Molecular indicators of warming and other climate stressors in larval Pacific cod

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Abstract

Recent marine heatwaves in the Gulf of Alaska negatively impacted Pacific cod (*Gadus macrocephalus*) through a series of failed year classes and poor recruitment to the fishery. Experimental work by Slesinger et al. (2024) corroborated the hypothesis that warming directly impacts recruitment by increasing larval mortality rates. In this companion study we applied transcriptomics with larvae from Slesinger et al. (2024) to better understand how warming affected their physiology and identify potential mechanisms contributing to mortality. RNASeq data reveal that warm-exposed larvae have unique gene expression profiles that may reflect high levels of inflammation, lipid dysregulation or depletion, and altered development of visual systems and neurological pathways. Warming may therefore cause a metabolic mismatch whereby energy-demanding activities (development, inflammation, growth) exceed energy production capacity despite access to prey. We also report the less pronounced transcriptional differences in larvae exposed to cold, acidification, and a combination of stressors reflecting

future climate scenarios. This information will guide future genetic and experimental work that will ultimately inform recruitment forecasts in years with conditions similar to those tested here.

Keywords: transcriptomics; functional genomics, Pacific cod, ocean warming, ocean acidification

Introduction

Climate change is causing complex changes in the world's oceans (Harley et al., 2006). Increasing concentrations of greenhouse gasses have contributed to higher average ocean temperatures and are linked to more frequent marine heatwaves (Frölicher et al., 2018). Increasing dissolved carbon dioxide levels are shifting carbonate parameters and reducing ocean pH (Doney et al., 2009). Climatologists predict that marine heatwaves may be more common in the future (Walsh et al., 2018) and that ocean acidification will intensify, particularly at high latitudes of the Arctic and Antarctica (Fabry et al., 2009). An unprecedented reorganization of marine communities has begun (Grebmeier et al. 2006) and will likely continue as conditions continue to change (Worm and Lotze, 2021). Notably, loss or decline of species that have large effects on trophic interactions may result in extensive changes to entire ecosystems. In the case of commercial fisheries, climate-induced changes may be further exacerbated by fishing pressure.

Pacific cod, *Gadus macrocephalus*, is a commercially fished marine species that is important both as predator and prey in the North Pacific, a region that is experiencing substantial environmental changes (Laurel et al., 2023; Mathis et al., 2014; Walsh et al., 2018). Pacific cod support the region's second most valuable fishery after walleye pollock (*Gadus chalcogrammus*), valued at $446 million in the Bering Sea and $113 million in Gulf of Alaska in 2021 (Abelman et al., 2023). Despite its importance, population declines have occurred across much of the Pacific cod range over the past several decades, concurrent with increases in climate stressors (e.g. Barbeaux et al., 2020). The southern end of the Pacific cod range, Puget

Sound, Washington State, USA, once supported a commercial fishery from which catches just under 1,000 tons (t) were taken annually from 1956 to 1967 (Alderdice and Forrester, 1971). Currently, Pacific cod are inconsistently encountered in the Puget Sound due to their low population size (Spies et al., 2020; Pacunski, R., WDFW, pers. comm.). Similarly, Pacific cod catches have declined by ~2 orders of magnitude from 1958 to 2021 off the coasts of Washington and Oregon (reviewed by Laurel et al. 2023). These declines are notable, as they occurred at the southern extent of the range, and thus may forecast future population changes in Alaska waters.

While it is difficult to draw a strong cause-and-effect relationship between environmental variables and the stock status of a single species, recent failed recruitment in Gulf of Alaska cod deserve mention. Between 2013 and 2016, Gulf of Alaska sea surface temperature was 3-4°C above the 1980-2010 average (Bond et al., 2015), and in the central Gulf of Alaska, heatwave conditions persisted over 89% of days from May 2014 to January 2017 (Barbeaux et al., 2020). Concurrently, Gulf of Alaska Pacific cod biomass declined by 58% between surveys conducted in 2015 and 2017 (Barbeaux et al., 2020). The 2017 biomass estimate was the lowest ever recorded from standardized surveys from 1984 to 2017 by more than half (Barbeaux et al., 2020). Beach seine surveys in the Central and Western Gulf of Alaska observed record low levels of age-0 juveniles arriving to summer nurseries during heatwave years (Abookire et al., 2022; Laurel et al., 2023; Hulson et al., 2023), suggesting that elevated water temperatures during spring increase egg and larval mortality.

Experimental work has shown that Pacific cod early life stages are sensitive to temperature. Temperature affects embryonic development and metabolic demand, driving growth and survival of Pacific cod larvae and egg development (Laurel et al., 2008). Successful egg development is optimal within a narrow temperature range (4-6°C), beyond which it declines (Bian et al., 2016; Laurel et al., 2008, 2023), which may reflect historically stable temperatures in the demersal environment where eggs incubate (Laurel and Rogers, 2020).

Larvae hatch at significantly larger sizes in cooler temperatures, despite hatching later (Laurel et al., 2008, 2012). The direct effects of ocean acidification on Pacific cod are less clear, in part because there is limited experimental data, but also because results vary among life stages (Hurst et al., 2019) and gadid species (Frommel et al., 2011; Hurst et al., 2012, 2013; Stiasny et al., 2016; Leo et al., 2017). As this experimental work progresses, it is important to also document the genetic control underlying the effects of climate stressors on Pacific cod. Identifying climate-sensitive genes and associated processes will guide researchers to pinpoint genetic variation within the species that could fuel climate-dependent selection.

Slesinger et al. (2024) recently reared Pacific cod from embryos to feeding-stage larvae in conditions resembling recent marine heat wave events in the Gulf of Alaska (10°C) and cold environments reflective of northward range shifts (3°C) compared to optimal temperature for growth and survival (6°C). They also investigated the effects of high pCO_2 levels projected for the northeast Pacific Ocean (~1,560 µatm, Pilcher et al., 2022) as a single exposure and when combined with suboptimal temperatures. Larval mortality was very high in warming (10°C) regardless of pCO_2 level (Slesinger et al., 2024). In this companion study, we examine molecular pathways that likely underlie the observed phenotypic effects reported by Slesinger et al. (2024) using gene expression analysis. Our primary goal is to present hypotheses as to why warming causes high larval mortality rates in order to understand the observed failed recruitment of Pacific cod during marine heatwaves. Secondarily, we describe processes that differ in cold and acidification-reared larvae that reflect acclimation but could carry over to affect physiology or recruitment at later stages.

The physiological effects of climate stressors presented here are derived from gene activity differences among treatments detected using RNASeq. RNASeq is a sequencing approach that simultaneously quantifies mature messenger RNA (mRNA, or transcripts) for all expressed genes in a tissue sample. For this study, 11-14 individual whole-body larvae were sequenced from each of six treatments. RNAseq does not directly measure standard

physiological metrics such as metabolic rate or lipid content. Instead, this approach provides a snapshot of gene activity, which reveals how energy is allocated and prioritized and thus describes more broadly the physiological state of each Pacific cod larva. Results of these gene expression data are interpreted in the context of larval performance indicators (Slesinger et al. 2024 and summarized here), and also reveal possible impacts of warming and acidification not apparent in phenotypic data, as well as providing direction for future studies to examine the genes and gene variants that could enable adaptation.

Methods

Larval Incubations

Larval husbandry and exposure to treatments are described in detail in Slesinger et al. (2024) and are summarized here. Gametes of wild-caught adult Pacific cod (1 female; 3 males) captured via pot gear set off Kodiak Island, AK, on April 4th, 2022 were used to generate embryos representing three half-sib families. Embryos were transported to NOAA's Alaska Fisheries Science Center laboratory in Newport, OR and reared in one of six experimental treatments encompassing a full cross of three temperatures (3°C, 6°C, 10°C) and two pCO_2 levels (ambient: ~360µatm; high ~1,560µatm) with four replicate tanks per treatment (24 tanks total). Embryos and larvae were reared in cylindrical 50-L experimental tanks in a flow-through upwelling system with a flow rate of 0.5 L min−1. Photoperiod was set to a 12:12 h schedule, and larvae were provided live rotifers *(Brachionus plicatilis*) twice daily at a density of 5 rotifers mL^{-1} with green water (RotiGreen Nanno; Instant Algae), which reflects high food levels used in prior laboratory experiments (Hurst et al. 2010; Laurel et al. 2011).

Source water was conditioned to maintain target temperatures and pH levels (Table 1). Target temperatures were achieved by mixing ambient water from Yaquina Bay (average

126 temperature ~11°C) with glycol-chilled water (average temperature ~2°C). Target pH levels
127 were achieved by CO_2 injections into three separate header tanks, one per temperature
128 treatment, which were monitored and controlled by Durafet III pH probes (Honeywell) and a dual
129 input analytical analyzer (Honeywell). Temperature and pH measurements were collected daily,
130 and discrete samples were fixed weekly to measure total alkalinity (TA) and dissolved inorganic
131 carbon (DIC). pH and pCO_2 were calculated from temperature and salinity measurements
132 (collected daily) and DIC and TA measurements (collected weekly). Daily mean temperatures
133 were maintained within 0.1°C of target conditions in all tanks, and weekly mean pCO_2 ranged
134 from 297 – 410 µatm in the ambient pCO_2 treatments and 1435 – 1780 µatm in the high pCO_2
135 treatments (Table 1).
136 Hatching occurred 8–9, 11–12, and 17–18 days after 2-day old embryos entered 10°C,
137 6°C, and 3°C, respectively. During the experiment, larvae were counted twice: once at hatch
138 and once at the end of the experiment. These counts were used to determine an instantaneous
139 daily mortality rate. Larvae were also sampled at regular intervals to calculate growth and
140 condition factors (15 larvae per replicate tank at each sampling timepoint for 60 larvae total per
141 treatment), which were analyzed using linear mixed effects models to account for potential
142 variation introduced by the replicate tanks (Slesinger et al. 2024). Two growth rates were
143 calculated, one based on standard length (G_L; mm day^{-1}) and one for dry weight (G_M; % day^{-1}).
144 Two condition factors were also calculated, which are morphometric-based condition (K_{MH}) that
145 reflects the relationship between myotome height and standard length, and weight-based
146 condition (K_{DW}) that reflects the measured dry weight relative to predicted dry weight based on
147 standard length. While in Slesinger et al. (2024) survival, growth and condition were assessed
148 across the entire study timeframe and at specific intervals, for this study values were assessed
149 on or up to the date of gene expression sampling (see below). Pairwise comparisons between
150 control conditions (6°C, 390 µatm pCO_2) and all other treatments were conducted with
151 condition, growth, and survival rates using pairwise t-tests with Bonferroni adjustment to control

for multiple testing. Effect sizes were estimated by Cohen's d (Cohen 1969) using pooled standard deviation. Total survival percentages were calculated for the period from hatch to gene expression sampling.

Fish were sampled for gene expression analyses at the post-yolk absorption, pre-flexion, feeding stage (~6-7 mm, Hurst et al. 2010), the timing of which varied among temperature treatments and occurred 13, 21, and 28 days post-hatch for the larvae exposed to 10°C, 6°C, and 3°C treatments, respectively (Table 2). This staggered sampling approach was necessary to ensure that all larvae were developmentally comparable, as developmental rate is affected by temperature. For each of the six treatments, 11-14 larvae (Table 2) were sampled in the morning prior to feeding and preserved individually for RNA-Seq by placing whole fish in RNAlater per manufacturer's instructions (Thermofisher Scientific, Waltham, MA). For most treatments, larvae were sampled from the four replicate tanks. For the cold-ambient pCO_2 treatment larvae were sampled from one tank (12 larvae total). Fewer fish were available for sampling in the cold treatment due to the slower larval development rate resulting in fewer larvae being at the specified developmental stage for all assays when sampling occurred, and lower cumulative survival compared to the control treatment (Table 3).

RNA Extraction, Sequencing, and Data Processing

RNASeq generates sequence data from all mature messenger RNA (mRNA) transcripts that were extracted from a tissue sample. The half-life of an mRNA molecule is short (~9 h as measured in mammals, Schwanhäusser et al. 2011), thus RNASeq measures the activity level of all recently transcribed genes and provides a snapshot of active processes in the tissue. For our study, RNA was isolated from homogenized whole-body larvae, thus the RNASeq analysis characterized gene activity and energy allocation across all tissues.

Detailed extraction, sequencing, and data processing methods are described in detail in the supplementary materials, and broadly here. After RNA isolation and library construction,

paired-end sequencing was conducted on one lane of a NovaSeq 6000 Sequencing System (Illumina, Inc., San Diego, CA) with 150 bp read length. Sequence data was processed to produce a gene count matrix, which quantifies the number of mRNA transcripts per gene per sample. First, raw reads were separated by sample (demultiplexed), trimmed to remove known sequences that were added for library prep and Illumina sequencing (adapters), and filtered to ensure quality. These trimmed/filtered reads were then mapped (i.e. aligned) to the Atlantic cod (*Gadus morhua*) genome assembly v3 (gadMor3.0, Genbank accession GCA_902167405.1). Once aligned, it is possible to quantify mRNA transcript levels for genome features of interest. Here, we quantified the number of read pairs (i.e. fragments) that mapped to protein-coding-genes, and are thus functionally important, which generated a gene count matrix. Gene functions were predicted by comparing gene coding sequences of the *G. morhua* genome, which are published along with the *G. morhua* genome, against gene sequences in the Uniprot/Swissprot database (UniProt Consortium, 2021), and filtering hits for sequences that are highly similar (e-value < 1^{-10}). The sequences contained in the Uniprot/Swissprot database are manually curated and derived from experimental work, largely in model organisms, and thus provide high-confidence predictions of protein functions based on sequences. Prior to comparing gene counts among treatments, genes were removed from the matrix if they contained very few reads across all samples (mean count <10 across all samples or those with counts <30 across at minimum 10% of the samples). The remaining gene count matrix was inspected for outlier samples using principal component analysis (PCA), and the total number of fragments was compared among treatments using ANOVA.

Controlling for length/development

Warm-reared larvae were smaller at the time of sampling than the other two temperature treatments (no difference in size in pCO_2 treatments), despite being collected at the same developmental stage (post-yolk absorption, pre-flexion, feeding) and at similar degree days

post-hatch (Table 1). Given the possible associations among larval size, development, and expression patterns, genes associated with larval length were removed from the dataset prior to the comparative analyses. The removal of length-associated genes did not affect the overall conclusions drawn about the effects of temperature and acidification. For a gene to be removed from the analysis because it was considered length-associated, it had to 1) be associated with length as per the core differential expression analysis, 2) respond linearly to length when all three treatments were considered, and 3) respond linearly to length without the warm treatment (see Supplemental Materials for more details). This final length-controlled gene count matrix was used in comparative gene expression analyses.

Global expression patterns

Genome-wide gene expression patterns were explored with PCA, using `prcomp` from the R package *vegan* v.2.5-7 on all gene counts that were transformed via variance-stabilization. Principal components that explained a significant amount of variance were identified using Cattell's rule (Cattell, 1966). Genome-wide differences among temperature and pCO_2 treatments were assessed by permutational pairwise permANOVA with `pairwise.adonis` from the *pairwiseAdonis* package, which is a wrapper for `adonis` from the *vegan* package. The level of biological variation in global gene expression was explored by calculating Euclidean distances to treatment centroids (means) in multivariate space (PC1 x PC2) and differences among treatments were assessed using ANOVA.

Differential gene expression analysis

Gene counts, i.e. the number of transcripts that mapped to each gene, were compared in each of the five experimental treatments (Table 2) relative to the control (6°C-390 µatm) to identify differentially expressed genes (DEGs). This was conducted using individual larval cod samples as replicates on a per-gene basis using *DESeq2* (Love et al., 2014), which models raw gene

counts with a negative binomial distribution, normalizes across samples based on overall sequencing depth, stabilizes dispersion estimates, estimates effect size (log-2 fold change, L_2FC) using Wald tests, and accounts for multiple comparisons using the Benjamini-Hochberg method. For each gene, outliers were handled using *DESeq2*'s built-in replacement method, where Cook's Distance was used to identify influential outliers and then the original count values were replaced with trimmed means, and using an additional iterative Leave-One-Out (iLOO) approach to identify and remove DEGs with outlier samples (n=887 genes) (George et al., 2015). In the five treatments for which samples from multiple tanks were available, we identified and removed genes from the DEG lists that were differentially expressed among replicate tanks within each treatment (total of 104 unique genes across five treatments, Table S6) using alpha=0.01 to ensure sizable tank effects. The resulting lists of differentially expressed genes represent those with differences in mean expression levels between control and each treatment, accounting for library size normalization and biological variability (n=11-14 replicate cod larvae per treatment).

Enrichment Analysis

We conducted enrichment analyses to identify biological processes that differed in larvae exposed to experimental treatments compared to control. Enrichment analysis leverages terms manually assigned to many genes (here, Uniprot Keywords & Gene Ontology Terms) to identify biological processes overrepresented in DEGs relative to all genes and processes that were captured by RNASeq. Two enrichment analyses were performed for each of the five experimental treatments to identify the functions of genes that were upregulated ($L_2FC > 0.5$) and downregulated ($L_2FC < -0.5$). For all DEG sets, genes were filtered for those that map to the Uniprot/Swissprot database (UniProt Consortium, 2021), and enriched biological process Uniprot Keywords and Gene Ontology Terms were identified by entering UniprotID's into the

Gene-Enrichment and Functional Annotation Tool from DAVID v2021 (Sherman et al., 2022), which were defined as those with at minimum three genes contributing to each process.

All analyses were performed in R v4.1.2 using RStudio interface v2021.09.1 (R Core Team, 2021; RStudio Team, 2020), and unless otherwise specified significance thresholds were alpha = 0.05.

Results

Mortality, growth, and condition

Larval phenotypic responses were previously reported by Slesinger et al. (2024) across multiple time points. Here, we report phenotypic differences in larvae exposed to treatments relative to control fish, focusing on measurements taken on or near (≤2 days) the day of sampling for gene expression (Table 3, see Slesinger et al. for broader effects, including time-series trends and embryonic effects). Warming increased daily larval mortality rates as a single stressor and when combined with acidification. Larvae reared in warm treatments grew and developed quickly such that they reached the developmental stage for sampling seven days earlier than control larvae (Table 2). However, their morphometric-based condition and mass-based growth rates were lower relative to the control larvae sampled at the same developmental stage seven days later. Cold temperature did not affect daily mortality rates, but it reduced both growth rates such that they reached the developmental stage for sampling seven days later than control larvae resulting in a lower cumulative survival rate at sampling. Cold temperature reduced condition indices as a single stressor and combined with acidification, except for the weight-based condition index, which was unaffected by combined cold and acidification. Acidification as a single stressor did not affect any phenotypic metric at the time of sampling. However, the broader timer-series of morphometric-based condition indicates that acidification-reared larvae

were becoming thinner, while control fish were not (Slesinger et al. 2024). For all measured metrics, there was minimal to no effect of replicate tank on the results (Slesinger et al. 2024). Overall, the effects of temperature were more pronounced than the effects of acidification.

RNA-Seq pre-processing

On average 49.0M ±6.8M RNASeq reads derived from larval Pacific cod uniquely aligned to the *G. morhua* genome (81.1% ±1.8% alignment rate), and 45.4M ±6.4M were assigned to gene-coding regions (75.2%±2.1). Reads mapped with sufficient depth to 21,076 of the genes in the *G. morhua* genome, 19,424 of which mapped to genes in the Uniprot/Swissprot database (e-value<$1e^{-10}$). Of those, we removed 6,664 genes that were associated with fish length, resulting in 14,412 genes for comparative analyses. Additional genes were later removed during the differential expression analysis as they were strongly influenced by outliers (n=606) or by tank-specific expression (n=104).

Global expression patterns

Global expression profiles of Pacific cod larvae were strongly influenced by temperature (Figure 1). Pairwise permANOVA tests detected significant differences in multivariate space among control temperature (6°C) and both cold (3°C: F(1)=6.0, *p*-adj=$3.0e^{-3}$) and warm (10°C: F(1)=4.7, *p*-adj=$3.0e^{-3}$) temperatures, which is evident from the biplot of principal components one and two, which combined explained 30.1% of variation in global gene expression (Figure 1). The scree test (Cattell, 1966) indicated that principal components 3, 4, 5, and 6 also explained a significant amount of variation (7.3%, 5.8%, 4.5%, and 3.9%, respectively) (Figure S1). Euclidean distances to treatment centroids (means) in PC space (PC1xPC2) did not differ significantly among treatments and were not affected by the number of replicate tanks from which larvae were sampled for RNASeq (one tank for the cold treatment, four tanks for other treatments Figure S18).

Differential gene expression analysis

Relative to control conditions, transcriptional differences were largest in response to warm temperature, and more moderate in response to acidification and cold conditions (Figure 2). The effect of acidification on gene expression was also highly dependent on temperature, as no genes were differentially expressed between ambient and high pCO_2 at 3°C or 10°C, which is evident from the overlap between ambient- and high pCO_2-exposed fish at 3°C and at 10°C in the PCA (Figure 1, contrasts not shown in Figure 2).

Larvae exposed to both warm temperature and high pCO_2 had fewer transcriptional differences from larvae reared in control conditions compared to those exposed to just warm temperature (Figure 2). Similarly, combined exposure to cold temperature and high pCO_2 had fewer DEGs than cold alone.

Functional analysis

Genes that were expressed at higher levels (henceforth “more active”) were enriched for biological processes associated with cell connectivity and signaling, vision, immune function, blood coagulation, and protein production (Figure 3). Cell adhesion and vision were more active in larvae reared in warming as a single stressor and when combined with acidification, and immunity, sensory transduction, and calcium transport were more active in warming as a single stressor (Figure 3). Inflammatory response genes were more active in acidification as a single stressor, and when combined with warming acidified conditions resulted in more active blood coagulation and hemostasis genes. Molecular chaperone stress-response genes (relating to heat shock protein activity) were more active in both cold conditions as a single stressor and when combined with acidification, and ribosome biogenesis and rRNA processing genes were more active in combined cold and acidified conditions.

Genes that were expressed at lower levels (henceforth “less active”) were largely enriched for lipid metabolism processes (Figure 3). Lipid metabolism was less active in four of

the five treatments (acidified, warm, warm + acidified, and cold conditions), lipid biosynthesis was less active in warm + acidified conditions, and steroid metabolism was less active in cold conditions. Digestion and electron transport (relating to mitochondrial energy production) was less active in acidified conditions as a single stressor and when combined with warming.

Discussion

In this companion study to Slesinger et al. (2024), we sought to identify possible mechanisms of mortality and low Pacific cod larval recruitment during marine heatwaves, and to deepen our understanding of how shifting ocean conditions will affect Pacific cod. Broadly, a large percentage of the transcriptome was expressed differently in larvae that were reared in warm conditions as a single stressor (3,411 genes, or 16%) and when combined with acidification (2,890 genes, or 14%). Processes enriched in these genes may provide insight into the mechanisms of warming-associated mortality, and hint at the potential for acidification to mitigate some molecular effects of heatwave conditions. Fewer genes were affected by acidification (9%), cold conditions (9%), and combined acidification and cold conditions (6%) than in warm conditions. Processes associated with cold and acidified conditions may point to causes of lower condition and growth rate, but also acclimatory responses that enable survival. In the remainder of this section, we compile biological processes that are impacted by warming, cold conditions, and acidification, including possible mechanisms underlying phenotypic effects of each environmental variable. Finally, we discuss how hypotheses generated here relate to observed and predicted changes in recruitment to the fishery.

Effects of warming

Warming may cause mortality due to depleted or dysregulated lipids

Genes associated with lipid metabolism were less active (i.e. fewer gene transcripts were present) in larvae reared in warming as a single stressor and when combined with acidification (Figure 3 & S3). Less active lipid metabolism-associated genes likely indicate a concomitant decrease in lipid processes. Genes involved in a variety of lipid components were affected, including those that store and catabolize lipids for energy, and those that incorporate lipids into cellular membranes and for use in signaling pathways. This broad, consistent effect of warming on lipid metabolism genes suggests that demand may have outpaced lipid availability or lipid mobilization capacity. We did not directly measure lipid levels or liver sizes, but by homogenizing whole-body larvae this RNASeq data captures system-wide lipid metabolism gene activity. From this data, we hypothesize that larval Pacific cod mortality under heatwave scenarios may relate to altered lipid utilization or depletion (similar repercussions of warming are hypothesized for adults; Barbeaux et al., 2020), even when prey densities are high. Gene expression data from Atlantic cod larvae reared in warming also suggests major shifts in energetic demand (Oomen et al., 2022). Both studies link high mortality rates to energy depletion.

Among the affected lipid metabolism genes were those involved in fatty acid processes, including beta-oxidation. Fatty acid beta-oxidation modeled across temperatures was highest in larvae reared at 6°C (control temperature, see non-linear response genes, Figure S12), which also produced larvae with high body condition and growth rates and low mortality rates. Fatty acid beta-oxidation expression can represent the contribution of fatty acids as a fuel source to generate energy via oxidative phosphorylation, which is an efficient source of ATP. Lower beta-oxidation activity in warm-reared larvae may reflect lower fatty acid availability (i.e. energetic limitation) due to depletion or reallocation (e.g. to immune functions, described below), a shift in

metabolic strategy, reduced mitochondrial efficiency at suboptimal temperatures, or varying tissue ratios (e.g. liver, muscle) (Norambuena et al. 2015; Yoon et al. 2022). Changes in larval fatty acid metabolism can be driven by prey lipid composition (Copeman and Laurel, 2010; Tocher 2003), although experimental larvae in our study were fed high densities of prey enriched with a complement of essential fatty acids, which has been used in previous larval studies (Hurst et al. 2010; Laurel et al. 2011). Therefore, the differential lipid metabolism gene expression was likely driven by warming rather than an artifact of the larval diet. That being said, quantifying lipid components alongside gene expression analyses with varying feeding levels and/or lipid composition (e.g. Hurst et al. 2019) would clarify any additional sensitivities to warming when prey quality and/or quantity is reduced.

Inflammation may exacerbate energetic limitations in warming

Fish immune systems are known to be sensitive to temperature (Bowden, 2008; Pérez-Casanova et al., 2008). Here, immune system gene activity increased with temperature and was particularly high in warm-reared larvae (Figure 3 & S4). While inflammation can successfully fight infection, it is energetically demanding, and can itself be a stressor. Larsen et al. (2018) demonstrated the cost of heightened immune activation in Atlantic cod: Interleukin 1β and other pro-inflammatory transcripts were higher in larvae exposed to warming with and without bacterial infection, and while warm-exposed larvae cleared bacterial pathogens faster than those held at control temperature, they died at higher rates (Larsen et al., 2018). Many of the affected genes identified here interact with proinflammatory interleukins (Figure S8, Table S2), which are cytokines that regulate the immune response and are often used in immune-response assays (Hu et al., 2018; Secombes et al., 2011), and indicate immune system hyperactivity in warm-reared larvae. Bacterial community shifts could also have affected immune activity in the warm treatments. Changes to immune function could therefore cause high mortality in warm-reared larvae directly due to the energetic costs of heightened immune system activation, or

393 indirectly due to increased infection rates or an ineffective immune response. High mortality and
394 low recruitment rates may in part be associated with inflammation, contributing to the
395 pathogenesis of warming in Pacific cod larvae.

396 *Warming may alter visual and neurological development rates relative to growth rates*

397 Genes involved in vision and cell adhesion were more active in response to warming (Figure 3,
398 S5 & S6). Visual opsin genes affected by warming are notable because they may provide
399 adaptive differences among Pacific cod populations (Spies et al. 2022) and Atlantic cod
400 ecotypes (Pamplouie et al. 2015) occupying varying light environments which often covary with
401 temperature. While it is tempting to suggest that warming may have triggered tuning of vision
402 components in larval Pacific cod, it is more likely that larvae reared in warming differed in their
403 visual and neurological development. More specifically, higher expression of specific genes
404 (e.g. green-cone sensitive opsin-2) which decrease throughout the larval stage in Atlantic cod
405 (Valen et al., 2016), indicates that the warm-reared larvae visual systems may have been less
406 developed. Other eye lens, retinal, phototransduction, and neurogenesis genes were expressed
407 at higher levels in warm-reared larvae, which probably reflects actively forming eyes. Visual
408 development appears to be decoupled from size-associated development, as we sampled
409 similarly sized larvae and excluded size-associated genes from our analysis (Supplemental
410 Materials). Measuring vision-related gene activity in later Pacific cod stages would clarify
411 whether visual plasticity is possible in response to warming.

412 A large number of cell adhesion genes were more active in response to warming (Figure
413 4, Figure S6). Cell adhesion is fundamental to the establishment and maintenance of tissue
414 structure and connectivity, and affected genes include core extracellular matrix proteins (e.g.
415 collagen, fibronectin), which could reflect cellular destabilization in warm-reared larvae. The
416 majority of affected adhesion genes are specific to nervous and visual systems and may be
417 linked to differential neurological development. Nevertheless, given the large effect of warming

on cell adhesion genes and their essential role in structural integrity, growth, and development, future genetic studies could explore cell adhesion genes identified here for signals of environment-dependent selection.

Effects of acidification

Acidification may affect larval lipid digestion

Acidification has been shown to affect lipid metabolism in marine organisms (e.g. Diaz-Gil et al. 2015; Gibbs et al. 2021; Strader et al. 2020), including gadids (Frommel et al. 2011, 2020; Hurst et al. 2019, 2021). However, the specific mechanism of action is often unclear, as impacts vary among developmental stages and lipid classes (Frommel et al., 2011; Hurst et al., 2019, 2021). Our gene expression data suggests that acidification reduces lipid digestion in pre-flexion staged Pacific cod larvae, which may explain why larvae became thinner over time in acidified conditions as reported by Slesinger et al. (2024). While our data provides indirect evidence of a link between acidification and altered digestion, more direct evidence has previously found that acidification reduces digestive enzyme activity in post-metamorphic flatfish larvae (Pimentel et al., 2015), and prolongs the specific dynamic action (SDA) in Atlantic cod, which the authors posited could explain lower feeding rates, growth, and condition (Tirsgaard et al., 2015a). Feeding efficiency and SDA are also modulated by temperature (Tirsgaard et al., 2015b), which may explain why digestion was not impacted by combined stressors in our study (Cominassi et al., 2020; Rosa et al., 2016). It is possible that acidification decreases feeding efficiency by lengthening digestion time and slowing stomach clearance rate, which in turn would lower food intake in larval Pacific cod. Alternative hypotheses are that acidification damaged gut tissue (Frommel et al., 2011) or affected intestinal development (Tillner et al., 2013), which would also likely affect digestion-related expression profiles. A handful of genes that negatively regulate inflammation were also expressed at higher levels, which suggests acidified-reared larvae were

actively suppressing inflammatory pathways, possibly to conserve energy or to prevent overactivation of immune responses (Figure 3). Overall, given that survival and growth remained high in the acidified treatment, the digestion and inflammatory effects were rather benign under experimental conditions, especially in contrast to the broad changes that occurred in the warm treatment. Negative effects may be exacerbated by more energy-demanding activities in the wild (e.g. predator avoidance) and under low-food conditions not tested here.

Acidification mutes some effects of warming on lipid and immune process, but may affect larval aerobic capacity

Acidification did not exacerbate effects of warming at the organismal level (Slesinger et al., 2024). Our gene expression data showed that acidification offset some impacts of warming. Broadly, the overall percentage of genes that were differentially expressed was lower in combined stressors (14%) compared to warming alone (16%), as was the effect size to differentially expressed genes (i.e. fold change, Table S1). Immune system and lipid processes, while still affected, were less so in combined warming and acidification compared to warming alone (Figure S3 & S5). Acidification may have depressed metabolic rate (Muller et al. 2021), partially counteracting the effects of higher metabolic rate that very likely occurred in response to warm temperature. Other possible explanations include less inflammation, possibly due to altered pathogen communities at lower pH levels or changes in mucosal immunity, and prolonged digestion impacting a cascade of energetic processes which are otherwise accelerated in warm conditions. These findings are somewhat counterintuitive because acidification is thought to elicit stress responses similar to warming in marine organisms (e.g. oxidative stress, Messina et al. 2023), and additive or synergistic effects are commonly predicted for organisms experiencing combined stressors. Despite the weaker transcriptional effects of combined stressors, the overwhelming effect of warming masks the signal of acidification at the organismal level (Table 3, Slesinger et al., 2024). In heatwave conditions

similar to those tested here, acidification will not likely affect larval mortality, growth, or body condition; however, it is possible that mitigating effects of acidification could offset heatwave impacts during later stages or in more natural settings.

One possible exception is that acidification and warming could impact larval aerobic capacity by impacting hemoglobin properties. Fish hemoglobins bind, transport, and deliver oxygen to peripheral tissues based on pH-dependent oxygen affinity (Wells, 2009). Gadid hemoglobin are particularly pH-sensitive (Barlow et al., 2016), and can polymerize in low pH levels, distorting red blood cell shape which is commonly referred to as sickling. Genes associated with mitochondrial electron transport and heme/iron binding were less active, and those associated with blood coagulation were more active (coagulation is a prominent feature in sickle-cell disease, Faes et al., 2018). While speculative, we suggest that combined warming and acidification could affect larval Pacific cod blood oxygen-carrying capacity and aerobic scope due to changes in hemoglobin properties. Validation is needed to confirm the hypothesized effects of combined warming and acidification on blood cells and how they might affect larval health.

Cold-reared larvae may acclimatize by increasing protein synthesis machinery and altering energy homeostasis

Understanding mechanisms associated with cold tolerance in Pacific cod is important as northern shifts have been observed in Pacific cod (Spies et al., 2020) and other fish species (Pinsky et al. 2013). Daily mortality rates were low in cold-reared larvae, while growth and development were prolonged and larvae were thinner (Table 3, Slesinger et al., 2024). Larval recruitment is not likely to be impacted by cold temperatures directly except at extremes (Zhuang and Chen 2021), but may be indirectly affected by slow growth and development. The most pronounced transcriptional differences in cold-reared larvae were changes in protein production and catabolism, indicative of higher protein concentration requirements. Expression

492 was higher for ribosomal biogenesis and molecular chaperone processes, which are
493 responsible for protein synthesis, folding, and stability. Higher protein production could be
494 needed to compensate for slower protein synthesis and enzymatic rates under cold conditions
495 (Long et al., 2012; Storch et al., 2005). This compensatory mechanism was clearly
496 demonstrated in cold-acclimated Atlantic cod, which had lower translation rates that were offset
497 by higher concentrations of RNA, resulting in no change to mean RNA activities (Foster et al.,
498 1992). Protein synthesis is energetically expensive and may have contributed to the slightly
499 lower condition of cold-reared larvae (Houlihan, 1991). Genes involved in multiple lipid
500 pathways were also affected (e.g. fatty acid biosynthesis, cholesterol metabolism, retinoic acid
501 response), which could enable larval fish to prioritize energy conservation and survival over
502 growth and development (Figure 3). Interestingly, lipid processes were not similarly affected in
503 larvae reared in the cold + acidified treatment, and protein synthesis-related gene expression
504 was higher (Figure S8). Larvae exposed to combined cold and acidified conditions were better
505 conditioned than those in cold alone (Table 3, Slesinger et al. 2024), which suggests that
506 acidification may stimulate or necessitate additional acclimatory mechanisms (e.g. protein
507 synthesis) that could affect energy homeostasis in cold temperatures. These data reveal genes
508 and processes that are important for larval Pacific cod acclimation to cold temperatures, which
509 could be monitored in cod exhibiting northward migration.

510 *Experimental considerations*

511 This study does not capture variability due to genetic, epigenetic, or parental carryover factors
512 (Almeida et al., 2024). Collecting thermal performances across multiple families or populations
513 could reveal genotype-specific tolerances to suboptimal conditions. Larvae were sampled for
514 gene expression after a 13-28-day larval incubation phase in which daily mortality rates were
515 high, although not uncommon for larval Pacific cod (Copeman & Laurel 2010; Laurel, Hurst, &
516 Cianelli 2011). Transcriptional patterns reported here therefore reflect those in surviving larvae,

517 and despite the relatedness of all larvae entering treatments (three half-sibling families)
518 survivors may have been genetically or phenotypically distinct. Larvae were sampled at similar
519 sizes and degree-days among temperature treatments (but different calendar days) to control
520 for development. Still, developmental differences are possible given the strong temperature-
521 dependent developmental rates. Warm-reared larvae were smaller than the other temperatures,
522 which could have resulted in size-specific expression. We removed genes that correlated
523 strongly with larval size, which is a good proxy for development. While this approach did not
524 affect overall conclusions, it could not control for developmental differences that are decoupled
525 from size and may not capture all processes affected by treatments. For instance, a number of
526 genes involved in ion transport, neurological activity, and circadian rhythm were more active in
527 response to warm temperatures in the unfiltered dataset but were removed due to their
528 association with length (Figure S17, Table S5). Future studies should strive to target more
529 genetically diverse cohorts, and to accommodate sampling at multiple time-points. These
530 approaches would enable descriptions of how molecular signatures change over the course of a
531 mortality event in developmentally similar larvae and to capture genotype x environment
532 interactions.

533 It must also be noted that larvae that were sampled for gene expression analysis from
534 the cold treatment were from a single tank (Table 2). Tank-specific responses can occur,
535 however the potential effect of replicate tank was assessed and was not found to influence the
536 measured phenotypic responses (Slesinger et al., 2024). In this study's gene expression
537 analysis, tank did not significantly influence expression in the treatments with samples from
538 multiple replicate tanks (Table S6), and expression variability among larvae in the cold
539 treatment did not differ from that observed in the other five treatments which included larvae
540 sampled from all four replicate tanks (Figure S18). Additionally, no genes were expressed at
541 significantly different levels in cold-exposed larvae (from a single tank) compared to larvae

542 exposed to cold + acidified conditions (from four tanks), which indicates that cold temperature
543 was the primary factor influencing gene expression.

544 ## Hypothesized impacts of climate stressors on recruitment

545 Pacific cod recruitment has significantly and consistently declined subsequent to heatwave
546 years in the Gulf of Alaska (e.g. Laurel et al., 2023; Abookire et al. 2024). Recent empirical data
547 (Slesinger et al., 2024) supports the theory that heat waves contributed to recruitment failure by
548 reducing larval survival. In this companion study, molecular data reveal that metabolic shifts and
549 inflammation are possible mechanisms of warming-associated mortality (Figure 4). Specifically,
550 we suggest that warming increases energetic demands due to rapid development, growth, and
551 higher metabolic rate, which likely outpaces lipid metabolism capacity. In an energy-depleted
552 condition, the costly process of mounting an immune response further reduces energy
553 mobilization and may decrease its effectiveness (Arts and Kohler, 2009). Larvae are equipped
554 with compensatory mechanisms that allow them to acclimate to and survive colder
555 temperatures, but recruitment rates are likely affected by a prolonged larval phase (Figure 4).
556 As reported by Slesinger et al. (2024), acidification as a single stressor would not likely reduce
557 recruitment directly by increasing larval mortality rates. However, acidification may reduce larval
558 body condition over time by possibly impacting lipid digestion, and effects may be more relevant
559 when prey is scarce (Figure 4). If acidification occurs during heatwave events or in cold
560 conditions, larval recruitment patterns will predominantly be determined by temperature.
561 Molecular data suggest that acidification may mitigate some negative effects of warming (e.g.
562 more muted immune and metabolic response) that could potentially benefit phenotypes in later
563 stages or in the natural environment, and may augment protein synthesis machinery in cold
564 temperatures which may actually improve larval condition.

565 Our findings and those from Slesinger et al. (2024) support the hypothesis that warming
566 events directly affect Pacific cod larval physiology and likely contribute to recruitment failures.

Larvae are more capable of acclimating to acidified and colder conditions, although predation rates and effects on growth and condition could become more impactful in later stages such as overwintering juveniles (Abookire et al. 2024). Taken together, results indicate it may be appropriate to apply higher natural mortality to early life stages in modeling efforts in response to heatwave conditions to capture expected declines in recruitment.

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Competing Interests

Competing interests: The authors declare there are no competing interests.

Data and Code Availability

Raw sequence data is published on NCBI under BioProject PRJNA1154236. Code and metadata is available in the GitHub repository, https://doi.org/10.5281/zenodo.15399957.

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832

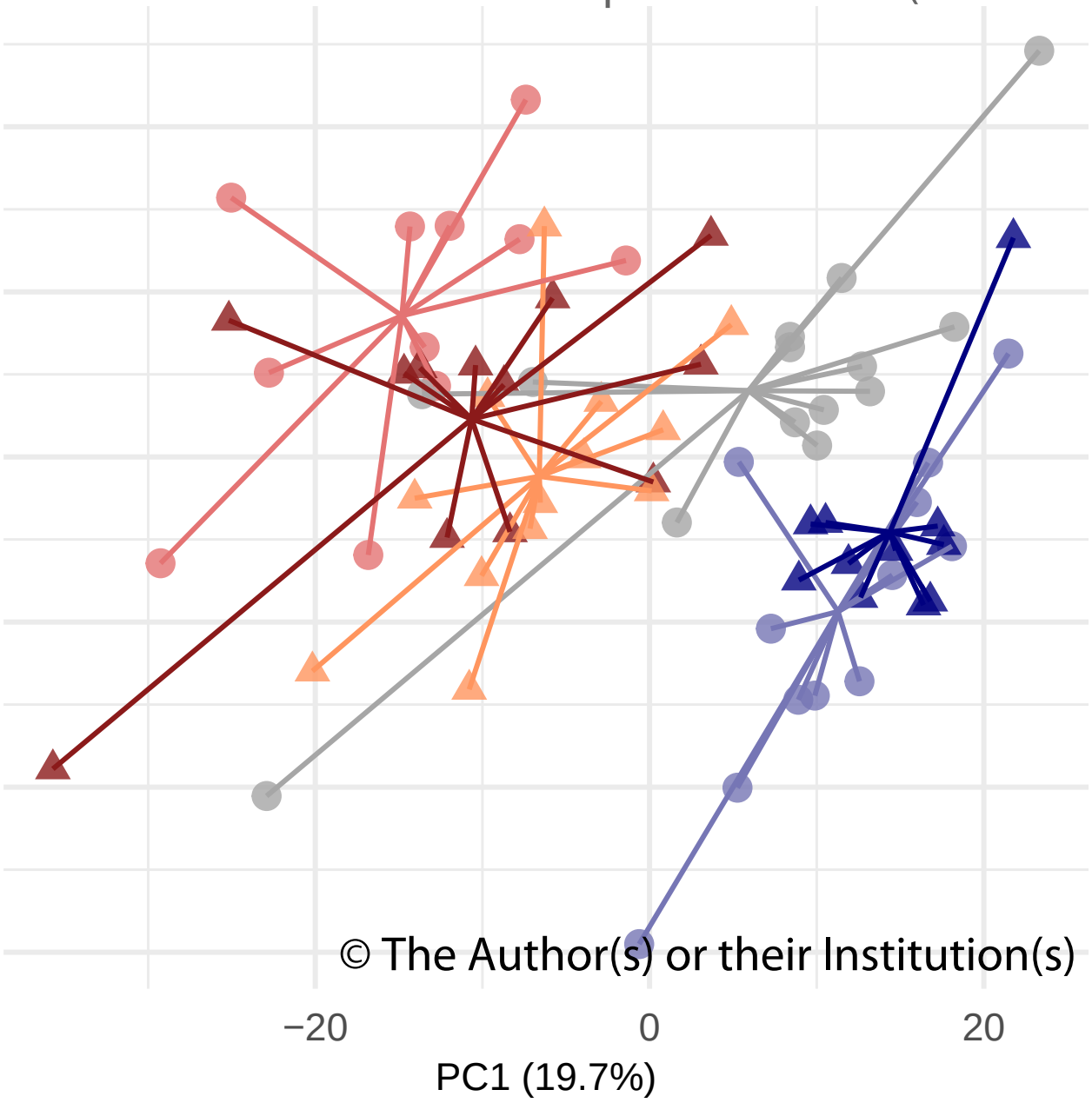
Figure Captions

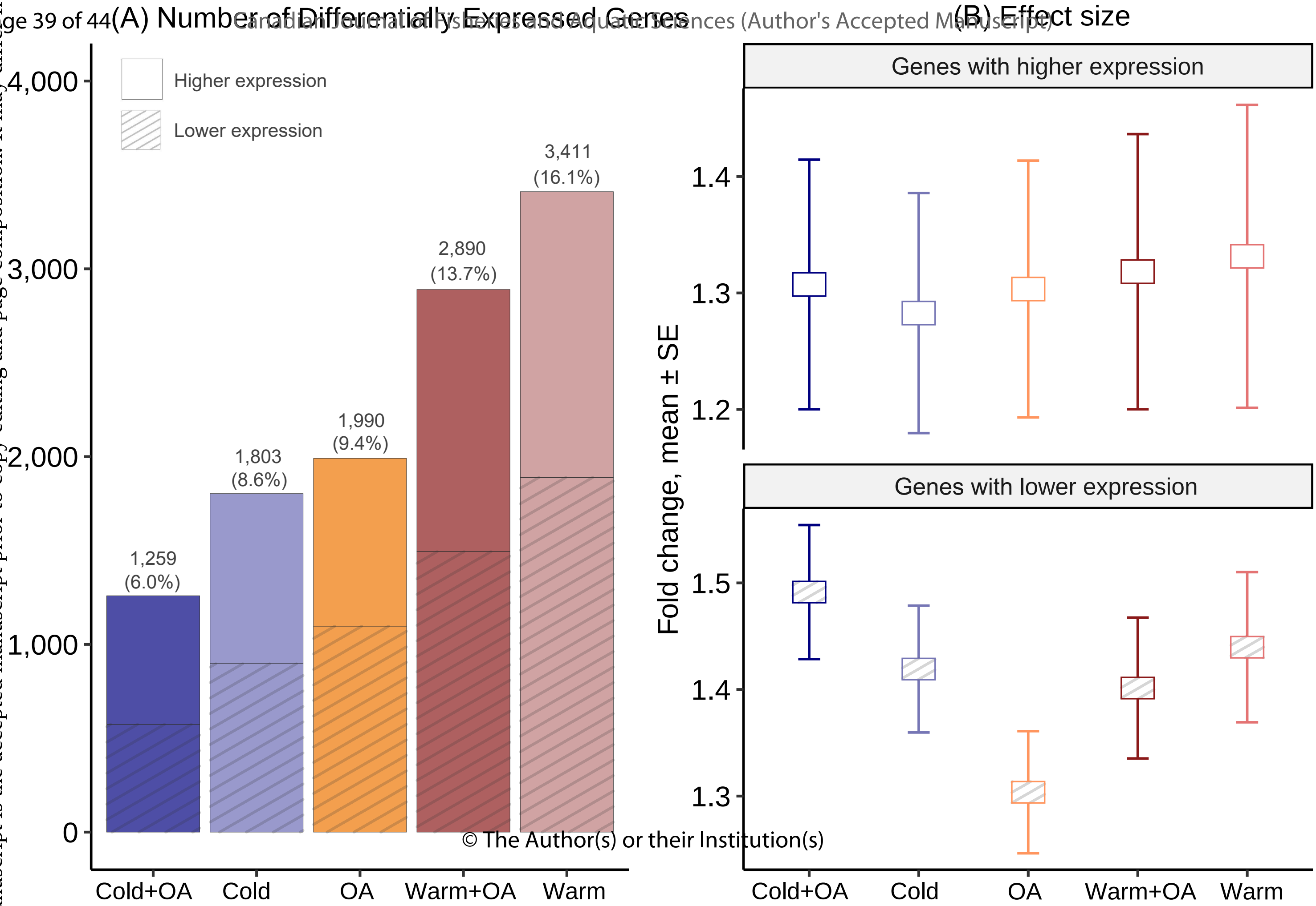
Figure 1. PCA biplot of principal components one and two, constructed from all examined genes. Points represent global expression patterns for individual larval fish reared in control conditions (6°C, 390µatm CO_2) and combinations of suboptimal temperatures (Cold=3°C, Warm=10°C) and acidification (1560µatm CO_2), and lines radiate out from treatment centroids.

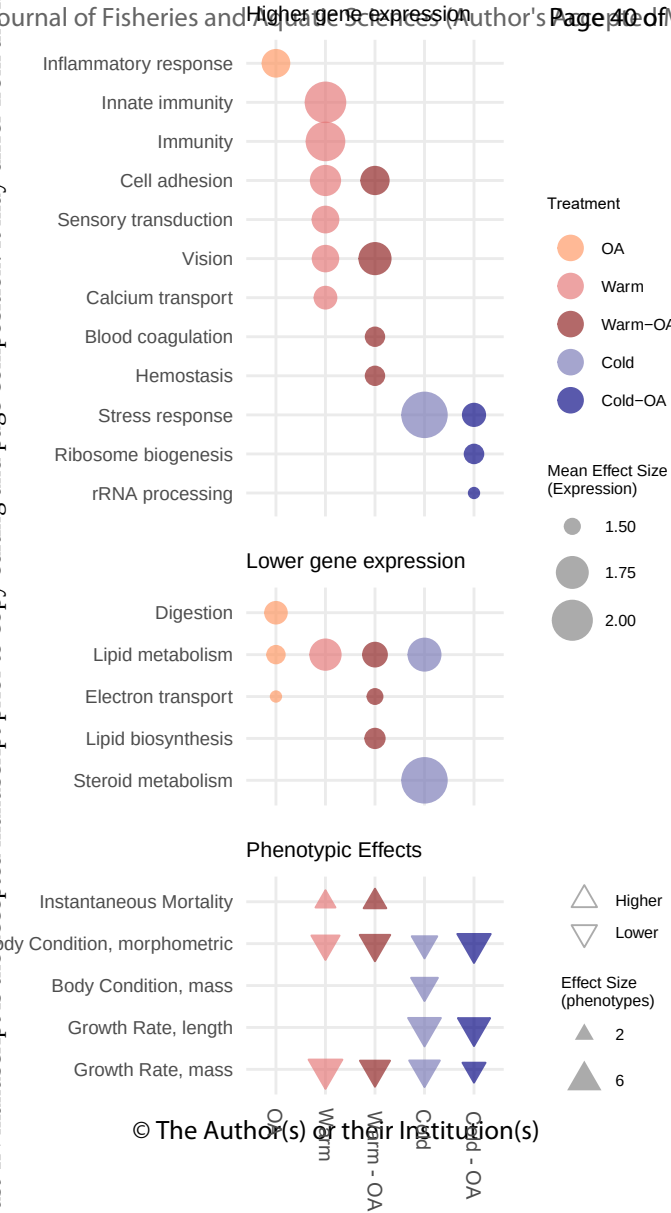
Figure 2. (A) The number of genes that were differentially expressed (DEGs) in response to temperature and acidification treatments as single stressors (Warm=10°C, Cold=3°C, OA = ~1,560 µatm pCO_2) and when combined, all compared to the control conditions (6°C, pCO_2 = ~390 µatm). Percentages are relative to all known genes detected in this study (n=21,076) (B) Mean effect size (fold change) of all DEGs that were expressed at higher levels (top) and lower levels (bottom) compared to control condition, where error bars represent the variability of effect size among DEGs within each treatment (SE). DEGs were determined from 11-14 individual larvae per treatment, sampled across four tanks for all treatments except the cold treatment (one tank).

Figure 3. Enriched biological processes (Uniprot keyword) in genes with higher expression (top) and lower expression (middle) in response to temperature and pCO_2 stressors alone (Cold=3°C, Warm=10°C, High pCO_2= ~1,560 µatm), and when combined, and phenotypic effects at the time of sampling for gene expression (bottom). All enriched processes and phenotypic effects are relative to the control conditions (6°C, pCO_2 ~390 µatm). Point sizes indicate the mean effect size of differentially expressed genes involved in each affected process (fold change), and effect size for phenotypes (Cohen's d). See Figures S3 and S4 and Table S2 for enriched Gene Ontology terms, and Slesinger et al. (2024) for phenotypic effects across multiple time periods.

858 **Figure 4:** Hypothesized molecular mechanism associated with phenotypic responses to climate
859 stressors and likely impacts on recruitment. Hypotheses are based on RNASeq-generated
860 transcriptomes from whole-body larvae that were exposed to warm (10°C), acidified (pH 7.47,
861 1,560 µatm pCO_2), cold (3°C), and combined warm + acidified and cold + acidified conditions.







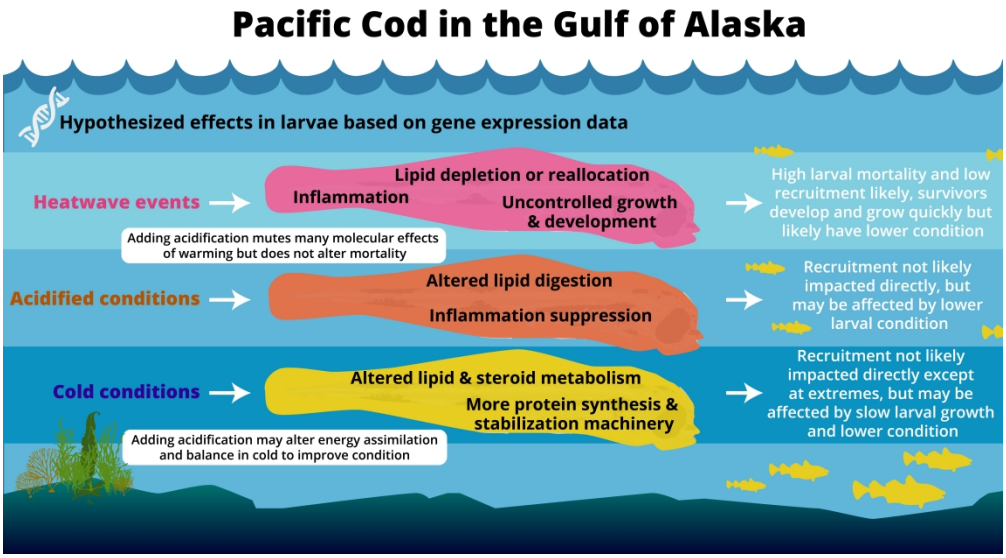


Figure 4. Hypothesized molecular mechanism associated with phenotypic responses to climate stressors and likely impacts on recruitment. Hypotheses are based on RNASeq-generated transcriptomes from whole-body larvae that were exposed to warm (10°C), acidified (pH 7.47, 1,560 µatm pCO2), cold (3°C), and combined warm + acidified and cold + acidified conditions.

1722x968mm (118 x 118 DPI)

Tables

Table 1. Seawater carbonate chemistry during larval incubations. Values indicate mean ± SD through time where temperature and salinity were measured daily, and dissolved inorganic carbon (DIC) and total alkalinity (TA) were measured from preserved bottle samples that were collected weekly. Weekly pH_{sws} and pCO_2 were calculated from temperature, salinity, DIC and TA. Reproduced from Slesinger et al. (2024).

Treatment	pH_{sws}	pCO_2 (µatm)	Temperature (°C)	Salinity	DIC (mol/kg)	TA (mol/kg)
Control	8.03 ± 0.11	394.28 ± 132.84	6.14 ± 0.29	29.84 ± 1.48	1943.38 ± 105.62	2063.08 ± 84.73
Acidified	7.47 ± 0.03	1480.80 ± 126.37	6.04 ± 0.51	29.36 ± 1.31	2078.14 ± 81.47	2042.54 ± 84.58
Warm	8.00 ± 0.03	409.60 ± 36.75	10.22 ± 0.24	29.63 ± 0.67	1911.35 ± 22.43	2040.80 ± 21.72
Warm + Acidified	7.40 ± 0.06	1780.01 ± 245.48	10.20 ± 0.37	29.63 ± 0.67	2076.27 ± 24.75	2038.04 ± 22.15
Cold	8.12 ± 0.07	297.20 ± 65.51	3.03 ± 0.39	30.21 ± 1.11	1936.76 ± 76.24	2074.63 ± 65.73
Cold + Acidified	7.48 ± 0.09	1434.95 ± 304.70	3.10 ± 0.18	29.77 ± 1.30	2093.99 ± 76.91	2050.55 ± 68.72

Table 2. Gene expression sampling scheme. For each treatment, gene expression data was generated from 11-14 individual whole-body larvae using separate RNASeq libraries, sampled across four replicate tanks in all but the cold treatment. Larvae from the cold treatment were sampled from one tank (see Methods and Supplemental for more details). Treatment duration from hatch until gene expression sampling are provided in both Julian days and Degree-Days (DD) calculated from average temperatures. Differential expression analysis was performed for all treatments relative to control conditions (6°C, 390 µatm pCO_2).

Treatment *Mean conditions*	Replicate larvae	Number of tanks sampled for RNASeq	Duration *Days (Degree Days)*	Larval length *Mean* ± *SE*
Control *6.1°C, 394 µatm pCO_2*	14	4	21 (129)	6.5±0.2
Acidified *6.1°C, 1,481 µatm pCO_2*	13	4	21 (127)	6.7±0.1
Warm *10.1°C, 410 µatm pCO_2*	11	4	13 (133)	5.7±0.1
Warm + Acidified *10.2°C, 1,780 µatm pCO_2*	12	4	13 (133)	6.0±0.1
Cold *3.0°C, 297 µatm pCO_2*	12	1	28 (85)	6.7±0.1
Cold + Acidified *3.1°C, 1,435 µatm pCO_2*	11	4	28 (87)	6.4±0.2

Table 3. Summary of phenotypic effects of treatments on mortality, growth, and condition during the larval stage, adapted from data previously reported in Slesinger et al. (2024). Phenotypic values represent measurements that were taken on or near (≤2 days) the day of sampling for gene expression (condition factors) or calculated between hatch and sampling date (growth and mortality), and are mean ± SD across three (†) or four replicate tanks. Values with asterisks (✱) indicate pairwise differences among each treatment and the control (6.1°C, 390 µatm). Temperature and pCO_2 represent treatment means across four replicate tanks.

Treatment	Control	Acidified	Warm	Warm + Acidified	Cold	Cold + Acidified
Temperature, pCO_2 (Mean + SD)	6.1 ± 0.3°C, 394 ± 133 µatm	6.0 ± 0.5°C, 1,481 ± 126 µatm	10.2 ± 0.2°C, 410 ± 37 µatm	10.2 ± 0.4°C, 1,780 ± 245 µatm	3.0 ± 0.4°C, 297 ± 66 µatm	3.1 ± 0.2°C, 1,435 ± 305 µatm
Total survival (%)	23.0 ± 20.8%	35.1 ± 15.0 ±	5.7 ± 4.2%	4.1 ± 2.0%	12.1 ± 13.3%	13.5 ± 4.9%
Instantaneous daily mortality rate (%)	0.097 ± 0.06	0.053 ± 0.02	0.24 ± 0.05 ✱	0.25 ± 0.04 ✱	0.11 ± 0.07	0.074 ± 0.01
Morphometric-based condition factor, K_{MH}	0.019 ± 0.02	0.014 ± 0.018	0.002 ± 0.02 ✱	-0.001 ± 0.019 ✱	-0.003 ± 0.022 †✱	-0.007 ± 0.021 ✱
Dry weight-based condition factor, K_{DW}	1.06 ± 0.08	1.05 ± 0.05	1.09 ± 0.11	1.08 ± 0.13	0.96 ± 0.07 †✱	1.00 ± 0.08
Growth rate, length-based G_L (mm/day)	0.071 ± 0.005	0.075 ± 0.01-	0.055 ± 0.014	0.062 ± 0.003	0.041 ± 0.007 †✱	0.046 ± 0.005 ✱
Growth rate, mass-based, G_M (%/day)	4.76 ± 0.40	4.78 ± 0.25	2.72 ± 0.68 ✱	3.77 ± 0.54 ✱	2.43† ± 0.41 ✱	3.37 ± 0.41 ✱