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Research article

Metabolic recovery and compensatory shell growth of juvenile Pacific geoduck *Panopea generosa* following short-term exposure to acidified seawater

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While acute stressors can be detrimental, environmental stress conditioning can improve performance. To test the hypothesis that physiological status is altered by stress conditioning, we subjected juvenile Pacific geoduck, *Panopea generosa*, to repeated exposures of elevated pCO_2 in a commercial hatchery setting followed by a period in ambient common garden. Respiration rate and shell length were measured for juvenile geoduck periodically throughout short-term repeated reciprocal exposure periods in ambient (~550 µatm) or elevated (~2400 µatm) pCO_2 treatments and in common, ambient conditions, 5 months after exposure. Short-term exposure periods comprised an initial 10-day exposure followed by 14 days in ambient before a secondary 6-day reciprocal exposure. The initial exposure to elevated pCO_2 significantly reduced respiration rate by 25% relative to ambient conditions, but no effect on shell growth was detected. Following 14 days in common garden, ambient conditions, reciprocal exposure to elevated or ambient pCO_2 did not alter juvenile respiration rates, indicating ability for metabolic recovery under subsequent conditions. Shell growth was negatively affected during the reciprocal treatment in both exposure histories; however, clams exposed to the initial elevated pCO_2 showed compensatory growth with 5.8% greater shell length (on average between the two secondary exposures) after 5 months in ambient conditions. Additionally, clams exposed to the secondary elevated pCO_2 showed 52.4% increase in respiration rate after 5 months in ambient conditions. Early exposure to low pH appears to trigger carryover effects suggesting bioenergetic re-allocation facilitates growth compensation. Life stage-specific exposures to stress can determine when it may be especially detrimental, or advantageous, to apply stress conditioning for commercial production of this long-lived burrowing clam.

Key words: stress conditioning, compensatory response, ocean acidification, geoduck, aquaculture

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Introduction

Sustainable food production minimizes overexploitation of wild populations and degradation of ecological health (Campbell *et al*., 1998; Shumway *et al*., 2003; Orensanz *et al*., 2004; Zhang and Hand, 2006). Shellfish aquaculture has expanded worldwide in recent decades to satisfy international trade (FAO 2018). However, early larval and juvenile rearing poses a production bottleneck. For example, early life histories are highly sensitive to biotic (e.g. harmful algae, pathogens; Prado *et al*., 2005; Rojas *et al*., 2015) and abiotic stressors (e.g. pH, salinity, thermal and hypoxic stress; Baker and Mann 1992; Przeslawski *et al*. 2015; Kroeker *et al*., 2010; Gimenez *et al*., 2018). These stressors are known to intensify in coastal marine systems (Cloern, 2001; Diaz and Rosenberg, 2001; Cai *et al*., 2011; Wallace *et al*., 2014) causing mass mortality for early-stage bivalves in wild or hatchery settings (Elston *et al*., 2008; Barton *et al*., 2015). Local and global anthropogenic stressors such as CO_2-induced changes in pH and carbonate mineral saturation states can reduce performance and normal shell development (White *et al*., 2013; Waldbusser *et al*., 2015; Kapsenberg *et al*., 2018).

Ocean acidification, or the decrease of oceanic pH due to elevated atmospheric partial pressures (µatm pCO_2), poses a threat to aquaculture (Barton *et al*., 2012; Froehlich *et al*., 2018; Mangi *et al*., 2018). Elevated pCO_2 and aragonite undersaturation ($\Omega_{aragonite} < 1$) generally have detrimental consequences for aerobic performance (Pörtner *et al*., 2004; Portner and Farrell, 2008) and shell biomineralization in marine calcifiers (Shirayama, 2005; Talmage and Gobler, 2010; Waldbusser *et al*., 2010, 2015; Gazeau *et al*., 2013). Responses to acidification can be species- (Ries *et al*., 2009) and population-specific (Lemasson *et al*., 2018), but it is widely established to be impactful during early life stages for bivalves (Dupont and Thorndyke, 2009; Gazeau *et al*., 2010; Kroeker *et al*., 2010; Gimenez *et al*., 2018). Experimental research is commonly focused on species with short generational times, (Parker *et al*., 2011, 2015; Lohbeck *et al*., 2012) limiting evidence for effects of acidification on long-lived mollusks important for food and economic security (Melzner *et al*., 2009).

The Pacific geoduck *Panopea generosa* is a large and long-lived infaunal clam of cultural and ecological importance (Dethier, 2006) with an increasing presence in sustainable shellfish industry (Cubillo *et al*., 2018). Geoduck production in Washington (USA) provides ∼90% of global supply (Shamshak and King, 2015) and alone constitutes 27% of the overall shellfish revenue in the state valued at >$24 million year^{-1} and >$14 pound^{-1} as of 2015 (Washington Sea Grant, 2015). Geoduck are known to live in dynamic CO_2-enriched low pH waters such as Hood Canal in Puget Sound, WA, where conditions in summer can reach $\Omega_{aragonite}$ 0.4 and pH 7.4 (Feely *et al*., 2010). Although *P. generosa* may be adapted and able to acclimatize to local stressors (Putnam *et al*., 2017; Spencer *et al*., 2018), acidification has caused massive losses of larval bivalves in hatcheries (Barton *et al*., 2015), identifying a critical need for assessment of physiological stress tolerance during early life stages.

Evidence of acclimatory mechanisms in response to acidification (Goncalves *et al*., 2018) and enhanced performance within and across generations (Parker *et al*., 2011, 2015; Putnam and Gates, 2015; Ross *et al*., 2016; Thomsen *et al*., 2017; Zhao *et al*., 2017) support conditioning as a viable strategy to mitigate the negative effects of stress exposure and enhance organismal performance under high pCO_2 (Parker *et al*., 2011; Dupont *et al*., 2012; Suckling *et al*., 2015; Foo and Byrne, 2016). Hormesis is a biphasic low-dose-stimulatory response, as identified in toxicological studies (Calabrese, 2008) and suggests beneficial carryover effects of moderate stress exposure (Calabrese *et al*., 2007; Costantini *et al*., 2010; Costantini, 2014; Putnam *et al*., 2018). Conditioning hormesis can explain patterns of intra- and transgenerational plasticity for organisms under environmental change (Calabrese and Mattson, 2011; Costantini *et al*., 2012; López-Martínez and Hahn, 2012; Putnam *et al*., 2018; Visser *et al*., 2018), but is understudied for stress resilience in bivalves likely due to generally negative physiological implications of acidification (Gazeau *et al*., 2013). In one example of early-life stage conditioning in bivalves, Putnam *et al*. (2017) found *P. generosa* exhibit compensatory shell growth after an acute exposure under elevated pCO_2. This finding suggests acute exposures may present a strategy for stress-hardening and enhancement of sustainable geoduck production. We therefore tested the hypothesis that repeated stress exposure under elevated pCO_2 can enhance intragenerational performance for Pacific geoduck. To this end, we measured the respiration rate and shell growth of juvenile geoduck in a commercial hatchery under repeated acute periods (∼6–10 days) of elevated pCO_2 and aragonite undersaturation, and the longer term (∼5 months) carryover effects.

Methods

Exposure of juveniles

Juvenile geoduck ($n = 640$; mean ± SEM initial size, 5.08 ± 0.66 mm shell length [measured parallel to hinge]) were reared in trays (Heath/Tecna water tray) with rinsed sediment for ∼16 weeks (pediveliger to juvenile stage) by Jamestown Point Whitney Shellfish Hatchery before allocated into eight trays for the experiment (Fig. 1; $n = 80$ clams per tray). During typical hatchery practice, geoduck are reared from 'setters' (pediveliger stage; 30 days old) to 'seed' (juvenile stage; 4–6 months old) in either downwellers or stacked trays; juveniles are then planted *in situ* to grow for several years until market size. Following aquaculture practice, trays were filled with a 5-mm depth of rinsed sand (35–45 µm grain size) that allowed juvenile geoduck to burrow and siphons could clearly be seen extended above the sediment throughout the experiments. To enable measurements of

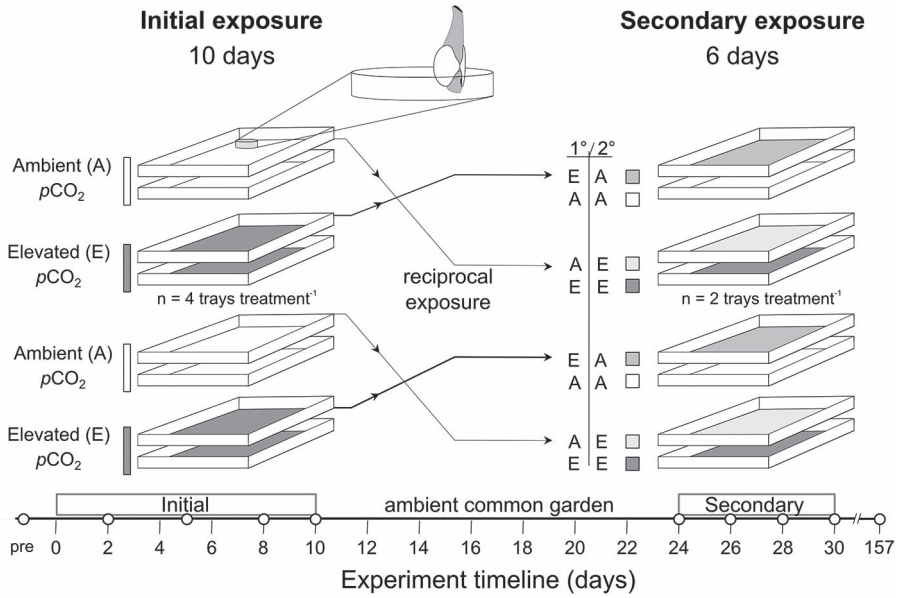

Figure 1: Schematic of the repeated exposure experimental design for two exposure trials, initial (10-day) and secondary (6-day), in ambient and elevated pCO_2 treatments. Timeline displays respiration and growth measurements as solid white circles

metabolic activity and shell growth, 30 geoduck were placed in an open circular dish (6.5 cm diameter and 3 cm height) with equal mesh size and sand depth submerged in each tray, the remaining 50 geoduck in each tray burrowed in the surrounding sediment. Seawater at the Jamestown Point Whitney Shellfish Hatchery (Brinnon, WA, USA) was pumped from offshore (100 m) in Quilcene Bay (WA, USA), bag-filtered (5 µm) and UV sterilized before fed to 250-L conical tanks at rate of 1 L min^{-1}. Four conical tanks were used as replicates for two treatments: elevated pCO_2 level of ~2300–2500 µatm and ~7.3 pH (total scale) and ambient hatchery conditions of ~500–600 µatm and ~7.8–7.9 pH (total scale). The elevated pCO_2 level was set with a pH-stat system (Neptune Apex Controller System; Putnam *et al.*, 2016) and gas solenoid valves for a target pH of 7.2 (NBS scale) and pH and temperature (°C) were measured every 10 s in conicals (Neptune Systems; accuracy: ± 0.01 pH units and ± 0.1°C, resolution: ± 0.1 pH units and ± 0.1°C). These treatments were delivered to replicate exposure trays, which were gravity fed seawater from conicals (Fig. 1; $n = 4$ per treatment). The experiment began with an initial exposure period of 10 days under elevated pCO_2 (2345 µatm) and ambient treatments (608 µatm; Table 1). Preliminary exposure was followed by 14 days in ambient common garden (557 ± 17 µatm; $pH_{t.s.}$ 7.9 ± 0.01; $\Omega_{aragonite}$ 1.46 ± 0.04, mean ± SEM) before secondary exposure for 6 days to reciprocal treatments of elevated pCO_2 (2552 µatm) and ambient treatments (506 µatm; Table 2). For the secondary exposure period, one tray was crossed to the opposite treatment to address both repeated and reciprocal exposure ($n = 2$ trays per initial×secondary pCO_2 treatment; Fig. 1). Following this, the juveniles were exposed to ambient conditions for 157 days within the replicate trays.

Juvenile geoduck were fed semi-continuously with a mixed algae diet (30% *Isochrysis galbana*, 30% *Pavlova lutheri* and 40% *Tetraselmis suecica*) throughout the 30-day experiment with a programmable dosing pump (Jebao DP-4 auto dosing pump). Large algae batch cultures were counted daily via bright-field image-based analysis (Nexcelom T4 Cellometer; Gurr *et al.*, 2018) to calculate a daily ration of 5×10^7 live algae cells day^{-1} individual^{-1}. Diet was calculated with an equation in Utting & Spencer (1991) catered for 5-mm clams: $V = (S \times 0.4) \div (7 \times W \times C)$; this equation accounts for a feed ration of 0.4 mg dried algae mg live animal weight^{-1} week^{-1}, the live animal weight (mg) of spat (S; estimated from regression of shell length and weight of Manilla clams in Utting & Spencer 1991), weight (mg) of one million algal cells (W) and cell concentration of the culture (cells µl^{-1}) to calculate the total volume (V) of each species in a mixed-algae diet. Tray flow rates (mean flow rate, ~480 ± 9 ml^{-1} min^{-1}) and food delivery were measured and adjusted daily.

All geoduck survived the exposure periods. Half of the remaining juveniles burrowed in each tray were maintained at the hatchery, positioned in the same replicate trays and stacked for continuous and high flow of ambient seawater (~8–10 L minute^{-1}). Stacked trays, commonly used for incubation of finfish, present a promising innovation for geoduck aquaculture; the experiment stack occurred along-

Table 1: pH, salinity and temperature measured with handheld probes and total alkalinity measured daily with 60 ml from each heath tray via Gran titration ($n = 4$ per treatment) during initial (10-day) and secondary (6-day) exposure trials. Seawater carbonate chemistry (CO_2, pCO_2, HCO_3^-, CO_3^{2-}, DIC, aragonite saturation state) was calculated with the seacarb R package (Gattuso *et al.*, 2018)

Treatment	Temperature	Salinity	Flow rate (mL min^{-1})	pH, total scale	CO_2 (µmol kg^{-1})	pCO_2 (µatm)	HCO_3 (µmol kg^{-1})	CO_3 (µmol kg^{-1})	DIC (µmol kg^{-1})	Total alkalinity (µmol kg^{-1})	Aragonite saturation state
Initial exposure											
Ambient	14.82 ± 0.12	29 ± 0.03	504 ± 21	7.8 ± 0.007	24 ± 0.5	608 ± 11	1842 ± 4	86 ± 1.4	1952 ± 3	2056 ± 1	1.35 ± 0.02
Low	14.91 ± 0.12	29 ± 0.04	484 ± 17	7.31 ± 0.004	91 ± 0.7	2345 ± 20	1992 ± 1	26 ± 0.20	2108 ± 1	2056 ± 1	0.41 ± 0.003
Ambient common garden											
Ambient	15.01 ± 0.22	29 ± 0.05	449 ± 18	7.89 ± 0.012	21 ± 0.7	561 ± 17	1821 ± 7	93 ± 2.6	1936 ± 5	2051 ± 1	1.45 ± 0.04
Secondary exposure											
Ambient	16.33 ± 0.22	28.67 ± 0.03	494 ± 29	7.93 ± 0.004	19 ± 0.3	506 ± 5	1781 ± 5	102 ± 1.4	1902 ± 4	2033 ± 2	1.60 ± 0.02
Low	16.40 ± 0.22	28.67 ± 0.04	471 ± 18	7.27 ± 0.007	95 ± 1.3	2551 ± 42	1972 ± 3	25 ± 0.3	2091 ± 3	2033 ± 3	0.39 ± 0.005

side prototype stacked growing trays stocked by Jamestown Point Whitney Shellfish. The juveniles were fed cultured algae *ad libitum* daily for 157 days before shell length and respiration rates were measured.

Respirometry and shell length measurements

Juvenile geoduck were measured on Days 2, 5, 8 and 10 of initial exposure, Days 0, 2, 4 and 6 (cumulatively as Days 24, 26, 28 and 30, respectively) of secondary exposure and 157 days after the exposure period (cumulatively as Day 187) to assess rates of oxygen consumption normalized to shell length. Calibrated optical sensor vials (PreSens, SensorVial SV-PSt5-4 ml) were used to measure oxygen consumption in 4 ml vials on a 24-well plate sensor system (Presens SDR SensorDish). Juveniles in each treatment dish were divided into three sensor vials (10 individuals vial^{-1} for exposure periods; 1 individual vial^{-1} at 157 days post-exposure), each filled with 0.2 µm filtered seawater from corresponding trays. Three blank vials per tray, filled only with 0.2 µm filtered seawater, were used to account for potential microbial oxygen consumption. Respiratory runs occurred within an incubator at 15°C, with the vials and sensor placed on a rotator for mixing. Each set of measurements lasted ~30 min, and trials ceased when oxygen concentration declined ~70–80% saturation to avoid hypoxic stress and isolate the effect of pCO_2 treatment on respiration rate. Siphons were observed pre and post-respirometry and were fully extended (~1–2 times shell length). Geoduck were subsequently photographed, and shell length (parallel to hinge) was measured using ImageJ with a size standard (1 mm stage micrometer).

Rates of respiration (oxygen consumption) were calculated from repeated local linear regressions using the R package LoLinR (Olito *et al.*, 2017). An initial criterion of fixed constants (from the LoLin R package) for weighting method ($L_{\%}$) and observations (alpha = 0.2) was run individually for each respirometry measurement over the full 30-min record as a 'reference' dataset. These are considered to be the most robust parameters as suggested by the R package authors (Olito *et al.*, 2017). Diagnostic plots (from the LoLin R package) were individually observed, and $L_{\%}$ and alpha were altered as necessary to best approximate the peak empirical distribution of local linear regressions (see doi: 10.5281/zenodo.3588326 for full details). To determine the optimal set of parameters, respiration data was calculated using three alpha values and data truncations (alpha = 0.2, 0.4 and 0.6; truncation = 10–20 min, 10–25 min and no truncation; weighting method = $L_{\%}$) and each was compared to the initial reference dataset with two curve fitting steps (local polynomial regressions) to calculate unbiased and reproducible rates of oxygen consumption similar to the reference (10-day exposure, $r^2 = 0.88$; 6-day exposure, $r^2 = 0.95$). Final metabolic rates of juvenile geoduck were corrected for vial volume, rates of oxygen change in the blank vials, and standardized by mean shell length (µg O_2 h^{-1} mm^{-1}).

Table 2: Two-way and three-way ANOVA tests for metabolic rate and shell length during initial and secondary exposures, respectively. A Welch's *t* test was used on day zero of secondary exposure to test for differences in mean respiration rate and shell length from initial treatments and a two-way ANOVA tested for treatment effects after 157 days. Significant effects are bolded for $P < 0.05$

		df	SS	MS	*F*	*P*
Initial exposure	*Two-way ANOVA*					
Respiration date	time	3	0.0323	0.011	0.822	0.485
	pCO_2	1	0.0983	0.098	7.512	**0.007**
	pCO_2 × time	3	0.0475	0.016	1.210	0.311
Shell length	time	3	4.250	1.415	3.392	
	pCO_2	1	0	0.0005	0.0012	0.973
	pCO_2 × time	3	0.170	0.058	0.138	0.937
Ambient common garden	*Welch two sample t-test*	df	t	P		
Respiration rate	pCO_2	19.833	2.673	**0.015**	-	-
Shell length	pCO_2	1.146	236.680	0.253	-	-
Secondary exposure	*Three-way ANOVA*					
Respiration rate	time	2	0.068	0.034	3.137	0.051
	$pCO_{2\ initial}$	1	0.021	0.021	1.916	0.171
	$pCO_{2\ secondary}$	1	0.032	0.032	2.926	0.092
	$pCO_{2\ initial}$ × $pCO_{2\ secondary}$	1	0.023	0.023	2.080	0.154
	$pCO_{2\ initial}$ × time	2	0.016	0.008	0.724	0.489
	$pCO_{2\ secondary}$ × time	2	0.002	0.001	0.103	0.903
	$pCO_{2\ initial}$ × $pCO_{2\ secondary}$ × time	2	0.035	0.017	1.608	0.209
Shell length	time	2	0.190	0.095	0.152	0.859
	$pCO_{2\ initial}$	1	9.910	9.910	15.821	**<0.001**
	$pCO_{2\ secondary}$	1	6.210	6.212	9.917	**0.002**
	$pCO_{2\ initial}$ × $pCO_{2\ secondary}$	1	0.060	0.063	1.100	0.752
	$pCO_{2\ initial}$ × time	2	0	0.01	0.002	0.998
	$pCO_{2\ secondary}$ × time	2	0.460	0.231	0.368	0.692
	$pCO_{2\ initial}$ × $pCO_{2\ secondary}$ × time	2	0.100	0.048	0.076	0.927
157 days post	*Two-way ANOVA*					
Respiration rate	$pCO_{2\ initial}$	1	0.003	0.002	0.011	0.919
	$pCO_{2\ secondary}$	1	3.037	3.037	13.008	**0.001**
	$pCO_{2\ initial}$ × $pCO_{2\ secondary}$	1	0.050	0.050	0.212	0.648
Shell length	$pCO_{2\ initial}$	1	10.600	10.597	5.228	**0.023**
	$pCO_{2\ secondary}$	1	0.21	0.214	0.105	0.746
	$pCO_{2\ initial}$ × $pCO_{2\ secondary}$	1	3.510	3.507	1.730	0.190

Significant *P* values (<0.05) are bolded.

Seawater carbonate chemistry

Total alkalinity (TA; μmol kg^{-1} seawater) water samples were collected from trays once daily during treatment periods, in combination with measurements of pH by handheld probe (Mettler Toledo pH probe; resolution: 1 mV, 0.01 pH; accuracy: ± 1 mV, ± 0.01 pH; Thermo Scientific Orion Star A series A325), salinity (Orion 013010MD Conductivity Cell; range 1 μS/cm–200 mS/cm; accuracy: ± 0.01 psu) and temperature (Fisherbrand Traceable Platinum Ultra-Accurate Digital Thermometer; resolution; 0.001°C; accuracy: ± 0.05°C). Seawater chemistry was measured for three consecutive days during the 14 days of ambient common garden between initial and secondary treatment periods. Quality control for pH data was assessed daily with Tris standard (Dickson Lab Tris Standard Batch T27) and handheld conductivity probes used for discrete measurements were calibrated every 3 days. TA was measured using an open cell titration (SOP 3b; Dickson *et al.*, 2007) with certified HCl titrant (~0.1 mol kg^{-1}, ~0.6 mol kg^{-1} NaCl; Dickson Lab) and TA measurements identified < 1% error when compared against certified reference materials (Dickson Lab CO_2 CRM Batches 137 and 168). Seawater chemistry was completed following Guide to Best Practices (Dickson *et al.*, 2007); daily measurements were used to calculate carbonate chemistry, CO_2, pCO_2, HCO^{3-}, CO_3 and $\Omega_{aragonite}$, using the SEACARB package (Gattuso *et al.*, 2018) in R v3.5.1 (R Core Team, 2018).

Data analysis

A two-way analysis of variance (ANOVA) was used to analyze the effect of time (fixed), pCO_2 treatment (fixed) and time×pCO_2 interaction for respiration and shell length during initial exposure. A *t* test was used to test the effect of initial pCO_2 treatment on respiration rate and shell length prior to the secondary exposure (last day of ambient common garden, cumulatively Day 24, Day 0). For the secondary exposure period, a three-way ANOVA was used to test the effects of time (fixed), initial pCO_2 treatment (fixed), secondary pCO_2 treatment (fixed) and their interactions on respiration rate and shell length. No significant differences in seawater chemistry were detected between trays of the same treatment (pH, pCO_2, TA, salinity and temperature; doi: 10.5281/zenodo.3588326); thus, tray effects were assumed negligible. Significant model effects were followed with pairwise comparisons with a Tukey's *a posteriori* HSD. We used a two-way ANOVA to analyze the effects of initial (fixed) and secondary (fixed) pCO_2 treatments on respiration and shell length after 157 days in ambient conditions. In all cases, model residuals were tested for normality assumptions with visual inspection of diagnostic plots (residual vs. fitted and normal *Q*–*Q*; Kozak and Piepho, 2018) and homogeneity of variance was tested with Levene's test. Model effects using raw data were robust to transformation(s) that resolved normality assumptions via Shapiro–Wilk test. Statistical tests were completed using R (v3.5.1; R Core Team, 2018). All data and code is available (doi: 10.5281/zenodo.3588326).

Results

Exposure 1

The respiration rate of juvenile clams (4.26 ± 0.85 mm shell length; mean ± SD) prior to exposure was 0.29 ± 0.16 μg O_2 h^{-1} mm^{-1} (mean ± SD). Elevated pCO_2 had a significant effect on respiration rate over the initial 10-day exposure (pCO_2 treatment, $F_{1,88} = 7.512$; $P < 0.01$) with a 25% reduction (averaged across all days) in respiration rate in elevated pCO_2 treatment relative to ambient (Fig. 2A). Juvenile geoduck grew significantly with time under the initial 10-day exposure (time, $F_{3,949} = 3.392$; $P = 0.018$) with a 3.6% increase in shell length between Days 2 and 10 (Fig. 2B), but there was no effect of pCO_2 treatment on shell length (Table 2). Significant differences in respiration rate from the initial pCO_2 treatment were still apparent after 14 days in ambient common garden and before the onset of the secondary exposure (Table 2 and Fig. 3A). In contrast, there was no significant change in shell length due to initial pCO_2 treatment after 14 days in ambient common garden (Table 2).

Exposure 2

There was no interaction between initial and secondary pCO_2 treatments nor between treatments and time on respiration rate or shell length (Table 2). There was a marginal effect of time on respiration rate (Table 2; time, $F_{2,60} = 3.137$; $P = 0.0506$) with a 31% increase in average respiration rate between Days 2 and 6. Initial pCO_2 treatment had a significant effect on shell length, with on average a ~4% reduction in shell size under high pCO_2 relative to ambient initial exposure (Fig. 3B; $pCO_{2\_initial}$, $F_{1,709} = 15.821$; $P < 0.001$). This same trend was present under the secondary high pCO_2 exposure, (Fig. 3B; $pCO_{2\_secondary}$, $F_{1,709} = 9.917$; $P = 0.002$) with 3.20% smaller shells for individuals exposed to elevated pCO_2 treatments. There were pairwise differences in shell size between animals only exposed to ambient and animals repeatedly exposed to elevated pCO_2 (Fig. 3B; Day 6, $P = 0.0415$; Day 6 ambient—Day 4 elevated, $P = 0.0406$).

Common garden after exposure periods

There was no interaction between initial and secondary pCO_2 treatments on respiration rate or shell length (Table 2). The initial exposure period had a significant effect on shell length of juveniles previously exposed to high pCO_2, after 157 days in ambient common garden (Fig. 4A; $pCO_{2\_initial}$, $F_{1,170} = 5.228$; $P = 0.023$), where average shell lengths were 5.8% larger in juveniles exposed to initial elevated pCO_2. Secondary 6-day exposure had a significant effect on

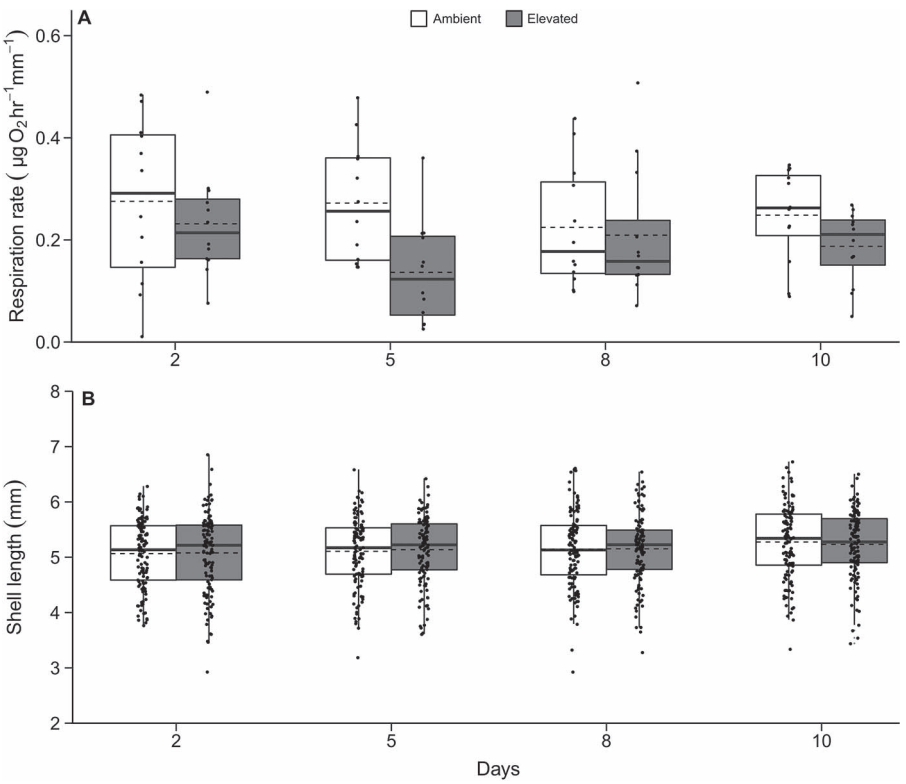

Figure 2: Respiration rates (**A**) and shell length (**B**) of juvenile geoduck under the initial 10-day exposure displayed as box whisker plots with mean (dashed line), median (solid line), 25–50% range (box) and interquartile range (whiskers). Small solid circles represent all data points

respiration rates after 157 days in ambient common garden (Fig. 4B; $pCO_{2\_seccondary}$, $F_{1,31} = 13.008$; $P = 0.001$) with an average of 52.4% greater respiration rates in juveniles secondarily exposed to elevated pCO_2. Visual examination during screening indicated low mortality (1–4 tray^{-1}) over the ~5-month grow-out period. Shell lengths of dead animals (as empty shells) were similar to the size of juvenile geoduck during the 30-day exposure period suggesting low mortality occurred at the start of the grow-out period possibly due to handling stress.

Discussion

Metabolic recovery and compensatory shell growth by juvenile *P. generosa* present a novel application of hormetic framework for resilience of a mollusc to acidification. To date, within-generation carryover effects remain poorly understood for marine molluscs (Ross *et al.*, 2016) with few examples of either positive and negative responses after stress challenges (Hettinger *et al.*, 2012; Gobler and Talmage, 2013; Putnam *et al.*, 2017). Further study on conditioning hormesis in response to pCO_2 stress must address cellular-level energy allocation, in addition to whole organism physiology, to account for essential functions with more holistic implications for stress resilience (Pan *et al.* 2015).

Metabolic depression and compensatory response

Metabolic depression, such as that found under initial exposure of geoduck to elevated pCO_2, has been suggested as an adaptive mechanism to extend survival (Guppy and Withers, 1999). Stress-induced metabolic depression has been documented for a variety of marine invertebrates in response to environmental stress. For example, in the New Zealand geoduck, *Panopea zelandica*, there was a 2-fold reduction in respiration rate under hypoxia (Le *et al.*, 2016). Prior work has shown metabolic reductions up to 60–95% of basal performance at rest for marine molluscs (Guppy and Withers, 1999). Here, depression of oxygen consumption rate by juvenile geoduck to ~25% in comparison with rates under ambient conditions suggests that *P. generosa* are relatively tolerant to short-term acidification and may have adaptive physiology to cope with environmental acidification and high pCO_2. Responsiveness to acidification is critical for pH-tolerant taxa to maintain buffering capacity and cope with acidosis (high intracellular pCO_2; (Melzner *et al.*, 2009). However, pH-induced metabolic depression to a similar degree found in this study has caused a permanent decrease in extracellular pH and increase in protein degradation and ammonia excretion in the Mediterranean mussel (*Mytilus galloprovincialis*) (Michaelidis *et al.*, 2005). Conversely, metabolic elevation is

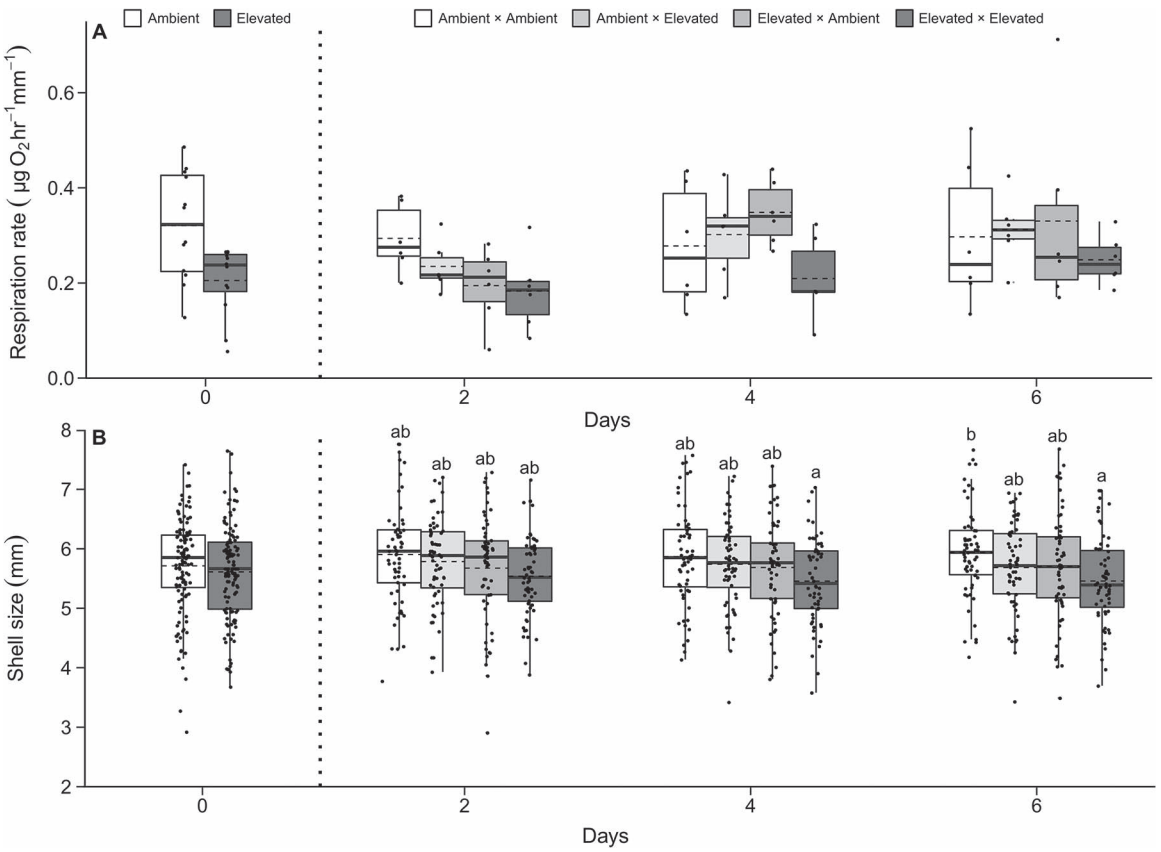

Figure 3: Respiration rates (**A**) and shell length (**B**) of juvenile geoduck under the secondary 6-day exposure displayed as box whisker plots with mean (dashed line), median (solid line), 25–50% range (box) and interquartile range (whiskers). Small solid circles represent all data points. Letters display significant post hoc effects ($P < 0.05$)

relatively common for early-life stage bivalves exposed to low pH and $\Omega_{aragonite}$ undersaturation and typically coincides with consequences for performance and survival (Michaelidis *et al.*, 2005; Beniash *et al.*, 2010; Thomsen and Melzner, 2010; Fernández-Reiriz *et al.*, 2011; Waldbusser *et al.*, 2015; Lemasson *et al.*, 2018). Whether depressed or elevated, stress-induced metabolic alterations are known to contribute to biochemical outcomes such as intracellular hypercapnia and hemolymph acidosis (Pörtner *et al.*, 2004; Spicer *et al.*, 2011) and increased ammonia excretion and reduced growth for invertebrate fauna (Michaelidis *et al.*, 2005; Beniash *et al.*, 2010; Lannig *et al.*, 2010; Thomsen and Melzner, 2010; Gazeau *et al.*, 2013). However, pCO_2 did not impair shell growth during the initial period further demonstrative of the pH/hypercapnia tolerance of *P. generosa*.

Juvenile geoduck repeatedly exposed to elevated pCO_2 showed possible stress 'memory' with rebound from metabolic depression under subsequent stress and higher respiration rate and compensatory shell growth after long-term recovery. Metabolic rebound supports a hormetic-like response by *P. generosa* (Calabrese *et al.*, 2007; Costantini, 2014) and prompts further investigation of energy budget, cellular and -omic measures under repeated reciprocal stress encounters to improve our understanding of the mechanism underpinning hormesis. Use of hormesis to conceptualize carryover effects of mild stress exposure is largely confined to model insects, plants and microorganisms (Lee *et al.*, 1987; Calabrese and Blain, 2009; López-Martínez and Hahn, 2012; Visser *et al.*, 2018). For example, Visser *et al.* (2018) found the Caribbean fruit fly, *Anastrepha suspensa*, exposed to oxidative stress early in life enhanced survivorship and investment in fertility and lipid synthesis under subsequent stress during adulthood. Mechanistic molecular and biochemical assessments under different and repeated stress intensities (i.e. magnitude, duration, and frequency) are planned to determine the threshold between low-dose stimulation and high-dose inhibition from stress conditioning.

Age and intensity dependence of shell growth

Metabolic recovery was coupled with reduced shell growth under a repeated stress encounter (Fig. 3) and compensatory shell growth after ~5 months in ambient conditions (Fig. 4).

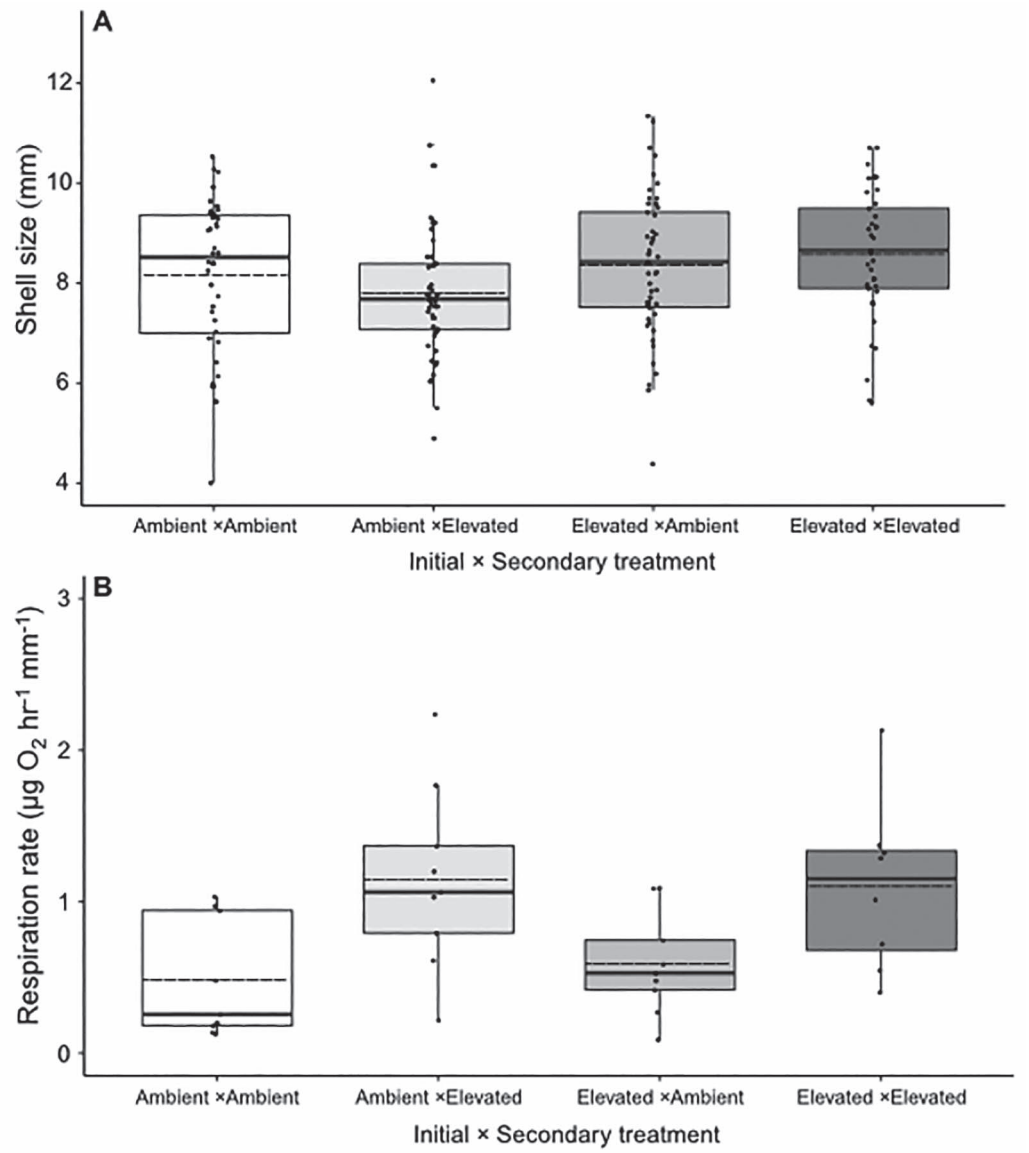

Figure 4: Shell length (**A**) and metabolic rates (**B**) of juvenile geoduck after 157 days in ambient common garden conditions post-exposure. Data is displayed as box whisker plots with mean (dashed line), median (solid line), 25–50% range (box) and interquartile range (whiskers). Small solid circles represent all data points

This could be explained by several hypotheses such as a carryover effect from metabolic depression under initial exposure to elevated pCO_2 (Fig. 2A), differing sensitivity to stress intensity (Table 1) and/or age dependence for environmental hardening, or the interaction with increasing temperature through the season (see Supplementary Fig. 1.). Bivalves known to exhibit metabolic suppression under acute and long-term acidification are often attributed with increased ammonia excretion rates and decreased ingestion and clearance rates as possible contributors to protein degradation and reduced growth (Michaelidis *et al.*, 2005; Thomsen and Melzner, 2010; Fernández-Reiriz *et al.*, 2011; Navarro *et al.*, 2013). Therefore, decreased shell length under secondary exposure may be a latent effect of metabolic depression during initial exposure. However, shell length was also reduced for clams initially exposed to the elevated treatment in the second exposure period (Table 2, Fig. 3B), indicating potential age dependence of calcification and bioenergetic effects for juvenile *P. generosa*. This reduction, however, could also be explained by the fact the secondary elevated pCO_2 treatment was on average ~0.04 pH units lower than the initial exposure (Table 1), suggesting possible sensitivity to increased stress intensity. It is likely that both temporal dynamics and stress thresholds influence intragenerational carryover effects and

further experimental efforts with repeated reciprocal design are needed.

Respiration rates and shell growth 5 months post-exposure show a latent enhancement for animals repeatedly stressed or exposed to a stress event earlier in life, emphasizing the importance of the severity, duration, and timing of intragenerational stress conditioning. These specific findings present a window in their life history where it may be advantageous to condition Pacific geoduck for enhancement of sustainable aquaculture.

Commercial and environmental applications of experimental findings

Our findings infer both positive and negative implications for aquaculture. Although advantageous to elicit carryover effects exhibited by stress-conditioned animals, results imply greater feed (ingestion rate) to sustain enhanced aerobic metabolism and compensatory shell growth; this can heighten labour and financial costs for industry, likely not incentivized by a marginal 5.8% increase in shell size. However, typical protocols for geoduck aquaculture yield 5-month-old juvenile clams in the hatchery before grown *in situ* for 5–7 years. Consequently, latency of enhanced performance in this study (~9-month-old juveniles), overlaid with the standard timeline for geoduck industry, does not present additional expenses. Further related tests on stress conditioning and production of resilient strains (i.e. phenotypes and/or epigenotypes) must account for distinct life-stages and species-specific attributes in aquaculture practice.

Shellfish farming has adapted in recent years to implement 'climate-proofing' technology to maintain production and combat both coastal and climate-related stressors (e.g. ocean acidification, sea-level rise, coastal development; Allison *et al*. 2011). For example, chemical buffering systems (e.g. mixing sodium bicarbonate) are increasingly common in shellfish industry to elevate aragonite saturation levels and reduce deleterious effects of ocean acidification; hatcheries report increases in productivity by 30–50%, offsetting the cost to maintain optimal carbonate chemistry year-round (Barton *et al*. 2015). Although buffering systems are advantageous to yield juvenile 'seed', alleviation of aragonite undersaturation in the short term may leave juveniles and adults unprepared to cope with the heterogeneity of environmental chemistry during long growing periods *in situ*. As conditions in coastal bays report deteriorating water quality (Cloern 2001; Feely *et al*. 2008; Melzner *et al*. 2013; Wallace *et al*. 2014), acclimatization and selective breeding posit alternate and more robust solutions to generate stress-resilience (Barton *et al*. 2015). Implementation and tests of effectiveness of stress conditioning remain uncommon for scientists and aquaculture; our novel findings collected in a hatchery setting provide incentive to fine-tune stress exposures and build a mechanistic understanding of physiological, cellular, and molecular responses. Critical questions to test the practical application of stress conditioning are: (i) what are the effects of repeated stress exposures on energy budget? (ii) what life-stages and/or pCO_2 stress intensity (i.e. magnitude and duration) optimizes establishment of resilient phenotypes and genotypes during hatchery-rearing? (iii) does stress history under elevated pCO_2 affect the stability and longevity of carryover effects later in life? Answers to these challenges will result in effective implementation of conditioning to both reduce pressure on wild stocks and sustain food security under environmental change.

Although this study was primarily focused on production enhancement in a hatchery setting, effects on shell growth and metabolism have important applications to natural systems. Seawater carbonate chemistry targeted for stress treatments was more severe than levels commonly used in experimental research (Gazeau *et al*., 2010; Navarro *et al*., 2013; Diaz *et al*., 2018), but relevant to summer subsurface conditions within the natural range of *P. generosa* (pH 7.4 and $\Omega_{aragonite}$ 0.4 in Hood Canal, WA; Feely *et al*., 2010). Thus, survival, metabolic recovery, and compensatory growth in *P. generosa* in this study demonstrates a resilience to short-term acidification in the water column. Enhanced growth rates during juvenile development can present benefits for burrowing behaviour (Green *et al*., 2009; Clements *et al*., 2016; Meseck *et al*., 2018) and survival due to decreased risk of predation and susceptibility to environmental stress (Przeslawski and Webb, 2009; Johnson and Smee, 2012). Specific to juvenile *P. generosa*, time to metamorphosis (to dissoconch), pre-burrowing time (time elapsed to anchor into substrate and obtain upright position), and burrowing depth are directly related to growth and survival (Goodwin and Pease, 1989; Tapia-Morales *et al*., 2015). Thus, stress conditioning under CO_2 enrichment and low pH may enhance survivorship of juvenile geoduck in natural systems. Water column carbonate chemistry may be critical for sustainable production of infaunal clams, such as *P. generosa*, that are outplanted for several years *in situ* on mudflats known to exhibit dynamic abiotic gradients (Green *et al*., 1993; Burdige *et al*., 2008) adjacent to seasonally acidified and undersaturated water bodies (Feely *et al*., 2010; Reum *et al*., 2014).

Conclusion

Data in this present study provides evidence of capacity to cope with short-term acidification for an understudied infaunal clam of high economic importance. Survival of all individuals over the 30-day experiment demonstrates the resilience of this species to low pH and reduced carbonate saturation. Juvenile geoduck exposed to low pH for 10 days recovered from metabolic depression under subsequent stress exposure and conditioned animals showed a significant increase in both shell length and metabolic rate compared to controls after 5 months under ambient conditions, suggesting stress 'memory' and compensatory growth as possible indicators of enhanced performance from intragenerational stress conditioning. Our focus on industry enhancement must expand to

test developmental morphology, physiology, and genetic and non-genetic markers over larval and juvenile stages in a multigenerational experiment to generate a more holistic assessment of stress hardening and the effects of exposure on cellular stress response (Costantini *et al*., 2010; Foo and Byrne, 2016; Eirin-Lopez and Putnam, 2018) for advancement of sustainable aquaculture (Branch *et al*., 2013). Advancements in genome sequencing will facilitate further research to synthesize -omic profiling (i.e. global DNA methylation and differential expression) with physiological responses throughout reproductive and offspring development under environmental stress (Gavery and Roberts, 2014; Li *et al*., 2019) to determine if these mechanisms are transferable among species. Stress conditioning within a generation at critical life stages may yield beneficial responses for food production and provide a baseline for other long-lived burrowing bivalves of ecological and economic importance.

Author contributions

S.J.G., B.V., S.B.R. and H.M.P. designed the experiments, S.J.G. conducted the experiments, S.J.G., B.V., S.B.R. and H.M.P. drafted, revised, read and approved the final version of the manuscript.

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