

RESEARCH ARTICLE

Effects of ocean acidification on early life-history stages of the intertidal porcelain crab *Petrolisthes cinctipes*

Lina Ceballos-Osuna¹, Hayley A. Carter¹, Nathan A. Miller¹ and Jonathon H. Stillman^{1,2,*}

¹Romberg Tiburon Center, San Francisco State University, 3150 Paradise Drive, Tiburon, CA 94920, USA and ²Department of Integrative Biology, University of California, Berkeley, Valley Life Sciences Building no. 3140, Berkeley, CA 94720-3140, USA

*Author for correspondence (stillmaj@sfsu.edu)

SUMMARY

Intertidal zone organisms naturally experience daily fluctuations in pH, presently reaching values beyond what is predicted for open ocean surface waters from ocean acidification (OA) by the year 2100, and thus present an opportunity to study the pH sensitivity of organisms that are presumably adapted to an acidified environment. The intertidal zone porcelain crab, *Petrolisthes cinctipes*, was used to study physiological responses to low pH in embryonic, larval and newly recruited juvenile life-history stages. In these crabs, embryonic development occurs in the pH-variable intertidal zone (pH6.9–9.5), larvae mature in the more stable pelagic environment (pH7.9–8.2), and juvenile crabs settle back into the pH-variable intertidal zone. We examined survival, cardiac performance, energetics and morphology in embryonic, larval and juvenile crabs exposed to two pH conditions (pH7.9 and 7.6). Embryos and larvae were split by brood between the pH treatments for 9 days to examine brood-specific responses to low pH. Hatching success did not differ between pH conditions, but ranged from 30% to 95% among broods. Larval survival was not affected by acidification, but juvenile survival was reduced by ~30% after longer (40 days) exposure to low pH. Embryonic and larval heart rates were 37% and 20% lower at low pH, and there was a brood-specific response in embryos. Embryos did not increase in volume under acidified conditions, compared with a 15% increase in ambient conditions. We conclude that sustained exposure to low pH could be detrimental to *P. cinctipes* embryos and larvae despite the fact that embryos are regularly exposed to naturally fluctuating hypercapnic water in the intertidal zone. Importantly, our results indicate that early life-history stage responses to OA may be brood specific through as yet undetermined mechanisms.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/216/8/1405/DC1>

Key words: crustacean larvae, embryo, juvenile, pH, hypercapnia, cardiac performance, heart rate, sub-lethal effect.

Received 24 July 2012; Accepted 12 December 2012

INTRODUCTION

Atmospheric CO₂ is predicted to be three to four times higher than pre-industrial levels by the end of the 21st century as a result of increased anthropogenic CO₂ emissions (Feely et al., 2008; Orr et al., 2005; Meehl et al., 2007). Increased atmospheric CO₂ leads to increased CO₂ dissolution in ocean surface waters, and is altering seawater carbonate chemistry and pH at rates not seen in the past 300 million years (Sabine et al., 2004), a phenomenon termed ocean acidification (OA) (Caldeira and Wickett, 2003; Meehl et al., 2007). How marine biota respond to OA will depend on their current tolerance to elevated CO₂ conditions, and the ecological ramifications of OA are considered to include changes in local biodiversity and community composition (Kleypas et al., 2006; Guinotte and Fabry, 2008; Pörtner and Farrell, 2008; Widdicombe and Spicer, 2008). OA may affect integrated physiological performance parameters such as metabolism, growth and reproduction (Whiteley, 2011; Melzner et al., 2009; Barry et al., 2011) as a consequence of fundamental requirements for intracellular pH homeostasis (Pörtner et al., 2011).

Organisms naturally experiencing high CO₂, such as intertidal zone species, facilitate the assessment of physiological performance under pH conditions that are more variable than those of offshore habitats (Pörtner et al., 2011; Hofmann et al., 2011; Findlay et al., 2009). Previous studies have shown tidepool pH values ranging from

6.5 to 9.5 (Morris and Taylor, 1983; Truchot, 1986; Wootton et al., 2008), thus reaching pH levels lower than global predictions for surface water during the next century (Morris and Taylor, 1983; Truchot, 1986; Wootton et al., 2008). In future climate change scenarios, tidepool and near-shore pH extremes may be further reduced by increased upwelling intensity of hypercapnic waters (Hofmann et al., 2011; Feely et al., 2008).

Reduced survival, growth, reproduction and metabolic rate have been observed under OA conditions in intertidal species of crustaceans and snails (Kurihara et al., 2008; Melatunan et al., 2011; Findlay et al., 2009). In contrast, physiological performance in other species, including teleost fish and some brachyuran crabs, is unaffected (Small et al., 2010; Melzner et al., 2009) or even enhanced by OA (Moulin et al., 2010; Dupont et al., 2010). For intertidal zone organisms, the largest responses to OA may be observed in early life-history stages (i.e. embryonic, larval or juvenile) (Kurihara, 2008; Ross et al., 2011) as these life stages are particularly energetically demanding (Barry et al., 2011). Though responses to OA in early life-history stages may vary, negative effects have been observed in mollusks, echinoderms and crustaceans, including reduced survival, growth and recruitment, as well as changes in developmental timing (Kurihara et al., 2008; Dupont et al., 2008; Crim et al., 2011; Findlay et al., 2009; Walther et al., 2010). The persistence or failure of a population will be determined by the

successful completion of all life stages (Byrne, 2011), emphasizing the importance of assessing sensitivity to OA in adult as well as early developmental life-history stages.

Porcelain crabs, genus *Petrolisthes* (Decapoda; Anomura), are common and abundant inhabitants of the California rocky shore (Haig, 1960). *Petrolisthes cinctipes* (J. W. Randall 1840) early life-history stages occur in distinct pH environments: embryos and newly settled juveniles live in the pH-variable intertidal zone, whereas larval stages spend around 45 days offshore in pelagic regions where the pH is more stable (Gonor, 1970; Gonor and Gonor, 1973; Shanks and Eckert, 2005). We hypothesized that embryonic and juvenile stages possess mechanisms to tolerate environmental acidification, and thus would have muted responses to low pH as compared with zoea I larval stages, which do not experience pH variability in their natural habitat. The specific aim of this study was to investigate whether responses to OA differ among developmental stages by evaluating survival, morphology and cardiac performance of embryonic, larval and juvenile crabs of *P. cinctipes* under continuous exposure to low pH water.

MATERIALS AND METHODS

Water chemistry

Seawater collected in Half Moon Bay, CA, USA, was delivered to the Romberg Tiburon Center by a commercial vendor (SeaPure Inc., El Granada, CA, USA). Water was kept in temperature-controlled insulated reservoirs (~500l) with continuous circulation and filtration (0.35 µm, no. FH803, Aquatic Eco-Systems Inc., Apopka, FL, USA). Water equilibrated with the atmosphere by continuously bubbling with air was used as the 'ambient pH' condition (pH 7.93±0.06). Water bubbled with CO₂ was used as the 'low pH' condition (pH 7.58±0.06) (modified from Widdicombe and Needham, 2007). A pH controller (Duo ORpH Controller no. CP2311, Captive Purity Inc., Marine Depot, Garden Grove, CA, USA; Accumet pH Submersible Combination Electrode, Fisher Scientific no. 13-620-AP56, Pittsburgh, PA, USA) connected to a solenoid valve on the CO₂ gas regulator was used to deliver CO₂ when the pH exceeded 7.6.

Total alkalinity (TA) was assessed weekly during the length of the experiments and calculated from linear Gran plots (Gran, 1952; Dickson, 1981) of potentiometric measurements (Metrohm dosimat 765 pH-Meter, Herisau, Switzerland) using certified Dickson references (<http://andrew.ucsd.edu>). Seawater pH was monitored daily with electrodes (see above) and measured spectrophotometrically twice per week using *m*-Cresol Purple sodium salt dye (PharmaSpec UV-1700, Shimadzu, Columbia, MD, USA; Sigma-Aldrich no. 211761, St Louis, MO, USA; pH reference: Tris buffer certified Dickson reference) using the modified protocol of DOE (DOE, 1994). Salinity and temperature were measured daily using a conductivity meter (YSI Model 30-25FT, Yellow Springs, OH, USA). The 'seacarb' package in R (v. 2.14.0) was used to calculate the carbonate chemistry of the water using TA and pH inputs, and applying the 'carb' and 'phinsi' functions (Lavigne and Gattuso, 2011) (Table 1).

Animal maintenance

Late-stage gravid females ($N=22$) and newly settled juveniles ($N=169$) of *P. cinctipes* were collected during low tide at Pacifica, CA, USA (37°35'48"N, 122°30'34"W) between March and June 2011 (supplementary material Table S1) and brought to the Romberg Tiburon Center, where they were maintained in ambient conditions (13±0.2°C, 33±1 salinity and pH 7.93±0.06). Gravid females were held individually in 385 ml acrylic cylinders (~7 cm

Table 1. Abiotic conditions measured or calculated during the experiments

	Variable	N	Ambient pH	Low pH
Measured	pH	20	7.93±0.06	7.58±0.06
	Salinity	84	32±0.9	32±1.0
	Alkalinity (µmol kg ⁻¹)	9	2380±98	2364±105
Calculated*	P _{CO₂} (µatm)	9	574±105	1361±199
	DIC (µmol kg ⁻¹)	9	2235±95	2338±104
	HCO ₃ ⁻ (µmol kg ⁻¹)	9	2124±122	2255±124
	CO ₃ ²⁻ (µmol kg ⁻¹)	9	118±14	57±9

Values are means ± s.d.

DIC, dissolved inorganic carbon.

*Calculations were made using the 'carb' function in 'seacarb' R package using 'flag 8'. Total pH scale, Kf (from Pérez and Fraga, 1987), k1, k2 (from Lueker et al., 2000) and Ks (from Dickson, 1990).

diameter and 24 cm height) with 500 µm Nytex mesh bottoms through which water flowed continuously. Embryo and larval experiments were performed in broods from $N=22$ females. Embryos were removed from females 2–4 weeks before hatching, based on embryo coloration and the appearance of eyespots, and split between treatments. Larval experiments were performed on newly hatched individuals. Broods were kept separate in all larval and embryonic experiments. Juvenile experiments were conducted on field-collected newly settled specimens that were roughly 2 mm carapace width. For all three life-history stages, individuals were placed in either acrylic cylinders or 50 ml conical tubes with mesh bottoms (as specified below per experiment) and randomly assigned to different sealed plastic boxes containing water at low and ambient pH. The number of individuals per cylinder was standardized as much as possible, but the number of cylinders per brood varied depending on the number of embryos or larvae in the brood (supplementary material Table S1). Daily 100% water changes were performed by transferring the cylinders with individuals to a new plastic box containing pre-equilibrated water from the reservoirs. Larvae and juveniles were fed daily *ad libitum* with a mixture of newly hatched *Artemia franciscana* nauplii (SF Bay strain, Brine Shrimp Direct, Ogden, UT, USA), lab-cultured live rotifers and Shellfish Diet 1800 (a mixture of four marine microalgae: 30% *Isochrysis*, 20% *Pavlova*, 20% *Tetraselmis* and 30% *Thalassiosira weissflogii*, Reed Mariculture Inc., Campbell, CA, USA).

Survival and hatching success

Hatching success, a proxy for embryonic survival, was determined separately in $N=6$ broods ($N=90$ – 200 embryos per brood per treatment) by placing 50 embryos per cylinder, with $N=3$ – 4 cylinders per treatment (i.e. three cylinders per treatment where used if the brood had at least 300 embryos) at ~8 days pre-hatching for each brood in each treatment, and counting the daily number of hatchlings (supplementary material Table S1). To determine larval survival, we used larvae from $N=10$ broods ($N=24$ – 123 larvae per brood per treatment) during a 9 day exposure to low pH (supplementary material Table S1). Newly hatched larvae were placed in 50 ml conical tubes (as described above) containing $N=3$ – 10 larvae per treatment and survival assessment and removal of dead individuals was performed daily. The total number of larvae screened was 473 in ambient pH and 468 in low pH. Juveniles were housed individually in 50 ml conical tubes and survival was determined by daily assessment during a ~40 day exposure ($N=84$ and 85 in ambient pH and low pH, respectively).

Cardiac performance

Heart rate (f_H) and stroke volume (V_S) were determined from video recordings of embryos and larvae. Calcification of juvenile carapaces prevented imaging of cardiac activity. Larvae were immobilized by attaching the rostral spine to a greased (petroleum jelly, Vaseline) glass capillary tube that was mounted to a glass microscope slide and placed in a Petri dish (o.d.=10 cm, Fisherbrand no. 08-757-913, Fisher Scientific, Pittsburgh, PA, USA) containing seawater at the treatment pH. Embryonic measurements did not require immobilization as each individual was simply placed in a dorsal view position using a scaled slide. The Petri dish was maintained at 13°C by placing it in a water-jacketed, aluminum block connected to a recirculating water bath (NESLAB RTE 7, Thermo Scientific, Pittsburgh, PA, USA). The block sat on the stage of a stereoscope (Scope Nikon SMZ1500, Nikon Instruments Inc., Melville, NY, USA). After a 10 min recovery period, each individual was imaged at 70× magnification and recorded at a rate of 25 frames s⁻¹ for 2 min using a digital camera (Nikon D3100, Nikon Inc.).

Videos were parsed into 10 s segments and slowed to 25% of original speed (Apple iMovie '11 software v. 9.0.4) to allow heartbeats to be accurately counted. For each individual, the heart rate was assessed in $N=5$ segments, and those rates were averaged to calculate an individual f_H . Specimens with a vertically aligned dorsal position were used to determine V_S by averaging $N=10$ cardiac cycles (heart beats) per individual using frame-by-frame analysis. The number of broods and individuals used per treatment is presented in supplementary material Table S1. V_S was calculated as the difference between the end-diastolic volume (EDV) and end-systolic volume (ESV) (Eqn 1), assessed using ImageJ (v. 1.45).

$$V_S = \text{EDV} - \text{ESV} \quad (1)$$

EDV and ESV were modeled as prolate spheroids (Eqn 2) (Harper and Reiber, 2004; Storch et al., 2009):

$$\text{Volume} = 4/3\pi ab^2, \quad (2)$$

where a is the radius of major diameter and b is the radius of minor diameter. Individual cardiac output (\dot{Q}) was determined as the product of V_S and f_H (Eqn 3) (Harper and Reiber, 2004):

$$\dot{Q} = V_S \times f_H, \quad (3)$$

where \dot{Q} is in nl min⁻¹, V_S is in nl beat⁻¹ and f_H is in beats min⁻¹.

Yolk consumption

Embryo yolk consumption rate was determined in $N=54$ embryos from $N=2$ broods (supplementary material Table S1) split between the two treatments and imaged after 5, 9 and 20 days in the experimental conditions (representing 20, 16 and 5 days pre-hatching). To estimate yolk volume at each time point, lateral and dorsal view photographs were analyzed using the color threshold function in ImageJ (Eqn 4) (supplementary material Fig. S1):

$$\text{Yolk volume} = 4/3\pi cd^2, \quad (4)$$

where c is the dorsal yolk area and d is the maximum radius of a lateral fitted ellipse.

Larval activity

Larval activity, defined as the rate of maxilliped movement, was determined in a subset of larvae randomly chosen from five broods (supplementary material Table S1). The same videography procedure used for the cardiac assessment was used to record the maxilliped movement. Videos with full lateral views of the maxillipeds were

used. For each individual, maxilliped beats were averaged across $N=5$ video segments of 10 s duration.

Morphometrics

Morphometric analyses were conducted from photographs of live specimens taken with the same stereoscope and camera used in the video recordings. Dimensions of distinctive structures [embryonic measurements: volume and ellipticity (major diameter/minor diameter); larval measurements: dorsal carapace length, anterior carapace width, rostrum spine width, telson length] were determined with ImageJ (supplementary material Figs S2, S3). Embryos were measured following exposure to ambient or acidified water for 5 or 9 days and larvae were measured after 9 days of exposure to their respective pH conditions.

Statistical analysis

Data were tested for normality and homoscedasticity using Shapiro–Wilk and variance tests, except for survival data, where a different approach was used (described below). If parametric requirements were met, one-way ANOVA tests were performed. If parametric requirements were not met, data transformation or non-parametric Kruskal–Wallis tests were applied. For heart rate analyses, larval and embryonic f_H data were square-root transformed and then analyzed using a nested one-way ANOVA, using brood as the nested variable. Embryonic morphometrics were analyzed by applying the Kruskal–Wallis test at each time point separately.

Survival was analyzed following previous methods (Bewick et al., 2004) and survival curves were created and compared between the treatments using the Kaplan–Meier log-rank test within the R ‘survival’ package (Therneau, 2011). Survival curves were constructed by pooling larval survival data from all 10 broods. Juvenile survival data were pooled across $N=4$ trials before analysis. Rate of yolk consumption was determined using linear regression. All statistical analyses and construction of plots were performed using R v. 2.14.0 (R Development Core Team, 2011) and the package ‘ggplot2’ (Wickham, 2009).

RESULTS

Hatching success and survival

Embryos hatched 6–10 days after placement in treatment conditions. Hatching success ranged from <30% to >95% among broods, independent of pH treatment (supplementary material Fig. S4A). No difference in mean hatching success was observed between pH conditions when embryos from all broods were pooled together (ANOVA $F_{1,10}=0.006$, $P=0.94$). A brood-specific response to acidification was observed; some broods had 40% lower hatching success relative to the mean survival at ambient pH, while others had 20% higher success at ambient pH (supplementary material Fig. S4B).

No significant differences in larval or juvenile survival were detected after 9 days in the treatments (Kaplan–Meier log-rank test, $\chi^2_1=2.7$, $P=0.102$, Fig. 1). However during the 9 days of observation, daily larval survival was routinely lower in the low pH treatment (Fig. 1A). At day 9 there was less than 50% larval survival in both conditions. Juveniles kept at low pH showed significantly lower survival than those in the ambient pH condition over the entire 40 day period (Kaplan–Meier log-rank test, $\chi^2_1=7.1$, $P=0.008$) (Fig. 1B). Almost no juvenile mortality was observed in either treatment during the first week.

Cardiac performance

Embryonic f_H was 37.4% lower in the acidified condition across all broods (ANOVA: $F_{1,45}=5.84$, $P=0.02$) (Fig. 2A). However, there was

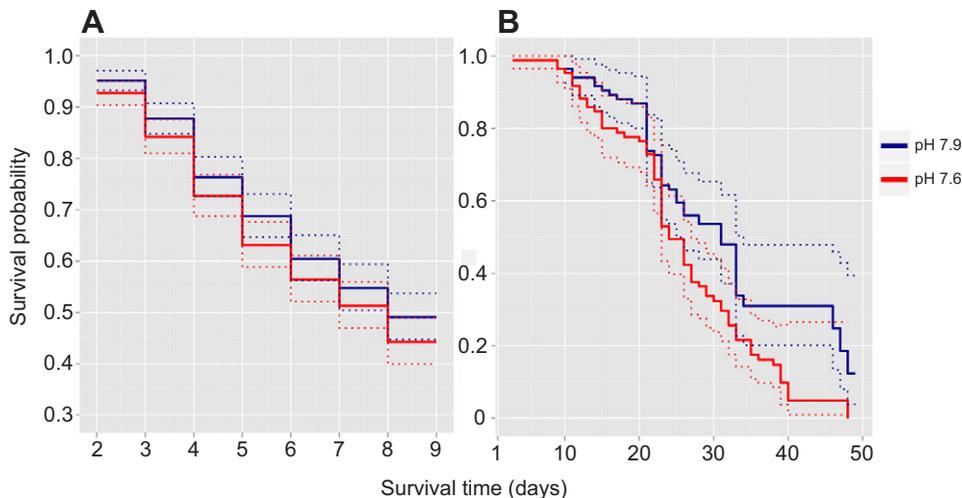


Fig. 1. Kaplan–Meier survival probability of *Petrolisthes cinctipes*. (A) Larvae from 10 broods (ambient pH $N_{t0}=473$, low pH $N_{t0}=468$, where N_{t0} is the number at time $t=0$). (B) Juveniles from four trials (ambient pH $N_{t0}=85$, low pH $N_{t0}=84$). Dotted line represents 95% confidence interval.

significant variation among broods in the degree of response to low pH, with embryonic f_H showing a 20–65% reduction across broods (Fig. 2B). In the embryonic stage, an interaction between the response and the brood was found (ANOVA nested by $N=5$ broods, $F_{8,45}=2.70$, $P=0.02$; ambient $N=26$, low $N=29$). Overall, larval f_H was 20.9% lower in the acidified condition (ANOVA: $F_{1,64}=4.64$, $P=0.04$) (Fig. 2A), and in several broods was reduced by 20–65% (Fig. 2B), though the overall effect of brood was not statistically significant.

Mean cardiac performance in embryonic and larval stages was reduced at low pH, though differences were not statistically significant (Fig. 3; supplementary material Table S2). Embryonic cardiac output (\dot{Q}) and stroke volume (V_S) were reduced by 52% and 14%, respectively, in low pH (ANOVA: \dot{Q} $F_{1,10}=2.88$, $P=0.12$; V_S $F_{1,8}=1.67$, $P=0.23$) compared with ambient pH (supplementary material Fig. S5). Larval cardiac performance was also affected, with \dot{Q} and V_S reduced by 20% and 7%, respectively (ANOVA: \dot{Q} $F_{1,14}=0.33$, $P=0.58$; V_S $F_{1,14}=0.17$, $P=0.69$; supplementary material Fig. S5).

Yolk consumption

Embryonic yolk was depleted at the same rate ($0.004 \text{ mm}^3 \text{ day}^{-1}$) in both pH treatments (time: t -value = -16.22 , $P < 0.001$; pH: t -value = 0.73 , $P = 0.47$). Yolk volume decreased in both conditions from $0.072 \pm 0.004 \text{ mm}^3$ (~20 days pre-hatching, 5 days in the treatments) to $0.012 \pm 0.001 \text{ mm}^3$ (~5 days pre-hatching, 20 days in the treatments; supplementary material Fig. S6).

Larval activity

Maxilliped activity ranged from 271 to 545 beats min^{-1} in the ambient pH treatment and was between 169 and 464 beats min^{-1} in low pH. Mean maxilliped frequency was 15% lower under acidified conditions, but the difference was not statistically significant (ANOVA: $F_{1,12}=1.17$, $P=0.30$; supplementary material Fig. S7).

Morphometrics

Embryo volume increased in the ambient pH condition from 0.27 ± 0.02 to $0.31 \pm 0.04 \text{ mm}^3$ between 6 days pre-hatching (5 days in the treatments) and 2 days pre-hatching (9 days in the treatments, Fig. 4). In contrast, no changes in embryo volume were observed under low pH ($0.28 \pm 0.03 \text{ mm}^3$ at 5 and 9 days, Fig. 4). Embryo ellipticity was not affected by pH, indicating that embryos maintained the same shape in both pH conditions. Non-parametric statistical analysis, treating the two time points independently, did not show a significant effect of pH on embryo volume

(supplementary material Table S3). Larval morphology was not affected by low pH after 9 days in acidified water (supplementary material Table S4).

DISCUSSION

In this study, we assessed physiological performance of porcelain crab early life-history stages (embryo, larval, juvenile) following exposure to acidified water in order to understand possible variation in responses to OA related to natural exposure to different pH environments (i.e. intertidal zone and offshore pelagic). Physiological responses to low pH are discussed in two broad categories: survival and metabolic performance. Overall, we conclude that future ocean pH decline may result in sub-lethal physiological rate reductions in *P. cinctipes* early life-history stages, and that long-term exposure to acidified water may be detrimental for organisms that are presently tolerant to natural pH variability. Additionally, there is a suggestion of brood-specific responses to low pH, highlighting the necessity of including the role of parental effects in early life-history stages in response to environmental change in future investigations.

Survival and hatching success

Increased sensitivity to OA during early developmental stages has been observed in shrimp, crabs, snails and other marine invertebrates (Kurihara et al., 2008; Walther et al., 2009; Ellis et al., 2009; Melzner et al., 2009). In our study, survival of embryonic, larval and juvenile porcelain crabs, *P. cinctipes*, was not affected by low pH after 9 days of continuous exposure. However, embryonic morphometrics data suggest suppressed development under elevated CO_2 as embryos did not increase their volume in low pH. Embryonic volume increase before hatching is the norm in most, if not all, crustaceans [e.g. *Hyas araneus* (Petersen and Anger, 1997); *Eupagurus bernhardus*, *Ligia oceanica*, *Artemia salina* (Pandian and Schumann, 1967); *Crangon crangon* (Pandian, 1970); and *Homarus gammarus* (Pandian, 1970)]. Pre-hatching volume increases are due to changes in water intake and/or production of metabolic water from lipid catabolism (Petersen and Anger, 1997). Our study suggests a disruption of this natural pre-hatching process caused by changes in the pH of the surrounding water, though the disruption did not alter hatching success.

Reduced survival of juveniles under low pH was only evident during a longer-term exposure (more than 40 days), demonstrating that exposure to a continuous low pH can be detrimental for

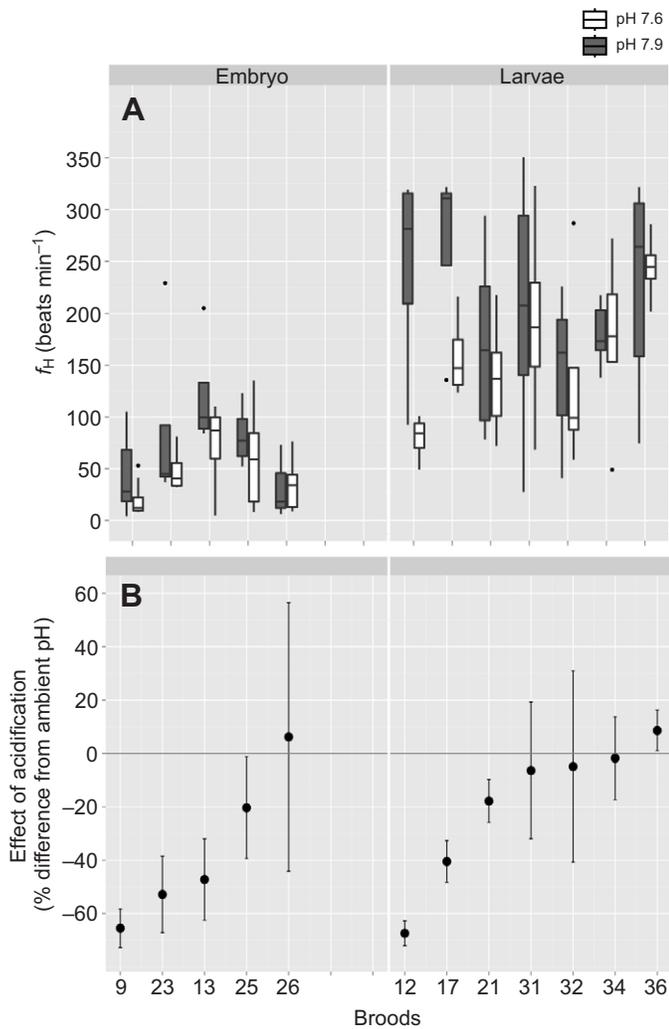


Fig. 2. (A) Heart rate (f_H) of *P. cinctipes* embryos ($N=26-29$ per treatment) and larvae ($N=38-40$ per treatment) pooled from five and seven broods, respectively, and measured after 8–10 days in the pH treatments. (B) Effect of low pH on heart rate of embryos and larvae ($N=4-8$ per brood) of independent broods after 8–10 days in the treatments, shown as the percentage change from ambient pH values. Means \pm s.e.m.

organisms that are tolerant of extreme pH variability in their natural habitat. Future OA and climate change scenarios predict prolonged, extreme upwelling events amplified by unusual weather conditions (Meehl et al., 2007). This threat is specifically relevant along the California coast, where acidified deep waters upwell close to the coast, directly affecting coastal ecosystems (Feely et al., 2008; Morgan et al., 2009; Yu et al., 2011). Our data suggest that long-term upwelling events and/or the superimposition of high levels of anthropogenic CO_2 could represent a threat to species that presently appear to be quite tolerant to natural short-term changes in their environment (Pörtner et al., 2011).

Metabolic performance

Environmental hypercapnia associated with ocean acidification affects physiological processes such as acid–base regulation, cardiovascular function and metabolic activity, and results in reductions in growth, reproduction and survival (Ishimatsu and Kita, 1999; Albright, 2011). Low external pH causes extracellular acidosis, reducing hemocyanin O_2 affinity and potentially damaging

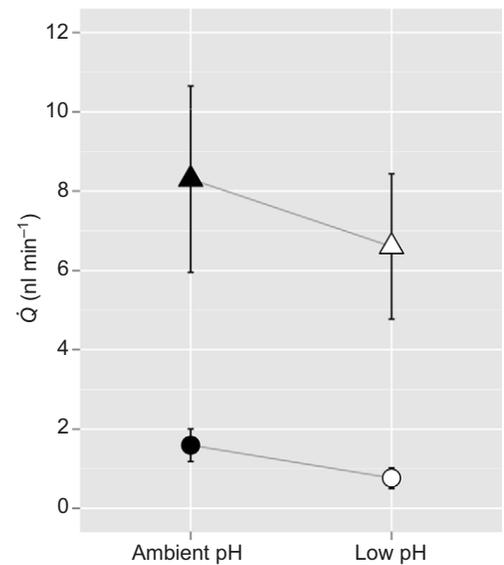


Fig. 3. Cardiac output (\dot{Q}) of *P. cinctipes* embryos (circles) and larvae (triangles) under two pH conditions: ambient (pH 7.9) and low (pH 7.6). Means \pm s.e.m., $N=5-10$ individuals per stage per treatment (from $N=4-6$ broods).

cardiac cells, leading to CO_2 toxicity, muscle contraction failure and metabolic depression (Pörtner et al., 2011; Ishimatsu et al., 2004; Ishimatsu et al., 2008; Widdicombe and Spicer, 2008; Gesser and Poupa, 1983). Embryos and larvae of *P. cinctipes* exhibited metabolic depression, as measured by a significantly reduced heart rate under acidification, which is likely due to extracellular acidosis and associated cardiac muscle failure. Many intertidal zone organisms use metabolic depression as a mechanism to survive short-term sub-optimal conditions; however, this mechanism could have negative effects during long-term exposure to stressful scenarios.

Extracellular pH homeostasis involves regulation of the concentration of bicarbonate in extracellular fluids by bicarbonate ion transport across the cell membrane or from the surrounding environment (Pörtner et al., 2011). Though active transport mechanisms require cellular energy, in this study no additional energetic demands were observed in embryonic crabs held at low pH, as inferred by equal embryonic yolk consumption rates across treatments. Thus, energy used for pH homeostasis-related transport may be allocated from other processes (e.g. heart rate) (Barry et al., 2011). The reallocation of energy during stressful periods is considered a first response and will allow organisms to maintain short-term homeostasis (Melzner et al., 2009; Pörtner et al., 2011), though if sustained may compromise growth or reproduction (Albright, 2011).

To survive and maintain homeostasis under stressful conditions, an organism must have the capacity to control cardiovascular function (Reiber, 1997). Recent studies have shown heart rate reduction under acidification in many marine organisms [e.g. *Littorina obtusata* (Ellis et al., 2009); *Hyas araneus* (Walther et al., 2009)], but only a few studies have analyzed the capacity of individuals to modify parameters of cardiac function independently (stroke volume, heart rate and cardiac output) in order to compensate for environmental change (Harper and Reiber, 2004; Orlando and Pinder, 1995; Spicer, 2001). A reduction in cardiac output under sustained stressful conditions was observed in embryos and larvae of *P. cinctipes*, driven primarily by a reduction in heart rate with no adjustment of stroke volume for compensation. This may result

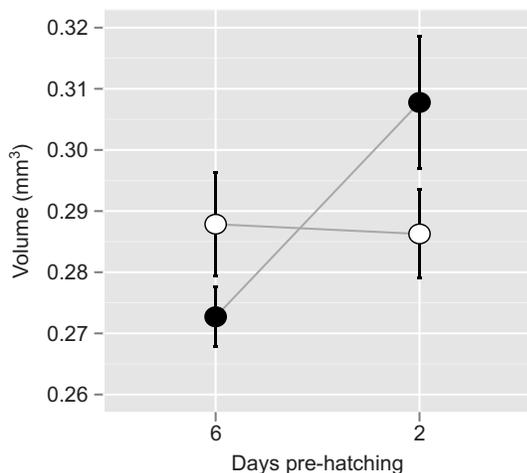


Fig. 4. *Petrolisthes cinctipes* embryo volume under two pH conditions (filled circles, ambient pH; open circles, low pH). Measurements were taken 6 and 2 days pre-hatching, representing 5 and 9 days in the treatments, respectively. Means \pm s.e.m., $N=13-15$ from three females per group per time point.

in lower oxygen availability and CO_2 removal at a cellular level, potentially contributing to an overall metabolic depression. Our results are consistent with additional findings (Carter et al., 2013) that the oxygen consumption rate of *P. cinctipes* embryos was reduced under acidification.

Consistent with reduced heart rate and a hypothesized compromise in muscle function under acidification, *P. cinctipes* larvae showed slower maxilliped activity. Reduced maxilliped beating may negatively impact important processes such as ventilation and swimming, potentially increasing vulnerability to predation and decreasing feeding performance (Larimer, 1964; Batterton and Cameron, 1978).

In many groups of organisms, the experiences of the breeding parents can influence the viability and quality of the offspring (Sibert et al., 2004; McCormick and Gagliano, 2008) and their tolerance of environmental stress (Parker et al., 2012; McCormick and Gagliano, 2008). Parental genetic input, energy allocated to eggs and maternal behavior (e.g. aeration, cleaning and protection) (Levi et al., 1999) during embryo brooding may determine the sensitivity of independent broods and their ability to tolerate extreme conditions. Factors like density-dependent stress in the breeding females of *Pomacentrus amboinensis* had a negative effect on larval size (McCormick, 2006). Adult shrimp *Palaemon pacificus* and copepods reduce egg production under acidified water (Kurihara et al., 2008; Zhang et al., 2011).

Consistent with previous studies, we observed that the response (heart rate) to low pH varied among females (broods) (e.g. Chan et al., 2011; Carter et al., 2013) and this variation was strongest during the embryonic stage, suggesting maternal and/or paternal effects on some physiological responses to elevated CO_2 . In our study design, treatment and brood were not truly independent, but the substantial variation between broods across multiple experiments provides considerable support for the possibility of brood-specific responses to OA. Different responses to acidification among broods could be related to microenvironmental variation within the intertidal zone. If females are exposed to diverse conditions (e.g. food availability, abiotic parameters) during gametogenesis or early embryogenesis, this could result in variation in genetic or energetic allocation and so alter the offspring's resilience to environment changes. Even

though many marine organisms have long generation times, reducing the adaptation capacity to face rapid changes in their environment, some recent studies have shown that the variability in the responses could represent potential for adaptation in some groups (Sunday et al., 2011; Parker et al., 2012). Variation in response to acidified water among broods observed in this study suggests a potential for adaptation; however, further analysis needs to be done in order to understand the heritability of tolerance capacities.

This study shows that continuous acidification may be detrimental to intertidal zone organisms that naturally experience fluctuating hypercapnic conditions. Greater understanding of sub-lethal responses, like the reduction in cardiac performance shown by *P. cinctipes*, and the interaction between parentage and the stress response are of great importance as we attempt to predict changes in intertidal zone community structure under future OA scenarios.

ACKNOWLEDGEMENTS

We thank Drs Anne Todgham, Tomoko Komada and Stephane Lefebvre for their important contributions to this study. Thanks to the members of Stillman Lab that participated in aspects of the project, and to the Romberg Tiburon Center and its staff for facilitating the experimental aspects of the project. Thanks to the San Francisco Bay National Estuarine Research Reserve for the use of their microscope.

AUTHOR CONTRIBUTIONS

L.C.-O. led all aspects of conception, design and execution of the study, interpretation of the findings, and drafting and revising the article. H.A.C., 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.

FUNDING

This research was supported by the National Science Foundation (NSF)-TREE Fellowship, the Maxwell Scholarship, the James C. Kelley Scholarship and a California State University (CSU) Council on Ocean Affairs, Science and Technology (COAST) Summer Award to L.C.-O., and CSU COAST Research and Travel Awards, a San Francisco State University College of Science and Engineering (COSE) Instructionally Related Award (IRA) Student Travel Award, a James C. Kelley Scholarship and a San Francisco Bay Scholarship (Romberg Tiburon Center) to H.A.C. This material is based upon work supported by the National Science Foundation [grant no. 1041225 to J.H.S.].

REFERENCES

- Albright, R. (2011). Reviewing the effects of ocean acidification on sexual reproduction and early life history stages of reef-building corals. *J. Mar. Biol.* **2011**, 473615.
- Barry, J. P., Widdicombe, S. and Hall-Spenser, J. M. (2011). Effects of Ocean Acidification on Marine Biodiversity and Ecosystem Function in Ocean Acidification. In *Ocean Acidification* (ed. J.-P. Gattuso and L. Hansson), pp. 192-209. Oxford: Oxford University Press.
- Batterton, C. V. and Cameron, J. N. (1978). Characteristics of resting ventilation and response to hypoxia, hypercapnia, and emersion in the blue crab *Callinectes sapidus*. *J. Exp. Zool.* **203**, 403-418.
- Bewick, V., Cheek, L. and Ball, J. (2004). Statistics review 12: survival analysis. *Crit. Care* **8**, 389-394.
- Byrne, M. (2011). Impact of ocean warming and ocean acidification on marine invertebrates life history stages: vulnerabilities and potential for persistence in a changing ocean. *Oceanogr. Mar. Biol.* **49**, 1-42.
- Caldeira, K. and Wickett, M. E. (2003). Oceanography: anthropogenic carbon and ocean pH. *Nature* **425**, 365.
- Carter, H. A., Ceballos-Osuna, L., Miller, N. A. and Stillman, J. H. (2013). Impact of ocean acidification on the metabolism and energetics of early life stages in the intertidal porcelain crab *Petrolisthes cinctipes*. *J. Exp. Biol.* **216**, 1412-1422.
- Chan, K. Y. K., Grünbaum, D. and O'Donnell, M. J. (2011). Effects of ocean-acidification-induced morphological changes on larval swimming and feeding. *J. Exp. Biol.* **214**, 3857-3867.
- Crim, R. N., Sunday, J. M. and Harley, C. D. G. (2011). Elevated seawater CO_2 concentrations impair larval development and reduce larval survival in endangered northern abalone (*Haliotis kamtschatkana*). *J. Exp. Mar. Biol. Ecol.* **400**, 272-277.
- Dickson, A. G. (1981). An exact definition of total alkalinity and a procedure for estimation of alkalinity and total inorganic carbon from titration data. *Deep Sea Res.* **A 28**, 609-623.

- Dickson A. G. (1990). Standard potential of the reaction: $\text{AgCl(s)} + 1/2\text{H}_2\text{(g)} = \text{Ag(s)} + \text{HCl(aq)}$, and the standard acidity constant of the ion HSO_4^- in synthetic sea water from 273.15 to 318.15 K. *J. Chem. Thermodyn.* **22**, 113-127.
- DOE (1994). *Handbook of Methods for the Analysis of the Various Parameters of the Carbon Dioxide System in Seawater*, Version 2 (ed. A. G. Dickson and C. Goyet Ornumiac), ORNL/CDIAC-74.
- Dupont, S., Havenhand, J., Thorndyke, W., Peck, L. and Thorndyke, M. (2008). Near-future of CO_2 -driven ocean acidification radically affects larval survival and development in the brittlestar *Ophiothrix fragilis*. *Mar. Ecol. Prog. Ser.* **373**, 285-294.
- Dupont, S., Ortega-Martinez, O. and Thorndyke, M. (2010). Impact of near-future ocean acidification on echinoderms. *Ecotoxicology* **19**, 449-462.
- Ellis, R. P., Bersey, J., Rundle, S., Hall-Spencer, J. M. and Spicer, J. I. (2009). Subtle but significant effects of CO_2 acidified seawater on embryos of the intertidal snail, *Littorina obtusata*. *Aquat. Biol.* **5**, 41-48.
- Feely, R. A., Sabine, C. L., Hernandez-Ayon, J. M., Janson, D. and Hales, B. (2008). Evidence for upwelling of corrosive 'acidified' water onto the continental shelf. *Science* **320**, 1490-1492.
- Findlay, H. S., Kendall, M. A., Spicer, J. I. and Widdicombe, S. (2009). Future high CO_2 in the intertidal may compromise adult barnacle (*Semibalanus balanoides*) survival and embryo development rate. *Mar. Ecol. Prog. Ser.* **389**, 193-202.
- Gesser, H. and Poupa, O. (1983). Acidosis and cardiac muscle contractility: comparative aspects. *Comp. Biochem. Physiol.* **76A**, 559-566.
- Gonor, S. L. (1970). The larval histories of four porcellanid anomurans (Crustacea, Decapoda) from Oregon. MSc Oceanography Thesis, Oregon State University, OR, USA.
- Gonor, S. L. and Gonor, J. J. (1973). Descriptions of the larvae of four North Pacific porcellanidae (Crustacea: Anomura). *Fish Bull.* **71**, 189-223.
- Gran, G. (1952). Determination of the equivalence point in potentiometric titrations. Part II. *Analyst* **77**, 661-671.
- Guinotte, J. M. and Fabry, V. J. (2008). Ocean acidification and its potential effects on marine ecosystems. *Ann. New York Acad. Sci.* **1134**, 320-342.
- Haig, J. (1960). *The Porcellanidae (Crustacea Anomura) of the Eastern Pacific*. Allan Hancock Pacific Expeditions 24. Los Angeles, CA: University of Southern California Press.
- Harper, S. L. and Reiber, C. L. (2004). Physiological development of the embryonic and larval crayfish heart. *Biol. Bull.* **206**, 78-86.
- Hofmann, G. E., Smith, J. E., Johnson, K. S., Send, U., Levin, L. A., Micheli, F., Paytan, A., Price, N. N., Peterson, B., Takeshita, Y. et al. (2011). High-frequency dynamics of ocean pH: a multi-ecosystem comparison. *PLoS ONE* **6**, e28983.
- Ishimatsu, A. and Kita, J. (1999). Effects of environmental hypercapnia on fish. *Jpn. J. Ichthyol.* **46**, 1-13.
- Ishimatsu, A., Kikkawa, T., Hayashi, M., Kyoung-Seon, L. and Kita, J. (2004). Effects of CO_2 on marine fish: larvae and adults. *J. Oceanogr.* **60**, 731-741.
- Ishimatsu, A., Masahiro, H. and Takashi, K. (2008). Fishes in high CO_2 , acidified oceans. *Mar. Ecol. Prog. Ser.* **373**, 295-302.
- Jensen, G. C. (1989). Gregarious settlement by megalopae of the porcellanid *Petrolisthes cinctipes* and *P. eriomerus* Stimpson. *J. Exp. Mar. Biol. Ecol.* **131**, 223-231.
- Kleypas, J. A., Feely, R. A., Fabry, V. J., Langdon, C., Sabine, C. L. and Robbins, L. L. (2006). *Impacts of Ocean Acidification on Coral Reefs and Other Marine Calcifiers: a Guide for Future Research*. Report of a workshop. St Petersburg, FL: NSF, NOAA and the USGS.
- Kurihara, H. (2008). Effects of CO_2 -driven ocean acidification on the early developmental stages of invertebrates. *Mar. Ecol. Prog. Ser.* **373**, 275-284.
- Kurihara, H., Matsui, M., Furukawa, H., Hayashi, M. and Ishimatsu, A. (2008). Long-term effects of predicted future seawater CO_2 conditions on the survival and growth of the marine shrimp *Palemon pacificus*. *J. Exp. Mar. Biol. Ecol.* **367**, 41-46.
- Larimer, J. L. (1964). The patterns of diffusion of oxygen across the crustacean gill membranes. *J. Cell. Comp. Physiol.* **64**, 139-148.
- Lavigne, H. and Gattuso, J. P. (2011). Seacarb: seawater carbonate chemistry with R. R package version 2.4.3. Available at: <http://CRAN.R-project.org/package=seacarb>.
- Levi, T., Barki, A., Hulata, G. and Karplus, I. (1999). Mother-offspring relationship in the red-claw crayfish *Cherax quadricarinatus*. *J. Crustac. Biol.* **19**, 477-484.
- Lueker, T. J., Dickson, A. G. and Keeling, C. D. (2000). Ocean pCO_2 calculated from dissolved inorganic carbon, alkalinity, and equations for K_1 and K_2 : validation based on laboratory measurements of CO_2 in gas and seawater at equilibrium. *Mar. Chem.* **70**, 105-119.
- McCormick, M. I. (2006). Mothers matter: crowding leads to stressed mothers and smaller offspring in marine fish. *Ecology* **87**, 1104-1109.
- McCormick, M. I. and Gagliano, M. (2008). Carry-over effects, the importance of a good start. In *Proceedings of the 11th International Coral Reef Symposium*, 7-11 July 2008. Fort Lauderdale, FL. Session Number 10.
- Meehl, G. A., Stocker, T. F., Collins, W. D., Friedlingstein, P. and Gaye, A. T. (2007). Contribution of working group I in the fourth assessment report of the intergovernmental panel on climate change. In *The Physical Science Basis*. Cambridge: Cambridge University Press.
- Melatun, S., Calosi, P., Rundle, S. D., Moody, A. J. and Widdicombe, S. (2011). Exposure to elevated temperature and PCO_2 reduces respiration rate and energy status in the periwinkle *Littorina littorea*. *Physiol. Biochem. Zool.* **84**, 583-594.
- Melzner, F., Gutowska, M. A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M. C., Bleich, M. and Pörtner, H. O. (2009). Physiological basis of high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* **6**, 2313-2331.
- Morgan, S. G., Fisher, J. L., Miller, S. H., McAfee, S. T. and Largier, J. L. (2009). Nearshore larval retention in a region of strong upwelling and recruitment limitation. *Ecology* **90**, 3489-3502.
- Morris, S. and Taylor, A. C. (1983). Diurnal and seasonal variation in physico-chemical conditions within intertidal rock pools. *Estuar. Coast. Shelf Sci.* **17**, 339-355.
- Moulin, L., Catarino, A. I., Claessens, T. and Dubois, P. (2010). Effects of seawater acidification on early development of the intertidal sea urchin *Paracentrotus lividus* (Lamarck 1816). *Mar. Pollut. Bull.* **62**, 48-54.
- Orlando, K. and Pinder, A. W. (1995). Larval cardiorespiratory ontogeny and allometry in *Xenopus laevis*. *Physiol. Zool.* **68**, 63-75.
- Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F. et al. (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* **437**, 681-686.
- Pandian, T. J. (1970). Yolk utilization and hatching time in the Canadian lobster *Homarus americanus*. *Mar. Biol.* **7**, 249-254.
- Pandian, T. J. and Schumann, K. H. (1967). Chemical composition and caloric content of egg and zoea of the hermit crab *Eupagurus bernhardus*. *Helgoländ. Wiss. Meer.* **16**, 225-230.
- Parker, L. M., Ross, P. M. and O'Connor, W. A. (2011). Populations of the Sydney rock oyster, *Saccostrea glomerata*, vary in response to ocean acidification. *Mar. Biol.* **158**, 689-697.
- Parker, L. M., Ross, P. M., O'Connor, W. A., Borysko, L., Raftos, D. A. and Pörtner, H. O. (2012). Adult exposure influences offspring response to ocean acidification in oysters. *Glob. Change Biol.* **18**, 82-92.
- Pérez, F. F. and Fraga, F. (1987). Association constant of fluoride and hydrogen ions in seawater. *Mar. Chem.* **21**, 161-168.
- Petersen, S. and Anger, K. (1997). Chemical and physiological changes during the embryonic development of the spider crab, *Hyas araneus* L. (Decapoda: Majidae). *Comp. Biochem. Physiol.* **117B**, 299-306.
- Pörtner, H. O. and Farrell, A. P. (2008). Ecology, physiology and climate change. *Science* **322**, 690-692.
- Pörtner, H. O., Gutowska, M., Ishimatsu, A., Lucassen, M., Melzner, F., and Seibel, B. (2011). Effects of ocean acidification on nektonic organisms. In *Ocean Acidification* (ed. J. P. Gattuso and L. Hansson), pp. 154-175. Oxford: Oxford University Press.
- R Development Core Team (2011). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available at: <http://www.R-project.org>.
- Reiber, C. I. (1997). Ontogeny of cardiac and ventilator function in the crayfish *Procambarus clarkii*. *Am. Zool.* **37**, 82-91.
- Ross, P. M., Parker, L., O'Connor, W. A. and Bailey, E. (2011). The impact of ocean acidification on reproduction, early development and settlement of marine organisms. *Water* **3**, 1005-1030.
- Sabine, C. L., Feely, R. A., Gruber, N., Key, R. M., Lee, K., Bullister, J. L., Wanninkhof, R., Wong, C. S., Wallace, D. W. R., Tilbrook, B. et al. (2004). The oceanic sink for anthropogenic CO_2 . *Science* **305**, 367-371.
- Shanks, A. and Eckert, G. L. (2005). Population persistence of California current fishes and benthic crustaceans: a marine drift paradox. *Ecol. Monogr.* **75**, 505-524.
- Sibert, V., Ouellet, P. and Brethes, J. C. (2004). Changes in yolk total proteins and lipid components and embryonic growth rates during lobster (*Homarus americanus*) egg development under a simulated seasonal temperature cycle. *Mar. Biol.* **144**, 1075-1086.
- Small, D., Calosi, P., White, D., Spicer, J. I. and Widdicombe, S. (2010). Impact of medium-term exposure to CO_2 enriched seawater on the physiological functions of the velvet crab *Necora puber*. *Aquat. Biol.* **10**, 11-21.
- Spicer, J. I. (2001). Development of cardiac function in crustaceans: patterns and processes. *Am. Zool.* **41**, 1068-1077.
- Storch, D., Santelices, P., Barria, J., Cabeza, K., Pörtner, H. O. and Fernández, M. (2009). Thermal tolerance of crustacean larvae (zoea I) in two different populations of the kelp crab *Talipes dentatus* (Milne-Edwards). *J. Exp. Biol.* **212**, 1371-1376.
- Sunday, J. M., Crim, R. N., Harley, C. D. and Hart, M. W. (2011). Quantifying rates of evolutionary adaptation in response to ocean acidification. *PLoS ONE* **6**, e22881.
- Therneau, T. (2011). Survival: survival analysis, including penalized likelihood. R package version 2.36-10. Available at: <http://CRAN.R-project.org/package=survival>.
- Truchot, J. P. (1986). Changes in the hemolymph acid-base state of the shore crab *Carcinus maenas*, exposed to simulated tidepool conditions. *Biol. Bull.* **170**, 506-518.
- Walther, K., Anger, K. and Pörtner, H. O. (2010). Effects of ocean acidification and warming on the larval development of the spider crab *Hyas araneus* from different latitudes (54° vs. 79°N). *Mar. Ecol. Prog. Ser.* **417**, 159-170.
- Whiteley, N. M. (2011). Physiological and ecological responses of crustaceans to ocean acidification. *Mar. Ecol. Prog. Ser.* **430**, 257-271.
- Wickham, H. 2009. *ggplot2: Elegant Graphics For Data Analysis*. New York, NY: Springer.
- Widdicombe, S. and Needham, H. R. (2007). Impact of CO_2 -induced seawater acidification on the burrowing activity of *Nereis virens* and sediment nutrient flux. *Mar. Ecol. Prog. Ser.* **341**, 111-122.
- Widdicombe, S. and Spicer, J. I. (2008). Predicting the impact of ocean acidification on benthic biodiversity: what can animal physiology tell us? *J. Exp. Mar. Biol. Ecol.* **366**, 187-197.
- Wootton, J. T., Pfister, C. A. and Forester, J. D. (2008). Dynamic patterns and ecological impacts of declining ocean pH in a high-resolution multi-year dataset. *Proc. Natl. Acad. Sci. USA* **105**, 18848-18853.
- Yu, P. C., Matson, P. G., Martz, T. R. and Hofmann, G. E. (2011). The ocean acidification seascape and its relationship to the performance of calcifying marine invertebrates: Laboratory experiments on the development of urchin larvae framed by environmentally-relevant pCO_2/pH . *J. Exp. Mar. Biol. Ecol.* **400**, 288-295.
- Zhang, D., Li, S., Wang, G. and Guo, D. (2011). Impacts of CO_2 -driven seawater acidification on survival, egg production rate and hatching success of four marine copepods. *Acta Oceanol. Sin.* **30**, 86-94.