

## RESEARCH ARTICLE

# Impact of ocean acidification on metabolism and energetics during early life stages of the intertidal porcelain crab *Petrolisthes cinctipes*

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### SUMMARY

**Absorption of elevated atmospheric CO<sub>2</sub> is causing surface ocean pH to decline, a process known as ocean acidification (OA). To date, few studies have assessed the physiological impacts of OA on early life-history stages of intertidal organisms, which transition from habitats with fluctuating pH (intertidal zone) to relatively stable (pelagic zone) pH environments. We used the intertidal crab *Petrolisthes cinctipes* to determine whether metabolic responses to year 2300 predictions for OA vary among early developmental stages and to examine whether the effects were more pronounced in larval stages developing in the open ocean. Oxygen consumption rate, total protein, dry mass, total lipids and C/N were determined in late-stage embryos, zoea I larvae and newly settled juveniles reared in ambient pH (7.93±0.06) or low pH (7.58±0.06). After short-term exposure to low pH, embryos displayed 11% and 6% lower metabolism and dry mass, respectively, which may have an associated bioenergetic cost of delayed development to hatching. However, metabolic responses appeared to vary among broods, suggesting significant parental effects among the offspring of six females, possibly a consequence of maternal state during egg deposition and genetic differences among broods. Larval and juvenile metabolism were not affected by acute exposure to elevated CO<sub>2</sub>. Larvae contained 7% less nitrogen and C/N was 6% higher in individuals reared at pH 7.58 for 6 days, representing a possible switch from lipid to protein metabolism under low pH; the metabolic switch appears to fully cover the energetic cost of responding to elevated CO<sub>2</sub>. Juvenile dry mass was unaffected after 33 days exposure to low pH seawater. Increased tolerance to low pH in zoea I larvae and juvenile stages may be a consequence of enhanced acid–base regulatory mechanisms, allowing greater compensation of extracellular pH changes and thus preventing decreases in metabolism after exposure to elevated  $P_{\text{CO}_2}$ . The observed variation in responses of *P. cinctipes* to decreased pH in the present study suggests the potential for this species to adapt to future declines in near-shore pH.**

Key words: carbon dioxide, pH, crustacean, respiration, protein, CHN, lipids, larvae, embryo.

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### INTRODUCTION

Ocean pH is declining as a consequence of absorbing atmospheric CO<sub>2</sub>, a process known as ocean acidification (OA) (Doney et al., 2009; Orr et al., 2005; Feely et al., 2004). Atmospheric CO<sub>2</sub> levels predicted by the end of the 21st century are estimated to decrease surface ocean pH by 0.2–0.4 units, and an additional 0.7 units by the year 2300, leading to associated changes in seawater carbonate chemistry and physiological processes of marine organisms (Feely et al., 2004; Orr et al., 2005; Meehl et al., 2007). The impacts of OA have been shown to vary by habitat (Watanabe et al., 2006; Pane and Barry, 2007; Clark et al., 2009), by taxonomic group (Ries et al., 2009; Maas et al., 2012), and between populations and individuals of the same species (Walther et al., 2010; Pistevo et al., 2011), providing strong evidence that future oceans will likely have ‘winners’ and ‘losers’ in response to changing ocean chemistry. To date, few studies have assessed the physiological impacts of OA on early life-history stages of coastal intertidal crustaceans, which transition from embryogenesis and settlement in habitats with fluctuating pH (intertidal zone) to larval periods in relatively stable (pelagic zone) pH environments.

While CO<sub>2</sub> is temporally constant in most oceanic surface waters (Hofmann et al., 2011), naturally high variability in  $P_{\text{CO}_2}$  levels can

occur in other marine systems, including coastal intertidal environments (Agnew and Taylor, 1986; Morris and Taylor, 1983; Wootton et al., 2008). Tidepool pH has been shown to fluctuate more than 0.24 pH units during daily cycles of respiration, photosynthesis and calcification, along with temperature and salinity, and can vary by as much as 1.5 pH units annually (Wootton et al., 2008). It is evident that animals adapted to low pH environments possess enhanced physiological tolerance to high  $P_{\text{CO}_2}$ . For example, elevated  $P_{\text{CO}_2}$  had a stronger impact on the mortality of copepods from surface oceans than on copepods living in high  $P_{\text{CO}_2}$ , deep-sea environments (Watanabe et al., 2006), and pteropods that migrate into CO<sub>2</sub>-rich oxygen minimum zones showed no response to elevated  $P_{\text{CO}_2}$  compared with non-migratory species (Maas et al., 2012). Similarly, larvae of Antarctic urchins occupying cold, low pH upwelled waters were less affected by high  $P_{\text{CO}_2}$  than were urchin larvae from tropical and temperate habitats (Clark et al., 2009).

The above-mentioned studies were conducted on a single life-history stage. However, less is known regarding physiological responses in coastal intertidal zone organisms that display life-history strategies that alternate between variable and stable CO<sub>2</sub> habitats during ontogeny. Such organisms face distinct energetic challenges to maintain homeostasis as, for example, embryonic and

juvenile intertidal zone crabs tolerate daily and seasonal variation in  $\text{CO}_2$ , while larvae occupy a pH/ $P_{\text{CO}_2}$  stable open ocean environment. Thus, evaluating energetic demands in response to low pH among multiple developmental stages is an important component of understanding influences of OA on near-shore ecosystems.

Here, we used an intertidal porcelain crab, *Petrolisthes cinctipes*, to explore how energetic processes are impacted at embryonic, larval and juvenile stages during continuous exposure to low pH seawater. Intertidal stages (embryos and juveniles) likely possess the ability to acclimate to changes in pH as they naturally occupy an environment with high variability in  $P_{\text{CO}_2}$ . However, larvae living in a more stable pH environment may display greater sensitivity to elevated  $P_{\text{CO}_2}$ . Studies of OA responses in crustaceans are limited and contradictory, though high  $\text{CO}_2$  has been reported to negatively impact multiple physiological processes in both adults and early developmental stages, including growth [adult shrimp (Kurihara et al., 2008); larval crab (Walther et al., 2010)], survival [adult barnacle (Findlay et al., 2009)], developmental rate [embryonic barnacle (Findlay et al., 2009); larval crab (Walther et al., 2010); larval shrimp (Bechmann et al., 2011)], immune response [adult lobster (Hernroth et al., 2012)], chemoreception [adult crab (de la Haye et al., 2012)] and swimming performance [adult shrimp (Dissanayake and Ishimatsu, 2011)], along with a narrowing of the thermal window [adult crab (Walther et al., 2009)]. Alternatively, several species displayed increased growth [adult barnacle (McDonald et al., 2009)] and calcification rates [adult crab, shrimp and lobster (Ries et al., 2009)], along with complete compensation of extracellular acid–base balance [adult crab (Pane and Barry, 2007); adult crab (Spicer et al., 2007)] after exposure to elevated  $\text{CO}_2$ . Some crustaceans may have greater hypercapnia tolerance because of their capacity to regulate the pH of their hemolymph with hemocyanins (a non-bicarbonate buffering system) and a  $\text{Na}^+$ -dependent  $\text{HCO}_3^-/\text{Cl}^-$  exchanger (Wheatly and Henry, 1992). However, the efficiency of crustacean buffering varies among species depending on their natural environment (Pane et al., 2008; Whiteley, 2011) and within life-history stages (Bouaricha et al., 1991). Thus, it is probable that early developmental stages of intertidal crustaceans vary in sensitivity to high  $\text{CO}_2$  as a consequence of physiological adaptations to their respective environments, with larval stages expected to be most vulnerable to future ocean acidification.

In this study, physiological energetic parameters including metabolic rates, total protein, dry mass, carbon and nitrogen content and total lipids were determined in individual *P. cinctipes* embryos, larvae and juveniles exposed to present-day near-shore  $P_{\text{CO}_2}$  and year 2300 predictions of ocean  $P_{\text{CO}_2}$  (Meehl et al., 2007).

## MATERIALS AND METHODS

### Study organism

Adult porcelain crabs *P. cinctipes* (J. W. Randall 1840) are common high-intertidal inhabitants of the temperate North Pacific (Stillman and Reeb, 2001; Haig and Abbott, 1980) and provide a model to study responses to OA because of their complex life cycle, in which different life stages are exposed to varying levels of environmental  $\text{CO}_2$  (Wootton et al., 2008; Hofmann et al., 2011). Embryos are brooded by adult females and develop in the intertidal zone for several months (Puls, 2001), later hatching into two planktonic zoea larval stages (Gonor and Gonor, 1973). After approximately 45 days in the water column (Shanks and Eckert, 2005), crabs return to the coast as megalopae, then undergo metamorphosis into juveniles, which begin benthic life in the intertidal zone (Jensen and Armstrong, 1991).

### Animal collection and maintenance

Gravid female porcelain crabs brooding late-stage embryos and newly settled juveniles were collected during low tide from the rocky intertidal zone in Pacifica, CA, USA (37°35'48"N, 122°30'34"W) during March 2011 to August 2011 and transported to laboratory facilities at the Romberg Tiburon Center. Gravid females and juveniles were held individually in 500  $\mu\text{m}$  mesh-bottomed, 385 ml acrylic cylinders (7 cm diameter, 24 cm height). Each cylinder had flow-through water from a recirculating natural seawater system (water collected from Half Moon Bay, 37°46'36"N, 122°42'75"W, SeaPure Inc., El Granada, CA, USA) at constant salinity (32.8 $\pm$ 1.1) and temperature (13.2 $\pm$ 0.2°C) and at ambient pH (7.93 $\pm$ 0.06). Embryos were selected for experimentation and removed from the abdomen of females when eye spots were visible and yolk mass was separated into two lobes, indicating embryos were within 2 weeks of hatching. Embryos ( $N=362$  individuals,  $N=15$  females), newly hatched larvae ( $N=197$  individuals,  $N=10$  females) and freshly settled field-collected juveniles ( $N=89$ ) were randomly assigned to one of two  $\text{CO}_2$  treatments ('ambient pH'=7.93 $\pm$ 0.06 or 'low pH'=7.58 $\pm$ 0.06). Embryos and larvae were maintained separately by brood in mesh-bottomed cylinders (at an approximate density of 100 and 20 individuals per cylinder, respectively), while juveniles were maintained individually in 50 ml mesh-bottomed conical tubes, each enclosed in sealed plastic containers (40 $\times$ 33 $\times$ 17 cm, Iris USA Inc., Pleasant Prairie, WI, USA) containing treatment seawater. Because of the relatively small number of eggs produced by female *P. cinctipes* (~400 eggs per brood on average) (Donahue, 2004), females used for hatching out larvae were different individuals from those from which we obtained embryos. With this limitation, we lacked the ability of observing carry-over effects and could not test for effects of 'stage' within female. Embryos were unfed, while larvae and juveniles were fed daily with a mixture of newly hatched *Artemia* sp. nauplii (San Francisco Bay Brand, Newark, CA, USA), rotifers (*Brachionus plicatilis*) and Shellfish Diet (Reed Mariculture Inc., Campbell, CA, USA) containing a mixture of 30% *Isochrysis* sp., 20% *Pavlova* sp., 20% *Thalassiosira weissflogii* and 30% *Tetraselmis* sp. Treatment seawater was changed daily and dead individuals removed from the cylinders. Survival was assessed in a subsample of individuals throughout experimentation. Embryonic survival (measured as the percentage of embryos hatching successfully) did not differ between pH treatments during the experiments, although hatching success varied from <30% to >95% among a subsample of six broods (Ceballos-Osuna et al., 2013). Larval survival did not differ between treatments, with <50% survival in both treatments after 9 days (Ceballos-Osuna et al., 2013). However, juvenile survival was significantly lower in low pH seawater after 40 days exposure, though almost no mortality occurred during the first week (Ceballos-Osuna et al., 2013).

### Water chemistry manipulation

The  $\text{CO}_2$  system was modeled after Widdicombe and Needham (Widdicombe and Needham, 2007). Each of two treatment tanks – designated as ambient pH and low pH – contained a pH control system consisting of an electrode (Accumet pH Submersible Combination Electrode no. 13-620-AP56, Fisher Scientific, no. 13-620-AP56, Pittsburgh, PA, USA) and digital relay controller (Duo ORpH Controller no. CP2311, Captive Purity Inc., Marine Depot, Garden Grove, CA, USA) to monitor and adjust the pH of natural seawater by bubbling with  $\text{CO}_2$  gas (Industrial grade, 99.5%, PraxAir, Danbury, CT, USA). An electrode within the low pH tank was connected to a  $\text{CO}_2$  regulator, which diffuse-bubbled pure  $\text{CO}_2$  gas when the pH exceeded 7.60. Once the pH was reduced to 7.60,

the CO<sub>2</sub> supply was discontinued *via* an automated feedback relay system. Each tank was bubbled with ambient air and maintained at constant salinity and temperature (ambient pH=13.1±0.2°C, low pH=13.3±0.2°C). Water was pumped from mixing tanks into treatment containers when the pH electrode indicated a pH of 7.60±0.03. Year 2300 *P*<sub>CO<sub>2</sub></sub> predictions were used rather than the more commonly applied year 2100 estimates because the year 2100 predictions are for the open ocean. There is some evidence that the intertidal zone currently experiences *P*<sub>CO<sub>2</sub></sub> values predicted for the open ocean in 2100 (Hofmann et al., 2011). Therefore, when studying intertidal organisms, a more accurate projection for the *P*<sub>CO<sub>2</sub></sub> they will experience in 2100 may actually be the *P*<sub>CO<sub>2</sub></sub> predicted for the open ocean in 2300. For this reason, we have applied IPCC predictions for 2300 in this study.

Seawater samples for pH were measured biweekly during the experiments and determined with a spectrophotometer at 25°C (Shimadzu UV-1700 PharmaSpec, Shimadzu, Columbia, MD, USA) following a protocol from SOP 6b (Dickson et al., 2007) using *m*-Cresol Purple sodium salt (90% dye content, Sigma-Aldrich no. 211761, St Louis, MO, USA). Samples for total alkalinity were collected weekly and measured by potentiometric titration with 0.05 mol l<sup>-1</sup> HCl (Metrohm dosimat 765 pH-Meter, Herisau, Switzerland) using the linear Gran procedure (Gran, 1952) (Table 1). Certified Dickson references were used as standards (<http://andrew.ucsd.edu>). Salinity and temperature were measured daily using a conductivity meter (YSI Model 30-25FT, Yellow Springs, OH, USA).

The statistical computing program R version 2.13.1 (R Development Core Team, 2011) package 'seacarb' version 2.4.3 (Lavigne and Gattuso, 2011) was used to calculate the pH at *in situ* temperature (13.1°C), along with additional parameters of the seawater carbonate chemistry including [HCO<sub>3</sub><sup>-</sup>], [CO<sub>3</sub><sup>2-</sup>] and *P*<sub>CO<sub>2</sub></sub> (Table 1). Seawater salinity, temperature, pH and alkalinity were used as input parameters (flag 8) using the total pH scale (pH<sub>T</sub>).

#### Determination of metabolic rate

Closed chamber respirometry was performed on single embryos from *N*=6 females (at 7, 8, 10 days in each treatment), single larvae from *N*=6 females (at 4, 7 and 10 days in each treatment) and freshly settled juvenile crabs (at 3 and 5 days in each treatment) to determine rates of oxygen consumption ( $\dot{V}_{O_2}$ ). Oxygen consumption rate was determined on *N*=2 broods per sampling day with *N*=4–10 individuals per brood per pH treatment for embryos and larvae, and

*N*=10–13 individuals per day per pH treatment for juveniles. Fiber optic micro-sensors connected to an oxygen meter (Microx TX2, PreSens Precision Sensing GmbH, Regensburg, Germany) were used to measure the percentage oxygen saturation of seawater. Micro-sensors were produced following previous methods (Klimant et al., 1995) by coating fiber optic cables (50 mm diameter, 100/140, ST-ST, Anixter, Grand Rapids, MI, USA) in oxygen-sensitive dye Pt(II) meso-Tetra(pentafluorophenyl) porphine (PtT975, Frontier Scientific Inc., Logan, UT, USA). Sensors were calibrated to a deoxygenated solution of 2% sodium sulfite and a 100% oxygen-saturated solution consisting of filtered ambient seawater shaken vigorously for 5 min and allowed to equilibrate with the atmosphere for 10 min. Temperature was measured with a Type-T thermocouple and entered manually during the calibration. Glass V-bottomed vials (0.3 ml, 11 mm, Wheaton, Cole-Palmer, Vernon Hills, IL, USA) were used as respirometry chambers for individual embryo, larval and juvenile crabs. A small magnetic cube (1.5 mm<sup>3</sup>, NdFeB Neodymium Magnet, K&J Magnetics, Jamison, PA, USA) was placed in each vial to allow mixing of the seawater before measurements by inversion of the vials. Individuals were placed in vials filled with 13°C equilibrated, filtered treatment seawater, with four vials containing only treatment seawater as controls. After 10 min of resting time, vials were capped with an airtight seal and submerged in a 13°C waterbath. The initial percentage air saturation of two controls was measured after all vials were sealed. Chambers were not allowed to drop below 79% air saturation. Endpoint percentage air saturation measurements were determined for embryos after approximately 2 h, larvae after approximately 1 h and juveniles after approximately 45 min, along with the two remaining blanks. Oxygen concentration per chamber was calculated from percentage oxygen saturation in seawater (Huber and Krause, 2006). After metabolic rate determination, individuals were removed from vials, rinsed briefly in deionized water, blotted dry with lint-free filter paper and stored individually in 1.5 ml tubes at -80°C until total protein determination.

#### Total protein determination

Total protein was quantified in all individuals from oxygen consumption rate experiments within a month of metabolic rate determination. Samples were removed from -80°C storage and thawed on ice, then a microplate-based Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA), optimized for 0.5–40 µg protein, was used to determine total protein in individual embryos and larvae. The Coomassie Bradford protein assay (Thermo Scientific) was used to determine total protein per individual in juvenile crabs. Standards were prepared using bovine serum albumin (2 mg ml<sup>-1</sup> ampules, no. 23209, Thermo Scientific).

#### Dry mass and elemental analyses

For dry mass and elemental analysis, randomly selected individuals from each stage were briefly rinsed in deionized water, blotted dry and stored at -80°C for later analysis. Embryos were sampled after 5, 6 and 7 days in pH treatments (*N*=6 broods, *N*=33–35 individuals per pH treatment), larvae (*N*=2 broods, *N*=15–19 individuals per pH treatment) were sampled after 6 days in pH treatments, and juveniles were sampled after 8, 13, 19 and 33 days in pH treatments (maternity unknown, *N*=4–6 individuals per pH treatment per day). Individuals were then placed in pre-weighed 5×9 mm tin capsules (Costech Analytical Technologies Inc., Valencia, CA, USA), dried at 60°C for 24 h, and cooled for an additional 24 h at room temperature in a sealed desiccation chamber containing anhydrous CaSO<sub>4</sub>. Individuals were weighed on a high precision microbalance

Table 1. Salinity, temperature and parameters of the carbonate system

Parameter	Control	Acidified
Salinity	32.8±0.9	32.7±1.4
Temperature (°C)	13.1±0.2	13.3±0.2
pH <sub>T</sub>	7.93±0.06	7.58±0.06
DIC*	2271±135	2338±104
TA (µequiv kg <sup>-1</sup> )	2380±98	2364±105
<i>P</i> <sub>CO<sub>2</sub></sub> (µatm) <sup>1</sup>	574±105	1361±199
[HCO <sub>3</sub> <sup>-</sup> ] (µmol kg <sup>-1</sup> )*	2124±122	2255±124
[CO <sub>3</sub> <sup>2-</sup> ] (µmol kg <sup>-1</sup> )*	118±14	57±9

Values (means ± s.d.) were measured/calculated during experiments investigating differences in carbonate chemistry between control and acidified treatments.

pH<sub>T</sub>, total pH scale; DIC, dissolved inorganic carbon; TA, total alkalinity.

\*Parameters calculated using R package 'seacarb' version 2.4.3 (Lavigne and Gattuso, 2011). Total pH scale, Kf (from Perez and Fraga, 1987), k1, k2 (from Lueker et al., 2000) and Ks (from Dickson, 1990).



(Sartorius SE2, Goettingen, Germany) and carbon (C) and nitrogen (N) content were measured in an elemental analyzer (ECS 4010 CHNSO Analyzer, Costech Analytical Technologies Inc.). Carbon to nitrogen ratio (C/N), a relative index of lipid to protein content, was then determined for each individual.

#### Total lipid determination

Randomly selected embryos and larvae sampled for total lipid determination were rinsed with Milli-Q ultrapure water, blotted dry with filter paper and stored at  $-80^{\circ}\text{C}$  until analysis. Embryos were sampled after 0, 6 and 13 days in pH treatments ( $N=3$  broods,  $N=30-32$  individuals per pH treatment per day) and larvae were sampled after 0 and 6 days in pH treatments ( $N=2$  broods,  $N=15-16$  individuals per pH treatment per day). Individual larval and embryo total lipid content was determined using a modified version of the spectrophotometric sulfophosphovanillin method (Barnes and Blackstock, 1973; Van Handel, 1985b). Samples were homogenized in 500  $\mu\text{l}$  of methanol:chloroform (1:1) using an ultrasonic probe (VirTis VirSonic 100 ultrasonic cell disruptor, Gardiner, NY, USA) and placed in a  $95^{\circ}\text{C}$  heat plate (Isotemp 125D, Isotemp Research Inc., Charlottesville, VA, USA) to allow the solvent to evaporate. Tubes were removed from the heat and cooled to room temperature; 300  $\mu\text{l}$  of concentrated sulfuric acid was then added to each sample and gently mixed. For standards, a serial dilution of 2  $\text{mg ml}^{-1}$  triglyceride (T7531-STD, Pointe Scientific, Canton, MI, USA) was prepared in 95% concentrated sulfuric acid in the range 0–200  $\mu\text{g}$ . Tubes containing sample or standard were heated for an additional 10 min at  $95^{\circ}\text{C}$  on a heat plate. Samples and standards were removed from the heat and cooled to room temperature. Colored reagent was prepared by dissolving vanillin (99%, Sigma-Aldrich ReagentPlus V1104) in 85% phosphoric acid and Milli-Q ultrapure water. In a separate 1.5 ml tube, 200  $\mu\text{l}$  of homogenate–sulfuric acid mixture was added to 1 ml colored reagent and vortexed. To a 96-well flat-bottomed microplate (Corning 96 Well Flat Clear Bottom Black, Fisher Scientific), 150  $\mu\text{l}$  of sample or standard was transferred to each well in triplicate and absorbance read at 540 nm in a  $22.2^{\circ}\text{C}$  temperature-controlled Tecan microplate spectrophotometer (Infinite F200, Männedorf, Switzerland). Net absorbance of the standards was plotted against concentration and a polynomial best-fit line was fitted to these values. Unknown sample concentrations were interpolated from the equation of the best-fit line. Concentrations were then multiplied by a dilution factor to obtain total lipid content per individual ( $\mu\text{g individual}^{-1}$ ).

#### Statistical analyses

In embryos and larvae, oxygen consumption rate, total protein and dry mass were normalized to the mean of each variable per brood in individuals from both control and acidified conditions to remove variation due to differences between broods in basal metabolism. Results were analyzed within each stage and checked for normality (Shapiro–Wilk test) and heteroscedasticity ( $F$ -test). When data did not pass these assumptions for an ANOVA, they were transformed [ $\log_{10}$  for larval and juvenile metabolism normalized to total protein,  $\log_{10}(x+1)$  for embryo metabolism normalized to total protein, and squared for juvenile metabolism per individual and juvenile total protein]. When data transformations could not yield normal distributions and equal variance, a non-parametric Kruskal–Wallis rank-sum test was performed (total protein, dry mass, embryo and larval elemental composition). For embryo and larval data in which maternity was known, a three-way ANOVA was performed to test for the effect of pH, exposure duration and brood, with brood nested within pH treatment. When the interaction between factors was

Table 2. Effect of pH on *Petrolisthes cinctipes* embryos

Metabolic rate					
ANOVA	d.f.	SS	MS	$F$	$P$
pH	1	0.06	0.06	12	<0.001
Days	1	0.0009	0.0009	0.20	0.66
pH–brood	9	0.15	0.02	3.6	<0.001
Residuals	97	0.45	0.004		
Metabolic rate per total protein					
ANOVA	d.f.	SS	MS	$F$	$P$
pH	1	0.05	0.05	9.6	<0.01
Days	1	0.001	0.001	0.21	0.65
pH–brood	9	0.16	0.02	3.6	<0.001
Residuals	97	0.47	0.005		
Total protein					
Kruskal–Wallis	d.f.	$\chi^2$			$P$
pH	1	0.81			0.37
Brood	5	67			<0.001
Dry mass					
Kruskal–Wallis	d.f.	$\chi^2$			$P$
pH	1	4.7			<0.05
Carbon (% mass)					
Kruskal–Wallis	d.f.	$\chi^2$			$P$
pH	1	1.6			0.20
Nitrogen (% mass)					
Kruskal–Wallis	d.f.	$\chi^2$			$P$
pH	1	3			0.08
C/N ratio					
Kruskal–Wallis	d.f.	$\chi^2$			$P$
pH	1	0.1			0.75
Lipid consumption rate					
Linear regression	d.f.	$t$ -value			$P$
Ambient pH–days	91	–13.4			<0.001
Low pH–days	90	–12.8			<0.001
ANOVA of linear models	d.f.	SS	RSS	$F$	$P$
pH	1	0.03	1.7	3.8	0.07

Results of ANOVA and Kruskal–Wallis rank-sum tests are shown.

significant, a Tukey HSD *post hoc* test was performed to detect differences between broods in response to pH. For time series data (embryo and larval total lipids, juvenile dry mass and elemental composition), an ANOVA was conducted on linear models within each stage to test for effects of pH. The  $\alpha$ -level to reject the null hypothesis was set to 0.05. All statistical analyses were performed using the statistical computing program R, version 2.13.1 (R Development Core Team, 2011).

## RESULTS

### $\dot{V}_{\text{O}_2}$ Embryo

After 7–10 days treatment, embryos pooled from six broods reared at low pH had an 11% mean metabolic rate reduction ( $1.7 \pm 0.1 \mu\text{mol O}_2 \text{ h}^{-1} \text{ individual}^{-1}$ ) compared with control individuals ( $1.9 \pm 0.1 \mu\text{mol O}_2 \text{ h}^{-1} \text{ individual}^{-1}$ ) (ANOVA,  $F_{1,97}=12$ ,  $P<0.001$ ; Table 2, Fig. 1A). There was no effect of exposure duration on metabolism (ANOVA,  $F_{1,0.20}=0.20$ ,  $P=0.66$ ; Table 2). With the mean  $\dot{V}_{\text{O}_2}$  per brood at control pH ranging from  $1.2 \pm 0.1 \mu\text{mol O}_2 \text{ h}^{-1} \text{ individual}^{-1}$  (female E) to  $2.7 \pm 0.1 \mu\text{mol O}_2 \text{ h}^{-1} \text{ individual}^{-1}$  (female C), oxygen consumption rates were highly variable among broods (ANOVA,  $F_{5,97}=24$ ,  $P<0.001$ ; Table 2, Fig. 2A). Additionally, the metabolic responses of embryos to low pH differed depending on the brood they were from, as evidenced by the statistically significant interaction between pH and brood (ANOVA,  $F_{1,9}=3.5$ ,  $P<0.001$ ; Table 2, Fig. 2A). Of six broods sampled, individuals from broods D and E experienced the largest metabolic decrease, with a  $43 \pm 5\%$  (brood D) (Tukey HSD,

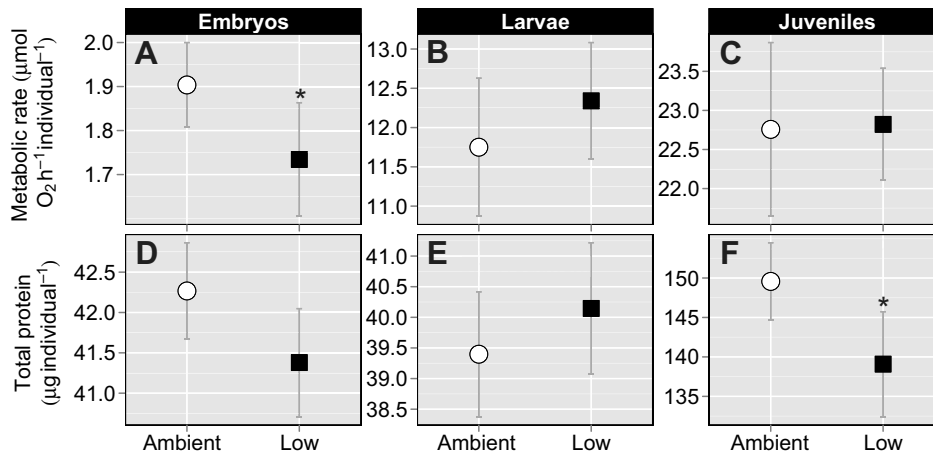


Fig. 1. (A–C) Oxygen consumption rate and (D–F) total protein at embryo, zoea I larvae and early juvenile stages of *Petrolisthes cinctipes* after short-term exposure (embryos 7–10 days, larvae 4–10 days, juveniles 3–5 days) to ambient pH ( $7.93 \pm 0.06$ ) or low pH ( $7.58 \pm 0.06$ ). Each point represents the mean  $\pm$  s.e.m. for  $N=54$ – $55$  individuals for embryos,  $N=50$  individuals for larvae and  $N=23$ – $24$  individuals for juveniles. Individuals were pooled from six broods for embryos and larvae (females used for hatching out larvae were different from those from which we obtained embryos). \*Significant difference ( $P \leq 0.05$ ) between pH treatments within a developmental stage. For embryos and larvae, statistical analyses were performed on data normalized to the mean oxygen consumption rate per brood to remove variation due to differences in basal metabolism among clutches.

$P < 0.05$ ) and  $54 \pm 9\%$  (brood E) (Tukey HDS,  $P < 0.0001$ ) reduction in mean oxygen consumption rate compared with control (Fig. 3). Furthermore, these two broods with the strongest negative responses to low pH also exhibited two of the lower control metabolic rates (Fig. 2A). Overall, the impact of low pH on metabolism per brood in embryos was largely negative compared with that in larvae (ANOVA,  $F_{1,92}=12$ ,  $P < 0.001$ , Fig. 3).

#### Larval

After 4–10 days in acidified seawater, zoea I larval metabolic rates from individuals pooled from six broods ( $12.2 \pm 0.7 \mu\text{mol O}_2 \text{h}^{-1} \text{individual}^{-1}$ ) did not differ significantly from control ( $12.0 \pm 0.9 \mu\text{mol O}_2 \text{h}^{-1} \text{individual}^{-1}$ ) (ANOVA,  $F_{1,99}=1.3$ ,  $P=0.25$ ; Table 3, Fig. 1B). However, similar to embryonic metabolism, larval  $\dot{V}_{\text{O}_2}$  was highly variable between broods, with control metabolic rates ranging from  $8.6 \pm 1 \mu\text{mol O}_2 \text{h}^{-1} \text{individual}^{-1}$  (brood 5) to  $19.5 \pm 1.6 \mu\text{mol O}_2 \text{h}^{-1} \text{individual}^{-1}$  (brood 2) (ANOVA,  $F_{1,105}=47.9$ ,  $P < 0.001$ ; Fig. 2B). When compared with the mean  $\dot{V}_{\text{O}_2}$  per brood of individuals reared in ambient pH, larvae from four of six broods had increased metabolic rate on average, with the largest metabolic increases per brood of 48% (brood 1) and 27% (brood 6) compared with control, suggesting a trend of elevated metabolism in response to  $\text{CO}_2$  in several broods (Fig. 3).

#### Juvenile

After 3 and 5 days in low pH seawater, juvenile oxygen consumption rates per individual ( $22.8 \pm 1.1 \mu\text{mol O}_2 \text{h}^{-1} \text{individual}^{-1}$ ) were not significantly different from those of juvenile crabs reared in control pH ( $22.8 \pm 0.7 \mu\text{mol O}_2 \text{h}^{-1} \text{individual}^{-1}$ ) (ANOVA,  $F_{1,44}=0.05$ ,  $P=0.83$ ; Table 4, Fig. 1C). We were unable to test for female effects on physiological parameters in juveniles as individuals were collected from the field and maternity was unknown.

#### Total protein Embryo

Total protein content per individual of embryos reared in low pH seawater for 7–10 days ( $41.4 \pm 0.6 \mu\text{g individual}^{-1}$ ) did not differ

significantly from that in control conditions ( $42.3 \pm 0.6 \mu\text{g individual}^{-1}$ ) (Kruskal–Wallis,  $\chi^2_1=0.81$ ,  $P=0.37$ ; Table 2, Fig. 1D). However, total protein content was highly variable among broods, and ranged from  $37.6 \pm 0.5 \mu\text{g individual}^{-1}$  (brood B) to  $48.3 \pm 1.0 \mu\text{g individual}^{-1}$  (brood E) (Kruskal–Wallis,  $\chi^2_1=67$ ,  $P < 0.0001$ ; Table 2; Fig. 2A). Normalizing embryo metabolic rate to individual total protein content did not change the outcome of  $\dot{V}_{\text{O}_2}$  analyses. Significantly reduced metabolic rate in individuals reared in low pH seawater ( $0.046 \pm 0.003 \mu\text{mol O}_2 \text{h}^{-1} \text{individual}^{-1}$ ) compared with control ( $0.044 \pm 0.003 \mu\text{mol O}_2 \text{h}^{-1} \text{individual}^{-1}$ ) (ANOVA,  $F_{1,97}=9.6$ ,  $P < 0.01$ ; Table 2), and an interaction of pH treatment and brood (ANOVA,  $F_{9,97}=3.6$ ,  $P < 0.001$ ; Table 2) were still evident after normalization to total protein.

#### Larval

Analysis of larval total protein content indicated no significant difference between individuals reared in low pH ( $40 \pm 1 \mu\text{g individual}^{-1}$ ) and ambient pH ( $39 \pm 1 \mu\text{g individual}^{-1}$ ) conditions after 4–10 days (Kruskal–Wallis,  $\chi^2_1=1.5$ ,  $P=0.23$ ; Table 3, Fig. 1E). Similar to embryos, total protein content was highly variable among females, with protein in control-reared larvae ranging from  $30 \pm 1$  to  $45 \pm 3 \mu\text{g individual}^{-1}$  (Kruskal–Wallis,  $\chi^2_1=53$ ,  $P < 0.001$ ; Table 3). Analysis of metabolic rate normalized to total protein content indicated equivalent results to metabolic rate per individual, in which rates of individuals reared at ambient pH ( $0.29 \pm 0.02 \mu\text{mol O}_2 \text{h}^{-1} \text{individual}^{-1}$ ) and low pH ( $0.30 \pm 0.02 \mu\text{mol O}_2 \text{h}^{-1} \text{individual}^{-1}$ ) did not differ significantly from one another (ANOVA,  $F_{1,99}=1$ ,  $P=0.32$ ; Table 3).

#### Juvenile

Juvenile *P. cinctipes* reared in low pH seawater contained 7% less protein than control individuals after 3 and 5 days (low pH  $139 \pm 7 \mu\text{g individual}^{-1}$ , control  $149 \pm 5 \mu\text{g individual}^{-1}$ ; Kruskal–Wallis,  $\chi^2_1=3.8$ ,  $P < 0.05$ ; Table 4, Fig. 1F). When juvenile  $\dot{V}_{\text{O}_2}$  was normalized to total protein content, metabolic rate did not significantly differ between control ( $0.15 \pm 0.02 \mu\text{mol O}_2 \text{h}^{-1} \mu\text{g protein}^{-1}$ ) and low pH conditions ( $0.18 \pm 0.02 \mu\text{mol O}_2 \text{h}^{-1} \mu\text{g protein}^{-1}$ ) (ANOVA,  $F_{1,42}=0.16$ ,  $P=0.69$ ; Table 4).

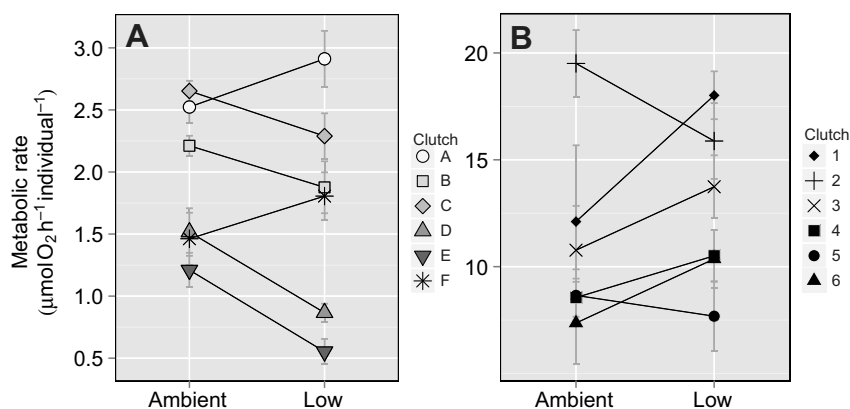


Fig. 2. *Petrolisthes cinctipes* oxygen consumption rate per brood of (A) embryos after 7–10 days exposure and (B) larvae after 4–10 days exposure to ambient pH (7.93±0.06) or low pH (7.58±0.06). Six separate broods were tested per stage (see key). Females used for hatching out larvae were different from those from which we obtained embryos. Each point represents the mean ± s.e.m. for  $N=4-10$  individuals. \*Significant difference ( $P\leq 0.05$ ) between pH treatments for a female's offspring within a developmental stage. Metabolic responses in embryos exhibited a significant interaction of pH treatment and brood (ANOVA,  $F_{9,91}=3.5$ ,  $P<0.0001$ ).

### Dry mass and elemental analysis

#### Embryo

Embryos reared in low pH seawater for 5–7 days ( $80.9\pm 10.0\mu\text{g individual}^{-1}$ ) had 6% lower mean dry mass than control individuals ( $85.8\pm 9.1\mu\text{g individual}^{-1}$ ) (Kruskal–Wallis,  $\chi^2_1=4.7$ ,  $P<0.05$ ; Table 2, Fig. 4A). As a percentage of dry mass, differences in mean carbon (low pH  $46.7\pm 1.3\%$  C, control  $44.5\pm 0.9\%$  C) and nitrogen (low pH  $10.3\pm 0.2\%$  N, control  $10.0\pm 0.2\%$  N) were non-significant (Kruskal–Wallis, carbon  $\chi^2_1=1.6$ ,  $P=0.20$ ; nitrogen  $\chi^2_1=3$ ,  $P=0.08$ ; Fig. 4C,E). The ratio of C/N – a relative index of lipid to protein content – did not vary between individuals in low pH conditions (pH  $4.6\pm 0.1$ ) and control treatments (pH  $4.5\pm 0.1$ ) (Kruskal–Wallis,  $\chi^2_1=0.1$ ,  $P=0.75$ ; Table 2, Fig. 4G).

#### Larval

Although no differences between treatments were observed in larval total protein per individual, nitrogen as a percentage of larval mass was significantly lower by 7% in individuals exposed to low pH water ( $9.3\pm 0.2\%$  N) compared with control ( $10.0\pm 0.2\%$  N), suggesting that larvae contain less protein under low pH conditions when values are considered per mass instead of per individual (Kruskal–Wallis,  $\chi^2_1=6.7$ ,  $P<0.01$ ; Table 3, Fig. 4F). Consequently, C/N ratio was significantly higher when individuals developed in low pH seawater (pH  $3.6\pm 0.08$ ) compared with control (pH  $3.4\pm 0.09$ ) (Kruskal–Wallis,  $\chi^2_1=7.4$ ,  $P<0.01$ ; Table 3, Fig. 4H). Mean dry mass of larvae from two broods reared in low pH ( $88.0\pm 16.5\mu\text{g individual}^{-1}$ ) or control ( $85.0\pm 12.2\mu\text{g protein}^{-1}$ ) seawater was not impacted by  $\text{CO}_2$  (Kruskal–Wallis,  $\chi^2_1=0.97$ ,  $P=0.32$ ; Table 3, Fig. 4B). Similarly, carbon levels (% mass) of larvae did not differ significantly between individuals reared in low pH ( $33.8\pm 0.8\%$  C) and ambient pH ( $33.1\pm 0.4\%$  C) conditions (Kruskal–Wallis,  $\chi^2_1=0.17$ ,  $P=0.68$ ; Table 3, Fig. 4D).

#### Juvenile

Juvenile dry mass after 8–33 days did not increase significantly with time in either treatment according to analysis of linear models (control  $t_{20}=0.8$ ,  $P=0.42$ , low pH  $t_{17}=1.5$ ,  $P=0.16$ ; Table 4) and there was no direct effect of  $\text{CO}_2$  (ANOVA,  $F_{1,38}=1.8$ ,  $P=0.18$ ; Table 4, Fig. 5A). Mean carbon content decreased with development by 10% in low pH (day 8,  $31.4\pm 1.1\%$  C; day 33,  $28.2\pm 1.7\%$  C;  $P<0.02$ ) and 4% in ambient pH treatments (day 8,  $30.6\pm 0.4\%$  C; day 33,  $29.5\pm 0.6\%$  C;  $P<0.03$ ), although no direct effect of  $\text{CO}_2$  on carbon content was observed (ANOVA,  $F_{1,38}=1.0$ ,  $P=0.32$ ; Table 4, Fig. 5B). Juvenile nitrogen content across all days was not affected by  $\text{CO}_2$  (ANOVA,

$F_{1,38}=0.27$ ,  $P=0.60$ ; Table 4, Fig. 5C). While the ratio of carbon to nitrogen did not vary across days for both treatments (control  $t_{20}=-1.0$ ,  $P=0.33$ , low  $t_{17}=1.9$ ,  $P=0.08$ ; Table 4), regression analysis suggested a small effect of  $\text{CO}_2$  on juvenile C/N (ANOVA,  $F_{1,38}=4.0$ ,  $P=0.05$ ; Table 4, Fig. 5D). On day 8, low pH-reared individuals had 7% lower mean C/N compared with control (low pH 4.0, control 4.3); however, differences disappeared on day 33.

### Total lipids

#### Embryo

Embryo total lipids decreased with developmental time for both treatments (control  $t_{91}=-13.4$ ,  $P<0.001$ ; low  $t_{90}=-12.8$ ,  $P<0.0012$ ; Table 2, Fig. 6A). After 13 days in treatment, mean total lipids of low pH-reared individuals decreased by 31% (day 0,  $6.7\pm 0.1\mu\text{g individual}^{-1}$ ; day 13,  $4.7\pm 0.1\mu\text{g individual}^{-1}$ ), while control individuals decreased by 34% (day 0,  $6.7\pm 0.1\mu\text{g individual}^{-1}$ ; day 13,  $4.4\pm 0.1\mu\text{g individual}^{-1}$ ). Linear regression analysis of embryo total lipid content per day (control  $-0.18\mu\text{g individual}^{-1}\text{ day}^{-1}$ ; low pH  $-0.16\mu\text{g individual}^{-1}\text{ day}^{-1}$ ) revealed no significant differences in lipid consumption between  $\text{CO}_2$  treatments (ANOVA,  $F_{1,182}=3.4$ ,  $P=0.07$ ; Table 2).

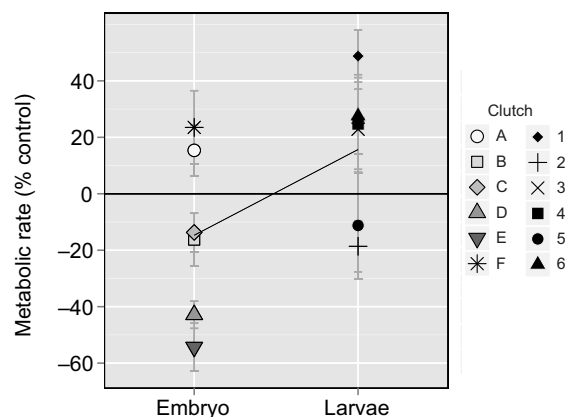


Fig. 3. Effect of changes in pH ( $\Delta\text{pH}$ ) on the metabolism of *P. cinctipes* embryos and zoea I larvae, presented as the percentage difference in oxygen consumption rate per individual between low pH (7.58±0.06)-reared individuals and ambient pH (7.93±0.06)-reared individuals for each brood. Effect of  $\Delta\text{pH}$  becomes significantly more positive from embryos to larvae (ANOVA,  $F_{1,92}=12.6$ ,  $P<0.0001$ ). Each point represents the mean ± s.e.m. for  $N=8-10$  low pH-reared individuals from one of six broods per stage. Females used for hatching out larvae were different from those from which we obtained embryos.

Table 3. Effect of pH on *Petrolisthes cinctipes* zoea I larvae

Metabolic rate					
ANOVA	d.f.	SS	MS	F	P
pH	1	0.24	0.24	1.3	0.25
Days	1	0	0	0	0.99
pH–brood	2	0.05	0.02	0.13	0.88
pH–days	1	0.2	0.2	1.1	0.29
pH–brood–days	2	0.25	0.12	0.68	0.51
Residuals	99	18	0.18		
Metabolic rate per total protein					
ANOVA	d.f.	SS	MS	F	P
pH	1	0.007	0.007	1	0.32
Days	1	0.02	0.02	2.6	0.11
pH–brood	2	0.004	0.002	0.27	0.76
pH–days	1	0.01	0.01	1.6	0.21
pH–brood–days	2	0.01	0.005	0.67	0.51
Residuals	99	0.71	0.007		
Total protein					
Kruskal–Wallis	d.f.	$\chi^2$			P
pH	1	1.5			0.23
Brood	5	53			<0.001
Dry mass					
Kruskal–Wallis	d.f.	$\chi^2$			P
pH	1	0.97			0.32
Carbon (% mass)					
Kruskal–Wallis	d.f.	$\chi^2$			P
pH	1	0.17			0.68
Nitrogen (% mass)					
Kruskal–Wallis	d.f.	$\chi^2$			P
pH	1	6.7			<0.01
C/N ratio					
Kruskal–Wallis	d.f.	$\chi^2$			P
pH	1	7.4			<0.01
Lipid consumption rate					
Linear regression	d.f.	t-value			P
Ambient pH–days	29	12.5			<0.001
Low pH–days	29	10.9			<0.001
ANOVA of linear models	d.f.	SS	MS	F	P
pH	1	0.0005	0.82	0.03	0.85

Results of ANOVA, linear regression analysis and Kruskal–Wallis rank-sum tests are shown.

#### Larval

Zoea I larval total lipids increased significantly with time from hatching for both treatments (ambient pH  $t_{29}=12.5$ ,  $P<0.001$ ; low pH  $t_{29}=10.9$ ,  $P<0.001$ ; Table 3, Fig. 6B). Mean total lipids increased by 48% in low pH-reared individuals (day 0,  $5.0\pm 0.2\ \mu\text{g individual}^{-1}$ ; day 6,  $7.5\pm 0.2\ \mu\text{g individual}^{-1}$ ) compared with 49% in control (day 0,  $5.0\pm 0.2\ \mu\text{g individual}^{-1}$ ; day 6,  $7.5\pm 0.2\ \mu\text{g individual}^{-1}$ ). However, lipid deposition rates did not vary between low pH and control treatments, with mean rates of 0.39 and 0.41  $\mu\text{g individual}^{-1}\text{ day}^{-1}$ , respectively (ANOVA,  $F_{1,60}=0.03$ ,  $P=0.85$ ; Table 3).

#### DISCUSSION

The intertidal porcelain crab *P. cinctipes* was used as a model to determine whether metabolic responses to ocean acidification vary during ontogeny in organisms that complete their life cycle in both  $\text{CO}_2$ -variable and -stable environments. We assessed metabolic rate, total protein, dry mass, total lipids and C/N to compare energetic demands between embryos, larvae and juveniles reared in ambient pH (7.93 $\pm$ 0.06) and low pH (7.58 $\pm$ 0.06) conditions. Significant effects of pH were observed in one or more metabolic performance characteristics at each stage; however, responses differed in magnitude and direction. Moreover, while previous physiological studies in crab early life-history stages have reported differences in dry mass and C/N in response to elevated  $\text{CO}_2$  (Walther et al., 2010), variable

Table 4. Effect of pH on *Petrolisthes cinctipes* juveniles

Oxygen consumption rate					
ANOVA	d.f.	SS	MS	F	P
pH	1	1915	1915	0.05	0.83
Residuals	44	1807951	41090		
Oxygen consumption rate per total protein					
ANOVA	d.f.	SS	MS	F	P
pH	1	0.002	0.002	0.16	0.69
Residuals	42	0.47	0.01		
Total protein					
Kruskal–Wallis	d.f.	$\chi^2$			P
pH	1	3.8			<0.05
Dry mass					
Linear regression	d.f.	t-value			P
Ambient pH–days	20	0.8			0.42
Low pH–days	17	1.5			0.16
ANOVA of linear models	d.f.	SS	RSS	F	P
pH	38	25846	537205	1.8	0.18
C (% mass)					
Linear regression	d.f.	t-value			P
Ambient pH–days	20	-2.7			<0.02
Low pH–days	17	-2.4			<0.03
ANOVA of linear models	d.f.	SS	RSS	F	P
pH	1	3.0	112	1.0	0.32
N (% mass)					
Linear regression	d.f.	t-value			P
Ambient pH–days	20	-1.0			0.32
Low pH–days	17	-3.2			<0.01
ANOVA of linear models	d.f.	SS	RSS	F	P
pH	1	0.1	12	0.27	0.60
C/N ratio					
Linear regression	d.f.	t-value			P
Ambient pH–days	20	-1.0			0.33
Low pH–days	17	1.9			0.08
ANOVA of linear models	d.f.	SS	RSS	F	P
pH	1	0.2	1.5	4.0	0.05

Results of ANOVA, linear regression analysis and Kruskal–Wallis rank sum tests are shown.

metabolic responses to  $\text{CO}_2$  among female broods have not been recorded in crustaceans. We expected zoea I larvae would display greater sensitivity to  $\text{CO}_2$  as they develop in a relatively  $\text{CO}_2$ -stable open-ocean environment. However, our data suggest embryos are likely the bottleneck in *P. cinctipes* development in sustained low pH, as observed metabolic reduction in several broods may lead to delayed development and have indirect effects on survival and fitness.

#### Embryo responses

Metabolic responses to low pH appear highly variable among embryo broods, as evidenced by the significant interaction between pH and brood (ANOVA,  $F_{1,9}=3.5$ ,  $P<0.001$ ), and could be a consequence of both genetic and environmental factors. While embryos were staged visually, it is improbable that observed differences are due to sampling individuals of different ages, as embryonic development occurs over approximately 3 months (Knudsen, 1964) and embryos were selected for experimentation after the appearance of eyespots (2 weeks from hatching), and thus they are likely within several developmental days of one another. In addition, though in our experimental design brood and treatment were not truly independent, the substantial variation between broods across multiple experiments provides considerable support for the existence of brood-specific responses to OA. In the sea urchin *Strongylocentrotus droebachiensis*, environment (collection depth)



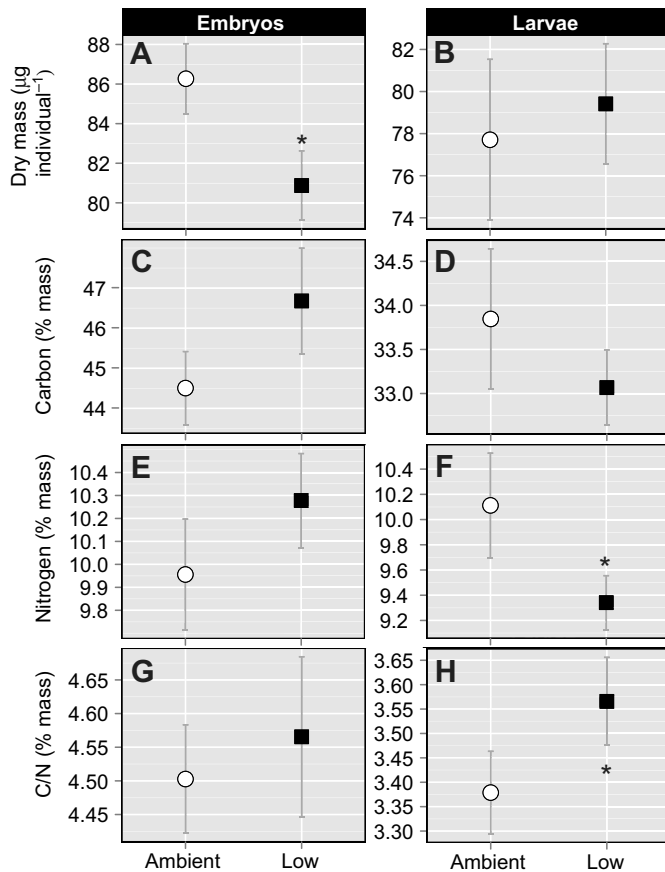


Fig. 4. (A,B) Dry mass, (C,D) carbon, (E,F) nitrogen and (G,H) carbon/nitrogen (C/N) ratio of *P. cinctipes* embryos and larvae reared in ambient pH ( $7.93 \pm 0.06$ ) or low pH ( $7.58 \pm 0.06$ ). Each point represents the mean  $\pm$  s.e.m. for  $N=33-35$  individuals for embryos (after 5–7 days exposure) and  $N=15-19$  individuals for larvae (after 6 days exposure). \*Significant difference ( $P \leq 0.05$ ) between pH treatments within a developmental stage.

influenced maternal nutritional state and impacted the quality and quantity of nutrient reserves deposited in the eggs and egg number/size, which affected growth rate in subsequent larval stages (Bertram and Strathmann, 1998). Thus, in *P. cinctipes* embryos, variation among broods in response to low pH may result from differences based on parental location in the intertidal zone, including differential exposure to thermal, osmotic and nutritional stress, and the associated adverse effects on maternal state during egg production. In both fish and oysters, adults exposed to elevated  $\text{CO}_2$  before reproduction produced offspring that were more tolerant of ocean acidification (Miller et al., 2012; Parker et al., 2011). However, the duration of adult acclimation to high  $\text{CO}_2$  may determine the magnitude of carry-over effects in the next generation, as has been observed in urchins (Dupont et al., 2012). It is plausible that some *P. cinctipes* broods are more tolerant of OA if mothers occupied tidepools with lower pH values during oogenesis, though this phenomenon has not yet been observed in crustaceans and merits further research. There is also an indication that genetic variation underlies differences in sensitivity to pH in some species. For example, clonal individuals of the bryozoan *Celleporella hyaline* displayed contrasting responses to pH (Pistevos et al., 2011). In the larval sand dollar *Dendraster excentricus*, morphological variation amongst maternal lineages in response to  $\text{CO}_2$  was not attributed

to differences in egg provisioning as egg size did not vary between female offspring and thus likely resulted from genetic differences among individuals (Chan et al., 2011). Interestingly, hypercapnia-induced metabolic reduction was largest in *P. cinctipes* embryo broods with lower control metabolic rates; thus, offspring with lower metabolic rates may be less tolerant of  $\text{CO}_2$ . If traits yielding variable responses to pH among *P. cinctipes* broods are genetically heritable, strong selection pressures may favor more tolerant conspecifics (i.e. females producing larger eggs, individuals with higher basal metabolism), leading to possible rapid adaptation to OA as has been observed in an urchin and mussel *via* artificial selection experiments (Sunday et al., 2011). Examining variation in gene expression among *P. cinctipes* families may allow us to differentiate between genetic (e.g. *cis*-regulatory region sequence variation, epigenetic modification) and environmental influences contributing to differences in  $\text{CO}_2$  tolerance between broods.

If mean responses are emphasized, embryonic *P. cinctipes* display the largest metabolic reduction following exposure to low pH. Metabolic depression associated with low pH has been reported in multiple species and is often coupled with a delay in development, activity or growth (Reipschläger and Pörtner, 1996; Michaelidis et al., 2005; Nakamura et al., 2011). In embryos of the barnacle *Semibalanus balanoides*, development was delayed at pH 7.70, accompanied by a 19 day delay to hatching (Findlay et al., 2009). Given the short duration of embryonic exposure to low pH in the current study (approximately 1/10 of total embryonic developmental time), it is unlikely *P. cinctipes* embryos in our experiments were greatly delayed compared with control individuals. However, decreased metabolic rate over the course of the entire embryonic period could result in developmental delay. While decreased metabolism may be a beneficial strategy for responding to an acute stress, sustained depression may come at the cost of energy budget reallocation and reduced energy available for basal maintenance, growth and activity and can have detrimental effects on a species' fitness (Langenbuch and Pörtner, 2004).

Hypercapnia-induced metabolic depression results from an uncompensated decrease in extracellular pH ( $\text{pH}_e$ ) (Pörtner et al., 1998). Many crustaceans can offset a decline in hemolymph  $\text{pH}_e$  by accumulating  $\text{HCO}_3^-$  *via*  $\text{Na}^+$ -dependent  $\text{HCO}_3^-/\text{Cl}^-$  exchangers (Wheatly and Henry, 1992); however, the observed metabolic reduction in *P. cinctipes* embryos suggests a reduced ability for acid–base regulation.  $\text{Na}^+/\text{K}^+$ -ATPases are responsible for re-establishing ion gradients driving  $\text{HCO}_3^-$  exchangers and require a substantial portion of cellular energy (Pörtner et al., 2011; Melzner et al., 2009). In the larval urchin *Strongylocentrotus purpuratus*,  $\text{Na}^+/\text{K}^+$ -ATPase gene expression decreased in individuals exposed to low pH, suggesting high  $\text{CO}_2$  may reduce these energetically expensive transporters (Todgham and Hofmann, 2009). In the estuarine shrimp *Callinassa jamaicense*,  $\text{Na}^+/\text{K}^+$ -ATPase activity increased with development from early to late embryonic stages (Felder et al., 1986), while in the green crab *Carcinus maenas*, ion-regulatory ability, including differentiation of ion-transport epithelia (gills) and  $\text{Na}^+/\text{K}^+$ -ATPase expression, was modified during the course of development from zoea I to juvenile crab instars in association with the osmoregulatory demands at each stage (Cieluch et al., 2004). In the present study, ontogenetic changes in gene expression and activity of enzymes involved in ion regulation may account for the greater metabolic sensitivity to  $\text{CO}_2$  observed at the embryonic stage and increased tolerance in larvae and juveniles, as later stages develop superior acid–base regulatory capacity. The pH sensitivity of  $\text{Na}^+/\text{K}^+$ -ATPase and its role in the ontogeny of ion regulation in crab embryos warrant further research.



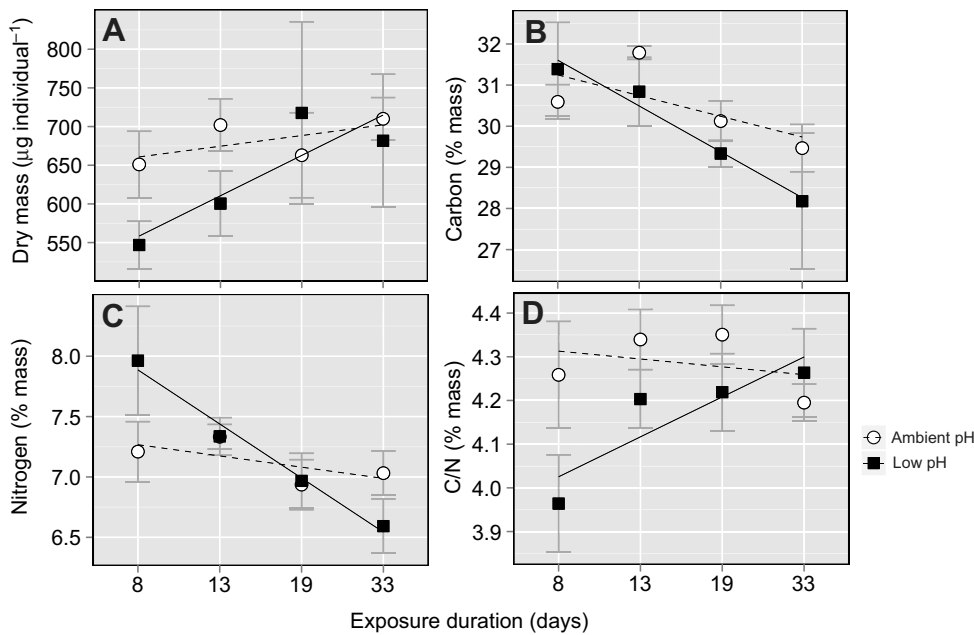


Fig. 5. Juvenile *P. cinctipes* (A) dry mass, (B) carbon content, (C) nitrogen content and (D) C/N of individuals reared in ambient pH ( $7.93 \pm 0.06$ ) or low pH ( $7.58 \pm 0.06$ ) (different individuals sampled for each time point). Each point represents the mean  $\pm$  s.e.m. for  $N=4-6$  individuals. Statistical analysis indicated a minor effect of pH on C/N (ANOVA,  $F_{1,36}=4.0$ ,  $P=0.05$ ).

For crustaceans, it is likely that their developmental mode may result in additional challenges for embryos during exposure to elevated seawater  $P_{CO_2}$ . Crab embryos, which develop inside a chorion (egg casing), may have difficulty in exchanging metabolic  $CO_2$  with the environment and face intracellular and extracellular hypercapnia as a natural part of their life cycle (Melzner et al., 2009). High  $CO_2$  concentrations (1300–4000  $\mu\text{atm}$ , pH as low as 7.2) have been reported in the fluid surrounding cephalopod *Sepia officinalis* embryos (Gutowska and Melzner, 2009), and it is likely *P. cinctipes* embryos experience similar ‘ontogenetic hypercapnia’ (Melzner et al., 2009). Additional increases in seawater proton levels may decrease the diffusional gradient for proton excretion, leading to further extracellular acidosis and metabolic depression observed in embryos in the present study. Additionally, the very low  $P_{O_2}$  levels that accompany high  $P_{CO_2}$  within crab egg masses (Fernández et al., 2000) may cause varying degrees of developmental delay within a brood, extending hatching over the course of several days. With high  $P_{CO_2}$ /low  $P_{O_2}$  expected to be more severe in future oceans, we predict that embryos that spend their entire embryonic development in such conditions may experience extended periods of larval release, the consequences of which are as yet unknown.

The lower dry mass observed in *P. cinctipes* embryos following low pH exposure suggests that responding to elevated  $CO_2$  may come with a bioenergetic cost, as has been shown with the reduced energy available for development in the urchin *S. purpuratus* (Stumpp et al., 2011). *Petrolisthes cinctipes* embryos allocate less energy to growth during short-term exposure to high  $CO_2$ , as evident in the observed embryonic metabolic reduction. A decrease in growth across the entire embryonic stage (approximately 3 months) could delay hatching and have indirect effects on survival, potentially via delayed recruitment (Pechenik et al., 1993). In the barnacle *S. balanoides*, the timing of larval release typically accompanied a spring bloom (Findlay et al., 2009); thus, a delay in embryonic development to hatching may be asynchronous with food supply, leaving late-hatching larvae at a competitive disadvantage.

#### Larval responses

Low pH seawater appeared to have a lesser effect in zoea I larval *P. cinctipes*, with observed impacts on their elemental composition

occurring only after short-term exposure. Carbon and nitrogen, elements highly correlated with the amount of total lipids and proteins, respectively, were used to establish how energy reserves were altered at each developmental stage after exposure to acidified seawater (Anger, 2001). Total lipids, constituting <20% of decapod crustacean biomass, play a key role in growth and development and can be mobilized to provide ATP for responding to environmental stressors (Anger, 2001). Protein synthesis is an energetically expensive process and typically declines in step with metabolic suppression in aquatic invertebrates (Hand and Hardewig, 1996). However, as we did not observe a decrease in larval metabolic rate (Fig. 1B), the observed decrease in nitrogen (increase in C/N) under elevated  $CO_2$  is likely due to an alteration in metabolic substrate preference to proteins. In the peanut worm *Sipunculus nudus*, hypercapnia induced an increase in protein/amino acid catabolism followed by increased plasma  $NH_4^+$  (Pörtner et al., 1998). While further investigation is necessary to determine whether crab larvae increase  $NH_4^+$  production under elevated  $CO_2$ , no differences were observed in lipid deposition rate between treatments, supporting the hypothesis that larvae utilize protein as a source of energy during exposure to low pH. Larvae may be fully able to cover the cost associated with responding to high  $CO_2$  in the short term by switching to protein catabolism, as differences in nitrogen and C/N are not accompanied by changes in growth, metabolism or survival (Ceballos-Osuna et al., 2013). Contrasting C/N results have been seen in larvae from other crab species. In the spider crab *Hyas araneus*, no differences were observed in larval C/N, though megalopa stages displayed decreased C/N with increasing  $CO_2$  only at an intermediate temperature. In the red king crab, *Paralithodes camtschaticus*, larvae had decreased C but not N following 7 days in acidified water, presumably related to faster lipid depletion leading to lower larval survival (Long et al., 2013). Thus, it would appear that there are taxon-specific responses to ocean acidification in crab early life history stages.

#### Juvenile responses

Similar to the larval stage, pH had a minimal effect on the physiology of juvenile *P. cinctipes*. While embryo and zoea I total protein content was not impacted by  $CO_2$ , juveniles contained less

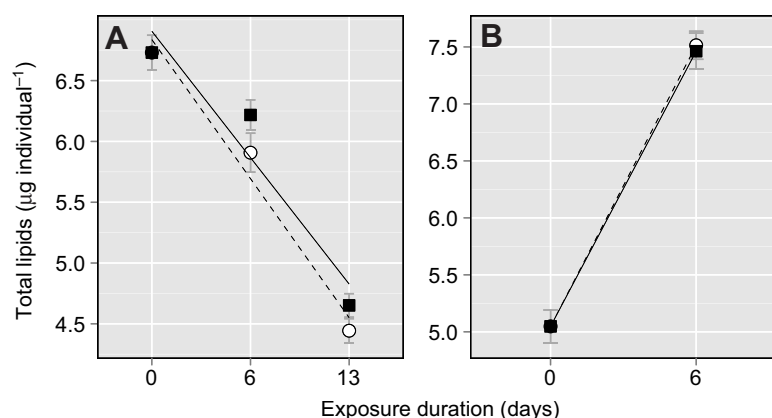


Fig. 6. *Petrolisthes cinctipes* total lipids in (A) embryos after 0, 6 and 13 days and (B) larvae after 0 and 6 days in individuals reared in ambient pH (7.93±0.06) or low pH (7.58±0.06). Each point represents the mean ± s.e.m. for  $N=30-32$  individuals for embryos and  $N=15-16$  individuals for larvae. Analysis indicated no significant differences between treatments for embryos (ANOVA,  $F_{1,181}=3.8$ ,  $P=0.07$ ) and larvae (ANOVA,  $F_{1,60}=0.03$ ,  $P=0.85$ ).

protein after short-term exposure to low pH. Caution should be taken when interpreting total protein data as they are reported per individual and may vary based on body size. While care was taken to ensure individuals of similar carapace width were used in experiments, it is likely that nitrogen content (as a percentage of individual mass) is more indicative of changes in rates of protein synthesis/catabolism. It is improbable that observed differences in total protein were due to disparity in initial juvenile size, as newly settled field-collected juveniles were randomly divided between treatments at the start of experiments. Decreased juvenile total protein may relate to a reduction in growth during initial short-term CO<sub>2</sub> exposure. In post-larval barnacle *S. balanoides*, growth rate and development was significantly reduced after 20 days exposure to elevated CO<sub>2</sub> (Findlay et al., 2009). Total protein reduction in *P. cinctipes* juveniles is not coupled with changes in metabolism; thus, it is likely that juveniles reallocate energy from aspects of growth to maintain homeostasis after short-term development in low pH. However, because no differences in elemental composition or dry mass were observed during longer term exposure, juveniles may have the capacity to compensate for initial adverse effects of low pH.

### CONCLUSIONS

The intensity and direction of physiological responses to low pH varied among early life-history stages of *P. cinctipes*, indicating the importance of elucidating effects of ocean acidification at different stages in coastal intertidal organisms with complex life-history cycles. High CO<sub>2</sub> negatively impacted embryonic metabolic rate and dry mass after short-term exposure, and it is likely that chronic metabolic depression can lead to adverse effects in subsequent stages; thus, longer duration exposures are necessary in future studies. Parental effects have the potential to influence embryo vulnerability to low pH, likely a consequence of both environmental and genetic factors. It is, perhaps, less important to consider mean responses but rather variation in responses across multiple broods when projecting impacts of ocean acidification across entire populations. By switching metabolic substrates from proteins to lipids, larval *P. cinctipes* appear less vulnerable to low pH, with no observed effects on metabolism, dry mass, protein or lipid content. Additionally, high CO<sub>2</sub> may impact juvenile growth during initial exposure as evidenced by decreased protein, though long-term exposure had no discernable effects. While *P. cinctipes* experience a large variation in CO<sub>2</sub> during development in the intertidal zone, our data suggest this does not translate to greater tolerance of the predicted seawater pH decline for their embryonic stage, though responses appeared to vary among broods. Our results suggest a potential for this species to adapt to low pH; thus, it is probable that

OA may select for more CO<sub>2</sub>-tolerant conspecifics and change the population structure of *P. cinctipes* in near-future oceans.

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### AUTHOR CONTRIBUTIONS

H.A.C. led all aspects of conception, design and execution of the study, interpretation of the findings, and drafting and revising the article. L.C.-O., N.A.M. and J.H.S. participated in aspects of conception, design and execution of the study, interpretation of the findings, and drafting and revising the article.

### COMPETING INTERESTS

No competing interests declared.

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