REPEAT EXPOSURE TO HYPERCAPNIC SEAWATER MODIFIES PERFORMANCE AND OXIDATIVE STATUS IN A TOLERANT BURROWING CLAM

Running title: Repeat $p\text{CO}_2$ stress and geoduck response

Samuel J. Gurr^{1*}, Shelly A. Trigg³, Brent Vadopalas², Steven B. Roberts³, Hollie M. Putnam¹

¹University of Rhode Island, College of the Environment and Life Sciences, 120 Flagg Rd, Kingston, RI 02881 USA

²University of Washington, Washington Sea Grant, 3716 Brooklyn Ave NE, Seattle, WA 98105 USA

³University of Washington, School of Aquatic and Fishery Sciences, 1122 NE Boat St, Seattle, WA 98105 USA

Corresponding author: Samuel J. Gurr; 120 Flagg Rd, Kingston, RI 02881 USA, samuel_gurr@uri.edu

Keywords: hormesis, ocean acidification, oxidative stress, phenotypic variation, stress acclimation, geoduck

Summary statement: Hypercapnic conditions during postlarval development improves physiological performance and oxidative status. This novel investigation of adaptive stress acclimation highlights the plasticity of bioenergetic and subcellular responses in *Panopea generosa*.

Abstract

Moderate oxidative stress is a hypothesized driver of enhanced stress tolerance and lifespan. Whereas thermal stress, irradiance, and dietary restriction show evidence of dose-dependent benefits for many taxa, stress acclimation remains understudied in marine invertebrates, despite being threatened by climate change stressors such as ocean acidification. To test for life-stage and stress-intensity dependence in eliciting enhanced tolerance under subsequent stress encounters, we initially conditioned pediveliger Pacific geoduck (*Panopea generosa*) larvae to (i) ambient and moderately elevated $p\text{CO}_2$ (920 μatm and 2800 μatm , respectively) for 110 days, (ii) secondarily applied a 7-day exposure to ambient, moderate, and severely elevated $p\text{CO}_2$ (750 μatm , 2800 μatm , and 4900 μatm , respectively), followed by 7 days in ambient conditions, and (iii) implemented a modified-reciprocal 7-day tertiary exposure to ambient (970 μatm) and moderate $p\text{CO}_2$ (3000 μatm). Initial conditioning to moderate $p\text{CO}_2$ stress followed by secondary and tertiary exposure to severe and moderate $p\text{CO}_2$ 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 to clams under initial ambient conditions, supporting the hypothesis that stress over postlarval-to-juvenile development affects oxidative status later in life. We posit two subcellular mechanisms underpinning stress-intensity-dependent effects on mitochondrial pathways and energy partitioning: i) stress-induced attenuation of mitochondrial function and ii) adaptive mitochondrial shift under moderate stress. Time series and stress intensity-specific approaches can reveal life-stages and magnitudes of exposure, respectively, that may elicit beneficial phenotypic variation.

1. Introduction

Ocean acidification (OA), or the decrease of oceanic pH, carbonate ion concentration, and aragonite saturation (Ω) due to elevated atmospheric partial pressures ($p\text{CO}_2$), 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).

Principles in ectotherm physiology (i.e. oxygen capacity-limited thermal tolerance: Pörtner, 2012; energy-limited tolerance to stress: Sokolova, 2013) describe aerobic performance “windows” under ‘optimum’, ‘pejus’ (moderate), and ‘pessimum’ (severe) environmental ranges defined by cellular and physiological modifications affecting energy homeostasis (Sokolova et al., 2012). 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 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 Frankenhuys, 2015) prompting investigation of environmental stress acclimation under a rapidly changing environment.

Dose-dependent stress response, or conditioning-hormesis (Calabrese et al., 2007), explains how stress priming can enhance tolerance limitations under subsequent encounters to similar or higher levels of stress intensity later in life (Costantini, 2014). Oxidative stress presents a common source of conditioning-hormesis (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, or an over-production of reactive oxygen species (ROS; superoxide, hydrogen peroxide, hydroxyl radical) primarily from mitochondrial oxidative phosphorylation, causes macromolecular damage. 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 ameliorate 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) due to a lifestyle of metabolic dormancy (when burrowed) and aerobic recovery (Abele et al., 2008). Further, Ivanina and Sokolova (Ivanina and Sokolova, 2016) found hypoxia-tolerant marine bivalves show anticipatory and compensatory upregulation of antioxidant proteins to mitigate oxidative bursts under hypoxia-reoxygenation. Such adaptive responses have yet to be explored under hypercapnic conditions to identify species tolerant to OA stress. Although bivalves are known to exhibit $p\text{CO}_2$ -induced oxidative damage and upregulated CSR (Tomanek et al., 2011; Matoo et al., 2013), studies have yet to investigate ROS-mediated activity in a hormetic framework (repeated dose exposures).

Pacific geoduck (*Panopea generosa*) are burrowing clams of ecological (Goodwin and Pease, 1987) and economic importance (Shamshak and King, 2015) and are a great candidate for investigating a hormetic framework for generation of stress-acclimated phenotypes. Juvenile geoduck have shown positive carryover effects after exposures under high $p\text{CO}_2$ /low Ω 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., 2020). The postlarval life stage presents an ecologically relevant and less susceptible window to investigate effects of $p\text{CO}_2$ 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 aragonite saturation and oxygen (Cai

et al., 2011). To investigate thresholds of hormesis and potential for beneficial stress acclimation, we investigated the effects of $p\text{CO}_2$ exposures of different intensity and at different time points in a repeated reciprocal fashion, on the physiological and subcellular phenotypes of juvenile Pacific geoduck.

2. Materials and Methods

2.1 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). Ambient seawater temperature, salinity, pH, and $p\text{CO}_2$ was 16-18 °C, 29 ppt, 7.7-7.8 pH, and ~800-950 μatm , respectively. 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-L 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.

2.2 Acclimation from pediveligers to juveniles

Initial Exposure (110 days): Pediveligers were placed into ambient and elevated $p\text{CO}_2$ conditions ($921 \pm 41 \mu\text{atm}$ and $2870 \pm 65 \mu\text{atm}$; Table 1; Fig. 1) for an initial exposure during the transition from pediveliger to the burrowing juvenile stage ($N=4$ trays treatment⁻¹; $N=1.5 \times 10^4$ pediveligers tray⁻¹). Seawater flowed into 250-L head tanks at a rate of 0.1 L min⁻¹ and replicate trays were gravity-fed from the head tanks. At the end of the initial exposure, respiration rate and shell growth was measured for 20 randomly selected juveniles from each of the eight trays as described below. Additionally, six animals from each tray were frozen in liquid nitrogen and stored at -80 °C for molecular analyses.

Table 1. Seawater carbonate chemistry. 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 , $p\text{CO}_2$, HCO_3^- , CO_3^{2-} , DIC, aragonite saturation state) was calculated with the seacarb R package (Gattuso et al., 2018).

Treatment	N	Salinity	Temperature	pH, Total Scale	CO_2 $\mu\text{mol kg}^{-1}$	$p\text{CO}_2$ μatm	HCO_3^- $\mu\text{mol kg}^{-1}$	CO_3^{2-} $\mu\text{mol kg}^{-1}$	DIC $\mu\text{mol kg}^{-1}$	Total Alkalinity $\mu\text{mol kg}^{-1}$	Aragonite Saturation state
Initial exposure (3-month conditioning)											
Ambient	27	29.3 ± 0.0426	16.8 ± 0.192	7.7 ± 0.0187	33.5 ± 1.36	921 ± 40.7	1850 ± 8.09	64.9 ± 2.6	1950 ± 7.63	2010 ± 6.55	1.02 ± 0.0405
Elevated	24	29.3 ± 0.0448	17.3 ± 0.205	7.22 ± 0.00921	103 ± 2.24	2870 ± 64.7	1950 ± 5.26	22.9 ± 0.454	2070 ± 6.09	2010 ± 5.29	0.361 ± 0.00722
Secondary exposure											
Ambient	33	29.2 ± 0.009	17.6 ± 0.087	7.78 ± 0.00777	27 ± 0.57	754 ± 15	1850 ± 4.81	79.1 ± 1.37	1950 ± 4.13	2040 ± 2.26	1.25 ± 0.0217
Moderate	33	29.2 ± 0.0138	17.6 ± 0.0895	7.24 ± 0.00467	98.1 ± 0.881	2750 ± 31.1	1980 ± 2.21	24.8 ± 0.195	2110 ± 2.5	2040 ± 2.46	0.392 ± 0.00295
Severe	33	29.2 ± 0.00833	17.6 ± 0.0862	7 ± 0.00416	176 ± 1.58	4940 ± 44.6	2010 ± 1.53	14.2 ± 0.143	2200 ± 2.57	2050 ± 1.77	0.225 ± 0.00228
Ambient recovery period											
Ambient	80	29.1 ± 0.0102	18.2 ± 0.0428	7.71 ± 0.00499	31.4 ± 0.392	896 ± 10.7	1890 ± 2.93	71.2 ± 0.822	1990 ± 2.5	2060 ± 1.18	1.13 ± 0.0132
Tertiary exposure											
Ambient	46	29.3 ± 0.0138	17.7 ± 0.0779	7.68 ± 0.00364	34.5 ± 0.368	967 ± 8.95	1920 ± 4.64	66.4 ± 0.609	2020 ± 4.66	2080 ± 4.09	1.05 ± 0.01
Moderate	45	29.2 ± 0.0156	17.8 ± 0.0596	7.21 ± 0.00311	108 ± 0.832	3030 ± 22.5	2020 ± 3.28	23.5 ± 0.165	2150 ± 3.76	2080 ± 3.22	0.372 ± 0.00262

Fig. 1. Schematic of the experimental design. Line color and shape refers to the $p\text{CO}_2$ treatments during the initial 110-day acclimation period (blue triangles, ambient $p\text{CO}_2$; red circles, elevated $p\text{CO}_2$). Shading is in reference to the secondary exposure conditions (light, ambient $p\text{CO}_2$; medium, moderate $p\text{CO}_2$; dark, severe $p\text{CO}_2$). Tertiary exposure conditions, following a 7-day ambient recovery period, are indicated by shape and line type (empty and solid, ambient $p\text{CO}_2$; filled and dashed, moderate $p\text{CO}_2$). Points indicate sampling days for respiration and shell growth measurements and fixed tissues.

2.3 Modified reciprocal exposure

Secondary Exposure:

To begin the secondary exposure, juvenile geoducks (~2,200 geoduck initial $p\text{CO}_2$ treatment⁻¹) were rinsed on a $3 \times 10^5 \mu\text{m}$ screen to isolate individuals and were divided equally in 36 175-ml plastic cups ($N=120$ animals cup⁻¹, $N=6$ cups treatment⁻¹) each with 50 ml rinsed sand (450-550 μm grain size). Seawater flowed into 250-L head tanks at a rate of 0.6 L min^{-1} and was pumped using submersible pumps to randomly interspersed cups each with a 1 gallon hour⁻¹ pressure compensating dripper (Raindrip). Flow rates from dripper manifolds to replicate cups averaged $0.012 \text{ liters minute}^{-1}$ (~8 cycles hour⁻¹ for 175-ml). Juveniles acclimated under ambient and elevated $p\text{CO}_2$ conditions were subjected to a secondary exposure period (7 days; Fig. 1) in three $p\text{CO}_2$ conditions: ambient ($754 \pm 15 \mu\text{atm}$), moderate ($2750 \pm 31 \mu\text{atm}$), and severe ($4940 \pm 45 \mu\text{atm}$; Table 1).

Ambient Recovery:

After secondary exposure, $p\text{CO}_2$ addition to head tank seawater ceased and all cups returned to ambient conditions ($896 \pm 11 \mu\text{atm}$, Table 1) for 7 days (Fig. 1).

Tertiary Exposure:

Replicate cups from the secondary exposure were split ($N=72$ cups) for subsequent tertiary exposure (7 days; Fig. 1) in two conditions: ambient ($967 \pm 9 \mu\text{atm}$) and moderate $p\text{CO}_2$ ($3030 \pm 23 \mu\text{atm}$; Table 1).

Animals were randomly chosen for respiration and growth measurements as described below ($N=3$ geoduck cup⁻¹) and fixed in liquid nitrogen ($N=6$ geoduck cup⁻¹) every three days and at the start of every treatment transition, cumulatively as days 1, 4, 7 (secondary $p\text{CO}_2$ exposure), 8, 11, 14 (ambient recovery), 15, 18, and 21 (tertiary $p\text{CO}_2$ exposure; Fig. 1). Geoduck were fed *ad libitum* a live mixed-algae diet of *Isocrysis*, *Tetraselmis*, *Chaetoceros*, and *Nano* throughout the experiment ($4\text{--}5 \times 10^4$ 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 hr⁻¹ tank⁻¹) with a programmable dosing pump (Jebao DP-4) to target 5×10^4 live algae cells ml⁻¹ in the 175-ml cups. Large algae batch cultures were counted daily via bright-field image-based analysis (Nexcelom T4 Cellometer; (Gurr et al., 2018) to calculate cell density of 2.5×10^4 live algae cells ml⁻¹ in the 250-L 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 four days.

2.4 Seawater chemistry

Elevated $p\text{CO}_2$ 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 elevated and moderate $p\text{CO}_2$ condition and pH of 6.8 for the severe $p\text{CO}_2$ condition (pH in NBS scale). pH and temperature (°C) were measured every 10 seconds 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; $\mu\text{mol kg}^{-1}$ seawater) of head tank, tray, and cup seawater was sampled in combination with pH (total scale) 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 $\mu\text{S/cm}$ - 200 mS/cm; accuracy: ± 0.01 psu), and temperature (Fisherbrand Traceable Platinum Ultra-Accurate Digital Thermometer; resolution: 0.001 °C; accuracy: ± 0.05 °C). Quality control for pH data was assessed on each day with Tris standard (Dickson Lab Tris Standard Batch T27). 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 $p\text{CO}_2$ 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 ($\sim 0.1 \text{ mol kg}^{-1}$, $\sim 0.6 \text{ mol kg}^{-1}$ 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 , $p\text{CO}_2$, HCO_3^- , CO_3 , and Ω_{arag} , using the SEACARB package (Gattuso et al., 2018) in R v3.5.1 (R Core Team, 2018).

2.5 Respiration rate and shell growth

Respiration rates (oxygen consumption per unit time) were estimated by monitoring oxygen concentration over time 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 $0.2 \mu\text{m}$ -filtered seawater from the corresponding treatment head tank. Oxygen consumption from microbial activity was accounted for by including 5-6 vials filled only with $0.2 \mu\text{m}$ -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 ($\mu\text{g O}_2 \text{ L}^{-1}$) was recorded every 15 seconds until concentrations declined to $\sim 50\text{-}70\%$ saturation (~ 20 minutes). 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 with suggested parameters 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-minute record. Final respiration rates of juvenile geoduck were corrected for blank vial rates and vial seawater volume ($\mu\text{g O}_2 \text{ hr}^{-1} \text{ individual}^{-1}$).

2.6 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 treatment¹) at the end of secondary exposure (36 total animals) and at the end of tertiary exposure (72 total animals). Whole animals were homogenized (Pro Scientific) with 300-500 μl cold $1\times\text{PBS}$ 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 OxiselectTM microplate protocol (STA-360) and standardized to the total protein content of the tissue lysate samples of the same individual ($\mu\text{M CRE mg protein}^{-1}$). Sample aliquots for total protein were solubilized by adding 10 μl 1 M NaOH preceding incubation at 50°C and 800 RPM for 4 hours and neutralized with 0.1 M HCl (pH 7). Total protein of tissue lysate samples was measured using the Pierce Rapid Gold assay with bovine serum albumin following the PierceTM microplate protocol (A53225). Total protein (mg) was standardized to organic biomass (mg protein mg AFDW) following ignition (4.5 hours at 450°C) subtracted by the dry weight (24 hrs at 75°C) and corrected for total homogenate volume.

2.7 Statistical Analysis

Welch's t-tests for unequal variances were used to analyze the effect of the initial 110-day $p\text{CO}_2$ acclimation (fixed) on respiration rate and shell length prior to the 21-day exposure period. A three-way analysis of variance (ANOVA) was used to analyze the effect of time (fixed) and initial and secondary $p\text{CO}_2$ exposures (fixed) on respiration rate and shell growth for both the secondary exposure and ambient recovery periods (days 1-7 and 8-14, respectively). A four-way ANOVA was used to analyze the effect of time (fixed) and initial, secondary, and tertiary $p\text{CO}_2$ exposures (fixed) on respiration rate and shell growth during the tertiary exposure period (days 15-21). Total antioxidant capacity, total protein, and organic biomass from samples on day 7 and day 21 were analyzed for effects of $p\text{CO}_2$ 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). 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; R Core Team, 2018).

3. Results

3.1 Initial stress acclimation and secondary exposure to hypercapnic seawater

There was no difference in respiration rate after 110 days of $p\text{CO}_2$ acclimation (Table 2; Welch's t-test; initial, $t=-0.602$, $df=31.725$, $P=0.5516$), however the shell length of geoduck under

elevated $p\text{CO}_2$ was significantly larger, by 2.6%, compared to those under ambient treatment (Table 2; Welch's t-test; initial, $t=-4.297$, $df=2884$, $P<0.0001$). Under subsequent secondary exposure, the initial stress acclimation had a marginal effect on respiration rate (three-way ANOVA; initial, $F_{1,89}=3.409$, $P=0.068$) with 12.4% greater respiration rates in animals under elevated $p\text{CO}_2$ compared to animals under ambient conditions (Table 2). This effect was primarily driven by a marginal interaction effect under secondary $p\text{CO}_2$ treatment (three-way ANOVA; initial $\times$ secondary, $F_{2,89}=2.824$, $P=0.0647$), with 31% greater respiration rates under moderate $p\text{CO}_2$ stress in animals acclimated under elevated $p\text{CO}_2$ (Fig. 2a). Shell length increased significantly with time (three-way ANOVA; time, $F_{2,306}=3.347$, $P=0.0365$; Tukey HSD, day 7 > day 4, $P=0.0236$) and independent of $p\text{CO}_2$ treatments (Table 2). Juvenile clams acclimated under elevated $p\text{CO}_2$ on average had significantly greater organic biomass (two-way ANOVA; initial, $F_{1,30}=9.313$, $P=0.0047$) at the end of the secondary exposure period (day 7) with 39% greater mg tissue AFDW individual⁻¹ compared to animals reared under ambient conditions (Table 3 and Fig. 3c). There was no significant effect from initial or secondary $p\text{CO}_2$ treatments on total protein or TAOC (Table 3 and Figs. 3a and 3b).

Table 2. Effects of $p\text{CO}_2$ stress exposures on mean respiration rate and shell growth of *Panopea generosa*. A Welch's t-test tested for effects of $p\text{CO}_2$ stress acclimation prior to the 21-day experiment. Three-way and four-way ANOVA tests were used under the secondary and tertiary exposure periods and a three-way ANOVA tested for treatment effects during the ambient recovery period. Significant effects are bolded for $P < 0.05$.

Effect	Respiration rate			Shell length		
	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>
Pre-experiment	<i>t-test</i>					
$p\text{CO}_2$ initial	31.725	-	0.5516	2884	-	<0.0001
Days 1-7	<i>Three-way ANOVA</i>					
$p\text{CO}_2$ initial	1,89	3.409	0.0681	1,306	3.442	0.0645
$p\text{CO}_2$ secondary	2,89	0.584	0.5596	2,306	0.137	0.8722
time	2,89	1.450	0.2400	2,306	3.447	0.0331
$p\text{CO}_2$ initial $\times$ $p\text{CO}_2$ secondary	2,89	2.824	0.0647	2,306	0.235	0.7909
$p\text{CO}_2$ initial $\times$ time	2,89	1.155	0.3199	2,306	0.018	0.9822
$p\text{CO}_2$ secondary $\times$ time	4,89	1.486	0.2131	4,306	0.145	0.9650
$p\text{CO}_2$ initial $\times$ $p\text{CO}_2$ secondary $\times$ time	4,89	0.573	0.6830	4,306	1.2870	0.2749
Days 8-14	<i>Three-way ANOVA</i>					
$p\text{CO}_2$ initial	1,87	0.149	0.7007	1,306	1.652	0.1995

$p\text{CO}_2$ secondary	2,87	2.328	0.1035		2,306	0.647	0.5242
time	2,87	3.719	0.0282		2,306	6.673	0.0015
$p\text{CO}_2$ initial $\times p\text{CO}_2$ secondary	2,87	1.004	0.3707		2,306	0.051	0.95
$p\text{CO}_2$ initial $\times$ time	2,87	1.127	0.3288		2,306	0.542	0.5821
$p\text{CO}_2$ secondary $\times$ time	4,87	1.325	0.2672		4,306	0.824	0.5105
$p\text{CO}_2$ initial $\times p\text{CO}_2$ secondary $\times$ time	4,87	0.597	0.6657		4,306	0.353	0.8417
Days 15-21	<i>Four-way ANOVA</i>						
$p\text{CO}_2$ initial	1,174	0.022	0.8800		1,606	8.421	0.0038
$p\text{CO}_2$ secondary	2,174	0.726	0.4583		2,606	1.172	0.3104
$p\text{CO}_2$ tertiary	1,174	0.556	0.4570		1,606	0.199	0.6560
time	1,174	0.088	0.9157		2,606	21.638	<0.0001
$p\text{CO}_2$ initial $\times p\text{CO}_2$ secondary	2,174	4.149	0.0174		2,606	0.423	0.6556
$p\text{CO}_2$ initial $\times p\text{CO}_2$ tertiary	1,174	0.747	0.3888		1,606	8.195	0.0044
$p\text{CO}_2$ secondary $\times p\text{CO}_2$ tertiary	2,174	0.339	0.7131		2,606	3.775	0.0235
$p\text{CO}_2$ initial $\times$ time	2,174	0.051	0.9502		2,606	1.001	0.3683
$p\text{CO}_2$ secondary $\times$ time	4,174	1.212	0.3077		4,606	1.393	0.2350
$p\text{CO}_2$ tertiary $\times$ time	2,174	0.023	0.9772		2,606	0.329	0.7199
$p\text{CO}_2$ initial $\times p\text{CO}_2$ secondary $\times p\text{CO}_2$ tertiary	2,174	1.812	0.1664		2,606	6.352	0.0019
$p\text{CO}_2$ initial $\times p\text{CO}_2$ secondary $\times$ time	4,174	1.689	0.1545		4,606	1.244	0.2910
$p\text{CO}_2$ initial $\times p\text{CO}_2$ tertiary $\times$ time	2,174	1.139	0.3227		2,606	0.301	0.7404
$p\text{CO}_2$ secondary $\times p\text{CO}_2$ tertiary $\times$ time	4,174	1.396	0.2373		4,606	0.770	0.5452
$p\text{CO}_2$ initial $\times p\text{CO}_2$ secondary $\times p\text{CO}_2$ tertiary $\times$ time	4,174	0.577	0.6798		4,606	0.499	0.7367
Significant P-values (< 0.05) are bolded; marginal P-values (<0.1) in <i>italics</i>							

Table 3. Effects of $p\text{CO}_2$ stress exposures on antioxidant capacity, total protein, and organic biomass (AFDW) of *Panopea generosa*. Two-way and three-way ANOVA tests for differences in physiological and cellular status on days 7 and 21 of the 21-day exposure period, respectively. Significant effects are bolded for $P < 0.05$.

Effect	Antioxidant capacity			Total protein			AFDW		
	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>
DAY 7	<i>Two-way ANOVA</i>								
$p\text{CO}_2$ initial	1,30	0.005	0.942	1,30	0.003	0.959	1,30	9.313	0.0047
$p\text{CO}_2$ secondary	2,30	0.143	0.867	2,30	0.866	0.431	2,30	2.536	0.096
$p\text{CO}_2$ initial $\times p\text{CO}_2$ secondary	2,30	1.007	0.377	2,30	2.136	0.136	2,30	0.158	0.8546
DAY 21	<i>Three-way ANOVA</i>								
$p\text{CO}_2$ initial	1,56	8.069	0.0063	1,56	2.365	0.13	1,56	12.899	<0.001
$p\text{CO}_2$ secondary	2,56	0.164	0.849	2,56	0.625	0.539	2,56	1.578	0.2153
$p\text{CO}_2$ tertiary	1,56	2.161	0.1471	1,56	1.272	0.264	1,56	3.298	<i>0.0747</i>
$p\text{CO}_2$ initial $\times p\text{CO}_2$ secondary	2,56	1.43	0.248	2,56	1.423	0.25	2,56	1.756	0.1822
$p\text{CO}_2$ initial $\times p\text{CO}_2$ tertiary	1,56	0.678	0.4136	1,56	2.25	0.139	1,56	0.453	0.5036
$p\text{CO}_2$ secondary $\times p\text{CO}_2$ tertiary	2,56	0.752	0.476	2,56	0.225	0.8	2,56	0.166	0.8906
$p\text{CO}_2$ initial $\times p\text{CO}_2$ secondary $\times p\text{CO}_2$ tertiary	2,56	0.141	0.8688	2,56	0.26	0.772	2,56	0.181	0.8353
Significant P-values (< 0.05) are bolded; marginal P-values (<0.1) in <i>italics</i>

Fig. 2. Respiration rate and shell length under secondary (a and b) and tertiary (c and d) exposure periods. Color, shapes, and fill are in reference to Figure 1. Significant *a posteriori* effects are shown as letters or an asterisk.

Fig. 3. Antioxidant response and physiology of fixed animals at the end of secondary (a-c; day 14) and tertiary treatments (d-f; day 21). Color, shapes, and fill are in reference to Figure 1.

3.2 Ambient common garden recovery under normocapnic conditions

During ambient recovery, respiration rate was not significantly affected by initial or secondary $p\text{CO}_2$ treatments, but was significantly affected by time (Table 2; three-way ANOVA; time, $F_{2,87}=3.719$, $P=0.0282$) with an increase in respiration rates over the 7-day period (Tukey HSD; day 14 > day 8; $P=0.0254$). Similarly, shell growth was not significantly affected by initial or secondary $p\text{CO}_2$ treatment, but showed a significant increase with time (Table 2; three-way ANOVA; time, $F_{2,306}=6.643$, $P=0.0015$; Tukey HSD; day 14 > day 8, $P=0.0013$; Tukey HSD, day 11 > day 8, $P=0.001$).

3.3 Tertiary exposure to hypercapnic seawater

The interaction of initial and secondary $p\text{CO}_2$ treatments had a significant effect on respiration rate under tertiary exposure (Table 2; four-way ANOVA; initial $\times$ secondary, $F_{2,174}=4.149$, $P=0.0174$), with this interaction primarily driven by a 20.4% greater respiration rate in $p\text{CO}_2$ stress-acclimated animals exposed to severe and ambient $p\text{CO}_2$ during the secondary period (Fig. 2c), although the post-hoc test was only marginally significant (Tukey HSD; moderate $\times$ severe > moderate $\times$ ambient, $P=0.0685$). Shell growth was affected by an interaction between initial, secondary, and tertiary $p\text{CO}_2$ treatments (Table 2 and Fig. 2d; four-way ANOVA; initial $\times$ secondary $\times$ tertiary, $F_{2,606}=6.352$, $P=0.0019$). Pairwise differences of the three-way treatment interaction showed 9.3% greater mean shell size by acclimated animals with secondary and tertiary exposure to severe and moderate $p\text{CO}_2$, respectively (Fig. 2d). At the end of the tertiary exposure period (day 21), initial stress acclimation under elevated $p\text{CO}_2$ increased organic biomass (Table 3; three-way ANOVA; initial, $F_{1,56}=12.899$, $P<0.001$) and tertiary exposure had a marginal increase (three-way ANOVA; tertiary, $F_{1,56}=3.298$, $P=0.0604$) with 51% and 28% greater organic biomass under stress treatments relative to ambient controls (Fig. 3f). There was a significant effect of initial stress acclimation on antioxidant activity (Table 3; three-way ANOVA; initial, $F_{1,56}=8.069$, $P=0.0063$) with 22% greater $\mu\text{M CRE mg protein}^{-1}$ by clams reared under ambient $p\text{CO}_2$ (Fig. 3d); there was no effect of $p\text{CO}_2$ treatment or two-way and three-way interactions of $p\text{CO}_2$ treatments on total protein (Table 3 and Fig. 3e).

4. Discussion

In the present study we evaluated the effects of postlarval stress acclimation and subsequent exposures to elevated $p\text{CO}_2$ on the physiological and oxidative stress response in juvenile geoduck.

Our findings suggest moderate hypercapnic conditions during postlarval development improves metrics of physiological performance and CSR. This novel investigation of adaptive stress acclimation demonstrates a high tolerance to $p\text{CO}_2$ regimes ($\sim 2500\text{--}5000 \mu\text{atm}$) and plasticity of bioenergetic and subcellular responses beneficial for later performance in *Panopea generosa*.

4.1 Stress-intensity- and life-stage-dependent effects

Survival under long-term stress exposure and positive physiological responses of acclimated animals under ‘moderate’ ($\sim 2900 \mu\text{atm } p\text{CO}_2$ 0.4Ω) and ‘severe’ ($\sim 4800 \mu\text{atm } p\text{CO}_2$ 0.2Ω) reciprocal exposures highlights the resilience of *Panopea generosa* to OA and suggests that stress acclimation (days) can induce beneficial effects during postlarval-juvenile development. Specifically, clams repeatedly exposed to the greatest intensity of stress (moderate $\times$ severe $\times$ moderate) had both greater respiration rates and shell size (Table 2; Fig. 2). Further, stress-acclimated individuals had greater organic biomass and lower amounts of antioxidant proteins relative to ambient controls (Figs. 3c, 3d, and 3f), 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 Louise 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 these findings, similar $p\text{CO}_2$ and Ω 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, $p\text{CO}_2$ tolerance limitations are likely species-specific, as well as life stage, duration, and stress-intensity specific.

$p\text{CO}_2$ -induced phenotypic variation over postlarval-juvenile development observed in this study suggests postlarval stages may be optimal for stress acclimation. Beneficial carryover effects herein are also corroborated with 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., 2020), further emphasizing life-stage dependence of $p\text{CO}_2$

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 $p\text{CO}_2$ 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 $p\text{CO}_2$ during gametogenesis in the Chilean mussel *Mytilus chilensis* and Sydney rock oyster *Saccostrea glomerata* increases the size of larval stages in progeny (Parker et al., 2012; Diaz et al., 2018). Future comparative studies should test stress responses of well-defined inter- and subtidal molluscs to determine if pre-conceived tolerance limitations are affected by repeated dose-responses post-settlement.

Our observation of beneficial effects in stress-acclimated clams suggest an adaptive resilience of *P. generosa* to hypercapnic conditions relevant to postlarval-juvenile development in both natural and aquaculture systems. *P. generosa* are likely capable of adaptive resilience particularly during this life stage because $p\text{CO}_2$ and Ω gradients naturally occur alongside the dramatic developmental transition from free-swimming larvae to sessile benthic juveniles. Habitat within the native range of *P. generosa* exhibits elevated $p\text{CO}_2$ and aragonite undersaturation with episodic/seasonal variation (surface water $\Omega < 1$ in winter months, Dabob Bay in Hood Canal, WA; Fassbender et al., 2018) and geographical ($> 2400 \mu\text{atm}$ and $\Omega < 0.4$ in Hood Canal, WA; Feely et al., 2010) and vertical heterogeneity (Reum et al., 2014) comparable to gradients within sub-surface sediments (Ω 0.4-0.6; Green et al., 2009). Meseck et al. (2018) found *in-situ* porewater pH increases model predictability of bivalve community composition, *Mya arenaria* and *Nucula* spp, suggesting sediment acidification and carbonate chemistry influence bivalve settlement. Our experimental timing and findings overlaid on common geoduck aquaculture practices suggest postlarval ‘settlement’ as an ecologically-relevant window to investigate the adaptive capacity for stress acclimatization.

4.2 Oxidative status and repeated stress encounters

Our results herein demonstrate activation of phenotypic variation after repeated stress encounters suggesting postlarval stress acclimation may have a critical role in subsequent stress response. A growing body of research supporting conditioning-hormesis challenges the paradigm of primarily deleterious effects of stress exposure (Calabrese et al., 2007; Costantini et al., 2012;

Gurr et al., 2020). Here we posit that hormetic priming could be occurring through moderate oxidative stress on protein homeostasis (patterns of differential expression), cellular signaling, and mitochondrially-mediated responses. For example, Constantini et al. (2012) found early-life exposure of the zebra finch *Taeniopygia guttata* to thermal-induced oxidative damage decreased oxidative stress under subsequent thermal exposure during adulthood. This 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.

In *Panopea generosa* in this study, compensatory antioxidant synthesis reduced performance in absence of prior stress acclimation suggesting an adaptive role of oxidative stress and conditioning-hormesis. pH- and CO₂-induced oxidative stress and antioxidant response are of growing interest to characterize stress resilience in bivalves (Tomanek et al., 2011; Matoo et al., 2013). Oxidative stress is intensified by acidosis (low intracellular pH) and hypercapnia either indirectly from low pH on ferrous iron enhancing the Fenton reaction (producing hydroxyl radicals), or a direct interaction of intracellular hypercapnia on free radical formation (Tomanek et al., 2011). pH- or CO₂-induced stress and mediative responses are species and stress-dependent (i.e. frequency, duration, and magnitude of stress exposures). Under short and prolonged exposure to subtle hypercapnic seawater (2-15 weeks; ~800 µatm, 7.9 pH, and 3 Ω), the Eastern oyster *Crassostrea virginica* and hard clam *M. mercenaria* differ in initial antioxidant production suggesting that CSR can determine interspecies vulnerability to hypercapnic seawater (Matoo et al., 2013). In more pronounced hypercapnic and aragonite undersaturated conditions, *C. virginica* upregulates antioxidant proteins (14 days; ~3500 µatm and 7.5 pH; Tomanek et al., 2011) and a similar response is also seen in the Yesso scallop *Patinopecten yessoensis* (duration = 14 days; ~2200 µatm, 7.5 pH, and 0.7 Ω; Liao et al., 2019) and mussel *Mytilus coruscus* (duration = 45 days; ~2800 µatm, 7.3 pH, and 0.5 Ω; Huang et al., 2018). Alternatively, a seemingly ‘preparatory’ energetic cost of antioxidant synthesis is found in a variety of taxa (Hermes-Lima and Zenteno-Savín, 2002; Ivanina and Sokolova, 2016) and suggests adaptive energy reallocation to scavenge ROS formed by aerobic recovery when a stressor is lifted.

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 a particularly low peroxidation-sensitive lipids (Munro and Blier, 2012) and high baseline antioxidant capacity throughout their lifespan suggesting an adaptive resilience to oxidative damage (Abele et al., 2008). Whereas intertidal molluscs (i.e. *Mytilus sp.*, *Crassostrea sp.*, and *Argopecten sp.*) have energetic deficits under dynamic environmental stress (i.e. elevated metabolic demand, ROS production, and antioxidant response; Kelley et al., 2017; Liao et al., 2019), *A. islandica* continues ventilation without anaerobic transition or overproduction of free radicals (Strahl et al., 2011). Thus, lower antioxidant production by stress-conditioned *P. generosa* could suggest adaptive subcellular mechanism(s) common across long-lived bivalves to maintain redox status under frequent or intermittent stress exposures. Altogether, the contrasting response of lower antioxidant proteins in stress-acclimated *P. generosa* suggests beneficial effects of prior exposure to hypercapnic seawater, which highlights the need for a mechanistic understanding of the role of oxidative stress in this process.

4.3 Mitochondrial and molecular mechanisms of moderate stress acclimation

Based on the effects of stress acclimation on antioxidant capacity and performance of *Panopea generosa* in this study, we describe two possible subcellular mechanisms underpinning stress-intensity-dependent effects on mitochondrial pathways and energy partitioning: 1) stress-induced attenuation of mitochondrial function and 2) adaptive mitochondrial shift under moderate stress (Fig. 4).

1) Stress-induced attenuation of mitochondrial function: Individuals initially exposed to ambient $p\text{CO}_2$ increased antioxidant proteins and decreased growth relative to stress-acclimated clams suggesting a greater demand for CSR leading to negative performance. Hypercapnia and acidosis can directly and indirectly drive increased formation of free radicals (Murphy, 2009; Tomanek et al., 2011) and elicit changes in the activity (Lambert and Brand, 2004) and expression of mitochondrial complexes (Dineshram et al., 2012; Dineshram et al., 2015), which altogether suggests diminished mitochondrial ATPs under environmental stress (Murphy 2009). Under normal aerobic respiration (Fig. 4a), complexes I (NADH dehydrogenase) and III (cytochrome *c* oxidoreductase) are dominant sources of mitochondrial ROS production by the electron transport chain (mETC). In contrast, mitochondrial dysfunction or alterations of mETC occur under environmental stress in which high pools of NADH in the mitochondrial matrix and reduced

coenzyme Q shift electron transport (reverse electron transport or RET) and proton gradient control to complex I, eliciting a deficit for ATPs and enhanced ROS production and CSR demand (Murphy, 2009); Fig 4b). Further, increased protonmotive force and pH gradient of the inner mitochondrial membrane increases RET and ROS production (Lambert and Brand, 2004; Miwa and Brand, 2003) suggesting a mechanism of pH- and CO₂-induced mitochondrial dysfunction. Altogether, the upregulation of antioxidant proteins and reduced growth in unconditioned *P. generosa* are likely outcomes of acclimation-dependent attenuation of mitochondrial function (Fig. 4b). In addition, uncoupling proteins (UCPs) are activated under oxidative damage to mediate mitochondrial stress although further decreasing the proton gradient and ATP production (Slocinska et al., 2016).

Fig. 4. Hypothesized model of stress acclimation through mitochondrial pathways: Diagram of inner mitochondrial membrane under normal conditions (a), dysfunction (b; i.e. reverse electrician transport), and the AOX pathway (c). Solid blue shapes highlight descriptors of modified pathways and green arrows display shifts in the direction of electrons (b and c). Legends with solid black arrows display the direction of changes relative to the mETC under normal conditions.

2) Adaptive mitochondrial shift under moderate stress: Alternative oxidase (AOX) is a regulatory protein of the inner mitochondrial membrane that receives electrons from ubiquinol to reduce oxygen and produce ATP without involving the cytochrome *c* mETC pathway (Figs. 4a and 4c). There is growing interest in AOX as a compensatory response that permits ATP synthesis and reduces ROS production during stress exposure (Tschischka et al., 2000; Sussarellu et al., 2013; Yusseppone et al., 2018). Although previously theorized as an exclusive mechanism in plant mitochondria (Vanlerberghe, 2013), molecular tools have helped define an evolutionary diversification of AOX in higher order animalia (McDonald and Vanlerberghe, 2006) with a particular prevalence in marine invertebrates and bivalve genomes (McDonald et al., 2009). AOX permits oxidative phosphorylation when complexes III and IV are inhibited (i.e. cyanide and sulphide inhibition), or when ROS is overproduced. A pH decline within the inner mitochondrial membrane can also activate the AOX pathway (Lima-Júnior et al., 2000). Although less efficient, with one-third of the membrane potential relative to cytochrome *c* mETC pathway (Millenaar and Lambers, 2003), AOX activity protects against free radical formation under abiotic and biotic disturbances (McDonald et al., 2009). Upregulated AOX coupled with decreased antioxidant protein synthesis under thermal stress suggests regulatory control of redox status, cellular signaling, and downstream energy allocation (Maxwell et al., 1999) are possible using this pathway. For example, *C. gigas* upregulates AOX in response to hypoxia-reoxygenation suggesting anticipatory dissipation of the proton gradient to avoid overproduction of free radicals upon reoxygenation (Sussarellu et al., 2013). An AOX-mediated response corroborates our hypothesis of a mechanism for hormesis that may outweigh energetic costs of the reduced ATP efficiency from this AOX pathway. A BLAST search identified AOX, mitochondrial carrier proteins (UCPs), and conserved mETC complexes within an annotated Pacific geoduck draft genome (Roberts et al., 2020). qPCR and metabolomics are planned to investigate these proposed mechanisms of hypercapnic-induced modification of mitochondrial function and link complexes I and III, AOX, reduced cofactors, UCPs, and ATP under acute and repeated stress encounters.

5. Conclusion

Postlarval 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 postlarval settlement and juvenile development. Thus, investigations of marine species responses to climate change should

consider adaptive dose-dependent regulation and effects post-acclimation. Further, alternative mitochondrial pathways can build an understanding of mechanisms underpinning hormesis to provide additional ‘climate-proofing’ strategies in aquaculture and conservation of goods and services in the Anthropocene.

Acknowledgments

We thank the Jamestown S’Klallam Tribe and Kurt Grinnell for providing the animals and facilities for this research. We also thank management staff and technicians at the Jamestown Point Whitney Shellfish Hatchery, Matt Henderson, Josh Valley and Clara Duncan, for their assistance. We also thank Emma Strand for analytical support.

Competing interests:

The authors declare no competing or financial interests.

Author contributions

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

Funding:

This work was funded in part through a grant from the Foundation for Food and Agriculture research; Grant ID: 554012, Development of Environmental Conditioning Practices to Decrease Impacts of Climate Change on Shellfish Aquaculture. The content of this publication is solely the responsibility of the authors and does not necessarily represent the official views of the Foundation for Food and Agriculture Research. Analysis and research materials were also supplemented by the Melbourne R. Carriker Student Research Grant.

Data availability

All raw data and statistical code are openly available as a Zenodo repository: <http://doi.org/10.5281/zenodo.3903019> (Gurr et al. 2020).

References

- 550
- 551
- 552 **Abele, D., Strahl, J., Brey, T. and Philipp, E. E. R.** (2008). Imperceptible senescence: ageing in
- 553 the ocean quahog *Arctica islandica*. *Free Radic. Res.* **42**, 474–480.
- 554
- 555 **An, M. I. and Choi, C. Y.** (2010). Activity of antioxidant enzymes and physiological responses in
- 556 ark shell, *Scapharca broughtonii*, exposed to thermal and osmotic stress: effects on
- 557 hemolymph and biochemical parameters. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*
- 558 **155**, 34–42.
- 559
- 560 **Barton, A., Hales, B., Waldbusser, G. G., Langdon, C. and Feely, R. A.** (2012). The Pacific
- 561 oyster, *Crassostrea gigas*, shows negative correlation to naturally elevated carbon dioxide
- 562 levels: Implications for near-term ocean acidification effects. *Limnol. Oceanogr.* **57**, 698–710.
- 563
- 564 **Brown, B., Dunne, R., Goodson, M. and Douglas, A.** (2002). Experience shapes the
- 565 susceptibility of a reef coral to bleaching. *Coral Reefs* **21**, 119–126.
- 566
- 567 **Brown, M. B. and Forsythe, A. B.** (1974). Robust tests for the equality of variances. *J. A. Stat.*
- 568 *Assoc.* **69**, 364–367.
- 569
- 570 **Cai, W. J., Hu, X., Huang, W.-J., Murrell, M. C., Lehrter, J. C., Lohrenz, S. E., Chou, W.-C.,**
- 571 **Zhai, W., Hollibaugh, J. T., Wang, Y., et al.** (2011). Acidification of subsurface coastal
- 572 waters enhanced by eutrophication. *Nat. Geosci.* **4**, 766–770.
- 573
- 574 **Calabrese, E. J., Bachmann, K. A., Bailer, A. J., Bolger, P. M., Borak, J., Cai, L.,**
- 575 **Cedergreen, N., Cherian, M. G., Chiueh, C. C., Clarkson, T. W., et al.** (2007). Biological
- 576 stress response terminology: Integrating the concepts of adaptive response and
- 577 preconditioning stress within a hormetic dose-response framework. *Toxicol. Appl. Pharmacol.*
- 578 **222**, 122–128.
- 579

580 **Costantini, D.** (2014). Oxidative stress and hormesis in evolutionary ecology and physiology.
581 Springer-Verlag, Berlin and Heidelberg, Germany. 978-3-642-54663-1.
582
583 **Costantini, D., Monaghan, P. and Metcalfe, N. B.** (2012). Early life experience primes resistance
584 to oxidative stress. *J. Exp. Biol.* **215**, 2820–2826.
585
586 **Diaz, R., Lardies, M. A., Tapia, F. J., Tarifeño, E. and Vargas, C. A.** (2018). Transgenerational
587 effects of pCO₂-driven ocean acidification on adult mussels *Mytilus chilensis* modulate
588 physiological response to multiple stressors in larvae. *Front. Physiol.* **9**, 1349.
589
590 **Dickson, A. G., Sabine, C. L. and Christian, J. R. eds.** (2007). *Guide to Best Practices for*
591 *Ocean CO₂ Measurements*. PICES Special Publication 3: 191
592
593 **Dineshram, R., Wong, K. K. W., Xiao, S., Yu, Z., Qian, P. Y. and Thiyagarajan, V.** (2012).
594 Analysis of Pacific oyster larval proteome and its response to high-CO₂. *Mar. Pollut. Bull.*
595 **64**, 2160–2167.
596
597 **Dineshram, R., Quan, Q., Sharma, R., Chandramouli, K., Yalamanchili, H. K., Chu, I. and**
598 **Thiyagarajan, V.** (2015). Comparative and quantitative proteomics reveal the adaptive
599 strategies of oyster larvae to ocean acidification. *Proteomics* **15**, 4120–4134.
600
601 **Fassbender, A. J., Alin, S. R., Feely, R. A., Sutton, A. J., Newton, J. A., Krembs, C., Bos, J.,**
602 **Keyzers, M., Devol, A., Ruef, W., et al.** (2018). Seasonal carbonate chemistry variability in
603 marine surface waters of the US Pacific Northwest. *Earth Syst. Sci. Data* **10**, 1367–1401.
604
605 **Fawcett, T. W. and Frankenhuys, W. E.** (2015). Adaptive explanations for sensitive windows in
606 development. *Front. Zool.* **12 Suppl 1**, S3.
607
608 **Feely, R. A., Alin, S. R., Newton, J., Sabine, C. L., Warner, M., Devol, A., Krembs, C. and**
609 **Maloy, C.** (2010). The combined effects of ocean acidification, mixing, and respiration on pH
610 and carbonate saturation in an urbanized estuary. *Estuar. Coast. Shelf S.* **88**, 442–449.

611

612 **Gattuso, J. P., Epitalon, J. M. and Lavine, H.** (2018). Seacarb: Seawater Carbonate Chemistry.

613

614 **Goodwin, C. L. and Pease, B.** (1987). The distribution of geoduck (*Panope abrupta*) size, density,

615 and quality to habitat characteristics such as geographic area, water depth, sediment type, and

616 associated flora and fauna in Puget Sound, Washington. Washington Department of Fisheries,

617 Shellfish Division, Olympia, 44 pp.

618

619 **Goodwin, C. L., and Pease, B.** (1989). Species profiles: Life histories and environmental

620 requirements of coastal fishes and invertebrates (Pacific Northwest). Pacific geoduck clam.

621 Biological Report 82 U.S. Army Corps of Engineers and U.S. Department of the Interior, 14

622 pp.

623

624 **Green, M. A., Waldbusser, G. G., Reilly, S. L., Emerson, K. and O'Donnell, S.** (2009). Death

625 by dissolution: Sediment saturation state as a mortality factor for juvenile bivalves. *Limnology*

626 *and Oceanography* **54**, 1037–1047.

627

628 **Gurr, S. J., Rollando, C., Chan, L. L., Vadopalas, B., Putnam, H. M. and Roberts, S. B.**

629 (2018). Alternative image-based technique for phytoplankton cell counts in shellfish

630 aquaculture (no. 1001481). Nexcelom.

631

632 **Gurr, S. J., Vadopalas, B., Roberts, S. B. and Putnam, H. M.** (2020). Metabolic recovery and

633 compensatory shell growth of juvenile Pacific geoduck *Panopea generosa* following short-

634 term exposure to acidified seawater. *Conserv. Physiol.* 8, coaa024

635

636 **Gurr, S. J., Trigg S. A., Vadopalas, B., Roberts, S. B. and Putnam, H. M.** (2020).

637 Repository for: Repeat exposure to hypercapnic seawater modifies performance and

638 oxidative status in a tolerant burrowing clam (version

639 20200622). <http://doi.org/10.5281/zenodo.3903019>

640

641 **Hermes-Lima, M. and Zenteno-Savín, T.** (2002). Animal response to drastic changes in oxygen
642 availability and physiological oxidative stress. *Comp. Biochem. Physiol. C.* **133**, 537–556.
643

644 **Hettinger, A., Sanford, E., Hill, T. M., Russell, A. D., Sato, K. N. S., Hoey, J., Forsch, M.,**
645 **Page, H. N. and Gaylord, B.** (2012). Persistent carry-over effects of planktonic exposure to
646 ocean acidification in the Olympia oyster. *Ecology* **93**, 2758–2768.
647

648 **Huang, X., Liu, Y., Liu, Z., Zhao, Z., Dupont, S., Wu, F., Huang, W., Chen, J., Hu, M., Lu,**
649 **W., et al.** (2018). Impact of zinc oxide nanoparticles and ocean acidification on antioxidant
650 responses of *Mytilus coruscus*. *Chemosphere* **196**, 182–195.
651

652 **Huo, Z., Rbbani, M. G., Cui, H., Xu, L., Yan, X., Fang, L., Wang, Y. and Yang, F.** (2019).
653 Larval development, juvenile survival, and burrowing rate of geoduck clams (*Panopea*
654 *japonica*) under different pH conditions. *Aquac. Int.* **27**, 1331–1342.
655

656 **Ivanina, A. V. and Sokolova, I. M.** (2016). Effects of intermittent hypoxia on oxidative stress and
657 protein degradation in molluscan mitochondria. *J. Exp. Biol.* **219**, 3794–3802.
658

659 **Jonsson, B. and Jonsson, N.** (2014). Early environment influences later performance in fishes. *J.*
660 *Fish Biol.* **85**, 151–188.
661

662 **Kapsenberg, L., Miglioli, A., Bitter, M. C., Tambutté, E., Dumollard, R. and Gattuso, J. P.**
663 (2018). Ocean pH fluctuations affect mussel larvae at key developmental transitions. *Proc.*
664 *Biol. Sci.* **285**, 20182381.
665

666 **Kelley, A. L., and Lunden, J. J.** (2017). Meta-analysis identifies metabolic sensitivities to ocean
667 acidification running title: ocean acidification impacts metabolic function. *Aims Environ. Sci.*
668 **4**, 709–729.
669

670 **Kozak, M. and Piepho, H. P.** (2018). What’s normal anyway? Residual plots are more telling
671 than significance tests when checking ANOVA assumptions. *J. Agron. Crop Sci.* **204**, 86–98.

672

673 **Kültz, D.** (2005). Molecular and evolutionary basis of the cellular stress response. *Annu. Rev.*

674 *Physiol.* **67**, 225–257.

675

676 **Kurihara, H., Kato, S. and Ishimatsu, A.** (2007). Effects of increased seawater pCO₂ on early

677 development of the oyster *Crassostrea gigas*. *Aquat. Biol.* **1**, 91–98.

678

679 **Lambert, A. J. and Brand, M. D.** (2004). Superoxide production by NADH:ubiquinone

680 oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner

681 membrane. *Biochem. J.* **382**, 511–517.

682

683 **Liao, H., Yang, Z., Dou, Z., Sun, F., Kou, S., Zhang, Z., Huang, X. and Bao, Z.** (2019). Impact

684 of ocean acidification on the energy metabolism and antioxidant responses of the yesso

685 scallop (*Patinopecten yessoensis*). *Front. Physiol.* **9**, 1967.

686

687 **Lima-Júnior, A., de Melo, D. F., Costa, J. H., Orellano, E. G., Jolivet, Y., Jarmuszkiewicz,**

688 **W., Sluse, F., Dizengremel, P. and Lima, M. S.** (2000). Effect of pH on CN-resistant

689 respiratory activity and regulation on *Vigna unguiculata* mitochondria. *Plant Physiol.*

690 *Biochem.* **38**, 765–771.

691

692 **Livingstone, D. R.** (2001). Contaminant-stimulated reactive oxygen species production and

693 oxidative damage in aquatic organisms. *Mar. Pollut. Bull.* **42**, 656–666.

694

695 **Matoo, O. B., Ivanina, A. V., Ullstad, C., Beniash, E. and Sokolova, I. M.** (2013). Interactive

696 effects of elevated temperature and CO₂ levels on metabolism and oxidative stress in two

697 common marine bivalves (*Crassostrea virginica* and *Mercenaria mercenaria*). *Comp.*

698 *Biochem. Physiol. A.* **164**, 545–553.

699

700 **Maxwell, D. P., Wang, Y. and McIntosh, L.** (1999). The alternative oxidase lowers

701 mitochondrial reactive oxygen production in plant cells. *Proc. Natl. Acad. Sci. U. S. A.* **96**,

702 8271–8276.

703

704 **McDonald, A. E. and Vanlerberghe, G. C.** (2006). Origins, evolutionary history, and taxonomic
705 distribution of alternative oxidase and plastoquinol terminal oxidase. *Comp. Biochem.*
706 *Physiol. D.* **1**, 357–364.

707

708 **McDonald, A. E., Vanlerberghe, G. C. and Staples, J. F.** (2009). Alternative oxidase in animals:
709 unique characteristics and taxonomic distribution. *J. Exp. Biol.* **212**, 2627–2634.

710

711 **Meseck, S. L., Mercaldo-Allen, R., Kuropat, C., Clark, P. and Goldberg, R.** (2018). Variability
712 in sediment-water carbonate chemistry and bivalve abundance after bivalve settlement in
713 Long Island Sound, Milford, Connecticut. *Mar. Pollut. Bull.* **135**, 165–175.

714

715 **Michaelidis, B., Ouzounis, C., Paleras, A. and Pörtner, H. O.** (2005). Effects of long-term
716 moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus*
717 *galloprovincialis*. *Mar. Ecol. Prog. Ser.* **293**, 109–118.

718

719 **Millenaar, F. F. and Lambers, H.** (2003). The alternative oxidase: in vivo regulation and
720 function. *Plant Biol.* **5**, 2–15.

721

722 **Miwa, S. and Brand, M. D.** (2003). Mitochondrial matrix reactive oxygen species production is
723 very sensitive to mild uncoupling. *Biochem. Soc. Trans.* **31**, 1300–1301.

724

725 **Munro, D. and Blier, P. U.** (2012). The extreme longevity of *Arctica islandica* is associated with
726 increased peroxidation resistance in mitochondrial membranes. *Aging Cell* **11**, 845–855.

727

728 **Murphy, M. P.** (2009). How mitochondria produce reactive oxygen species. *Biochem. J* **417**, 1–
729 13.

730

731 **Navarro, J. M., Torres, R., Acuña, K., Duarte, C., Manriquez, P. H., Lardies, M., Lagos, N.**
732 **A., Vargas, C. and Aguilera, V.** (2013). Impact of medium-term exposure to elevated pCO₂

733 levels on the physiological energetics of the mussel *Mytilus chilensis*. *Chemosphere* **90**,
734 1242–1248.
735
736 **Olito, C., White, C. R., Marshall, D. J. and Barneche, D. R.** (2017). Estimating monotonic rates
737 from biological data using local linear regression. *J. Exp. Biol.* **220**, 759–764.
738
739 **Pan, T.-C. F., Applebaum, S. L. and Manahan, D. T.** (2015). Experimental ocean acidification
740 alters the allocation of metabolic energy. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 469
741
742 **Parker, L. M., O'Connor, W. A., Raftos, D. A., Pörtner, H.-O. and Ross, P. M.** (2015).
743 Persistence of positive carryover effects in the oyster, *Saccostrea glomerata*, following
744 transgenerational exposure to ocean acidification. *PLoS One* **10**, e0132276.
745
746 **Parker, L. M., Ross, P. M., O'Connor, W. A., Borysko, L., Raftos, D. A. and Pörtner, H.-O.**
747 (2012). Adult exposure influences offspring response to ocean acidification in oysters. *Glob.*
748 *Change Biol.* **18**, 82–92.
749
750 **Pörtner, H. O.** (2012). Integrating climate-related stressor effects on marine organisms: unifying
751 principles linking molecule to ecosystem-level changes. *Mar. Ecol. Prog. Ser.* **470**, 273–290.
752
753 **Putnam, H. M., Davidson, J. M. and Gates, R. D.** (2016). Ocean acidification influences host
754 DNA methylation and phenotypic plasticity in environmentally susceptible corals. *Evol. Appl.*
755 **9**, 1165–1178.
756
757 **Putnam, H. M., Roberts S., Spencer L. H.** (2017). Capacity or adaptation and acclimatization to
758 ocean acidification in geoduck through epigenetic mechanisms. Poster, Figshare.
759 <https://doi.org/10.6084/m9.figshare.4990889.v1>
760
761 **R Core Team** (2018). A language and environment for statistical computing.
762

763 **Reum, J. C. P., Alin, S. R., Feely, R. A., Newton, J., Warner, M. and McElhany, P.** (2014).
764 Seasonal carbonate chemistry covariation with temperature, oxygen, and salinity in a fjord
765 estuary: implications for the design of ocean acidification experiments. *PLoS ONE* **9**, e89619.
766

767 **Ristow, M. and Schmeisser, K.** (2014). Mitohormesis: promoting health and lifespan by increased
768 levels of reactive oxygen species (ROS). *Dose-Response* **12**, dose-response.1.
769

770 **Roberts, S., White, S., Trigg, S. A., and Putnam, H.M.** (2020). *Panopea_generosa_genome*.
771 OSF. June 24. doi:10.17605/OSF.IO/YEM8N.
772

773 **Shamshak, G. L., and King, J. R.** (2015). From cannery to culinary luxury: the evolution of the
774 global geoduck market. *Mar. Policy* **55**: 81–89.
775

776 **Slocinska, M., Barylski, J. and Jarmuszkiewicz, W.** (2016). Uncoupling proteins of
777 invertebrates: A review. *IUBMB Life* **68**, 691–699.
778

779 **Sokolova, I. M.** (2013). Energy-limited tolerance to stress as a conceptual framework to integrate
780 the effects of multiple stressors. *Integr. Comp. Biol.* **53**, 597–608.
781

782 **Sokolova, I. M., Frederich, M., Bagwe, R., Lannig, G. and Sukhotin, A. A.** (2012). Energy
783 homeostasis as an integrative tool for assessing limits of environmental stress tolerance in
784 aquatic invertebrates. *Mar. Environ. Res.* **79**, 1–15.
785

786 **Strahl, J., Brey, T., Philipp, E. E. R., Thorarinsdóttir, G., Fischer, N., Wessels, W. and Abele,**
787 **D.** (2011). Physiological responses to self-induced burrowing and metabolic rate depression in
788 the ocean quahog *Arctica islandica*. *J. Exp. Biol.* **214**, 4223–4233.
789

790 **Sussarellu, R., Dudognon, T., Fabioux, C., Soudant, P., Moraga, D. and Kraffe, E.** (2013).
791 Rapid mitochondrial adjustments in response to short-term hypoxia and re-oxygenation in the
792 Pacific oyster, *Crassostrea gigas*. *J. Exp. Biol.* **216**, 1561–1569.
793

794 **Tanner, R. L. and Dowd, W. W.** (2019). Inter-individual physiological variation in responses to
795 environmental variation and environmental change: Integrating across traits and time. *Comp.*
796 *Biochem. Physiol. A.* **238**, 110577.

797

798 **Timmins-Schiffman, E., Guzmán, J. M., Thompson, R. E., Vadopalas, B., Eudeline, B. and**
799 **Roberts, S. B.** (2019). Dynamic response in the larval geoduck (*Panopea generosa*) proteome
800 to elevated pCO₂. *Ecol. Evol.* **10**, 185–197.

801

802 **Tomanek, L., Zuzow, M. J., Hitt, L., Serafini, L. and Valenzuela, J. J.** (2012). Proteomics of
803 hyposaline stress in blue mussel congeners (genus *Mytilus*): implications for biogeographic
804 range limits in response to climate change. *J. Exp. Biol.* **215**, 3905–3916.

805

806 **Tomanek, L., Zuzow, M. J., Ivanina, A. V., Beniash, E. and Sokolova, I. M.** (2011). Proteomic
807 response to elevated PCO₂ level in eastern oysters, *Crassostrea virginica*: evidence for
808 oxidative stress. *J. Exp. Biol.* **214**, 1836–1844.

809

810 **Tschischka, K., Abele, D. and Pörtner, H. O.** (2000). Mitochondrial oxyconformity and cold
811 adaptation in the polychaete *Nereis pelagica* and the bivalve *Arctica islandica* from the Baltic
812 and White Seas. *J. Exp. Biol.* **203**, 3355–3368.

813

814 **Vanlerberghe, G. C.** (2013). Alternative oxidase: a mitochondrial respiratory pathway to maintain
815 metabolic and signaling homeostasis during abiotic and biotic stress in plants. *Int. J. Mol. Sci.*
816 **14**, 6805–6847.

817

818 **Visser, B., Williams, C. M., Hahn, D. A., Short, C. A. and López-Martínez, G.** (2018).
819 Hormetic benefits of prior anoxia exposure in buffering anoxia stress in a soil-pupating insect.
820 *J. Exp. Biol.* **221**, jeb167825.

821

822 **Waldbusser, G. G., Hales, B., Langdon, C. J., Haley, B. A., Schrader, P., Brunner, E. L.,**
823 **Gray, M. W., Miller, C. A. and Gimenez, I.** (2015). Saturation-state sensitivity of marine
824 bivalve larvae to ocean acidification. *Nat. Clim. Change* **5**, 273–280.

825

826 **Walsh, P. J. and Louise Milligan, C.** (1989). Coordination of metabolism and intracellular acid–
827 base status: ionic regulation and metabolic consequences. *Can. J. Zool.* **67**, 2994–3004.

828

829 **White, M. M., McCorkle, D. C., Mullineaux, L. S. and Cohen, A. L.** (2013). Early exposure of
830 bay scallops (*Argopecten irradians*) to high CO₂ causes a decrease in larval shell growth.
831 *PLoS ONE* **8**, e61065.

832

833 **Wojtczyk-Miaskowska, A. and Schlichtholz, B.** (2018). DNA damage and oxidative stress in
834 long-lived aquatic organisms. *DNA Repair* **69**, 14–23.

835

836 **Yusseppone, M. S., Rocchetta, I., Sabatini, S. E., Luquet, C. M., de Molina, M. del C. R.,**
837 **Held, C. and Abele, D.** (2018). Inducing the alternative oxidase forms part of the molecular
838 strategy of anoxic survival in freshwater bivalves. *Front. Physiol.* **9**, 100.

839

840 **Zhang, Y., Humes, F., Almond, G., Kavazis, A. N. and Hood, W. R.** (2018). A mitohormetic
841 response to pro-oxidant exposure in the house mouse. *Am. J. Physiol. Regul. Integr. Comp.*
842 *Physiol.* **314**, R122–R134.