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STRESS CONDITIONING TO HYPERCAPNIC SEAWATER

IN THE PACIFIC GEODUCK *PANOPEA GENEROSA*

BY

SAMUEL J. GURR

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOLOGICAL AND ENVIRONMENTAL SCIENCES,

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OF

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ABSTRACT:

Expansion and enhancement of sustainable shellfish production is necessary to prevent overexploitation of wild stock and satisfy international trade, but hatchery rearing poses a critical production bottleneck due partially to environmental stressors such as ocean acidification. Given that stress conditions exacerbated by anthropogenic activity are projected to intensify in the near-future, long-lived molluscs, such as Pacific geoduck *Panopea generosa* (known lifespan up to 168 years), may rely on intragenerational acclimation to buffer against rapid environmental change. While acute stressors can be detrimental, environmental stress conditioning can improve performance. For example, moderate oxidative stress (i.e. temperature, irradiance, and dietary restriction) shows evidence of dose-dependent benefits for many taxa, however stress acclimation remains understudied in marine invertebrates, despite being threatened by climate change stressors. To test the hypothesis that physiological status is altered by stress conditioning, we first subjected juvenile geoduck clams to repeated exposures of elevated pCO₂ in a commercial hatchery setting followed by a period in ambient common garden. Our initial experiment found early exposure to low pH elicits compensatory carryover effects suggesting bioenergetic re-allocation facilitates growth compensation and metabolic recovery. Further, to test for life-stage and stress-intensity dependence in eliciting enhanced tolerance under subsequent stress encounters, we acclimatized post-larval geoduck for >100 days before re-exposure under two reciprocal periods of moderate and severe elevated pCO₂. Stress acclimation followed by secondary and tertiary exposure to severe and moderate elevated pCO₂ increased respiration rate, organic biomass, and shell size suggesting a stress-intensity-dependent effect on energetics. Moreover, 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. Transcriptomics was completed to better understand molecular underpinnings of emergent physiological phenotypes from this repeated reciprocal stress challenge. The naïve phenotype showed a high transcriptional demand involving fatty-acid degradation and glutathione components, highlighting mobilization of endogenous lipids, primarily for β -oxidation, as a favored energy source affecting somatic growth. In contrast, the transcriptome profile was more diverse and responsive to environmental changes (e.g. low pH: cellular quality control and immune defense; ambient recovery: energy metabolism and biosynthesis) and under putative control of transcriptional modifiers (e.g. histone methyltransferases and transcription factors) in the stress-acclimated phenotype, corroborating physiological traits of emergent phenotypes to propose molecular mechanisms underpinning beneficial developmental acclimation and stress resilience. Altogether, the summation of dissertation findings suggests early-life stress can trigger beneficial phenotypic variation. Thus, investigations of marine species responses to climate change should consider adaptive dose-dependent regulation and effects post-acclimation.

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Thank you, Steven Roberts, Brent Vadopalas, and Shelly Trigg, for your mentorship and support among many meetings and trips to the hatchery. It was an honor to be a part of this exceptional team; your uplifting and constructive feedback enhanced my motivation and excitement at each milestone. Thank you for devoting your time to this collaborative effort.

Hollie Putnam, thank you for believing in me - it was a gift to witness your incredible mind and attentive care for the well-being and academic progress of your students. Our paths crossing was transformative for my outlook on science and sense of belonging - I am honored to call you my PhD mother.

DEDICATION

To my parents (Johnna and Ronald Gurr) for each supportive moment from toddlerhood beach excursions to Sunday suppers. Mom, your insightful patience in life permeates each moment of joy and hardship as I grow stronger. Pops, your grit and devotion to family inspires me to make strides while finding happiness in each day. I hope you see in me what I know derives from you.

To my partner (Cara Keifer), only as a team could I navigate the changing seas of the before, now, and what is to come.

PREFACE

The most intrinsically motivating topics in my life are those that lie in plain sight but remain largely unseen. New Haven Harbor's invasive species and depleting marshes sparked this interest from a young age. Estuaries are the ideal laboratory; their essential value for society yet susceptibility to teeming human populations writes the perfectly ironic recipe. On the global scale, our current Anthropocene is often portrayed with pessimism and acceptance of an inevitability. I acknowledge our unprecedented effects on the environment, while I am captured by the ways life strives to persist. "*What does not kill you makes you stronger*", is an anthropomorphic trope that charismatically describes the hypothesis and findings of my research and the inspiration behind this dissertation.

This dissertation was written in 'Manuscript format', as each chapter is published or in preparation for submission.

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**METABOLIC RECOVERY AND COMPENSATORY SHELL GROWTH OF
JUVENILE PACIFIC GEODUCK *PANOPEA GENEROSA* FOLLOWING
SHORT-TERM EXPOSURE TO ACIDIFIED SEAWATER**

Samuel J. Gurr¹, Brent Vadopalas², Steven B. Roberts³ and Hollie M. Putnam¹

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ABSTRACT

While acute stressors can be detrimental, environmental stress conditioning can improve performance. To test the hypothesis that physiological status is altered by stress conditioning, we subjected juvenile Pacific geoduck, *Panopea generosa*, to repeated exposures of elevated $p\text{CO}_2$ in a commercial hatchery setting followed by a period in ambient common garden. Respiration rate and shell length were measured for juvenile geoduck periodically throughout short-term repeated reciprocal exposure periods in ambient ($\sim 550 \mu\text{atm}$) or elevated ($\sim 2400 \mu\text{atm}$) $p\text{CO}_2$ treatments and in common, ambient conditions, 5 months after exposure. Short-term exposure periods comprised an initial 10-day exposure followed by 14 days in ambient before a secondary 6-day reciprocal exposure. The initial exposure to elevated $p\text{CO}_2$ significantly reduced respiration rate by 25% relative to ambient conditions, but no effect on shell growth was detected. Following 14 days in common garden, ambient conditions, reciprocal exposure to elevated or ambient $p\text{CO}_2$ did not alter juvenile respiration rates, indicating ability for metabolic recovery under subsequent conditions. Shell growth was negatively affected during the reciprocal treatment in both exposure histories; however, clams exposed to the initial elevated $p\text{CO}_2$ showed compensatory growth with 5.8% greater shell length (on average between the two secondary exposures) after 5 months in ambient conditions. Additionally, clams exposed to the secondary elevated $p\text{CO}_2$ showed 52.4% increase in respiration rate after 5 months in ambient conditions. Early exposure to low pH appears to trigger carryover effects suggesting bioenergetic re-allocation facilitates growth compensation. Life stage-specific exposures to stress can determine when it may be

especially detrimental, or advantageous, to apply stress conditioning for commercial production of this long-lived burrowing clam.

INTRODUCTION

Sustainable food production minimizes overexploitation of wild populations and degradation of ecological health (Campbell *et al.*, 1998; Shumway *et al.*, 2003; Orensanz *et al.*, 2004; Zhang and Hand, 2006). Shellfish aquaculture has expanded worldwide in recent decades to satisfy international trade (FAO 2018). However, early larval and juvenile rearing poses a production bottleneck. For example, early life histories are highly sensitive to biotic (e.g. harmful algae, pathogens; Prado *et al.*, 2005; Rojas *et al.*, 2015) and abiotic stressors (e.g. pH, salinity, thermal and hypoxic stress; Baker and Mann 1992; Przeslawski *et al.* 2015; Kroeker *et al.*, 2010; Gimenez *et al.*, 2018). These stressors are known to intensify in coastal marine systems (Cloern, 2001; Diaz and Rosenberg, 2001; Cai *et al.*, 2011; Wallace *et al.*, 2014) causing mass mortality for early-stage bivalves in wild or hatchery settings (Elston *et al.*, 2008; Barton *et al.*, 2015). Local and global anthropogenic stressors such as CO₂-induced changes in pH and carbonate mineral saturation states can reduce performance and normal shell development (White *et al.*, 2013; Waldbusser *et al.*, 2015; Kapsenberg *et al.*, 2018).

Ocean acidification, or the decrease of oceanic pH due to elevated atmospheric partial pressures ($\mu\text{atm } p\text{CO}_2$), poses a threat to aquaculture (Barton *et al.*, 2012; Froehlich *et al.*, 2018; Mangi *et al.*, 2018). Elevated $p\text{CO}_2$ and aragonite undersaturation ($\Omega_{\text{aragonite}} < 1$) generally have detrimental consequences for aerobic performance (Pörtner *et al.*, 2004; Portner and Farrell, 2008) and shell biomineralization in marine calcifiers (Shirayama, 2005; Talmage and Gobler, 2010; Waldbusser *et al.*, 2010, 2015; Gazeau *et al.*, 2013). Responses to acidification can be species- (Ries *et al.*, 2009) and population-specific (Lemasson *et al.*, 2018), but it is widely established to be impactful during early

life stages for bivalves (Dupont and Thorndyke, 2009; Gazeau *et al.*, 2010; Kroeker *et al.*, 2010; Gimenez *et al.*, 2018). Experimental research is commonly focused on species with short generational times, (Parker *et al.*, 2011, 2015; Lohbeck *et al.*, 2012) limiting evidence for effects of acidification on long-lived mollusks important for food and economic security (Melzner *et al.*, 2009).

The Pacific geoduck *Panopea generosa* is a large and long-lived infaunal clam of cultural and ecological importance (Dethier, 2006) with an increasing presence in sustainable shellfish industry (Cubillo *et al.*, 2018). Geoduck production in Washington (USA) provides 90% of global supply (Shamshak and King, 2015) and alone constitutes 27% of the overall shellfish revenue in the state valued at >\$24 million year⁻¹ and >\$14 pound⁻¹ as of 2015 (Washington Sea Grant, 2015). Geoduck are known to live in dynamic CO₂-enriched low pH waters such as Hood Canal in Puget Sound, WA, where conditions in summer can reach $\Omega_{\text{aragonite}}$ 0.4 and pH 7.4 (Feely *et al.*, 2010). Although *P. generosa* may be adapted and able to acclimatize to local stressors (Putnam *et al.*, 2017; Spencer *et al.*, 2018), acidification has caused massive losses of larval bivalves in hatcheries (Barton *et al.*, 2015), identifying a critical need for assessment of physiological stress tolerance during early life stages.

Evidence of acclimatory mechanisms in response to acidification (Goncalves *et al.*, 2018) and enhanced performance within and across generations (Parker *et al.*, 2011, 2015; Putnam and Gates, 2015; Ross *et al.*, 2016; Thomsen *et al.*, 2017; Zhao *et al.*, 2017) support conditioning as a viable strategy to mitigate the negative effects of stress exposure and enhance organismal performance under high pCO₂ (Parker *et al.*, 2011; Dupont *et al.*, 2012; Suckling *et al.*, 2015; Foo and Byrne, 2016). Hormesis is a biphasic

low-dose-stimulatory response, as identified in toxicological studies (Calabrese, 2008) and suggests beneficial carryover effects of moderate stress exposure (Calabrese *et al.*, 2007; Costantini *et al.*, 2010; Costantini, 2014; Putnam *et al.*, 2018). Conditioning hormesis can explain patterns of intra- and transgenerational plasticity for organisms under environmental change (Calabrese and Mattson, 2011; Costantini *et al.*, 2012; López-Martínez and Hahn, 2012; Putnam *et al.*, 2018; Visser *et al.*, 2018), but is understudied for stress resilience in bivalves likely due to generally negative physiological implications of acidification (Gazeau *et al.*, 2013). In one example of early-life stage conditioning in bivalves, Putnam *et al.* (2017) found *P. generosa* exhibit compensatory shell growth after an acute exposure under elevated $p\text{CO}_2$. This finding suggests acute exposures may present a strategy for stress-hardening and enhancement of sustainable geoduck production. We therefore tested the hypothesis that repeated stress exposure under elevated $p\text{CO}_2$ can enhance intragenerational performance for Pacific geoduck. To this end, we measured the respiration rate and shell growth of juvenile geoduck in a commercial hatchery under repeated acute periods (6–10 days) of elevated $p\text{CO}_2$ and aragonite undersaturation, and the longer term (5 months) carryover effects.

METHODS

Exposure of juveniles

Juvenile geoduck ($n = 640$; mean SEM initial size, 5.08 ± 0.66 mm shell length [measured parallel to hinge]) were reared in trays (Heath/Tecna water tray) with rinsed sediment for 16 weeks (pediveliger to juvenile stage) by Jamestown Point Whitney Shellfish Hatchery before allocated into eight trays for the experiment (Fig. 1; $n = 80$ clams per tray). During

typical hatchery practice, geoduck are reared from ‘setters’ (pediveliger stage; 30 days old) to ‘seed’ (juvenile stage; 4–6 months old) in either downwellers or stacked trays; juveniles are then planted *in situ* to grow for several years until market size. Following aquaculture practice, trays were filled with a 5-mm depth of rinsed sand (35–45 µm grain size) that allowed juvenile geoduck to burrow and siphons could clearly be seen extended above the sediment throughout the experiments. To enable measurements of metabolic activity and shell growth, 30 geoduck were placed in an open circular dish (6.5 cm diameter and 3 cm height) with equal mesh size and sand depth submerged in each tray, the remaining 50 geoduck in each tray burrowed in the surrounding sediment. Seawater at the Jamestown Point Whitney Shellfish Hatchery (Brinnon, WA, USA) was pumped from offshore (100 m) in Quilcene Bay (WA, USA), bag- filtered (5 µm) and UV sterilized before fed to 250-L conical tanks at rate of 1 L min⁻¹. Four conical tanks were used as replicates for two treatments: elevated *p*CO₂ level of ~2300–2500 µatm and 7.3 pH (total scale) and ambient hatchery conditions of 500–600 µatm and 7.8–7.9 pH (total scale). The elevated *p*CO₂ level was set with a pH- stat system (Neptune Apex Controller System; Putnam *et al.*, 2016) and gas solenoid valves for a target pH of 7.2 (NBS scale) and pH and temperature (°C) were measured every 10 s in conicals (Neptune Systems; accuracy: ± 0.01 pH units and ± 0.1°C, resolution: ± 0.1 pH units and ± 0.1°C). These treatments were delivered to replicate exposure trays, which were gravity fed seawater from conicals (Fig. 1; *n* = 4 per treatment). The experiment began with an initial exposure period of 10 days under elevated *p*CO₂ (2345 µatm) and ambient treatments (608 µatm; Table 1). Preliminary exposure was followed by 14 days in ambient common garden (557 ± 17 µatm; pH_{t.s.} 7.9 ± 0.01; $\Omega_{\text{aragonite}}$ 1.46 ± 0.04, mean SEM) before secondary exposure for 6 days to reciprocal treatments of

elevated $p\text{CO}_2$ (2552 μatm) and ambient treatments (506 μatm ; Table 2). For the secondary exposure period, one tray was crossed to the opposite treatment to address both repeated and reciprocal exposure ($n = 2$ trays per initial secondary $p\text{CO}_2$ treatment; Fig. 1). Following this, the juveniles were exposed to ambient conditions for 157 days within the replicate trays. Juvenile geoduck were fed semi-continuously with a mixed algae diet (30% *Isochrysis galbana*, 30% *Pavlova lutheri* and 40% *Tetraselmis suecica*) throughout the 30-day experiment with a programmable dosing pump (Jebao DP-4 auto dosing pump). Large algae batch cultures were counted daily via bright-field image-based analysis (Nexcelom T4 Cellometer; Gurr *et al.*, 2018) to calculate a daily ration of 5×10^7 live algae cells day $^{-1}$ individual $^{-1}$. Diet was calculated with an equation in Utting & Spencer (1991) catered for 5-mm clams: $V = (S \times 0.4) \div (7 \times W \times C)$; this equation accounts for a feed ration of 0.4 mg dried algae mg live animal weight $^{-1}$ week $^{-1}$, the live animal weight (mg) of spat (S ; estimated from regression of shell length and weight of Manilla clams in Utting & Spencer 1991), weight (mg) of one million algal cells (W) and cell concentration of the culture (cells μl^{-1}) to calculate the total volume (V) of each species in a mixed-algae diet. Tray flow rates (mean flow rate, $\sim 480 \pm 9 \text{ ml}^{-1} \text{ min}^{-1}$) and food delivery were measured and adjusted daily.

All geoduck survived the exposure periods. Half of the remaining juveniles burrowed in each tray were maintained at the hatchery, positioned in the same replicate trays and stacked for continuous and high flow of ambient seawater ($\sim 8\text{--}10 \text{ L minute}^{-1}$). Stacked trays, commonly used for incubation of finfish, present a promising innovation for geoduck aquaculture; the experiment stack occurred alongside prototype stacked growing trays stocked by Jamestown Point Whitney Shellfish. The juveniles were fed cultured algae *ad libitum* daily for 157 days before shell length and respiration rates were measured.

Respirometry and shell length measurements

Juvenile geoduck were measured on days 2, 5, 8, and 10 of initial exposure, days 0, 2, 4, and 6 (cumulatively as day 24, 26, 28, and 30, respectively) of secondary exposure, and 157 days after the exposure period (cumulatively as day 187) to assess rates of oxygen consumption normalized to shell length. Calibrated optical sensor vials (PreSens, SensorVial SV-PSt5-4ml) were used to measure oxygen consumption in 4 ml vials on a 24-well plate sensor system (Presens SDR SensorDish). Juveniles in each treatment dish were divided into three sensor vials (10 individuals vial⁻¹ for exposure periods; 1 individual vial⁻¹ at 157-d post-exposure), each filled with 0.2 µm-filtered seawater from corresponding trays. Three blank vials per tray, filled only with 0.2 µm-filtered seawater, were used to account for potential microbial oxygen consumption. Respiratory runs occurred within an incubator at 15°C, with the vials and sensor placed on a rotator for mixing. Each set of measurements lasted ~30 minutes and trials ceased when oxygen concentration declined ~70-80% saturation to avoid hypoxic stress and isolate the effect of pCO₂ treatment on respiration rate. Siphons were observed pre and post-respirometry and were fully extended (~1-2 times shell length). Geoduck were subsequently photographed and shell length (parallel to hinge) was measured using Image J with a size standard (1 mm stage micrometer).

Rates of respiration (oxygen consumption) were calculated from repeated local linear regressions using the R package LoLinR (Olito *et al.*, 2017). An initial criterion of fixed constants (from the LoLin R package) for weighting method (L%) and observations (alpha = 0.2) was run individually for each respirometry measurement over the full 30-

minute record as a “reference” dataset. These are considered to be the most robust parameters as suggested by the R package authors (Olito *et al.*, 2017). Diagnostic plots (from the LoLin R package) were individually observed and L% and alpha were altered as necessary to best approximate the peak empirical distribution of local linear regressions (see https://github.com/SamGurr/Juvenile_geoduck_OA/releases/tag/version_20191210 for full details). To determine the optimal set of parameters, respiration data was calculated using three alpha values and data truncations (alpha = 0.2, 0.4, and 0.6; truncation = 10-20 minutes, 10-25 minutes, and no truncation; weighting method = L%) and each was compared to the initial reference dataset with two curve fitting steps (local polynomial regressions) to calculate unbiased and reproducible rates of oxygen consumption similar to the reference (10-day exposure, $r^2=0.88$; 6-day exposure, $r^2=0.95$). Final metabolic rates of juvenile geoduck were corrected for vial volume, rates of oxygen change in the blank vials, and standardized by mean shell length ($\mu\text{g O}_2 \text{ hr}^{-1} \text{ mm}^{-1}$).

Seawater carbonate chemistry

Total alkalinity (TA; $\mu\text{mol kg}^{-1}$ seawater) water samples were collected from trays once daily during treatment periods, in combination with measurements of pH by handheld probe (Mettler Toledo pH probe; resolution: 1 mV, 0.01 pH ; accuracy: ± 1 mV, ± 0.01 pH; Thermo Scientific Orion Star A series A325), salinity (Orion 013010MD Conductivity Cell; range 1 $\mu\text{S/cm}$ - 200 mS/cm; accuracy: ± 0.01 psu), and temperature (Fisherbrand Traceable Platinum Ultra-Accurate Digital Thermometer; resolution; 0.001°C; accuracy: ± 0.05 °C). Seawater chemistry was measured for three consecutive days during the 14 days of ambient common garden between initial and secondary treatment periods. Quality

control for pH data was assessed daily with Tris standard (Dickson Lab Tris Standard Batch T27) and handheld conductivity probes used for discrete measurements were calibrated every three days. TA was measured using an open cell titration (SOP 3b; Dickson *et al.*, 2007) with certified HCl titrant (~ 0.1 mol kg $^{-1}$, ~ 0.6 mol kg $^{-1}$ NaCl; Dickson Lab) and TA measurements identified $<1\%$ error when compared against certified reference materials (Dickson Lab CO₂ CRM Batches 137 and 168). Seawater chemistry was completed following Guide to Best Practices (Dickson *et al.*, 2007); daily measurements were used to calculate carbonate chemistry, CO₂, $p\text{CO}_2$, HCO $^{3-}$, CO₃, and $\Omega_{\text{aragonite}}$, using the SEACARB package (Gattuso *et al.*, 2018) in R v3.5.1 (R Core Team, 2018).

Data Analysis

A two-way Analysis of Variance (ANOVA) was used to analyze the effect of time (fixed), $p\text{CO}_2$ treatment (fixed), and time $\times p\text{CO}_2$ interaction for respiration and shell length during initial exposure. A t-test was used to test the effect of initial $p\text{CO}_2$ treatment on respiration rate and shell length prior to the secondary exposure (last day of ambient common garden, cumulatively day 24, day 0). For the secondary exposure period, a three-way ANOVA was used to test the effects of time (fixed), initial $p\text{CO}_2$ treatment (fixed), secondary $p\text{CO}_2$ treatment (fixed), and their interactions on respiration rate and shell length. No significant differences in seawater chemistry were detected between trays of the same treatment (pH, $p\text{CO}_2$, TA, salinity, and temperature; doi: 10.5281/zenodo.3588326), thus tray effects were assumed negligible. Significant model effects were followed with pairwise comparisons with a Tukey's *a posteriori* HSD. We used a two-way ANOVA to analyze the effects of initial (fixed) and secondary (fixed) $p\text{CO}_2$ treatments on respiration

and shell length after 157 days in ambient conditions. In all cases, model residuals were tested for normality assumptions with visual inspection of diagnostic plots (residual vs. fitted and normal Q-Q; Kozak and Piepho, 2018) and homogeneity of variance was tested with Levene's test. Model effects using raw data were robust to transformation(s) that resolved normality assumptions via Shapiro-Wilk test. Statistical tests were completed using R (v3.5.1; R Core Team, 2018). All data and code are available (doi: 10.5281/zenodo.3588326).

RESULTS

Exposure 1

The respiration rate of juvenile clams (4.26 ± 0.85 mm shell length; mean ± SD) prior to exposure was 0.29 ± 0.16 µg O₂ hr⁻¹ mm⁻¹ (mean ± SD). Elevated pCO₂ had a significant effect on respiration rate over the initial 10-day exposure (*p*CO₂ treatment, $F_{1,88} = 7.512$; $P < 0.01$) with a 25% reduction (averaged across all days) in respiration rate in elevated *p*CO₂ treatment relative to ambient (Fig. 2A). Juvenile geoduck grew significantly with time under the initial 10-d exposure (time, $F_{3,949} = 3.392$; $P = 0.018$) with a 3.6% increase in shell length between days 2 and 10 (Fig. 2B), but there was no effect of *p*CO₂ treatment on shell length (Table 2). Significant differences in respiration rate from the initial *p*CO₂ treatment were still apparent after 14 days in ambient common garden and before the onset of the secondary exposure (Table 2 and Fig. 3A). In contrast, there was no significant change in shell length due to initial *p*CO₂ treatment after 14 days in ambient common garden (Table 2).

Exposure 2

There was no interaction between initial and secondary $p\text{CO}_2$ treatments nor between treatments and time on respiration rate or shell length (Table 2). There was a marginal effect of time on respiration rate (Table 2; time, $F_{2,60} = 3.137$; $P = 0.0506$) with a 31% increase in average respiration rate between days 2 and 6. Initial $p\text{CO}_2$ treatment had a significant effect on shell length, with on average a ~4% reduction in shell size under high $p\text{CO}_2$ relative to ambient initial exposure (Fig. 3B; $p\text{CO}_{2\text{-initial}}$, $F_{1,709} = 15.821$; $P < 0.001$). This same trend was present under the secondary high $p\text{CO}_2$ exposure, (Fig. 3B; $p\text{CO}_{2\text{-secondary}}$, $F_{1,709} = 9.917$; $P = 0.002$) with 3.20% smaller shells for individuals exposed to elevated $p\text{CO}_2$ treatments. There were pairwise differences in shell size between animals only exposed to ambient and animals repeatedly exposed to elevated $p\text{CO}_2$ (Fig. 3B; day 6, $P = 0.0415$; day 6 ambient - day 4 elevated, $P = 0.0406$).

Common garden after exposure periods

There was no interaction between initial and secondary $p\text{CO}_2$ treatments on respiration rate or shell length (Table 2). The initial exposure period had a significant effect on shell length of juveniles previously exposed to high $p\text{CO}_2$, after 157 days in ambient common garden (Fig. 4A; $p\text{CO}_{2\text{-initial}}$, $F_{1,170} = 5.228$; $P = 0.023$), where average shell lengths were 5.8% larger in juveniles exposed to initial elevated $p\text{CO}_2$. Secondary 6-day exposure had a significant effect on respiration rates after 157 days in ambient common garden (Fig. 4B; $p\text{CO}_{2\text{-secondary}}$, $F_{1,31} = 13.008$; $P = 0.001$) with an average of 52.4% greater respiration rates in juveniles secondarily exposed to elevated $p\text{CO}_2$. Visual examination

during screening indicated low mortality ($1\text{-}4 \text{ tray}^{-1}$) over the ~5-month grow-out period. Shell lengths of dead animals (as empty shells) were similar to the size of juvenile geoduck during the 30-d exposure period suggesting low mortality occurred at the start of the grow-out period possibly due to handling stress.

DISCUSSION

Metabolic recovery and compensatory shell growth by juvenile *P. generosa* present a novel application of hormetic framework for resilience of a mollusc to acidification. To date, within-generation carry-over effects remain poorly understood for marine molluscs (Ross *et al.*, 2016) with few examples of either positive and negative responses after stress challenges (Hettinger *et al.*, 2012; Gobler and Talmage, 2013; Putnam *et al.*, 2017). Further study on conditioning-hormesis in response to $p\text{CO}_2$ stress must address cellular-level energy allocation, in addition to whole organism physiology, to account for essential functions with more holistic implications for stress resilience (Pan *et al.* 2015).

Metabolic depression and compensatory response

Metabolic depression, such that was found under initial exposure of geoduck to elevated $p\text{CO}_2$, has been suggested as an adaptive mechanism to extend survival (Guppy and Withers, 1999). Stress-induced metabolic depression has been documented for a variety of marine invertebrates in response to environmental stress. For example, in the New Zealand geoduck, *Panopea zelandica*, there was a 2-fold reduction in respiration rate under hypoxia (Le *et al.*, 2016). Prior work has shown metabolic reductions up to 60-95% of basal performance at rest for marine molluscs (Guppy and Withers, 1999). Here,

depression of oxygen consumption rate by juvenile geoduck to ~25% in comparison with rates under ambient conditions suggests *P. generosa* are relatively tolerant to short-term acidification and may have adaptive physiology to cope with environmental acidification and high $p\text{CO}_2$. Responsiveness to acidification is critical for pH-tolerant taxa to maintain buffering capacity and cope with acidosis (high intracellular $p\text{CO}_2$; (Melzner *et al.*, 2009). However, pH-induced metabolic depression to a similar degree found in this study has caused a permanent decrease in extracellular pH and increase in protein degradation and ammonia excretion in the Mediterranean mussel (*Mytilus galloprovincialis*) (Michaelidis *et al.*, 2005). Conversely, metabolic elevation is relatively common for early-life stage bivalves exposed to low pH and $\Omega_{\text{aragonite}}$ undersaturation and typically coincides with consequences for performance and survival (Michaelidis *et al.*, 2005; Beniash *et al.*, 2010; Thomsen and Melzner, 2010; Fernández-Reiriz *et al.*, 2011; Waldbusser *et al.*, 2015; Lemasson *et al.*, 2018). Whether depressed or elevated, stress-induced metabolic alterations are known to contribute to biochemical outcomes such as intracellular hypercapnia and hemolymph acidosis (Pörtner *et al.*, 2004; Spicer *et al.*, 2011) and increased ammonia excretion and reduced growth for invertebrate fauna (Michaelidis *et al.*, 2005; Beniash *et al.*, 2010; Lannig *et al.*, 2010; Thomsen and Melzner, 2010; Gazeau *et al.*, 2013). However, $p\text{CO}_2$ did not impair shell growth during the initial period further demonstrative of the pH/hypercapnia-tolerance of *P. generosa*.

Juvenile geoduck repeatedly exposed to elevated $p\text{CO}_2$ showed possible stress “memory” with rebound from metabolic depression under subsequent stress and higher respiration rate and compensatory shell growth after long-term recovery. Metabolic rebound supports a hormetic-like response by *P. generosa* (Calabrese *et al.*, 2007;

Costantini, 2014) and prompts further investigation of energy budget, cellular, and -omic measures under repeated reciprocal stress encounters to improve our understanding of the mechanism underpinning hormesis. Use of hormesis to conceptualize carry-over effects of mild stress exposure is largely confined to model insects, plants, and microorganisms (Lee *et al.*, 1987; Calabrese and Blain, 2009; López-Martínez and Hahn, 2012; Visser *et al.*, 2018). For example, Visser *et al.* (2018) found the Caribbean fruit fly, *Anastrepha suspensa*, exposed to oxidative stress early in life enhanced survivorship and investment in fertility and lipid synthesis under subsequent stress during adulthood. Mechanistic molecular and biochemical assessments under different and repeated stress intensities (i.e. magnitude, duration, and frequency) are planned to determine the threshold between low-dose stimulation and high-dose inhibition from stress-conditioning.

Age and intensity dependence of shell growth

Metabolic recovery was coupled with reduced shell growth under a repeated stress encounter (Fig. 3) and compensatory shell growth after approximately five months in ambient conditions (Fig. 4). This could be explained by several hypotheses such as: carry-over effect from metabolic depression under initial exposure to elevated $p\text{CO}_2$ (Fig. 2A), differing sensitivity to stress intensity (Table 1), and/or age dependence for environmental hardening, or the interaction with increasing temperature through the season (see Supplementary Figure 1.). Bivalves known to exhibit metabolic suppression under acute and long-term acidification are often attributed with increased ammonia excretion rates and decreased ingestion and clearance rates as possible contributors to protein degradation and reduced growth (Michaelidis *et al.*, 2005; Thomsen and Melzner, 2010; Fernández-Reiriz

et al., 2011; Navarro *et al.*, 2013). Therefore, decreased shell length under secondary exposure may be a latent effect of metabolic depression during initial exposure. However, shell length was also reduced for clams initially exposed to the elevated treatment in the second exposure period (Table 2, Fig. 3B), indicating potential age-dependence of calcification and bioenergetic effects for juvenile *P. generosa*. This reduction however, could also be explained by the fact the secondary elevated $p\text{CO}_2$ treatment was on average ~0.04 pH units lower than the initial exposure (Table 1), suggesting possible sensitivity to increased stress intensity. It is likely that both temporal dynamics and stress thresholds influence intragenerational carry-over effects and further experimental efforts with repeated reciprocal design are needed.

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

Commercial and environmental applications of experimental findings

Our findings infer both positive and negative implications for aquaculture. Although advantageous to elicit carry-over effects exhibited by stress-conditioned animals, results imply greater feed (ingestion rate) to sustain enhanced aerobic metabolism and compensatory shell growth; this can heighten labor and financial costs for industry, likely not incentivized by a marginal 5.8% increase in shell size. However, typical protocols for

geoduck aquaculture yield 5-month-old juvenile clams in the hatchery before grown *in-situ* for 5-7 years. Consequently, latency of enhanced performance in this study (~9-month-old juveniles), overlaid with the standard timeline for geoduck industry, does not present additional expenses. Further related tests on stress conditioning and production of resilient strains (i.e. phenotypes and/or epigenotypes) must account for distinct life-stages and species-specific attributes in aquaculture practice.

Shellfish farming has adapted in recent years to implement “climate-proofing” technology to maintain production and combat both coastal and climate-related stressors (e.g. ocean acidification, sea-level rise, coastal development; Allison et al. 2011). For example, chemical buffering systems (e.g. mixing sodium bicarbonate) are increasingly common in shellfish industry to elevate aragonite saturation levels and reduce deleterious effects of ocean acidification; hatcheries report increases in productivity by 30-50%, offsetting the cost to maintain optimal carbonate chemistry year-round (Barton et al. 2015). Although buffering systems are advantageous to yield juvenile ‘seed’, alleviation of aragonite undersaturation in the short-term may leave juveniles and adults unprepared to cope with the heterogeneity of environmental chemistry during long growing periods *in-situ*. As conditions in coastal bays report deteriorating water quality (Feely et al. 2008; Wallace et al. 2014; Cloern 2001; Melzner et al. 2013), acclimatization and selective breeding posit alternate and more robust solutions to generate stress-resilience (Barton et al. 2015). Implementation and tests of effectiveness of stress conditioning remain uncommon for scientists and aquaculture; our novel findings collected in a hatchery setting provide incentive to fine-tune stress exposures and build a mechanistic understanding of physiological, cellular, and molecular responses. Critical questions to test the practical

application of stress conditioning are: (1) what are the effects of repeated stress exposures on energy budget? (2) what life-stages and/or $p\text{CO}_2$ stress intensity (i.e. magnitude and duration) optimizes establishment of resilient phenotypes and genotypes during hatchery-rearing? (3) does stress history under elevated $p\text{CO}_2$ affect the stability and longevity of carry-over effects later in life? Answers to these challenges will result in effective implementation of conditioning to both reduce pressure on wild stocks and sustain food security under environmental change.

Although this study was primarily focused on production enhancement in a hatchery setting, effects on shell growth and metabolism have important applications to natural systems. Seawater carbonate chemistry targeted for stress treatments was more severe than levels commonly used in experimental research (Gazeau *et al.*, 2010; Navarro *et al.*, 2013; Diaz *et al.*, 2018), but relevant to summer subsurface conditions within the natural range of *P. generosa* (pH 7.4 and $\Omega_{\text{aragonite}}$ 0.4 in Hood Canal, WA; Feely *et al.*, 2010). Thus, survival, metabolic recovery, and compensatory growth in *P. generosa* in this study demonstrates a resilience to short-term acidification in the water column. Enhanced growth rates during juvenile development can present benefits for burrowing behavior (Green *et al.*, 2009; Clements *et al.*, 2016; Meseck *et al.*, 2018) and survival due to decreased risk of predation and susceptibility to environmental stress (Przeslawski and Webb, 2009; Johnson and Smee, 2012). Specific to juvenile *P. generosa*, time to metamorphosis (to dissoconch), pre-burrowing time (time elapsed to anchor into substrate and obtain upright position), and burrowing depth are directly related to growth and survival (Goodwin and Pease, 1989; Tapia-Morales *et al.*, 2015). Thus, stress conditioning under CO₂-enrichment and low pH may enhance survivorship of juvenile geoduck in

natural systems. Water column carbonate chemistry may be critical for sustainable production of infaunal clams, such as *P. generosa*, that are out-planted for several years *in-situ* on mudflats known to exhibit dynamic abiotic gradients (Green *et al.*, 1993; Burdige *et al.*, 2008) adjacent to seasonally acidified and undersaturated water bodies (Feely *et al.*, 2010; Reum *et al.*, 2014).

CONCLUSION

Data in this present study provides evidence of capacity to cope with short-term acidification for an understudied infaunal clam of high economic importance. Survival of all individuals over the 30-d experiment demonstrates the resilience of this species to low pH and reduced carbonate saturation. Juvenile geoduck exposed to low pH for 10 days recovered from metabolic depression under subsequent stress exposure and conditioned animals showed a significant increase in both shell length and metabolic rate compared to controls after five months under ambient conditions, suggesting stress “memory” and compensatory growth as possible indicators of enhanced performance from intragenerational stress-conditioning. Our focus on industry enhancement must expand to test developmental morphology, physiology, and genetic and non-genetic markers over larval and juvenile stages in a multi-generational experiment to generate a more holistic assessment of stress hardening and the effects of exposure on cellular stress response (Costantini *et al.*, 2010; Foo and Byrne, 2016; Eirin-Lopez and Putnam, 2018) for advancement of sustainable aquaculture (Branch *et al.*, 2013). Advancements in genome sequencing will facilitate further research to synthesize -omic profiling (i.e global DNA methylation and differential expression) with physiological responses throughout

reproductive and offspring development under environmental stress (Gavery and Roberts, 2014; Li *et al.*, 2019) to determine if these mechanisms are transferable among species. Stress conditioning within a generation at critical life stages may yield beneficial responses for food production and provide a baseline for other long-lived burrowing bivalves of ecological and economic importance.

Table 1. pH, salinity and temperature measured with handheld probes and total alkalinity measured daily

Treatment	Temperature	Salinity	Flow rate mL min ⁻¹	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
Initial exposure											
Ambient	14.82 ± 0.12	29 ± 0.03	504 ± 21	7.86 ± 0.007	24 ± 0.5	608 ± 11	1842 ± 4	86 ± 1.4	1952 ± 3	2056 ± 1	1.35 ± 0.02
Low	14.91 ± 0.12	29 ± 0.04	484 ± 17	7.31 ± 0.004	91 ± 0.7	2345 ± 20	1992 ± 1	26 ± 0.20	2108 ± 1	2056 ± 1	0.41 ± 0.003
Ambient common garden											
Ambient	15.01 ± 0.22	29 ± 0.05	449 ± 18	7.89 ± 0.012	21 ± 0.7	561 ± 17	1821 ± 7	93 ± 2.6	1936 ± 5	2051 ± 1	1.45 ± 0.04
Secondary exposure											
Ambient	16.33 ± 0.22	28.67 ± 0.03	494 ± 29	7.93 ± 0.004	19 ± 0.3	506 ± 5	1781 ± 5	102 ± 1.4	1902 ± 4	2033 ± 2	1.60 ± 0.02
Low	16.40 ± 0.22	28.67 ± 0.04	471 ± 18	7.27 ± 0.007	95 ± 1.3	2551 ± 42	1972 ± 3	25 ± 0.3	2091 ± 3	2033 ± 3	0.39 ± 0.005

Table 2. Two-way and three-way ANOVA tests for metabolic rate and shell length during initial and secondary exposures, respectively.

			df	SS	MS	F	P
Initial exposure							
Respiration rate		<i>Two-way ANOVA</i>					
	time		3	0.0323	0.011	0.822	0.485
	p CO ₂		1	0.0983	0.098	7.512	0.007
	p CO ₂ × time		3	0.0475	0.016	1.210	0.311
Shell length		time	3	4.250	1.415	3.392	0.018
	p CO ₂		1	0	0.0005	0.0012	0.973
	p CO ₂ × time		3	0.170	0.058	0.138	0.937
Ambient common garden							
Respiration rate		<i>Welch Two Sample t-test</i>		df	t	P	
	p CO ₂		19.833	2.673	0.015	-	-
Shell length		p CO ₂	1.146	236.680	0.253	-	-
Secondary exposure							
Respiration rate		<i>Three-way ANOVA</i>					
	time		2	0.068	0.034	3.137	0.051
	p CO ₂ initial		1	0.021	0.021	1.916	0.171
	p CO ₂ secondary		1	0.032	0.032	2.926	0.092
	p CO ₂ initial × p CO ₂ secondary		1	0.023	0.023	2.080	0.154
	p CO ₂ initial × time		2	0.016	0.008	0.724	0.489
	p CO ₂ secondary × time		2	0.002	0.001	0.103	0.903
	p CO ₂ initial × p CO ₂ secondary × time		2	0.035	0.017	1.608	0.209
Shell length		time	2	0.190	0.095	0.152	0.859
	p CO ₂ initial		1	9.910	9.910	15.821	<0.001
	p CO ₂ secondary		1	6.210	6.212	9.917	0.002
	p CO ₂ initial × p CO ₂ secondary		1	0.060	0.063	0.100	0.752
	p CO ₂ initial × time		2	0	0.001	0.002	0.998
	p CO ₂ secondary × time		2	0.460	0.231	0.368	0.692
	p CO ₂ initial × p CO ₂ secondary × time		2	0.100	0.048	0.076	0.927
157 days post							
Respiration rate		<i>Two-way ANOVA</i>					
	p CO ₂ initial		1	0.003	0.002	0.011	0.919
	p CO ₂ secondary		1	3.037	3.037	13.008	0.001
	p CO ₂ initial × p CO ₂ secondary		1	0.050	0.050	0.212	0.648
Shell length		p CO ₂ initial	1	10.600	10.597	5.228	0.023
	p CO ₂ secondary		1	0.210	0.214	0.105	0.746
	p CO ₂ initial × p CO ₂ secondary		1	3.510	3.507	1.730	0.190

Significant P-values (< 0.05) are bolded.

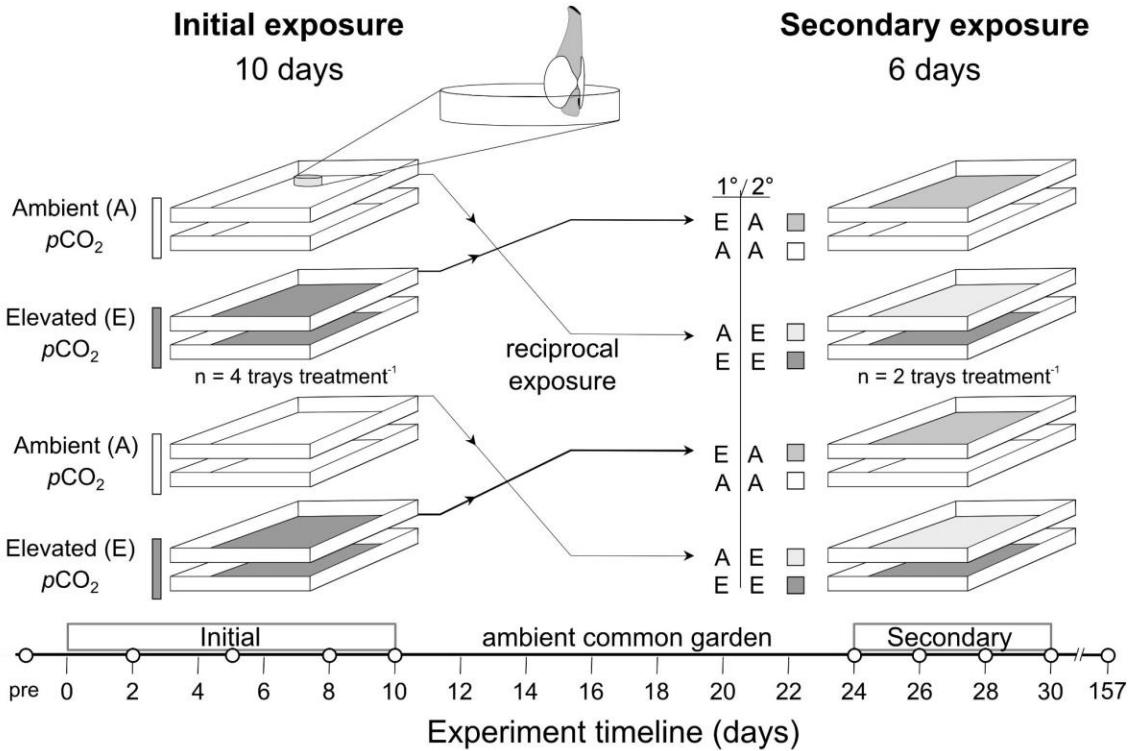


Figure 1. Schematic of the repeated exposure experimental design for two exposure trials.

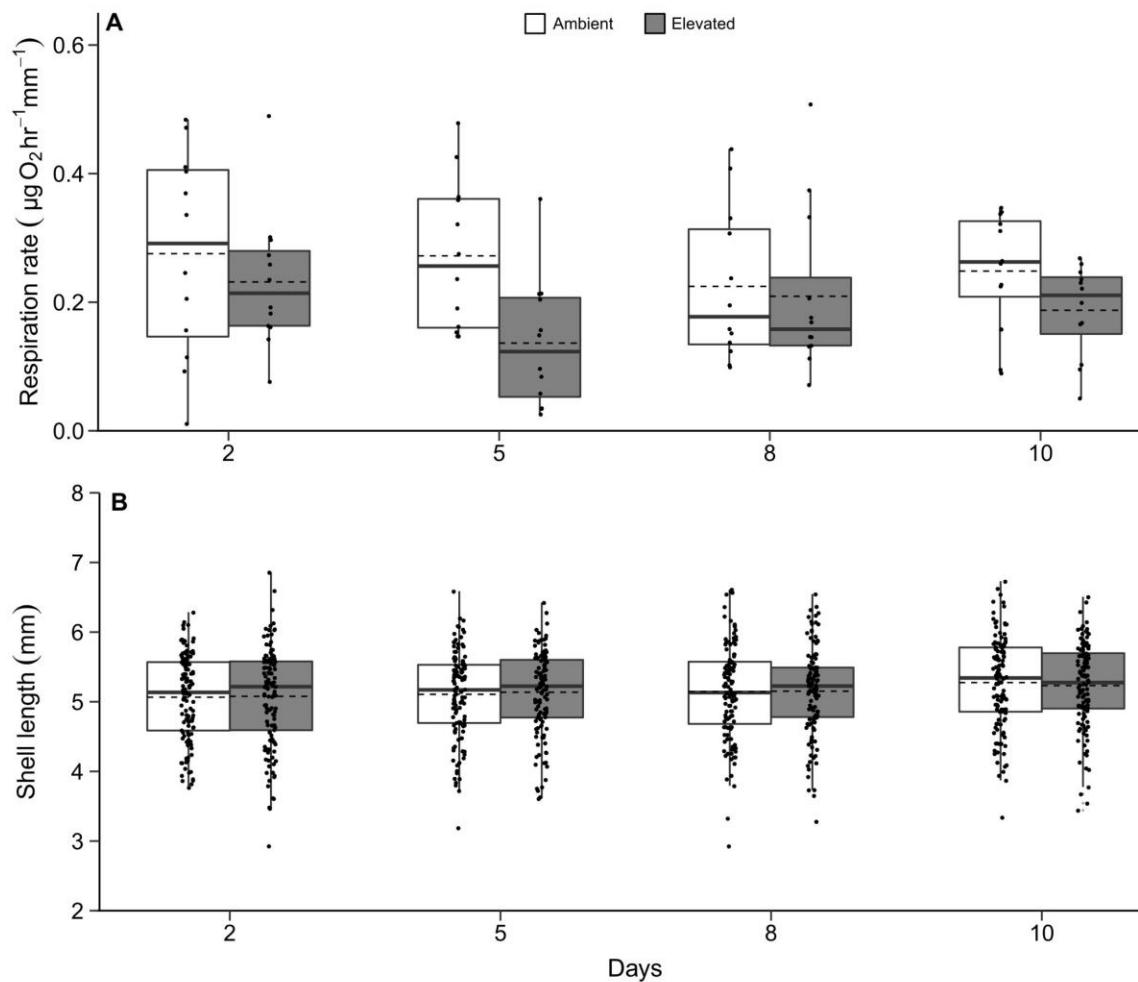


Figure 2. Respiration rates (A) and shell length (B) of juvenile geoduck under the initial 10-day exposure.

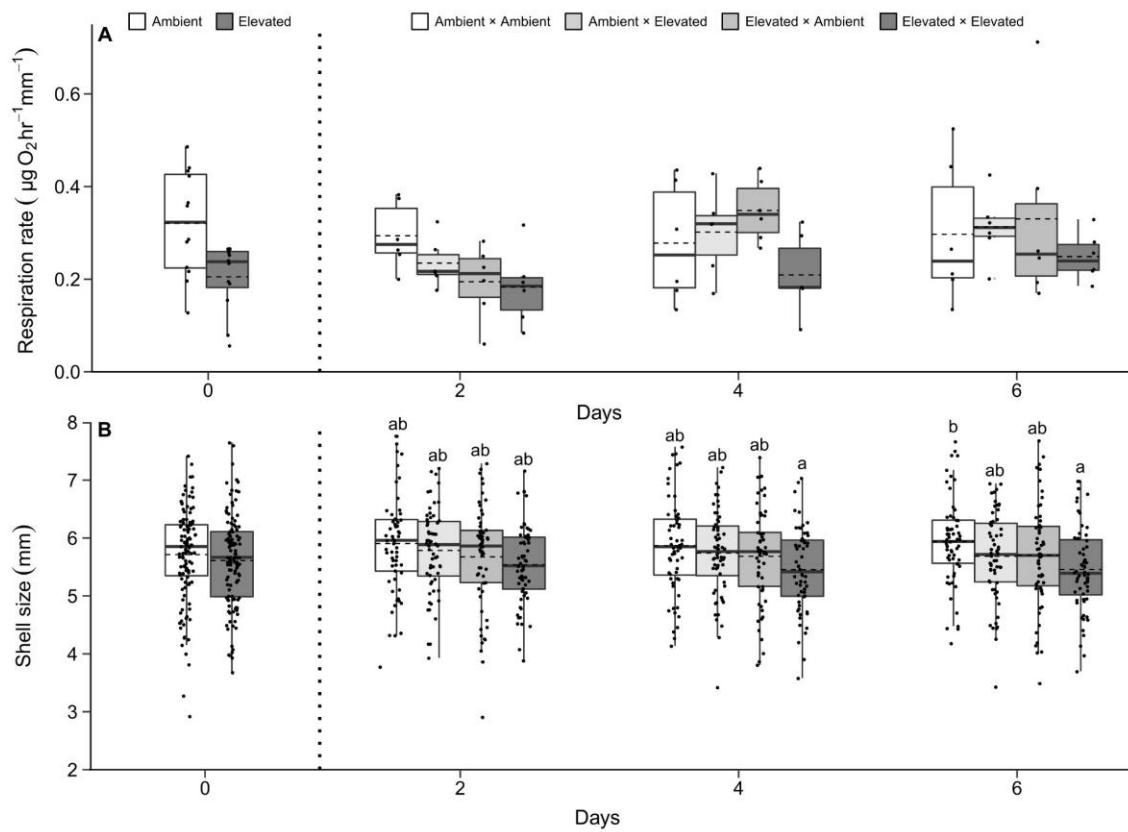


Figure 3. Respiration rates (A) and shell length (B) of juvenile geoduck under the secondary 6-day exposure.

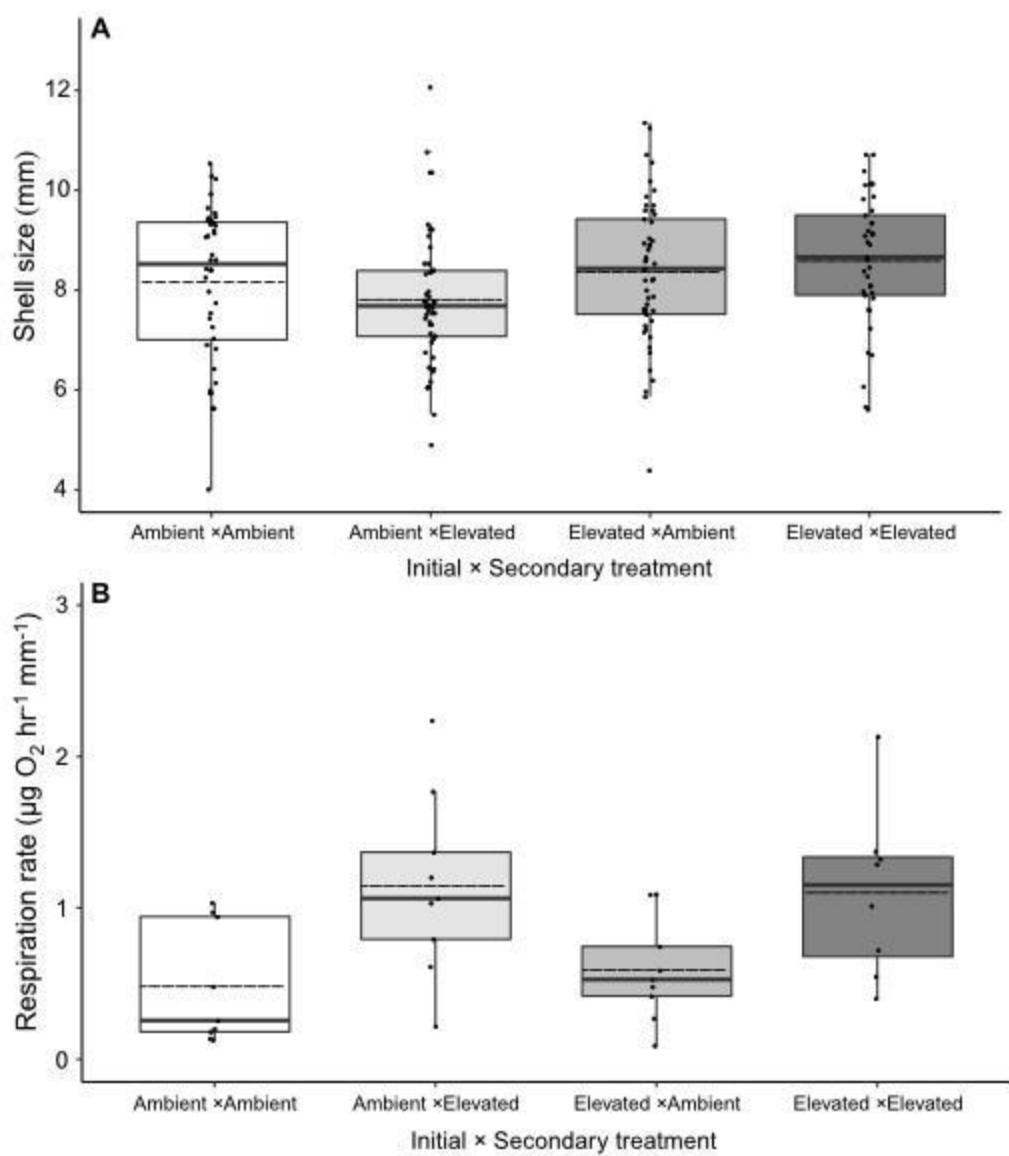


Figure 4. Shell length (A) and metabolic rates (B) of juvenile geoduck after 157 days in ambient common garden conditions post-exposure.

**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 PCO_2 (920 μatm and 2800 μatm , respectively) for 110 days. Then, clams were exposed to ambient, moderate or severely elevated PCO_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 PCO_2 (3000 μatm). Initial conditioning to moderate PCO_2 stress followed by second and third exposure to severe and moderate PCO_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

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_{CO₂}), 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 Frankenhuys, 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 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 PCO₂-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 PCO₂/low Ω_{arg} conditions, including compensatory respiration rates and shell growth (Gurr et al., 2020a). 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 PCO₂ 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 PCO₂ 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 PCO₂ treatments

Ambient hatchery conditions and local buoy data contextualize the choices of PCO₂ to test responses under ‘pejus’ and ‘pessimum’ range (Sokolova et al., 2012; Sokolova, 2021). First, as control PCO₂ conditions, incoming hatchery ambient seawater temperature, salinity, pH, and PCO₂ 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’ PCO₂ (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, 1991) 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’ PCO₂ (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_{CO₂} 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=1.5×10⁴ 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_{CO₂} condition.

Modified reciprocal exposure

Second exposure

To begin the second exposure, juvenile geoducks (~ 2200 geoducks per initial PCO_2 treatment) were rinsed on a $3 \times 10^5 \mu\text{m}$ 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 PCO_2 conditions were subjected to a second exposure period (7 days; Fig. 1) in three PCO_2 conditions: ambient ($754 \pm 15 \mu\text{atm}$), moderate ($2750 \pm 31 \mu\text{atm}$) or severe ($4940 \pm 45 \mu\text{atm}$; Table 1).

Ambient recovery

After the second exposure, PCO_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).

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 ($967 \pm 9 \mu\text{atm}$) or moderate PCO_2 ($3030 \pm 23 \mu\text{atm}$; 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 PCO_2 exposure), 8, 11, 14 (ambient recovery), 15, 18 and 21 (third PCO_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. 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 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 PCO_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 PCO_2 condition and pH of 6.8 for the severe PCO_2 condition (pH in NBS scale). pH and temperature ($^{\circ}\text{C}$) were measured every 10 s by logger probes (Neptune Systems; accuracy: ± 0.01 pH units and $\pm 0.1^{\circ}\text{C}$; resolution: ± 0.1 pH units and $\pm 0.1^{\circ}\text{C}$) 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 (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:

$1 \mu\text{S cm}^{-1}$ to 200 mS cm^{-1} ; accuracy: $\pm 0.01 \text{ psu}$) and temperature (Fisherbrand Traceable Platinum Ultra-Accurate Digital Thermometer; resolution; 0.001°C ; accuracy: $\pm 0.05^\circ\text{C}$). 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 PCO_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 CO₂ 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, CO₂, PCO_2 , HCO^{3-} , CO₃, Ω_{arag} and Ω_{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 $0.2 \mu\text{m}$ -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 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 ($\mu\text{g O}_2 \text{ l}^{-1}$) 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 by the package authors (Olito et al., 2017) and following Gurr et al. (2020a) 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 ($\mu\text{g O}_2 \text{ h}^{-1} \text{ individual}^{-1}$).

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 (μ moles CRE mg protein $^{-1}$). Sample aliquots for total protein were solubilized by adding 10 μ l 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 PCO_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 PCO_2 treatments and random effect of time during the second PCO_2 exposure, ambient recovery, and third PCO_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 PCO_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 PCO_2 acclimation (Table S1; Welch's t-test; primary, $t=-0.602$, $df=31.725$, $P=0.5516$); however, the shell length of geoducks under moderate PCO_2 was significantly larger, by 2.6%, compared with those under ambient treatment (Table S1; Welch's t-test; primary, $t=-4.297$, $df=2884$, $P<0.0001$). Under the second exposure, there was no significant effect of PCO_2 treatments on respiration rate and shell length. Juvenile clams acclimated under moderate PCO_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 PCO_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 PCO_2 treatments (Table S1).

Third exposure to hypercapnic seawater

The interaction of primary and second PCO_2 treatments had a significant effect on respiration rate under the third exposure period (Table S1; LME; primary \times second,

$F_{2,198}=3.810$, $P=0.024$), with this interaction primarily driven by a 20.4% greater respiration rate in PCO_2 stress-acclimated animals exposed to severe PCO_2 than ambient PCO_2 during the second period (Fig. 3), although the post hoc test was only marginally significant (Tukey HSD; moderate \times severe $>$ moderate \times ambient, $P=0.0992$). Shell growth was affected by an interaction between primary, second and third PCO_2 treatments (Table S1 and Fig. 3; four-way ANOVA; primary \times second \times 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 PCO_2 , respectively (Fig. 3). At the end of the third exposure period (day 21), primary exposure under moderate PCO_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-way ANOVA; primary, $F_{1,56}=8.069$, $P=0.0063$) with 22% greater $\mu\text{mol CRE}_{\text{red}} \text{ g}^{-1}$ protein by clams reared under ambient PCO_2 (Fig. 2); there was no effect of PCO_2 treatment or two-way and three-way interactions of PCO_2 treatments on total protein (Table S2 and Fig. 2). The effects of PCO_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 PCO_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 PCO_2 regimes ($\sim 2500\text{--}5000 \mu\text{atm}$) 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’ ($\sim 2900 \mu\text{atm} \text{PCO}_2 0.4 \Omega_{\text{arg}}$) and ‘severe’ ($\sim 4800 \mu\text{atm} \text{PCO}_2 0.2 \Omega_{\text{arg}}$) reciprocal exposures highlight 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 \times severe \times moderate) had both greater respiration rates and shell size (Table S1; Fig. 2). Furthermore, stress-acclimated individuals had greater organic biomass and lower amounts of antioxidant proteins relative to ambient controls (Fig. 3), 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., 2020a). Contrary to our findings, similar PCO_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, PCO_2 tolerance limitations are likely species specific, as well as life stage, duration and stress-intensity specific.

PCO_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., 2020a). 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 PCO_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 PCO_2 leads to persistent negative effects (i.e. reduced shell growth and development) in Pacific oyster *Crassostrea 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 PCO_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. PCO_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 PCO_2 and aragonite undersaturation with episodic/seasonal variation (surface water $\Omega_{\text{arg}} < 1$ in winter months, Dabob Bay in Hood Canal, WA; Fassbender et al., 2018) and geographical ($> 2400 \mu\text{atm}$ and $\Omega_{\text{arg}} < 0.4$ in Hood Canal, WA; Feely et al., 2010) and vertical heterogeneity (Reum et al., 2014) comparable to gradients within sub-surface sediments ($\Omega_{\text{arg}} 0.4\text{--}0.6$; 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.

Table 1. Seawater carbonate chemistry.

Treatment	<i>N</i>	Salinity	Temperature	pH (total scale)	CO_2 ($\mu\text{mol kg}^{-1}$)	P_{CO_2} (μatm)	HCO_3 ($\mu\text{mol kg}^{-1}$)	CO_3 ($\mu\text{mol kg}^{-1}$)	DIC ($\mu\text{mol kg}^{-1}$)	Total alkalinity ($\mu\text{mol kg}^{-1}$)	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	24.6±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

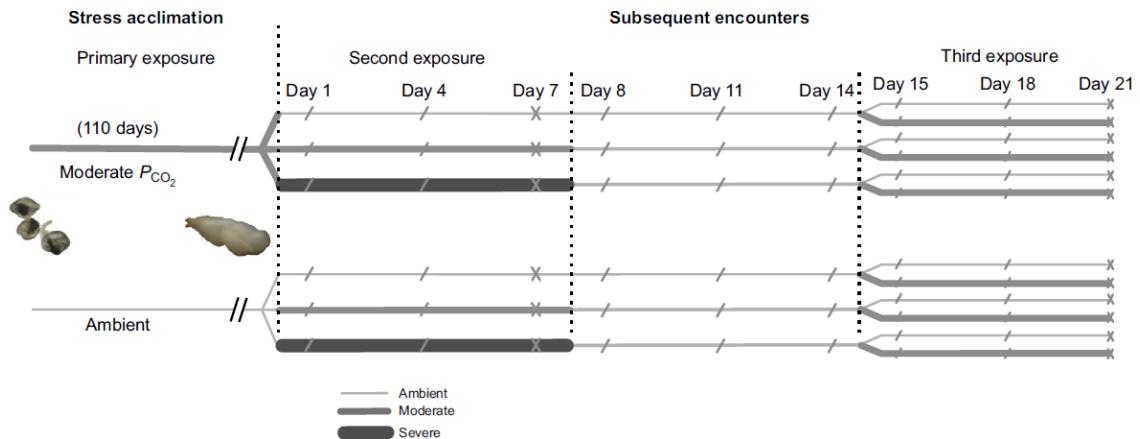


Figure 1. Schematic of the experimental design.

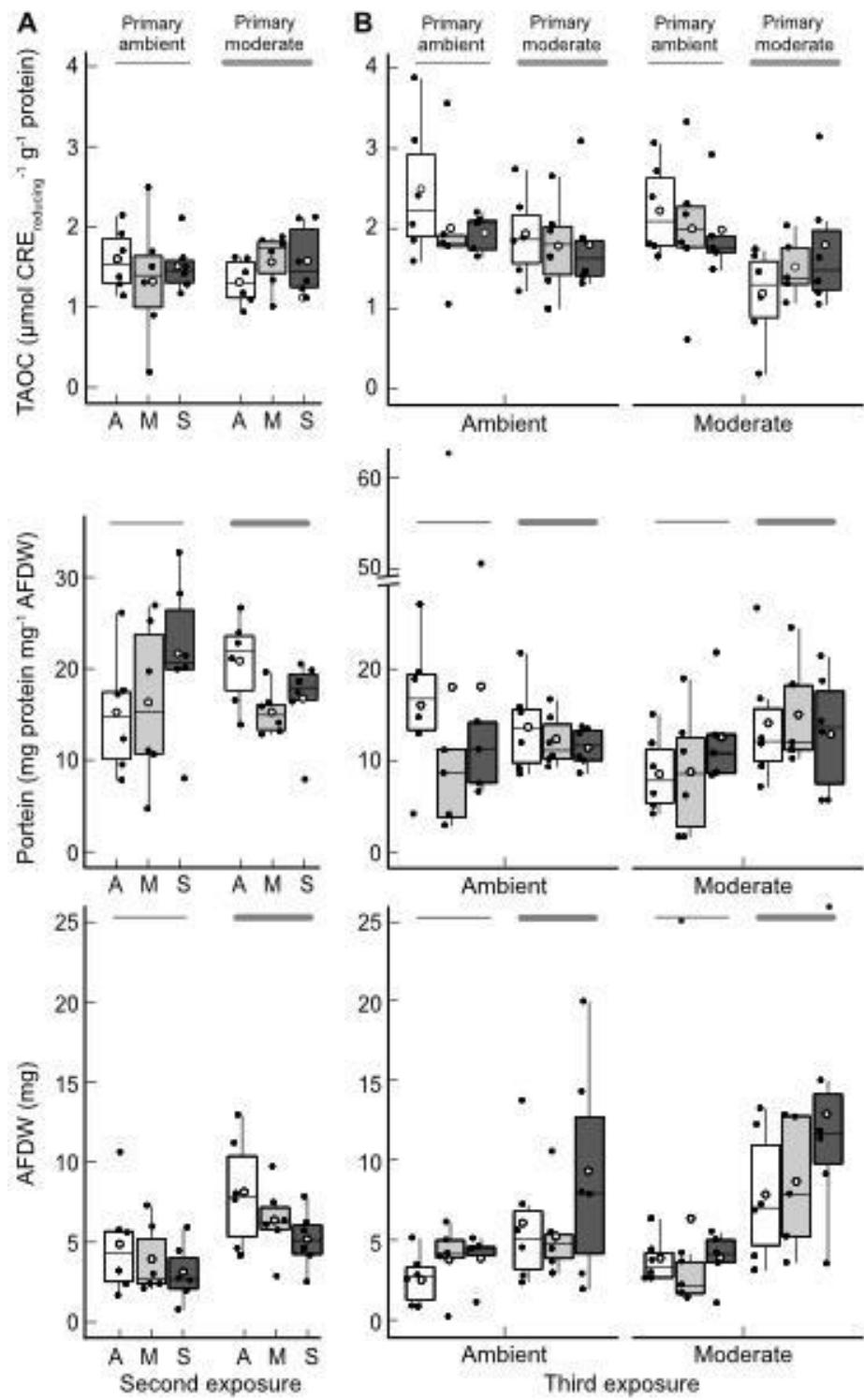


Figure 2. Antioxidant response and physiology of fixed Pacific geoduck (*Panopea generosa*) at the end of second and third exposure periods.

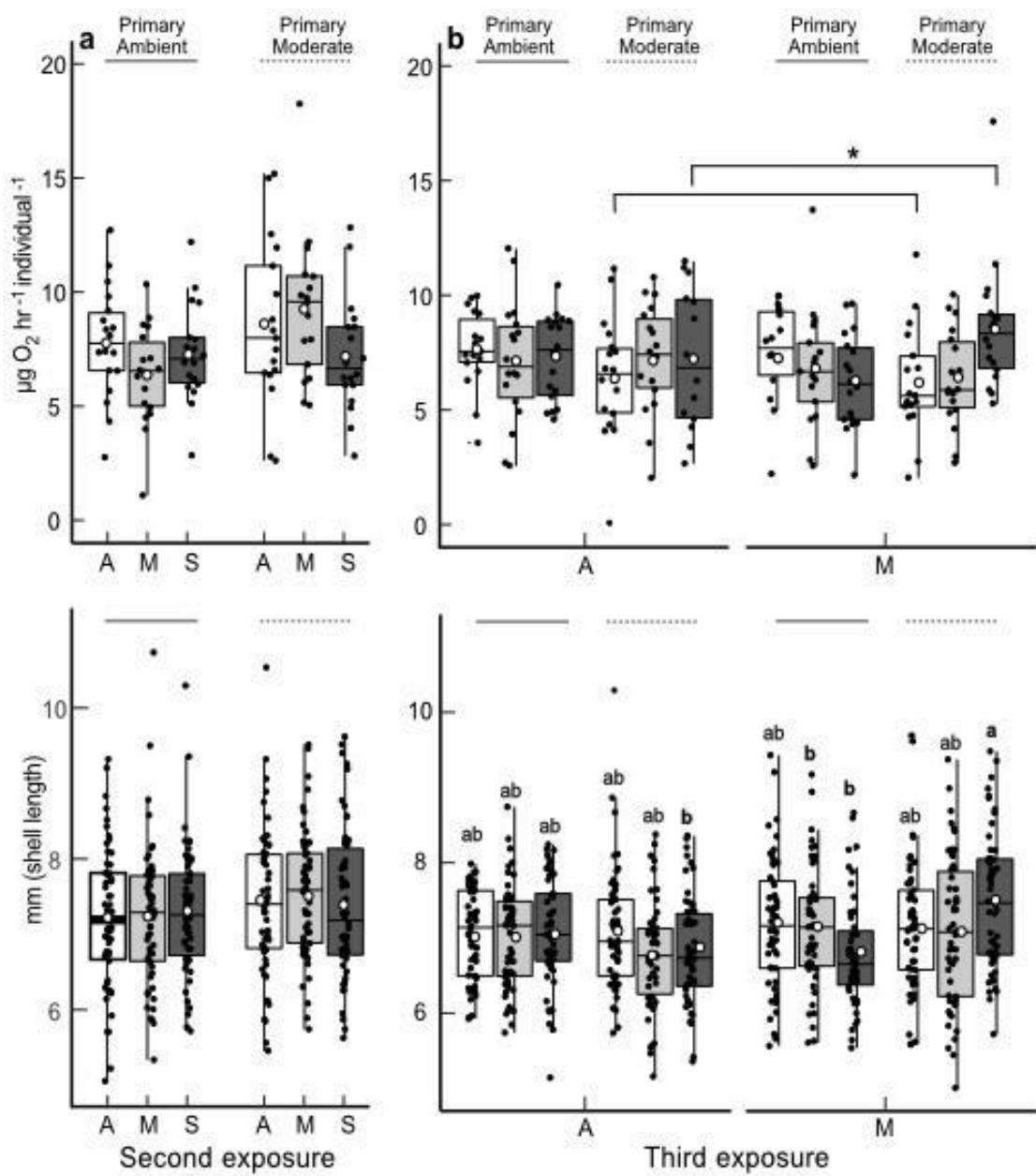


Figure 3. Respiration rate and shell length of geoducks under second and third exposure periods.

Supplementary Table S1. Effects of $p\text{CO}_2$ stress exposures on mean respiration rate and shell growth of *P. generosa*.

Effect	Respiration rate			Shell length		
	df	F	P	df	F	P
Pre-experiment	<i>t-test</i>					
$p\text{CO}_2$ primary	31.725	-	0.552	2884	-	<0.0001
Days 1-7	<i>Three-way ANOVA</i>					
$p\text{CO}_2$ primary	1.99	3.503	0.064	1,316	3.489	0.063
$p\text{CO}_2$ second	2.99	0.593	0.554	2,316	0.139	0.871
$p\text{CO}_2$ primary \times $p\text{CO}_2$ second	2.99	2.870	0.067	2,316	0.238	0.788
Days 8-14	<i>Three-way ANOVA</i>					
$p\text{CO}_2$ primary	1.97	0.112	0.739	1,316	1.678	0.196
$p\text{CO}_2$ second	2.97	2.232	0.113	2,316	0.656	0.520
$p\text{CO}_2$ primary \times $p\text{CO}_2$ second	2.97	0.969	0.383	2,316	0.052	0.950
Days 15-21	<i>Four-way ANOVA</i>					
$p\text{CO}_2$ primary	1,198	0.019	0.890	1,628	7.786	0.005
$p\text{CO}_2$ second	2,198	0.728	0.484	2,628	1.200	0.302
$p\text{CO}_2$ third	1,198	0.544	0.462	1,628	0.296	0.587
$p\text{CO}_2$ primary \times $p\text{CO}_2$ second	2,198	3.810	0.024	2,628	0.412	0.662
$p\text{CO}_2$ primary \times $p\text{CO}_2$ third	1,198	0.891	0.347	1,628	8.399	0.004
$p\text{CO}_2$ second \times $p\text{CO}_2$ third	2,198	0.340	0.671	2,628	3.853	0.022
$p\text{CO}_2$ primary \times $p\text{CO}_2$ second \times $p\text{CO}_2$ third	2,198	1.772	0.173	2,628	6.360	0.002
Significant P-values (< 0.05) are bolded; marginal P-values (<0.1) in <i>italics</i>						

Supplementary Table S2. Effects of $p\text{CO}_2$ stress exposures on antioxidant capacity, total protein, and organic biomass (AFDW) of *P. generosa*.

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>								
$p\text{CO}_2$ primary	1,30	0.005	0.942	1,30	0.003	0.959	1,30	9.313	0.0047
$p\text{CO}_2$ second	2,30	0.143	0.867	2,30	0.866	0.431	2,30	2.536	0.096
$p\text{CO}_2$ primary \times $p\text{CO}_2$ second	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>								
$p\text{CO}_2$ primary	1,56	8.069	0.0063	1,56	0.009	0.926	1,56	12.899	<0.001
$p\text{CO}_2$ second	2,56	0.164	0.849	2,56	0.018	0.983	2,56	1.578	0.2153
$p\text{CO}_2$ third	1,56	2.161	0.1471	1,56	135.1	0.245	1,56	3.298	0.0747
$p\text{CO}_2$ primary \times $p\text{CO}_2$ second	2,56	1.43	0.248	2,56	0.309	0.735	2,56	1.756	0.1822
$p\text{CO}_2$ primary \times $p\text{CO}_2$ third	1,56	0.678	0.4136	1,56	3.605	<i>0.063</i>	1,56	0.453	0.5036
$p\text{CO}_2$ second \times $p\text{CO}_2$ third	2,56	0.752	0.476	2,56	0.038	0.963	2,56	0.166	0.8906
$p\text{CO}_2$ primary \times $p\text{CO}_2$ second \times $p\text{CO}_2$ third	2,56	0.141	0.8688	2,56	0.105	0.901	2,56	0.181	0.8353
Significant P-values (< 0.05) are bolded; marginal P-values (<0.1) in <i>italics</i>									

**ENVIRONMENTAL CONDITIONING OF CLAMS TO LOW PH ENHANCES
ROBUSTNESS TO ENVIRONMENTAL STRESS
THROUGH ACCLIMATORY GENE EXPRESSION**

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ABSTRACT

Post-larval acclimation to hypercapnic seawater improves performance in terms of growth and oxidative status of juvenile Pacific geoduck *Panopea generosa*, indicating the potential for transcriptional shifts to drive modified organismal and cellular phenotypes. Following three-months of conditioning immediately post-settlement under ambient and moderately-elevated $p\text{CO}_2$, repeated hypercapnia and an ambient depuration period elicited variation of transcriptome profiles between stress-acclimated and naïve juvenile geoducks. Stress-acclimated geoducks were rapidly responsive to change, showing fine-tuned gene expression for quality control of mitochondria and immune defense during hypercapnia, and increased gene expression involved in energy metabolism and biosynthesis during ambient recovery. Furthermore, continuous gene ontology enrichment included histone methyltransferases and transcription factors, illustrating that moderate-stress history may frontload transcriptional modifiers. In contrast, the naïve animals showed greater transcriptional demand and continuous enrichment for fatty-acid degradation and glutathione components suggesting unsustainable energetic requirements if changes in carbonate chemistry exacerbated or persisted. Altogether, transcriptomic findings complement physiological phenotypes, supporting beneficial gene-expression regulation and cellular maintenance by the acclimatized phenotype as opposed to putative depletion of endogenous fuels to supply broad transcription in absence of prior stress experience. Post-larval acclimatory periods, predecessor to episodic changes, can enhance robustness to environmental stress in juvenile *P. generosa*.

INTRODUCTION

Climate change exerts growing pressures to marine life and is projected to intensify in the near-future. In particular, global environmental phenomena such as ocean acidification, or the reduction of ocean pH due to absorption of atmospheric CO₂, and increasing marine heat waves are devastating marine life and thus ocean ecosystems (Lotze *et al.*, 2006). Moreover, low pH conditions are magnified in coastal systems and co-occur with multiple stressors (Cai *et al.*, 2011; Melzner *et al.*, 2013) presenting a growing concern for aquaculture (Barton *et al.*, 2012, 2015). In isolation, OA affects essential cellular processes (e.g. acid-base homeostasis and energy metabolism; (Michaelidis *et al.*, 2005; Dineshram *et al.*, 2013) and shell formation and survival for calcifying organisms, especially during early development and metamorphosis (Kurihara *et al.*, 2007; Waldbusser *et al.*, 2015; Kapsenberg *et al.*, 2018). Thus, understanding species' capacity for acclimation is important, but remains understudied.

Species success and environmental resilience depends on integration of predictable environmental cues by developmentally modulated phenotypes. Environmental variation (spatial and temporal) shapes phenotypes (Dowd *et al.*, 2015) and numerous studies support an acclimatory capacity for marine invertebrates to cope with episodic (Suckling *et al.*, 2015; Détrée and Gallardo-Escárate, 2018; Gurr *et al.*, 2020; Li *et al.*, 2020) and cross-generational periods of elevated pCO₂ (Parker *et al.*, 2015; Goncalves *et al.*, 2016). Furthermore, although larvae are highly susceptible to changes in the surrounding environment, early life presents an ideal “window” for developmental acclimation due to the importance of environmental information in setting the stage for subsequent phenotypic outcomes (Burton and Metcalfe, 2014; Fawcett and Frankenhuys, 2015). Thus, the timing

and stress magnitude of environmental change likely have a joint effect on plasticity (Donelson *et al.*, 2018). Beneficial or maladaptive carryover of stress history (Parker *et al.*, 2012; Hettinger *et al.*, 2013) will have ecological and evolutionary implications, so it is essential to understand how external signals, such as those from climate change stressors, are transduced to elicit acclimatory patterns under repeated stress.

Gene expression regulation is key to homeostasis, thus has important consequences. In molluscs, gene-expression regulation during early development contributes to emergent phenotypes (Riviere *et al.*, 2017; Fellous *et al.*, 2019). Thus, transcriptomics is a broad and sensitive approach to assess global gene expression and expand upon genomic markers and economic traits in aquaculture (Chandhini and Kumar, 2019). Transcriptome profiling of clams and oysters found differential regulation of mitochondrial complexes, antioxidants, and lipid degradation in response to environmental change (Chapman *et al.*, 2011; Goncalves *et al.*, 2017; López-Landavery *et al.*, 2021; Teng *et al.*, 2021), suggesting that external abiotic conditions can affect metabolism and shift substrates for bioenergetics. Furthermore, pre-preparatory transcript accumulation, or gene frontloading, is a proposed mechanism to cope with unpredictable or novel changes in the environment (Barshis *et al.*, 2013). For instance, limpets (*Lottia sp.*) occupying the high intertidal upregulate heat-shock proteins relative to low-intertidal individuals suggesting a preemptive gene-level response (Dong *et al.*, 2008). Acclimatory patterns are commonly used to infer divergent evolutionary adaptations among populations, whereas rapidly-induced variation of transcriptome profiles remains understudied.

Geoduck clams (*Panopea sp.*) are long-lived molluscs of high economic value and recent studies corroborate their particular resilience to low pH (Spencer *et al.*, 2019; Gurr

et al., 2020, 2021). Transcriptome profiles of larval geoduck (*Panopea globosa*) and Pacific geoduck (*Panopea generosa*) reared under hypercapnic seawater (elevated $p\text{CO}_2$) found umbonate-stage larvae regulate energy production and acid/base homeostasis (López-Landaverry *et al.*, 2021), as opposed to delayed metamorphosis and molecular metabolic shifts during early stages post-fertilization (Huo *et al.*, 2019; Timmins-Schiffman *et al.*, 2020). Post-settlement, juvenile *P. globosa* upregulate genes for DNA repair and transcriptional regulation during chronic thermal stress (Juárez *et al.*, 2018) and intermittent exposures of juvenile *P. generosa* under OA conditions elicits compensatory growth and metabolism (Gurr *et al.*, 2020) and differential DNA methylation (Putnam *et al.* 2017). In a previous study by Gurr et al. (Gurr *et al.*, 2021), *P. generosa* at settlement-competency (pediveliger stage) were acclimated under hypercapnic and ambient seawater before juveniles were exposed under repeated hypercapnia; the stress-acclimated phenotype was larger (tissue biomass and shell length) and decreased total antioxidant capacity supporting a physiological benefit of stress history. Transcriptome profiling of *P. generosa* has provided critical molecular insight on negative effects of low-pH exposure (Timmins-Schiffman *et al.*, 2020), opening interest in transcriptomics to expand upon findings in Gurr et al. (2021) and determine mechanisms underpinning developmental acclimatization in the following questions: Does stress acclimation affect transcriptome profiles under repeated exposure(s)? Are there distinct gene functions and pathways underpinning phenotypic benefits of early-life stress?

METHODS

pCO₂ exposure experiment and tissue sampling

Larval Pacific geoduck were reared from gametes at the Jamestown Point Whitney Shellfish Hatchery (Brinnon, WA) following standard industry practice before a 110-day stress-acclimation period followed by a 21-day full-factorial repeat exposure to hypercapnic (high *pCO₂*) conditions (detailed in Gurr et al. 2021). In brief, once animals reached settlement competency (~30 days post-fertilization), pediveliger larvae were exposed to ambient and elevated *pCO₂* conditions ($921 \pm 41 \mu\text{atm}$ and $2870 \pm 65 \mu\text{atm}$) for an initial 110-day acclimatory period targeting the metamorphic transition from pediveliger to the burrowing juvenile stage (Table S1; $N=4$ trays treatment⁻¹ and $N=1.5 \times 10^4$ pediveligers tray⁻¹). Juveniles acclimated under ambient and elevated *pCO₂* were divided at equal density into 36 replicate cups ($N=6$ cups treatment⁻¹), and subjected to a secondary 7-day period under three *pCO₂* conditions (ambient *pCO₂*= $754 \pm 15 \mu\text{atm}$, moderate *pCO₂*= $2750 \pm 31 \mu\text{atm}$, and severe *pCO₂*= $4940 \pm 45 \mu\text{atm}$) followed by 7 days of ambient recovery ($896 \pm 11 \mu\text{atm}$) before replicates were split into 72 cups ($N=6$ cups treatment⁻¹) for a 7-day third exposure in two conditions (ambient *pCO₂*= $967 \pm 9 \mu\text{atm}$ and moderate *pCO₂*= $3030 \pm 23 \mu\text{atm}$; Table S1). Note these are all elevated *pCO₂* relevant to the native range of *P. generosa* as they correspond to values at local sites and sediment conditions where the clams live (e.g. Hood Canal in Puget Sound, WA; (Feely *et al.*, 2010; Reum *et al.*, 2014). As previously described in Gurr et al. (2021), the stress-acclimated phenotype reduced total antioxidant capacity and increased shell growth and tissue biomass under subsequent stress encounters as evidence supporting the pediveliger-to-juvenile ‘window’ for adaptive developmental plasticity. In this study, samples were sequenced at the same

timepoints in Gurr et al (2021) to investigate transcriptome profiles attributed with phenotypic outcomes. Whole juveniles from each replicate tray and cup were snap frozen in liquid nitrogen between 9:00-11:00 on the final day of the initial stress-acclimation period ($N = 8$; Fig. 1 day 0; after 110-day acclimation period) and secondary exposure ($N = 36$; Fig. 1 day 7), ambient recovery ($N = 35$; Fig. 1 day 14), and third exposure ($N = 62$; Fig. 1 day 21); note that days 14 and 21 do not contain the maximum replication (6 sampling treatment⁻¹) due to lost or unavailable samples for extractions.

TagSeq data

Whole juvenile geoduck samples ($N = 141$) were thawed individually in 1 ml DNA/RNA shield and homogenized with 0.25 ml 0.5 mm glass beads (vortexed for ~1 minute). Total RNA was extracted from whole tissue homogenate using the Quick-DNA/RNA Kit (Zymo) according to manufacturer's instructions. RNA quantity was determined using RNA Broad Range Assay Kit with Qubit fluorometer (ThermoFisher) and quality was ascertained using 4200 TapeStation System for ribosomal bands (Agilent Technologies). RNA samples (10 ng μl^{-1}) were used for TagSeq, a 3' short transcript method that allows cost-effective and accurate estimation of transcript abundances relative to traditional RNAseq (Lohman et al. 2016). Library preparation was adapted for sequencing on two lanes of Illumina NovaSeq 6000 SR100 targeting standard coverage of 3-5 million 100 bp single-end reads (University of Texas Austin, Genomic Sequencing and Analysis Facility). Raw reads were trimmed of Illumina adapters, poly-A, and quality filtered with fastp; quality control for filter optimization was completed using MultiQC.

The *P. generosa* reference genome contains 34,947 putative coding sequences, 42% (14,671) with gene name and gene ontology (GO) annotation (doi:10.17605/OSF.IO/YEM8N; Roberts et al. 2020). Reads were mapped to the *P. generosa* reference transcriptome using HISAT2 with a mapping efficiency of ~30%. Unique counts averaged $810,290 \pm 165,381$ reads sample $^{-1}$ (mean \pm SD; $N = 114,250,931$ total reads) representing a total of 29,335 unigenes with at least a single read, or 83.94% of the reference. Stringtie2 was used to quantify reads and assemble a count matrix (using prepDE.py) for analysis in R v3.5.1 (<https://www.r-project.org>).

Gene expression analysis

Four raw read matrices for each sampling period (days 0, 7, 14, 21; Fig. 1) were filtered of genes with <10 counts per million ('edgeR' in R) in 50% of samples; these parameters account for the variability in reads sample $^{-1}$ and assume genes unexpressed due to the binary initial acclimation period (ambient and moderate $p\text{CO}_2$) remain for transcriptomic analysis. Pre-filtering resulted in $7.5\text{--}8.5 \times 10^5$ reads sample $^{-1}$ and $8,700 \pm 348$ unigenes matrix $^{-1}$, $72.12 \pm 0.004\%$ contained gene name and GO annotation.

Gene expression in response to stress acclimation and repeated stress encounters was analyzed with Weighted Gene Co-expression Network Analysis (WGCNA; 'WGCNA' in R) to assess expression patterns (Zhang and Horvath, 2005). Considering the full-factorial experimental design, co-expression network allows an assessment of broad expression-level directionality and the influence of compounding treatment history as opposed to pairwise differential expression analysis. Each matrix applied a soft threshold (scale-free topology $r^2 > 0.9$), minimum module size of 100, and a 'signed' adjacency

matrix. $p\text{CO}_2$ treatments were applied as categorical variables to compute eigenegenes and gene ‘modules’, or genes with common expression patterns. Modules were merged based on observed similarities within cluster dendograms and eigengene trees. Significant correlations with the treatment variables, or co-expression modules, contained a p-value <0.05. Variance-stabilizing transformation (‘*varianceStabilizingTransformation*’ in R) was applied to expression data to visualize gene expression patterns of co-expression modules. Enriched ‘molecular function’ and ‘biological process’ terms were computed with GOseq using Wallenius approximation preceding goSlim to condense significant GO terms ($p<0.05$) into hierarchical GO bins. goSlim applied a filtering criteria of >10 genes for each significant ‘biological process’ GO term and ≥ 2 genes for each significant ‘molecular function’ GO term. To understand higher-level functional processes of co-expression modules, blastx (diamond v.2.0.0) with a *P. generosa* genome query against a Pacific oyster *Crassostrea gigas* protein database (Zhang *et al.*, 2012) to acquire KEGG Orthology (KO) annotation based on sequence relatedness. *C.gigas* proteins annotated as ‘uncharacterized protein/family’ were omitted and KOs with the highest bit score (lowest e-value) were chosen for each *P. generosa* gene. The best hits contained 55 ± 18 percent identity and 129 ± 111 bitscore and accounted for 87.3% of the *P. generosa* genes. Enriched pathways (adjusted p-value<0.05) were computed using KEGG for *C.gigas* KOs (‘crg’; ‘*KEGGprofiler*’ in R) and online ‘KEGG Mapper’ was used to investigate gene functions in enriched pathways. Following analysis using all individuals, a parallel analysis was completed for animals under subsequent exposure to elevated $p\text{CO}_2$ (ambient $p\text{CO}_2$ omitted) to compare gene-expression patterns affected by subsequent $p\text{CO}_2$ stress.

To complement co-expression analysis and investigate pairwise $p\text{CO}_2$ effects, a second approach employed differential gene expression (DGE; using Bioconductor ‘*DESeq2*’ in R). Each pairwise DGE model applied a false discovery rate threshold of 5% (alpha=0.05); in all cases, histograms were observed for the distribution of p-values. Differentially expressed genes (DEGs) contained an adjusted p-value <0.05 and log-fold change $|x| > 0$. Pairwise models investigated the effects of acclimation (ambient v. moderate), second exposure (ambient v. moderate, ambient v. severe, and moderate v. severe) and third exposure (ambient v. moderate) and grouped contrasts to determine changes in gene expression due to cumulative treatment history (day 7 and 14: primary×second; day 21: primary×second×third). Functional enrichment of DEGs was computed with GOseq ($p<0.05$) preceding goSlim of terms into hierarchical GO bins; a less conservative criteria than WGCNA (≥ 2 for genes per bin) was applied for function functional interpretation of DEGs.

Raw sequence data is available on NCBI (BioProject: PRJNA740307, Title: ‘Transcriptome profiles of *Panopea generosa* under hypercapnic seawater’, Accessions: SAMN19838979-SAMN19839120) and analysis is publicly available in an open repository (osf: <https://osf.io/ydmt5/>; github: https://github.com/SamGurr/Pgenerosa_TagSeq_Metabolomics).

RESULTS

Co-expression network analysis overview

Network analysis resulted in three significant co-expression modules on day 7 (‘brown’, ‘yellow’, and ‘green’; Fig. 2), four on day 14 (‘brown’, ‘black’, ‘pink’, and

‘magenta’; Fig. 3), and seven on day 21 (‘blue’, ‘magenta’, ‘yellow’, ‘red’, ‘black’, ‘pink’, and ‘turquoise’; Fig. 4); $72 \pm 6\%$ of module-associated genes contained gene name and GO annotation. Modules on each sampling day were correlated with primary $p\text{CO}_2$ treatment and represented (1) ambient-effect modules or modules showing higher mean \pm SE gene expression due to ambient acclimation (day 7 ‘brown’, day 14 ‘brown’ and day 21 ‘blue’ and ‘magenta’) and (2) moderate-effect modules or modules showing higher mean \pm SE gene expression due to moderate $p\text{CO}_2$ acclimation (day 7 ‘yellow’, day 14 ‘black’ and day 21 ‘yellow’; Figs. 2-4). All ambient and moderate-effect modules, except day 21 ‘yellow’, were additionally correlated with cumulative $p\text{CO}_2$ history (Figs. 2-4). For example, genes within day 7 ‘brown’ were abundantly expressed by ambient-acclimated animals subsequently exposed to moderate $p\text{CO}_2$ exposure (‘AM’, Fig. 2). In contrast, genes within day 7 ‘yellow’ were more abundantly expressed by moderate $p\text{CO}_2$ -acclimated animals subsequently exposed to severe $p\text{CO}_2$ (‘MS’, Fig. 2). All other significant modules were not correlated with primary exposure, but with cumulative treatment interactions (day 7 and 14: primary \times second; day 21: primary \times second \times third). For example, genes within day 7 ‘green’ demonstrate a primary \times second $p\text{CO}_2$ treatment interaction with higher expression by naïve clams subsequently exposed to severe $p\text{CO}_2$, relative to stress-acclimated clams subsequently exposed to moderate $p\text{CO}_2$ (AS > MM, Fig. 2). All follow-up analysis (functional and pathway enrichment) and data interpretation focused on ambient-effect and moderate-effect modules to determine transcriptome profiles underpinning $p\text{CO}_2$ acclimation and divergent phenotypes in Gurr et al. (2021). Network analysis using only subsequently exposed animals (ambient treatment omitted) found significant co-expression in four modules on day 7, two modules on day 14, and four modules on day 21 (Figs. S1-

S3). Several of these modules showed similar expression patterns to modules applying all samples (e.g. Fig. S1: day 7 ‘turquoise’, ‘blue’, ‘brown’, and ‘greenyellow’; Fig. S2: day 14 ‘blue’; day 21: ‘blue’) for meaningful expansion of the transcriptome profiles post-acclimation.

GO analysis of primary-effect modules: persistent functions

Continuously regulated functions were determined as persistent GO terms and/or bins significantly enriched on all sampling days. Ambient-effect modules were continuously enriched for the following hierarchical GO bins and associated terms: ‘transport’ (i.e. intracellular protein transport), ‘lipid binding’ and ‘ion binding’ (i.e. sterol, fatty acid, cholesterol, and phosphatidylinositol binding), ‘peptidase activity’ (cysteine and serine-type endopeptidase activity), and ‘oxidoreductase activity’ (i.e. glutathione peroxidase activity, acyl-CoA oxidase, alcohol dehydrogenase, and carbonyl reductase activity; Fig. 5, S4A, and S5). The following genes were largely attributed with ambient-effect modules: ‘transport’: WASH complex subunits, AP-2 complex subunits, ras-related proteins, and sorting nexins; ‘ion binding’: components of fatty-acid metabolism (acyl-CoA dehydrogenases, peroxisomal acyl-coenzyme A, and fatty-acid binding proteins) and sorting nexins; ‘lipid binding’: apolipoprotein D; ‘oxidoreductase activity’: components of β -oxidation and fatty acid degradation pathways (e.g. alcohol dehydrogenase, peroxisomal acyl-coenzyme A, peroxisomal bifunctional enzyme), glutathione components (e.g. glutaredoxin-1, glutathione S-transferase omega-1 and glutathione peroxidases), and cytochrome P450; ‘peptidase activity’: cathepsins.

Moderate effect modules lacked persistent GO term-level enrichment, but hierarchical GO bins were continuously enriched throughout the experiment for ‘ion binding’ (e.g. copper and metal ion binding), ‘cellular nitrogen compound metabolic process’ (e.g. positive and negative regulation of transcription), ‘methyltransferase activity’ (e.g. histone methyltransferase activity), ‘transcription factor binding’ (e.g. myogenic regulatory factors and NF-kappa β binding), and ‘RNA binding’ (Fig. 5, S4B and S6). The following genes or gene families were largely attributed with moderate-effect modules; ‘ion binding’: chromatin modifiers (histone methyltransferases and chromatin-remodeling ATPase), E3 ubiquitin-protein ligases, ion exchange (e.g. sodium/calcium exchanger 3 and sodium/potassium-transporting ATPase), kinases (mitogen-activated protein kinases and serine/threonine-protein kinases), and zinc finger proteins; ‘cellular nitrogen compound metabolic processes’: transcriptional regulators (e.g. post-translational modifications [histone methyltransferases, histone acetyltransferases, chromatin-remodeling ATPase, and sirtuin 1], transcription factors [e.g. protein max, forkhead box protein O, hypoxia-inducible factor 1-alpha, homeobox protein SIX4, AT-rich interactive domain-containing protein 4B], zinc finger proteins, and proteasome activity [e.g. 26S and E3 ubiquitin-protein ligases]), mRNA export (e.g. nuclear pore complex protein Nup85 and transcription and mRNA export factor ENY2), and signaling (e.g. NF-kappa β activation and innate immune response genes); ‘methyltransferase activity’: histone-lysine-N-methyltransferases (e.g. EHMT1, NSD2, SETD5, and ASH1L). ‘transcription factor binding’: general regulation of transcription (e.g. transcription initiation factor IIA subunit 1, Krueppel-like factor 5, CCR4-NOT transcription complex subunit 1), alternative

splicing (SNW domain-containing protein 1), and histone acetylation (e.g. CREB-binding protein and breast carcinoma-amplified sequence 3).

GO analysis of primary-effect modules: transient functions

Remaining results of GO analysis demonstrated transient patterns in three categorical groups: (1) ‘Stress-induced and recovery’ represents enriched functions on days 7 or shared between day 7 and 14, (2) ‘recovery and preparatory regulation’ represents enriched functions on day 14 or shared between days 14 and 21, and (3) ‘rapidly induced under stress exposures’ represents enriched functions on days 7 and 21 (Fig. 5).

‘Stress-induced and recovery’ represents a response to hypercapnic/low-pH seawater and subsequent carry-over during ambient conditions. In this category, ambient-effect modules were enriched for ‘immune system response’ (neutrophil degranulation) and ‘enzyme binding’ (mitogen-activated protein kinase, Rab GTPase, protein kinase, RNA polymerase, and ubiquitin-specific protease binding) and moderate-effect modules were enriched for ‘signal transduction’ (serine/threonine-protein kinases, mitogen kinase signaling, and Wnt signaling), ‘cellular protein modification’ (E3 ubiquitin-protein ligases, serine/threonine-protein, mitogen-activated kinases, and kelch-like proteins), and ‘enzyme binding’ (Fig. 5, and S4-S6).

Second, ‘recovery and preparatory regulation’ represents both a depuration of hypercapnia/acidosis and putative indication of prepreatory gene frontloading. Ambient-effect modules were enriched for ‘response to stress’ (e.g. blood coagulation) on day 14 and ‘lipid metabolic process’ (fatty acid and cholesterol metabolic processes), ‘catabolic process’ (autophagy and proteolysis), ‘enzyme regulatory activity’ (ATPase and

endopeptidase inhibitors and GTPase activator activity) and ‘transmembrane activity’ (i.e. proton exporting/transporting ATPase activity) on days 14 and 21 (Fig. 5 and Figs S4A and S5). Moderate-effect modules were enriched on day 14 for ‘oxidoreductase activity’ (e.g. laccase 1 and 10, putative tyrosinase-like protein tyr-3, ferric-chelate reductase, and NADH dehydrogenase activity) and ‘transmembrane transporter activity’ (e.g. sodium:bicarbonate symporter and ATP synthase activity) and lacked GO bins unique to days 14 and 21 (Figs. S4B and S6).

Lastly, ‘rapidly induced under stress exposures’ confers a repeated response to hypercapnia. Ambient-effect modules lacked representation of this category (Figs. 5, S4A, and S5). In contrast, moderate-effect modules involved ‘response to stress’ and ‘immune system response’ (e.g. NF-kappa β activation [toll-like receptors 2, 3 and 4, TNF receptor-associated factor 6, MyD88, B-cell lymphoma 3 protein, and death-associated inhibitor of apoptosis 2], E3 ubiquitin ligases [HERC2, rnf168, TRIP12, and XIAP], and antiviral and antibacterial activity), ‘cell death’ (e.g. apoptotic and negative regulation of apoptotic processes), ‘cell motility’ (e.g. cell migration), ‘cytoskeletal protein binding’ (e.g. actin and beta-tubulin binding), ‘enzyme binding/regulatory activity’ (e.g. ubiquitin protein ligase, Rab GTPase, and small GTPase binding), and ‘kinase activity’ (e.g. tyrosine-, serine/threonine-, and mitogen-activated protein kinases) (Figs. 5, S4B, and S6).

KEGG pathway enrichment of primary-effect modules

Pathways analysis of ambient-effect modules found persistent enrichment on days 7, 14, and 21 for fatty-acid degradation ($N=11\pm 1$) and fatty acid metabolism ($N=12\pm 2$), peroxisome ($N=15\pm 3$), lysosome ($N=23\pm 4$), and endocytosis ($N=24\pm 8$) (Table S2 and Fig.

5). The following genes or gene families were continuously attributed with these pathways: ‘fatty-acid degradation’ and ‘fatty-acid metabolism’: acyl-CoA synthetase, dehydrogenases (alcohol, estradiol 17-beta, hydroxyacyl-CoA, medium-/long-chain specific acyl-CoA) very-long-chain 3-oxoacyl-CoA reductase, peroxisomal acyl-CoA oxidases 1 and 3, and carnitines (e.g. carnitine O-palmitoyltransferase 1 and 2 and carnitine O-acetyltransferase); ‘peroxisome’: 2-hydroxyacyl-CoA lyase 1, catalase, D-aspartate oxidase, peroxisomal membrane proteins, peroxisomal and acyl-CoA oxidases; ‘lysosome’: alpha-galactosidase A, AP-1 complex subunit beta 1, cation-independent mannose-6-phosphate receptor, clathrin light chain A, cathepsins (B and L1), epididymal secretory protein E1, galactocerebrosidase, ganglioside GM2 activator, sialin, and V-type proton ATPases’; ‘endocytosis’: actin-related proteins, ras-related proteins (i.e. Rab 8A), charged multivesicular body proteins, RUN and FYVE domain-containing protein 2, sorting nexins 2, 3 and 6, and vacuolar protein sorting-associated protein 29. Lastly, non-continuous pathways were significantly enriched for retinol metabolism on day 7 ($N=5$) and carbon metabolism on day 14 ($N=17$). Furthermore, co-expression modules without ambient exposures, day 7 ‘turquoise’ and day 21 ‘blue’, were also enriched for fatty-acid degradation ($N=7$ and 8) and peroxisome ($N=12$ and 13) (Table S3 and Figs. S1. and S3) with few genes expanding those enriched in ambient-effect modules (i.e. peroxisomal 2,4-dienoyl-CoA reductase and enoyl-CoA hydratase).

In contrast, moderate-effect modules were transiently enriched for endocytosis ($N=14$) on days 7, several pathways during ambient recovery on day 14 [pentose phosphate pathway ($N=5$), glycolysis / gluconeogenesis ($N=7$), carbon metabolism ($N=12$), proteasome ($N=6$), and biosynthesis of amino acids ($N=9$)], and mitophagy ($N=7$) on day

21 (Table S2 and Fig. 5). The following examples of genes or gene families were associated with enriched pathways: ‘endocytosis’: E3 ubiquitin-protein ligases (CBL-B and WWP1), receptor proteins (e.g. TNF, mannose-6-phosphate, G protein) and protein trafficking and transport (e.g. ADP-ribosylation factor 4, AP-2 complex subunit, charged multivesicular body proteins 2b and 5); ‘pentose phosphate pathway’ and ‘glycolysis / gluconeogenesis’: glycolytic enzymes (ATP-dependent 6-phosphofructokinase, glucose-6-phosphate isomerase, fructose-bisphosphate aldolase, phosphoglucomutase-1); ‘carbon metabolism’: glycolytic enzymes, citrate cycle (malate and isocitrate dehydrogenase), and non-oxidative phase of pentose phosphate cycle (transketolase-like protein 2); ‘proteasome’: ATP-dependent degradation of ubiquitinated proteins (26S proteasome non-ATPase regulatory subunits 1, 4, 13, and 14) and proteasome subunit beta type-5; ‘biosynthesis of amino acids’: glycolytic enzymes, non-oxidative pentose phosphate cycle, aminotransferases (aspartate and alanine, and methionine synthase; ‘mitophagy’: PINK1-Parkin components (serine/threonine-protein kinases TBK1 and PINK1), autophagy receptors (optineurin, sequestosome-1, tax-1 binding protein 1 homolog B), activation of ras-related rab-7a for lysosomal degradation (TBC1 domain family member 15), and forkhead box protein o transcription factor. Furthermore, co-expression modules computed without ambient exposures resemble expression patterns of moderate-effect modules and were enriched for additional functions (Table S3 and Figs. S1-S3). For instance, module day 7 ‘brown’ (Fig. S1) shows the same expression pattern to the full moderate-effect module day 7 ‘yellow’ (Fig. 2B) with additional pathways enriched for autophagy ($N=20$), mitophagy ($N=9$), and FoxO signaling ($N=11$) (Table S3 and Fig. S1C). Enrichment for FoxO pathway included mitogen-activated protein kinase signaling (e.g. mitogen-activated protein kinase 1,

GTPase HRas,growth factor receptor-bound protein, epidermal growth factor receptor), insulin signaling pathway (insulin receptor substrate 1, PTEN, and 3-phosphoinositide-dependent protein kinase), and cell apoptosis (tumor necrosis factor sf10). Furthermore, module ‘pink’ on day 7 (Fig. S1) reflects the gene expression pattern in moderate-effect module day 7 ‘black’ (Fig. 2B) and was additionally enriched for ‘oxidative phosphorylation’ ($N=12$; Table S3), involving NADH dehydrogenases, ATPases, and cytochrome c oxidase (Table S3).

Differential gene expression

DGE on Day 0 totaled 14 DEGs with fewer expressed by stress-acclimated individuals (A \times M, 3 down regulated genes) relative to naive, ambient exposed, individuals (A \times M, 11 upregulated genes; Table S4 and Fig. S7). Only four DEGs contained gene name and GO annotation and were upregulated for E3 ubiquitin-protein ligase rnf213-alpha and helicase with zinc finger domain and downregulated for putative isoforms for von Willebrand factor D protein.

Subsequent exposures on days 7, 14, and 21 showed greater transcriptional variation due to $p\text{CO}_2$ acclimation (primary treatment) than second or third $p\text{CO}_2$ treatments (Tables S4 and Fig. S7). Pairwise DE of primary ambient versus moderate $p\text{CO}_2$ acclimation yielded 108 DEGs on day 7 (62 upregulated and 49 downregulated), 429 DEGs on day 14 (317 upregulated and 112 downregulated), and 155 DEGs on day 21 (101 upregulated and 52 downregulated; Table S4). In summary, the majority of main-effect DEGs (primary exposure) in this study were upregulated by the naïve phenotype (70%), especially in response to ambient recovery (85% of upregulated DEGs; Fig. S7).

Upregulated genes were enriched for glutathione components (dehydrogenase, peroxidase, and transferase), endopeptidases, lipid metabolism, and transmembrane regulatory activity. Functional enrichment of downregulated genes showed common functions for signaling, oxidoreductase activity, stress response (transforming growth factor beta binding), and metal ion binding (Fig. S7). There were 22 (14 upregulated and 8 downregulated) DEGs that occurred on all sampling days with the same directionality, deemed as ‘persistent DEGs’ (Fig. S7). Thirteen of the 22 persistent DEGs had putative gene annotation; immune system response to bacteria was a common function among persistent DEGs (e.g. mucin-1, chitotriosidase-1, and defensin; Table S5). Upregulated genes notably differed in their functional annotation for lipid and calcium binding, signal transduction, and catabolic processes (e.g. apolipoprotein D, regucalcin, neuroendocrine convertase 1). Persistent downregulated genes, although fewer, were additionally associated with cobalt transport (cobalamin) and protease inhibition (CD109 antigen and BPTI/Kunitz domain-containing protein 4-like; Table S5).

Pairwise DGE models addressing second and third $p\text{CO}_2$ yielded minimal expression-level differences (0-13 total DEGs), with the exception of the second $p\text{CO}_2$ treatment on day 7 (106 total DEGs: 14 upregulated and 92 downregulated; Table S4). GO analysis of downregulated genes found enrichment of cell adhesion, plasminogen activation, and endopeptidase activity. Results of cumulative treatment histories on day 7 (primary \times second), found MA v. AM outweighed DE relative to other pairwise models ($N=16$) with 168 total DEGs (31 upregulated and 137 downregulated; Table S6); upregulated genes were enriched for actin filament polymerization, cell migration, and

cilium assembly and downregulated DEGs were primarily associated with plasminogen activation, cell adhesion, and proteolysis.

DISCUSSION

Postlarval acclimation to hypercapnic seawater affected transcriptome profiles. In Gurr et al. (2021), stress-acclimated clams increased somatic growth (tissue biomass and shell length) and decreased total antioxidant capacity relative to clams without previous exposure to elevated $p\text{CO}_2$. In this study, the naïve phenotype showed greater overall gene expression (65% of genes in primary-effect modules; >70% DEGs upregulated) suggesting that changes in carbonate chemistry increased transcriptional demand. Moreover, abundant transcripts in the naïve phenotype involved fatty-acid metabolism and glutathione components, highlighting degradation of endogenous fuels, primarily by peroxisome β -oxidation, as a favored energy source affecting somatic growth. In contrast, the transcriptome profile was fine-tuned for cellular homeostasis (e.g. cellular quality control, immune defense, and energy metabolism) and under putative control of transcriptional modifiers (e.g. histone methyltransferases and transcription factors) in the stress-acclimated phenotype. Altogether, this study corroborates physiological traits of emergent phenotypes (Gurr et al. 2021), to propose molecular mechanisms underpinning beneficial developmental acclimation and stress resilience.

Naïve profile: Endogenous lipids supply high transcriptional demand

A growing body of research suggests that environmental stress, such as low pH, increases energy partitioning toward protein biosynthesis (Langenbuch and Pörtner, 2002;

Pan *et al.*, 2015), conferring costs for somatic growth and storage retention (Stumpp *et al.*, 2011; Sokolova, 2013). In agreement, the main difference between transcriptome profiles was a higher transcriptional load by the naïve phenotype attributed with fatty-acid degradation and glutathione components (Figs. 5-6 and S7). Persistent gene enrichment for peroxisome activity (β -oxidation), acetyltransferase to mitochondria, and bioremediation of free radicals illustrates elevated use of endogenous metabolic fuel to satisfy broad transcriptional demand. Mobilization of endogenous reserves, primarily lipids, is essential to meet energetic requirements of early development (Waldbusser *et al.* 2003; Liu *et al.* 2020), but also plays a vital role in rapid provisions during stress exposure (Sokolova *et al.*, 2012; Teng *et al.* 2015; Ivanina *et al.* 2013). In marine calcifiers, exposure to elevated $p\text{CO}_2$ causes shell malformations and delayed settlement competency coupled with lipid loss and altered fatty-acid metabolism (Timmings-Schiffman *et al.*, 2014; Talmage and Gobler, 2010; Dickinson *et al.*, 2012; Liu *et al.*, 2020). For instance, elevated $p\text{CO}_2$ affects shell biomineralization and fatty-acid metabolism in the pearl oyster *Pinctada fucata* (Li *et al.*, 2016a, 2016b) and reorganizes the lipid profile in purple-hinge rock scallop *Crassadoma gigantea* (Alma *et al.*, 2020). Furthermore, upregulation of long-chain specific acyl-CoA dehydrogenase in the coral *Acropora millepora* and barnacle *Balanus amphitrite* (Wong *et al.*, 2011; Kaniewska *et al.*, 2012) and peroxiredoxins and carnitine O-acetyltransferase in larval oysters *Crassostrea virginica* and *Crassostrea hongkongensis* (Tomanek *et al.*, 2011; Dineshram *et al.*, 2015), highlights the importance of lipid degradation and peroxisome activity under elevated $p\text{CO}_2$. Pediveliger oysters *Crassostrea hongkongensis* demonstrate differential methylation of genes (exon regions) associated with fatty-acid metabolism (hypomethylated) and carbohydrate metabolism

(hypermethylated) under OA stress, suggesting that methylation patterns may drive this shift for lipid oxidation. In contrast, elevated $p\text{CO}_2$ may not affect fatty-acid metabolism (Matson *et al.*, 2012; Timmins-Schiffman *et al.*, 2014) or may interact with multiple stressors on lipid use (e.g. dietary restriction; (Gibbs *et al.*, 2021), testament to an array of contingencies affecting metabolic shifts (e.g. species, timing, stress type(s) and intensity). Analysis of the lipidome (totality of lipids in an organism) can expand upon the importance of lipid metabolism on physiological success (Laudicella *et al.*, 2020) and expanded efforts should consider the tissue-specificity of proteomic and gene expression patterns (Elowitz, 2002; Wei *et al.*, 2015), requiring fine-scale sampling in contrast to whole-tissue homogenates sequenced herein. Altogether, the transcriptome profile of naïve *P. generosa* suggests that fatty-acid degradation may ensure short-term survival and satisfy compensatory transcriptional requirements during hypercapnia; however depletion of endogenous storages confers an unsustainable mismatch between energy demand and supply if hypercapnic conditions exacerbated or persisted (i.e. ‘pessimum’ range; Sokolova *et al.*, 2012; Sokolova, 2021), suggesting that the adaptive environmental range of *P. generosa* is conditional upon early-life experience. Beyond the scope of this study, standing and cryptic genetic variation may underlie heritable plasticity to environmental change (Paaby and Rockman, 2014). For example, normal development of the purple sea urchin *Strongylocentrotus purpuratus* under elevated $p\text{CO}_2$ may be attributed to allele variation in genes for lipid metabolism (Pespeni *et al.*, 2013). Future studies should further examine transcriptome profiles and genome markers affecting selection.

Pre-preparatory upregulation, or gene frontloading, is an adaptive mechanism to cope with unpredictable and dynamic environments, however this response typically

concerns divergent populations (Dong *et al.*, 2008; Barshis *et al.*, 2013; Shiel *et al.*, 2017). This study highlighted a ‘cryptic release’ of expression-level variation upon environmental change, suggesting that transcriptional plasticity can be rapidly induced during early development. In particular, a greater magnitude of gene expression and variation between stress histories occurred during ambient recovery (Fig. S4 and Table S4). Similarly, mussels *Mytilus galloprovincialis* submitted to episodic stress increase transcription during stress depuration (Détrée and Gallardo-Escárate, 2018). Considering that deployed pCO₂ levels mimicked the natural habitat of *P. generosa* (Feely *et al.*, 2010; Reum *et al.*, 2014), expanded research should investigate adaptive responses during intermittent encounters. Moreover, the short timescale of this experiment relative to the lifespan of *P. generosa* (up to 168 years; Dominique Bureau *et al.*, 2002) constrains adaptive or maladaptive implications of increased transcriptional demand, as slower growers may supersede compensatory responses (Gurr *et al.* 2020).

Stress-acclimated profile: Fine-tuned and responsive to episodic hypercapnia

Stress-acclimated *P. generosa* expressed a muted transcriptome profile relative to naïve geoducks, albeit fine-tuned to regulate cellular quality control and homeostasis (Figs. 5-6 and S7). A general decrease in transcription may be attributed with adaptive benefits under environmental stress (Bultelle *et al.*, 2021). For instance, mussels *Mytilus californianus* decrease gene expression when acclimated to dynamic thermal stress as opposed to acute isothermal conditions, highlighting a lower transcriptional demand during episodic exposures (Connor and Gracey, 2020). Environmental history can have positive carryover effects (Ross *et al.*, 2016), especially when the current condition matches the

perceived cue (Burggren, 2015; Zhao *et al.*, 2018). Stress-acclimated *P. generosa* showed distinct pH-responsive signatures when faced with subsequent matches stress, heightening expression in cellular quality control, signaling, protein modifications, and stress and immune system responses (Figs. 5, S4B, and S6). Hypercapnia/acidosis affects mitochondrial integrity and can enhance free radical production (Miwa and Brand, 2003; Lambert and Brand, 2004; Tomanek *et al.*, 2011), therefore removal of damaged mitochondria, or mitophagy, may regulate cellular homeostasis during repeated stress. For example, offspring Sydney rock oysters *Saccostrea glomerata* of *pCO₂*-conditioned broodstock upregulate PINK1 during hypercapnia (Goncalves *et al.*, 2017), an essential kinase of the PINK1-Parkin pathway for efficient clearance of mitochondria (Wu *et al.*, 2015). In this study, stress-acclimated *P. generosa* exhibited mitophagy during second and third exposures to elevated *pCO₂* (Figs. 5 and S3 and Tables S2-S3), involving PINK1 protein kinase (Vives-Bauza *et al.*, 2010; Wu *et al.*, 2015), autophagy receptors (optineurin, sequestosome 1, tax1-binding protein 1; Moore and Holzbaur, 2016), amplification of autophagy signaling (TBK1; Manford and Rape, 2015), and regulation of autophagosomes (TBC1D15; Yamano *et al.*, 2014). Stress-acclimated *P. generosa* also expressed genes essential for protein turnover, 26S proteasome, E3 ubiquitinases, and caspase; (Voges *et al.*, 1999; Goldberg, 2003), that are otherwise unaffected by low pH in other bivalve species (*Crassostrea virginica* and *Mercenaria mercenaria*; Götze *et al.*, 2014) likely due to energy-limitations of environmental stress (Ivanina *et al.*, 2016). Since stress-acclimated *P. generosa* grew larger than naïve clams (Gurr *et al.*, 2021), early-life priming may render hypercapnic seawater less energetically-limiting to express these transcriptome signatures.

Signaling was also a core component of pH-responsive patterns, as stress-acclimated *P. generosa* expressed genes that activate NF-kappa β (Fig. 5 and S6; e.g. mitogen-activated protein kinase, toll-like receptors 2, 3 and 4, TNF receptor-associated factor 6, MyD88, B-cell lymphoma 3 protein, and death-associated inhibitor of apoptosis 2), a transcription factor involved in immune deficiency signaling cascade in defense of pathogens (Leulier *et al.*, 2006), and the FoxO signaling pathway (Fig. S1C). A growing body of research highlights the general importance of NF-kappa β in the innate immune response in bivalves (Li *et al.*, 2015; Huang *et al.*, 2021) and elevated $p\text{CO}_2$ can have synergistic and antagonistic effects on immunomodulation (Castillo *et al.*, 2017; Cao *et al.*, 2018). For example, upregulated expression of NF-kappa β in the mussel *Mytilus coruscus* may improve immune defenses compensatory for weakened shell strength under low pH (Zhao *et al.*, 2020). Moreover, the blood clam *Tegillarca granosa* downregulates NF-kappa β activity during hypercapnia, rendering greater susceptibility to disease (Liu *et al.*, 2016). After an initial stress encounter, *Mytilus galloprovincialis* reduces transcription of immune-related proteins, however insufficient to counteract decreased growth (Détréé and Gallardo-Escárate, 2018). Altogether, early-life experience heightened critical signaling and immune-related proteins potentially enhancing resilience to subsequent hypercapnia in the acclimated phenotype.

Stress-acclimated *P. generosa* showed pre-emptive signatures during stress depuration for enhanced energy metabolism (glycolysis and oxidative phosphorylation) and biosynthesis (pentose phosphate pathway) (Figs. 5, S2C, and S5). Stimulation of the electron transport chain increases energy production under low-pH conditions (Evans *et al.*, 2017), however altered expression of mitochondrial complexes may also confer

metabolic suppression (Murphy, 2009). For example, geoduck *P. globosa*, Pacific oyster *C. gigas*, and eastern oyster *C. virginica* upregulate NADH dehydrogenase suggesting an increase ATP production (Chapman *et al.*, 2011; Wei *et al.*, 2015; López-Landaverry *et al.*, 2021), whereas oysters *C. gigas* and *C. hongkongensis* downregulate cytochrome c oxidase and ATP synthase suggesting metabolic suppression (Dineshram *et al.*, 2012, 2013). Increased expression of complexes I, IV, and V (NADH dehydrogenase, cytochrome c oxidase, and ATPase) suggests an opportunistic increase in energy production by stress-acclimated *P. generosa* under optimal conditions. Moreover, enrichment for glycolysis and the non-oxidative pentose phosphate pathway suggests the stress-acclimated geoducks also favored carbohydrate metabolism and nucleotide biosynthesis during stress depuration. GO term enrichment during ambient recovery also included iron binding proteins and phenoloxidases ('oxidoreductase activity' in Fig S6). Expression of ferric-chelate reductase may improve iron homeostasis and prevent excess iron-induced toxicity (Li *et al.*, 2019), converting ferric iron to an 'active' electron-donor state (ferrous iron) required for biological processes (Connolly *et al.*, 2003). Since antioxidants were not abundant, excess iron-induced toxicity (Fenton reaction enhancing free radicals) was likely negligible for stress-acclimated geoducks. In contrast, stressed mud snails *Littorea littoriae* upregulate antioxidants and ferritin (English and Storey 2003), a ferroxidase essential for storing iron, suggesting taxa-specific flux of iron constituents during stress. Lastly, laccase and tyrosinase are phenoloxidases of growing interest as biomarkers of immune response and detoxification (Luna-Acosta *et al.*, 2017) and were expressed during the ambient recovery period by stress-acclimated geoducks. Future study is needed to determine the

role of divergent transcriptome profiles during episodic acidosis and stress depuration in marine invertebrates.

Transcriptional control suggests ‘memory’ post-acclimation

Understanding how the environment triggers biological responses that lead to gene expression regulation and thus environmental ‘memory’ is key, however potentially transient and interdependent molecular mechanisms affecting phenotype remain poorly understood (Adrian-Kalchhauser et al. 2020). Growing evidence suggests that modulation of post-translational and non-genetic markers may affect gene expression in marine taxa (e.g. oysters, coral, and fish; Gavery and Roberts, 2013; Putnam *et al.*, 2016; Ryu *et al.*, 2018) and participate in phenotypic acclimatization to novel changes (Liew *et al.*, 2018; Eirin-Lopez and Putnam, 2019). Stress ‘memory’, or stored information from initial stress enhancing robustness to future encounters, is a largely plant-based phenomenon (Bruce *et al.*, 2007) with growing support in invertebrate models. Molecular mechanisms underpinning memory may manifest as modulated non-genetic markers, transcription factors, and key signaling metabolites with cascading implications for performance. For example, sustained expression of the transcription factor Nrf2 co-occurs with improved antioxidant defense systems in cold-primed tunicates *Ciona robusta* (Li *et al.*, 2020). In this study, hypercapnia-primed *P. generosa* expressed histone methyltransferases (HMTs) at a higher abundance than animals without priming (Fig. 5 and S6). Each abundant HMT (SETD5, ASH1L, and NSD2) affected histone H3 tri/dimethylateion of lysine residue 36 (H3K36me3 and H3K36me2; An *et al.*, 2011; Greer and Shi, 2012), a chromatin-carrying marker affecting recruitment of gene-body DNA methylation (Dhayalan *et al.*, 2010; Nanty

et al., 2011), alternative splicing (de Almeida *et al.*, 2011), and co-participating in histone acetylation (Osipovich *et al.*, 2016). Akin to larval oysters *Crassostrea hongkongensis*, upregulated HMTs may fine-tuned transcription, controlling normal development under low pH (Dineshram *et al.*, 2015). Thus, sustained/accumulated HMTs may regulate DNA accessibility for transcription and contribute to the emergent phenotype in stress-acclimated geoducks (Fig. 6), consistent with the well-established role of histone modifications in stress ‘memory’ and improved performance (Mozgova *et al.*, 2019).

Genome-wide epigenetic and post-translational modifications can mediate phenotypic variation (Liew *et al.*, 2020; Putnam *et al.*, 2016; Anastasiadi *et al.*, 2017) and fine-tune transcription (Liew *et al.*, 2018), although in some cases this mechanism can be subtle (Downey-Wall *et al.*, 2021). *P. generosa* with a history of low pH exposure have demonstrated epigenetic signatures of differentially methylated genes linked to a beneficial phenotype of compensatory growth and resilience when challenged with low pH again, in comparison to more sensitive clams exposed to low pH for the first time (Putnam *et al.* 2017). As an expansion of this finding, expression of HMTs may control accessibility of DNA with cascading effects on essential biological processes (e.g. signal transduction, cell proliferation, growth, and cell death; Greer and Shi, 2012). Moreover, pediveliger larvae *Crassostrea hongkongensis* exposed to OA stress increase hypomethylation of genes related to lipid metabolism in contrast to hypermethylated of NADH dehydrogenase and ATP synthases, suggesting that lipids oxidation is an alternative or compensatory energy source to cope with hypercapnia (Lim *et al.* 2021). Further research is needed to elucidate molecular mechanisms underpinning rapid gene-expression regulation under OA stress. Moreover, standing genetic variation, such as cryptic genetic variation, may be contingent

on environmental change (i.e. environment-to-genotype; Paaby and Rockman, 2014), as evidenced from sufficient genetic variation in the Mediterranean mussel *Mytilus galloprovincialis* for adaptive directional selection under low pH (Bitter *et al.*, 2019). Expanded efforts require long-term tracking and interdisciplinary approaches (multi - omics) to understand how plasticity affects species and populations with different susceptibilities (Fox *et al.*, 2019).

‘Anticipatory training’ to improve hatchery-propagated seed

Transcriptome-to-phenome results in this study highlighted a fine-tuned response to low pH encounters post-acclimation (Fig. 6). We propose ‘anticipatory training’ as an approach for aquaculture enhancement, applying core concepts of developmental acclimatization (e.g. early-life programming ‘windows’; Fawcett and Frankenhuys, 2015) and mild stress-priming (i.e. conditioning hormesis and oxidative-stress hypothesis; Calabrese *et al.*, 2007; Costantini, 2014) to minimize the negative effects of domestication selection and increase resilience in hatchery-propagated seed. Aquaculture is projected to surpass wild capture to satisfy global seafood demand (FAO 2020); therefore the irreversible nature of global acidification and rapid changes in coastal and benthic zones (Gruber *et al.*, 2012) require societal actions (e.g. policies and public awareness; Kelly *et al.*, 2011) and novel strategies for improvement of food security (Nascimento-Schulze *et al.*, 2021). Domestication selection is an issue for hatcheries, in which standardized conditions ensure survival (e.g. abiotic variables, ration, stock density; Marshall *et al.*, 2014), but artificially propagate seedstocks unprepared for dynamic environments over long *in-situ* growth periods (Nascimento-Schulze *et al.*, 2021). Alternatively, moderate

stress challenges may elicit molecular foresight to cope with environmental change. Marine invertebrates present a rapid buffering capacity in response to the external environment, evidenced by improved performance due to multi-generational (Parker *et al.*, 2012; Suckling *et al.*, 2014; Goncalves *et al.*, 2016; Thomsen *et al.*, 2017; Kong *et al.*, 2019) and intragenerational carryover effects (Parker *et al.*, 2015; Gurr *et al.*, 2020), potentially driven by adaptive genetic and non-genetic markers (e.g. differential methylation, chromatin state, and standing genetic variation; Bitter *et al.*, 2019; Eirin-Lopez and Putnam, 2019). Transcriptome profiling, as showcased in this study (Fig 6), can expand genomic resources in aquaculture by identifying genes or gene-expression patterns associated with stress-resilient or fast-growing economic traits (Chandhini and Kumar, 2019).

CONCLUSION

In this study, we investigated the transcriptome profiles of juvenile geoduck post-acclimation and under episodic stress to understand molecular underpinnings of emergent physiological benefits in Gurr *et al.* (2021). In absence of moderate $p\text{CO}_2$ priming, transcriptome profiles showed an enhanced energetic requirement for transcriptional loading, fueled by persistent fatty-acid metabolism. In contrast, moderate stress history conferred gene-expression control, such that stress-acclimated *P. generosa* fine-tuned transcription during subsequent pH changes. Altogether, this study demonstrates the importance of gene-expression regulation on positive developmental acclimatization, and further study is required to disentangle the roles of genetic and non-genetic drivers across long-term and multi-generational timescales.

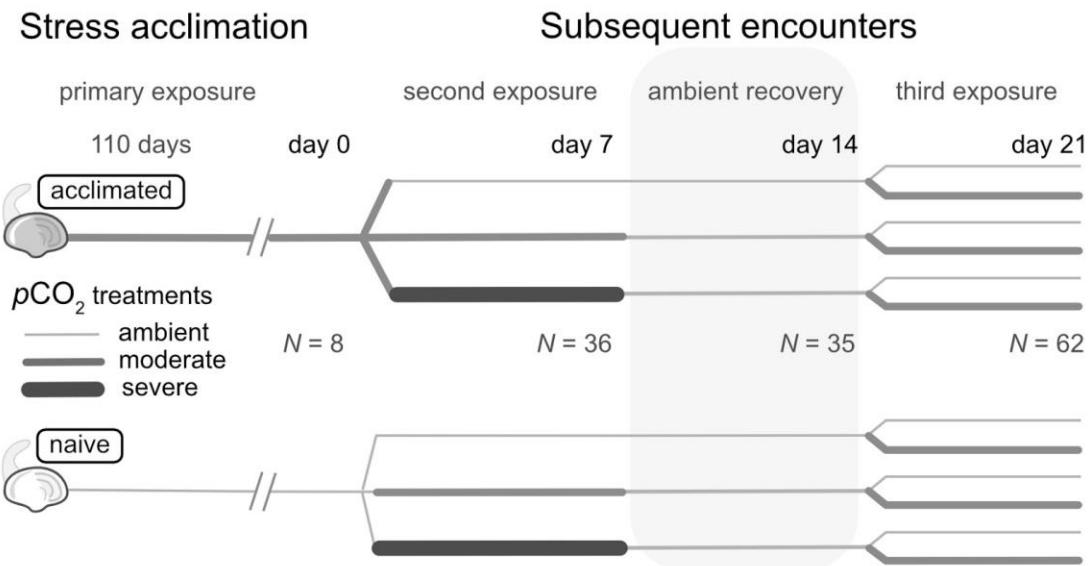


Figure 1. Experimental design for whole tissue sampling.

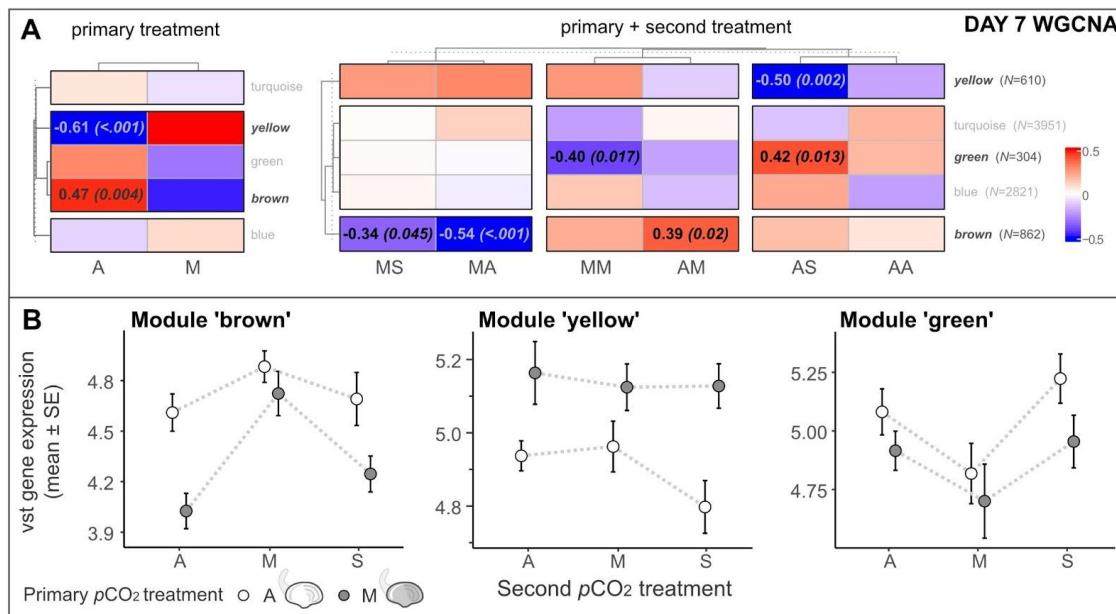


Figure 2. WGCNA results for samples on day 7 of the experiment.

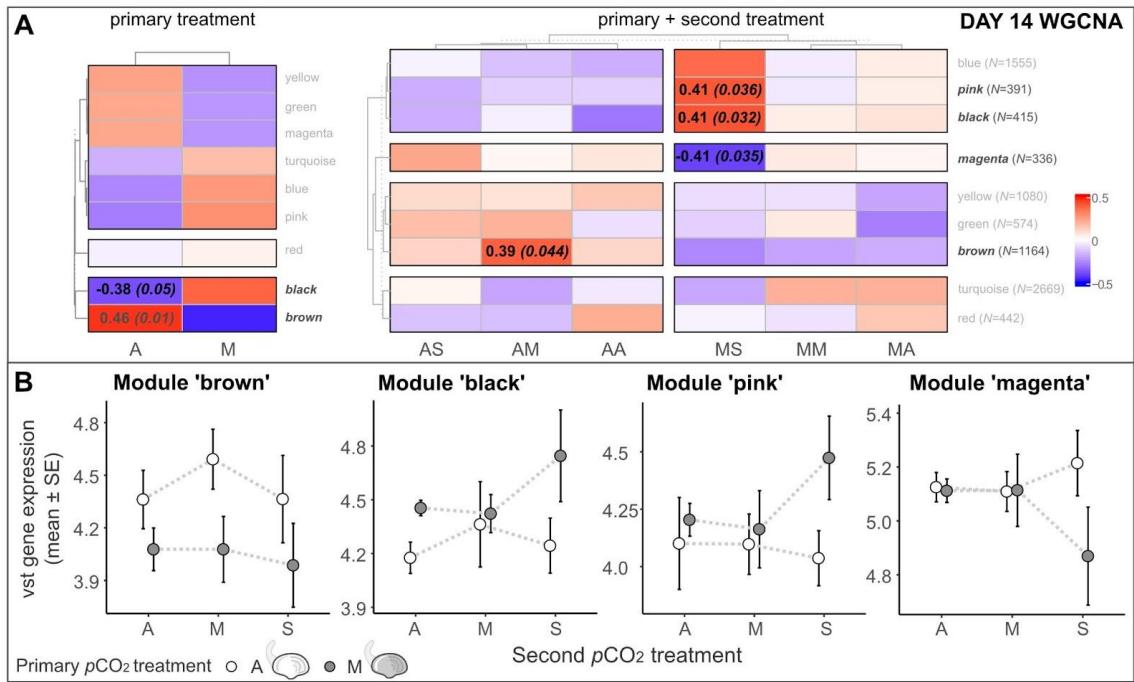


Figure 3. WGCNA results for samples on day 14 of the experiment.

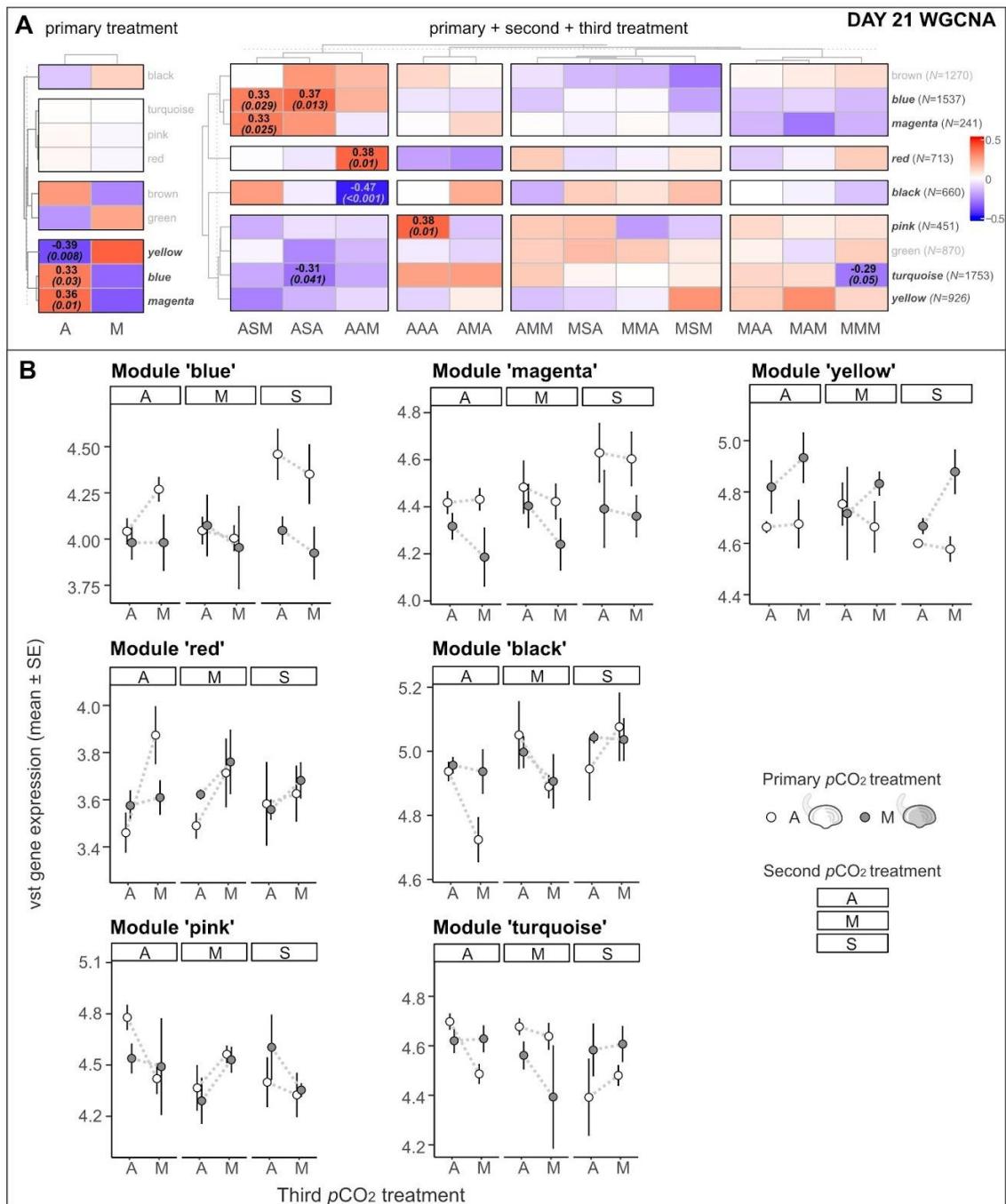


Figure 4. WGCNA results for samples on day 21 of the experiment.

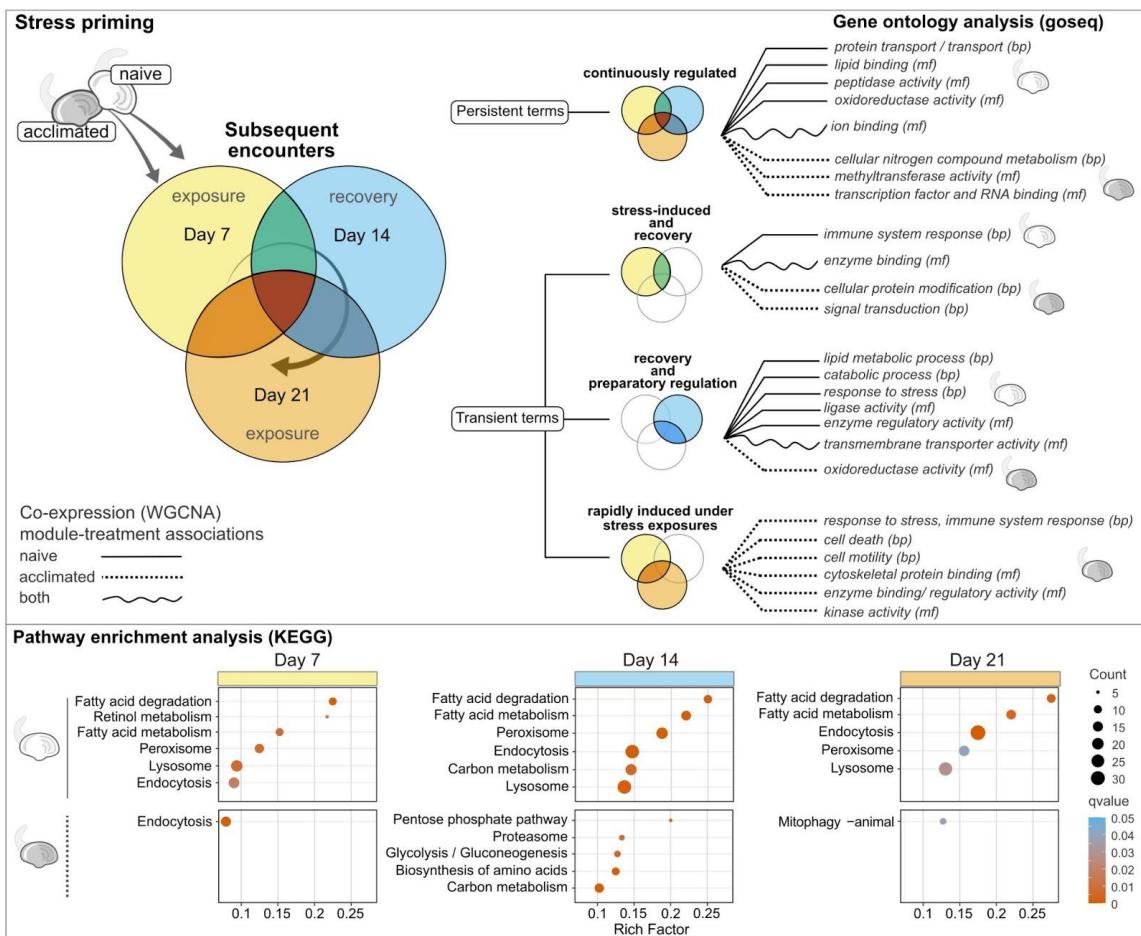


Figure 5. GO and pathway enrichment analysis of ambient-effect modules (naive) and moderate-effect modules (acclimated).

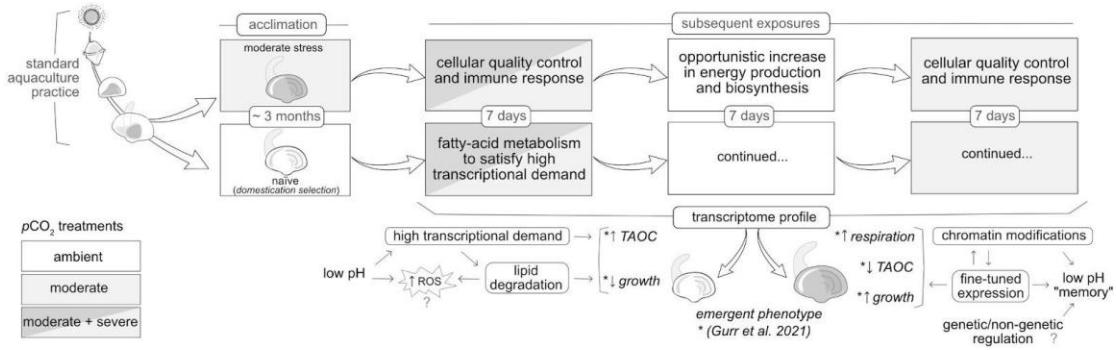


Figure 6. Summary of transcriptome profiles of stress-acclimated and naïve juvenile geoducks under episodic hypercapnia. Transcriptome-to-phenome mechanisms are proposed, synthesizing gene function and pathway enrichment patterns and the emergent physiological phenotype from Gurr et al. (2021) (asterisks, total antioxidant capacity ‘TAOC’; Gurr et al. 2021).

Supplementary Table. S1. Seawater carbonate chemistry.

Review Table 1 in Chapter 2 - sample table applied here

Supplementary Table. S2. Results of KEGG pathway enrichment analysis (using ‘KEGGprofile’ in R) of full-treatment WGCNA modules.

Pathway enrichment analysis								
period	WGCNA module	genes module ⁻¹ (number annotated)	% aligned to <i>C. gigas</i>	KEGG Enrichment Analysis	count	rich factor	description	
Day7	brown	862 (624)	85.1	0.001	9	0.225	Fatty acid degradation	
				0.023	5	0.217	Retinol metabolism	
				0.008	9	0.153	Fatty acid metabolism	
				0.008	12	0.125	Peroxisome	
				0.008	18	0.094	Lysosome	
				0.020	16	0.090	Endocytosis	
	yellow	610 (431)	84.7	0.001	14	0.079	Endocytosis	
				na	na	na	na	
	green	304 (187)	85	na	na	na	na	
Day14	brown	1164 (848)	84.8	0.001	10	0.250	Fatty acid degradation	
				0.001	13	0.220	Fatty acid metabolism	
				<0.001	18	0.188	Peroxisome	
				<0.001	26	0.147	Endocytosis	
				0.007	17	0.145	Carbon metabolism	
				0.001	26	0.136	Lysosome	
	black	415 (304)	85.9	0.005	5	0.200	Pentose phosphate pathway	
				0.009	6	0.133	Proteasome	
				0.005	7	0.127	Glycolysis / Gluconeogenesis	
				0.001	9	0.125	Biosynthesis of amino acids	
				0.001	12	0.103	Carbon metabolism	
	magenta	336 (238)	86.1	0.002	8	0.057	Spliceosome	
	pink	391 (252)	82.6	na	na	na	na	
Day21	blue	1537 (1155)	86.5	0.003	11	0.275	Fatty acid degradation	
				0.005	13	0.220	Fatty acid metabolism	
				<0.001	31	0.175	Endocytosis	
				0.046	15	0.156	Peroxisome	
				0.034	25	0.131	Lysosome	
	magenta	241 (193)	88.1	0.001	10	0.071	Protein processing in endoplasmic reticulum	
	yellow	926 (632)	81.5	0.040	7	0.127	Mitophagy - animal	
	black	660 (547)	88.7	<0.001	15	0.190	Ribosome biogenesis in eukaryotes	
				<0.001	19	0.135	Spliceosome	
				<0.001	19	0.132	RNA transport	
				0.049	13	0.092	Protein processing in endoplasmic reticulum	
	pink	451 (283)	81.6	na	na	na	na	
	red	713 (563)	84.5	na	na	na	na	
	turquoise	1753 (1235)	86.3	na	na	na	na	
mean ± SD				85 ± 2				

Supplementary Table. S3. Results of KEGG pathway enrichment analysis (using ‘KEGGprofile’ in R) of significant WGCNA modules computed for only samples subsequently exposed under elevated $p\text{CO}_2$ treatments.

Pathway enrichment analysis							
period	WGCNA module	genes module $^{-1}$ (number annotated)	% aligned to <i>C. gigas</i>	KEGG Enrichment Analysis			
				p-value $_{\text{adj}}$	count	rich factor	description
Day7	turquoise	1126 (808)	85	0.040	7	0.175	Fatty acid degradation
				0.040	12	0.125	Peroxisome
				0.040	12	0.120	Phagosome
				0.017	20	0.113	Endocytosis
				0.023	20	0.105	Lysosome
	brown	1495 (1023)	83.7	<0.001	20	0.182	Autophagy - animal
				0.043	9	0.164	Mitophagy - animal
				0.043	11	0.139	FoxO signaling pathway
				0.043	19	0.107	Endocytosis
	greenyellow	1776 (1243)	81.1	<0.001	53	0.373	Ribosome
				0.009	19	0.194	Oxidative phosphorylation
	blue	1343 (957)	87.6	na	na	na	na
Day14	blue	1554 (1140)	86.6	<0.001	35	0.246	Ribosome
				0.021	21	0.179	Carbon metabolism
				0.010	30	0.169	Endocytosis
				0.012	31	0.162	Lysosome
	pink	532 (385)	81.3	0.001	12	0.122	Oxidative phosphorylation
Day21	blue	956 (707)	87.8	0.012	8	0.200	Fatty acid degradation
				0.012	10	0.169	Fatty acid metabolism
				0.004	15	0.150	Phagosome
				0.012	13	0.135	Peroxisome
				0.001	25	0.131	Lysosome
	magenta	827 (570)	81.2	<0.001	45	0.317	Ribosome
				0.009	13	0.133	Oxidative phosphorylation
	pink	448 (316)	85	na	na	na	na
	yellow	664 (451)	82	na	na	na	na
		mean \pm SD	84 \pm 3				

Supplementary Table. S4. Count and directionality of differentially expressed genes from each main pairwise treatment model.

Experiment data sampling day	pCO ₂ treatment	DE Analysis pairwise <i>p</i> CO ₂	total DE	upregulated genes	downregulated genes
0	Primary	A_v_M	14	11	3
7	Primary	A_v_M	108	62	46
7	Second	A_v_M	106	14	92
7	Second	A_v_S	2	0	2
7	Second	M_v_S	13	9	4
14	Primary	A_v_M	429	317	112
14	Second	A_v_M	0	0	0
14	Second	A_v_S	0	0	0
14	Second	M_v_S	0	0	0
21	Primary	A_v_M	155	101	54
21	Second	A_v_M	1	0	1
21	Second	A_v_S	1	0	1
21	Second	M_v_S	0	0	0
21	Third	A_v_M	0	0	0

Supplementary Table. S5. Persistent DEGs

Gene annotation	Up/down-regulated	Log ₂ fold change
Four-domain proteases inhibitor (McaPI)	+	5.78 ± 1.04
Apolipoprotein D	+	5.54 ± 2.52
Chitotriosidase-1	+	4.83 ± 1.63
Neuroendocrine convertase 1	+	4.53 ± 1.63
Coadhesin	+	4.52 ± 1.56
Mucin-1	+	4.45 ± 1.63
Prostasin	+	4.39 ± 1.79
Complement C1q tumor necrosis factor-related protein 3	+	4.07 ± 1.04
Regucalcin	+	3.88 ± 0.41
Cobalamin binding intrinsic factor	-	-14.1 ± 5.80
Defensin	-	-11.88 ± 6.57
BPTI/Kunitz domain-containing protein 4-like	-	-9.16 ± 5.69
CD109 antigen	-	-7.50 ± 1.52

Supplementary Table. S6. Count and directionality of differentially expressed genes for all pairwise interactions on day 7.

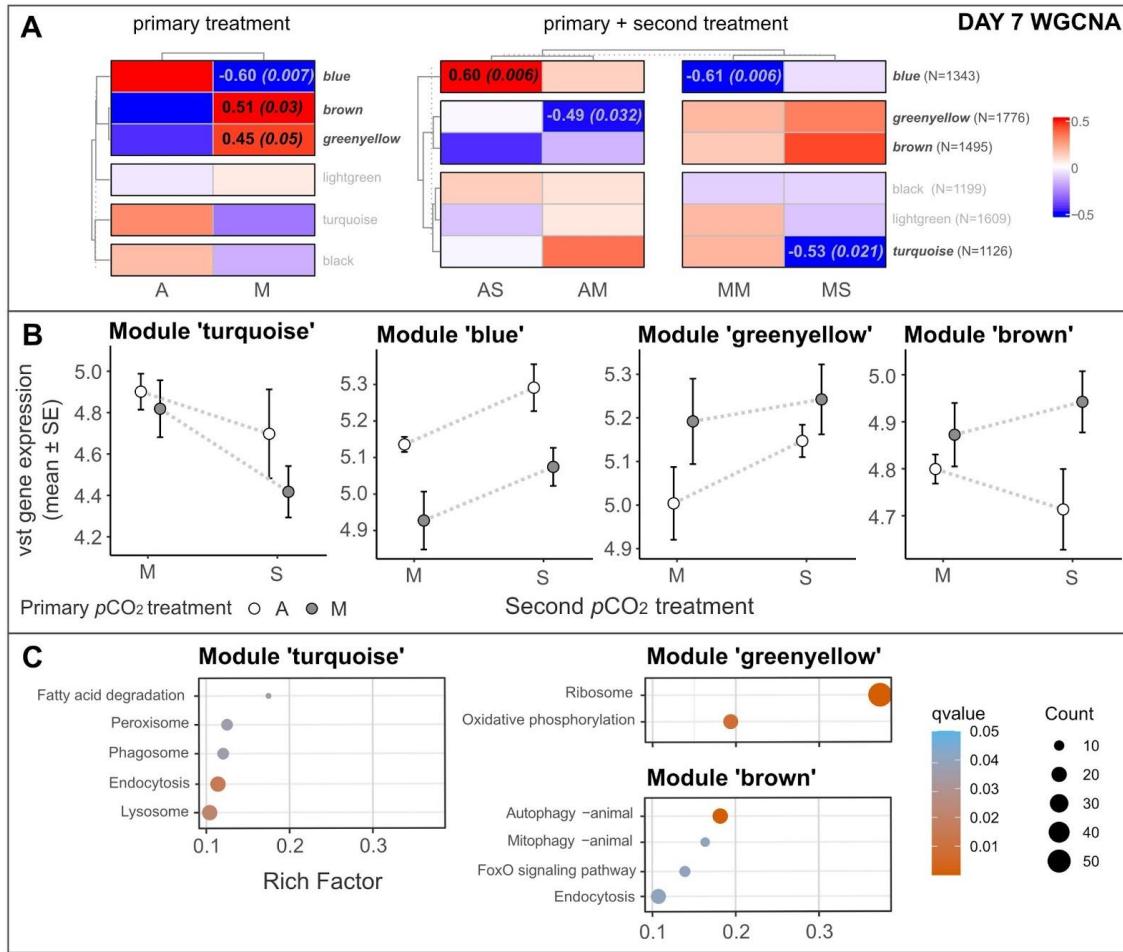
Experiment data sampling day	pCO ₂ treatment	DE Analysis			upregulated genes	downregulated genes
		pairwise p CO ₂	total DE			
7	groups	AA_AM	1	0	1	
7	groups	AA_AS	10	0	10	
7	groups	AA_MA	10	2	8	
7	groups	AA_MM	20	4	16	
7	groups	AA_MS	6	1	5	
7	groups	AM_AS	1	0	1	
7	groups	AM_MA	168	137	31	
7	groups	AM_MM	5	0	5	
7	groups	AM_MS	60	52	8	
7	groups	AS_AM	1	1	0	
7	groups	AS_MA	30	21	9	
7	groups	AS_MM	31	23	8	
7	groups	AS_MS	15	9	6	
7	groups	MA_MM	36	4	32	
7	groups	MA_MS	1	0	1	
7	groups	MS_MM	3	2	1	

Supplementary Table. S7. Count and directionality of differentially expressed genes for all pairwise interactions on day 14.

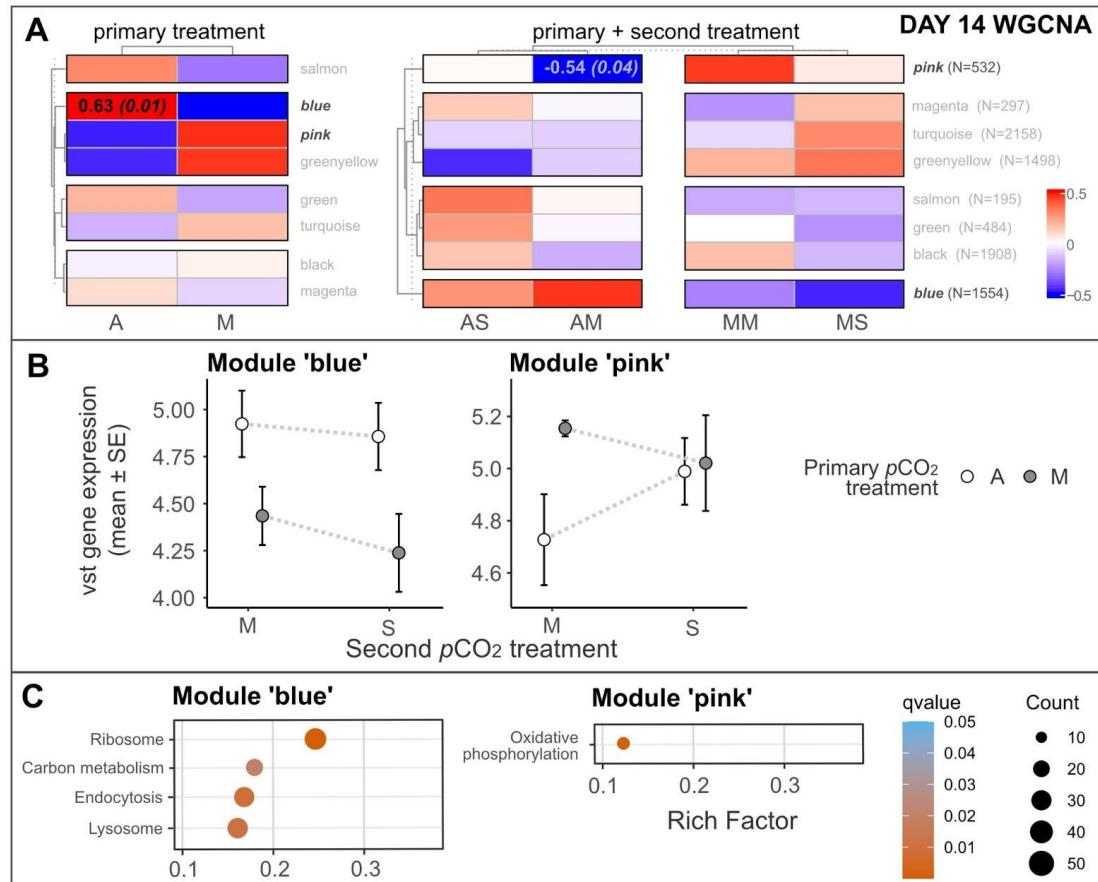
Experiment data sampling day	pCO ₂ treatment	DE Analysis pairwise p CO ₂	total DE	upregulated genes	downregulated genes
14	groups	AA_AM	1	1	0
14	groups	AA_AS	1	1	0
14	groups	AA_MA	5	3	2
14	groups	AA_MM	10	6	4
14	groups	AA_MS	41	10	31
14	groups	AM_AS	1	1	0
14	groups	AM_MA	12	7	5
14	groups	AM_MM	10	9	1
14	groups	AM_MS	1	0	1
14	groups	AS_AM	1	0	1
14	groups	AS_MA	8	7	1
14	groups	AS_MM	2	2	0
14	groups	AS_MS	2	1	1
14	groups	MA_MM	1	0	1
14	groups	MA_MS	1	0	1
14	groups	MS_MM	1	0	1

Supplementary Table. S8. Count and directionality of differentially expressed genes for all pairwise interactions on day 21.

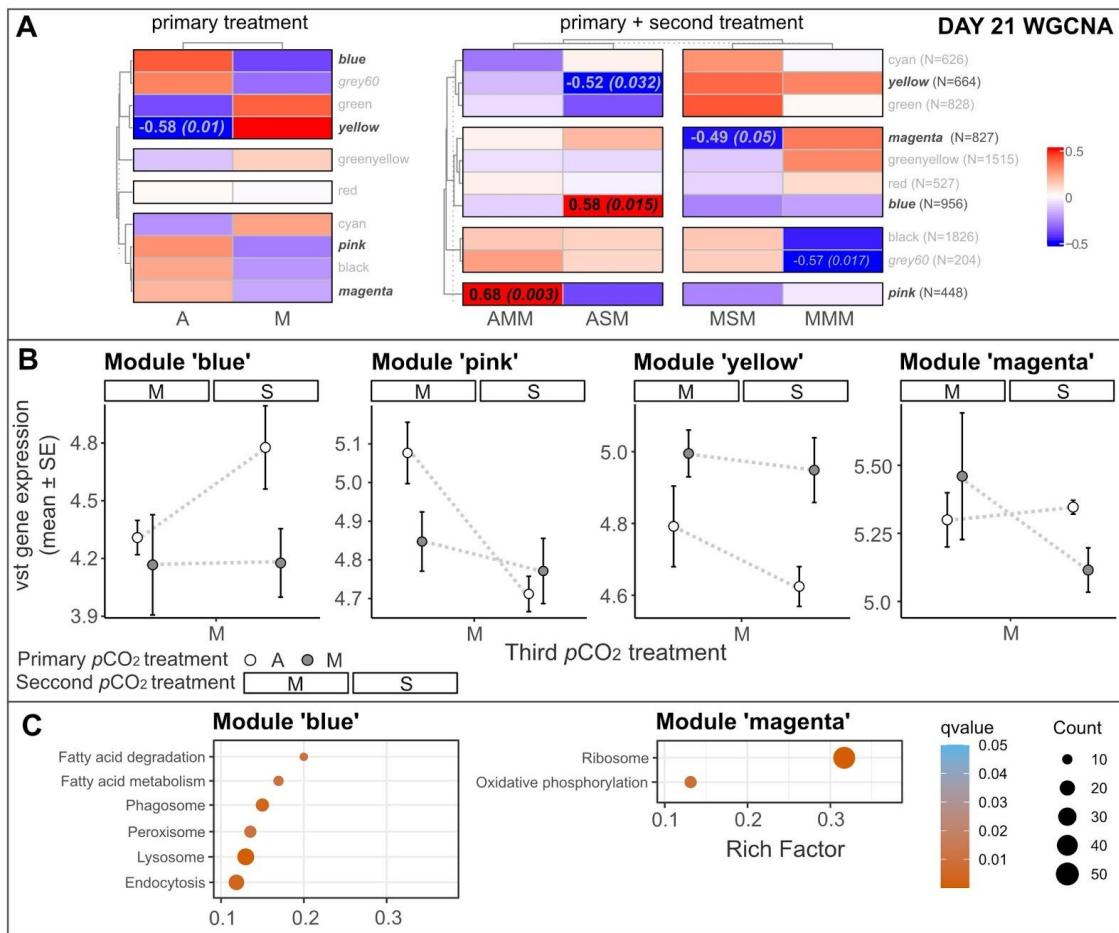
Experiment data sampling day	pCO2 treatment	DE Analysis pairwise p CO2	total DE	upregulated genes	downregulated genes
21	groups	AAA_AAM	1	0	1
21	groups	AAA_AMA	1	1	0
21	groups	AAA_AMM	13	13	0
21	groups	AAA ASA	1	1	0
21	groups	AAA ASM	1	0	1
21	groups	AAA MAA	68	31	37
21	groups	AAA MAM	15	9	6
21	groups	AAA MMA	1	1	0
21	groups	AAA MMM	1	0	1
21	groups	AAA MSA	2	0	2
21	groups	AAA MSM	1	0	1
21	groups	AAM AMA	1	1	0
21	groups	AAM AMM	12	12	0
21	groups	AAM ASA	3	3	0
21	groups	AAM ASM	1	1	0
21	groups	AAM MAA	110	37	73
21	groups	AAM MAM	9	4	5
21	groups	AAM MMA	1	1	0
21	groups	AAM MMM	1	1	0
21	groups	AAM MSA	1	0	1
21	groups	AAM MSM	1	1	0
21	groups	AMA AMM	1	1	0
21	groups	AMA ASA	2	1	1
21	groups	AMA ASM	1	0	1
21	groups	AMA MAA	15	5	10
21	groups	AMA MAM	13	5	8
21	groups	AMA MMA	1	1	0
21	groups	AMA MMM	18	6	12
21	groups	AMA MSA	21	1	20
21	groups	AMA MSM	1	1	0
21	groups	AMM ASA	1	1	0
21	groups	AMM ASM	1	0	1
21	groups	AMM MAA	20	0	20
21	groups	AMM MAM	2	0	2
21	groups	AMM MMA	1	1	0
21	groups	AMM MMM	26	1	25
21	groups	AMM MSA	116	10	106
21	groups	AMM MSM	1	1	0
21	groups	ASA ASM	1	0	1
21	groups	ASA MAA	7	1	6
21	groups	ASA MAM	2	0	2
21	groups	ASA MMA	2	0	2
21	groups	ASA MMM	15	2	13
21	groups	ASA MSA	42	6	36
21	groups	ASA MSM	1	0	1
21	groups	ASM MAA	1	1	0
21	groups	ASM MAM	5	2	3
21	groups	ASM MMA	1	1	0
21	groups	ASM MMM	23	10	13
21	groups	ASM MSA	60	10	50
21	groups	ASM MSM	1	1	0
21	groups	MAA MAM	26	20	6
21	groups	MAA MMA	31	28	3
21	groups	MAA MMM	78	53	25
21	groups	MAA MSA	187	76	111
21	groups	MAA MSM	26	24	2
21	groups	MAM MMA	1	1	0
21	groups	MAM MMM	1	0	1
21	groups	MAM MSA	1	0	1
21	groups	MAM MSM	2	2	0
21	groups	MMA MMM	1	0	1
21	groups	MMA MSA	1	0	1
21	groups	MMA MSM	1	0	1
21	groups	MMM MAA	78	25	53
21	groups	MMM MAM	1	1	0
21	groups	MMM MSA	3	1	2
21	groups	MMM MSM	1	1	0
21	groups	MSA MSM	1	0	1



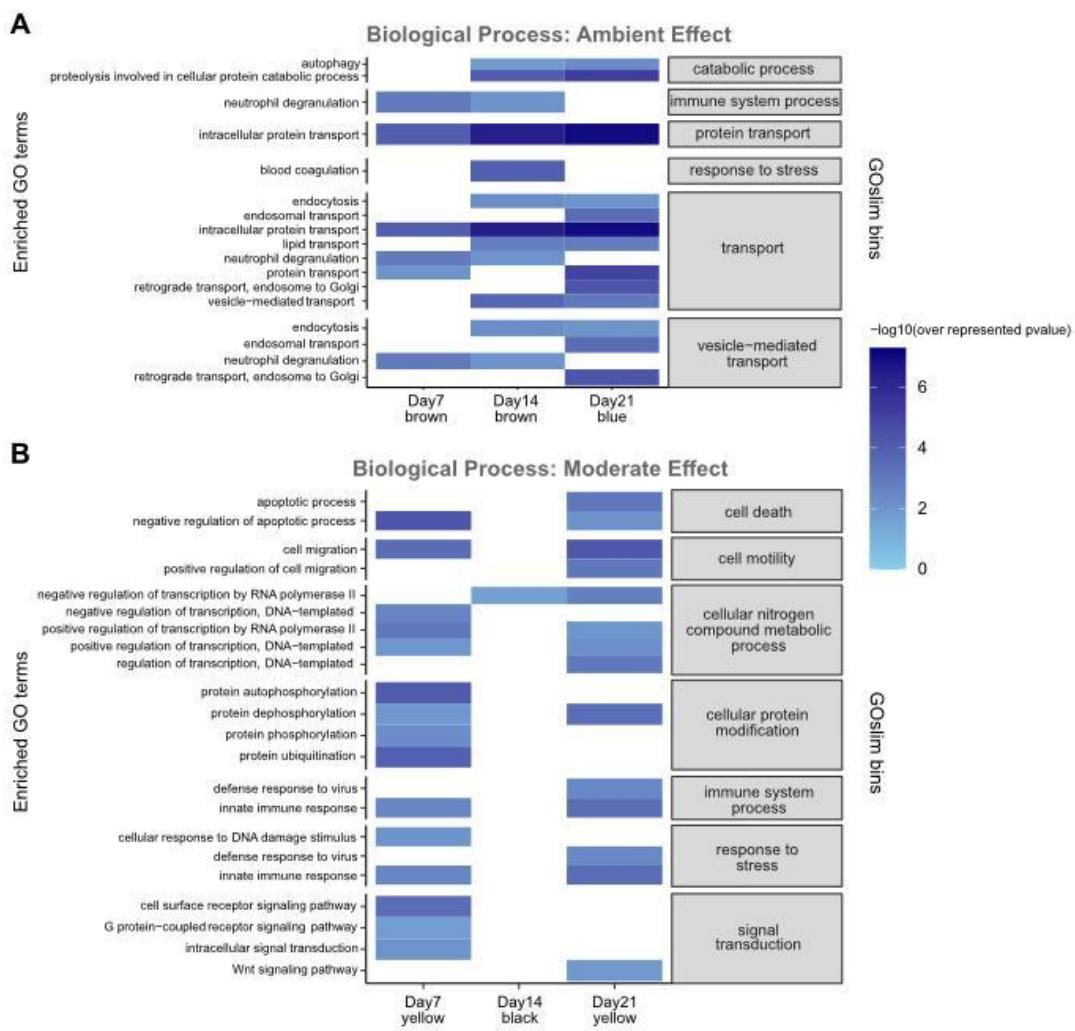
Supplementary Fig. S1. Day 7 WGCNA analysis for samples exposed to elevated $p\text{CO}_2$ during subsequent challenges (without ambient treatment).



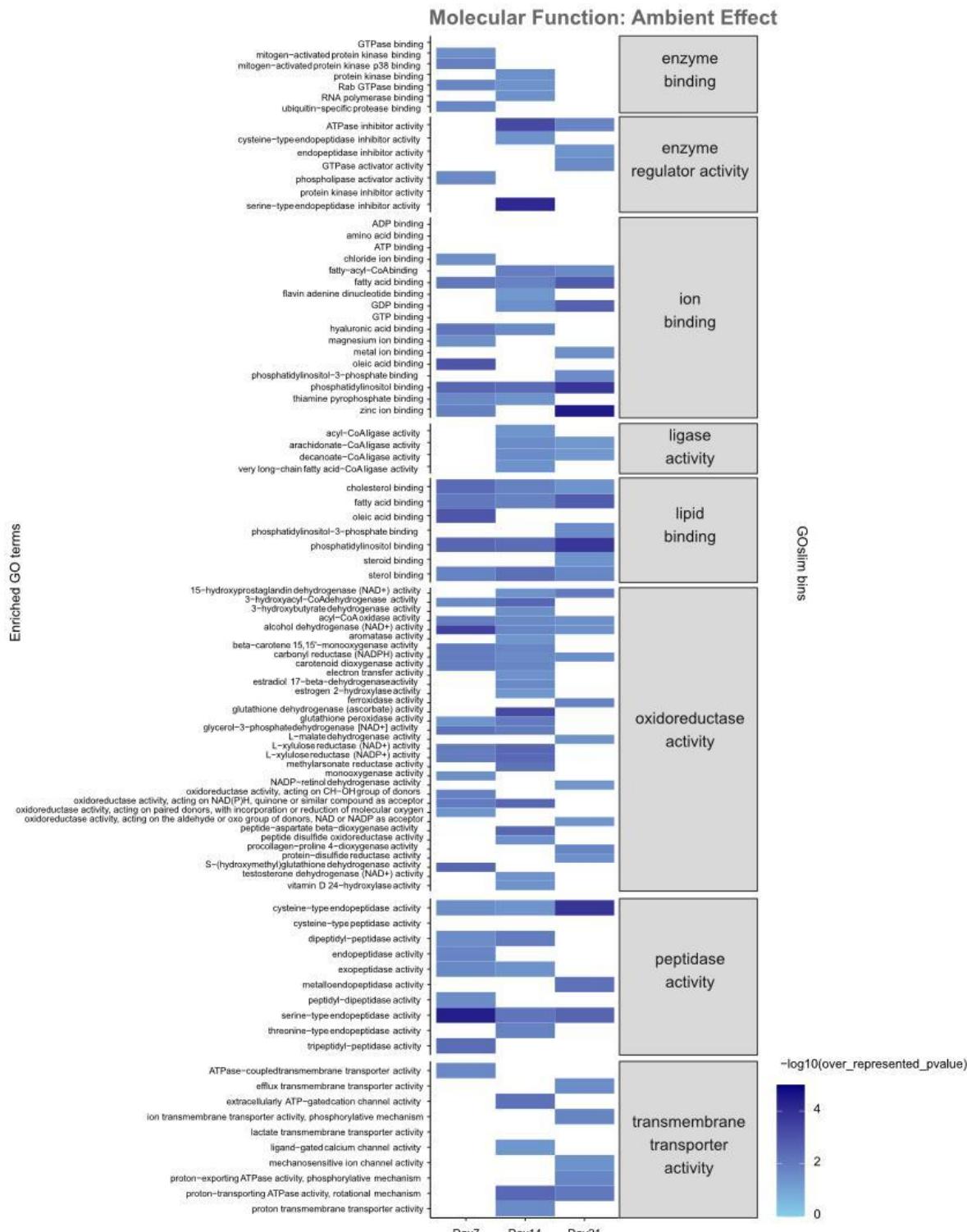
Supplementary Fig. S2. Day 14 WGCNA analysis for samples exposed to elevated $p\text{CO}_2$ during subsequent challenges (without ambient treatment).



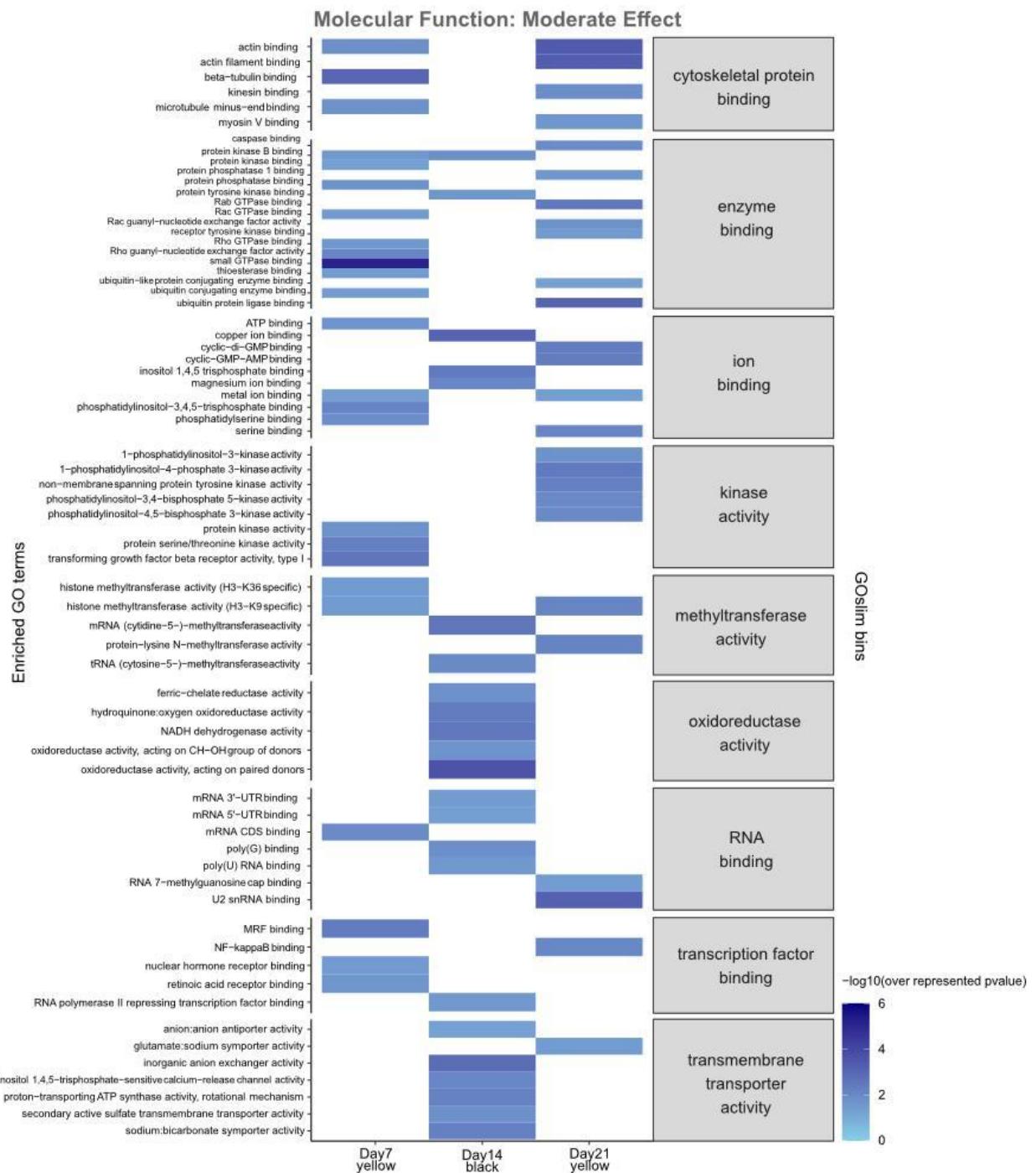
Supplementary Fig. S3. Day 21 WGCNA analysis for samples exposed to elevated $p\text{CO}_2$ during subsequent challenges (without ambient treatment).



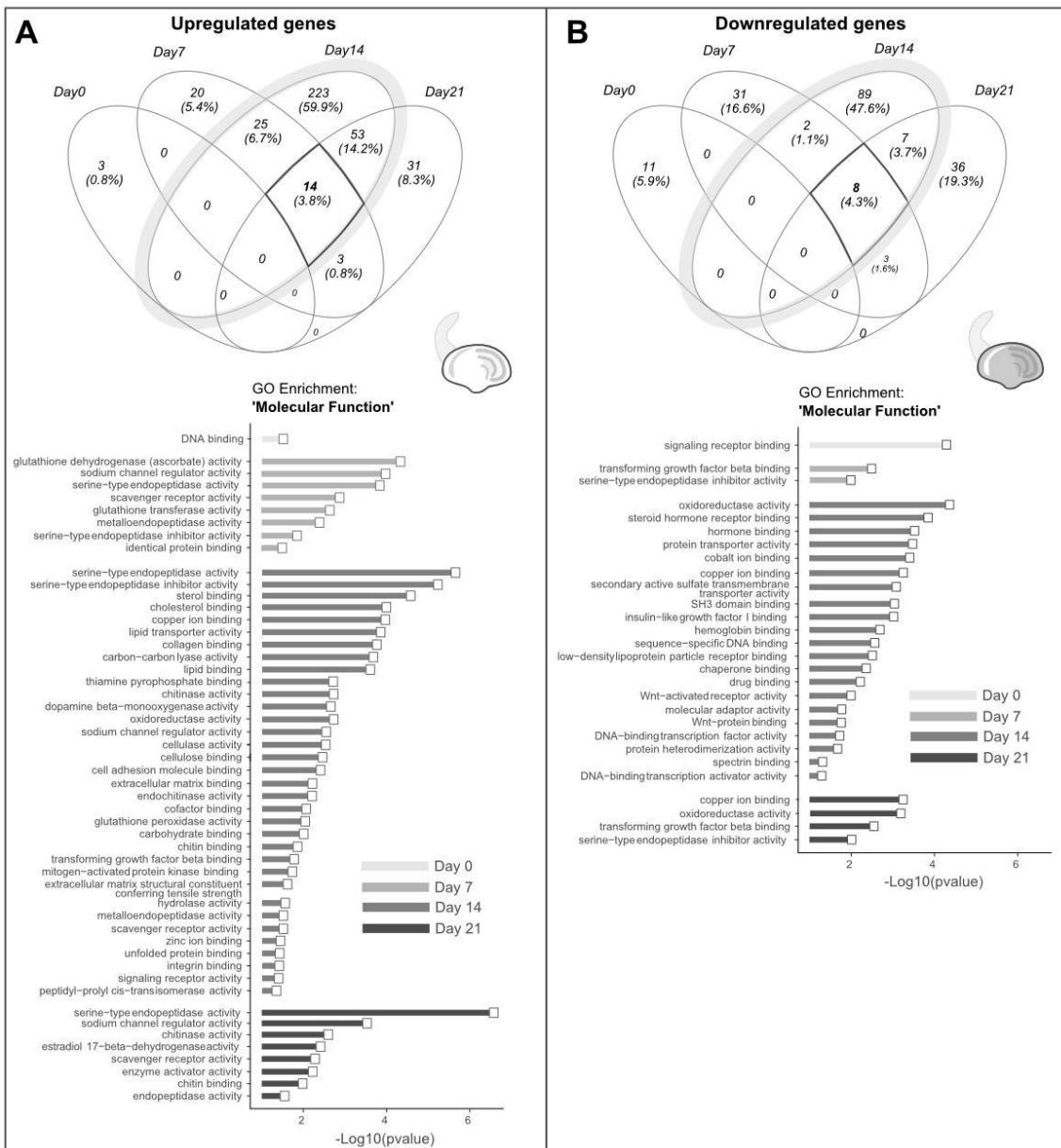
Supplementary Fig. S4. Enriched ‘biological process’ GO terms and corresponding hierarchical GO bins (from ;GOslim’) of significant co-expression modules



Supplementary Fig. S5. Enriched ‘molecular function’ GO terms and corresponding hierarchical GO bins (‘GOslim’) of significant ambient-effect modules.

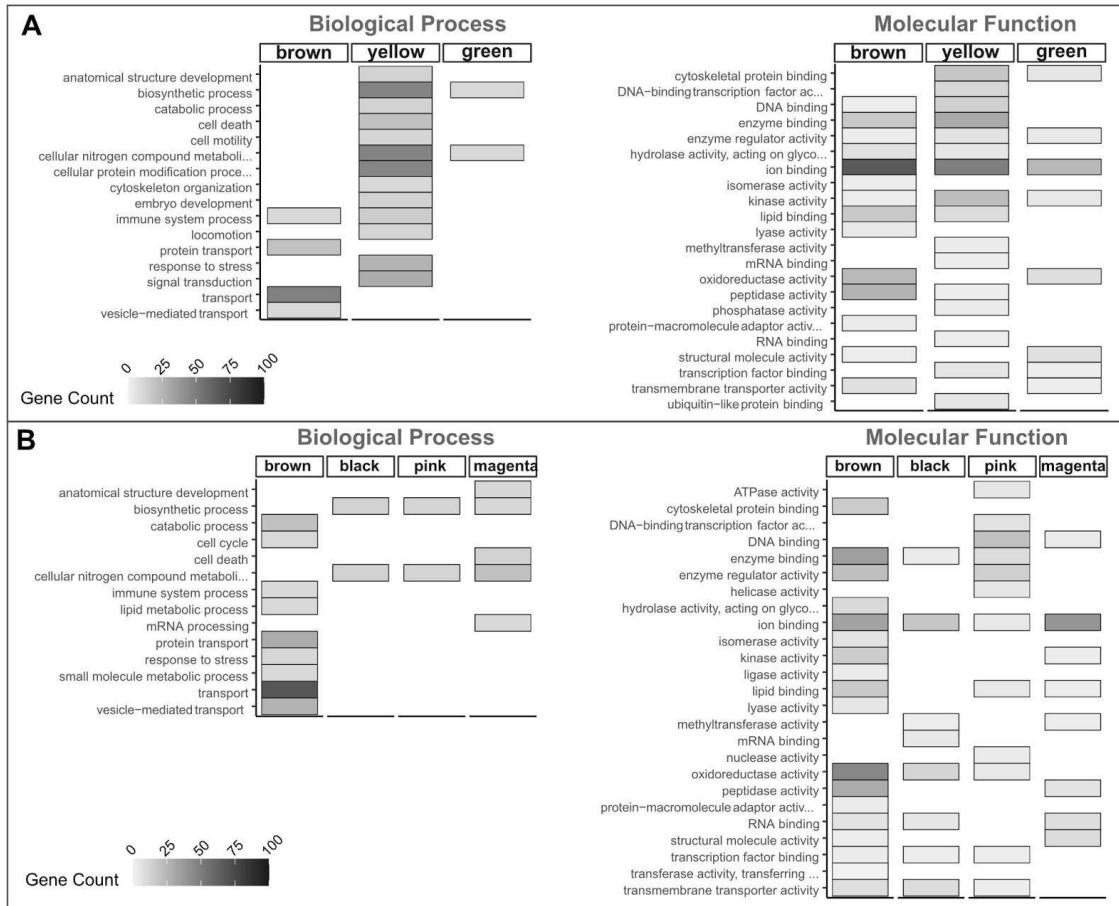


Supplementary Fig. S6. Enriched ‘molecular function’ GO terms and corresponding hierarchical GO bins (‘GOslim’) of significant moderate-effect modules.

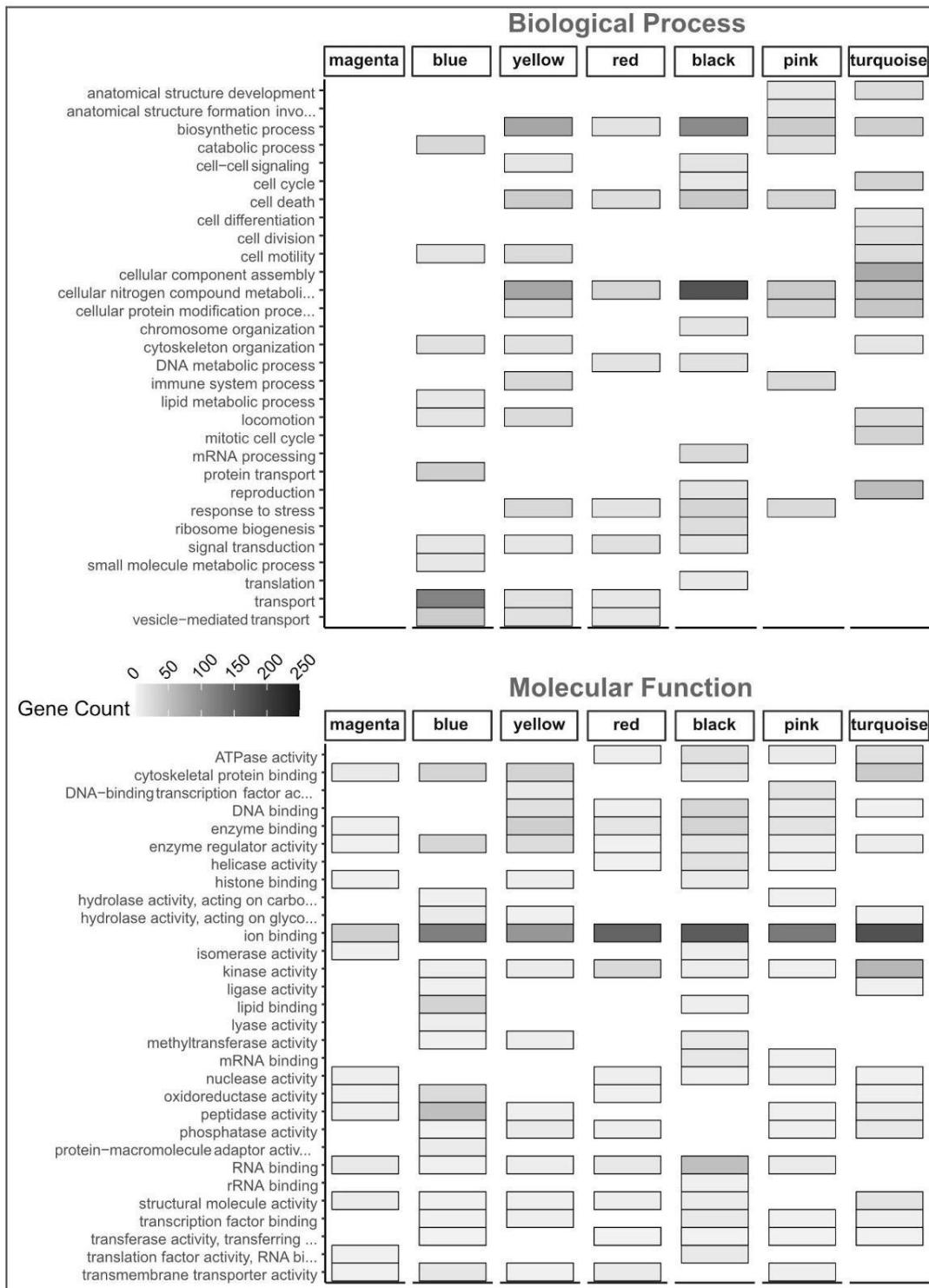


Supplementary Fig. S7. Effect of primary ambient versus moderate pCO₂ acclimation

on DGE.



Supplementary Fig. S8. GO enrichment analysis of day 7 (A) and day 14 (B) WGCNA data showing gene counts for each significant co-expression module.



Supplementary Fig. S9. GO enrichment analysis of day 21 WGCNA data showing gene counts for each significant co-expression module.

Appendices

A. Introduction and review of the problem

Expansion and enhancement of sustainable shellfish production is necessary to prevent overexploitation of wild stock (Campbell et al. 1998; Shumway et al. 2003; Orensanz et al. 2004; Zhang and Hand 2006) and satisfy international trade (FAO, 2018), but hatchery rearing poses a critical production bottleneck due partially to environmental stressors such as OA (Barton et al. 2015). OA, or the decrease of ocean pH and aragonite saturation state ($\Omega_{\text{aragonite}}$) due to elevated atmospheric partial pressures ($p\text{CO}_2$), poses a global threat with magnified intensity in productive coastal marine systems (Cai *et al.*, 2011). OA is known to cause acidosis, extra- and intracellular hypercapnia, and oxidative stress particularly for marine calcifiers (Burnett, 1997; Tomanek *et al.*, 2011; Tomanek, 2014). These challenges elicit broad downstream consequences for assimilation (i.e. aerobic metabolism, calcification, somatic growth, and storage retention; Pörtner *et al.*, 2004; Michaelidis *et al.*, 2005; Shirayama, 2005; Talmage and Gobler, 2010; Waldbusser *et al.*, 2010, 2015; Gazeau *et al.*, 2013), acid/base regulation (Portner and Farrell 2008), and energy-consuming processes (i.e. protein synthesis; Lannig *et al.*, 2010; Dickinson *et al.*, 2012; Mukherjee *et al.*, 2013; Wei *et al.*, 2015; Goncalves *et al.*, 2016). Thus, OA is a major concern for bivalve aquaculture (Waldbusser et al. 2015; Barton et al. 2015), demanding effective strategies to elicit adaptive resistance or resilience (Adelsman and Binder 2012; Barton et al. 2015).

Known as the “Pacific Northwest Seedstock Crisis” aragonite undersaturation and/or elevated $p\text{CO}_2$ can impair early stages of fertilization, larval development, and metamorphosis of bivalves (Kurihara, 2008; Kroeker *et al.*, 2013) and is responsible for

production declines and documented mass-mortalities within hatcheries in the Pacific coast of North America (Barton *et al.*, 2012, 2015). Pacific geoduck (*Panopea generosa*) are long-lived hiatellid clams of ecological importance (Dethier 2006) and a major contributor to both the total infaunal biomass of Puget Sound (Goodwin and Pease 1989) and annual shellfish revenue of Washington state (Shamshak and King, 2015). Geoduck production in Washington provides ~90% of global supply (Shamshak and King, 2015) and alone constitutes 27% of the overall shellfish revenue in the state valued at >\$24 million yr⁻¹ and >\$14 pound⁻¹ as of 2015 (Grant, 2015). Current knowledge of *P. generosa* is largely foundational (e.g. life history, distribution, wild/farm aquaculture requirements, growth/aging; Goodwin and Pease 1989; Bureau *et al.* 2012; Calderon-Aguilera *et al.*, 2010; Vadopalas *et al.*, 2010; Marshall *et al.*, 2012, 2014; McDonald *et al.*, 2015) with sparse laboratory experimentation (Tapia-Morales *et al.*, 2015; Nava-Gómez *et al.*, 2018) and only recent inquiries on stress response (Putnam *et al.*, 2017; Juárez *et al.*, 2018; Huo *et al.*, 2019; Spencer *et al.*, 2019; Timmins-Schiffman *et al.*, 2019), relative to other bivalves prominent in commercial industry (reviewed in Gazeau *et al.*, 2013; e.g. oysters, scallops, hard-shell clams, and mussels). Given that stress conditions exacerbated by anthropogenic activity are projected to intensify in the near-future (Feely *et al.*, 2009), long-lived molluscs, such as *P. generosa* (known lifespan up to 168 years; Bureau *et al.*, 2002), may rely on intragenerational acclimation under rapid environmental change. Recent findings describe the potential for acute pCO₂ to elicit compensatory growth and establishment of epigenetic markers in *P. generosa* (Putnam *et al.*, 2017). However, evidence of a negative effect during larval development suggests OA responses are

contingent on life-stage and stress intensity (Huo *et al.*, 2019; Timmins-Schiffman et al., 2019).

B. Limitations, speculative discussion, and theoretical implications

It is essential to admit the limitations that exist in this dissertation to sustain scientific transparency and rigor and guide expanded efforts. First, the timing and duration of hypercapnia exposures and ambient recovery periods was based on limited evidence, commonly estimated from ionregulatory activity (e.g. bicarbonate levels; Pörtner 2004). Whereas marine animals (invertebrates and fish) can reestablish acid-base homeostasis and foraging behavior 24-48h after exposure to acidified seawater (Holeton et al. 1983; Spicer et al. 2007; Leung et al. 2015), compromised acid-base status can persist >8 days after exposure in aquatic molluscs (Pynnönen 1994). Thus, we considered stress periods in the span of days (e.g. 6-14 days) as sufficient to modify subcellular status and infer a stressed and basal state during exposure to elevated $p\text{CO}_2$ and ambient seawater, respectively. Respiration rates of juvenile *P. generosa* in Chapter 1 showed metabolic suppression continued after the 14-day recovery period (day 0 of second exposure), suggesting this duration was insufficient to amend aerobic metabolism to rates prior to exposure. In contrast, gene expression from Chapter 3 found a putative increase in energy metabolism (expression of mitochondrial complexes) by low-pH acclimated clams during 7-days of ambient recovery, as opposed to continued expression for fatty-acid degradation by clams naive to low pH. Altogether, future research is needed to disentangle the effects of episodic stress, as evidence from this dissertation suggests acclimation preceding episodic exposures can improve responsiveness and may decrease the time required to recover from stress. Second, physiological measurements in Chapter 1 were pseudo-replicated.

Sacrificial pseudoreplication occurs when the same individuals within each experimental unit are measured repeatedly through time and are addressed statistically as independent units (Hurlbert 1984). An additional caveat to this experiment was the random interspersion of experimental units (trays) constrained by gravity-fed seawater perpendicular to mixing tanks. An alternative to this design in Chapters 2 and 3 employed random interspersion, destructive sampling (data collection preceded removal of those animals from the experiment), and true biological replication ($N=6$).

Our production-oriented message and methodologies provide a foundation for future tests on the industry scale. Aquaculture facilities run autonomous flow-through systems with high larval densities and avoid semi-continuous and non-autonomous systems subject to human error and profit loss. Cornwall and Hurd (2016) propose five approaches to avoid pseudoreplication in OA research, but only one design uses semi-autonomous and flow-through seawater. This method uses a three-step system: (1) one seawater storage tank (2) one CO_2 mixing tank (3) and header tanks for each culture tank (Fig 3b in Cornwall and Hurd 2016). I propose the following method to attempt stress-conditioning using raceway tanks (long and shallow tanks with sediment) and repurposed larval-rearing conicals. Raceways are growing in popularity to rear geoducks post-settlement (pediveliger larvae to juvenile seed) and are indicative of the seasonal transition when larvae reared over winter months have reached settlement competency, leaving larval-rearing conicals unused. Repurposing these conicals as $p\text{CO}_2$ mixing tanks can condition seawater at no additional spatial or material cost for the hatchery. $p\text{CO}_2$ -conditioned seawater can be fed to header tanks positioned above raceways where algae feed is mixed before gravity-fed.

Similar to experiments in this dissertation, this approach strives for autonomous control and feasibility under the constraints of a small-business hatchery.

Common improvement methods in bivalve aquaculture include seawater and ration control, culling, and selective breeding. Domestication selection is an understudied problem in which environmental control ensures larval survival, but artificially propagates animals with high sensitivity to *in-situ* conditions. Long-term out growth from seed to harvest (5-7 years) presents a high likelihood for domestication selection to affect geoduck aquaculture. ‘*Anticipatory training*’ (as discussed in Chapter 3) proposes an alternative and more naturalistic perspective, applying stress exposures that reflect species’ natural habitat and developmental timing. In synthesis of chapters 2 and 3, geoducks acclimated under hypercapnic seawater increased tissue biomass and shell size and fine-tuned gene expression during subsequent stress exposures suggesting a moderate dose of stress can trigger robust phenotypes. In contrast, naive animals under OA grew slower and gene expression patterns indicated catabolism of endogenous storages (fatty-acid degradation) and high transcriptional demand. Distinct divergences between the naive and acclimated phenotypes highlight putative benefits of early-life stress, but the long-term implications of ‘*anticipatory training*’ remain speculative. Carryover effects beyond the hatchery period are only represented in chapter 1; despite the caveats of this chapter (described above), we found negative effects of $p\text{CO}_2$ stress preceded compensatory shell growth and metabolism in ~10-month geoducks. Future research is needed to test the long-term effects of stress conditioning and determine whether beneficial outcomes of ‘*anticipatory training*’ can offset the costs to integrate CO_2 -control systems in hatchery rearing.

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