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RESEARCH ARTICLE

Repeat exposure to hypercapnic seawater modifies growth and oxidative status in a tolerant burrowing clam

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ABSTRACT

Although low levels of thermal stress, irradiance and dietary restriction can have beneficial effects for many taxa, stress acclimation remains little studied in marine invertebrates, even though they are threatened by climate change stressors such as ocean acidification. To test the role of life-stage and stress-intensity dependence in eliciting enhanced tolerance under subsequent stress encounters, we initially conditioned pediveliger Pacific geoduck (*Panopea generosa*) larvae to ambient and moderately elevated P_{CO_2} (920 µatm and 2800 µatm, respectively) for 110 days. Then, clams were exposed to ambient, moderate or severely elevated P_{CO_2} (750, 2800 or 4900 µatm, respectively) for 7 days and, following 7 days in ambient conditions, a 7-day third exposure to ambient (970 µatm) or moderate P_{CO_2} (3000 µatm). Initial conditioning to moderate P_{CO_2} stress followed by second and third exposure to severe and moderate P_{CO_2} stress increased respiration rate, organic biomass and shell size, suggesting a stress-intensity-dependent effect on energetics. Additionally, stress-acclimated clams had lower antioxidant capacity compared with clams under ambient conditions, supporting the hypothesis that stress over postlarval-to-juvenile development affects oxidative status later in life. Time series and stress intensity-specific approaches can reveal life-stages and magnitudes of exposure, respectively, that may elicit beneficial phenotypic variation.

KEY WORDS: Ocean acidification, Oxidative stress, Phenotypic variation, Stress acclimation, Geoduck

INTRODUCTION

Ocean acidification (OA), including the decrease of oceanic pH, carbonate ion concentration and aragonite saturation state (Ω_{arg}) due to elevated atmospheric partial pressures (P_{CO_2}), poses a global threat with magnified intensity in coastal marine systems (Cai et al., 2011). Marine molluscs are particularly susceptible to OA, with negative physiological impacts in aerobic performance (Navarro et al., 2013), calcification, growth and development (Waldbusser et al., 2015), acid/base regulation (Michaelidis et al., 2005) and energy-consuming processes (i.e. protein synthesis; Pan et al., 2015).

It is posited for ectotherm physiology (i.e. oxygen capacity-limited thermal tolerance: Pörtner, 2012; energy-limited tolerance to stress: Sokolova, 2013) that cellular and physiological modifications affecting energy homeostasis describe aerobic performance 'windows' under 'optimum' (ambient), 'pejus' (moderate) and 'pessimum' (severe) environmental ranges (Sokolova et al., 2012; Sokolova, 2021). The conserved defense proteome, or cellular stress response (CSR), is the hallmark of cellular protection but comes at an energetic cost (Kültz, 2005). Whereas the CSR is unsustainable if harmful conditions exacerbate or persist (Sokolova et al., 2012), episodic or sublethal stress encounters can induce adaptive phenotypic variation (Tanner and Dowd, 2019). A growing body of research suggests that moderate or intermittent stress (e.g. caloric restriction, irradiance, thermal stress, oxygen deprivation, etc.) can elicit experience-mediated resilience for a variety of taxa (i.e. fruit fly, coral, fish, zebra finch, mice) increasing CSR, fitness and compensatory/anticipatory responses under subsequent stress exposures (Brown et al., 2002; Costantini et al., 2012; Jonsson and Jonsson, 2014; Visser et al., 2018; Zhang et al., 2018). Further, early-life development presents a sensitive stage to elicit adaptive phenotypic adjustments (Fawcett and Frankenhuis, 2015), prompting investigation of environmental stress acclimation under a rapidly changing environment.

Hormetic priming describes the beneficial effects of pre-exposure enhancing the ability to cope with subsequent encounters of similar or higher levels of stress later in life, as opposed to individuals without previous experience or primed under severe stress (Costantini, 2014). Mild oxidative stress presents a common source of hormetic priming (Costantini, 2014) and is a hypothesized driver of longevity (Ristow and Schmeisser, 2014; Wojtczyk-Miaskowska and Schlichtholz, 2018). For example, early-life exposure to moderate oxidative stress in the Caribbean fruit fly *Anastrepha suspensa* and zebra finch *Taeniopygia guttata* decreases cellular damage and increases proteomic defense, energy assimilation and survival under a subsequent stress encounter during adulthood (Costantini et al., 2012; Visser et al., 2018). Oxidative stress causes macromolecular damage and can occur from an over-production of reactive oxygen species (ROS such as superoxide, hydrogen peroxide or hydroxyl radical) primarily from mitochondrial oxidative phosphorylation, or changes to antioxidant systems that disrupt ROS scavenging. In marine invertebrates, oxidative stress can intensify under environmental stressors such as hypoxia and emersion (Abele et al., 2008), hyposalinity (Tomanek et al., 2012), thermal stress (An and Choi, 2010), pollutants and contaminants (Livingstone, 2001), and OA (Tomanek et al., 2011; Matoo et al., 2013). Protein families that are involved in the CSR function in signaling, avoidance and mediation of oxidative damage. Specifically, antioxidant proteins (i.e. superoxide dismutase, catalase, glutathione peroxidase, etc.) are widely conserved across phyla to scavenge ROS and regulate redox status at the expense of energy homeostasis (Kültz, 2005). Adaptive cellular defense against oxidative damage is thought to have an important evolutionary role in the longevity of the ocean quahog *Arctica islandica* (lifespan >400 years) as a result of a

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lifestyle of metabolic dormancy (when burrowed) and aerobic recovery (Abele et al., 2008). Further, hypoxia-tolerant marine bivalves show anticipatory and compensatory upregulation of antioxidant proteins to mitigate oxidative bursts under hypoxia–reoxygenation (Ivanina and Sokolova, 2016). Such adaptive responses have yet to be explored under hypercapnic conditions to identify species tolerant to OA stress. Although bivalves are known to exhibit P_{CO_2}-induced oxidative damage and upregulated CSR (Tomanek et al., 2011; Matoo et al., 2013), studies have yet to investigate oxidative stress response (i.e. antioxidant capacity) in a hormetic framework (repeated exposures).

Pacific geoduck (*Panopea generosa* Gould 1850) is a burrowing clam of ecological (Goodwin and Pease, 1987) and economic importance (Shamshak and King, 2015) and is a great candidate for investigating hormetic priming for generation of stress-acclimated phenotypes. Juvenile geoduck have shown positive carryover effects after exposure to high P_{CO_2}/low Ω_{arg} conditions, including compensatory respiration rates and shell growth (Gurr et al., 2020). In contrast, larval performance is negatively impacted under OA exposure (Timmins-Schiffman et al., 2019). The postlarval life stage presents an ecologically relevant and less susceptible window to investigate effects of P_{CO_2} stress acclimation. 'Settlement' in bivalves is a developmental transition from free-swimming larvae in an oxygen-saturated water column to an increasingly sedentary or burrowed life in the benthos (Goodwin and Pease, 1989) where stratification, bacterial carbon mineralization and reduced buffering capacity drives down calcium carbonate saturation and oxygen levels (Cai et al., 2011). To investigate the potential for early stress to elicit beneficial responses under subsequent encounters, we investigated the effects of P_{CO_2} exposures of different intensity and at different time points in a repeated reciprocal approach (multiple and crossed treatment periods), on the physiological and subcellular phenotypes of juvenile Pacific geoduck.

MATERIALS AND METHODS

Environmental context for chosen P_{CO_2} treatments

Ambient hatchery conditions and local buoy data contextualize the choices of P_{CO_2} to test responses under 'pejus' and 'pessimum' range (Sokolova et al., 2012; Sokolova, 2021). First, as control P_{CO_2} conditions, incoming hatchery ambient seawater temperature, salinity, pH, and P_{CO_2} was 16–18°C, 29 ppt, 7.7–7.8 pH, and ∼800–950 µatm, respectively. These data correspond with local conditions obtained from data buoys (i.e. Dabob Bay in Hood Canal, WA; Fassbender et al., 2018). As the 'pejus' range, a pH 7.2 and Ω_{arg} 0.4 or 'moderate' P_{CO_2} (2800–3000 µatm) was used in this study. Hood Canal is a known habitat for *P. generosa* (Mcdonald et al., 2015) and demonstrates seasonal patterns of low pH and undersaturated conditions with respect to aragonite (Fassbender et al., 2018) especially at depth (i.e. 50 m, pH 7.4 and Ω_{arg} 0.4; Feely et al., 2010). Moreover, the deep benthic range (i.e. 110 m; Goodwin and Pease, 1989) and infaunal lifestyle of *P. generosa* further suggests that exposure to severe low pH and aragonite undersaturated conditions may be common for geoduck. Thus, as a 'pessimum' range, a pH 7.0 and Ω_{arg} 0.2 and 'severe' P_{CO_2} (4940 µatm) was chosen.

Experimental setup

Larval Pacific geoduck were reared from gametes at the Jamestown Point Whitney Shellfish Hatchery (Brinnon, WA) following standard shellfish aquaculture industry practices, using bag-filtered (5 µm) and UV-sterilized seawater pumped from offshore (27.5 m depth) in Dabob Bay (WA, USA). Larvae reached settlement competency, characterized by a protruding foot and larval shell length >300 µm, at 30 days post-fertilization. Approximately 15,000 larvae were randomly placed into each of eight 10-liter trays (Heath/Tecna) containing a thin layer of sand to simulate the natural environment and enable metamorphosis from veliger larvae to pediveliger larvae, and subsequently to the burrowing and sessile juvenile stage.

Acclimation from pediveligers to juveniles (primary exposure)

Pediveligers were placed into ambient or moderate P_{CO_2} conditions (921±41 or 2870±65 µatm; Table 1; Fig. 1) for an initial exposure during the transition from pediveliger to the burrowing juvenile stage (N=4 trays per treatment; N=1.5×10^4 pediveligers per tray). Seawater flowed into 250 liter head tanks at a rate of 0.1 liters min^{-1} and replicate trays were gravity-fed from the head tanks. At the end of the primary exposure after 110 days, respiration rate and shell growth were measured for 20 randomly selected juveniles from each of the 8 trays as described below. Additionally, 6 animals from each tray were frozen in liquid nitrogen and stored at −80°C for biochemical analysis. Observations at the end of the acclimation period estimated ∼30% survival (4000–5000 juveniles per tray) regardless of P_{CO_2} condition.

Modified reciprocal exposure

Second exposure

To begin the second exposure, juvenile geoducks (∼2200 geoducks per initial P_{CO_2} treatment) were rinsed on a 3×10^5 µm screen to isolate individuals and were divided equally in 36 plastic cups (175 ml) (N=120 animals per cup, N=6 cups per treatment) each with 50 ml rinsed sand (450–550 µm grain size). Seawater flowed into 250 liter head tanks at a rate of 0.6 liters min^{-1} and was pumped using submersible pumps to randomly interspersed cups each with a ∼0.06 liters min^{-1} (1 gallon h^{-1}) pressure compensating dripper (Raindrip). Flow rates from dripper manifolds to replicate cups averaged 0.012 liters min^{-1} (∼8 cycles h^{-1} for 175 ml). Juveniles acclimated under ambient and moderate P_{CO_2} conditions were subjected to a second exposure period (7 days; Fig. 1) in three P_{CO_2} conditions: ambient (754±15 µatm), moderate (2750 ±31 µatm) or severe (4940±45 µatm; Table 1).

Ambient recovery

After the second exposure, P_{CO_2} addition to head tank seawater ceased and all cups returned to ambient conditions (896±11 µatm, Table 1) for 7 days (Fig. 1).

Third exposure

Replicate cups from the second exposure were split (N=72 cups) for subsequent third exposure (7 days; Fig. 1) in two conditions: ambient (967±9 µatm) or moderate P_{CO_2} (3030±23 µatm; Table 1).

Animals were randomly chosen for respiration and growth measurements as described below (N=3 geoducks per cup) and fixed in liquid nitrogen (N=6 geoducks per cup) every 3 days and at the start of every treatment transition, cumulatively as days 1, 4, 7 (second P_{CO_2} exposure), 8, 11, 14 (ambient recovery), 15, 18 and 21 (third P_{CO_2} exposure; Fig. 1). Geoducks were fed *ad libitum* a live mixed-algae diet of *Isocrysis*, *Tetraselmis*, *Chaetoceros* and *Nannochloropsis* throughout the experiment (4–5×10^4 cells ml^{-1}). Live algae cells were flowed into head tanks during the 21-day modified reciprocal exposure at a semi-continuous rate (2.0×10^3 ml h^{-1} per tank) with a programmable dosing pump (Jebao DP-4) to target 5×10^4 live algae cells ml^{-1} in the 175 ml cups.

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Table 1. Seawater carbonate chemistry

Treatment	*N*	Salinity	Temperature	pH (total scale)	CO_2 (μmol kg^{-1})	P_{CO_2} (μatm)	HCO_3 (μmol kg^{-1})	CO_3 (μmol kg^{-1})	DIC (μmol kg^{-1})	Total alkalinity (μmol kg^{-1})	Aragonite saturation state	Calcite saturation state
Primary exposure (110 day conditioning)												
Ambient	27	29.3±0.04	16.8±0.19	7.7±0.02	33.5±1.36	921±40.7	1850±8.09	64.9±2.60	1950±7.63	2010±6.55	1.02±0.04	1.61±0.06
Elevated	24	29.3±0.04	17.3±0.21	7.22±0.01	103±2.24	2870±64.7	1950±5.26	22.9±0.45	2070±6.09	2010±5.29	0.361±0.01	0.568±0.01
Second exposure												
Ambient	33	29.2±0.01	17.6±0.09	7.78±0.01	27±0.57	754±15	1850±4.81	79.1±1.37	1950±4.13	2040±2.26	1.25±0.02	1.96±0.03
Moderate	33	29.2±0.01	17.6±0.09	7.24±0.005	98.1±0.88	2750±31.1	1980±2.21	24.8±0.20	2110±2.5	2040±2.46	0.392±0.003	0.616±0.005
Severe	33	29.2±0.01	17.6±0.09	7±0.004	176±1.58	4940±44.6	2010±1.53	14.2±0.14	2200±2.57	2050±1.77	0.225±0.002	0.353±0.004
Ambient recovery period												
Ambient	80	29.1±0.01	18.2±0.04	7.71±0.005	31.4±0.39	896±10.7	1890±2.93	71.2±0.82	1990±2.5	2060±1.18	1.13±0.01	1.77±0.02
Third exposure												
Ambient	46	29.3±0.01	17.7±0.08	7.68±0.004	34.5±0.37	967±8.95	1920±4.64	66.4±0.61	2020±4.66	2080±4.09	1.05±0.01	1.65±0.02
Moderate	45	29.2±0.02	17.8±0.06	7.21±0.003	108±0.83	3030±22.5	2020±3.28	23.5±0.17	2150±3.76	2080±3.22	0.372±0.003	0.584±0.004

pH, salinity, and temperature measured with handheld probes and total alkalinity (via Gran titration) measured with 60 ml from trays and tanks during the 110 day acclimation period (weekly) and during the 21 day experiment, respectively. Seawater carbonate chemistry (CO_2, P_{CO_2}, HCO_3^-, CO_3^{2-}, DIC, aragonite saturation state and calcite saturation state) was calculated with the SEACARB R package (http://CRAN.R-project.org/package=seacarb).

Large algae batch cultures were counted daily via bright-field image-based analysis (Nexcelom T4 Cellometer) to calculate cell density of 2.5×10^4 live algae cells ml^{-1} in the 250 liter head tanks; the closed-bottom cups retained algae to roughly twice the head tank density and algal density was analyzed in three cups via bright field image-based analysis every 4 days.

Seawater chemistry

Elevated P_{CO_2} levels in head tanks were controlled with a pH-stat system (Neptune Apex Controller System; Putnam et al., 2016) and gas solenoid valves for a target pH of 7.2 for the moderate P_{CO_2} condition and pH of 6.8 for the severe P_{CO_2} condition (pH in NBS scale). pH and temperature (°C) were measured every 10 s by logger probes (Neptune Systems; accuracy: ±0.01 pH units and ±0.1°C; resolution: ±0.1 pH units and ±0.1°C) positioned in header tanks and trays.

Total alkalinity (TA; μmol kg^{-1} seawater) of head tank, tray and cup seawater was sampled in combination with pH (mV) 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^{-1} to 200 mS cm^{-1}; accuracy: ±0.01 psu) and temperature (Fisherbrand Traceable Platinum Ultra-Accurate Digital Thermometer; resolution; 0.001°C; accuracy: ±0.05°C). pH data was assessed on each day with Tris standard (Dickson Lab Tris Standard Batch T27) for quality control and calculation of pH in total scale (Dickson et al., 2007). Carbonate chemistry was recorded weekly for each replicate tray during the 110-day acclimation period and daily during the 21-day experiment for three randomized cups representative of each P_{CO_2} treatment (days 1–7 and 8–15, *N*=9 cups; days 15–21, *N*=6 cups). Additionally, carbonate chemistry of all cups was measured once weekly during each 7 day period (days 1–7 and 8–15, *N*=32 cups; days 15–21, *N*=72 cups). 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, Batches A15 and A16) and TA measurements identified <1% error when compared against certified reference materials (Dickson Lab CO_2 CRM Batch 180). Seawater chemistry was completed following guide to best practices (Dickson et al., 2007); TA and pH measurements were used to calculate carbonate chemistry, CO_2, P_{CO_2}, HCO^{3-}, CO_3, Ω_{arag} and $\Omega_{calcite}$ using the SEACARB package (http://CRAN.R-project.org/package=seacarb) in R v3.5.1 (https://www.r-project.org/).

Respiration rate and shell growth

Respiration rates (oxygen consumption per unit time) were estimated by monitoring oxygen concentration using calibrated optical sensor vials (PreSens, SensorVial SV-PSt5-4ml) on a 24-well plate sensor system (Presens SDR SensorDish). Vials contained three individuals per cup filled with 0.2 μm-filtered seawater from the corresponding treatment head tank. Oxygen consumption from microbial activity was accounted for by including 5-6 vials filled only with 0.2 μm-filtered treatment seawater. Respiration rates were measured in an incubator set at 17°C, with the vials and plate sensor system fixed on a rotator for mixing. Oxygen concentration (μg O_2 l^{-1}) was recorded every 15 s until concentrations declined to ~50-70% saturation (~20 min). Vial seawater volume was measured and clams from each vial were photographed with a size standard (1 mm stage micrometer) to measure shell length (parallel to hinge; mm) using Image J. Respiration rates were calculated using the R package LoLinR (https://github.com/colin-olito/LoLinR) with suggested parameters

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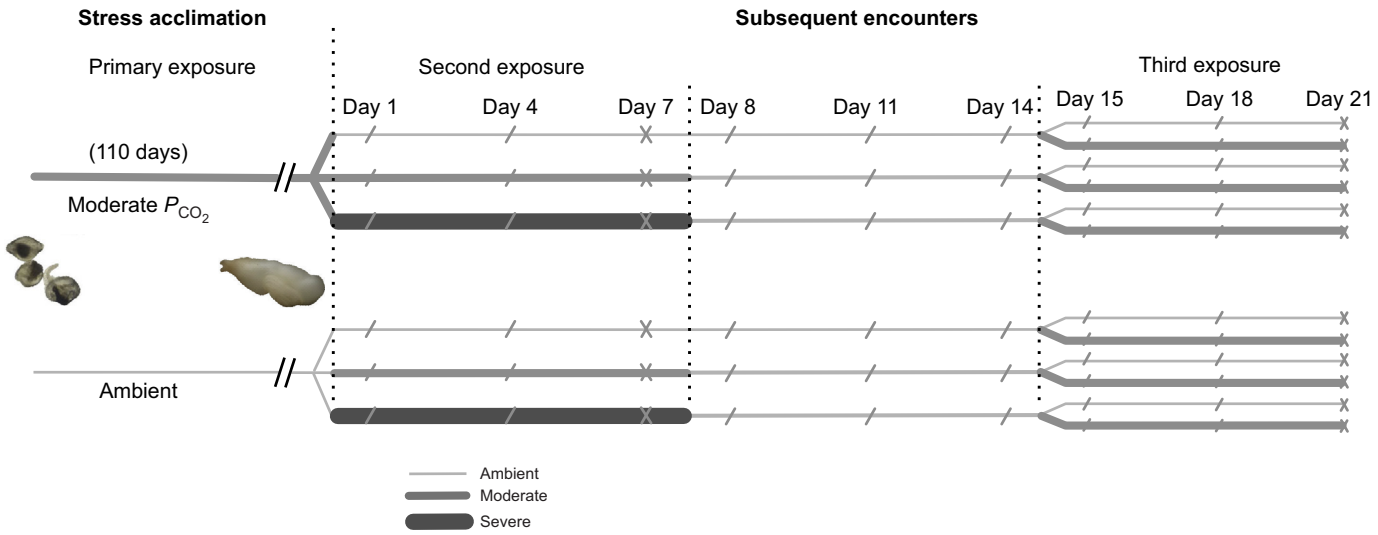

Fig. 1. Schematic of the experimental design. Line width represents the P_{CO_2} treatment during the primary exposure period and subsequent 7 day exposure periods (thin line, ambient P_{CO_2}; mid line, moderate elevated P_{CO_2}; wide line, severe P_{CO_2}). Single dashes '/' indicate sampling days for respiration and shell growth measurements and '×' indicates sampling days for both respiration and growth measurements and fixed tissues for subcellular analysis.

by the package authors (Olito et al., 2017) and following Gurr et al. (2020) with minor adjustments: fixed constants for weighting method ($L_{\%}$) and observations (alpha=0.4) over the full 20 min record. Final respiration rates of juvenile geoduck were corrected for blank vial rates and vial seawater volume (μg O_2 h^{-1} $individual^{-1}$).

Physiological assays

Total antioxidant capacity (TAOC), total protein and ash free dry weight (AFDW; organic biomass) was measured for one animal from each biological tank replicate (N=6 animals per treatment) at the end of the second exposure (total of 36 animals) and at the end of the third exposure (total of 72 animals). Whole animals were homogenized (Pro Scientific) with 300–500 µl cold 1×PBS and total homogenized volume (µl) was recorded. Homogenates were aliquoted for TAOC and total protein assays and the remaining homogenate was used to measure organic biomass. TAOC was measured in duplicate as the reduction capacity of copper reducing equivalents (CRE) following the Oxiselect™ microplate protocol (STA-360) and standardized for volume and to the total protein content of the tissue lysate samples of the same individual (µmoles CRE mg $protein^{-1}$). Sample aliquots for total protein were solubilized by adding 10 µl 1 mol l^{-1} NaOH preceding incubation at 50°C and 800 RPM for 4 h and neutralized with 0.1 mol l^{-1} HCl (pH 7). Total protein of tissue lysate samples was measured using the Pierce Rapid Gold assay with bovine serum albumin following the Pierce™ microplate protocol (A53225). Total protein (mg) was standardized to organic biomass (mg protein mg AFDW) following ignition (4.5 h at 450°C) subtracted by the dry weight (24 h at 75°C) and corrected for total homogenate volume.

Statistical analysis

Welch's t-tests for unequal variances were used to analyze the effect of the primary exposure, or initial 110-day P_{CO_2} acclimation period (fixed), on respiration rate and shell length prior to the 21-day exposure period. Over the 21 day exposure, respiration rate and shell size were assessed with ANOVA based on linear mixed effects (LMEs) to analyze the fixed effects of P_{CO_2} treatments and random effect of time during the second P_{CO_2} exposure, ambient recovery, and third P_{CO_2} exposure periods (days 1–7, 8–14 and 14–21, respectively). Total antioxidant capacity, total protein, and organic biomass from samples on day 7 and day 21 were analyzed for effects of P_{CO_2} treatments (fixed) with two-way and three-way ANOVAs, respectively. In all cases, normality assumptions were tested 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 (Brown and Forsythe, 1974). Results of three-way ANOVAs on day 21 total protein and day 21 organic biomass were robust to outlier removal and transformation(s) that resolved normality via Shapiro-Wilk test. A pairwise Tukey's *a posteriori* Honestly Significant Difference test was applied to significant model effects. All data analysis was completed using R (v3.5.1; https://www.r-project.org/).

RESULTS

Stress acclimation, second exposure to hypercapnic seawater and ambient recovery

There was no difference in respiration rate after 110 days of P_{CO_2} acclimation (Table S1; Welch's t-test; primary, t=−0.602, d.f.=31.725, P=0.5516); however, the shell length of geoducks under moderate P_{CO_2} was significantly larger, by 2.6%, compared with those under ambient treatment (Table S1; Welch's t-test; primary, t=−4.297, d.f.=2884, P<0.0001). Under the second exposure, there was no significant effect of P_{CO_2} treatments on respiration rate and shell length. Juvenile clams acclimated under moderate P_{CO_2} on average had significantly greater organic biomass (two-way ANOVA; primary, $F_{1,30}$=9.313, P=0.0047) at the end of the second exposure period (day 7) with 39% greater individual mg tissue AFDW compared with animals reared under ambient conditions (Table S2 and Fig. 2). There was no significant effect from the primary or second P_{CO_2} treatments on total protein or TAOC (Table S2 and Fig. 2). During ambient recovery, respiration rate and shell length were not significantly affected by the primary or second P_{CO_2} treatments (Table S1).

Third exposure to hypercapnic seawater

The interaction of primary and second P_{CO_2} treatments had a significant effect on respiration rate under the third exposure period (Table S1; LME; primary×second, $F_{2,198}$=3.810, P=0.024), with this

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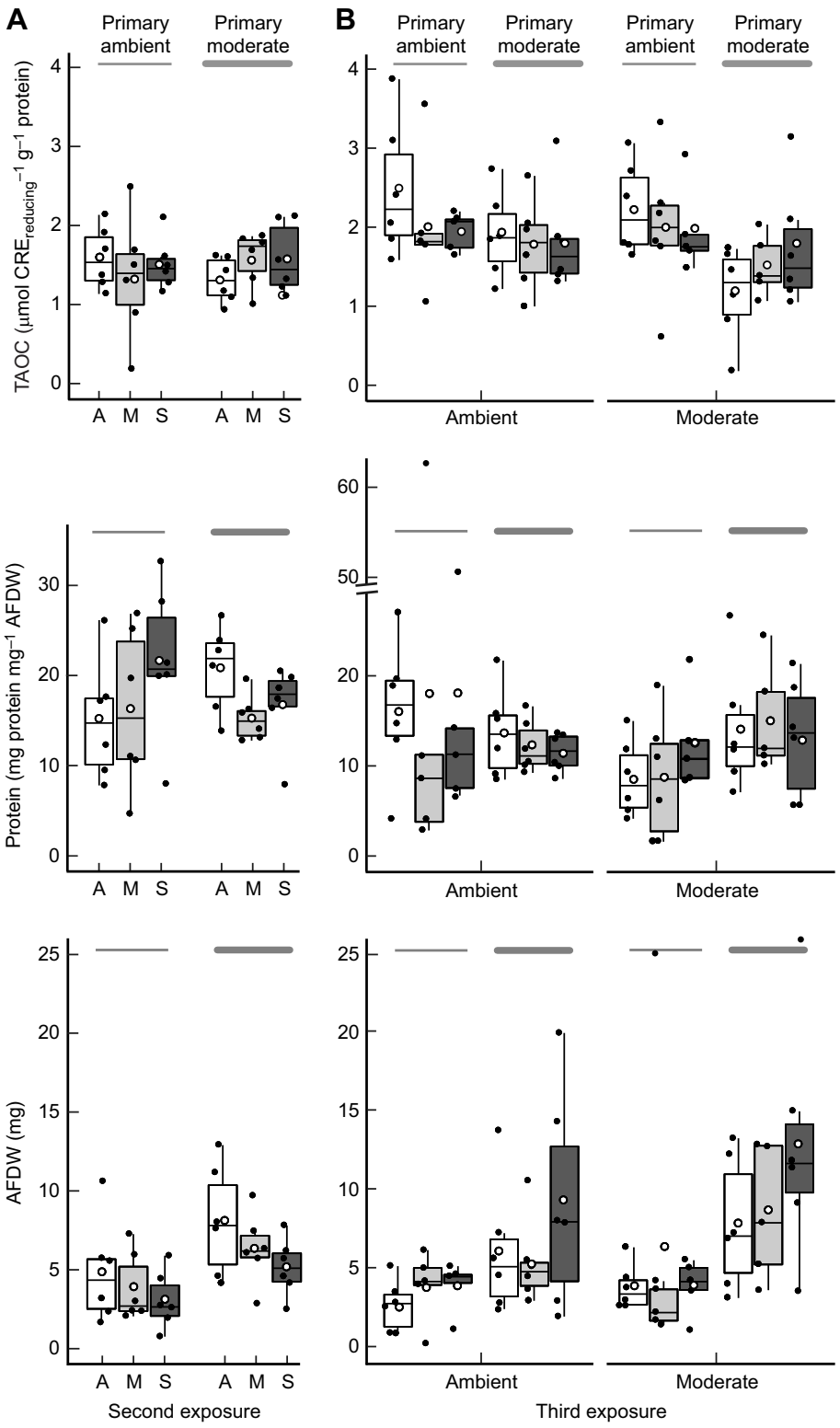

Fig. 2. Antioxidant response and physiology of fixed Pacific geoduck (*Panopea generosa*) at the end of second and third exposure periods. Box plots show the 25th and 75th percentiles (boxes), 1.5× interquartile range (whiskers), median (horizontal line) and mean (white circles) for TAOC (top), protein (middle) and AFDW (bottom) for (A) second exposure and (B) third exposure period. Black circles represent individual measurements. Shading represents the three P_{CO_2} treatments during the second exposure period [white, ambient (A); grey, moderate elevated P_{CO_2} (M); dark grey, severe elevated P_{CO_2} (S)]. Solid horizontal lines indicate the primary treatment history in ambient and moderate P_{CO_2}.

interaction primarily driven by a 20.4% greater respiration rate in P_{CO_2} stress-acclimated animals exposed to severe P_{CO_2} than ambient P_{CO_2} during the second period (Fig. 3), although the *post hoc* test was only marginally significant (Tukey HSD; moderate×severe> moderate×ambient, P=0.0992). Shell growth was affected by an interaction between primary, second and third P_{CO_2} treatments (Table S1 and Fig. 3; four-way ANOVA; primary×second×third, $F_{2,628}$=6.360, P=0.002). Pairwise differences of the three-way treatment interaction showed 9.3% greater mean shell size by acclimated animals with a second and third exposure to severe and moderate P_{CO_2}, respectively (Fig. 3). At the end of the third exposure period (day 21), primary exposure under moderate P_{CO_2} increased organic biomass (Table S2; three-way ANOVA; primary, $F_{1,56}$=12.899, P<0.001) with 51% greater AFDW under stress treatment relative to ambient controls (Fig. 2). There was a significant effect of primary exposure on antioxidant activity (Table S2; three-

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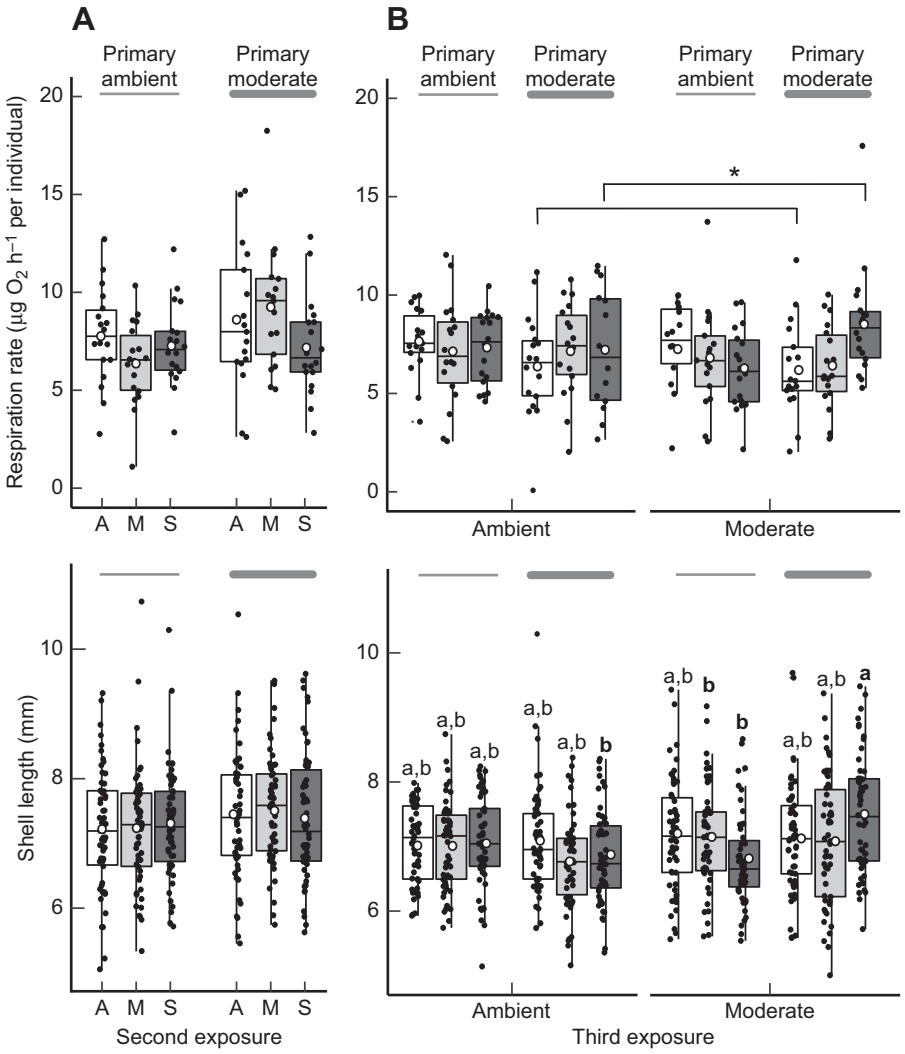

Fig. 3. Respiration rate and shell length of geoducks under second and third exposure periods. Box plots show the 25th and 75th percentiles (boxes), 1.5× interquartile range (whiskers), median (horizontal line) and mean (white circles) for respiration rates in (A) second exposure and (B) third exposure period. Black circles represent individual measurements. Shading represents the three P_{CO_2} treatments during the second exposure period [white, ambient (A); grey, moderate elevated P_{CO_2} (M); dark grey, severe elevated P_{CO_2} (S)]. Solid horizontal lines indicate primary treatment history under ambient and moderate P_{CO_2}. Significant *a posteriori* effects are shown as letters or asterisks.

way ANOVA; primary, $F_{1,56}$=8.069, P=0.0063) with 22% greater µmol CRE_{red} g^{-1} protein by clams reared under ambient P_{CO_2} (Fig. 2); there was no effect of P_{CO_2} treatment or two-way and three-way interactions of P_{CO_2} treatments on total protein Table S2 and Fig. 2). The effects of P_{CO_2} on survival over the 21-day exposure period was negligible as there were no observed cases of mortality.

DISCUSSION

In the present study we evaluated the effects of post-larval stress acclimation and subsequent exposures to elevated P_{CO_2} on the physiological and biochemical stress response in juvenile geoduck. Our findings suggest moderate hypercapnic conditions during post-larval development improve metrics of physiological performance and CSR. This novel investigation of beneficial effects of early-life stress demonstrates a high tolerance to P_{CO_2} regimes (~2500–5000 µatm) and plasticity of bioenergetic and subcellular responses in *P. generosa*.

Stress-intensity- and life-stage-dependent effects

Survival under long-term stress exposure and positive physiological responses of acclimated animals under 'moderate' (~2900 µatm P_{CO_2} 0.4 Ω_{arg}) and 'severe' (~4800 µatm P_{CO_2} 0.2 Ω_{arg}) reciprocal exposures highlights the resilience of *P. generosa* to OA and suggests that stress acclimation can induce beneficial effects during post-larval to juvenile development. Specifically, clams repeatedly exposed to the greatest intensity of stress (moderate×severe×moderate) had both greater respiration rates and shell size (Table S1; Fig. 3). Furthermore, stress-acclimated individuals had greater organic biomass and lower amounts of antioxidant proteins relative to ambient controls (Fig. 2), suggesting optimized tissue accretion and energy partitioning, coupled with decreased costs for cytoprotection. Previous studies describe metabolic compensation and regulation of CSR during hypercapnia as attributes of a well-adapted stress response to control acid–base status and normal development/metamorphosis (Walsh and Milligan, 1989; Dineshram et al., 2015). Indeed, prior work on juvenile *P. generosa* also demonstrates positive acclimatory carryover effects, with increased shell length and metabolic rate after repeat exposures to hypercapnic and undersaturated conditions with respect to aragonite (Gurr et al., 2020). Contrary to our findings, similar P_{CO_2} and Ω_{arg} levels decrease metabolic rate and scope for growth in the mussel *Mytilus chilensis* (Navarro et al., 2013), cause a three-fold increase in mortality rate in juvenile hard clam *Mercenaria mercenaria* (Green et al., 2009), and alter metamorphosis and juvenile burrowing behavior in *Panopea japonica* (Huo et al., 2019). Thus, P_{CO_2} tolerance limitations are likely species specific, as well as life stage, duration and stress-intensity specific.

P_{CO_2}-induced phenotypic variation over post-larval to juvenile development observed in this study suggests postlarval stages may

be optimal for stress acclimation. A growing body of research posits an adaptive role of early life as a 'programming window' owing to the importance of environmental information in setting the stage for subsequent phenotypic outcomes (Fawcett and Frankenhuis, 2015). Beneficial carryover effects in the present study are also corroborated by compensatory physiology and differential DNA methylation of juvenile *P. generosa* in other studies (Putnam et al., 2017; Gurr et al., 2020). In contrast, OA can have deleterious effects on growth/development, settlement and proteomic composition of larval *P. generosa* (Timmins-Schiffman et al., 2019), further emphasizing the life-stage dependence of P_{CO_2} stress exposure. Mollusc larvae are widely established to have enhanced susceptibility to OA with impacts on shell growth and developmental transition (Kurihara et al., 2007; Kapsenberg et al., 2018). For example, larval exposure to elevated P_{CO_2} leads to persistent negative effects (i.e. reduced shell growth and development) in Pacific oyster *Crassostreas gigas*, Olympia oyster *Ostrea lurida* and bay scallop *Argopecten irradians* (Barton et al., 2012; Hettinger et al., 2012; White et al., 2013). Beneficial responses to OA are also possible, especially in longer term and carryover-effect studies (Parker et al., 2015). For example, elevated P_{CO_2} during gametogenesis in the Chilean mussel *Mytilus chilensis* (Diaz et al., 2018) and Sydney rock oyster *Saccostrea glomerata* (Parker et al., 2012) increases the size of larval stages in progeny. Future comparative studies should test molluscs resilient and susceptible to environmental stressors to determine if these associations are impacted by early-life stress acclimation and subsequent stress encounters post-settlement. Further, the adaptive or maladaptive implications of early-life hormetic priming likely depend on the stress type and intensity experienced later in life, demanding long-term investigations under matched/mismatched environments (Costantini et al., 2014).

Our observation of beneficial effects in stress-acclimated clams suggests an adaptive resilience of *P. generosa* to hypercapnic conditions relevant to post-larval to juvenile development in both natural and aquaculture systems. P_{CO_2} and Ω_{arg} gradients naturally occur alongside the developmental transition from free-swimming larvae to sessile benthic juveniles suggesting *P. generosa* may be capable of adaptive resilience particularly during this life stage. Furthermore, habitat within the native range of *P. generosa* exhibits elevated P_{CO_2} and aragonite undersaturation with episodic/seasonal variation (surface water $\Omega_{arg}<1$ in winter months, Dabob Bay in Hood Canal, WA; Fassbender et al., 2018) and geographical (>2400 µatm and $\Omega_{arg}<0.4$ in Hood Canal, WA; Feely et al., 2010) and vertical heterogeneity (Reum et al., 2014) comparable to gradients within sub-surface sediments (Ω_{arg} 0.4–0.6; Green et al., 2009). Therefore, the population of adult broodstock spawned in this experiment may be better suited for a low-pH environment. Relevant to aquaculture, the findings and experimental timing of this study suggest that postlarval 'settlement' is an ecologically relevant life stage to investigate stress conditioning.

Oxidative status and repeated stress encounters

Our results herein demonstrate activation of phenotypic variation after repeated stress encounters suggesting post-larval acclimation may have a critical role in subsequent stress response. A low-dose stimulatory effect of oxidative stress is well characterized (i.e. under calorie restriction, hypoxia and exercise; Ristow and Schmeisser, 2014) for a wide range of taxa (Costantini et al., 2012; Visser et al., 2018; Zhang et al., 2018), but remains poorly understood in response to OA conditions. Here, we posit that hormetic priming can be both stress-intensity and life-stage dependent affecting physiology and total antioxidant capacity over subsequent stress encounters; however, further research is required to determine the role of oxidative stress in this process (i.e. oxidative damage, ROS signaling pathways, etc.).

Intermittent oxidative stress may have evolutionary importance in stress resilience of long-lived marine bivalves. The ocean quahog *Arctica islandica* is the oldest known non-colonial animal; their substantial longevity is hypothesized to be driven by intermittent metabolic-quiescence (dormancy when burrowed) demanding resilience to ROS overproduction (oxidative bursts) and resistance to cell death upon subsequent aerobic recovery (Abele et al., 2008). Interestingly, *A. islandica* have lipids with low sensitivity to peroxidation (Munro and Blier, 2012) and high baseline antioxidant capacity throughout their lifespan suggesting an adaptive resilience to oxidative damage (Abele et al., 2008). The lower antioxidant production by stress-conditioned *P. generosa* in the present study could suggest adaptive subcellular mechanism(s) that differ from other long-lived bivalves but may similarly function in maintaining homeostasis under frequent or intermittent stress exposures.

Effects of stress acclimation on antioxidant capacity and performance of *P. generosa* infers potential subcellular and mitochondrial pathways and the need for a mechanistic understanding of the role of oxidative stress. Furthermore, pre-emptive frontloading of stress-related transcripts can promote stress resilience (Barshis et al., 2013), but remains poorly understood in response to hormetic priming. Alternative oxidase is a regulatory mitochondrial pathway in bivalves that permits ATP synthesis and reduces ROS production during stress (Tschischka et al., 2000; Sussarellu et al., 2013; Yusseppone et al., 2018) and frontloading of genes in this pathway could enhance tolerance. Further experiments are needed to elucidate molecular mechanisms of adaptive phenotype variation in response to hormetic priming.

Conclusion

Post-larval acclimation under moderate hypercapnia can elicit beneficial phenotypes under subsequent stress encounters. This acclimatory capacity is likely contingent on stress intensity (i.e. magnitude, duration, frequency of stress periods) and timing during post-larval settlement and juvenile development. Thus, investigations of marine species responses to climate change should consider adaptive dose-dependent regulation and effects post-acclimation (i.e. carryover). A holistic understanding of cellular and molecular mechanisms can advance understanding of hormetic priming and provide additional 'climate-proofing' strategies in aquaculture and conservation of goods and services in the Anthropocene.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.J.G., S.A.W., B.V., S.B.R., H.M.P.; Methodology: S.J.G., H.M.P.; Formal analysis: S.J.G., H.M.P.; Investigation: S.J.G., S.A.W.; Resources: B.V., S.B.R., H.M.P.; Writing - original draft: S.J.G., S.A.W., B.V., S.B.R., H.M.P.; Writing - review & editing: S.J.G., S.A.W., B.V., S.B.R., H.M.P.; Visualization: S.J.G.; Supervision: B.V., S.B.R., H.M.P.; Project administration: B.V., S.B.R., H.M.P.; Funding acquisition: B.V., S.B.R., H.M.P.

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Data availability
All raw data and statistical code are openly available in the Zenodo repository at: http://doi.org/10.5281/zenodo.3903019.

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