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Acclimatory gene expression of primed clams enhances robustness to elevated pCO_2

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ABSTRACT

Sub-lethal exposure to environmental challenges may enhance ability to cope with chronic or repeated change, a process known as priming. In a previous study, pre-exposure to seawater enriched with pCO_2 improved growth and reduced antioxidant capacity of juvenile Pacific geoduck *Panopea generosa*, suggesting that transcriptional shifts may drive phenotypic modifications post-priming. To this end, juvenile clams were sampled and TagSeq gene expression data analyzed after 1) a 110-day acclimation under ambient (921 µatm, naïve) and moderately-elevated pCO_2 (2870 µatm, pre-exposed); then following 2) a second 7-day exposure to three pCO_2 treatments (ambient: 754 µatm; moderately-elevated: 2750 µatm; severely-elevated: 4940 µatm), a 7-day return to ambient pCO_2, and a third 7-day exposure to two pCO_2 treatments (ambient: 967 µatm; moderately-elevated: 3030 µatm). Pre-exposed geoducks frontloaded genes for stress and apoptosis/innate immune response, homeostatic processes, protein degradation, and transcriptional modifiers. Pre-exposed geoducks were also responsive to subsequent encounters, with gene sets enriched for mitochondrial recycling and immune defense under elevated pCO_2 and energy metabolism and biosynthesis under ambient recovery. In contrast, gene sets with higher expression in naïve clams were enriched for fatty-acid degradation and glutathione components, suggesting naïve clams could be depleting endogenous fuels, with unsustainable energetic requirements if changes in carbonate chemistry persist. Collectively, our transcriptomic data indicates pCO_2 priming during post-larval periods could, via gene expression regulation, enhance robustness in bivalves to environmental change. Such priming approaches may be beneficial for aquaculture, as seafood demand intensifies concurrent with increasing climate change in marine systems.

Keywords: Transcriptomics, Phenotypic plasticity, Ocean acidification, Molluscs, Stress priming

INTRODUCTION

Climate change exerts environmental pressures on marine life and is projected to continue to intensify in the near-future (Lotze *et al.*; IPCC 2021). In particular, ocean acidification (OA), or the reduction of ocean pH due to absorption of atmospheric CO_2, posits a major ecosystem and economic concern within highly-eutrophic coastal estuarine regions (Ekstrom *et al.*, 2015). 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 (Kleypas *et al.* 2006; Fabry *et al.* 2008), especially during early development and metamorphosis (Kurihara *et al.*, 2007; Waldbusser *et al.*, 2015; Kapsenberg *et al.*, 2018). Exacerbation of low pH conditions in coastal systems (Cai *et al.*, 2011; Melzner *et al.*, 2013) presents a growing concern for aquaculture (Barton *et al.* 2012) prompting interventions through water quality buffering and selective breeding programs (Barton *et al.* 2015). These actions optimize conditions for survival, however this can lead to domestication selection by propagating environmentally-sensitive cohorts (Araki *et al.* 2007; Nascimento-Schulze *et al.* 2021). A growing body of study proposes moderate stress priming to increase adaptive plasticity (Costantini *et al.* 2010; Hackerott *et al.* 2021), such that repeated challenges initiate beneficial responses (Hawkins and Warner 2017; Georgoulis *et al.* 2021) that may help marine organisms acclimatize to climate change.

Organismal environmental resistance depends on integration of predictable environmental cues into acclimatory phenotypes (Ghalambor *et al.* 2007; Snell-Rood *et al.* 2018). Although larvae of marine metazoans are highly susceptible to changes in the surrounding environment, early life presents an ideal window for developmental acclimatization due to the importance of environmental information in setting the stage for subsequent phenotypic outcomes (Burton and Metcalfe, 2014; Fawcett and Frankenhuis, 2015). Environmental variation (both spatial and temporal) shapes phenotypes (Dowd *et al.*, 2015) and numerous studies support an acclimatory capacity for marine invertebrates to cope with intermittent periods of thermal stress (Hraoui *et al.* 2021) and elevated pCO_2 within a generation (Suckling *et al.*, 2015; Détrée and Gallardo-Escárate, 2018; Gurr *et al.*, 2020; Li *et al.*, 2020), as well as across a generation (Parker *et al.*,

2015; Goncalves *et al.*, 2016). Thus, the timing and magnitude of environmental change is likely to have a joint effect on plasticity (Donelson *et al.*, 2018). Carryover effects of environmental history have physiological (Parker *et al.* 2012; Espinel-Velasco *et al.* 2021), ecological (Costantini *et al.* 2014; Hettinger *et al.* 2013), and evolutionary implications (Thomsen *et al.* 2017). In light of this, it is essential to understand how intermittent or repeated environmental signals, such as the challenges posed from climate change, are transduced to elicit acclimatory mechanisms.

Gene expression regulation is key to homeostasis and phenotypic plasticity. Both constitutive and inducible gene expression responses can enhance acclimatory capacity (Georgoulis *et al.* 2021; Barshis *et al.*, 2013). Transcriptome profiling of clams and oysters exposed to changing environmental conditions has identified differential regulation of mitochondrial complexes, antioxidants, and proteins related to lipid degradation (Chapman *et al.*, 2011; Goncalves *et al.*, 2017; López-Landavery *et al.*, 2021; Teng *et al.*, 2021), indicating that external abiotic conditions can affect metabolism and shift substrates for bioenergetics. Furthermore, enhanced constitutive expression, or gene frontloading, is a proposed mechanism to cope with challenging but predictable environmental signals (Barshis *et al.*, 2013). For instance, limpets (*Lottia sp.*) occupying the high intertidal transcribe heat-shock proteins at a higher level relative to low-intertidal individuals, suggesting an acclimated response (Dong *et al.*, 2008). Thus, transcriptomics provides a broad and sensitive means to assess the role of gene expression in environmental priming and the molecular underpinnings of important economic traits in aquaculture species (Chandhini and Kumar, 2019).

Geoduck clams (*Panopea sp.*) are long-lived molluscs of high economic value and recent studies corroborate their particular resilience to OA (Spencer *et al.*, 2019; Gurr *et al.*, 2020, 2021). Transcriptome profiles of geoduck clams reared under OA conditions found regulation of energy production and acid/base homeostasis in umbonate-stage larvae of Mexican geoduck *Panopea globosa* (López-Landavery *et al.*, 2021), whereas similar exposures delay metamorphosis in the Japanese geoduck *Panopea japonica* (Huo *et al.*, 2019). Transcriptome profiling of *P. generosa* has provided critical molecular insight on effects of low-pH exposure, highlighting molecular metabolic shifts over larvae-to-juvenile development and the

informative capacity of transcriptomics for examining responses to low pH in this species (Timmins-Schiffman *et al.*, 2020). There is also evidence that repeated 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 light of this, we posited that pre-exposure, or priming, generates changes in transcriptome profiles under repeated encounters. Specifically, we hypothesized that there will be frontloading of distinct gene functions and pathways underpinning adaptive phenotypes from early-life priming. To this end, we examined gene expression patterns underlying high-pCO_2 priming in postlarval *P. generosa* that resulted in larger (tissue biomass and shell length) juvenile clams with reduced total antioxidant capacity after repeat exposures (Gurr *et al.*, 2021). Gene expression data was analyzed for juvenile *P. generosa* acclimated at the pediveliger stage under ambient and moderately-elevated pCO_2 conditions for 110 days (day 0) and then were subsequently reexposed in a reciprocal fashion to a second exposure of either ambient, moderate-elevated pCO_2, or severely-elevated pCO_2 for 7 days (day 7), a 7-day return to ambient pCO_2 (day 14), and a third 7-day exposure to ambient or moderately-elevated pCO_2 (day 21; Fig. 1). Review Gurr *et al.* (2021) for details regarding the rationale (i.e. timing and magnitude of pCO_2 conditions) and limitations of this hormetic framework.

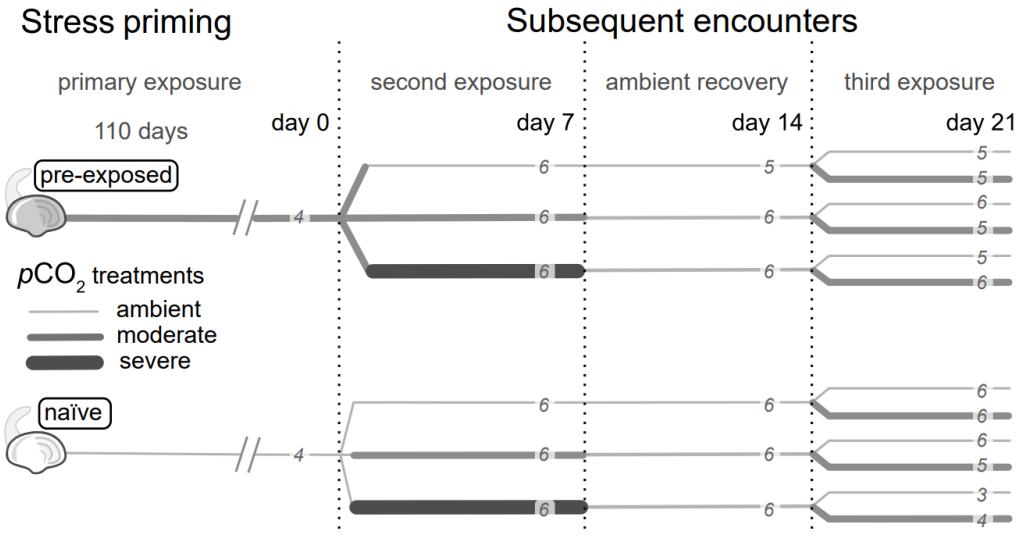

Figure 1. Experimental design for sampling. Line thickness indicates the pCO_2 treatments. Whole individual geoduck samples were snap frozen in liquid nitrogen at ~10:00 am on each of days 0, 7, 14, and

21 and subsequently sequenced for gene expression (italicized numbers). White and grey geoduck cartoons correspond to primary exposure under ambient (naïve) and moderate elevated pCO_2 (pre-exposed), respectively. This figure is adapted from Gurr *et al.* (2021).

METHODS

pCO_2 exposure experiment and tissue sampling

Larval Pacific geoduck clams were reared from gametes at the Jamestown Point Whitney Shellfish Hatchery (Brinnon, WA) following standard industry practice (i.e. live-algal feed regime, larvae runts culled periodically, etc.). Once animals reached settlement competency (30 days post-fertilization), pediveliger larvae were exposed to ambient and elevated pCO_2 conditions (921 ± 41 µatm and 2870 ± 65 µatm) for an initial 110-day conditioning period targeting the metamorphic transition from pediveliger to the burrowing juvenile stage (N=4 trays treatment^{-1} and N=1.5×10^4 pediveliger geoduck tray^{-1}). The timing for primary exposure has naturalistic relevance, as postlarval development represents a transition from a free-swimming stage to a sedentary life in the benthos (Goodwin and Pease, 1989), where bacterial carbon mineralization and low buffering capacity elevate pCO_2 and decrease calcium carbonate saturation (Cai *et al.* 2011). Survivorship over the pediveliger-to-juvenile transition was ~30% regardless of pCO_2 condition (4-5×10^3 juvenile geoduck tray^{-1}; Gurr *et al.* 2021). Juveniles acclimated under ambient and elevated pCO_2, hereafter referred to as naïve and pre-exposed clams, were divided at equal density into 36 replicate vessels (N=120 animals per vessel, N=6 vessels treatment^{-1}), and subjected to a secondary 7-day period under three pCO_2 conditions (ambient pCO_2=754 ± 15 µatm, moderate pCO_2=2750 ± 31 µatm, and severe pCO_2=4940 ± 45 µatm) followed by 7 days of ambient recovery (896 ± 11 µatm) before replicates were split into 72 vessels (N=6 vessels treatment^{-1}) for a 7-day third exposure in two conditions (ambient pCO_2=967 ± 9 µatm and moderate pCO_2=3030 ± 23 µatm; Fig. 1); the time to reach target treatments (i.e. ambient to moderately-elevated pCO_2) occurred more rapidly (~3 hours) than the return to ambient conditions from elevated pCO_2 levels (~ 6-8 hours). Note these pCO_2 values are higher than pCO_2 in the open ocean because they are designed to be relevant to the native range of *P. generosa*, as they correspond to measurements at local estuarine sites and sediment conditions where the clams live (e.g. >2400 µatm and Ω_{arag} <0.4 in Hood Canal,

WA: Feely *et al.*, 2010; Reum *et al.*, 2014; Ω_{arag} 0.4–0.6 in sub-surface sediments: Green *et al.*, 2009). Geoduck were fed a live mixed-algae diet *ad libitum* with a programmable dosing pump (Jebao DP-4), targeting 5×10^4 cells ml^{-1} in each vessel. Furthermore, marine bivalves can reestablish acid-base homeostasis 24-48 hours after exposure to acidified seawater (Michaelidis *et al.* 2005; Spicer *et al.* 2007), therefore we considered a span of 7 days as sufficient to infer a stable state during exposure to elevated pCO_2 and ambient seawater. Additional details on geoduck rearing and experimental conditions are outlined in Gurr *et al.* (2021). As previously described in Gurr *et al.* (2021), the pre-exposed clams displayed a phenotype of reduced total antioxidant capacity and increased shell growth and tissue biomass under subsequent pCO_2 challenges, which provided evidence supporting the pediveliger-to-juvenile window for adaptive developmental plasticity. In this study, samples were sequenced at the same time points as physiological samples collected in Gurr *et al.* (2021) to investigate transcriptome profiles and their linkages to the differing phenotypic outcomes. Whole juveniles from each replicate tray and vessel were snap frozen in liquid nitrogen at ~10:00 am on the final day of the initial priming period ($N = 8$ treatment^{-1}; Fig. 1 day 0; after 110-day primary exposure), secondary exposure ($N = 6$ treatment^{-1}; Fig. 1 day 7), ambient recovery ($N = 6$ treatment^{-1} except one instance where N=5; Fig. 1 day 14), and third exposure ($N = 3$-6 treatment^{-1}; Fig. 1 day 21).

Gene expression

Individual whole juvenile geoduck samples were homogenized in DNA/RNA shield (1 ml) with 0.25 ml 0.5 mm glass beads by vortexing for 1 minute. Total RNA was extracted from whole tissue homogenate using the Quick-DNA/RNA Kit (Zymo) according to manufacturer's instructions. RNA was quantified using the Qubit RNA Broad Range Assay Kit with fluorometer (ThermoFisher) and quality was ascertained using a 4200 TapeStation System (Agilent Technologies) to visualize ribosomal bands, keeping in mind that the bands co-migrate in geoduck clams into a single peak. 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 and sequencing of the

141 samples was conducted at the University of Texas Austin, Genomic Sequencing and Analysis Facility. Sequencing was completed on two lanes of Illumina NovaSeq 6000 SR100, targeting standard coverage of 3-5 million 100 bp single-end reads per sample. Raw TagSeq reads were trimmed of Illumina adapters, poly-A sequence, and quality filtered with fastp (Chen *et al.* 2018); quality control for filter optimization was completed using MultiQC (Ewels *et al.* 2016). Following quality control, reads were mapped to the *P. generosa* reference genome (Putnam et al. 2022) using HISAT2 (Kim *et al.* 2015) with a mapping efficiency of ~30%; the majority of unmapped reads aligned to ribosomal rRNAs (i.e. 18S and 28S) in geoduck clams (i.e. *Panopea globosa*). Stringtie2 (Pertea *et al.* 2015) 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). All code and data is publicly available (Gurr *et al.* 2021; doi:10.5281/zenodo.6908630).

Gene expression analysis

We chose a combined analytical approach to first test for gene expression frontloading (sensu Barshis *et al.* 2013) and fine-tuning or responsiveness to pCO_2 change by examining modules of co-expression via network analysis and their functional enrichment (Fig. 2) and second to test for differential expression in pairwise treatment groups at each time point (i.e. differentially expressed genes; DEGs) and their functional enrichment.

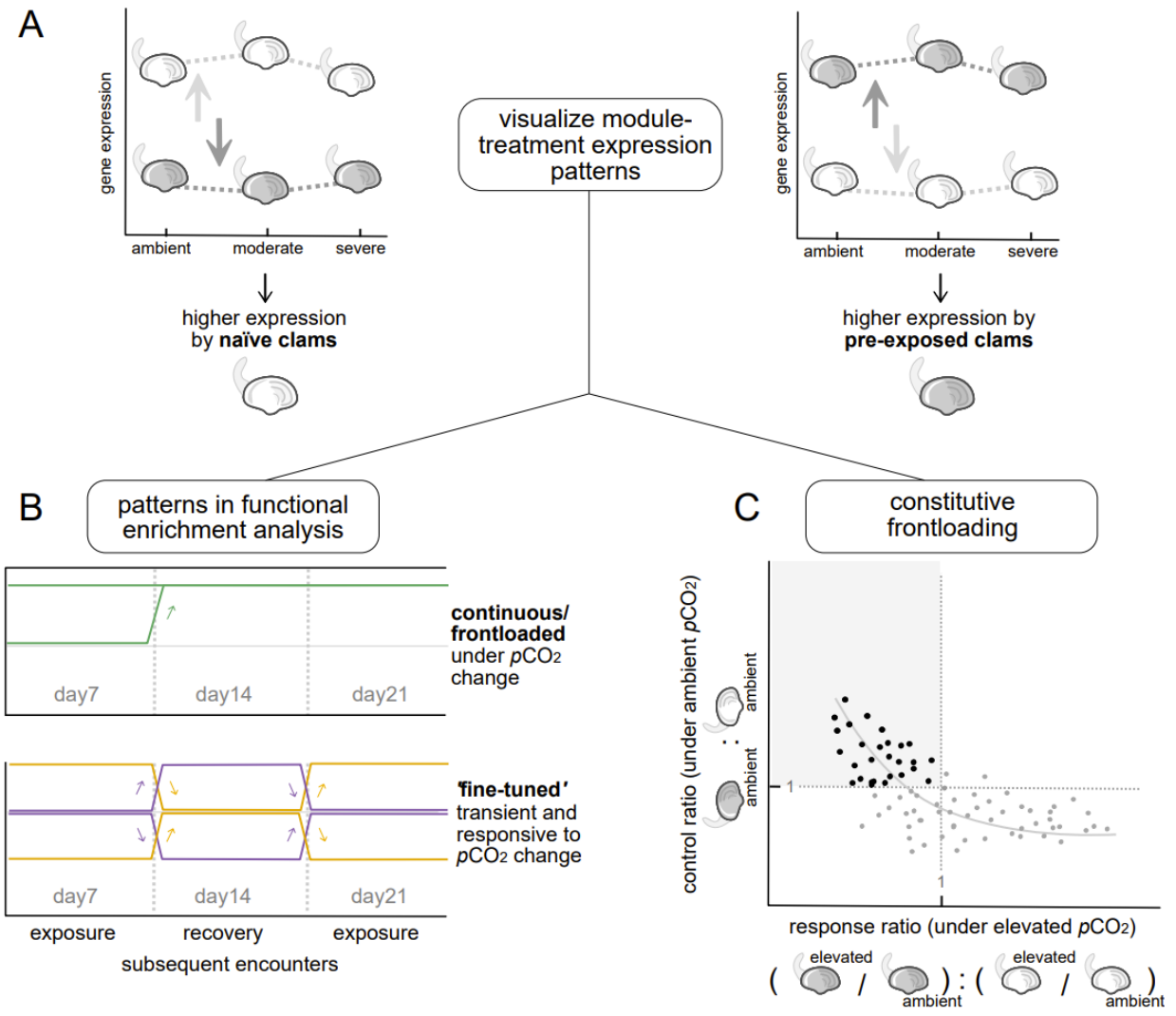

Figure 2. Conceptual guide on our statistical approach to identifying gene frontloading and fine-tuning using co-expression analysis. White and gray geoduck cartoons represent the averaged expression data of naïve and pre-exposed clams. Shown is the directionality of module-treatment associations strongly correlated with the primary exposure period (A), containing groups of genes with similar expression patterns across time points. These putatively frontloaded genes were investigated for continuous or transient functional enrichment (B). Further, constitutive frontloading (sensu Barshis *et al.* 2013) defined by the quantitative criteria of genes with higher constitutive expression and lower response to reciprocal exposure, was used following co-expression network analysis. Genes that fall in the upper left quadrant are thus defined as "frontloaded" in their expression (C).

First, Weighted Gene Co-expression Network Analysis (WGCNA; Zhang and Horvath 2005; Langfelder and Horvath 2008) was used to identify gene expression patterns and test for frontloading of transcripts (constitutive changes in expression, Fig 2C), or fine-tuning of transcripts under pCO_2 change (dynamic changes across repeated exposures), due to conditioning history by calculating gene co-expression modules

at each time point. Co-expression network construction allows an assessment of broad expression-level directionality and the influence of compounding treatment history (e.g. initial × second exposure × third exposure), as opposed to typical approaches of pairwise differential expression analysis. Second, we used DESeq2 to test for differential expression in pairwise group comparisons. While pairwise group comparisons are not able to examine the interactive effects, pairwise models investigated the effects of initial 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 (not interactive) pCO_2 treatment history.

Gene expression in response to pre-exposure and repeated encounters was analyzed with WGCNA using the Bioconductor (v.3.13) package '*WGCNA*' (v.1.70-3; Langfelder and Horvath 2008, Langfelder and Horvath 2012) in R (R Core Team 2021) to assess co-expression patterns (Zhang and Horvath, 2005). Following co-expression module assignment of each read matrix (days 0, 7, 14, 21; Fig. 1), gene expression was transformed using variance-stabilizing transformation ('*varianceStabilizingTransformation*' DESeq2 v1.12.3; Anders and Huber 2010; discussed as vst normalized gene expression) to visualize gene expression patterns associated with significant module-treatment associations. At each time point, the treatments represent the exposure history until that time point (e.g., Day 0 = A and M; Day 7 and Day 14 = AA, AM, AS, MA, MM, MS; and Day 21 = AAA, AAM, AMA, AMM, ASA, ASM, MAA, MAM, MMA, MMM, MSA, MSM). This full analysis identified that the primary exposure (A vs M) was a major driver of the expression response. Thus we plotted heatmaps of module-treatment correlations for both the full analysis (all treatments) and based on the primary treatments only (the same gene set visualized and analyzed with the main effect of primary history only). To determine the functional patterns associated with pCO_2 treatment, co-expression modules significantly correlated with treatment variables were assessed for significantly-enriched 'molecular function' and 'biological process' GO terms ($p<0.05$) and GOslim assignment was used to place significantly enriched GO terms into hierarchical bins of broader function. Lastly, Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa and Goto 2000) was applied to understand higher-level functional processes of co-expression modules and the web interface '*KEGG*

Mapper' (Kanehisa and Sato 2020) was used to manually investigate genes involved in enriched pathways. Additional details regarding read filtration and gene expression analysis (WGCNA and DESeq2) are provided in the supplement (Supporting Information).

Co-expression module-treatment associations significantly correlated with the primary exposure period (Figs 2A) were categorized as "frontloaded" or "fine-tuned" with respect to their expression patterns. Gene sets were identified as either consistently higher in expression through time (Fig. 2B and 2C) were identified as putatively frontloaded, and those that were dynamic across repeated exposures (Fig 2B) were identified as putatively fine-tuned. Quantitatively, gene frontloading was assessed as described in Barshis *et al.* (2013), where frontloaded genes are defined as those with a higher constitutive expression due to priming and less responsive to a subsequent environmental challenge. The term 'frontloaded' was therefore assigned to those genes with higher expression under ambient pCO_2 by pre-exposed clams ('control ratio'; Fig. 2C Y-axis) and lower response ratio by pre-exposed clams ('response ratio' = expression in elevated pCO_2: expression in ambient pCO_2; Fig. 2C X-axis). Frontloaded genes can then be defined as those with control ratio >1 and response ratio <1 (i.e., top left quadrant Fig 2C). A one-way ANOVA was used to test differences in vst normalized gene expression of frontloaded genes between primary×second pCO_2 treatments. In contrast to frontloaded genes, fine-tuned expression was defined by first identifying the genes in modules correlated with exposure to the primary exposure period, and then second by tracking these genes underlying the GO terms that were uniquely responsive to subsequent exposures (Fig 2B).

Raw sequence data is available on NCBI (BioProject: PRJNA740307). Analytical code and data files (i.e. gene lists and enrichment analysis) are publicly available in an open repository (Gurr *et al.* 2021; doi:10.5281/zenodo.6908630).

RESULTS

Co-expression network analysis overview

Network analysis using WGCNA (Zhang and Horvath 2005; Langfelder and Horvath 2008) resulted in groups of genes that shared expression at a time point, termed modules. These modules are each

named with a color based on the convention of Horvath and Langfelder (Zhang and Horvath 2005; Langfelder and Horvath 2008). Given our goal to examine the interaction of repeated exposures, we analyzed gene co-expression at each time point, resulting in modules named with colors at each point. To differentiate these, we have used the following nomenclature: "Day X module color". The modules were then tested for significant correlation with treatment groups via module eigengene-treatment correlations (using the Pearson method in the *cor* function in WGCNA). These correlations quantify the strength and direction of the association between the gene expression profile of a module and a specific treatment. If a module eigengene-treatment correlation is positive, the expression of the module is greater within that treatment. Conversely if a module eigengene-treatment correlation is negative, the expression of the module is lower within that treatment.

Day 0

Network analysis after the 110-day acclimation period (primary exposure, day 0) resulted in six co-expression modules with between 414 and 2818 genes (Fig. S1), excluding the module containing only one gene (the module 'grey' which contains unassigned genes). One module (Day 0 midnightblue) was significantly correlated with the primary exposure treatment and represented genes with higher vst normalized gene expression values in the naïve clams (Fig. S1). It must be noted that the authors of WGCNA recommend larger sample matrices (e.g. >15 samples) to yield robust and biologically relevant data (Langfelder and Horvath 2008); because eight samples were sequenced on day 0, these results must be interpreted with caution and are presented in the supplement (Fig. S1).

Genes within Day 0 module midnightblue were significantly enriched for 'pentose phosphate pathway' (*N*=8), 'glycolysis / gluconeogenesis' (*N*=14), 'carbon metabolism' (*N*=28), 'foxO signaling pathway' (*N*=16), and 'ubiquitin mediated proteolysis' (*N*=24; Fig S1). Gene families associated with enriched pathways included, but not limited to, enzymes involved in glycolysis, citric acid cycle, immune response, protein ubiquitination, mitogen-activated protein kinase signaling, and oxidative stress response.

Day 7

Modules correlated with initial treatment

Network analysis of expression following second exposure (day 7) resulted in five co-expression modules containing between 304 and 3951 genes (Fig. S2A). Of these five modules, two were significantly correlated with the primary exposure treatment (Day 7 module brown, and Day 7 module yellow Fig. S2A). Day 7 module brown (*N*=862 genes) showed higher vst normalized gene expression values in the naïve clams relative to the pre-exposed clams, whereas Day 7 module yellow (*N*=610 genes) had higher vst normalized gene expression in the pre-exposed clams (Fig. S2B).

Day 7 module brown, representing genes highly expressed by naïve clams, was enriched for pathways 'fatty acid degradation' (*N*=9), 'fatty-acid metabolism (*N*=9), 'retinol metabolism' (*N*=5), 'peroxisome' (*N*=12), 'lysosome' (*N*=18), and 'endocytosis' (*N*=16; Table S1 and Fig 3). Further, GO analysis of Day 7 module brown resulted in enriched terms in the following GOslim categories: ion binding, protein transport and transport, lipid binding, peptidase activity, immune system response, enzyme binding, and oxidoreductase activity (Figs. 3, S5A, S6, and S8A). Gene families associated with enriched pathways and GO terms included, but not limited to, enzymes involved in mitochondrial and peroxisomal *B*-oxidation, carnitine acyltransferases, acetate metabolism, proteases, oxidative stress response, intracellular trafficking and endocytosis, regulation of apoptosis, and transcriptional regulators.

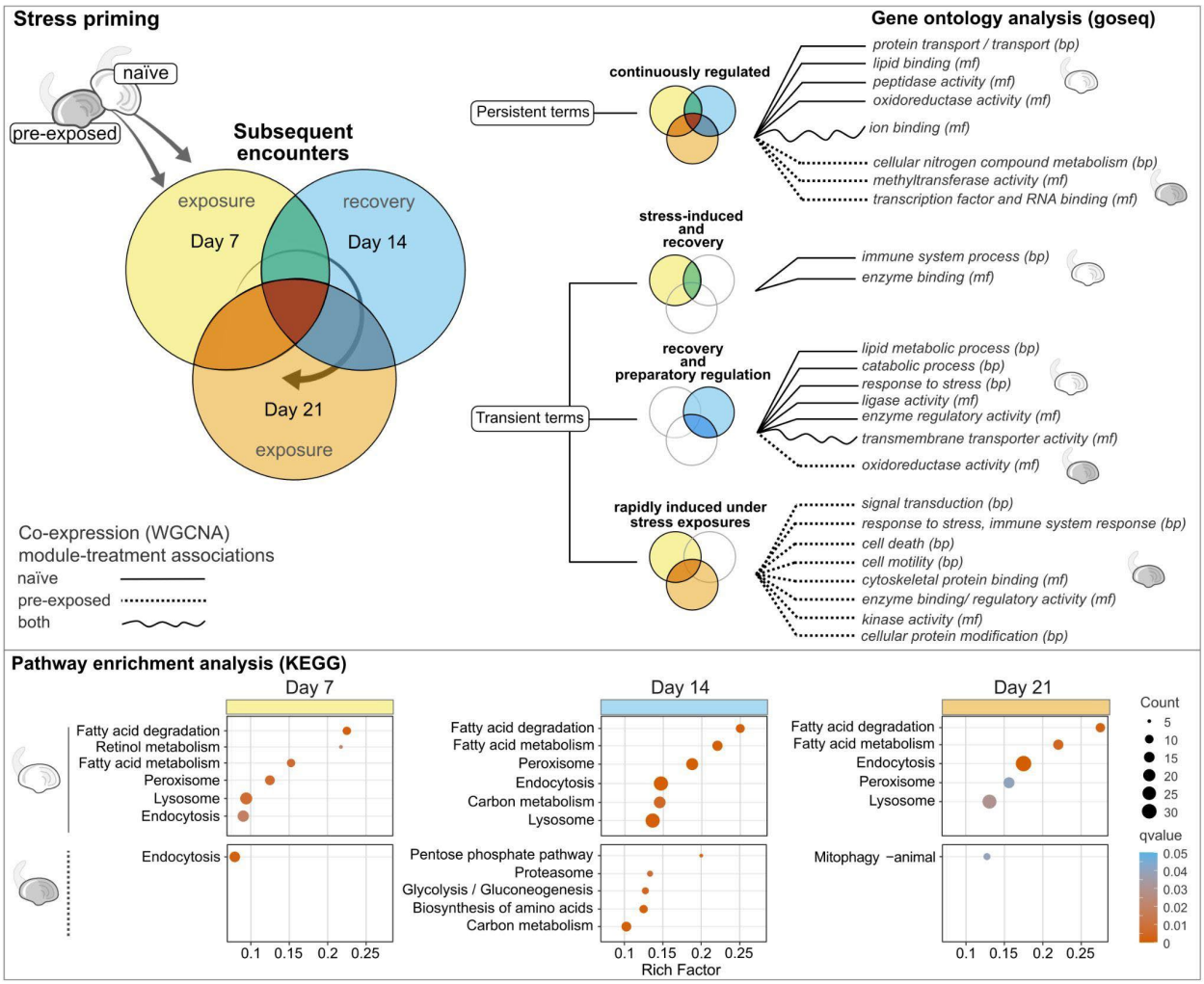

Figure 3. GO and pathway enrichment analysis of modules representing higher expression by naïve clams (white geoduck symbol, WGCNA modules = Day 7 brown, Day 14 brown, and Day 21 blue and magenta) and pre-exposed clams (grey geoduck symbol, WGCNA modules = Day 7 yellow, Day 14 black, and Day 21 yellow). Branches show enriched hierarchical GO terms ('bp' = biological process; 'mf' = molecular function) unique to naïve and pre-exposed clams (solid and dashed lines) and those that were shared (wavy lines). GO terms are divided into two major groups: (1) 'persistent terms' are enriched functions throughout the experiment; (2) 'transient terms' are enriched unique to or partially shared between the second exposure and ambient recovery period as 'stress-induced and recovery', ambient recovery and third exposure period as 'recovery and preparatory regulation', and both elevated pCO_2 periods as 'rapidly induced under stress exposures'. Enriched pathways (p_{adj}<0.05) are shown for each module representing higher expression by naïve and pre-exposed clams.

Day 7 module yellow, representing genes highly expressed by pre-exposed clams, was enriched for terms in the pathway 'endocytosis' (*N*=14; Table S2) and the following genes or gene families were associated with this pathway: E3 ubiquitin-protein ligases, receptor proteins, and protein trafficking and transport. Further, GO analysis of Day 7 module yellow resulted in enriched GO terms in the following

GOslim categories: ion binding, cellular nitrogen compound metabolic process, methyltransferase activity, transcription factor and RNA binding, response to stress, immune system response, cell death, cell motility, cytoskeletal protein binding, enzyme binding / regulatory activity, and kinase activity (Figs. 3, S5B, S7, and S8A). Gene families associated with enriched GO terms included, but not limited to, chromatin modifiers, transcription factors, stress response proteins, innate immune/antiviral response/signaling cascade, MAP kinases, ion transport, E3 ubiquitin-protein ligases, and serine/threonine-protein kinases.

Modules correlated with interactions of treatments

One module was only significantly correlated with the combined primary and second treatments (Day 7 module green; Fig. S3) and suggests that primary acclimation treatments and second exposures under moderate and severe pCO_2 lead to divergent expression patterns. Day 7 module green (*N*=304 genes) showed low gene expression by pre-exposed clams reexposed under moderate pCO_2, whereas naïve clams had high expression values when exposed under severe pCO_2 ('MM' < 'AS'; Fig. S3). This module was enriched for cellular nitrogen compound metabolic process and included transcription factors and other regulators of transcription. There were no pathways enriched for Day 7 module green.

Frontloaded genes

Of the full Day 7 module yellow (*N*=610 genes), naïve clams increased expression of 346 and 184 genes under moderately-elevated and severely-elevated pCO_2 of which 243 and 138 genes were assigned as 'frontloaded' by pre-exposed clams; 106 genes were frontloaded under both moderately and severely-elevated pCO_2 (Fig. 4). Frontloaded genes were attributed with, but not limited to, stress response and apoptosis/innate immune response (heat shock 70 kDa protein, toll-like receptors, caspase-10, antiviral innate immune response receptor RIG-I, and MY88), homeostatic processes (sodium/calcium exchanger), protein ubiquitination/degradation (E3 ubiquitin ligases XIAP, TRAF6, and rnf168), transcription factors (i.e. Mafk, SNW domain-containing protein 1, Kruppel-like factor, TNF-receptor-associated factor, and bile acid receptor), and histone and chromatin-binding proteins (i.e. histone-binding protein N1/N2,

histone-lysine N-methyltransferase, and chromatin remodeling ATPase INO80). Frontloaded genes were significantly affected by pCO_2 treatment (one-way ANOVA; $p < 0.001$) and *a posteriori* pairwise differences reinforced the constitutive gene frontloading criteria (Fig. 4).

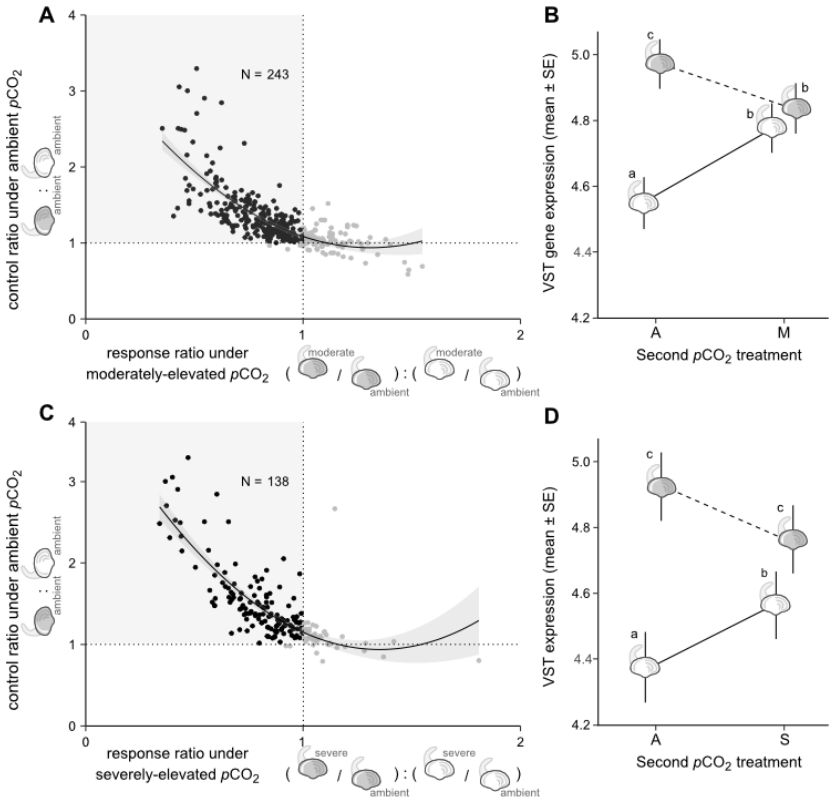

Figure 4. Frontloaded genes, as solid black points, under moderate and severe pCO_2 change (A and C), each supplemented visually with a loess curve (solid line with 95% CI). Right panels display the mean ± standard error vst gene expression values of frontloaded genes under moderate and severe pCO_2 change (B and D) with letters for Tukey's *a posteriori* significant differences ($p < 0.05$). White and grey geoduck cartoons represent the average vst gene expression.

Day 14

Modules correlated with initial treatment

Network analysis of expression following ambient recovery (day 14) resulted in nine co-expression modules containing between 391 and 2669 genes (Fig 3A). Of these nine modules, two were significantly correlated with primary exposure treatment (Day 14 module brown and Day 14 module black; Fig. S3A). Day 14 module brown (*N*=1164 genes) showed higher vst normalized gene expression values in the naïve

clams, whereas Day 14 module black (*N*=415 genes) showed higher vst normalized gene expression values in the acclimated clams (Fig. S3B).

Day 14 module brown, representing genes highly expressed by naïve clams, was enriched for 'fatty acid degradation' (*N*=10), 'fatty-acid metabolism' (*N*=13), 'peroxisome' (*N*=18), 'endocytosis' (*N*=26), 'carbon metabolism' (*N*=17), and 'lysosome' (*N*=26; Table S1 and Fig 3). In addition to pathways enriched in Day 7 module brown (also representing higher expression by naïve clams), gene families of enriched pathways in Day 14 module brown involved the citric acid cycle and glucose and glycine metabolism. GO analysis of Day 14 module brown included enriched terms in several GOslim categories also in Day 7 module brown (Figs. 3. S5A, S6, and S8B) with additional glutathione components, proteases, endocytosis and trafficking-related proteins, and lipid catabolic proteins. Day 14 module brown was also enriched for transmembrane transporter activity, lipid metabolic process, catabolic process, response to stress, ligase activity, and enzyme regulatory activity (Figs. 3. S5A, S6, and S8B); these terms included genes for stress response, protein recycling, and trypsin inhibition.

Day 14 module black, representing genes highly expressed by pre-exposed clams, was enriched for 'pentose phosphate pathway' (*N*=5), 'proteasome' (*N*=6), 'glycolysis / gluconeogenesis' (*N*=7), 'biosynthesis of amino acids' (*N*=9), and 'carbon metabolism' (*N*=12; Table S1 and Fig 3). Gene families associated with enriched pathways included, but not limited to, glycolytic enzymes, citric acid cycle, protein recycling, and aminotransferases. GO analysis of Day 14 module black included enriched terms in several GOslim categories also in Day 7 module yellow (also representing higher expression by pre-exposed clams; Figs. 3. S5B, S7, and S8) and additional transcriptional regulators and immune signaling proteins. Further, Day 14 module black was enriched for oxidoreductase activity and transmembrane transporter activity (Figs. 3. S5B, S7, and S8); these terms included proteins/complexes of the mitochondrial electron transport chain, iron-binding proteins, and regulation of sodium/bicarbonate and calcium ion channels.

Modules correlated with interactions of treatments

Four modules were significantly correlated with the combined primary and second treatments (Day 14 modules brown, black, pink, and magenta; Fig. S3A) and each suggest primary acclimation treatments and second exposures under moderate and severe pCO_2 lead to divergent expression patterns. Of these four modules, two were not significantly correlated with the initial acclimation treatment (Day 14 module pink, and Day 14 module magenta; Fig. S3A). Day 14 module magenta (*N*=336 genes), representing genes with low expression values by pre-exposed clams reexposed under severe pCO_2, was enriched for 'spliceosome' (*N*=8; Table S2) involving alternative splicing proteins. GO analysis of Day 14 module magenta showed enriched terms in GOslim categories for cellular nitrogen compound metabolic process and ion binding (Fig. S8B) and included components of the spliceosome, E3 ubiquitin-protein ligases, tubulin proteins, and Ras-related proteins. Day 14 module pink (*N*=391 genes), representing genes highly expressed by pre-exposed clams reexposed under severe pCO_2 (Fig. S3), did not show pathway enrichment (Table S1), however GO terms were enriched in GOslim categories of cellular nitrogen compound metabolic process, DNA binding, enzyme regulator activity, and oxidoreductase activity (Fig. S8B) including homeodomain transcription factors, superoxide dismutase, and Rho-GTPase activating proteins.

Day 21

Modules correlated with initial treatment

Network analysis of gene expression following the third exposure (day 21) resulted in nine co-expression modules containing between 451 and 1753 genes (Fig. S4). Of these nine modules, three were significantly correlated with the primary exposure treatment (Day 21 modules blue, magenta and yellow; Fig. S4A). Day 21 modules blue (*N*=1537 genes) and magenta (*N*=241 genes) showed higher vst normalized gene expression values in naïve clams, whereas Day 21 module yellow (*N*=926 genes) showed higher expression values in the pre-exposed clams (Fig. S4B).

Day 21 module blue, representing genes highly expressed by naïve clams, was enriched for 'fatty acid degradation' (*N*=11), 'fatty-acid metabolism' (*N*=13), 'endocytosis' (*N*=31), 'peroxisome' (*N*=15), and

'lysosome' (*N*=25; Table S1 and Fig 3), involving the same genes and gene families enriched in earlier modules with the same expression pattern (i.e. Day 7 module brown and Day 14 module brown). Day 21 module magenta was enriched for 'protein processing in endoplasmic reticulum' (*N*=10; Table S2) and contained genes for protein quality control and trafficking and stress of the endoplasmic reticulum. All enriched GO terms for Day 21 modules blue and magenta were in GOslim categories that were also enriched in earlier modules representing the same expression pattern (i.e. Day 7 module brown and Day 14 module brown) with the exception of enzyme binding and immune system response (Figs. S5A, S6, and S9). These additional terms included genes for endosomal cargo trafficking and transport, regulation of ion channels, serine/threonine-protein kinases, E3 ubiquitin ligases, and zinc finger proteins.

Day 21 module yellow, representing genes highly expressed by pre-exposed clams, was enriched for 'mitophagy' (*N*=7; Table S1 and Fig 3) and included PINK1-Parkin components, autophagy receptors, lysosomal degradation, and forkhead box protein o transcription factor. All enriched GO terms for in Day 21 module yellow were in GOslim categories that were also enriched in earlier modules representing the same expression pattern (i.e. Day 7 module yellow and Day 14 module black; Figs. S5B, S7, and S9). Enriched terms included additional genes such as autophagy receptors, tyrosine-protein kinases, histone modifiers, and transcriptional regulators.

Modules correlated with interactions of treatments

Six modules were significantly correlated with the combined primary, second, and third treatments (Day 21 modules blue, magenta, red, black, pink, turquoise; Fig. S4). Of these six modules, two were significantly correlated with the primary exposure treatment (Day 21 modules blue and yellow) and four were only correlated with the combined treatment history (Day 21 modules red, black, pink, turquoise; Fig. S4A). Day 21 module black (*N*=660), representing genes expressed at a low abundance by naïve animals under moderately-elevated pCO_2 during the third exposure (Fig 4A), was enriched for 'ribosome biogenesis in eukaryotes' (*N*=15), 'spliceosome' (*N*=19), 'RNA transport' (*N*=19), and 'protein processing in

endoplasmic reticulum' (*N*=13; Table S1). There were no pathways enriched for Day 21 modules pink (*N*=451 genes), red (*N*=713 genes), or turquoise (*N*=1753 genes; Table S1).

Differential gene expression

Differential gene expression analysis on Day 0 resulted in 14 DEGs between treatments with higher expression level of eleven genes by naïve clams and three genes by pre-exposed clams (Table S2 and Fig. 5). Only four DEGs contained gene name and functional annotation and included higher expression levels under ambient for E3 ubiquitin-protein ligase rnf213-alpha and helicase with zinc finger domain and higher expression levels under elevated pCO_2 for putative isoforms for von Willebrand factor D protein.

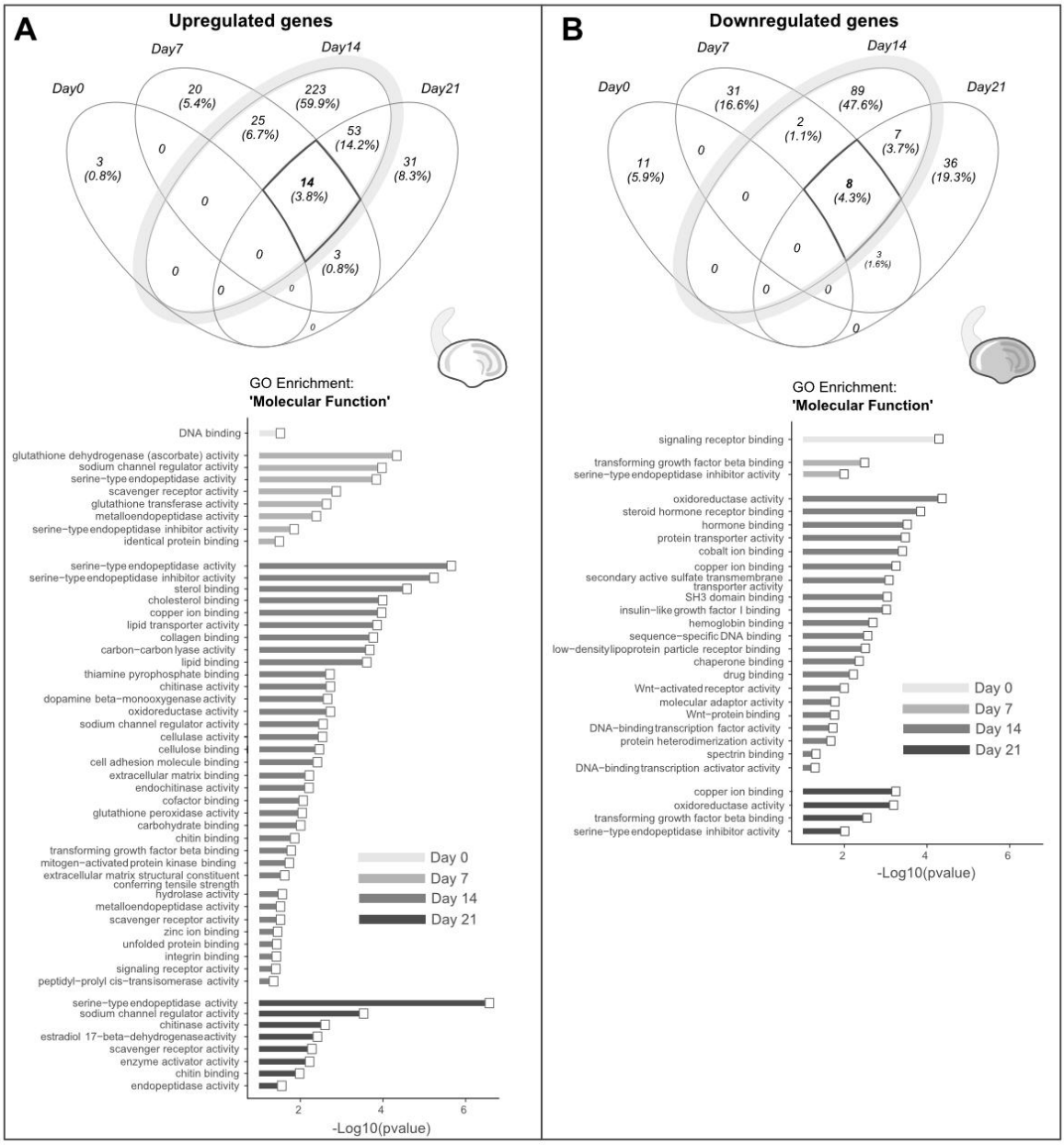

Figure 5. Effect of ambient and moderate-elevated pCO_2 priming on differential gene expression. Venn diagrams show the number of genes upregulated (A, higher expression level in naïve clams) and downregulated (B, higher expression level in pre-exposed clams) and unique to and shared between experiment days; featured counts in bold represent persistent DEGs shared among days 7, 14, and 21. Segment charts represent significant 'molecular function' GO terms ($-\log_{10}$p-value) in grayscale for time points.

Subsequent exposures on days 7, 14, and 21 showed greater differential expression due to pCO_2 priming than second or third pCO_2 treatments (Tables S2). Pairwise comparison of primary ambient versus moderately-elevated pCO_2 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 S2 and Fig. 5). In summary, the majority of DEGs resulting from primary exposure in this study were upregulated in the naïve phenotype (70%), especially following ambient recovery (85% of upregulated DEGs; Table S2 and Fig. 5). Functional annotation of genes highly expressed by naïve clams involved glutathione components (dehydrogenase, peroxidase, and transferase), endopeptidases, fatty-acid binding and lipid metabolism, and transmembrane regulatory activity and genes highly expressed by pre-exposed clams were enriched for signaling, oxidoreductase activity, stress response (transforming growth factor beta binding), and metal ion binding (Fig. 5). There were 22 DEGs (14 upregulated and 8 downregulated) that occurred on all sampling days deemed as 'persistent DEGs' (Fig. 5). 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 S3). Persistent DEGs with higher expression level by naïve clams notably differed in their functional annotation for response to stress, lipid and calcium binding, and protease inhibition (apolipoprotein D, regucalcin, mucin-1, four-domain protease inhibition, etc.; Table S3). Persistent DEGs with higher expression level by pre-exposed clams, although fewer, were additionally associated with antimicrobial activity, cobalt transport (cobalamin) and protease inhibition (defensin, CD109 antigen, and BPTI/Kunitz domain-containing protein 4-like; Table S3).

Pairwise models addressing second and third pCO_2 exposures yielded minimal expression-level differences (0-13 total DEGs), with the exception of the second pCO_2 treatment on day 7 (106 total DEGs: 14 upregulated and 92 downregulated; Table S2). GO analysis of downregulated genes showed enrichment of cell adhesion, plasminogen activation, and endopeptidase activity. Results of cumulative treatment histories on day 7 (primary×second; N=16 pairwise models), found 168 total DEGs in the pairwise model MA v. AM (31 upregulated and 137 downregulated; Table S4); upregulated genes were enriched for actin filament polymerization, cell migration, and cilium assembly and downregulated genes were primarily associated with plasminogen activation, cell adhesion, and proteolysis. Results of cumulative treatment models for day 14 and 21 are provided in supplementary tables (Tables S5 and S6).

DISCUSSION

Post-larval acclimation to elevated pCO_2 affected transcriptome profiles. Specifically, priming to moderately-elevated pCO_2 involved frontloaded and continuous expression of stress/innate immune response proteins, ubiquitin ligases, transcription factors, and chromatin modifiers as well as transient expression of genes affecting cellular homeostasis (e.g. cellular quality control, mitophagy, immune signaling/defense, and energy metabolism) during periods of reexposure to elevated pCO_2 and ambient recovery. Moreover, functional annotation suggests putative control via transcriptional modifiers (e.g. transcription factors and histone methyltransferases) in the pre-exposed phenotype. In contrast, the naïve phenotype showed higher overall gene expression (74% of genes in treatment-module correlations showing higher expression in naïve clams and >70% DEGs with higher expression level in naïve clams) with functional annotation for fatty-acid metabolism, primarily by peroxisome β-oxidation, and glutathione metabolism. This transcriptomic pattern in naïve clams suggests increased oxidative stress and a catabolic shift (from carbohydrate metabolism; Fig S1B) favoring lipid degradation to putatively supply energy for a higher transcription under elevated pCO_2, at a cost for organismal physiology. Our results suggest beneficial gene-expression regulation via frontloading, but also the capacity for fine-tuned, or responsive, gene expression patterns. Fine-tuning, as expeditious/dynamic gene expression, may bolster tolerance to external environmental changes. Altogether, this study corroborates physiological traits of emergent organismal and cellular phenotypes (Gurr *et al.* 2021) with transcriptomics, highlighting how early life priming events can rapidly induce transcriptional plasticity which have the potential to enhance resilience under subsequent environmental challenges later in life.

Naïve profile: Endogenous lipids supply high transcriptional demand

A growing body of research suggests that environmental changes, such as elevated pCO_2/low pH conditions, increases energy partitioning toward protein biosynthesis (Langenbuch and Pörtner, 2002; Pan *et al.*, 2015), conferring energetic tradeoffs for somatic growth and storage retention (Stumpp *et al.*, 2011; Sokolova, 2013). Our results support this, as a key difference between transcriptome profiles was a higher

transcript abundance attributed with fatty-acid degradation and glutathione components in the naïve phenotype (Figs. 3 and 5). Persistently enriched pathways included peroxisome activity (β-oxidation), acetyltransferase to mitochondria (carnitines), and bioremediation of free radicals illustrating elevated use of endogenous metabolic fuel to satisfy greater transcriptional demand. Mobilization of endogenous reserves, primarily lipids, is essential to meet energetic requirements of early development (Waldbusser *et al.* 2013; Liu *et al.* 2020), but also plays a vital role in rapid energy provisioning during stress exposure (Sokolova *et al.*, 2012; Teng *et al.* 2015) and may be advantageous to sustain acid-base homeostasis (Evans *et al.*, 2017). Naïve juveniles were enriched for carbohydrate metabolism (i.e. pentose phosphate pathway, glycolysis, and carbon metabolism) prior to pCO_2 challenge, further representing greater energetic requirements and the reliance for lipid sources to satisfy demand under pCO_2 change.

Across multiple marine calcifiers, exposure to elevated pCO_2 causes lipid loss and altered fatty-acid metabolism coupled with shell malformations and delayed settlement competency (Timmins-Schiffman *et al.*, 2020; Talmage and Gobler, 2010; Dickinson *et al.*, 2012; Liu *et al.*, 2020). For instance, elevated pCO_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). Gurr *et al.* (2021) found naïve geoduck clams decreased organic biomass and shell length under elevated pCO_2, linking physiological responses with gene-expression patterns of naïve clams in this study. Similarly, marine invertebrates are known to increase lipid degradation and peroxisome activity under elevated pCO_2, such as higher expression levels 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). In contrast, elevated pCO_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), which is a 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 assess the importance of lipid

catabolism on physiological success (Laudicella *et al.*, 2020) and future efforts should consider the tissue-specificity of proteomic and gene expression patterns (e.g. Elowitz, 2002; Wei *et al.*, 2015), requiring fine-scale sampling in contrast to whole-tissue homogenates sequenced herein.

Altogether, the gene expression patterns of naïve *P. generosa* suggest that fatty-acid degradation is a primary response to OA to ensure short-term survival and may compensate for the additional transcriptional requirements; however depletion of endogenous storages confers an unsustainable mismatch between energy demand and supply if the pCO_2 challenge exacerbated or persisted (i.e. 'pessimum' range; Sokolova *et al.*, 2012; Sokolova, 2021). Thus, the transcriptomic profile of pre-exposed *P. generosa* illustrates the importance of early-life environmental cues for eliciting molecular resistance. Beyond the scope of this study, standing and cryptic genetic variation may underlie adaptive capacity and heritable plasticity to environmental change (Bitter *et al.* 2019). For example, survival of the Yesso scallop *Patinopecten yessoensis* (Yang *et al.* 2021) and normal development of the purple sea urchin *Strongylocentrotus purpuratus* (Pespeni *et al.*, 2013) under elevated pCO_2 is linked to allele variation of candidate genes associated with energy and lipid metabolism, respectively. Future studies should further examine genomic markers affecting transcriptome profiles and selection.

A substantial increase in differentially expressed genes during repeat exposures, relative to immediate post-acclimation, suggests that phenotypic differences are most evident upon environmental change. In particular, a greater magnitude of change in gene expression occurred during ambient recovery (Fig. 5 and Table S2). Similarly, mussels *Mytilus galloprovincialis* submitted to intermittent challenges increase transcription during depuration (Détrée and Gallardo-Escárate, 2018). Expanded research should consider the relevant magnitude of elevated pCO_2 (i.e. *P. generosa* in Puget Sound, WA (USA); Feely *et al.*, 2010; Reum *et al.*, 2014) and the dynamic and intermittent behavior of current and future OA projections. Moreover, the short timescale of this experiment relative to the lifespan of *P. generosa* (up to 168 years; Bureau *et al.*, 2002) limits the selective implications of transcriptome profiles on fitness, as *P. generosa* have shown evidence of negative effects of elevated pCO_2 exposure (i.e. shell growth) precursor to compensatory responses later in life (Gurr *et al.* 2020).

Primed profile: Fine-tuned response to intermittent OA

Pre-exposed *P. generosa* expressed fewer genes at a higher abundance relative to naïve geoducks, albeit functional annotation of these genes showed diverse regulatory processes in cellular quality control and homeostasis (Fig. 3). A general decrease in transcription may be attributed with adaptive benefits under environmental change (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 intermittent exposures (Connor and Gracey, 2020). Moreover, 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). For example, pre-exposed clams persistently downregulated genes involved in chitin degradation (i.e. chitotriosidase-1) and calcium homeostasis (i.e. regucalcin). Although critical for shell biomineralization, *Mytilus edulis* and *Crassostrea gigas* suppress expression of chitinases and regulation under high pCO_2 (Wei *et al.* 2014 and Hüning *et al.* 2013) suggesting an adjustment of energy and ion retention under chitin and calcium-demanding conditions. Pre-exposed *P. generosa* also showed distinct treatment-responsiveness when faced with subsequent pCO_2 exposures, both frontloading and heightening expression in cellular quality control, signaling, transcriptional modifiers, and stress and innate-immune response genes (Fig. 3). Constitutive frontloading suggests that pre-exposed clams already expressed key proteins at an optimal abundance to cope with OA. For example, heat shock 70kDa is a common indicator of resilience and pre-emptive and acclimatory expression in marine invertebrates (Dong *et al.* 2018; Moya *et al.* 2015; Barshis *et al.* 2013) and was constitutively expressed by pre-exposed *P. generosa* under both moderate and severe pCO_2 change. Moreover, apoptotic and immune signaling genes (i.e. caspase-10, toll receptors, MyD88) were also frontloaded, whereas downregulation of these genes can compromise immune activity and development under elevated pCO_2 (Todgham and Hoffmann 2009; Liu *et al.* 2016). Functional enrichment of gene sets expressed by pre-exposed *P. generosa* also linked to mitophagy during second and third exposures under elevated pCO_2 (Fig. 3 and Table S1), 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). Similarly, offspring Sydney rock oysters *Saccostrea glomerata* of pCO_2-conditioned broodstock upregulate PINK1 under elevated pCO_2 (Goncalves *et al.*, 2017), an essential kinase of the PINK1-Parkin pathway for efficient clearance of mitochondria (Wu *et al.*, 2015). Hypercapnia/acidosis affects mitochondrial integrity and can enhance free radical production (Miwa and Brand, 2003; Lambert and Brand, 2004; Tomanek *et al.*, 2011), thus removal of damaged mitochondria, or mitophagy, may regulate cellular homeostasis during repeated pCO_2 challenge. Pre-exposed *P. generosa* also expressed higher levels of genes essential for protein turnover, such as 26S proteasome, E3 ubiquitin ligases, and caspase (Voges *et al.*, 1999; Goldberg, 2003), that are otherwise unaffected by low pH in other species (i.e. *C. virginica* and *Mercenaria mercenaria*; Götze *et al.*, 2014) likely due to energetic limitations of exposure to environmental change (Ivanina *et al.*, 2016).

Pre-exposed *P. generosa* increased expression of genes for energy metabolism (NADH dehydrogenase, cytochrome c oxidase, and ATPase) and biosynthesis (pentose phosphate pathway) during ambient recovery, suggesting an opportunistic or pre-emptive increase in energy production under optimal conditions (Figs. 3 and 6).

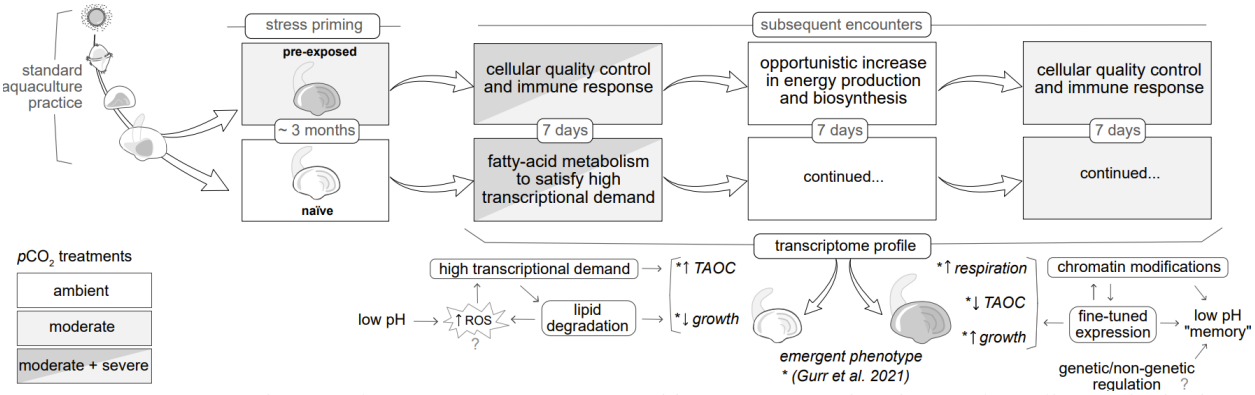

Figure 6. Summary of transcriptome patterns expressed by pre-exposed and naïve juvenile geoduck clams under repeat exposure to elevated pCO_2. Putative mechanisms are shown based on synthesis of the enriched terms (functions and pathways) and differential expression in this study, alongside phenotypic data described in Gurr *et al.* 2021 (indicated by asterisks and *italics;* 'TAOC' total antioxidant capacity); recommended topics for future research are proposed ('?').

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, under elevated pCO_2, geoduck *P. globosa* (López-Landavery *et al.*, 2021), Pacific oyster *C. gigas* (Wei *et al.*, 2015), and eastern oyster *C. virginia* (Chapman *et al.*, 2011) upregulate NADH dehydrogenase suggesting increased ATP production, whereas lower levels of gene expression for cytochrome c oxidase in *C. gigas* (Dineshram *et al.* 2012) and ATP synthase in *C. hongkongensis* (Dineshram *et al.*, 2013) suggest metabolic suppression. Thus, increased expression of mitochondrial complexes by pre-exposed geoduck clams illustrates an opportunistic increase in energy production. Moreover, enrichment for glycolysis and the non-oxidative pentose phosphate pathway suggests the pre-exposed clams metabolized carbohydrate fuels and increased nucleotide biosynthesis during ambient recovery. This demonstrates expeditious rescue by primed clams, as naïve clams were enriched for carbohydrate metabolism under ambient priming but not post-challenge during ambient common garden. GO term enrichment during ambient recovery also included iron binding proteins and phenoloxidases ('oxidoreductase activity'; 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 abundantly-expressed by pre-exposed clams, excess iron-induced toxicity (Fenton reaction enhancing free radicals) was likely negligible. In contrast, stressed mud snails *Littorea littoriea* increase expression levels of antioxidants and ferritin (English and Storey 2003) 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 pre-exposed clams. Future study is needed to determine the role of divergent transcriptome profiles during intermittent acidosis in marine invertebrates.

Lastly, immunomodulation and signaling were key transcriptional components of pre-exposed *P. generosa* following the acclimatory period, including genes for activation and inhibition of NF-kappa β (Figs. S5B, 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). Similarly, immune priming under abiotic (i.e. temperature) and biotic (i.e. synthetic and non-synthetic viral exposure) challenges bolster antiviral defenses associated with upregulated expression of apoptotic, signaling, and immune-related genes in the Pacific oyster *C. gigas* (Lafont et al. 2020; Delisle et al. 2020) and Pacific abalone *Haliotis discus hannai* (Zhang et al. 2022). A growing body of research highlights the general importance of NF-kappa β for the innate immune response in bivalves (Li *et al.*, 2015; Huang *et al.*, 2021) and elevated pCO_2 can have synergistic and antagonistic effects on immunomodulation (Castillo *et al.*, 2017; Cao *et al.*, 2018). For example, increased levels 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* decreases gene expression of NF-kappa β signaling during hypercapnia, rendering clams more susceptible 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 (Détrée and Gallardo-Escárate, 2018). Altogether, early-life experience heightened critical signaling and immune-related proteins potentially enhancing resilience to subsequent pCO_2 change in primed clams. Considering that pre-exposed *P. generosa* grew larger than naïve clams (Gurr *et al.*, 2021), early-life priming to elevated pCO_2 likely affects organismal responses to subsequent encounters demonstrated herein from 'fine-tuned' transcript abundance during repeated exposure.

Transcriptional control suggests 'memory' post-acclimation

Understanding how the environment triggers biological responses that lead to gene expression regulation is key to understanding environmental 'memory'. However, potentially transient and interdependent molecular mechanisms affecting regulation of gene expression remain poorly understood (Adrian-Kalchhauser *et al.* 2020). Growing evidence suggests that post-translational and non-genetic markers 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.*, 2020; Eirin-Lopez and Putnam, 2019). Stress 'memory', or stored/imprinted/regulatory information from initial stress enhancing robustness to future encounters, has largely been studied as a plant-based phenomenon (Bruce *et al.*, 2007), with growing support in invertebrate models. Molecular mechanisms underpinning memory may manifest as 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 defence systems in cold-primed tunicates *Ciona robusta* (Li *et al.*, 2020). In our study, pre-exposed *P. generosa* frontloaded several transcription factors suggesting an influence of stress history on general regulatory transcription. Moreover, histone methyltransferases (HMTs) were expressed at a higher abundance by pre-exposed clams (i.e. SETD5, ASH1L, and NSD2) each affecting histone H3 tri/dimethylateion of lysine residue 36 (H3K36me3 and H3K36me2; An *et al.*, 2011; Greer and Shi, 2012), a chromatin-carrying marker for 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). Gene expression frontloading can enhance the ability for marine invertebrates to cope with dynamic environments (Dong *et al.*, 2008; Barshis *et al.*, 2013; Shiel *et al.*, 2017), and may be rooted in epigenetic processes. For example, normal development of larval oysters *C. honkongensis* under low pH co-occurred with upregulation of HMTs and fine-tuned transcription (Dineshram *et al.*, 2015). Thus, the continuous expression of histone modifiers by pre-exposed *P. generosa* in this study suggests a rapid and inducible mechanism to support gene frontloading. Altogether, sustained/accumulated HMTs may regulate DNA accessibility for transcription and contribute to the fine-tuned and responsive gene expression and emergent phenotype in juvenile geoduck clams, 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 is subtle (Downey-Wall *et al.*, 2020). *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 larval oysters *C. hongkongensis* exposed to OA show hypermethylation of genes related to lipid metabolism in contrast to hypomethylation of genes related to carbohydrate metabolism (e.g. NADH dehydrogenase and ATP synthases) suggesting that lipid fuel acts as an alternative energy source to cope with elevated pCO_2 (Lim *et al.* 2021). Further research is needed to elucidate molecular mechanisms underpinning gene-expression regulation, and 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). Moreover, standing genetic variation may drive adaptive directional selection under environmental change, as evidenced by sufficient genetic variation for rapid evolution in the marine molluscs (Thomsen *et al.* 2017; Bitter *et al.*, 2019; Yang *et al.* 2021) and echinoderms (Pespeni *et al.* 2013) to cope with elevated pCO_2.

'Stress priming' to improve hatchery-propagated seed

This study highlights frontloaded and fine-tuned gene expression post-priming and under reciprocal encounters. We propose priming as an approach for ecological conservation and aquaculture enhancement. Applying concepts of developmental acclimatization (e.g. early-life programming 'windows'; Fawcett and Frankenhuis, 2015) and mild hormesis (e.g. conditioning hormesis and oxidative-stress hypothesis; Calabrese *et al.*, 2007; Costantini, 2014) may help to minimize effects of domestication selection and improve environmental 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) requires mitigation through societal actions (e.g. management interventions, policies and public awareness; Kelly *et al.*, 2011)

and aquaculture adaptations to improve food security (Nascimento-Schulze *et al.* 2021; Reid *et al.* 2019). Transcriptome profiling, as showcased in this study, can expand genomic resources for ecosystem conservation and aquaculture by identifying candidate genes and 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 intermittent pCO_2 change to test the hypothesis that priming during a developmental window elicits transcriptional frontloading and an expeditious response to environmental challenges. In absence of priming, naïve clams exhibited higher transcriptional demand during experimental exposures attributed with a putative catabolic shift favoring primarily lipids. In contrast, acclimatization conferred gene frontloading and gene-expression control, such that pre-exposed *P. generosa* contained lower levels of expression, however frontloaded and fine-tuned under subsequent pCO_2/pH changes. The pre-exposed transcriptome profile was attributed with heightened expression for cellular quality control, signaling, transcriptional modifiers, and stress and innate-immune response genes under subsequent pCO_2 challenge and putative markers of enhanced energy production during recovery. Altogether, this study demonstrates the importance of gene-expression regulation on positive developmental acclimatization.

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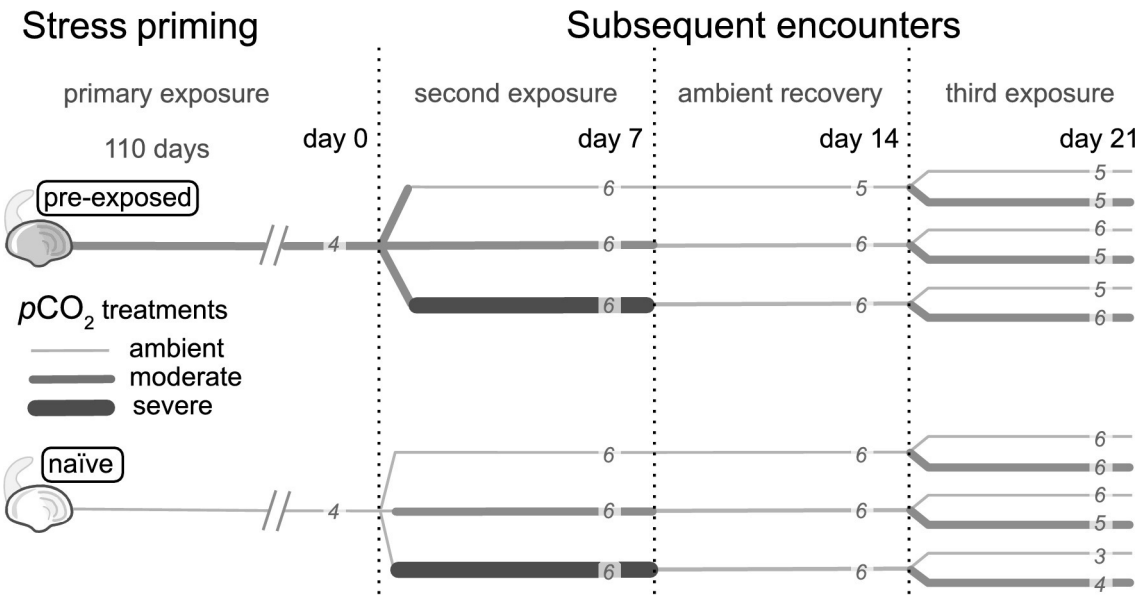
DATA ACCESSIBILITY:

- Raw sequence reads are deposited in the SRA (Accession: PRJNA740307; BioProject: Transcriptome profiles of Panopea generosa under hypercapnic seawater)
- All data analysis is submitted as a public Zenodo repository (doi:10.5281/zenodo.6908630)

AUTHOR CONTRIBUTIONS

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



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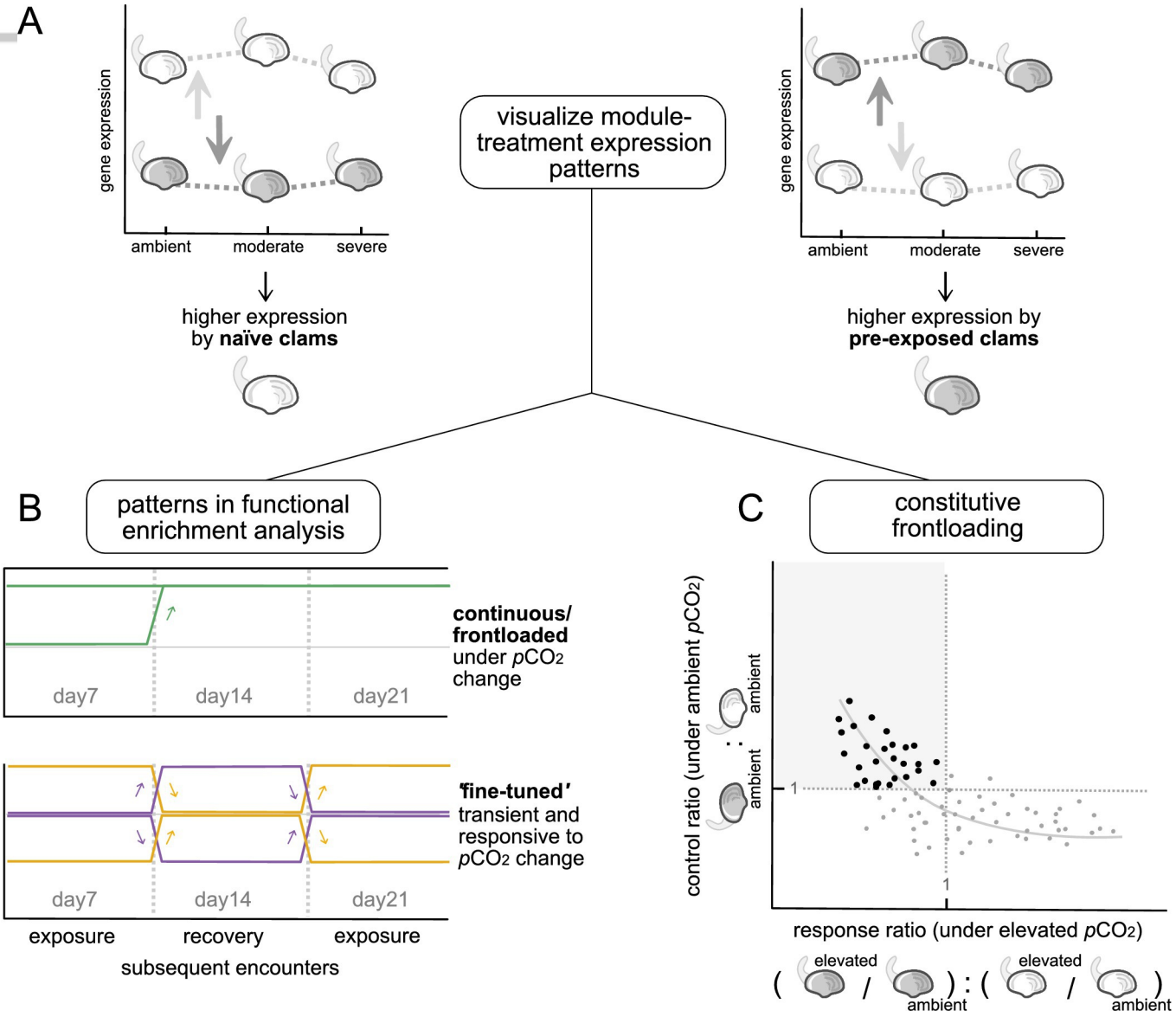

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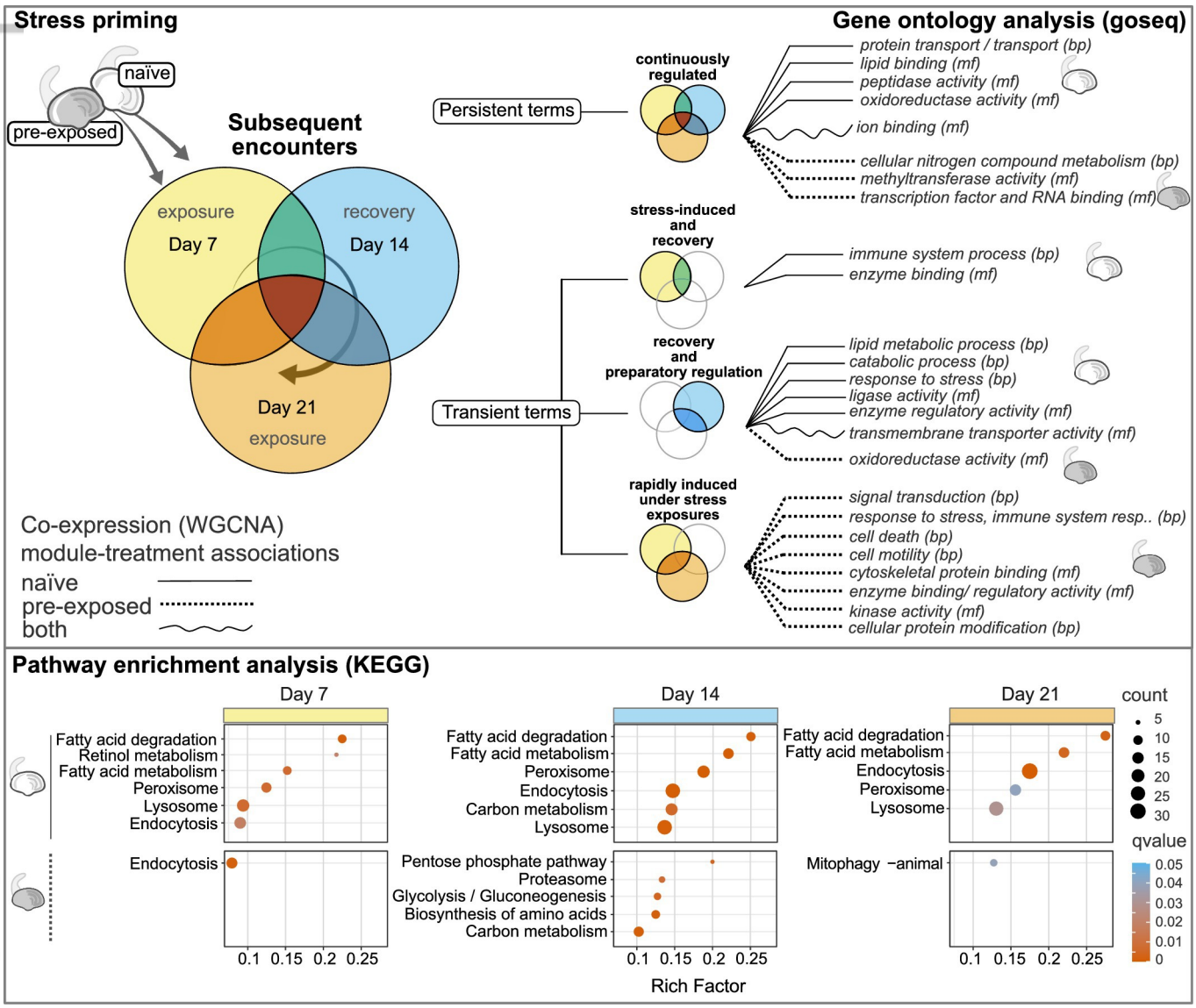

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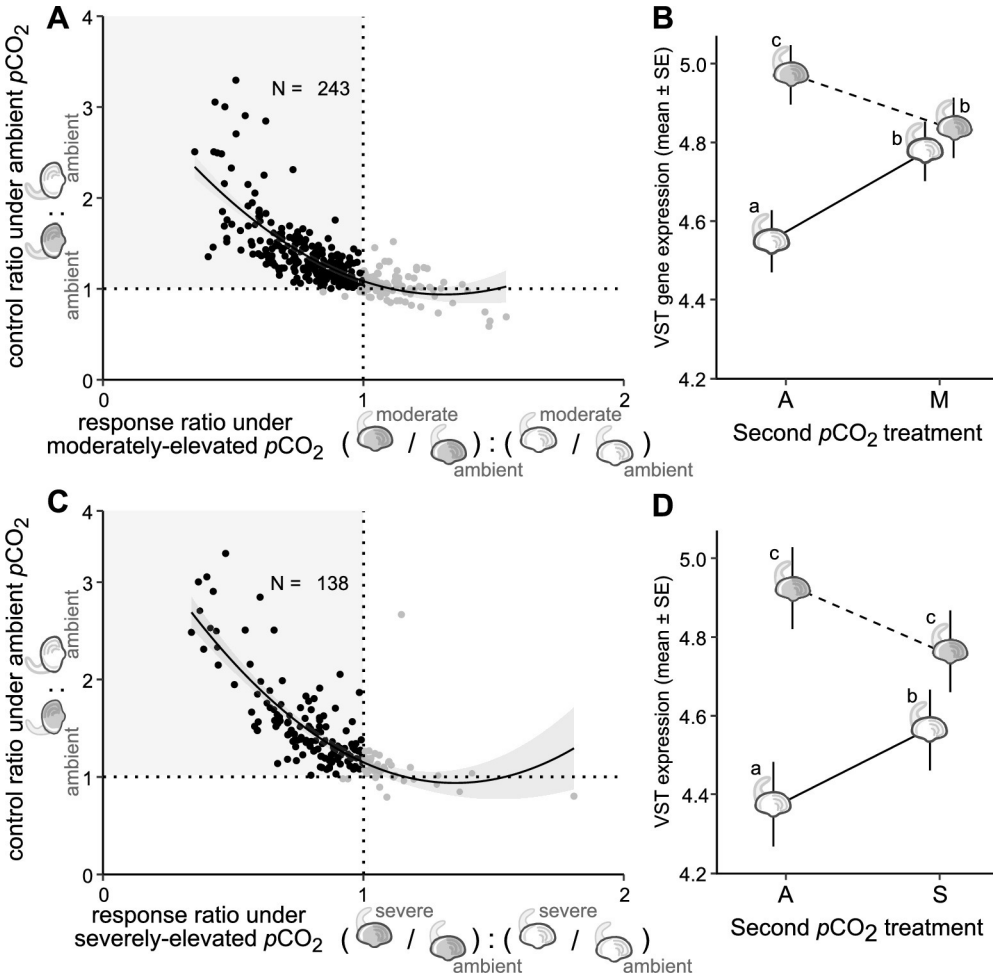

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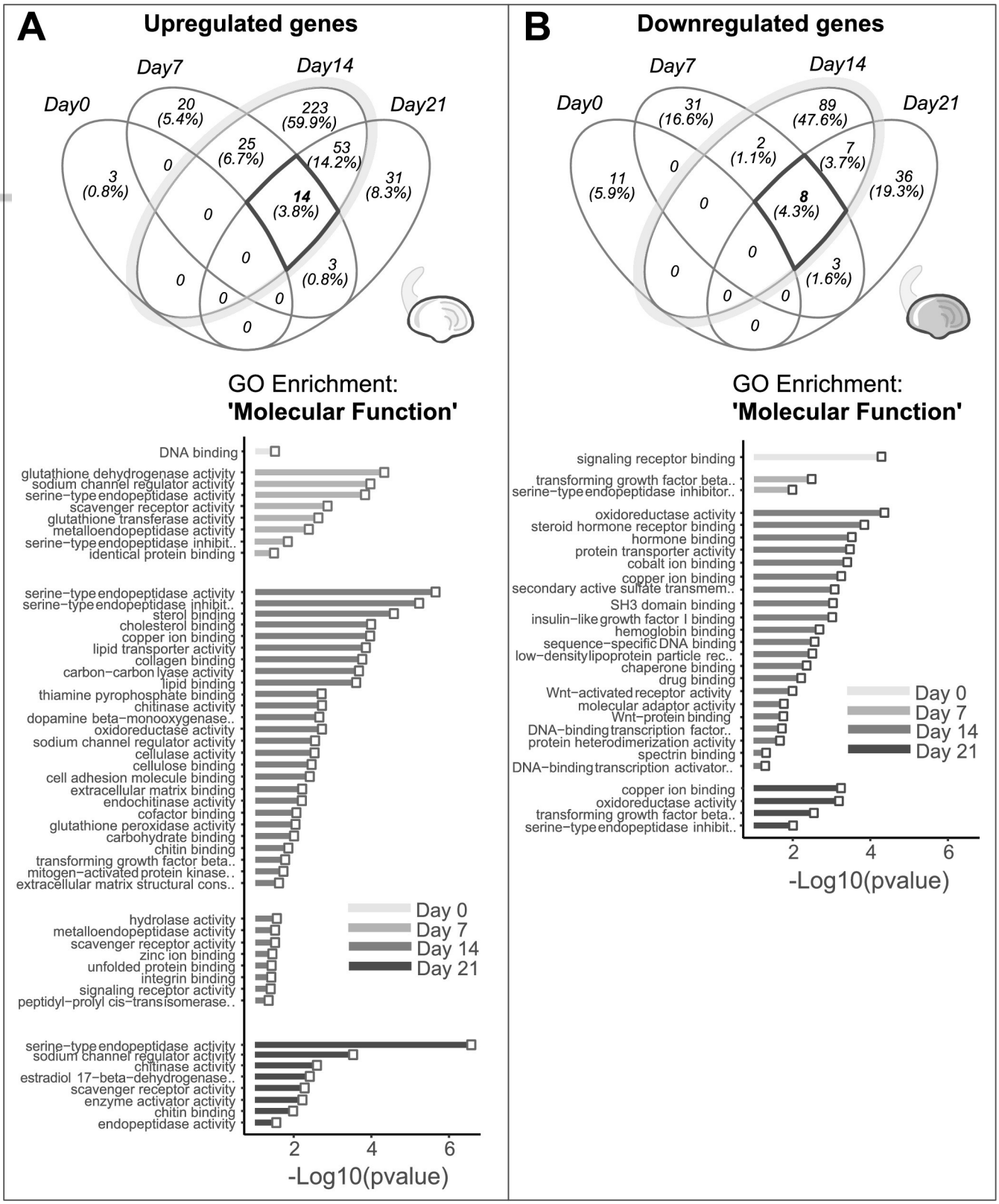

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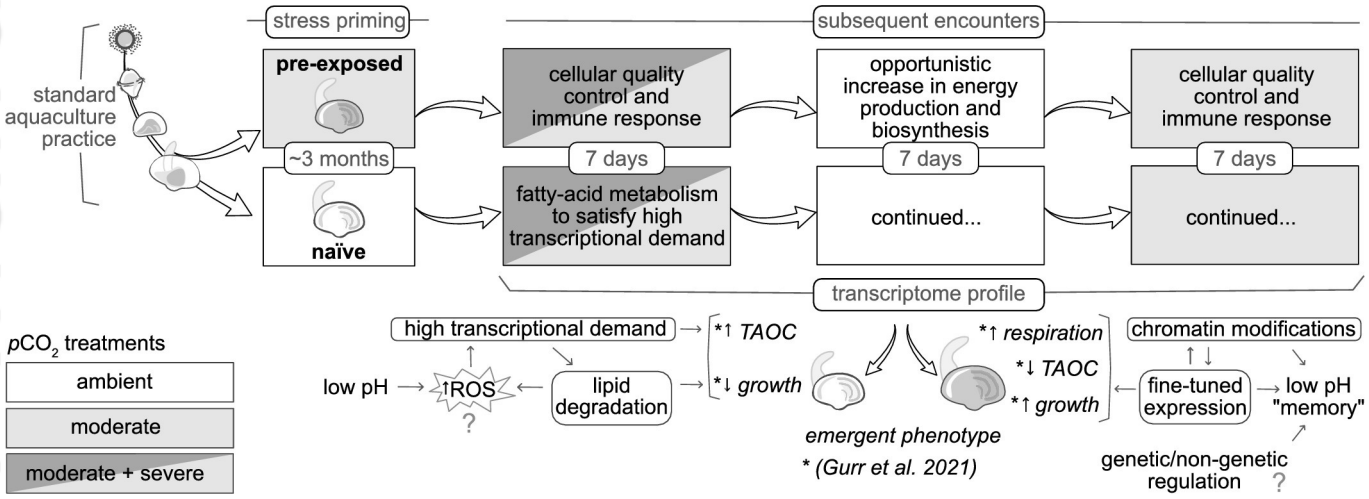

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