.03 (0.00)	2.17 (0.03)	2.12 (0.03)	0.41 (0.02)	0.65 (0.03)

pH (free scale) and temperature were measured daily (year 1 $n = 316$ and year 2 $n = 412$ per treatment); DIC, salinity, and alkalinity were measured weekly (year 1 $n = 40–41$ and year 2 $n = 61–63$ per treatment); and all other parameters were calculated.

Table 2. Embryonic developmental stages modified from Moriyasu and Lanteigne (1998) descriptions for *Chionoecetes opilio*. See Moriyasu and Lanteigne (1998) for detailed descriptions.

Stage no.	Embryonic developmental stage	Stage description
1	Prefuniculus formation	The egg is filled with yolk until stage 6. The funiculus is not formed.
2	Funiculus formation	The funiculus is formed.
3	Cleave and blastula	Yolk segmentation goes from two cells to the blastula.
4	Gastrula	The blastophore appears as a disc-shaped structure on the surface of the gastrula.
5	Lateral ectodermal band	The germinal disc with three rudiments appears as a U-shaped structure.
6	Prenauplius	The percentage of yolk diminishes steadily until stage 12. The rudiments of larval structures appear. A clear area representing the developing embryo is visible.
7	Nauplius	The antennae are biramous and the antennae and antenules are rod-shaped and separated from the centre. The segments forming the upper region are fused.
8	Maxilliped formation	The rudiments of the first and second maxillipeds appear. Crescent-shaped carapace rudiments are present in the anterior part of the thoracic abdomen.
9	Metanauplius	The rudiments of the first and second maxillae and the third maxillipeds appear. The labrum is triangular. The telson is forked.
10	Late metanauplius	The rudiments of the dorsal spine are a disc-shaped structure in the middle of the yolk. The optic lobes are well developed and a thin pigmented crescent of the compound eye is visible.
11	Eye-pigment formation	Eye pigments spread. All the larval appendages have formed. The rudiments of the dorsal spine separate the yolk into two parts.
12	Chromotophore formation	Dot-like chromatophores appear on the maxillipeds. The lateral spines elongate and separate the yolk into four parts. The eye pigment spreads and become teardrop-shaped.
13	Reduced yolk	Almost no yolk is visible. The rudiments of the dorsal and lateral spines become wider. The thoracic abdomen forms six segments including the telson.
14	Prehatching	The eyes are completely pigmented and no yolk is visible.

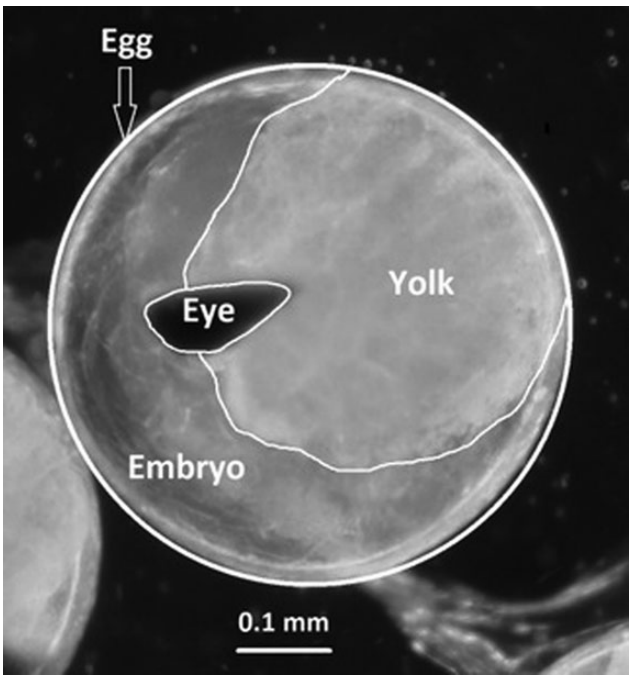

Figure 2. Tanner crab egg, embryo, yolk, and eye sections measured for embryonic morphometric analysis.

seawater was mixed with ambient seawater in the 170-l treatment head tanks via peristaltic pumps controlled by Honeywell controllers and Durafet III pH probes. The ambient head tank did not receive any pH 5.5 water. Waters from the pH 7.8 and pH 7.5 treatment head tanks were then supplied to the individual tubs containing females. pH_F (free scale) and temperature were measured daily in each experimental tub using a Durafet III pH probe calibrated using a TRIS buffer solution and 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. Weekly water samples from each treatment were taken, poisoned with mercuric chloride, and sent to one of the two analytic laboratories for dissolved inorganic carbon (DIC) and total alkalinity (TA) analysis. The two laboratories used similar, but slightly different instruments. 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 Institute, San Diego, CA, USA; 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 with a 5012 Coulometer (UIC Inc.) using Certified Reference Material from the Dickson Laboratory (Scripps Institute) and the procedures in DOE (1994). Target pHs were achieved throughout the experiment (Table 1). Alkalinity decreased with decreasing pH, and DIC increased with decreasing pH (Table 1). Aragonite was supersaturated in the ambient treatment, but undersaturated in the pH 7.8 and pH 7.5 treatments. Calcite was supersaturated in the ambient and pH 7.8 treatments, and undersaturated in the pH 7.5 treatment (Table 1).

Statistical analysis

The effects of seawater pH on the development of embryos were analysed using principal component analysis (PCA) in Primer 6.1.15 (Primer-E Ltd, Lutton, UK); each year was analysed separately. Embryo measurements were normalized before analysis. We retained all PCs that were necessary to explain at least 90% of the variation in the data each year. Each retained PC was analysed with an ANOVA with pH treatment fully crossed with month and female nested in treatment crossed with month as factors. The second PC in the second year failed to meet the assumption of homogeneity of variance, so we analysed the average PC2 score for each female on each date (which met the assumption) using an

ANOVA with pH treatment fully crossed with month and female nested in treatment as factors.

The effects of different seawater pH on the mean number of viable larvae hatched, hatching success, mean hatch duration (2012 only), mean brooding duration, and calcification were examined using one-way ANOVAs or Kruskal–Wallis tests with pH as the factor. The 2013 mean hatch duration was examined with a two-sample *t*-test because hatching duration could only be estimated for one female in the pH 7.5 treatment, so this treatment was not included in the analysis. Anderson–Darling test for normality and Levene's test for homogeneity of variance were used to determine if data meet the assumptions of ANOVA and when data failed these test, the non-parametric Kruskal–Wallis test was used. When significant differences were detected with ANOVAs, Tukey's HSD *post hoc* multiple comparisons tests were used to examine the differences between pH treatments. When significant differences were detected with Kruskal–Wallis tests, Conover–Inman tests for all pairwise comparisons were used to examine the differences between pH treatments. Statistical analyses were conducted in SYSTAT 10.00.05 (Systat Software, Inc., Chicago, IL, USA).

We modelled female mortality using a binomial distribution of errors assuming a constant mortality rate such that $N_t = N_0 e^{-rt}$, where N_t is the number surviving at time t (in days), N_0 is the initial number, and r is the mortality rate. We fit the survival data to three models using maximum likelihood in R (V2.14.0, Vienna, Austria), one in which the mortality rate did not differ among treatments, one in which it differed among all treatments, and one in which the rate in the Ambient treatment differed from the two experimental treatments. We selected the best model using the Akaike's information criterion corrected for small sample size (AIC_c). Models whose AIC_c differed by <2 were considered to explain the data equally well (Burnham and Anderson, 2002).

Results

Mean embryo stage by date did not differ among pH treatments in either year 1 or 2 of the experiment (Figure 3). In year 1, stage 14, which is the prehatching stage (Table 2), was not observed during the monthly sampling because after the April 2012 sampling females were either hatching their larvae or they had finished hatching and extruded a new clutch.

Embryonic morphometrics were significantly different among pH treatments in both years, and PCA results were qualitatively similar between the years. In both years, the first two PCs explained >90% of the variance and were the only two retained (Tables 3 and 4). The first PCs were negatively associated with egg, embryo, and eye size, and positively associated with yolk size, and are interpreted as embryo maturity, with more mature embryos having smaller PC1 scores. The second PCs were positively associated with egg and yolk size. In both years, there was a significant interaction between month and treatment on the PC1 scores (Tables 3 and 4, and Figure 4); early in development, there were no differences among the treatments, but there began to be significant differences starting in December in the first year and in November the second year. These differences persisted through hatching (Tables 3 and 4, and Figure 4); the lack of a difference in May in the second year was because this sampling took place during the hatching period, and 70% of the ambient females and 40% of the pH 7.8 females had completed hatching (none of the pH 7.5 females had completed hatching) and the remaining embryos were at the prehatching stage (Table 2). In general, embryos in the ambient and pH 7.8 treatments differed from those in the pH 7.5 treatment, but not from each other.

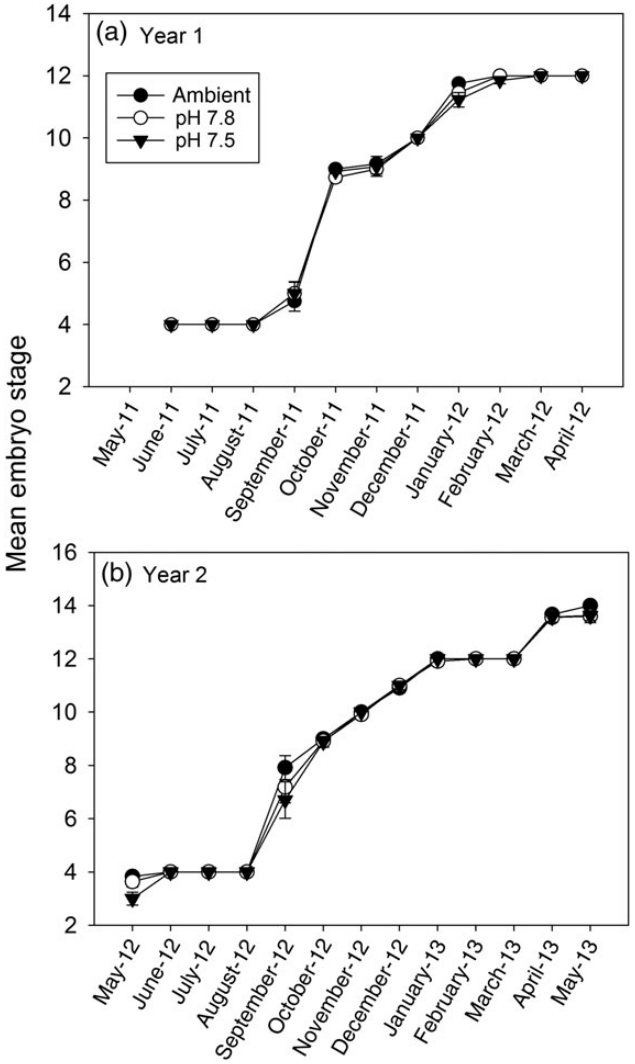

Figure 3. Comparison of Tanner crab mean embryo stage by pH treatment for (a) year 1 and (b) 2 of the experiment. See Table 2 for embryo stage descriptions. Values are means and vertical bars represent standard errors.

PC2 showed similar trends. In the first year, there were significant differences among the pH treatments with the ambient and pH 7.8 treatments differing from the pH 7.5 treatment, but not from each other whereas in the second year this was the case in most, but not all, months leading to a significant interactive effect (Tables 3 and 4, and Figure 4). In both years, the pH 7.5 embryos had larger yolks and smaller eyes and embryos. Although there were statistically significant differences among the treatments, the effect size in year 1 was slight; the average difference in the measurements between the ambient and pH 7.5 embryos was only 3.6% in April 2012, with a maximum difference in any one measurement of 7.1%. In the second year, effect sizes were larger; embryos in the pH 7.5 treatment had 10.1% larger yolks and 6.3% smaller embryos than those in the ambient treatment.

The mean number of viable larvae hatched in year 1 of the experiment did not differ with pH treatment (Kruskal–Wallis, $H = 2.253$, $p = 0.324$; Figure 5a). Furthermore, there was not a significant difference in the mean number of viable larvae hatched in year 2 among the ambient and pH 7.8 treatments, however, on average,

Table 3. PCA of Tanner crab embryo morphometrics in year 1.

PC	Eigenvalues	Percentage variation	Cumulative percentage variation
1	10.8	82.8	82.8
2	1.32	10.2	92.9
	Eigenvectors		
Variable	**PC1**	**PC2**	
Egg area	−0.273	0.377	
Egg maximum diameter	−0.231	0.45	
Egg minimum diameter	−0.269	0.275	
Egg average diameter	−0.272	0.378	
Yolk area	0.276	0.334	
Yolk maximum diameter	0.256	0.365	
Yolk minimum diameter	0.268	0.282	
Yolk average diameter	0.277	0.327	
Embryo area	−0.301	−0.06	
Eye area	−0.291	0.025	
Eye maximum diameter	−0.29	−0.039	
Eye minimum diameter	−0.297	−0.01	
Eye average diameter	−0.297	−0.024	
ANOVA			
Variable	**Factor**	***F***	***p***
PC1	Treatment	77.291	<0.0005
	Month	21,702.58	<0.0005
	T * M	10.025	<0.0005
	Crab(T * M)	9.027	<0.0005
PC2	Treatment	12.355	<0.0005
	Month	28.304	<0.0005
	T * M	0.931	0.548
	Crab(T)	10.286	<0.0005

The first two PCs, representing 93% of the cumulative variation, are retained. ANOVA analysis with treatment (T) fully crossed with month (M), and crab (nested) was performed for the first two eigenvectors.

Table 4. PCA of Tanner crab embryo morphometrics in year 2.

PC	Eigenvalues	Percentage variation	Cumulative percentage variation
1	10.5	80.6	80.6
2	1.73	13.3	93.9
	Eigenvectors		
Variable	**PC1**	**PC2**	
Egg area	−0.261	0.398	
Egg maximum diameter	−0.231	0.429	
Egg minimum diameter	−0.256	0.331	
Egg average diameter	−0.259	0.405	
Yolk area	0.284	0.271	
Yolk maximum diameter	0.249	0.367	
Yolk minimum diameter	0.282	0.254	
Yolk average diameter	0.275	0.313	
Embryo area	−0.305	−0.084	
Eye area	−0.299	−0.050	
Eye maximum diameter	−0.291	−0.013	
Eye minimum diameter	−0.302	−0.045	
Eye average diameter	−0.300	−0.030	
ANOVA			
Variable	**Factor**	***F***	***p***
PC1	Treatment	104.60	<0.0005
	Month	16,523.18	<0.0005
	T * M	5.318	<0.0005
	Crab(T * M)	13.807	<0.0005
PC2	Treatment	121.16	<0.0005
	Month	297.588	<0.0005
	T * M	5.607	<0.0005
	Crab(T * M)	11.576	<0.0005

The first two PCs, representing 94% of the cumulative variation, are retained. ANOVA analysis with treatment (T) fully crossed with month (M), and crab (nested within treatment crossed with month) was performed for the first four eigenvectors.

71% less viable larvae hatched from the pH 7.5 treatment in year 2 (ANOVA, $F_{2,\ 27} = 5.796$, $p = 0.008$; Figure 5b). For all three treatments, less viable larvae hatched in year 2 than in year 1. In the ambient pH treatment, on average, 47% less viable larvae hatched in year 2 than in year 1, in the pH 7.8 treatment 54% less viable larvae hatched in year 2, and in the pH 7.5 treatment 83% less viable larvae hatched in year 2 than in year 1 (Figure 5).

Hatching success, measured as the per cent of viable larvae hatched, did not differ in year 1 among pH treatments and averaged 99% (Kruskal–Wallis, $H = 0.265$, $p = 0.876$; Figure 6). In year 2, hatching success in the pH 7.5 treatment was, on average, 46% and significantly lower than the average 87% hatching success observed in the ambient treatment; the ambient and pH 7.8 treatments did not differ statistically (Kruskal–Wallis, $H = 7.988$, $p = 0.018$; Figure 6). Overall fewer viable larvae were hatched in year 2 than in year 1 as 99% hatching success was observed in year 1 and in year 2 hatching success averaged 46–87% depending on the pH treatment (Figure 6). More non-viable larvae hatched in year 2, averaging 3–15% depending on pH treatment (Figure 6), compared with <1% observed in year 1. Likewise, on average, more eggs did not hatch in year 2, 10–39% depending on pH treatment (Figure 6), compared with <1% observed in year 1.

In year 1, mean larval hatching duration did not differ with pH treatment (Kruskal–Wallis, $H = 3.783$, $p = 0.151$) nor did it differ in year 2 (t-test, $t = 0.515$, $p = 0.616$). In year 1, average larval hatching duration was 6 (SE = 0.182) days and ranged from 4 to 8 days. In year 2, average larval hatching duration was longer and more variable averaging 16 (SE = 3.537) days, and ranged from 6 to 59 days with only one female from treatment pH 7.5 completing hatching. Mean brood duration, which was only estimated for year 2 of the experiment, did not differ with pH treatment (Kruskall–Wallis, $H = 0.800$, $p = 0.670$) and averaged 356 (SE = 1.150) days.

In the best fit model, the mortality rate of adult females throughout the 2 years of the experiment differed among all treatments and increased under high pCO_2 conditions (Figure 7 and Table 5). At the end of the experiment, adult females from the ambient pH treatment had the highest survival rate (63%) followed by females in the 7.5 pH treatment (44%), and survival was lowest in the 7.8 pH treatment (38%); however, the difference in survival between the 7.8 and 7.5 pH treatments was only one crab (Figure 7). The per cent dry weight of calcium in the females' carapace at the end of the experiment was significantly less in the pH 7.5 treatment than in either the ambient or pH 7.8 treatments which did not differ (Kruskal–Wallis, $H = 11.041$, $p = 0.004$; Figure 8a). The per cent dry weight of magnesium contained in the carapace did not differ with pH treatment (ANOVA, $F_{2,16} = 0.929$, $p = 0.415$; Figure 8b). The ratio of magnesium and calcium differed between the ambient and 7.5 pH treatments, but did not differ among the other pH treatments (ANOVA, $F_{2,16} = 5.108$, $p = 0.019$; Figure 8c). The carapaces of females held in the pH 7.5 treatment were noticeably more pliable than the females reared in the other pH treatments.

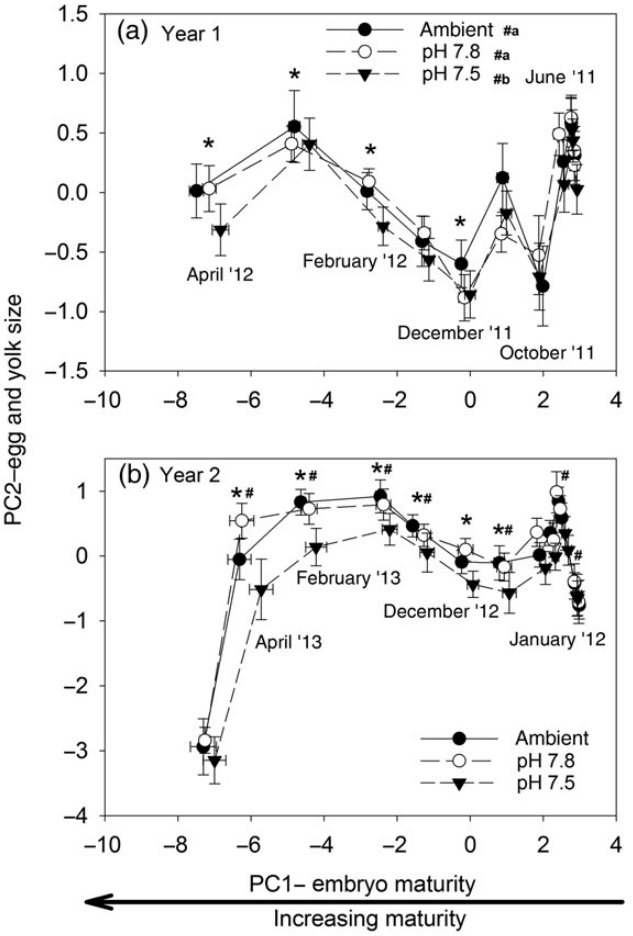

Figure 4. Average principal component (PC) 1 and 2 scores ± standard errors for Tanner crab embryos measured monthly during (a) year 1 and (b) year 2. The scores were averaged among females within each treatment, so sample size varied with the number of females surviving each month and varied from 16 at the beginning of the experiment to as low as 6 in the pH 7.8 treatment at the end. PC1 is associated with embryo maturity and PC2 is associated with egg and embryo size. Every other month is labelled. When there were significant differences among treatments within a month, it is indicated with asterisks for PC1 and an hash for PC2. When there were differences among the treatments in all months, it is indicated in the figure legend using the same symbols; treatments with the different letters next to them are significantly different.

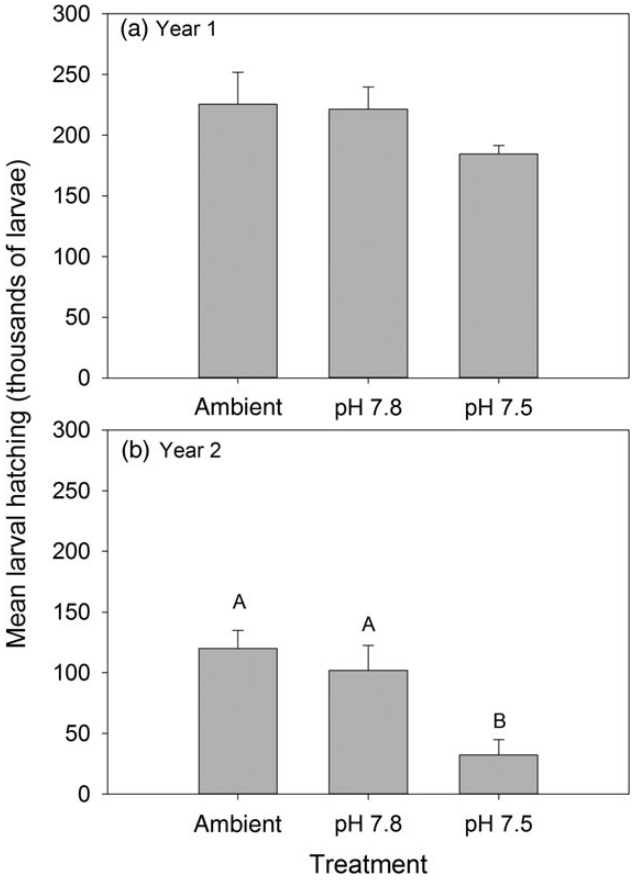

Figure 5. Comparison of the mean number of viable Tanner crab larvae hatched by pH treatment in (a) year 1 and (b) year 2. Bars are mean with standard error. Bars with different letters above them differ significantly.

Discussion

Decreased pH had a slight effect on Tanner crab embryonic development and no effect on larval hatching in year 1 of this study, suggesting that Tanner crab embryonic development and larval hatching are not sensitive to high pCO_2 if oocytes are developed under ambient (not high pCO_2) conditions. Under high pCO_2 conditions, significant effects were detected in embryonic development, the number of viable larvae hatched, and hatching success in year 2 of this study, which suggests that Tanner crab reproductive success is most susceptible to the effects of high pCO_2 during oocyte development and that these effects carryover into embryonic development and larval hatching. Variability in the sensitivity of different life stages and carryover effects between stages to acidified waters has been found for other crab species. For the great spider crab *H. araneus*, larval mortality was significantly higher and developmental delays observed under acidified conditions when larvae were hatched from embryos developed in acidified waters compared with larvae exposed to acidified conditions, but embryos that did not develop under acidified conditions (Schiffer *et al.*, 2014). For the porcelain crab *Petrolisthes cinctipes*, the embryo stage is more sensitive to acidified waters than the larval and juveniles stages (Carter *et al.*, 2013). Acidified waters did not affect embryonic development rate, heart rate, or oxygen consumption of the Norway lobster *Nephrops norvegicus* after 4 months of exposure (Styf *et al.*, 2013). Embryonic development for red king crab, *Paralithodes camtschaticus*, is sensitive to acidified waters, even with only short-term exposure (2 months; Long *et al.*, 2013a). These studies highlight the need for ocean acidification research to examine the potential effects of acidification on all stages and for different species (Kurihara, 2008).

Tanner crab embryos that developed in pH 7.5 waters had larger yolks and smaller embryos than embryos reared in pH 7.8 and ambient waters, suggesting that development was delayed for embryos reared in the lowest pH treatment. Hatching success was low among pH 7.5 embryos in year 2 of the study and differences in embryo development may be due to these embryos not developing normally and ultimately not hatching. Regardless, the developmental differences were not large enough to cause a difference in the mean monthly embryo stages among the treatments or brood

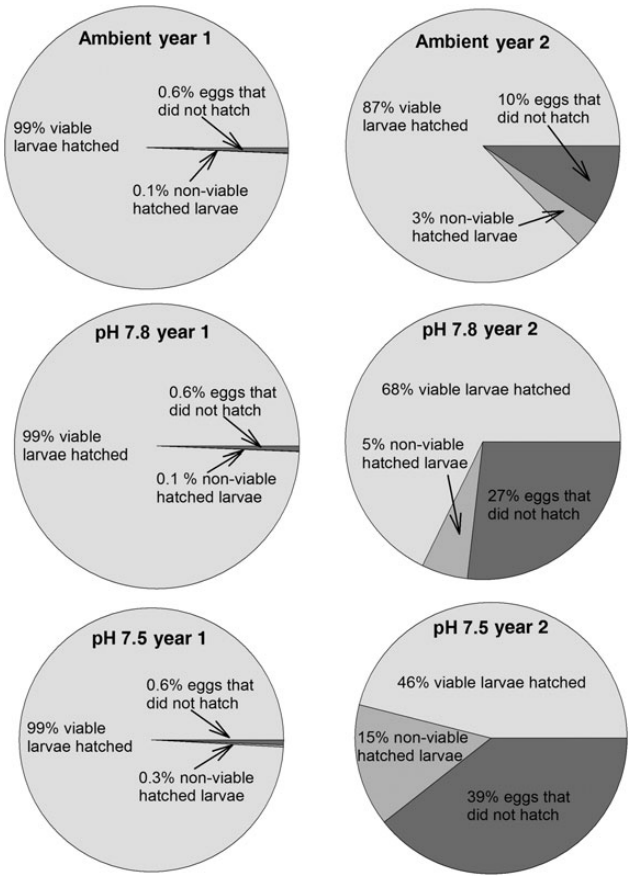

Figure 6. Comparison of the mean per cent viable Tanner crab larvae hatched, non-viable larvae hatched, and eggs that did not hatch in year 1 and year 2 among ambient, pH 7.8, and pH 7.5 treatments.

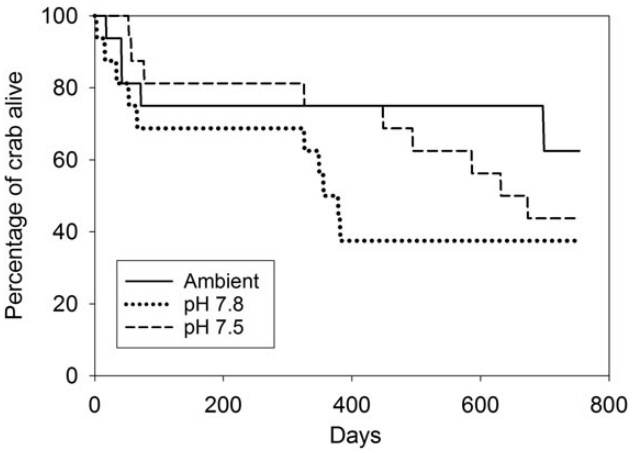

Figure 7. Daily percentage survival of female Tanner crab by treatment for the 2-year experiment.

duration among females that had a successful hatch. Similarly, exposure of porcelain crab embryos to acidified waters (pH 7.6) resulted in lower metabolism and dry mass which may cause delayed development (Carter *et al.*, 2013). In contrast, red king crab embryos that developed in acidified waters (pH 7.7) had larger embryos and smaller yolks, suggesting an increase in the development rate under acidified conditions (Long *et al.*, 2013a).

Table 5. Ranking of models of adult female Tanner crab mortality using AIC_c.

Model	*K*	AIC_c	ΔAIC_c	Likelihood	AIC_c weight
m(T)	3	7993.72	0.00	1.00	1.00
m(T2)	2	8773.83	780.11	0.00	0.00
m	1	9497.13	1503.42	0.00	0.00

Model indicates how the mortality rate, *r*, was modelled. T indicates the rate varied among all treatments and T2 indicates the rate differed between the ambient and experimental treatments (see text for full model description). *K* is the number of parameters in each model.

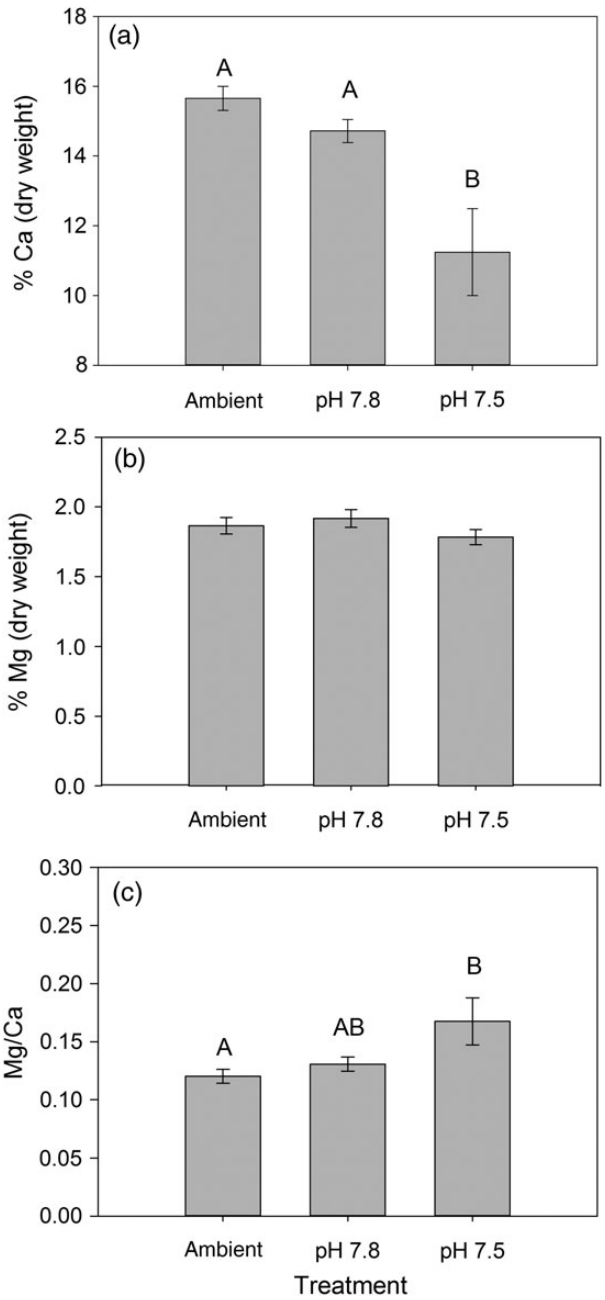

Figure 8. Comparison of the mean (a) per cent dry weight calcium, (b) per cent dry weight magnesium, and (c) magnesium calcium ratio from carapaces of female Tanner crab reared in ambient, 7.8 pH and 7.5 pH treatments for 2 years. Bars are mean with standard error. Bars with different letters above them differ significantly.

High pCO_2 waters had a pronounced effect on the reproductive output in year 2 of this study; females reared in pH 7.5 waters hatched on average 71% fewer viable larvae than the other two treatments, and hatching success in the pH 7.5 treatment was 40% less than in the ambient treatment. The significant reduction in the reproductive output of females reared in the pH 7.5 waters will likely have a substantial impact on Tanner crab population size and fisheries under these conditions. Likewise, acidified waters (pH 7.89) reduce the reproductive output of the marine shrimp *Palaemon pacificus* by reducing the number of clutches individual females' brood (Kurihara *et al.*, 2008).

For all the treatments, fewer viable larvae hatched and hatching success were lower in year 2 than in year 1 of the study. This may have occurred due to senescence and/or laboratory affects. The closely related snow crab, *C. opilio*, is thought to have a maximum post-terminal molt lifespan of 6–7 years in the eastern Bering Sea (Ernst *et al.*, 2005), and become senescent with age (Kon *et al.*, 2010). Longevity information on Tanner crab is lacking (North Pacific Fishery Management Council, 2013), but if Tanner crab are similar to snow crab, the reduction in viable larvae hatched and hatching success in year 2 of the experiment may have been due to senescence. The females used in this study were multiparous in year 1, so they were at a minimum of 3 years post-terminal molt during year 2 of the study. Regardless of the reason for reduced numbers of viable larvae hatched and hatching success in year 2, we are confident that the significant reduction in reproductive output in the pH 7.5 treatment is real since all the crab were randomly assigned to treatments, and in the laboratory for the same amount of time so laboratory effects should be the same for all treatments.

High pCO_2 water did not affect Tanner crab larval hatching duration in either year of the study, suggesting that this process is not sensitive to high pCO_2 conditions for this species. These results support the notion that hatching duration is innate, heritable, and not easily altered by most environmental parameters (Morgan, 1995). It has been hypothesized that Tanner crab hatching is controlled by an endogenous biological clock, and that hatching corresponds to the period of greatest tidal exchange to optimize larval transport (Stevens, 2003). Hatching was longer and more variable for all treatments in year 2 of the study, which may have been the result of senescence or laboratory effects. With the crab being in the laboratory for 2 years, their biological clocks may have been affected by the crab not having direct environmental influences, such as tidal fluctuations, which appear important cues for hatching. In contrast, red king crab hatching duration was 33% longer in acidified waters (pH 7.7; Long *et al.*, 2013a). King crab have a longer hatching duration than Tanner crab (~22 days), and it is thought that the longer hatching duration is an evolutionary response to conditions that were highly variable and unpredictable. A longer hatching duration increases that chance that some larvae will be released during a time of high food availability which will improve larval survival (Stevens, 2006). For red king crab, acidified waters may be perceived as increased environmental variability resulting in the longer hatching duration observed (Long *et al.*, 2013a).

Based on the few adult decapod species examined, acidified waters have either a positive or no effect on calcification (Ries *et al.*, 2009; Landes and Zimmer, 2012; Long *et al.*, 2013a). In this study, the reduced calcification (per cent dry weight of calcium) in the 7.5 pH treatment is unprecedented. The difference between our results and other studies may simply be that, in our experiment, the crab were reared in high pCO_2 water for 2 years, whereas the previous studies were shorter (maximum of 5 months) in duration and effects may not be seen due to the shorter duration of the studies. Long-term ocean acidification studies are needed to see if other decapod species experience reduced calcification or if this response is unique to Tanner crab. Alternately, Tanner crab physiological responses to acidification may differ from other decapod species, regardless of exposure duration, as was observed in a long-term study (nearly 200 days) comparing the responses of juvenile Tanner and red king crab with ocean acidification (Long *et al.*, 2013b). Juvenile Tanner crab had decreased calcium content and an unaffected condition index (dry body mass divided by carapace width or length cubed), whereas juvenile red king crab calcium content was unaffected, but the condition index was reduced (Long *et al.*, 2013b). Ries *et al.* (2009) suggest that organisms, such as crustaceans, expend energy to physiologically elevate pH at the site of calcification and depending on the efficiency of their specific proton-regulating mechanism end up with similar conditions at the site of calcification as they currently experience. It is unclear why Tanner crab have reduced calcium content under high pCO_2 conditions, but they may not be as efficient at increasing pH at the site of calcification as other decapods. Alternatively, the calcium carbonate in the carapace may be dissolved internally in an attempt to buffer internal pH.

Among crustaceans, typically when calcium content is altered under acidified conditions, magnesium content is also altered (Arnold *et al.*, 2009; Long *et al.*, 2013a), but that was not the case with adult Tanner crab in this study or juvenile Tanner crab (Long *et al.*, 2013b). Adult and juvenile Tanner crab had reduced calcium content under high pCO_2 conditions, but magnesium did not vary, and thus, the Mg/Ca ratio was higher under high pCO_2 conditions. Because the strength of calcite is proportional to the Mg/Ca ratio (Magdans and Gies, 2004), the increase in the Mg/Ca ratio may be an adaptive response to reduced calcification in that it may reduce the overall loss of carapace strength although the mechanism is unclear. In this study, we observed more pliable shells under high pCO_2 conditions, so although the shell may be stronger than they would have been if calcium and magnesium were reduced in the same proportion, the amount of calcium content lost under high pCO_2 conditions was high enough to result in adults with more pliable shells in the pH 7.5 treatment. Additional research is needed to better understand the effects of ocean acidification on Tanner crab calcification, including both calcium and magnesium content and their effects on shells strength.

Projected ocean pH levels within the next two centuries will likely have a pronounced negative effect on Tanner crab populations unless the crab are able to adapt as conditions change. Tanner crab appear resilient to medium term (1 year) exposure to high pCO_2 waters. However, the distressing effects of high pCO_2 on Tanner crab populations exposed to acidified water for the entire reproductive cycle (from oocyte development to hatching) are apparent by the relatively small number of viable larvae hatched among females reared in the pH 7.5 treatment year 2 of this study. Furthermore, calcification was reduced among females reared in the pH 7.5 treatment and their carapaces were noticeably more pliable than females reared in the other treatments. Softer carapaces may result in reduced defences against predators, and a reduction in the ability to feed on prey with shells such as bivalves. The components of an organism's energy budget are linked together with stressed organisms requiring additional energy for somatic maintenance, thus reducing or eliminating the amount of energy available for activities such as growth and reproduction (Sokolova

et al., 2012; Sara *et al.*, 2014). The decreased hatching success and calcification observed in this study were likely the result of high pCO_2 altering the crab's energy budgets. In fact, crab in the pH 7.5 treatment were stressed to the point that they appear to not have had enough energy to maintain the integrity of their carapaces. Results from this study highlight the need for long-term ocean acidification research that encompasses many life stages, so potential carryover effects can be observed.

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