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Integrated organismal responses induced by projected levels of CO_2 and temperature exposures in the early life stages of lake sturgeon

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Abstract

Atmospheric CO_2 and temperature are rising concurrently, and may have profound impacts on the transcriptional, physiological and behavioural responses of aquatic organisms. Further, spring snowmelt may cause transient increases of pCO_2 in many freshwater systems. We examined the behavioural, physiological and transcriptomic responses of an ancient fish, the lake sturgeon (*Acipenser fulvescens*) to projected levels of warming and pCO_2 during its most vulnerable period of life, the first year. Specifically, larval fish were raised in either low (16°C) or high (22°C) temperature, and/or low (1000 μatm) or high (2500 μatm) pCO_2 in a crossed experimental design over approximately 8 months. Following overwintering, lake sturgeon were exposed to a transient increase in pCO_2 of 10,000 μatm, simulating a spring melt based on data in freshwater systems. Transcriptional analyses revealed potential connections to otolith formation and reduced growth in fish exposed to high pCO_2 and temperature in combination. Network analyses of differential gene expression revealed different biological processes among the different treatments on the edges of transcriptional networks. Na^+/K^+-ATPase activity increased in fish not exposed to elevated pCO_2 during

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development, and mRNA abundance of the β subunit was most strongly predictive of enzyme activity. Behavioural assays revealed a decrease in total activity following an acute CO_2 exposure. These results demonstrate compensatory and compounding mechanisms of pCO_2 and warming dependent on developmental conditions in lake sturgeon. Conserved elements of the cellular stress response across all organisms provide key information for how other freshwater organisms may respond to future climate change.

KEYWORDS
behaviour, climate change, Na^+/K^+-ATPase, physiology, RNA-seq, transcriptional network

1 | INTRODUCTION

The impact of rising atmospheric CO_2 on marine biota is undeniable, but the highly variable chemistry of freshwater ecosystems, with over an order of magnitude greater CO_2 variability over time than marine ecosystems, complicates our understanding of how organisms may respond to increased CO_2 in freshwater environments (Chan et al., 2017; Cole et al., 2007; Fleet et al., 2024; Hasler et al., 2016; Jeffrey et al., 2018; Ou et al., 2015; Quijada-Rodriguez et al., 2021; Tripp et al., 2022; Weiss et al., 2018). This increase in CO_2 can be especially prominent in flowing freshwater, which is often supersaturated with respect to the atmosphere (Cole et al., 2007; Toavs et al., 2023). Moreover, increases in atmospheric CO_2 coincide with elevation in global temperatures (Anderson et al., 2016; Shakun et al., 2012). Increasing temperatures and temperature variability in freshwater, such as fluctuations in daily river temperatures, will also be concurrent with increasing air temperatures with climate change (Morash et al., 2021; Van Beek et al., 2012; Van Vliet et al., 2011). The combined effect of increased temperature and the partial pressure of CO_2 in the environment (environmental pCO_2), may induce cumulative or novel responses in aquatic ectotherms through stressor interactions not elicited by each factor individually (Todgham & Stillman, 2013). These combined effects are likely to be profound throughout organismal development, but our understanding of the long-term impacts of acute exposures to elevated CO_2 remains limited (Crocker & Cech Jr., 1998; Huynh et al., 2011; Nawata & Wood, 2008). To better understand the effects of climate change in natural systems, it is necessary to determine how pCO_2 and temperature act synergistically over extended timescales throughout a freshwater organism's life.

Early life is the most critical period in an individual's development, with environmental changes causing profound and potentially long-lasting effects on an organism's capacity to respond to future environmental perturbations (i.e. developmental plasticity) (Burggren, 2021). In addition, young freshwater fish may have a lower ability to move away from a stressor than adults (Lechner et al., 2016; Voesenek et al., 2018), necessitating physiological plasticity in early development. Along with increasing atmospheric CO_2, spring snowmelts can transiently increase pCO_2 in freshwater, reaching 10,000 μatm of pCO_2 in systems where it is consistently around 1000 μatm (Telmer & Veizer, 1999). These transient increases depend on local geochemical conditions and buffering capacity, and are common in Boreal forest ecosystems (Bottomley et al., 1986; Jeffries et al., 1979; Telmer & Veizer, 1999). While increasing temperatures may decrease the duration and intensity of spring snowmelts, along with decreasing the saturation point of CO_2 in water, atmospheric CO_2 is projected to increase throughout the 21st century in plausible climate scenarios (Anderson et al., 2016; Schwalm et al., 2020). This increase in CO_2 will, therefore, lead to continued transient increases in pCO_2 in freshwater systems as a result of spring snowmelts. High environmental pCO_2 and low pH can lead to acidosis, but a majority of fish are efficient at regulating acid–base balance by retention and uptake of bicarbonate mainly by gill and renal tissue (Heuer & Grosell, 2014; Wood et al., 1999). However, extended periods of hypercapnia can lead to chronically increased activity and Na^+/K^+-ATPase activity (Deigweiher et al., 2008), indicating long-term shifts in energy balance as well as ion and acid–base regulation that may interact with transient periods of intense pCO_2 exposure such as after snowmelt.

As climate change will lead to fishes developing in environments with higher thermal and hypercapnic baselines, we hypothesized that increased pCO_2 and temperature exposure throughout early development could alter the capacity of aquatic organisms to tolerate a transient pCO_2 challenge. Multiple response directions are reasonable in this circumstance (Betts et al., 2021): either fish exposed to a more stressful developmental environment may be more tolerant to a later pCO_2 challenge because they adjusted their phenotypes during development, or the developmental environment decreased tolerance to the later challenge due to reduced physiological capacity from chronic environmental stress responses (Earhart et al., 2022; Pottier et al., 2022).

In this study, we used the lake sturgeon (*Acipenser fulvescens*), an ancient, long-lived fish with extensive phenotypic plasticity in response to environmental stressors (Brandt et al., 2022; Bugg et al., 2020; Dammerman et al., 2016; Yoon et al., 2022; Yoon, Deslauriers, & Anderson, 2019). Because their capacity for plasticity is unusually high among vertebrates, lake sturgeon are valuable for studying mechanistic responses to a broad range of stressors at different magnitudes. The reasons for this plasticity remain unknown, but large genome size may be a factor (Fontana et al., 2004;

Knight, 2005; Murren et al., 2015). We studied the interacting effects of temperature and pCO_2 throughout early development, using both current and projected levels of pCO_2 in freshwater at the end of the 21st century (Loeppky et al., 2021; Raymond et al., 2013; Telmer & Veizer, 1999; Weiss et al., 2018), followed by an overwintering period. After overwintering, we used an acute transient increase in pCO_2 of 10,000 μatm for 7 days to simulate spring snowmelt near the end of the sturgeon's first year of life (Figure 1) (Telmer & Veizer, 1999; Toavs et al., 2023).

After the transient acute pCO_2 increase, lake sturgeon behaviour was analysed for both boldness and alarm cue responses, in crossed rearing and exposure trial conditions. To test the effects of elevated long-term pCO_2 and temperature exposures independently and together, changes in energy metabolism and acid–base regulation were examined using measurements of Na^+/K^+-ATPase activity, haematocrit, ammonia excretion and metabolic rate at different timepoints following the transient pCO_2 increase. Messenger RNA (mRNA) sequencing was used to develop a greater understanding of gill transcriptional responses, which was also assessed at different timepoints following the transient pCO_2 increase. The same individuals were used in mRNA sequencing and measurements of Na^+/K^+-ATPase activity, allowing for a direct integration of transcriptional and enzymatic results. Gill tissue was selected for this study based on its key roles in environmental responses and gas exchange, and has been shown to be effective in other transcriptomic research (Jeffries et al., 2021). A network analysis was performed among genes in which transcript abundance changed over time within each experimental group, to analyse potential regulatory processes in network hubs versus processes on the edges of gene regulatory networks. Therefore, this work connected long-term developmental conditions with climate change-related stressors at multiple biological levels (i.e. behavioural, whole organism and cellular scales) in a non-model fish, which will have implications for the mechanisms of climate change responses in freshwater organisms.

2 | MATERIALS AND METHODS

2.1 | Animal rearing

In the spring of 2017, gametes were collected from crosses of four female and at least two male lake sturgeon from the Winnipeg River, Manitoba and were transported to the University of Manitoba for fertilization (Earhart et al., 2020). While we recognized family effects on phenotypic development (Deslauriers et al., 2023), we chose to use full- and half-siblings from wild crosses in our study due to logistical constraints. Following de-adhesion, embryos were incubated at 16°C and either 1000 or 2500 μatm pCO_2 in one of three 12 L

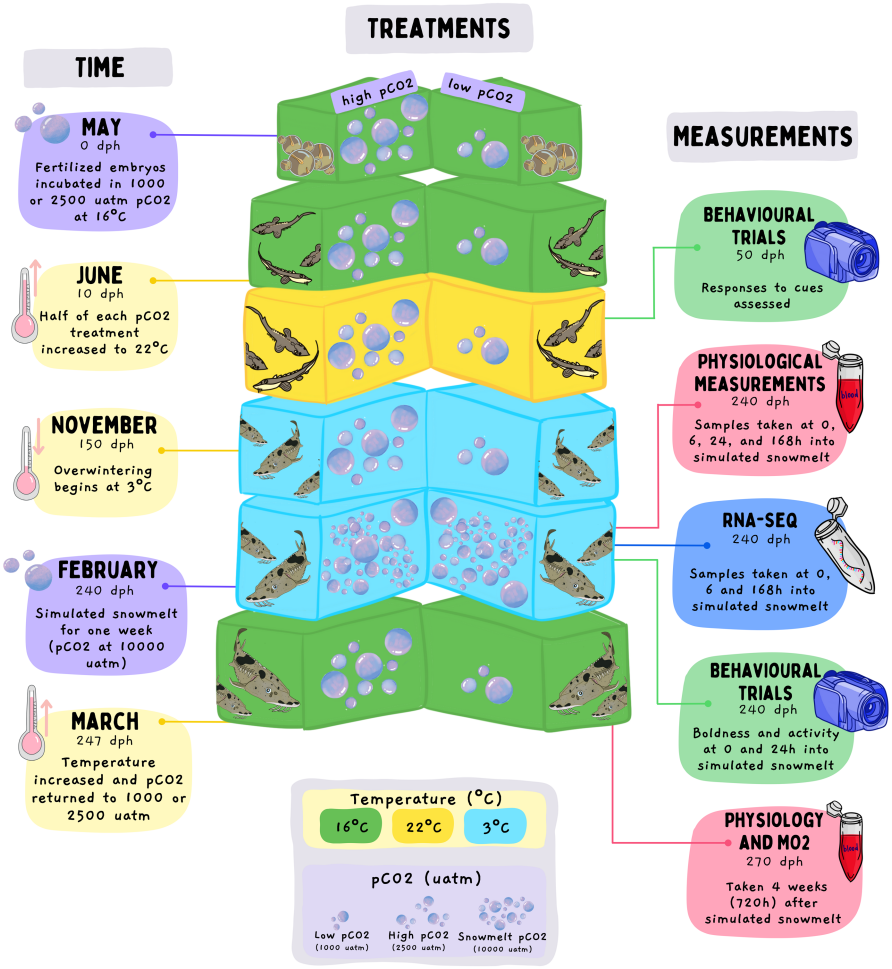

FIGURE 1 Conceptual diagram of rearing environments, transient pCO_2 increase simulating snowmelt, and sampling times. A timeline is provided on the left, and measurements taken at different times are shown on the right. Boxes correspond to discrete groups of fish, colours represent temperature at a given time for a group of fish while bubbles represent pCO_2 in μatm. Days post-hatch (dph) values are provided within each label. Illustration by Madison L. Earhart.

aquaria per treatment group with a photoperiod of 12L:12D. All fish aquaria were provided constant aeration and flow of dechlorinated tap water. Water source was the tap water of the city of Winnipeg filtered by charcoal and sand with oxygen above 8 ppm (~90% air saturation) throughout the study period. Live artemia (Brine Shrimp Direct; Ogden, Utah) was provided three times a day beginning at 15 days post-fertilization, and 3 weeks after exogenous feeding, food was transitioned to bloodworms (Hikari USA; Hayward, California, USA) twice a day for the remainder of this experiment other than the overwinter food restriction period (Earhart et al., 2020). We ensured that fish were fed to satiation by visually confirming leftover feed after 15 min and any excessive feed was cleaned after each meal. Also, rearing density was accounted for and reduced as fish grew at a density of 15 larvae·L^{-1} (see below).

The exposures of 1000 or 2500 μatm pCO_2 represent current levels of *p*CO2 and projected levels at the end of the 21st century respectively (Loeppky et al., 2021; Raymond et al., 2013; Telmer & Veizer, 1999; Weiss et al., 2018). The 1000 μatm pCO_2 group was, thus, treated as a relative control against which the elevated pCO_2 group was compared. To test the effects of elevated temperature on development, a random subset of larvae from both pCO_2 treatments was transferred to a second set of holding tanks 10 days post-hatch, and the temperature was slowly raised to 22°C (1°C·day^{-1}), the highest temperature that wild lake sturgeon currently experience during summer months in the northern part of their range (Bugg et al., 2020). At this time, fish were transferred to one of four 170 L tanks. Thus, larvae from all four treatments – 16°C 1000 μatm (control), 16°C 2500 μatm (high pCO_2), 22°C 1000 μatm (elevated temperature) and 22°C 2500 μatm (high temperature and pCO_2) – were held in these conditions for 5 months until they were exposed to overwintering conditions. An IKS Aquastar system with pH monitoring electrodes (IKS Aquastar Computer Systeme GmbH, Karlsbad, Germany) was used to inject a controlled amount of CO_2 into tanks. Spectrophotometric assays of total alkalinity (Sarazin et al., 1999) were used in conjunction with temperature and pH (NBS scale) measurements, K1 and K2 constants and pressure effects in freshwater (Millero, 1979) and the KSO_4 dissociation constant (Dickson, 1990) in the software CO2calc to estimate total pCO_2 throughout the present experiments (Robbins et al., 2010).

For the overwintering experiment, fish were transferred to one of four 250 L flow-through tanks per treatment situated in a controlled environment chamber (CMP 6050, Conviron, Manitoba, Canada). Overwintering was simulated by decreasing the temperature in all treatments (1°C·day^{-1}) until water temperature reached 3°C, following established protocols (Yoon, Deslauriers, Enders, et al., 2019). The sturgeon were then held at 3°C for 3 months, with complete food restriction in the final 2 months to simulate Manitoba winters.

Following the overwintering experiment (i.e. 8 months post-hatch), sturgeon were exposed to a simulated acute spring snowmelt, where all treatments were exposed to an acute transient increase in pCO_2 of 10,000 μatm for 7 days. This value was chosen based on prior work on pCO_2 increases in fresh water following spring snowmelts (Telmer & Veizer, 1999; Toavs et al., 2023). Water temperatures in all tanks were then slowly increased to 16°C at a rate of 1°C·day^{-1}. Once all tanks were at 16°C, pCO_2 was decreased to developmental conditions of 2500 or 1000 μatm. Sampling for mRNA and enzyme activity was conducted with respect to the start of the acute transient increase to 10,000 μatm of pCO_2. The sex of individual fish was unable to be assessed. All animals used in this study were reared and sampled according to animal use and care guidelines established by the Canadian Council for Animal Care and approved by the Animal Care Committee at the University of Manitoba (protocol #F15-007).

2.2 | Messenger RNA sequencing and bioinformatics

Lake sturgeon haphazardly sampled for this study were netted and euthanized by immersion in an overdose of tricaine methanesulfonate solution (250 mg·L^{-1}; MS-222, Syndel Laboratory, Vancouver, Canada). In all four treatments, lake sturgeon were terminally sampled at 0, 6 and 168 h post-transient pCO_2 increase. Between $n=4$ and 6 individuals were used within each experimental group and timepoint ($n=4$ for control fish at 168 h post-transient pCO_2 increase and elevated temperature fish 0 h post-pCO_2; $n=5$ for control and pCO_2-only fish 0 h post-transient pCO_2 increase; $n=6$ for all other treatments; $N=66$ total). Individuals were sampled haphazardly, and RNA extractions were completed in a randomized manner among treatments and timepoints. While MS-222 induces transcriptional changes such as in oxidative stress and apoptosis (Félix et al., 2020), all fish in the present study were treated with it in the same way and sampled within 10 min of MS-222 exposure. Therefore, analyses of differences between timepoints or treatments (e.g. differential expression) account for any potential impacts of MS-222 exposure. Gill tissue was then extracted from each fish and preserved in RNA*later* (Invitrogen; Carlsbad, California, USA), held at 4°C for 24 h and stored at −80°C prior to RNA extraction. Total RNA was extracted from the gill tissue by homogenization in 500 μL of lysis buffer (PureLink RNA Mini Kit; Invitrogen; Ambion Life Technologies; Waltham, MA, USA) for 10 min at 50 Hz using a TissueLyser II (Qiagen; Germantown, Maryland, USA) and then using a PureLink RNA Mini Kit (Invitrogen; Ambion Life Technologies) following manufacturer's instructions. Total RNA purity and concentration were evaluated for all samples using a NanoDrop One (Thermo Fisher Scientific; Waltham, Massachusetts, USA) as well as gel electrophoresis to assess RNA integrity. Samples were then stored at −80°C prior to preparation for sequencing. Samples were normalized to 50 ng·μL^{-1} and messenger RNA isolation was done with NEBNext Poly(A) Magnetic Isolation Modules (New England Biolabs) followed by the production of stranded cDNA libraries with NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs). All samples had a minimum RNA Integrity Number of 8.0. Individual barcodes were applied with NEBNext dual adaptors (New England Biolabs) for each library prior to sequencing. Paired-end, 100 base

pair reads were sequenced on a NovaSeq 6000 Sequencing System (Illumina) at the Génome Québec sequencing facility (https://genomequebec.com/en/).

A mean of 50.6 million raw reads was sequenced per individual (standard deviation 16.3 million raw reads) (Table S1). The program fastp v0.20.1 was used for adapter trimming and read filtering, with a minimum phred quality of 15, a maximum of 40% of unqualified bases allowed in a read before read filtering, a minimum length of 100 base pairs and polyG read tails were trimmed (Chen et al., 2018). MultiQC v1.11 was used for collating sequencing and quality reports from fastp (Ewels et al., 2016). Salmon v1.7.0 was used to quantify trimmed and filtered raw reads against a lake sturgeon gill transcriptome (Patro et al., 2017; Thorstensen et al., 2023). The flags validateMappings, seqBias and gcBias were included with the program to enable more selective alignments, correct for sequence-specific biases and address potential GC biases respectively. A published gill transcriptome was used as an already-developed, high-quality resource for lake sturgeon work developed from the same population as used in the present study. Mapping rates were consistent with the gill transcriptome being a high-quality resource (mean mapping rate 84.05%, 0.99% standard deviation; Table S1). Salmon was used as a rapid quasi-mapping-based approach for transcript quantification that results in accurate maximum likelihood-based estimates of transcript abundance (Patro et al., 2017).

The R package tximport v1.24.0 was used to import Salmon estimates of transcript abundance for differential gene expression analyses by edgeR v3.38.4, while tidyverse v1.3.2 was useful for data management in R (Chen et al., 2016; McCarthy et al., 2012; R Core Team, 2023; Robinson et al., 2010; Soneson et al., 2016; Wickham et al., 2019). The package edgeR was chosen as a well-established approach to differential expression, and for its flexibility in specifying contrasts with *makeContrasts* (Robinson et al., 2010). Transcripts were not summarized to the level of gene models, because the polyploid lake sturgeon genome may have led to a fragmented transcriptome (Thorstensen et al., 2023). Therefore, we chose not to speculate about paralogous or ohnologous gene expression because of the lack of present genomic resources (Singh & Isambert, 2019). Transcripts were filtered for those present in any individual sample, and pairwise contrasts were drawn between timepoints within groups. This approach allowed a greater number of transcripts to be used during model fitting, but significance was still assessed in group-wise comparisons – thus, transcripts with low counts in most individuals were considered non-significant. Transcripts that changed in abundance between any two timepoints in the control conditions were subtracted from transcripts in the other groups to identify transcripts that were specific to the environmental effects over time of increased temperature, pCO_2 or both. For each comparison between two groups, a gene-wise negative binomial generalized linear model with quasi-likelihood test was used (glmQLFit) where transcripts significant at Benjamini–Hochberg adjusted *p*-values (q) < .05 were accepted as significant (Soneson et al., 2016).

Significant transcripts were associated with gene annotations from the gill transcriptomes and used in gene set enrichment analyses with enrichR v3.1 (Chen et al., 2013; Kuleshov et al., 2016; Xie et al., 2021). The enrichment database Biological Process 2021 was used in each analysis. Gene ontology terms were accepted as significant at $q < .05$, and considered in terms of combined scores, which were calculated by the enrichR software using both *p*-values from a Fisher's exact test and *z*-scores for deviations from expected rank (Chen et al., 2013). The combined score was chosen for prioritizing gene ontology terms for analysis because it incorporates both significance and the proportion of genes within a term (as opposed to, for example, only the absolute number of genes in a term). For a gene ontology term that was of particular interest for its presence in both the specific and unique pCO_2 and temperature enrichment analyses (negative regulation of organ growth; GO: 0046621), gene function and counts per million among timepoints and experimental groups were further investigated. This term was potentially important in the context of other work on CO_2 exposures in fish (Ou et al., 2015).

In addition, a network analysis of genes annotated to transcripts that were specific to each treatment (temperature, pCO_2, or both) was run with OmicsNet 2.0 using the zebrafish (*Danio rerio*) annotation database and GeneBankIDs (Zhou et al., 2022; Zhou & Xia, 2019). Betweenness centrality scores (based on the number of shortest paths that pass through a gene, with higher scores more central in a network) from genes within each network were assessed for skewness to quantify the distribution in centrality for genes in different networks using the moments v0.14.1 package in R. Genes were plotted in order of decreasing betweenness, omitting genes with values of 0, to visualize the distribution of betweenness values in each experimental treatment. In addition, the R package ComplexUpset v1.3.5 was used to visualize the intersections of each network in terms of genes unique or shared among them, as identified with OmicsNet 2.0. EnrichR was also used with the Biological Process 2021 database to analyse gene ontology terms significant within network analyses for each treatment, where terms were prioritized by combined score. However, two enrichment analyses were run on each treatment: one on genes where betweenness centrality was >0, or the 'hubs' of the networks, and one on genes where betweenness centrality = 0, or the 'edges' of the networks. Betweenness centrality scores are higher on nodes that are on shorter paths between pairs of nodes in the network, and are thus likely more connected (Ewald et al., 2024; Zhou et al., 2022). Therefore, this analysis was developed to distinguish between more connected and less connected genes, among differentially expressed transcripts within each treatment. This partition in each treatment was done to distinguish between terms comprised regulatory genes versus others comprised of genes that were more proximal to biological processes.

2.3 | Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity has been shown to be responsive to hypercapnic conditions in fishes, and is an indicator of cellular acid–base

and ion regulation (Deigweiher et al., 2008). Activity of this enzyme was assayed in duplicate for each individual haphazardly sampled following protocols published in Kissinger et al. (2017), with $n = 8$ per treatment and timepoint (except increased pCO_2 at 24 h, which had $n = 7$; $N = 159$). Here, the four experimental treatments were assessed over 0, 6, 24, 168 and 720 h post-transient pCO_2 increase. A total of 80 different measurement runs were performed, with four measurements assayed per run except for two, which had three measurements each. The fish used in mRNA sequencing were a subset of the individuals sampled for Na^+/K^+-ATPase activity. A Bayesian approach was used to statistically assess Na^+/K^+-ATPase activity in each experimental treatment and timepoint with the R package brms v2.18.0 (Bürkner, 2017, 2018, 2021). The model formula used was Na^+/K^+-ATPase activity dependent on experimental group as a fixed effect, with measurement run and individual specified as crossed random intercepts because individuals were measured in duplicate and measurement run may have introduced bias into the results. Variance was allowed to be unequal among experimental groups. Each model in the present study was run with a skew-normal distribution over 5000 Hamiltonian Monte Carlo warm-up iterations and 15,000 sampling iterations, with four separate chains used for sampling (60,000 sampling iterations total). Model fits were assessed with the potential scale reduction statistic $\hat{R}$ (also called Rhat) and trace plots to estimate how well chain sampling and convergence progressed, as well as posterior predictive checks to visualize how well predicted results from the model matched real data. The model was accepted only if $\hat{R}$ was 1.00 for all parameters. All model priors and distributions used in physiological analyses, along with changes to adapt delta or tree depth to increase the efficiency of model fits, are included in Table S2.

Differences in Na^+/K^+-ATPase activity were assessed with 95% highest posterior density intervals using the R package emmeans v1.8.1–1, while Tidybayes v3.0.2 was used to visualize model results (Searle et al., 1980). Specifically, posterior distributions of Na^+/K^+-ATPase activity in each experimental group were compared in a pairwise manner with estimated marginal means to generate 95% highest posterior density intervals (HPD) of contrasts between groups. Estimated marginal means were also used to visualize posterior distributions for each experimental group. This modelling approach of fitting the dependent variable to experimental groups, choosing uninformative to weakly informative priors, controlling for variables when possible with random intercepts, checking models, using HPD intervals to make pairwise comparisons and visualizing posterior distributions with estimated marginal means was applied to all physiological and behavioural models in the present study. Because all individuals used in mRNA sequencing were also assayed for Na^+/K^+-ATPase activity ($N = 66$ for this analysis), correlations between Na^+/K^+-ATPase activity and mRNA transcript abundance were assessed with Bayesian models of activity dependent on transcript abundance, fit to Student's *t*-distributions, which are more robust to outliers than Gaussian distributions (Andrade, 2023). One model was run for each of 33 transcripts annotated to subunits of the Na^+/K^+-ATPase gene. Expected log pointwise predictive densities were calculated with leave one out cross-validation for each model with the R package loo v2.6.0 (Vehtari et al., 2017). These values were summarized to the level of overall Na^+/K^+-ATPase subunit, α or β, to estimate the predictive power of gene transcription on overall enzyme activity with respect to each subunit. The Na^+/K^+-ATPase α subunit contains the active site of ion exchange, while the β subunit plays a role in membrane binding and intracellular adhesion (Mobasheri et al., 2000; Vagin et al., 2006).

2.4 | Haematocrit

Haematocrit, the percentage of red blood cells in whole blood, was measured in lake sturgeon haphazardly netted and euthanized as described above. To collect blood, the tail was severed and blood was collected in haematocrit tubes, and centrifuged in a micro-haematocrit centrifuge (CritSpin) (Baker et al., 2008). Samples were $n = 8$ within each treatment and timepoint for the first day after the transient pCO_2 increase, $n = 10$ for all treatments and timepoints at 168 h after the transient increase, and $n = 12$ each at 720 h after the transient increase ($N = 184$ total; Table S3). A Bayesian approach with brms was also used for assessing haematocrit values among different experimental groups. The model formula used was haematocrit dependent on the random slope of experimental group within timepoint, with variance allowed to be unequal using the same random effect formula.

2.5 | Ammonia excretion

For ammonia excretion measurements, lake sturgeon were haphazardly sampled and placed in a 1 L Tupperware container with 400 mL of aerated water corresponding to the fish's respective treatment (pCO_2 and/or temperature). After a 60-min period to allow the fish to relax after transfer, two 1-mL water samples were collected at 0 and 1 h. Ammonia in the collected water samples was measured spectrophotometrically by the colorimetric salicylate-hypochlorite assay described in other work (Verdouw et al., 1978). Ammonia excretion rate was then calculated based on the change in ammonia concentration between the 0- and 1-h sample. Each treatment and timepoint had $n = 8$ fish in it, except for the combined pCO_2 and higher temperature treatment at time 0 with $n = 7$ fish ($N = 127$ total). Results were assessed with a Bayesian model, with ammonia excretion dependent on experimental group, and variance allowed to be unequal among experimental groups.

2.6 | Metabolic rate at 720 h post-pCO_2 exposure

Metabolic rate was indirectly assessed by measuring whole-body oxygen consumption rate with intermittent flow respirometry (Loligo Systems, Viborg, Denmark). The protocol of measuring metabolic rate was performed as previously described (Yoon

et al., 2021), but logistic constraints limited data collection to only the 720-h timepoint. The respirometry consisted of eight acrylic chambers (639.0 ± 15.5 mL; mean ± standard deviation.) and non-oxygen permeable tubing (63.3 ± 13.3 mL) submerged in a 250-L oval-shaped tank. Fish were fasted overnight and haphazardly captured prior to the measurement of metabolic rate. Black curtains were hung around the setup to minimize visual disturbance. Fish were left in the metabolic chambers for 24 h, after which fish were removed from the chambers and chased by gentle prodding to the tail for 15 min. Fish were immediately returned to the chambers and measured for two additional measurement cycles. Before and after each trial, oxygen consumption was measured without fish to estimate background respiration, and two data points were linearly interpolated overtime during the sampling period and all the metabolic rate data were corrected (Rodgers et al., 2016). Only slopes with $R^2 > .9$ were used for analysis. Routine metabolic rate (RMR) was calculated by averaging the metabolic rate data following the exclusion of the first 6 h of data as an acclimation. Maximum metabolic rate (MMR) was the highest observation following the chase protocol. Each measure of metabolic rate was recorded in units of mg $O_2 kg^{-1} h^{-1}$. The average ratio between body mass and chamber volume was 81.8 ± 26.1, and the average ratio between background respiration and metabolic rate was 0.17 ± 0.29. A total of $n = 8$ fish were used per treatment ($N = 32$ total). Models were used with either routine or maximum metabolic rate dependent on experimental group, a random intercept of date the measurement was taken, and variance was allowed to differ among experimental groups.

2.7 | Alarm Cue responses at 0 and 24 h post-pCO_2 exposure

Alarm cue response is an anti-predatory response that is triggered by injury of conspecifics, which is particularly important for young fish to avoid predation. Fish were individually exposed to an alarm cue in a double-flume experiment with laminar flow immediately after the simulated spring snowmelt experiment of exposure to 10,000 μatm of pCO_2 and 24 h post-exposure, in all four rearing groups (control, temperature, pCO_2 and pCO_2 combined with temperature). A minimum of $n = 15$ fish were used for each treatment, with $N = 132$ individuals used in total for this experiment ($n = 18$ for control and combined pCO_2 and increased temperature at 0 h, $n = 17$ for each of temperature and pCO_2 separately at 0 h, $n = 16$ for pCO_2 only and pCO_2 combined with increased temperature at 24 h and $n = 15$ for control and increased temperature at 24 h). Skin and muscle tissue from similarly aged euthanized lake sturgeon larvae (with 250 mg·L^{-1} MS-222, Syndel Laboratory, Vancouver, Canada) were used as an alarm cue at a concentration of 0.1 g tissue·mL water $^{-1}$, following a previously established protocol (Ou et al., 2015). Fish were recorded by Sony Hi8 CCD-TR700 camcorder (Oradell, New Jersey, USA) during the experiment. Proportion of time fish spent in the alarm cue was assessed in a blinded manner with experimental treatment and timepoint unknown to one assessor via randomized video identification numbers. At time 0 in each treatment, $n = 17$ (increased temperature, increased pCO_2) or $n = 18$ (control, combined temperature and pCO_2) fish were used. At 24 h post-exposure, $n = 15$ (control, increased temperature) or $n = 16$ (increased $pCO2$, combined temperature and pCO_2) fish were used ($N = 132$ total). To model the proportion of time that fish spent in the alarm cue, treatment was modelled as a random slope with timepoint as a random intercept, nested random intercepts of treatment tank ID within cue application side and the date of the experiment was performed as a random intercept. Variance was allowed to differ among a combined variable of treatment and timepoints.

2.8 | Boldness and activity in crossed rearing and trial groups

To assess the effects of rearing environment on behavioural responses to future increases in temperature, pCO_2, or both, an experiment with crossed rearing and trial exposures was performed with fish exposed individually to a novel stimulus between 50 and 53 days post-hatch. The novel stimulus was a plastic object previously unencountered by study subjects. Each cue of a control, alarm, food or alarm and food was added to a 9-L tank and mixed around the object as previously conducted (Ou et al., 2015). Alarm cues used in this protocol were identical to those used above (0.1 g of skin and muscle tissue·mL water $^{-1}$). Food cues were 0.1 g of bloodworm tissue·mL water $^{-1}$, a cue intended to simulate a feeding session for the fish. A single fish from one of the four rearing groups was then added to the thigmotaxis (outer) zone of the tank. The experimenter then left the testing area within 20 s of adding the cue. Recording proceeded for 5 min using a Hi8 CCD-TR700 camcorder (Sony), while data were only recorded and analysed from the middle 3 min of video to reduce disturbances or behavioural abnormalities at the beginning or end of each trial. Boldness was assessed in terms of the total time, proportion of time and activity near the novel object relative to the thigmotaxis zone further from the object. Time near the novel object, time in the thigmotaxis zone, total activity in any area of the tank, activity near the object and activity in the thigmotaxis zone were measured by the experimenter in blinded recordings via randomized video identification numbers to mitigate bias in recording data. Total distance moved was recorded as the number of total length changes in position multiplied by individual's total length in centimetres. After fish behaviour was recorded, the randomized video identification numbers were used to re-associate individual measurements to experimental treatments. In this experiment, $n = 64$ fish were used in all combinations of rearing and trial groups ($N = 640$ total). All behavioural models were run with the behaviour in question dependent on a combined variable of rearing and trial group modelled with a random slope with cue as a random intercept, treatment tank nested within rearing tank as a random intercept and the interaction of length and mass as a fixed effect. Variance was allowed to differ among groups, except in the model

of activity near the novel object. As with other models, 95% highest posterior density intervals were used to assess pairwise differences among combined rearing and trial groups with the R package emmeans v1.8.1–1 (Searle et al., 1980).

3 | RESULTS

3.1 | Transcriptomics

A multidimensional scaling analysis using pairwise distances representing the top 10% of transcripts (16,256) by leading $\log_2$-fold change between samples (i.e. the root mean-square average of the top 10% transcripts with largest $\log_2$ fold changes between two given samples) (Ritchie et al., 2015) revealed variation consistent with pCO_2 and temperature separately on dimension 1, and variation largely driven by time on dimension 2 (Figures S1 and S2). Pairwise contrasts among timepoints within treatments revealed the greatest number of differentially expressed transcripts between the elevated temperature-only treatment at 168 h versus 6 h (14,684 transcripts), and the fewest transcripts in the control group between 6 h versus 0 h (31 transcripts) (Table S4). After subtracting transcripts that had significant differential expression among any timepoints in the control group from those that had significant differential expression among timepoints in the other treatments, 13,033 transcripts remained in the pCO_2-only group, 13,243 remained in the temperature-only group and 13,943 remained in the combined pCO_2 and temperature group (Table S5). Within gene ontology terms specific to the treatment groups, microautophagy was prominent in the elevated temperature-only treatment (e.g. lysosomal microautophagy (GO:0016237), combined score (score) 1,669,824) (Table S6). This potential microautophagy was concurrent with RNA and protein regulation in other terms, such as in cytoplasmic translation (GO:0002181) with a score of 1645 and rRNA processing (GO:0006364) with a score of 795 (Table S6). Among the terms specific to the elevated pCO_2 treatment, plus-end-directed vesicle transport along microtubule (GO:0072383) was the term with the highest score of 902,620 (Table S7). Terms such as positive regulation of pinocytosis (GO:0048549) and caveola assembly (GO:0070836), with scores of 438 and 187, respectively, were also present (Table S7).

Negative regulation of organ growth (GO:0046621) had the highest score of 921,306 for the combined pCO_2 and warm temperature treatment (Table S8). This term was notable for its connections to decreases in growth in response to elevated CO_2 observed in other work (Ou et al., 2015). One gene in this term, SLC6A4, is sodium-dependent serotonin transporter that terminates the action of and recycles serotonin, thus regulating serotonergic signalling (Uniprot). Messenger RNA transcript abundance associated with *slc6a4* in the data (transcript ID TR49140|c2_g1_i4) decreased in the combined pCO_2 and temperature treatment, with limited differences in the other groups (Figure S3). The next highest term (regulation of organ growth; GO:0046620) had a score of 380 (Table S8). Ear development (GO:0043583) was the third-highest term present in the combined pCO_2 and temperature treatment with a score of 316, which was notable given potential connections of the stressors with otolith formation (Loeppky et al., 2021).

A prominent term shared among all three treatments was hepatobiliary system development (GO:0061008; minimum score 28.6 across the three experimental treatments) (Tables S6–S8). Other terms were related to protein modifications, such as cellular protein modification process (GO:0006464; minimum score 28.5) or gene expression, such as regulation of translation (GO:0006417; minimum score 13.4). Several less prominent shared terms were related to DNA repair and organization, such as chromatin organization (GO:0006325; minimum score 12.5) and DNA repair (GO:0006281; minimum score 9.2).

Temperature exhibited the most diffuse response in terms of network centrality, while pCO_2 and temperature showed a response more focused on relatively fewer central genes (Figure 2; Figure S4). Temperature also had the greatest number of transcripts unique to it among the three networks at 265, while 72 and 70 were unique to the combined pCO_2 and temperature treatment and pCO_2-only treatment respectively (Figure 2). Similar terms were present when analysing genes more central in the networks of all three treatments. For example, the term SRP-dependent co-translational protein targeting to membrane (GO:0006614) had the highest score in each of the isolated pCO_2 and temperature treatments (27,487 and 11,298 respectively), and the second highest score in the combined pCO_2 and temperature treatment (16,191) (Tables S9–S11). This term, and others with high combined scores in genes more central to networks among the three treatments, was characterized by the extensive presence of ribosomal protein genes (i.e. RPL and RPS genes; e.g. RPS7 in all three treatments).

However, less consistent processes were apparent when analysing ontology terms from genes on the edge of each network, which we reasoned may be more proximate to biological outcomes of regulatory pathways. Terms from the pCO_2 treatment were related to splicing (e.g. spliceosomal conformational changes to generate catalytic conformation; GO:0000393; score 1,715,914) or protein processing (e.g. proteasomal ubiquitin-independent protein catabolic process; GO:0010499; score 871) (Table S12). Meanwhile, genes on the edge of the temperature treatment network were characterized by metabolism (e.g. regulation of cellular amine metabolic process; GO:0033238; score 1438) or cell cycling (e.g. negative regulation of cell cycle G2/M phase transition; GO:1902750; score 1385) (Table S13). In the combined pCO_2 and temperature treatment, genes along the edges of the network were characterized by translation regulation (e.g. cytoplasmic translation; GO:0002181; score 851) or cell death-related processes (e.g. positive regulation of intrinsic apoptotic signalling pathway by p53 class mediator; GO:1902255; score 540) (Table S14). Therefore, a shared transcriptional response may have driven the networks initiated by all three experimental manipulations. However, pCO_2 responses may have led to alternative splicing and protein ubiquitination, temperature responses to cell cycling and metabolism and the combination of pCO_2 and temperature to protein synthesis and cell death.

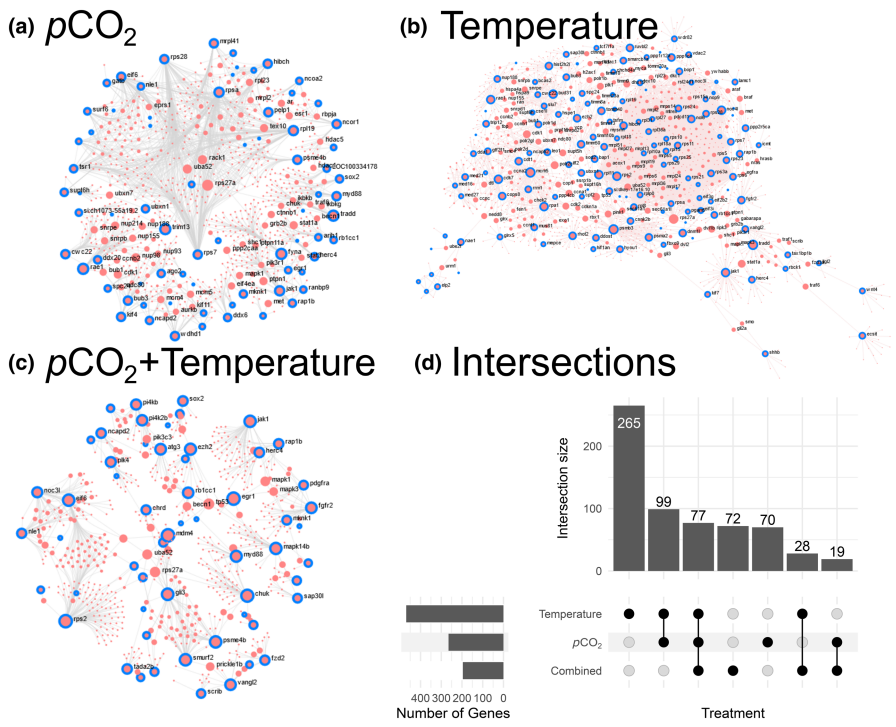

FIGURE 2 Transcriptional networks from genes with differential expression specific to each group following a simulated snowmelt and transient pCO_2 increase of 10,000 µatm. Gene IDs were analysed using OmicsNet 2.0 against the zebrafish (*Danio rerio*) protein interaction database. Panel (a) represents genes specific to fish reared in elevated pCO_2 only, panel (b) represents genes specific to fish reared in elevated temperature only and panel (c) represents genes specific to fish reared in both elevated pCO_2 and temperature. Panel (d) shows the intersection of genes shared and unique among the different networks, with shared genes indicated by lines connecting the treatments and unique genes indicated by single dots.

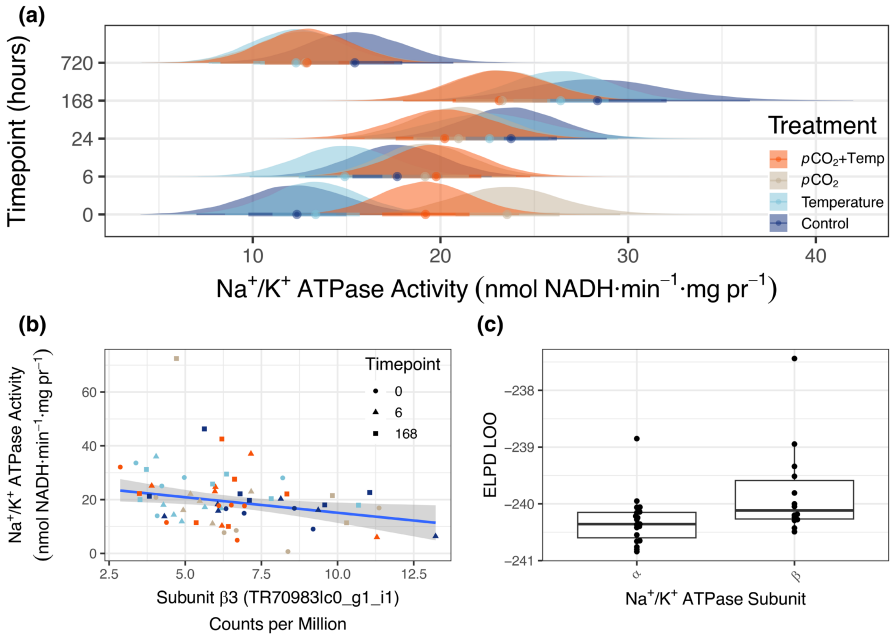

FIGURE 3 Posterior distributions, linear models, and expected log pointwise predictive densities calculated with leave one out cross validation (ELPD LOO) for Na^+/K^+-ATPase activity and associated transcript abundance. Panel (a) represents Bayesian posterior estimates of Na^+/K^+-ATPase activity calculated for each group, at each timepoint. Panel (b) represents the model of Na^+/K^+-ATPase activity dependent on Na^+/K^+-ATPase mRNA transcript abundance with the lowest ELPD LOO value and highest R^2 value out of 33 models of mRNA transcript abundance tested. Panel (c) shows ELPD LOO values for all 33 models of Na^+/K^+-ATPase mRNA transcript abundance correlated with enzyme activity, with Na^+/K^+-ATPase subunit annotations at the scale of the overall enzyme subunit (α or β) on the horizontal axis. Colours are consistent for treatment groups between panels (a and b).

3.2 | Physiology

Na^+/K^+-ATPase activity increased between 0 and 168 h in both the temperature and control groups, before returning to 0-h levels at 720 h post-acute pCO_2 exposure (Figure 3; Table S15). Na^+/K^+-ATPase activity did not notably change in the pCO_2 or combined pCO_2 and temperature groups. There was no apparent trend in haematocrit in the experimental treatments, except it was lower in the control group at 0 h than at 720 h (Figure S5; Table S16). Ammonia excretion was highest in the control group at 0 h, but increased in all groups at 720 h, especially

the pCO_2-only group (Figure S6; Table S17). At 720 h, routine metabolic rate was lower in the combined pCO_2 and temperature treatment than in the individual pCO_2 and temperature treatments, a condition potentially linked to respiratory alkalosis (Figure S7; Table S18) (Perry & Wood, 1989). Similarly, maximum metabolic rate was slightly higher in the pCO_2-only group relative to the combined pCO_2 and temperature group (Figure S7; Table S19).

We correlated the abundance of transcripts annotated to subunits of the gene Na^+/K^+-ATPase to enzyme activity. A total of 33 unique transcripts were annotated to subunits of the gene, with 19 annotated to the alpha subunit and 14 annotated to the beta subunit (Table S20). Bayesian models were used to correlate Na^+/K^+-ATPase activity to transcript abundance. By comparing expected log pointwise predictive densities across models, we found that mRNA abundance of beta subunits tended to have higher predictive power for enzyme activity than alpha subunits (Figure 3).

3.3 | Behaviour

Alarm cue responses at 0 and 24 h post-pCO_2 exposure did not differ among experimental groups, both within and between the 0-h and 24-h timepoints used (Figure S8; Table S21). No differences were observed among any rearing group or trial group combination, and no effect of cue was observed in time near the novel object (Figure S9; Table S22). However, the group reared in high temperature but trialled in control conditions spent the highest proportion of time in the thigmotaxis zone, while the group reared in elevated pCO_2 but trialled in control conditions trended towards less time in the thigmotaxis zone (Figure S10; Table S23). There was no effect of food or alarm cue on time in the thigmotaxis zone. Boldness and activity in crossed rearing and trial groups showed elevated temperature and the combination of temperature and pCO_2 exhibited greater total activity than the others, including overall, activity near the novel object and activity near the thigmotaxis zone (Figures S11–S13; Tables S24–S26). Meanwhile, the control and elevated pCO_2-reared fish trialled in elevated pCO_2 conditions trended towards less total activity (measured in number of total length changes in position) and total distance moved, especially when exposed to an alarm cue (Figure 4, Figure S13; Table S27). This decrease in distance moved was mitigated by elevated temperatures during trial conditions (Figure 4). There was little effect of alarm or food cue on total distance moved overall.

Overall, the cue delivered had limited or no effect on fish responses, while acute pCO_2 exposure may have decreased activity. This effect of decreased activity from pCO_2 exposure may have been masked by temperature, which likely increased activity, especially in the combined pCO_2 and temperature trial groups. Raw data from the behavioural experiments, along with length and mass for the boldness and activity in the crossed rearing and trial groups, are provided in Tables S28 and S29.

4 | DISCUSSION

The present results revealed that increased developmental temperature and pCO_2 independently as cumulative stressors influenced the responses to a transient 10,000 μatm pCO_2 exposure in a simulated spring snowmelt. These findings hold several implications

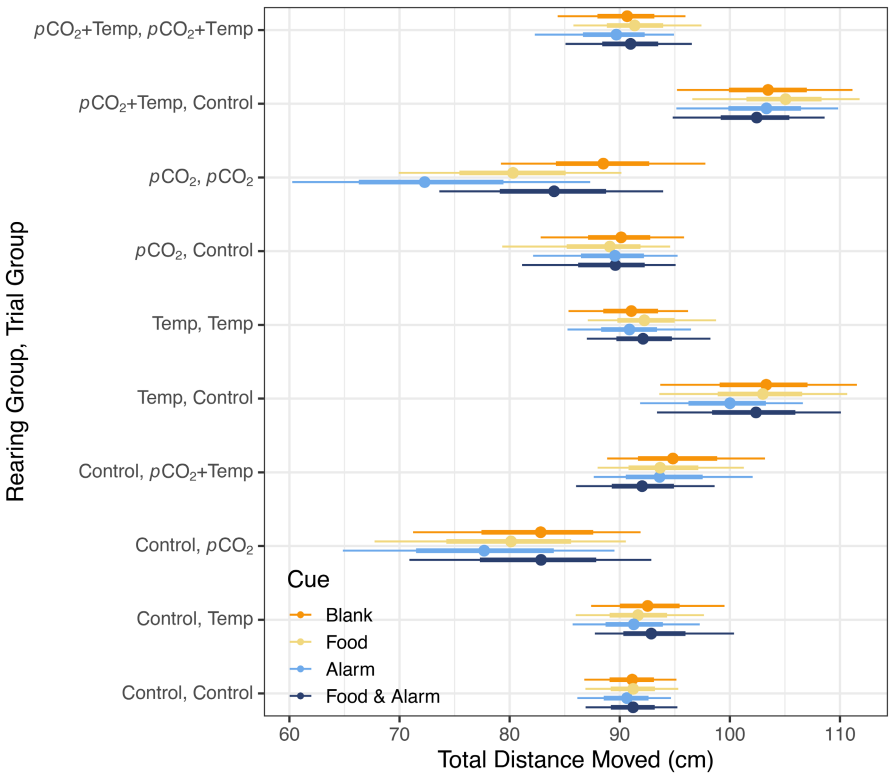

FIGURE 4 Credible intervals of distance moved (cm) in crossed rearing and trial groups with respect to cue. Thin lines represent 95% credible intervals, while bold lines represent 66% credible intervals. Recordings were done for 5 min in the trial group conditions, with the middle 3 min of video used for analysis.

for freshwater fishes responding to climate change. Behaviourally, activity decreased in response to acute pCO_2 exposure, regardless of rearing condition. Fish reared in elevated pCO_2 and temperature exhibited transcriptional changes consistent with a decrease in organ growth, a pattern associated with lowered growth in response to CO_2 in other freshwater species (Ou et al., 2015). One gene potentially involved in the decrease in organ growth, sodium-dependent serotonin transporter, was involved in blood flow regulation and oxygen chemoreception in gill neuroepithelial cells (Amador & McDonald, 2018; Porteus et al., 2012). In addition, mechanisms potentially consistent with a lowered routine metabolic rate were observed in the combined group. The transcriptional signal of a decrease in organ growth was consistent with the lower routine metabolic rate in fish reared in elevated pCO_2 and temperature compared to a control group, 720 h after the transient pCO_2 exposure. The present work demonstrates that physiological and transcriptional processes remain different for at least 7 days post-exposure to transient pCO_2 increase. Therefore, spring snowmelt with increasing atmospheric CO_2 may have more pronounced effects on fish in watersheds characterized by heavy flows from snowpacks, depending on local geochemistry (Schindler, 1988). Thus, future research on the effects of climate change on seasonal acclimation in aquatic freshwater organisms should integrate the effects of CO_2 and temperature over extended periods, as the factors together induce unique and prolonged physiological responses. Lastly, pCO_2 will need to be considered in conservation management as it can induce responses independent from temperature, despite the two being tightly linked environmental variables.

4.1 | Transcriptomics and physiology

The contrast between gene ontology terms specific to the warm treatment or the pCO_2 treatment separately highlights the different biological mechanisms likely active under each condition. For example, the microautophagy-dominated terms in the warm temperature treatment were notable in the context of micronucleophagy, where portions of the nucleus are degraded and recycled without killing a cell (Roberts et al., 2003; Wang et al., 2023). Autophagy generally has been implicated in cellular stress and hyperthermic responses in yeast and mammal cells (Kroemer et al., 2010; Zhang & Calderwood, 2011), and was identified as a potential mechanism in transcriptional network hubs in lake sturgeon responses to high-temperature exposures during development (Bugg et al., 2023). Therefore, microautophagy may play an important role in the fish exposed to elevated temperatures over extended periods of time. In conjunction with the terms related to translation, such as cytoplasmic translation, protein turnover may be a key mechanism for responding to temperatures elevated over the long term. Terms related to microtubule activity in the treatment with elevated pCO_2, along with pinocytosis regulation, vesicle transport along microtubules, and organelle transport along microtubules hint at a role for transport within the gills in response to CO_2. The observation that these mechanisms are not present in the combined warm and pCO_2 treatment shows that other regulatory processes may be favoured in response to cumulative stressors.

Temperature alone exhibited the transcriptional network with highest connectivity based on betweenness centrality scores, while the combination of both pCO_2 and temperature had the least connectivity. Shared regulatory mechanisms were at the centre of each network among the three treatments. One prominent set of shared regulatory mechanisms was in ribosomal protein genes, such as in the presence of RPS7 centrally in the networks of all three treatments. This finding is consistent with protein translation regulation in a stress response context, such as in Arctic charr (*Salvelinus alpinus*) (Quinn et al., 2011; Zencir et al., 2020). These data add to the broad literature on how gene transcription and protein translation regulation are characteristic elements of the vertebrate stress response (Thorstensen et al., 2022), by contextualizing these patterns more centrally in gene regulatory networks. However, differences in the outcomes of responses were apparent by analysing the edges of networks, which are likely related to mechanisms specific to each treatment. These transcriptional mechanisms included alternative splicing and protein ubiquitination in response to pCO_2 only, and cell cycling and metabolism changes in response to elevated rearing temperature. Signals of apoptosis and cell death from the combination of pCO_2 and temperature during rearing may not be a compensatory mechanism, but instead the outcome of cumulative stressors following the transient increase in pCO_2.

As a metabolic compensation strategy for acidosis, plasma HCO_3^- increases to support acid–base balance. As a result, increased availability of plasma HCO_3^- may lead to increased otolith growth (Heuer & Grosell, 2014). However, in a concurrent study using the same experimental design, otolith composition was affected by temperature but not pCO_2 (Loeppky et al., 2021), indicating robust compensatory mechanisms for acid–base regulation in this fish species. Similarly, both the control and temperature groups had increased Na^+/K^+-ATPase activity following the transient pCO_2 increase, but not the two groups reared in elevated pCO_2 conditions. This result suggests that compensatory mechanisms for responding to acute pCO_2 may have been present in the groups that developed in increased pCO_2, but not in the other individuals, highlighting the potential for developmental plasticity affecting later physiological responses. Based on models of transcript abundance and enzyme activity, β subunits tended to be most predictive of Na^+/K^+-ATPase enzyme activity, a result consistent with work in other systems (Mobasheri et al., 2000). Therefore, in the context of elevated temperature, Na^+/K^+-ATPase activity might be regulated by enzyme movement to cell membranes rather than differential production of the binding site, because of the β subunit's role in membrane binding (Vagin et al., 2006).

4.2 | Behaviour

Food and alarm cue responses did not differ among rearing or trial conditions, while total distance moved was lower in all groups

exposed to acute pCO_2. The reduction in activity in response to acute pCO_2 may be a result of anaesthetic effect of high CO_2 (Oberg et al., 2015). Interestingly, the total activity of control and pCO_2 rearing conditions were not different from each other following acute pCO_2 exposure. Therefore, the exposure to pCO_2 itself rather than developmental environment led to the observed differences. Elevated temperature could have mitigated the effects of pCO_2 on total distance moved despite reduced olfactory responses to acidification (Kimmel et al., 2023; Ou et al., 2015; Porteus et al., 2018; Velez et al., 2019). Therefore, periods of high transient pCO_2 but low temperature could influence the fish energy budgets, with the potential for negative impacts on foraging and growth (Boisclair & Sirois, 1993; Strobbe et al., 2011). Increased pCO_2 may have led to reductions in the olfactory sensitivity of lake sturgeon directly and by lowering freshwater pH (Velez et al., 2021), providing a potential link between total distance moved and olfactory sensitivity. Marine acidification likely has inconsistent effects on fish behaviour (Clark et al., 2020), and our results support those observations in lake sturgeon.

4.3 | Conclusions

Variability of pCO_2 in flowing freshwater over time can be more than an order of magnitude greater than in marine systems (Chan et al., 2017; Cole et al., 2007; Ko et al., 2021; Takeshita et al., 2015; Telmer & Veizer, 1999). As a consequence, researchers may expect robust adaptations to CO_2 fluctuations in freshwater organisms (Fleet et al., 2024; Quijada-Rodriguez et al., 2021; Tripp et al., 2022), but variability has nevertheless been shown to induce physiological responses in the lake sturgeon and other species (Ou et al., 2015). Lake sturgeon, like other sturgeons, exhibit exceptional phenotypic plasticity to various environmental stressors (Bugg et al., 2023; Haukenes et al., 2008; Penny et al., 2023). This high plasticity makes sturgeons valuable for studying responses to environmental change at multiple biological scales, while their status as an ancient fish enables analyses of potentially conserved traits (Thorstensen et al., 2023). Despite a broad geographic distribution, lake sturgeon have exhibited extensive phenotypic plasticity even in genetically distinct populations (Bugg et al., 2023; Yoon et al., 2023). Therefore, the present results are likely representative of lake sturgeon in other parts of their range, with potential limitations to applicability for more southern populations such as those in Mississippi (USA) (Moore et al., 2021).

Ultimately, we observed extensive transcriptional and physiological plasticity in response to ecologically relevant changes in pCO_2 and temperature, based on projections at the end of the 21st century (Anderson et al., 2016; Schwalm et al., 2020). As increasing temperature will lower the saturation point of CO_2 in water, concurrent increases of atmospheric and thus dissolved CO_2 may nevertheless lead to continued impacts from spring snowmelt and transient increases in pCO_2. Thus, the present results, analysing the effects of pCO_2 and temperature both in combination and isolation, will be relevant for many freshwater organisms in the near future. While other freshwater organisms may not have the capacity to marshal such a plastic response, elements of the cellular stress response are deeply conserved across all organisms (Horne et al., 2014; Kültz & Somero, 2020). Therefore, these may be critical response mechanisms for freshwater organisms faced with climate change.

AUTHOR CONTRIBUTIONS

L.D.B., A.R.Q.R., W.S.B., G.R.Y., A.R.L., D.W., K.M.J., and W.G.A. conceived of the study and designed its methodology. L.D.B., A.R.Q.R., W.S.B., G.R.Y., A.R.L., G.J.P.A., A.N.S., M.L.E., C.B., and J.L.A. contributed to animal rearing, performed experiments, and collected data. D.W., K.M.J., and W.G.A. provided resources, project administration, and supervision. M.L.E. created the conceptual diagram. M.J.T. curated data, conducted formal analyses, provided and implemented software, visualized results, and wrote the original draft of the manuscript with assistance from A.R.Q.R., W.S.B., G.R.Y., and W.G.A. All authors contributed to manuscript review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at [https://doi.org/10.6084/m9.figshare.24043269.v1].

DATA AVAILABILITY STATEMENT

Raw messenger RNA sequencing data are available at the NCBI Sequence Read Archive (SRA Accession: PRJNA953937). Raw behavioural data tables and representative video files are available on Figshare (https://doi.org/10.6084/m9.figshare.24043269.v1). All

bioinformatic and statistical codes used for the present analyses are available and annotated on GitHub (https://github.com/BioMatt/lkst_pCO2).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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