The impact of thermal stress on the gene expression, plasma lactate, osmolality, and growth of juvenile bull trout (*Salvelinus confluentus*)

by

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Abstract

Bull trout (*Salvelinus confluentus*) is a cold-water stenotherm that occupies a cold thermal niche relative to many freshwater fishes in North America. Consequently, populations at the southern range extent have been impacted by warming streams and many are listed as Threatened; however, it is unclear if populations further north will respond similarly. This study investigates the effects of thermal stress on gene expression, plasma lactate, osmolality, and growth in juvenile bull trout from a source population in Smith-Dorrien Creek, Alberta. One-year-old bull trout were acclimated to three ecologically-relevant temperatures (9°C, 15°C, and 21°C) for 60 days. Transcript abundance of genes involved in heat stress, growth-metabolism, immune response, and ion transport were measured as cellular stress indicators in gill and liver tissues using high-throughput qPCR, while plasma lactate and osmolality were assessed as physiological stress indicators. Osmolality and lactate decreased with temperature, suggesting compromised osmoregulatory function at elevated temperatures and an increased reliance on anaerobic metabolism. Condition factor and growth were highest at 9°C, with reduced condition despite continued growth at 15°C; fish held at 21°C exhibited markedly reduced growth, poorer body condition, and the highest mortality after 60 d. Sex had no significant effect on growth or physiological metrics. Gill and liver showed a decreased abundance of genes involved in growth-metabolism, ion transport, and immune function, while abundance of heat stress genes increased. The combined physiological and transcriptional evidence suggests that 15°C represents a sub-lethal

stress threshold, while 21°C approaches the upper functional thermal limit for this population.

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1. INTRODUCTION

Climate change is intensifying the frequency and duration of extreme heat events worldwide, pushing numerous species beyond their physiological limits (Welbergen et al. 2008; Hoegh-Guldberg and Bruno 2010; De La Fuente et al. 2025), and models project substantial reductions to biodiversity as increasing temperatures lead to a decrease in suitable habitat (Isaak et al. 2017; Sayer et al. 2025). Freshwater ecosystems are particularly vulnerable due to shifting precipitation patterns and high anthropogenic dependence for intensive agriculture, industrial development, and increasing urbanization (Schindler and Donahue 2006). While magnitude of disturbance varies among ecoregions and between species, reduced ice cover and albedo effects are predicted to elevate water temperatures in northern, high altitude freshwater systems (Hansen et al. 2006; Råman Vinnå et al. 2021), leading to cumulative downstream effects on water temperature and quality (Gooseff et al. 2005; Poesch et al. 2016). Ectotherms, such as freshwater fishes, experience disproportionate impacts from climate warming as they retain little metabolic heat and thus rely on behaviour to regulate their internal body temperature with the external environment (Brett 1956). Inability to behaviourally thermoregulate or physiologically adjust to thermal stress may lead to long-term fitness reductions that can result in population-level effects (Schulte 2014). Additionally, warming often compounds with existing anthropogenic pressures, such as habitat fragmentation, invasive species, and overexploitation, to further augment the effects of thermal stress (Dudgeon et al. 2006).

Fishes in the Salmonidae family (hereafter salmonids; including salmon, trout, whitefish, and char) are key indicators of climate change-mediated impacts (Isaak et al. 2015), as many are cold-adapted species whose physiology, distribution, and survival are often constrained by temperature (Wehrly et al. 2007), in addition to their substantial cultural and economic importance (Noble et al. 2016; Carothers et al. 2021). Many salmonids are increasingly at risk from climate warming due to relatively narrow thermal tolerance across broad ranges of habitat and life-stages (Zillig et al. 2021), with relatively limited acclimatization potential compared to thermal generalists (Beitinger and Bennett 2000; Somero 2010). Eurythermal fishes like sea lamprey (*Petromyzon marinus* Linnaeus, 1758) can tolerate a wider range of temperatures (e.g., 0 to 31°C , Potter and Beamish 1975; Hansen et al. 2016) in contrast to stenotherms, which occupy less variable environments (e.g., Arctic nototheniids -2 to 6°C, Somero and DeVries 1967). Stenothermal salmonids, like bull trout (*Salvelinus confluentus* Suckley, 1859), occupy a cold thermal niche and are generally most abundant in streams with mean summer temperatures between 6-11°C, relative to other cold-water species (Mochnacz et al. 2023) such as brook trout (*Salvelinus fontinalis* Mitchill, 1814) that tend to occupy streams between 12-17°C (Isaak et al. 2017).

Bull trout inhabit streams and rivers across montane watersheds with relatively stable thermal regimes (i.e., low seasonal variation) and many southern populations are considered to be constrained by warm-edge boundaries (Isaak et al. 2015; Mochnacz et al. 2023). Their preference for cold, clean, structurally complex, and well-connected stream habitats across all life stages (Mochnacz et al. 2013) makes them highly

vulnerable to anthropogenic disturbances, including climate-driven warming, and many southern populations are listed as extirpated, vulnerable, or threatened due to substantial declines (USFWS 1998; COSEWIC 2012). As obligate cold-water spawners, dispersal and recruitment are strongly constrained by climate and limited access to natal headwater streams in the spring and fall due to low summer streamflow (Rieman and Dunham 2000); these limitations are exacerbated by late maturation and infrequent (often non-consecutive) annual spawning (McPhail and Baxter 1996). A recent study by Mochnacz et al. (2025) suggests that the distribution of northern populations may be constrained by cold-edge boundaries, whereby populations occupy very narrow thermal niches and thermal variability in these habitats is mediated by perennial groundwater, with climate responses strongly dependent on adaptive capacity. Spatially explicit stream-temperature modeling further highlights the potential for widespread reduction of thermally suitable habitat under warming scenarios into the 2080s (Rieman et al. 2007; Isaak et al. 2015). To that end, the integration of distribution modelling with transcriptional profiling may be an important tool for advancing conservation management of bull trout given the limitations to lethal sampling in the wild (Lazaro-Côté et al. 2025).

Transcriptional analysis can provide insight into the mechanistic drivers of environmental stress by tying changes at the molecular level to broader physiological responses (Connon et al. 2018). Changes in gene expression can identify sub-lethal thresholds beyond which recovery is possible but continued homeostatic maintenance represents a significant challenge to long-term fitness (Schulte 2014; Jeffries et al. 2018).

While sub-lethal conditions may not cause immediate mortality, increased metabolic demand to manage thermal stress at the cellular level suggests a reduced ability to tolerate additional stress and may further exacerbate population declines in species living close to their thermal limits (Jeffries et al. 2016). Assessing gene expression in metabolically active tissues such as gill and liver can shed light on cellular stress responses in fishes, as these tissues integrate external conditions with internal metabolic regulation (Emam et al. 2022). Gills are multi-functional respiratory organs composed of complex vasculature and high-surface area epithelium that are primarily responsible for gas exchange, in addition to regulation of ion balance, body fluid pH, immune response, and nitrogenous waste excretion (Evans et al. 2005). As gill filaments maintain continuous contact with the external environment, gill tissue is sensitive to acute stressors that necessitate rapid transcriptional adjustments, such as the production of molecular chaperones to prevent protein misfolding and maintain ionoregulatory function with acute thermal stress (Buckley et al. 2006). Altered gill function can therefore impose downstream energetic and metabolic demands that are integrated by the liver, which functions as a central metabolic hub that coordinates energy storage, endocrine signalling and nutrient allocation in support of growth and maintenance (Bruslé and Gonzàlez i Anadon 2017).

Temperature is known to influence various physiological process in fishes, including development, growth rates, and neuroendocrine pathways involved in reproduction (Alix et al. 2020; Lindmark et al. 2022). Previous bull trout growth studies reported a significant decline in growth and feed intake above 16°C, in addition to 100% mortality at

acclimation temperatures above 22°C (24, 25 and 28°C) after 60 days (Selong et al. 2001). Transcriptional analysis in young-of-the-year (YOY) bull trout across a broad range of acclimation temperatures (6, 9, 12, 15, 18 and 21°C) found that the transcript abundance of genes associated with growth and lipid oxidation metabolism decreased significantly and concurrently with growth at temperatures above 15°C (Best et al. 2025). Growth correlates positively with fecundity in fishes (Tsoukali et al. 2016) and larger females produce larger alevins with more yolk that are more likely to survive due to competitive advantages (Beacham and Murray 1985; Moffett et al. 2006). Additionally, production of testosterone (T) and 17β-estradiol (E_2) sex hormones necessary for spawning are influenced by temperature (Jeffries et al. 2012a), and thermal stress can inhibit gametogenesis by reducing the circulation of E_2 and T, dampening the synthesis and sequestration of hepatic vitellogenin necessary for oocyte yolk formation (King et al. 2003; Pankhurst and King 2010). Therefore, temperature-induced challenges to growth and reproduction suggest the potential for reduced recruitment and may be driving declines in southern bull trout populations already experiencing thermal stress (Rieman et al. 2007; Eby et al. 2014). Linking these physiological constraints with patterns of habitat availability is therefore essential for identifying and protecting cold-water habitat critical to the species' persistence.

Osmolality and lactate are common indicators of a secondary stress response in fishes as they reflect downstream physiological consequences of stress, including disruptions to ion balance and shifts in energy metabolism that arise as organisms attempt to maintain internal stability under challenging environmental conditions

(Kieffer et al. 1994; Wendelaar Bonga 1997). Osmolality reflects the balance between ion and water flux across a permeable epithelia, and thermal stress can disrupt this balance by altering gill permeability, ion transporter activity, and cardiac output, thereby increasing the energetic cost of osmoregulation and compounding metabolic stress (Evans et al. 2005). Concurrently, warming increases metabolic oxygen demand while reducing oxygen solubility in water, constraining aerobic scope and promoting lactate accumulation as cardiorespiratory capacity becomes limiting near upper thermal limits (Farrell et al. 2009; Eliason et al. 2011). Elevated or prolonged lactate levels are associated with delayed recovery from stress, reduced swimming performance, and impaired growth, and often reflect energetic trade-offs between maintaining homeostasis and sustaining aerobic metabolism (Jeffries et al. 2012b; Eliason et al. 2020). Additionally, many salmonids exhibit sex-specific physiological responses to stress, such as prolonged recovery from elevated lactate and cortisol (Eliason et al. 2020) and increased mortality when exposed to thermal stress (Jeffries et al. 2012a). Different responses to acute and chronic thermal stress indicate divergent physiological demands that may translate into sex-biased performance limits and survival (Minke-Martin et al. 2018).

1.1 Research objectives & hypotheses

This study contributes to the characterization of molecular and physiological responses to thermal stress in bull trout, with potential implications for conservation management. Although thermal limits exhibit some plasticity with acclimation in fishes

(Schulte et al. 2011), the decline of acclimation response ratios with temperature in juvenile bull trout suggests limited acclimation capacity and tolerance beyond 18°C (Best et al. 2025). These patterns reflect the broad-scale distribution and occupancy patterns of bull trout in the wild, where mean summer temperatures above 12°C are inversely linked with occurrence (Isaak et al. 2015; Mochnacz et al. 2023). However, little data exists for the transcriptional and physiological thermal stress response in bull trout beyond these life stages.

To redress this gap, patterns of transcript abundance in liver and gill tissues were integrated with morphological (length, weight) and physiological (lactate, osmolality) data to provide insight on whole-organism changes in response to temperature in a population of juvenile bull trout from the central range of their distribution. Response and sensitivity to thermal stress varies across life-stages (Del Rio et al. 2021) and exposure to stressful temperatures can influence thermal plasticity in adulthood (Penney et al. 2022). Growth is also of concern due to potential implications for reduced reproductive success and offspring survival with warming temperatures (Beacham and Murray 1985; Tsoukali et al. 2016). Consequently, establishing which life-stages are most resilient to thermal stress is crucial to understanding population-level vulnerabilities to warming. Identifying cellular biomarkers that are diagnostic of stress, such as the differential regulation of heat-shock proteins in response to thermal stress (Houde et al. 2019; Semeniuk et al. 2022) can then be applied to broader population and ecological function (Semeniuk et al. 2022) and thus used to identify critical habitats for protection, recovery, or provide rationale for management policy, such as restricted fishing efforts

where temperatures exceed thermal stress thresholds (Macdonald et al. 2010; Jeffries et al. 2016; Connon et al. 2018).

I set out to test the following hypotheses:

H_0: Higher temperatures do not alter sex-specific responses, whole-body metrics, transcript abundance of growth genes, and blood plasma concentrations relative to cooler temperatures in bull trout.

H_1: Higher temperatures do alter sex-specific responses, whole-body metrics, transcript abundance of growth genes, and blood plasma concentrations relative to cooler temperatures in bull trout.

P_1: Bull trout acclimated to higher temperatures will have lower transcript abundance of growth genes than bull trout acclimated to lower temperatures as growth transcript abundance is inversely related to temperature in bull trout.

P_2: Bull trout acclimated to higher temperatures will have lower condition factor and growth than bull trout acclimated to lower temperatures because growth declines with temperature.

P_3: Bull trout acclimated to higher temperatures will have higher blood plasma lactate and osmolality than bull trout acclimated to lower temperatures reflecting a secondary stress response to temperature.

P_4: Bull trout females will have higher blood plasma lactate and osmolality than bull trout males due to sex-specific stress responses common to salmonids because female salmonids exhibit higher lactate concentrations due to thermal stress.

2. METHODS & MATERIALS

2.1 Animal husbandry

The initial study occurred in association with Fisheries and Oceans Canada (DFO) at the Freshwater Institute (FWI) in Winnipeg, Manitoba, Canada. Milt and eggs were collected on 15 September 2021 from three male and three female bull trout in Smith-Dorrien Creek, Alberta. Gametes were placed in a cooler with ice and shipped in a pressurized compartment by airplane (~2h) to the FWI. Eggs were fertilized at the FWI on 16 September 2021 (after an 18–22h delay from harvest) using a ratio of 200 mL eggs for each female to 100 µL milt cocktail mix from two males (Kissinger et al. 2025). Milt from a third male was omitted due to concerns regarding container integrity (Kissinger et al. 2025). Gametes from an additional three males and three females were fertilized *in situ* at Smith-Dorrien on 16 September 2021 and then shipped to the FWI, where they were received on the same day ~10–11h post-harvest and fertilization (Kissinger et al. 2025).

Embryos separated by treatment were placed in Heath trays within a vertical-flow stack incubator (Legend Brands Inc., Burlington, WA, USA) held at 4°C and warmed to 5°C during hatch until 23 February 2022, where temperature then increased 1°C/day up to 8°C. Alevins remained in trays until 15 March 2022 where they were transferred to tanks before swim-up in April-May 2022. Following swim-up, fish were moved to 100L tanks with a flow-through system of de-chlorinated water and independent aeration for long-term holding, and temperatures were then increased gradually to 10°C ± 1°C (Kissinger et al. 2025). No significant differences in mortality and survival were detected

between fertilization treatment groups, and the two populations were subsequently combined after 23 August 2022 (Kissinger et al. 2025). Maintenance followed a 12:12h photoperiod (65 min dawn/dusk, full light at 0705, and full dark at 1905) and a daily feed ratio of 0.5% average tank body mass of commercial pellets (#2 crumble EWOS Pacific: Complete Fish Feed for Salmonids, Cargill, Winnipeg, MB, CA).

Animal care, handling, collection and experimental protocols were approved and performed within guidelines set by the Fisheries and Oceans Canada Ontario & Prairies Animal Care Committee (OPA-ACC-2023-32), and in accordance with the standards set by the Canadian Council for Animal Care.

2.2 Experimental design and tissue sampling

A random selection of ~1 year old bull trout were acclimated to three temperatures (9°C, 15°C, and 21°C) in increments of 2°C per day using a TempReg device (Loligo® Systems, Tjele, DK). Temperatures were selected from an ecologically-relevant range already encountered by bull trout in the wild (9°C and 15°C) in addition to the species' known upper thermal tolerance (21°C) (Selong et al. 2001; Best et al. 2025), beyond the preferential range where core populations typically occur (Isaak et al. 2015; Mochnacz et al. 2023). Replicates were grouped into three blocks (A, B and C) of three tanks (one per temperature), and each tank contained ~20-22 fish. Acclimation started on 12 April 2023 and occurred over eight days in 570L tanks with a de-chlorinated flow-through system and independent temperature control via heaters or chillers. Tank conditions (e.g. temperature, conductivity, and dissolved oxygen percent and concentration in mg L^{-1}),

health, mortality, and fish behaviour (e.g. activity, spatial distribution, and feeding response) were recorded daily. Oxygen saturation was maintained above 80% with independent aeration via bubblers in each tank.

Feeding began 21 Apr 2023 after measuring length and weight of all fish on 20 April (day 0) until terminal sampling after 60 days on 19 June 2023 at the FWI. All tanks were fed to satiation daily at 30 min intervals (n = 6) across a 3 h period, with feed amount (g) per interval fluctuating by decreased or increased appetite (i.e., presence or absence of pellets in the tank). After 3 h, all waste and uneaten pellets were collected from each tank, and wet and dry mass (g) were collected to calculate feed consumption and efficiency.

After 60 d, fish were euthanized within a bath of 300 mg L^{-1} tricaine methanesulfonate (MS-222) buffered with 600 mg L^{-1} sodium bicarbonate, for 10 min. Blood was extracted from the caudal peduncle via capillary tubes, collected in vials, and iced for 5 min before centrifugal fractioning of the plasma, buffy coat, and erythrocytes. Isolated plasma was pipetted into separate vials for all sampled individuals and then frozen at -80°C for later analysis of plasma parameters. Gill and liver tissue samples were collected (n = 19 for 9°C; n = 18 for 15 and 21°C) from the left second gill arch and left lobe, respectively. Tissue samples were placed in 1 mL of RNA*later*™ (Invitrogen™, Carlsbad, CA, USA) at 4°C for 24hr before freezing at -80°C for future transcriptional analysis.

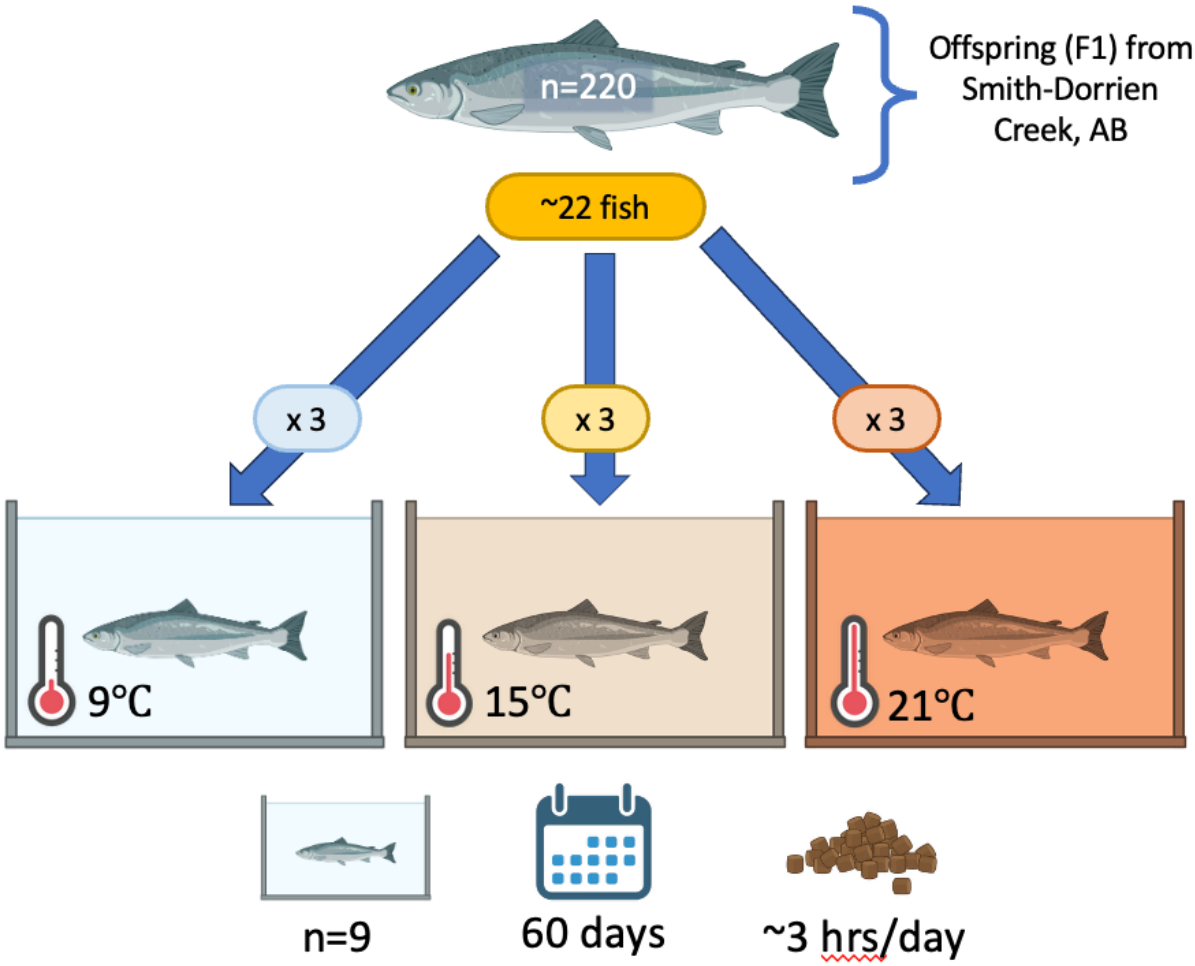

Figure 1. A visual representation of the growth study design. A total of 220 juvenile bull trout (*S. confluentus*) were distributed across temperature treatments, 9°C, 15°C and 21°C. Approximately 22 *S. confluentus* were assigned to each tank, with three replicate tanks per treatment (tank n = 9). All tanks were fed to satiation over a 3 h period for 60 days. Figure created using BioRender assets.

2.3 Measurement of blood plasma parameters

Plasma lactate concentrations (ng μL^{-1}) were determined with an enzymatic assay kit (Sigma-Aldrich L-Lactate Assay Kit, Cat no. MAK570; Merck KGaA, Darmstadt, DE). Plasma samples (15 µL, n = 53) were individually deproteinated with a 6% perchloric acid solution (7.5 µL) to remove lactate dehydrogenase, centrifuged for 5 mins at 13.4 rpm for separation of the precipitate and deproteinated supernatant, and then neutralized by 6 µL of 3M potassium bicarbonate ($KHCO_3$). Neutral deproteinized samples (6 µL) were diluted 1:20 with an assay buffer (114 µL) provided in the kit. Sample dilutions (50 µL) were added in duplicate to a clear 96-well plate before the addition of 50 µL reaction mix containing assay buffer, enzymes, and probes (Sigma-Aldritch L-Lactate Assay Kit, Cat no. MAK570). Reactions occurred at room temperature for 30 min before absorbance was measured (570 nm) by a microplate spectrophotometer (BioTek Powerwave; BioTek, Winooski, VT, USA) with Gen5 software (Biotek). Sample lactate concentrations were determined against a standard curve of known lactate concentration using a synthesized lactate molecule (lactic acid sodium sulfate). Osmolality (mmol kg^{-1}) was measured in 10 µL of sample (n = 54) with a VAPRO vapour pressure osmometer (model 5520; Wescor, Inc., Logan, UT, USA). Samples with an insufficient plasma volume were omitted (n = 2 for lactate and 1 for osmolality, respectively, all from 9°C tanks; see Appendix A, Table 2 for raw data).

2.4 Measurement of whole-body metrics for growth and survival

Whole-body measurements of all individuals were collected at experiment day 0 (20 April 2023), in 13-18 d intervals, and before terminal sampling. Feeding stopped 24 h before individuals were placed in an anesthetic bath of buffered MS-222 to collect mass (g) and fork length (mm, from snout tip to centre of the caudal fin) for morphometric assessment of growth. Tanks were monitored daily for mortalities at the start of acclimation (12 April 2023) and until experiment end, and all mortalities were recorded by individual tank. Fish that did not pass a scored visual assessment for health (e.g., external body condition factors such as presence of eye bulging, spinal deformation, emaciation, scale erosion, fin condition, etc., ranked in severity from 0–3) were euthanized with buffered MS-222 according to body weight and considered a mortality.

Relative daily growth (%) was calculated with the formula $(W_2 – W_1) / (Y_1 – t) * 100\%$, where W_2 and W_1 represent the final and initial average weight per treatment (at a given time point), respectively, and t is the number of days (Selong et al. 2001). Fulton's condition factor (K) can be used to assess fish health through the relationship between individual mass and length, where a higher value indicates greater mass relative to length and suggests a better overall condition. Condition factor was calculated using $K = 100 * W / L^3$, where W represents individual mass and L represents fork length.

2.5 Sex determination

Following the protocols of Webster et al. (2021), individuals were genotypically sexed with a qPCR protocol using a highly conserved salmonid master-sex determining gene sdY (Yano et al. 2013), in addition to a reference gene (18s, see Amish et al. 2022)

to rule out amplification error. For qPCR, genomic DNA was extracted from gill tissue and purified using a QIAGEN DNeasy Blood & Tissue Kit according to manufacturer protocols (QIAGEN, Hilden, DE). Genomic DNA from a different cohort of experimental bull trout that were visually sexed after dissection were used to determine the primer efficacy; qPCR assays had an 80% success rate for identifying an individual's phenotypic sex. Presence of a melt curve and fluorescence of qPCR product in agarose gel electrophoresis were used to identify genetic males (Appendix B, Fig. 6 and 7), as both indicate amplification of the sdY gene (Anglès d'Auriac et al. 2014).

2.6 Measurement of transcript abundance

Total RNA from the left second gill arch and the left liver lobe tissue samples was extracted and purified using a QIAGEN RNeasy Plus Mini Kit according to manufacturer protocols (QIAGEN). Assessment of RNA quality and quantity were conducted with a NanoDrop One Spectrophotometer (Thermo Fisher Scientific) to measure RNA purity (with 260:280 and 260:230 ratios above 1.9), and agarose gel electrophoresis to visually assess RNA strand integrity (inspection of 18S and 28S rRNA bands), respectively. Tissue RNA extractions were normalized to 1 µg μL^{-1} for conversion to cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Waltham, MA, USA) after removal of genomic DNA (ezDNase, Invitrogen) according to manufacturer protocols, and then stored in -20°C until qPCR analysis.

Gene expression was quantified from cDNA amplification on a custom OpenArray high-throughput qPCR chip (Thermo Fisher Scientific) containing 56 salmonid genes for

23 individual samples and a no-template control (NTC) run in duplicate, totaling 48 subarrays and 2688 TaqMan® PCR reactions on one chip. Target genes were selected according to involvement in general fish health and thermal stress (Islam et al. 2026), in addition to four reference genes. Samples were selected for chip loading according to treatment temperature (n = 8 for 15°C, 21°C and n = 7 for 9°C) for each tissue (gill and liver) and further split by sdY amplification for sex-specific comparison with an equal sex ratio (i.e., n = 4 males and females per treatment). However, only 2 males were identified among the 9°C tank populations which resulted in an imbalanced sex ratio (n = 2 males and 5 females).

Samples were first assembled in a 384-well plate, with each well containing 5 µL reactions consisting of 2.5 µL 2x OpenArray master mix (Thermo Fisher Scientific) and 2.5 µL 5x diluted cDNA (1:5 ratio of Nuclease-Free H_2O to cDNA). Reactions were then loaded onto the custom OpenArray plate by the OpenArray Acufill System (Thermo Fisher Scientific) with each 5 µL reaction corresponding to one sub-array containing all 56 assays. Plates were filled with immersion oil after lid placement and then sealed within ~90 sec of loading to prevent evaporation, according to manufacturer protocol. Plates were loaded separately by tissue type and then run on the QuantStudio™ 12K Flex qPCR System (Thermo Fisher Scientific). QuantStudio Design and Analysis v1.5.2. and ExpressionSuite software v1.3 (Thermo Fisher Scientific) generated raw amplification data for quality screening.

2.7 Data and statistical analyses

All analyses and figures were created using Rstudio v. 4.5.2 and plotted using *ggplot2* (Wickham 2016; R Core Team 2025). For blood plasma variables (osmolality and lactate) and condition factor, statistical differences between sexes and temperature treatments were assessed using a two-factor ANOVA with temperature and sex as factors. For relative daily growth, statistical differences across temperatures over time were assessed using a two-factor ANOVA with date and temperature as factors. All data were assessed for normality and homogeneity of variances with a Shapiro-Wilk test and Levene's test, respectively, using base R and the *car* package (Fox and Weisberg 2019; R Core Team 2025); lactate values were log-transformed to meet normality assumptions and are presented as back-transformed estimated marginal means (EMMs) ± SE (standard error). Post-hoc comparisons of EMMs were assessed using the *emmeans* package in R (Lenth and Piaskowski 2017). Pairwise differences among treatment groups were evaluated using Tukey's Honestly Significant Difference (HSD) adjustment to control the family-wise error rate for multiple comparisons. Adjusted p-values < 0.05 were considered statistically significant.

Mean amplification efficiencies for 56 genes in each tissue were assessed using LinRegPCR (Appendix A, Table 3) (Ramakers et al. 2003). The qPCR efficiency scores describe the exponential increase in target DNA per amplification cycle and are estimated from the slope of a standard curve relating quantification cycle (Cq) values (where TaqMan® reporter fluorescence crosses the detection threshold) to the log of starting template concentration. These scores reflect the effectiveness of primer amplification for each gene and are inversely related to abundance. Efficiencies below

70% indicate poor or inconsistent amplification, while efficiencies above 130% likely reflect technical artifacts rather than true amplification; a broader efficiency tolerance was applied given the variation in amplification efficiency across multiple genes and between tissue types, especially for low-expression or tissue-specific target genes. Genes outside of the efficiency threshold range (<70% and >130%) were removed from subsequent statistical analyses. Further quality control checks removed NTCs and samples with >50% amplification fail, amplification threshold <1.24, Cq confidence threshold <0.8, or a standard deviation >0.7. Omissions in the liver include *casp9*, *hk1*, *il1β*, *il8*, *ldha*, *ldhb*, *lepr*, *slc2a1a*, and *vegfc*; in the gill, *pck1* (see Appendix A, Table 2 for a list of gene names and descriptions).

For abundance analyses, raw Crt values were converted to counts using *cq2counts* and analyzed with Poisson-lognormal generalized linear mixed models in a Bayesian framework using *MCMC.qpcr* (Matz 2013). Models were run for 13,000 iterations, where the first 3,000 iterations were discarded, and parameters thinned by retaining every 10th iteration. Each model included gene, temperature, and sex, in addition to their interaction as categorical fixed effects, and sample ID was included as a random effect to account for technical replicates (Matz et al. 2013). Presence of global effects and reference gene stability (*ef1a*, *rpl7*, *rpl13a*, *rps9*) were assessed by fitting naïve models. Selection of endogenous controls requires consistent amplification with low standard deviation across all temperature treatments to ensure unbiased, consistent normalization between groups; *ef1a* was subsequently removed as a control due to high variability across each temperature treatment (see Appendix B, Fig. 8). Confirmed stable

reference genes were used as priors to create a second informed model and diagnostic plots were generated with *diagnostic.mcmc* to visually assess model fit.

Principal component analysis (PCA) plots were generated from *MCMC.qpcr* converted count data using the package *FactoMinR* (Lê et al. 2008) with code adapted from Best et al. (2025). Loadings (e.g., top contributing genes for each PCA) were generated by dividing variable coordinates by the eigenvalue's square root for each dimension. Pairwise differences by sex and temperature for each gene were generated using the *HPDsummary* function in *MCMC.qpcr* (Matz 2013) and plotted as 95% credible intervals according to significance (geneWise $p < 0.05$).

3. RESULTS

3.1 Plasma parameters

Osmolality varied with acclimation temperature, whereas sex differences were statistically negligible (Fig. 2A). Significant differences in osmolality were detected between temperature groups ($F_{2, 48} = 4.18$, $p < 0.05$), while no significant effect of sex ($F_{1, 48} = 1.40$, $p > 0.05$) or the interaction of sex and temperature ($F_{2, 48} = 0.76$, $p > 0.05$) were observed. Post-hoc comparisons indicated osmolality differed moderately between 9°C and 15°C ($p = 0.035$) and strongly between 9°C and 21°C ($p = 0.0015$). Although the overall effect of sex was not significant among all other groups, a pairwise comparison indicated osmolality in males at 21°C differed moderately from females at 9°C (p = 0.049). Osmolality tended to be highest at 9°C (335.7 ± 6.8 mmol kg^{-1} mean ± SE), with median values declining slightly at 15°C (317.4 ± 4.9 mmol kg^{-1}) and again at 21°C (305.7

± 3.9 mmol kg^{-1}). At 9 °C, males tended to exhibit higher osmolality than females (354.0 ± 23 mmol kg^{-1} to 333.4 ± 7.2 mmol kg^{-1} respectively), whereas both sexes showed similar values with overlapping distributions at 15°C (316.8 ± 8.3 mmol kg^{-1} in females and 318.0 ± 5.8 mmol kg^{-1} in males) and 21°C (307.3 ± 6.3 mmol kg^{-1} in females and 303.6 ± 4.1 mmol kg^{-1} in males).

Acclimation temperature influenced lactate with negligible sex differences within and across treatments (Fig. 2B). Differences were identified across temperature groups ($F_{2, 47}$ = 9.14, $p < 0.001$) with no significant differences between sexes ($F_{1, 47}$ = 0.019, $p > 0.05$) and their interaction ($F_{2, 47}$ = 0.21, $p > 0.05$). Post-hoc comparisons identified differences in 21°C relative to 9°C and 15°C (p = 0.003 and 0.0001, respectively). While sex was similarly not significant in all groups, pairwise comparisons indicated significant differences in lactate among 9°C females and 21°C males (p = 0.004), and in 15°C females and 21°C males (p = 0.004). Fish held at 15 °C exhibited the highest lactate concentrations (537.2 ± 24.5 ng μL^{-1}), whereas those at 9 °C had moderately elevated but more variable levels (447.8 ± 31.5 ng μL^{-1}), and fish at 21 °C showed the lowest overall concentrations (316.6 ± 26.5 ng μL^{-1}). Females consistently displayed higher concentrations than males at 9 °C (460.78 ± 31.5 ng μL^{-1} to 356.6 ± 139.1 ng μL^{-1}, respectively) and 15 °C (547.4 ± 32.0 ng μL^{-1} to 525.8 ± 39.7 ng μL^{-1}, respectively). However, at 21 °C this pattern weakened, as male and female lactate concentrations converged (292.5 ± 38.8 ng μL^{-1} and 335.9 ± 35.0 ng μL^{-1}, respectively) with broad variability in both groups.

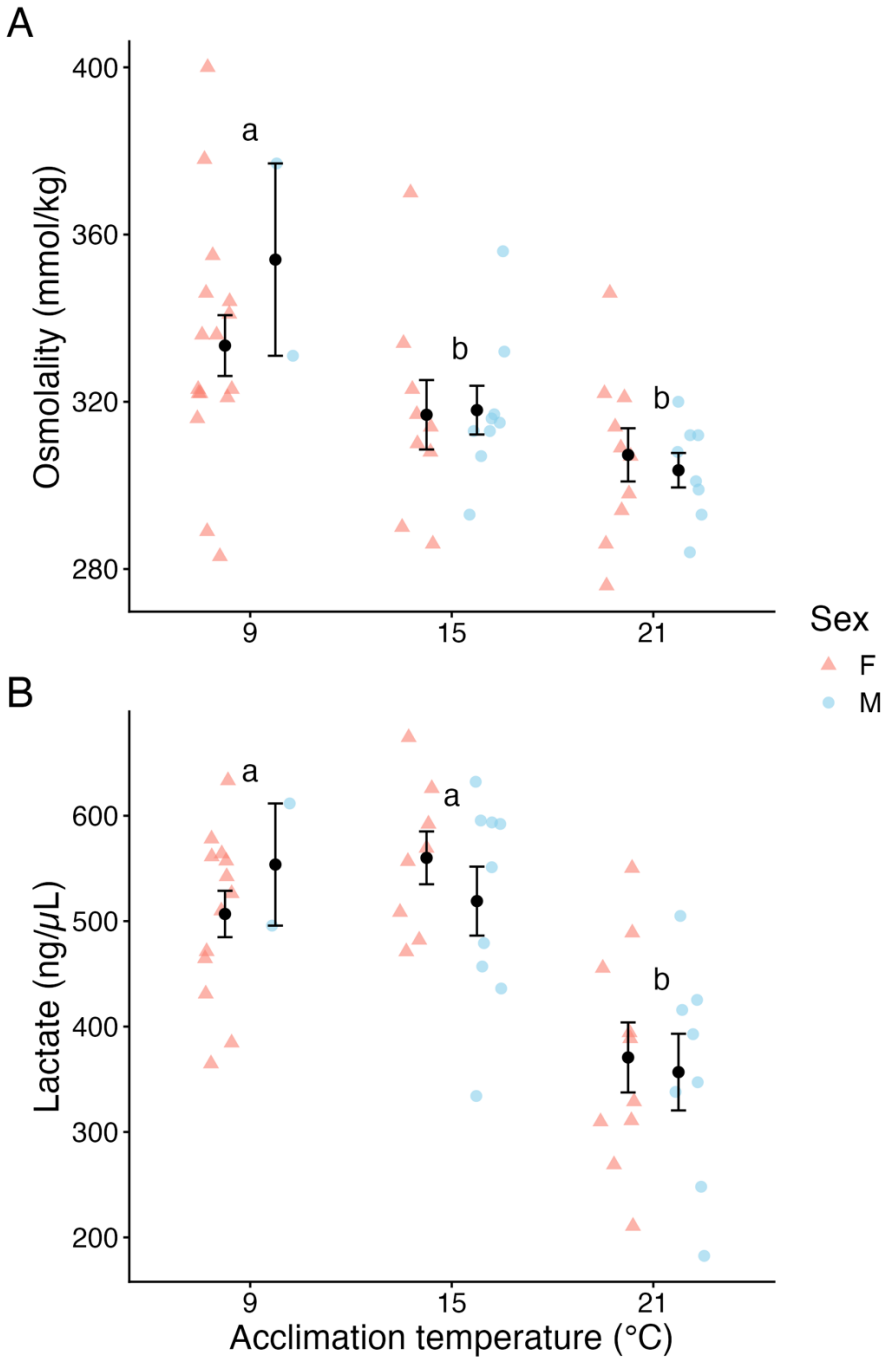

Figure 2. Blood plasma **(A)** osmolality (mmol kg^{-1}, n= 54) and **(B)** lactate (ng μL^{-1}, n = 53) from juvenile bull trout (*S. confluentus*) held at three temperature treatments (9°C, 15°C, and 21°C, n = 9) across 60 days. Each circle represents an individual grouped by acclimation temperature (°C) and sex (F = pink, M = blue). Mean and SEM are represented by points and error bars in black, respectively. Groups with different lowercase letters are significantly different from one another ($p < 0.05$) relative to groups with shared letters.

3.2 Growth and survival

Condition factor varied by temperature but not sex (Fig. 3A), with a significant difference between acclimation temperatures ($F_{2, 49} = 6.02$, $p < 0.01$) but not sex ($F_{1, 49} = 0.37$, $p > 0.05$) or their interaction ($F_{2, 49} = 0.016$, $p > 0.05$). Post-hoc tests identified differences between 15°C and 21°C relative to 9°C ($p = 0.032$ and $p = 0.024$, respectively), and no significant difference between 15°C and 21°C ($p = 0.985$). Mean condition factor for all tanks was highest in the 9°C treatment (1.15 ± 0.17) and lowest at both 15°C (1.07 ± 0.79) and 21°C (1.07 ± 0.84). Sex differences were small in 9°C (1.14 ± 0.19 in females; 1.18 ± 0.003 in males), 15°C (1.03 ± 0.02 in females; 1.10 ± 0.02 in males), and 21°C (1.07 ± 0.01 in females; 1.06 ± 0.04 in males).

Relative daily growth varied by temperature and sample time (Fig. 3B). Differences in growth were evident by acclimation temperature ($F_{2, 24} = 50.4$, $p < 0.001$) and date ($F_{3, 24} = 0.76$, $p < 0.05$) but not their interaction ($F_{6, 24} = 0.42$, $p > 0.05$). While temporal patterns were consistent across all groups ($p < 0.05$), post-hoc tests revealed significant reductions in growth at 21°C relative to 9°C ($p < 0.001$) and 15°C ($p < 0.01$) across all dates. Average growth at 9°C was higher (>1%) than 21 °C (<1%) across 60 d. Fish held at 15°C showed >1% average relative growth at 18 and 46 d, although trends suggest an overall downward trajectory relative to 9°C.

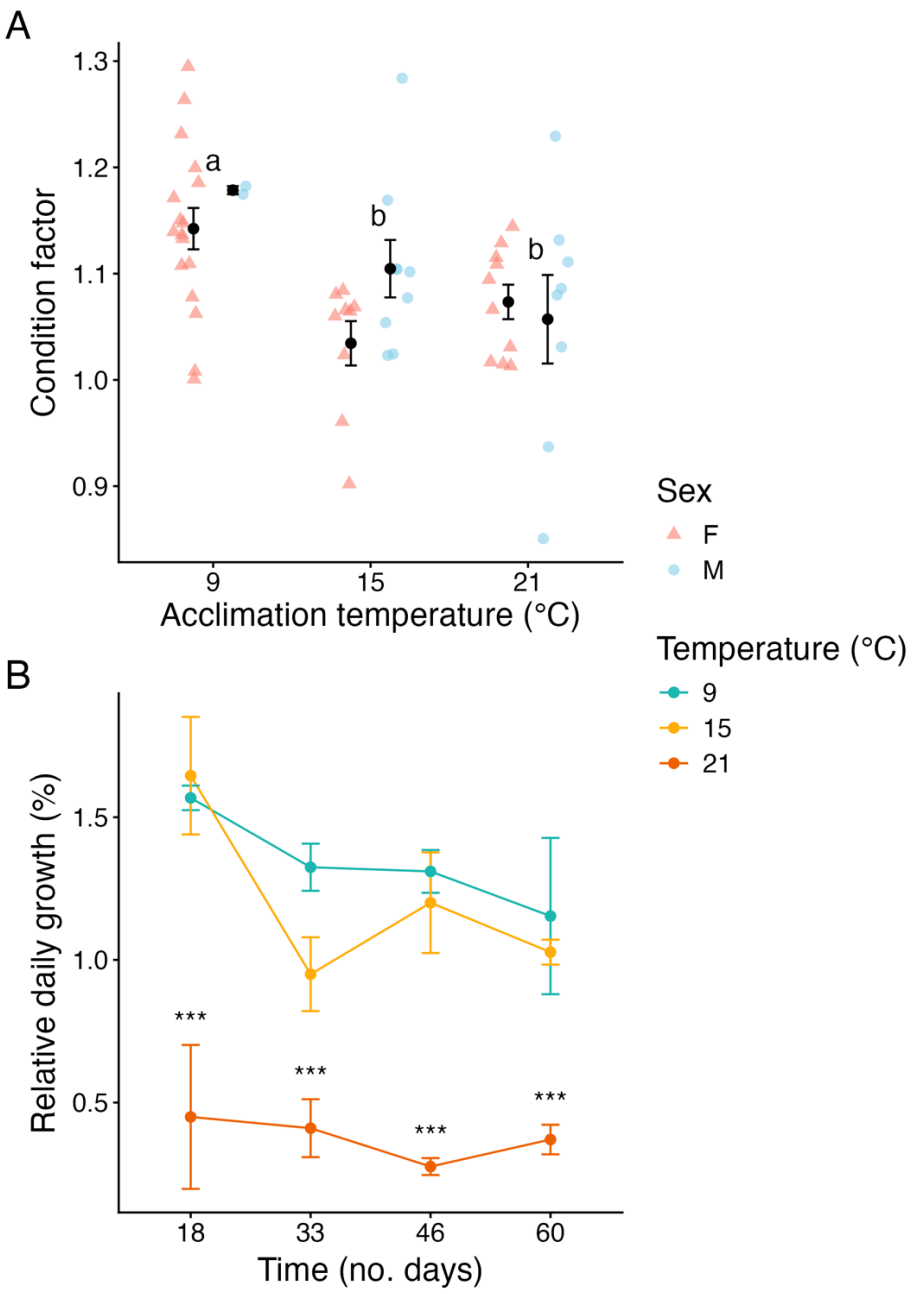

Figure 3. Whole body metrics of juvenile bull trout (*S. confluentus*) held at three acclimation temperatures across 60 days (20 Apr to 19 Jun 2023). **(A)** Fulton's condition factor calculated from terminal measurements of fork length (cm) and mass (g) by sex (F = pink, M = blue). Groups with different lowercase letters are significantly different from one another ($p < 0.05$) relative to groups with shared letters. **(B)** Relative daily growth (%) over time (no. days) by temperature treatment (9°C = teal, 15°C = orange, and 21°C = red; n = 9); asterisks represent a significant difference ($p < 0.001$). Mean and SEM are represented by points and error bars in black and by temperature colours, respectively.

Mortality was lowest in the intermediate temperatures and highest in the upper temperatures, with 5% at 15°C and 18% at 21°C, respectively (Table 2). Deaths in the 9°C were largely contained to one tank (5 from block C, tank 9); controlling for potential tank effects would reduce overall 9°C mortality to 3 (5%). Starting average tank mass was highest at 9°C (31.93 ± 0.60 g) and lowest at 21°C (29.80 ± 0.52 g) with a similar pattern at the experiment end (66.70 ± 1.55 g at 9°C and 37.25 ± 1.35 g at 21°C, respectively). Average tank mass at 15°C remained intermediate from experiment start (30.67 ± 0.52 g) to end (59.95 ± 2.03 g).

Table 1. Mortality (%), population by temperature (pop. total), and mass for juvenile bull trout (*S. confluentus*) across three temperature treatments (9°C, 15°C and 21°C, n=9) for 60 days. Mortality includes cumulative tank deaths (no. deaths) by tank and temperature from the start of acclimation. Note that 9°C mortality was largely contained to one tank (9, block c) and deaths in the remaining two 9°C tanks totaled 3 (5%).

Group	Days growth	Start mass (g. mean ± SE)	End weight (g. mean ± SE)	Pop. total	No. deaths	Mortality % (% mean ± SE)
9°C	60	31.93 ± 0.60	66.7 ± 1.55	62	8	13% ± 0.11
15°C	60	30.67 ± 0.52	59.95 ± 2.03	62	3	5% ± 0.07
21°C	60	29.80 ± 0.52	37.25 ± 1.35	63	11	18% ± 0.14

3.3 Transcript abundance

Assessment of transcript abundance for 51 target genes in the gill indicated differences in gene expression profiles by acclimation temperature (Fig. 4A). Assessment by PCA showed that PC1 explained 25.84% of variance, while PC2 explained 20.14% of variance (Fig. 4A). Individuals acclimated to 21°C clustered toward negative PC1 values, whereas those acclimated to 9°C were distributed toward positive PC1 values, with 15°C samples occupying an intermediate position. The broader ellipse associated with the 9°C group suggests greater dispersion in transcriptional profiles at the lowest temperature. No clear separation between sexes was evident in the ordination. Transcripts with the largest loadings (positive and negative) contribute more to variation on each principal component axis and reflect a higher contribution to trends in transcriptional changes (increased or decreased expression). Transcripts with the largest positive loadings on PC1 included genes associated with metabolism, ion transport, immune function, and growth; the largest negative loadings indicate further separation by cellular stress genes (Appendix B, Fig. 9). The largest loadings in PC2 include transcripts involved in cellular stress, apoptosis, cortisol response, and immune function (Appendix B, Fig. 9).

Gene-level analyses indicated temperature-dependent changes in several transcripts. Bayesian Poisson–lognormal mixed models identified significant interactions between temperature and sex for expression of *cpt1a*, *hsd11b2*, *hsp70a*, *il1β*, *il8*, *ldha*, *mmp2* and *mmp9* (geneWise $p < 0.05$) in the gill (Fig. 4B). Heat shock protein *hsp70a* abundance was highest at 21°C. In contrast, extracellular matrix remodeling genes *mmp2* and *mmp9* tended to decrease at the highest temperature relative to 9°C and 15°C. Inflammatory

markers displayed mixed responses: expression of *il8* and *il1b* was highest in females at 9°C and declined in *il8* with warming, whereas *il1b* showed relatively modest variation across temperatures. Metabolic genes also varied with temperature; *ldha* increased toward 21°C, while *cpt1a* showed relatively minor differences among treatments but significant differences by sex between treatments. The metabolic gene *hsd11b2* was lowest at 15°C and higher at both 9°C and 21°C.

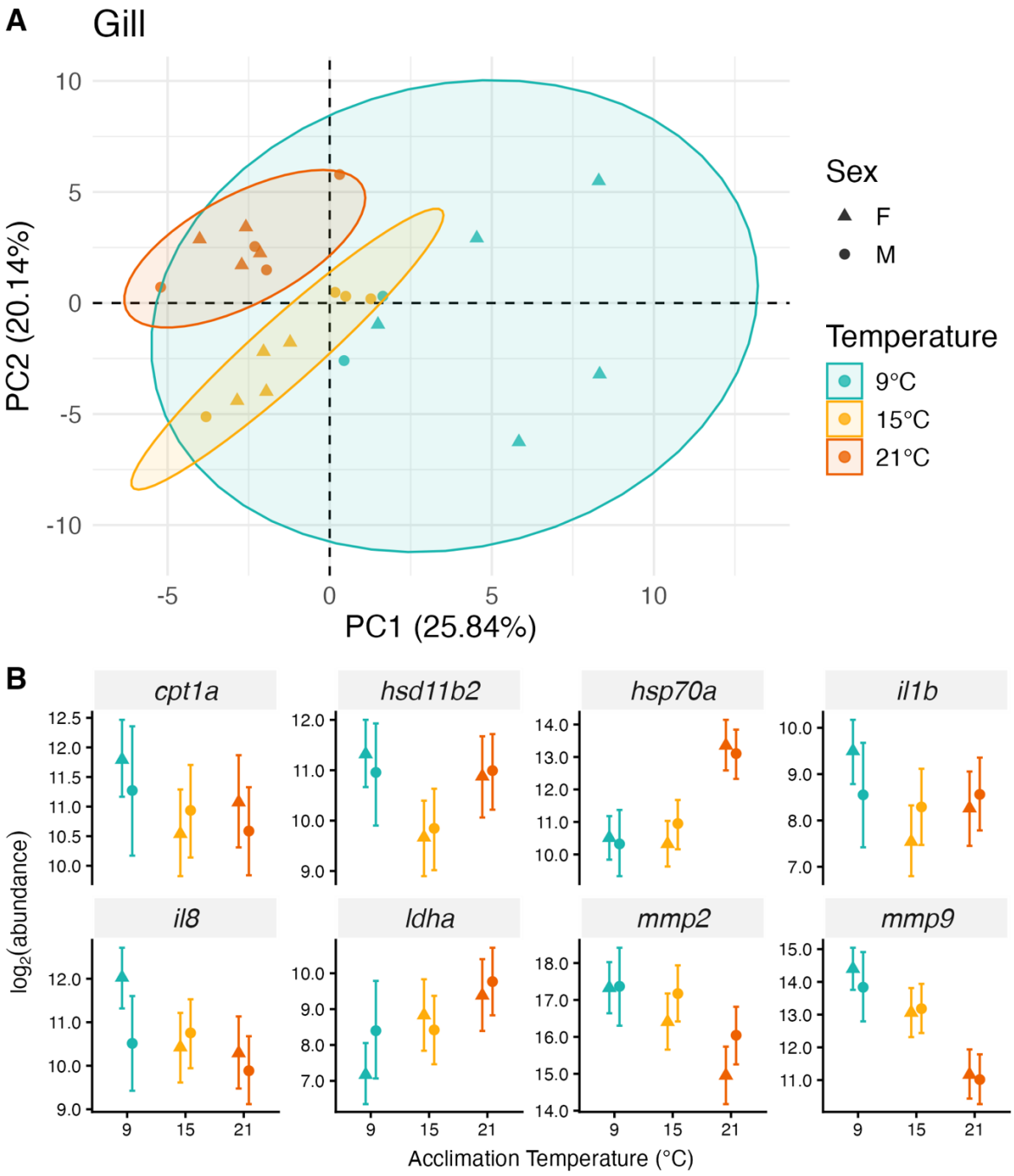

Figure 4. Gill transcript abundance patterns of juvenile bull trout (*S. confluentus*) by sex (shape) after 60 d in three acclimation temperatures (colour). **(A)** Principal component analysis (PCA) of gill mRNA transcripts for 51 genes, where points represent individual fish (9°C n = 7, 15 and 21°C n = 8) and ellipses are 95% confidence ellipses by group. The explaination of variance for each PC is indicated in brackets. **(B)** Transcript abundance (log_2(abundance)) changes in gill by acclimation temperature and sex (F = triangle, M = circle) in genes with significant post-hoc variation between pairwise groups ($p < 0.05$). Points represent means and error bars represent 95% credible intervals. Bars that do not overlap are considered statistically significant.

In the liver, 44 target genes meeting sufficient quality and quantity standards for analysis (see Methods 2.7 for exclusion criteria and list of omitted genes) showed similar differences in gene expression profiles by acclimation temperature (Fig. 5A). Assessment by PCA showed that PC1 explained 33.51% of variance, while PC3 explained 10.55% of variance (Fig. 5A). Although PC2 explained more variance overall (22.74%), PC3 was retained for further analysis because it better captured variation and separation between treatment groups. Individuals acclimated to 9°C were distributed toward positive PC1 values, whereas those acclimated to 21°C were shifted toward negative PC1 values and higher PC3 scores. Samples from 15°C clustered near the centre, suggesting intermediate transcriptional profiles relative to 9°C and 21°C. No clear separation between sexes was evident in the ordination. The broader ellipse associated with the 9°C group suggests greater dispersion in transcriptional profiles at the lowest temperature. The largest loadings in PC1 included transcripts involved with metabolism and cellular stress (Appendix B, Fig. 10), similar to the largest loadings in PC3 in addition to cortisol response, growth, and apoptosis (Appendix B, Fig. 10).

Gene-level analyses revealed temperature-dependent changes in several metabolic and stress-related transcripts. Significant interactions between temperature and sex were identified by Bayesian Poisson–lognormal mixed models for the expression of *aldoaa*, *ampka1*, *casp3ab*, *cpt1a*, *cyp1a*, *fasn*, *gstp1*, *hsp70a*, *igf1*, *igf2*, *lipeab*, *lpl*, *mmp2*, *mmp9*, *mta*, *mtb*, *pck1*, and *sod1* (geneWise $p < 0.05$) in the liver (Fig. 5B). Markers associated with lipid metabolism showed distinct patterns: *cpt1a* decreased at 15 °C relative to 9 and 21°C, whereas *lpl* expression increased toward 21°C, and *fasn* and

lipeab generally declined with increasing temperature. Glycolytic gene *aldoaa* and gluconeogenic gene *pck1* tended to show lower expression at 15°C relative to 9°C, with partial increases at 21°C. Expression of the heat shock gene *hsp70a* increased at 21°C, consistent with elevated cellular stress under warm acclimation. Growth-related genes also varied with temperature: *igf1* expression was highest at 9 and 15°C but declined at 21°C, while *igf2* showed more modest variation across treatments but likewise declined at 21°C. Transcripts associated with extracellular matrix remodeling decreased with warming, with both *mmp2* and *mmp9* showing lower expression at 21°C relative to 9 and 15°C. Oxidative stress defense gene *sod1* and transcriptional regulators *mta* and *mtb* displayed moderate variation among temperature treatments but without strong directional shifts.

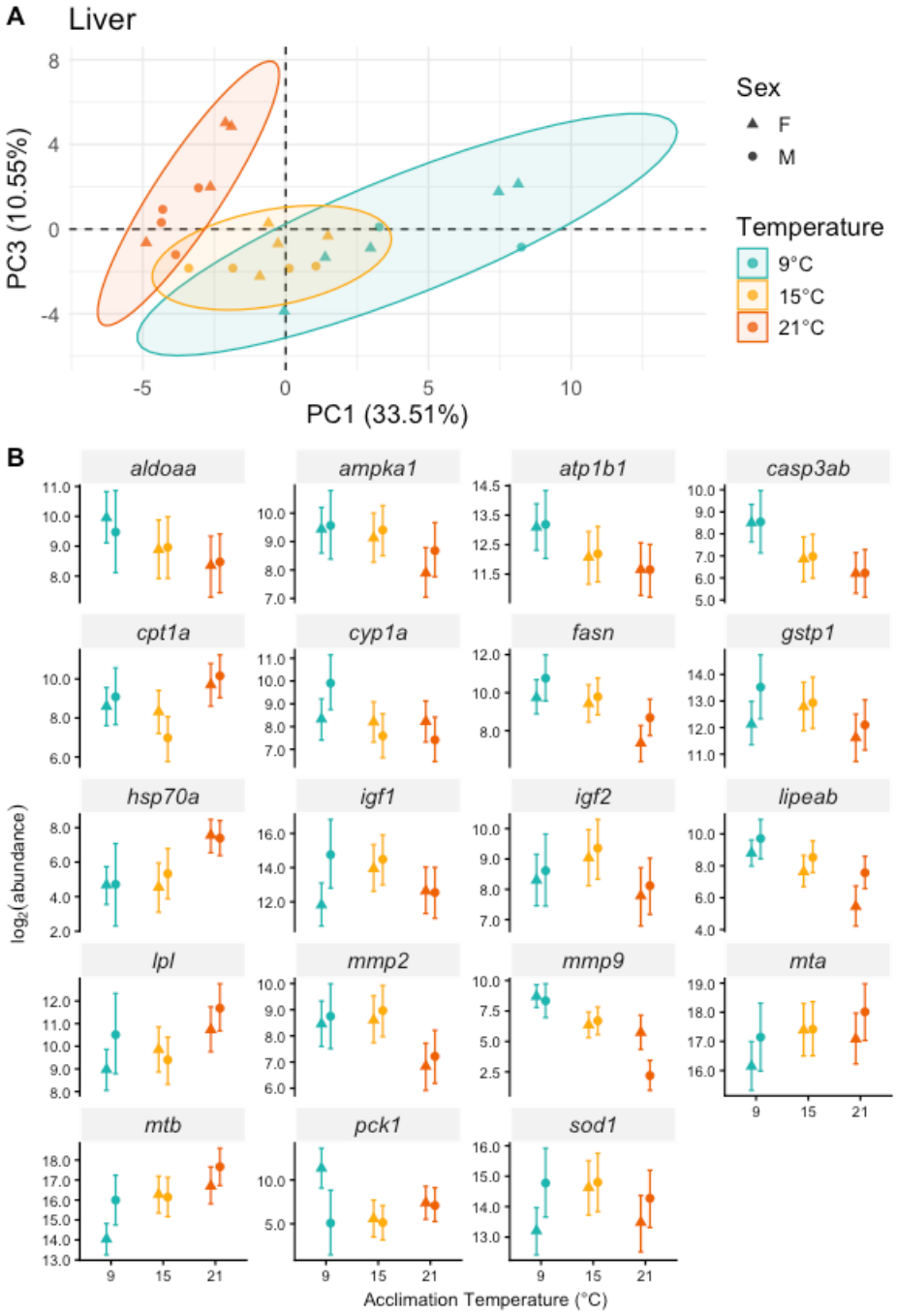

Figure 5. Liver transcript abundance patterns of juvenile bull trout (*S. confluentus*) by sex (shape) after 60 d in three acclimation temperatures (colour). **(A)** Principal component analysis (PCA) of liver mRNA transcripts for 44 genes, where points represent individual fish (9°C n = 7, 15 and 21°C n = 8) and ellipses are 95% confidence ellipses by group. The explaination of variance for each PC is indicated in brackets. **(B)** Transcript abundance (log_2(abundance)) changes in liver by acclimation temperature and sex (F = triangle, M = circle) in genes with significant post-hoc variation between pairwise groups ($p < 0.05$). Points represent means and error bars represent 95% credible intervals. Bars that do not overlap are considered statistically significant.

4. DISCUSSION

My results show that transcriptional responses occurred alongside clear physiological changes in juvenile bull trout over 60 days, including declines in relative growth, condition factor, plasma osmolality, and lactate concentration, with increasing acclimation temperature. Reduced growth and condition at higher temperatures likely reflect energetic trade-offs between maintenance and somatic growth, a pattern frequently reported in fishes experiencing chronic thermal stress (Das and Majhi 2015; Best et al. 2025; Abdel-Tawwab et al. 2025). Elevated temperature increases metabolic demand and can reduce energy available for growth and energy storage, resulting in lower growth performance and reduced condition factor (Beuvard et al. 2022). The concurrent transcriptional changes observed in liver metabolism genes and growth-related pathways (e.g. *igf* signalling) are consistent with this shift in energy allocation. Gill transcriptional profiles indicate a shift at higher temperatures toward increased cellular stress and metabolic demand alongside reduced expression of genes associated with tissue remodeling and growth (e.g., *mmp2*, *mmp9*), suggesting diminished epithelial plasticity and osmoregulatory capacity that may contribute to the observed declines in condition, growth, lactate, and plasma osmolality. Significant decreases in osmolality and lactate at 21°C may reflect altered metabolic and osmoregulatory processes associated with long-term acclimation to warmer conditions. While juvenile bull trout at 15°C sustained growth, the poor condition factor, low osmolality, and declining transcriptional profiles for growth-metabolism and tissue remodelling pathways align with previous studies that suggest 15°C is an important sub-lethal threshold where cellular stress

becomes a challenge to homeostasis (Best et al. 2025). Combined, my results suggest that chronic warming induces integrated organismal responses that include altered metabolic regulation, activation of cellular stress pathways, and reduced investment in growth at temperatures beyond 15°C.

4.1 Physiological responses to temperature

Temperature-dependent reductions to osmolality showed that thermal conditions influence osmotic regulation in juvenile bull trout. Elevated metabolic demand in response to temperature necessitates an increase in ventilation and blood flow to the gill to meet oxygen demand (Clark et al. 2008). However, the functional characteristics that favour high oxygen uptake in the gill (e.g. thin epithelia and increased blood flow) also facilitate faster passive diffusion of ions and water across the gill surface, known as the osmorespiratory compromise (Randall et al. 1972; Wood and Eom 2021). When challenged with a bacterial infection, YOY bull trout subjected to exhaustive exercise at 15°C exhibited no significant differences in plasma osmolality relative to control fish, although mortality was considerably higher (~95%; Jones and Moffitt 2004) and this temperature is still within the known sub-lethal limit (Best et al. 2025). Reduction of osmolality across each temperature treatment potentially reflects temperature-dependent changes in osmoregulatory efficiency, including enhanced ion pump activity and altered membrane permeability that lead to declines of osmolality due to impaired ion uptake (Vargas-Chacoff et al. 2020; Yang et al. 2023). These changes are further supported by a high loading of transcripts associated with ion transport in the gill,

indicating ion regulation is an important component for separation between temperatures.

Bull trout at 21°C exhibited significantly lower lactate levels relative to other treatment temperatures. Shifts in lactate concentration can indicate an imbalance between tissue oxygen demand and oxygen delivery in the blood, often associated with anaerobic metabolism and a secondary stress response in fishes subject to exhaustive exercise (Wood et al. 1983; Kieffer et al. 1994). Lactate is synthesized from pyruvate, a metabolite supplied by the breakdown of glycogen stores in the liver and can serve as an alternative fuel when glucose supplies are insufficient (Talarico et al. 2023). Sustained thermal stress mobilizes energy reserves such as glycogen in muscle and liver (Vijayan and Moon 1992), though prolonged or repeated stress can deplete these stores. In juvenile Chinook salmon (*Oncorhynchus tshawytscha* Walbaum, 1792), repeated acute stress caused cumulative increases in plasma glucose and a greater depletion of hepatic glycogen with repeated disturbances (Barton et al. 1986). Once glycogen availability is limited, the capacity for anaerobic glycolysis declines, potentially reducing lactate production even under continued stress (Scarabello et al. 1991). Brook trout acclimated to 23°C exhibited lower levels of lactate concentrations after 24 h of recovery from acute exhaustive exercise relative to cooler temperature treatments, potentially due to increased clearance rates or utilization of glycogenesis pathways within the muscle to recover lost energy stores (Milligan 1996; Mackey et al. 2021). Metabolic depression to conserve energy by suppressing non-essential functions (e.g. protein synthesis), metabolic rate, and oxygen consumption, may be an adaptive response to cope with

chronic thermal stress and reduce the accumulation of anaerobic end-products (Viant et al. 2003; Mackey et al. 2021). In our study, lower lactate concentration and reduced growth in juvenile bull trout at 21°C suggests severe depletion of glycogen stores. Lack of regenerative substrate may be an important contributor to compromised osmoregulatory capacity due to the substantial metabolic expense required for ion regulation in a hypo-osmotic environment. Conversely, the peak in lactate concentration at 15°C suggests heightened metabolic activity or greater reliance on anaerobic pathways at intermediate temperatures (Morrison et al. 2020), and may reflect a transition zone where metabolic rate increases substantially but cardiovascular oxygen delivery capacity lags behind (Balasubramanian et al. 2025). A growing body of work points to 15°C as a sub-lethal threshold in bull trout (Isaak et al. 2015; Mochnacz et al. 2023; Best et al. 2025), and may represent a plateau where tissue oxygen demand begins to outpace oxygen supply, driving increased anaerobic glycolysis and lactate production.

Sex showed no statistically significant interactions with temperature in both osmolality and lactate, although there were moderate and inconsistent differences among select groups. The higher osmolality observed in males at 9°C suggests greater osmotic sensitivity at colder temperatures, whereas females' elevated lactate at 9°C and 15°C points to increased metabolic demand or activity, potentially linked to sex-specific energetic allocation (Houde 1997; Ricklefs and Wikelski 2002). The convergence of both metrics at 21°C suggests that thermal stress constrains both sexes similarly, consistent with evidence that approaching upper thermal limits significantly reduces physiological performance (Jeffries et al. 2012a). However, it should be noted that the 9°C population

had less males than females, and therefore it may be difficult to elucidate differential trends given the limited sample size.

4.2 Whole-body responses to temperature

Differences in growth and condition factor at 21°C compared to 9 and 15°C are stark and suggest that chronic thermal stress can compromise long-term performance. Growth rates are influenced by individual competitive ability, metabolic rate, and prevailing abiotic conditions, and considerable variation may be present seasonally and within populations (Claireaux and Lefrançois 2007; Xu et al. 2010). Warm-water habitat is an important resource for cold-water species, as short forays into warmer, more productive habitat can increase growth potential and provide an important refugia for overwintering (Armstrong et al. 2021; Mochnacz et al. 2025). Compensatory growth in response to suboptimal conditions has been recorded in wild populations of cutthroat trout (*Oncorhynchus clarkii* Richardson, 1836), where lower condition individuals exhibited faster and higher growth in mass across all seasons, although high condition fish demonstrated an overall length advantage (Al-Chokhachy et al. 2019). However, rapid compensatory growth may throttle other physiological capabilities required for swim performance (Perez and Munch 2015), decrease life-span (Lee et al. 2013), and reduce life-time fecundity (Auer et al. 2010), suggesting compensation may not fully overcome periods of poor growth during developmentally sensitive life-stages. Higher relative growth in juvenile bull trout at 15°C aligns with well-established thermal optima for growth in many cold-water fishes (Brett 1971), whereas reduced growth and poorer

condition at 21°C suggests that the energetic demands at warmer temperatures may exceed intake or assimilation capacity (Mesa et al. 2013). Consistent reduction of condition factor at 21°C across all measurement periods may reflect chronic metabolic stress and reduced feeding efficiency near upper thermal thresholds (Claireaux and Lefrançois 2007). Given the relationship between fecundity, size, and onset of sexual maturity in fishes (McMillan et al. 2012; Tsoukali et al. 2016), reduced growth at 21°C suggests that sexual maturation and offspring survival may be constrained in juvenile bull trout exposed to elevated temperature over long periods of time. Additionally, survival is known to decline after 60 d in temperatures beyond the species' known thermal limits at 20.9°C, an ultimate upper incipient lethal temperature 1-5°C lower than other salmonids (Selong et al. 2001), likely reflecting the combined effects of reduced food intake, cellular stress, and high cost of homeostatic maintenance. Notably, mortalities and declining growth in the 9°C tanks suggests the presence of tank effects and may not accurately reflect true temperature responses.

4.3 Transcriptional responses to temperature

Temperature acclimation produced coordinated changes in transcriptional profiles in the gill, indicating a broad transcriptional response to mitigate tissue damage and maintain ionoregulatory function. As the primary site of ion regulation, gas exchange, and acid–base balance, the gill is particularly sensitive to environmental stressors that challenge both oxygen uptake and osmotic homeostasis during thermal stress (Evans et al. 2005; Wood and Eom 2021). Matrix metalloproteinases (*mmp2*, *mmp9*) are key

mediators of tissue remodeling and the immune response in teleosts, facilitating epithelial restructuring, immune cell migration, and repair processes (Chadzinska et al. 2008). Because gill remodeling can alter ionocyte distribution, in turn modifying epithelial permeability and ion flux (Bradshaw et al. 2012), reduced expression of these genes may reflect the constrained osmoregulatory capacity evident at 15 and 21°C. Similar patterns of decreased *mmp* abundance were observed in YOY bull trout after 3 weeks of thermal stress (Best et al. 2025), although decreases to the lipid metabolism gene *cpt1a* in the current study further implicates osmoregulatory challenges given the importance of lipids in regulating ion flux and maintaining membrane integrity (Tkatcheva et al. 2007). Heat stress is known to suppress abundance of the pro-inflammatory gene *il1b* in salmonids (Frenette et al. 2023), and the decline in transcript abundance for genes involved in immune function beyond 9°C indicates suppression or dysregulation of pathways needed to manage cellular damage. Up-regulation of *ldha* (lactate dehydrogenase a, an enzyme that can convert lactate to pyruvate) corresponded with the reduced lactate concentration at 21°C, suggesting increased turnover and potentially reflecting elevated energetic demand. Sustained exposure to chronic thermal stress appears to shift gill physiology toward metabolic compensation while constraining immune and tissue repair processes. This reorganization of transcriptional priorities may therefore limit the capacity for effective osmoregulation and tissue repair at temperatures beyond 15°C.

Liver transcriptional profiles varied across acclimation temperatures and suggest that chronic warming alters metabolic regulation and growth signalling in juvenile bull

trout. Our results show that several genes associated with lipid and carbohydrate metabolism, including *fasn*, *cpt1a*, *aldoaa*, and *pck1*, displayed temperature-related differences in transcript abundance. The liver plays a central role in coordinating metabolic responses to environmental stress by regulating carbohydrate storage, lipid synthesis, and energy mobilization (Bruslé and Gonzàlez i Anadon 2017). Increased metabolic demand can drive reorganization of these pathways to support maintenance metabolism rather than somatic growth (Pörtner 2010). Reduced growth and condition in juvenile bull trout at 21°C coincided with altered expression profiles of lipid and carbohydrate metabolic genes and lower expression of growth-related transcripts such as *igf1*, suggesting that prolonged exposure to warm temperatures may suppress anabolic pathways while prioritizing energy homeostasis. Similar transcriptional adjustments have been observed in other salmonids exposed to chronic thermal stress, where warming induces shifts in hepatic metabolism and oxidative stress responses that reflect increased energetic demand and reduced physiological performance (Jeffries et al. 2012b; Nuez-Ortín et al. 2018). Additionally, variation in genes associated with oxidative stress defence and heat shock chaperone proteins, including *sod1* and *hsp70a*, indicates activation of protective pathways that mitigate cellular damage under prolonged thermal stress (Feder and Hofmann 1999). Heightened energetic demand at the cellular level to maintain homeostasis likely contributes to constrained growth and may play a significant role in reduced performance in bull trout experiencing prolonged heat stress.

4.4 Conclusion

Collectively, my findings suggest that prolonged exposure to elevated temperatures imposes energetic and physiological constraints that limit the ability of bull trout to cope with additional stressors, with important implications for their persistence under ongoing climate warming. The onset of a coordinated, multi-level reorganization of physiology and transcriptional profiles in juvenile bull trout at 15°C aligns with declining occupancy above 11°C in wild populations (Isaak et al. 2015; Mochnacz et al. 2023), where increasing temperature shifts energy allocation away from growth and toward maintenance of homeostasis. Although warm-water habitat provides necessary resources and seasonal refugia for cold-water species, a growing portfolio of research suggests that prolonged exposure is detrimental to bull trout (Selong et al. 2001; Best et al. 2025). However, these results are limited to one population and life stage raised in a controlled environment. Intraspecific variation in thermal tolerance is consistent with historic conditions in salmonids (Eliason et al. 2011) and northern, sub-Arctic bull trout populations appear to experience differential thermal constraints to their distribution when compared with southern populations (Isaak et al. 2015; Mochnacz et al. 2025). Additionally, the lack of males at 9°C limits interpretation given the relatively small sample size (n = 2) relative to other temperature treatments. Sex-based characterization of thermal stress and physiology in more northerly populations would improve our understanding of potential climate impacts across the species' range, given the accelerated warming rates at higher latitudes (Rantanen et al. 2022) and performance variation between sexes in sexually mature salmonids (Minke-Martin et al. 2018).

Incorporating transcriptomic indicators of stress and metabolic limitation into bull trout distribution models may improve predictions of habitat suitability by capturing sub-lethal physiological thresholds that precede mortality, while identifying these thresholds is critical for conservation planning, as they define temperature ranges where performance is impaired even if survival is still possible.

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Appendix A

Table 2. Raw data for individual (fish ID) juvenile bull trout (*S. confluentus*) held at three temperature treatments (temp °C) in nine tanks across 60 days (n = 7 for 9°C; n = 8 for 15°C and 21°C); sex, fork length (FL), total length (TL), mass, condition factor, osmolality and lactate. Columns marked with "--" are due to insufficient blood plasma volumes.

Fish ID	Temp (°C)	Tank	Sex	FL (cm)	TL (cm)	Mass (g)	Condition factor	Osmolality (mmol/kg)	Lactate (ng/µL)
F018	9	1	F	16.9	17.5	52.03	1.078	355	1365.909
F021	9	1	F	18.3	18.9	75.47	1.231	322	471.263
F023	9	1	F	18.5	19.5	72.84	1.150	346	364.808
F026	9	1	F	17.6	18.5	65.40	1.200	283	561.239
F028	9	1	M	17.9	18.5	67.81	1.182	377	495.762
F030	9	1	F	18.5	19.2	72.14	1.139	378	1230.217
F035	9	1	F	19.5	20.5	96.01	1.295	336	633.404
F039	15	2	F	17.8	18.4	60.27	1.069	317	508.518
F041	15	2	M	15.0	16.2	37.26	1.104	313	593.688
F043	15	2	M	20.7	21.6	103.71	1.169	315	595.489
F045	15	2	F	18.8	19.5	72.04	1.084	290	625.961
F048	15	2	M	19.0	19.7	72.29	1.054	313	479.141
F050	15	2	F	18.3	18.7	65.24	1.065	334	1114.346
F065	21	3	M	15.3	15.9	36.93	1.031	301	338.009
F067	21	3	F	15.5	16.0	38.39	1.031	276	210.685
F070	21	3	F	15.0	15.7	38.62	1.144	346	388.702
F071	21	3	F	18.5	19.2	71.48	1.129	298	328.848
F074	21	3	M	17.2	18.0	54.95	1.080	299	347.179
F076	21	3	F	18.0	18.9	65.04	1.115	294	309.803
F081	9	4	F	18.6	19.4	81.32	1.264	316	564.034
F084	9	4	F	17.2	17.9	60.33	1.186	321	464.552
F085	9	4	F	16.0	16.5	40.99	1.001	289	542.351
F087	9	4	F	18.0	18.7	66.07	1.133	--	--
F090	9	4	F	16.7	17.4	51.59	1.108	322	509.865
F095	9	4	F	16.2	16.5	42.86	1.008	341	526.299
F100	15	5	F	18.5	19.3	67.48	1.066	314	471.082
F102	15	5	F	16.0	16.6	43.42	1.060	370	556.792
F103	15	5	M	20.9	21.6	100.58	1.102	293	632.257
F108	15	5	M	18.4	19.0	68.80	1.104	356	592.239
F110	15	5	F	14.4	15.0	28.69	0.961	323	674.313
F112	15	5	M	14.4	15.0	30.59	1.024	307	334.133
F125	21	6	M	16.2	17.0	52.27	1.229	312	425.293
F127	21	6	F	16.3	16.8	47.40	1.094	286	455.468
F130	21	6	F	16.0	16.5	41.57	1.015	307	269.019
F131	21	6	M	15.5	16.0	42.14	1.132	308	504.955
F132	21	6	F	17.4	17.8	53.37	1.013	321	550.312
F134	21	6	F	16.8	17.6	50.56	1.066	322	394.375
F143	15	7	M	16.0	16.6	44.12	1.077	316	436.122
F144	15	7	F	17.2	17.7	54.98	1.080	308	592.215
F147	15	7	M	18.1	18.7	60.67	1.023	317	551.166
F149	15	7	F	14.5	15.0	27.50	0.902	286	569.370
F150	15	7	F	15.3	15.8	36.66	1.024	310	482.163
F153	15	7	M	15.0	15.7	43.33	1.284	332	456.979
F163	21	8	M	14.2	14.8	26.83	0.937	320	415.777
F166	21	8	M	13.5	13.9	20.93	0.851	312	182.470
F167	21	8	F	15.5	16.1	41.29	1.109	314	488.962
F172	21	8	M	18.9	19.7	75.01	1.111	284	248.056
F178	21	8	F	16.2	16.8	43.23	1.017	309	310.881
F181	21	8	M	16.3	17.1	47.03	1.086	293	392.790
F188	9	9	F	17.0	17.8	54.50	1.109	400	384.599
F189	9	9	F	18.1	18.9	68.05	1.148	336	--
F191	9	9	F	18.5	19.3	74.16	1.171	344	578.112
F192	9	9	M	18.0	19.0	68.51	1.175	331	611.678
F194	9	9	F	18.1	19.1	63.00	1.062	323	431.040
F195	9	9	F	17.8	18.6	64.10	1.137	323	557.210

Table 3. Genes used in the OpenArray qPCR reactions with mean efficiencies (eff. %) by tissue type (gill, liver) from juvenile bull trout (*S. confluentus*) held at three temperature treatments across 60 days (n = 7 for 9°C; n = 8 for 15°C and 21°C). Genes marked with three stars (***) did not pass the efficiency threshold and were thus omitted from all subsequent analyses; those marked with one star (*) did not pass additional QA/QC checks and were likewise omitted (see Methods 2.7 Data and statistical analyses). Format and descriptions adapted from Best et al. (2025).

Gene ID	Name	Gill eff. (%)	Liver eff. (%)	Pathway	Description
aldoaa	aldolase A	112.7%	98.2%	metabolism (glycolysis)	enzyme - converts frucose-1,6-bisphosphate to 3C compounds that become pyruvate
ampka1	AMP-activated protein kinase	101.1%	100.7%	metabolism (other)	cellular energy sensor - rises when ratio of AMP/ADP to ATP increases and acts to boost ATP
atp1b1	sodium-potassium ATPase (NKA)	97.9%	100.2%	ion transport	maintain ionic balance in body fluids, particularly involved in ion exchange in the gill
cam	calmodulin	109.5%	107.4%	metabolism (other)	binds to calcium ions; regulates calcium-dependent processes
casp3ab	caspase 3 cysteine peptidase a and b	96.5%	77.5%	apoptosis	enzyme involved in activation and execution of apoptosis in response to stress
casp9	caspase 9	93.4%	***	apoptosis	cystein aspartic protease involved in apoptosis (initiator caspase - activates caspase 3)
cat	catalase	86.4%	94.0%	cellular stress (antioxidant)	enzyme in peroxisomes that degrades the metabolic byproduct hydrogen peroxide
chmp5ab	charged multivescular body protein 5	83.9%	93.5%	apoptosis	involved in degrading surface receptor proteins via endocytosis, also anti-apoptic
cirbpa	cold-inducible RNA binding protein	119.4%	114.3%	cellular stress (thermal)	regulates transcriptional response to cold (and other stressors)
clock1a	clock circadian regulator a	92.1%	94.6%	circadian rhythm	regulates circadian rhythm and daily locomotor activity; linked to wound repair
cpt1a	carnitine palmitoyl transferase I	93.1%	109.1%	metabolism (lipid)	enzyme involved in fatty acid transport into mitochondria for beta-oxidation
cry1ab	cyrptochrome circadian regulator 1a and b	87.5%	91.7%	circadian rhythm	photoreceptors that respond to environmental light cues; linked to metabolism and immune response
cs	citrate synthase	99.5%	91.7%	metabolism (other)	citric acid cycle enzyme, joins acetyl-coA with oxaloacetate
ctsd	cathepsin D	95.9%	99.7%	metabolism (other)	ubiquitous lysosomal protease
cyp1a	cytochrome P450 1A	92.2%	96.8%	immune	enzyme that binds to xenobiotics, hormones, and fatty acids
ef1a	elongation factor 1-alpha	98.4%	101.4%	metabolism (other)	elongation during protein synthesis
fasn	fatty acid synthase	90.0%	93.8%	metabolism (lipid)	uses acetyl-coA to synthesize long chain fatty acids, sensitive to hormones/food/devt
ghr	growth hormone receptor	93.7%	94.6%	growth	catabolic/lipolytic type growth hormone receptor (as opposed to anabolic/somatic growth)
gr2	glucocorticoid receptor 2	76.6%	85.3%	cortisol response (stress)	receptor for cortisol, more sensitive that gr1 and likely mediates basal cortisol functions
gstp1	glutathione-s-transferase pi isoform	106.6%	109.9%	cellular stress (antioxidant)	enzyme that catalyzes the conjugation of glutathione with other compounds (detox/antioxidant)

hk1	hexokinase 1	79.6%	***	metabolism (glycolysis)	first step in glycolysis, uses ATP to phosphorylate glucose
hsd11b2	11 beta hydroxysteroid dehydrogenase 2	83.4%	94.2%	cortisol response	converts cortisol into its active form, cortisone
hsf1	heat shock transcription factor 1	113.2%	101.5%	cellular stress (thermal)	binds to HSE (heat shock elements) during cellular stress to change transcription (e.g. hsp)
hsp70a	heat shock protein 70a (hspa1)	100.5%	83.8%	cellular stress (thermal)	binds other proteins to assist in folding, repair
hsp7c	heat shock cognate 71 kDa protein	97.6%	105.4%	cellular stress (thermal)	constitutively expressed heat shock protein, involved in protein folding during translation
hsp90ba	heat shock protein 90	94.4%	95.2%	cellular stress (thermal)	inducible hsp90, involved in protein folding/chaperoning/aggregate prevention
hspa4	heat shock protein a4 (hsph2)	107.6%	109.3%	cellular stress (thermal)	possible nucleotide exchange factor for HSPA family (removes ADP after ATP is used)
igf1	insulin-like growth factor 1	94.6%	108.6%	growth	produced by the liver in response to GH, major anabolic agent for tissue growth
igf2	insulin-like growth factor 2	98.1%	101.6%	growth	contributes to growth through paracrine/autocrine effects
igfbp1	igf-binding protein 1a	79.2%	97.0%	growth	binds to IGF to regulate growth, also involved in catabolism during fasting/stress
il1b	interleukin-1 beta	76.5%	***	immune	pro-inflammatory cytokine involved in inflammation and pain signalling; also regulates cell proliferation, differentiation and apoptosis
il8	interleukin 8	94.9%	***	immune	pro-inflammatory cytokine that promotes blood vessel formation and wound healing
ldha	lactate dehydrogenase a	84.2%	***	metabolism (glycolysis)	enzyme that converts pyruvate to lactate when oxygen is low
*ldhb**	lactate dehydrogenase b	102.7%	82.4%	metabolism (glycolysis)	enzyme that converts lactate to pyruvate (Cori cycle in liver, muscle lac > pyru > glucose)
lepr	leptin receptor	91.8%	***	metabolism (other)	transmembrane receptor important in lipid and glucose mobilization, fasting, appetite
lipeab	lipase E (hormone-sensitive lipase)	103.0%	120.3%	metabolism (lipid)	enzyme that converts stored fat (triglycerides) to FFA, considered rate limiting step in lipolysis
lpl	lipoprotein lipase	98.8%	114.1%	metabolism (lipid)	enzyme that breaks down lipoprotein triglycerides, also mediates FFA uptake into tissue
mhci	major histocompatibility complex class I	125.8%	117.6%	immune	proteins which signal cell health found on most nucleated cells
mmp2	matrix metalloproteinase 2	114.5%	107.8%	growth	collagenase acting in the extracellular matrix - tissue remodelling, devt, disease states
mmp9	matrix metalloproteinase 9	96.5%	91.3%	growth	collagenase acting in the extracellular matrix - tissue remodelling, devt, disease states

mr	mineralocorticoid receptor	87.5%	90.6%	cortisol response	mediates effect of cortisol
mta	metallothionein a	106.8%	108.0%	cellular stress (antioxidant)	small cysteine-rich proteins that bind and protect against metals, and scavenge free radicals
mtb	metallothionein b	109.4%	118.3%	cellular stress (antioxidant)	small cysteine-rich proteins that bind and protect against metals, and scavenge free radicals
nfe2l2a	nuclar factor erythroid 2 like 2	109.8%	99.6%	cellular stress (antioxidant)	binds to ARE (antioxidant response element) - usually degraded but persists in oxidative stress
pck1	phosphoenolpyruvate carboxykinase (cytosolic)	***	78.5%	metabolism (glycolysis)	converts oxaloacetate into PEP as the rate-limiting step in gluconeogenesis
pdcd10ab	programmed cell death protein 10 (ccm3)	102.2%	98.1%	apoptosis	stabilizes brain vasculature, enhances apoptosis, other functions
pgk	phosphoglycerate kinase	101.4%	101.6%	metabolism (glycolysis)	enzyme in glycolysis, producing ATP (1,3-bisphosphoglycerate to 3-phsphoglycerate)
rpl13a	ribosomal protein L13a	106.4%	115.1%	reference	ribosomal protein, catalyze protein synthesis - reference gene
rpl7	ribosomal protein L7	100.6%	101.9%	reference	ribosomal protein, catalyze protein synthesis - reference gene
rps9	ribosomal protein S9	109.4%	119.6%	reference	ribosomal protein, catalyze protein synthesis - reference gene
serpinh1	serine proteinase inhibitor H1 (hsp47)	85.0%	92.8%	growth	aids the maturation of fresh collagen secreted from ER, may be involved in fibrosis (excess collagen)
slc2a1a	solute carrier family 2, member 1a	100.4%	***	growth	involved in glucose import
sod1	superoxide dismutase 1 (Cu-Zn type)	102.7%	107.2%	cellular stress (antioxidant)	enzyme that converts superoxide radicals to H2O2 and oxygen to counteract oxidative stress
sod2	superoxide dismutase 2 (Mn type)	71.8%	96.6%	cellular stress (antioxidant)	enzyme that converse superoxide radicals to H2O2 and oxygen to counteract oxidative stress
stat1	signal transducer and activator of transcription 1	93.8%	94.3%	immune	regulates transcriptional responses in the immune system; also involved in controlled cell growth and apoptosis
vegfc	vascular endothelial growth factor c	74.8%	***	growth	growth factor involved in vessel formation (lympatic - debated in fish) and angiogenesis

Appendix B

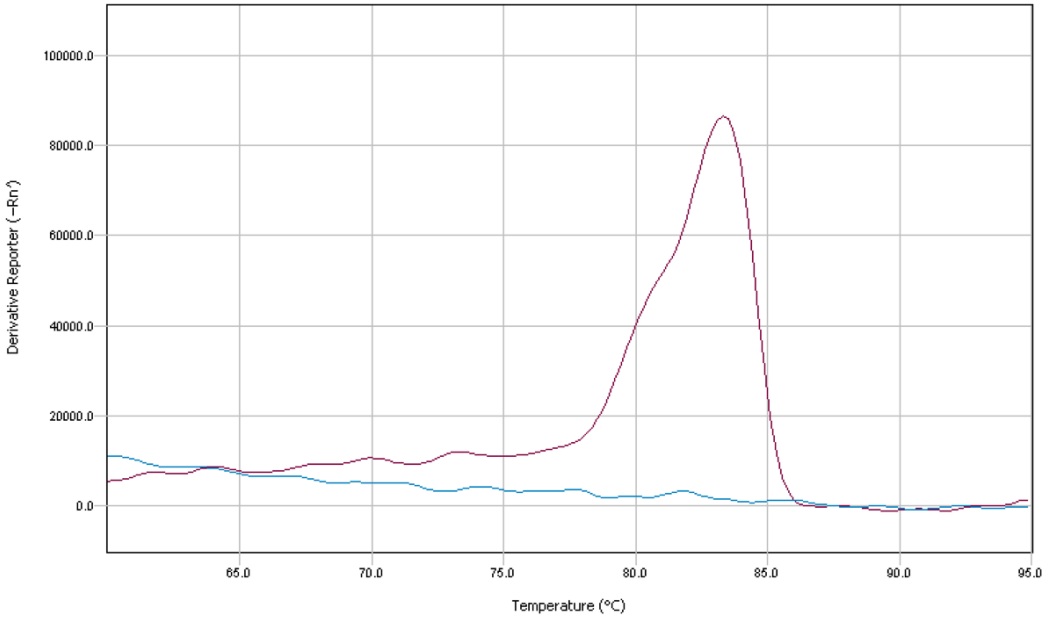

Figure 6. A qPCR melt curve analysis for sdY from two samples of *S. confluentus* gill tissue. Presence of a melt curve in E1 (purple) indicates sdY amplification (male). Curve absence in E2 (blue) indicates no sdY amplification (female).

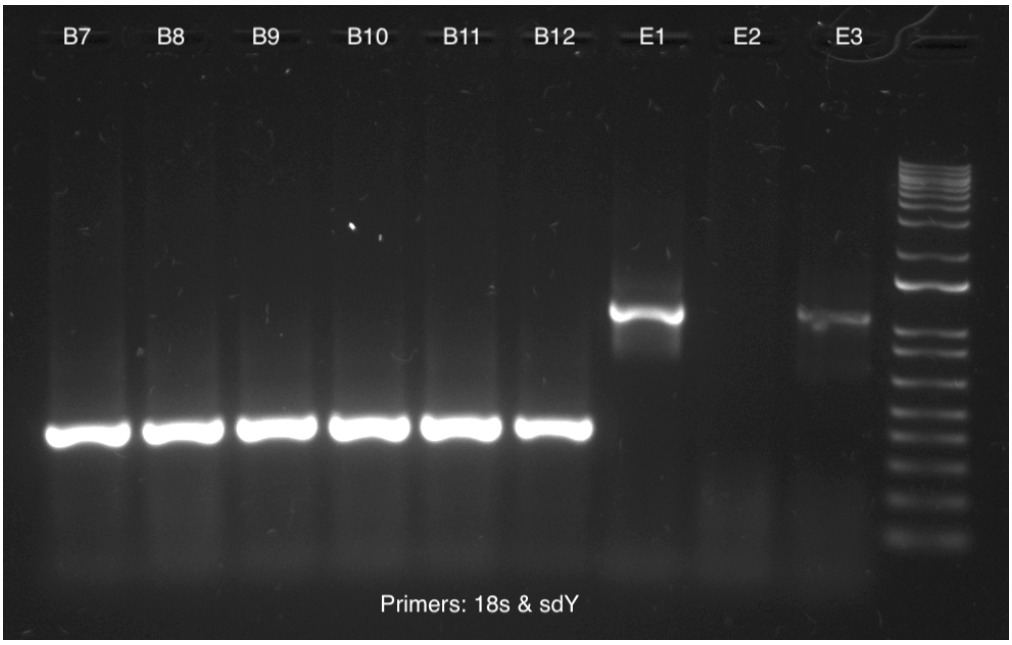

Figure 7. Agarose gel electrophoresis of qPCR products for the primers 18s and sdY with *S. confluentus* gill tissue samples, from wells B7 to B12 (primer 18s) and E1 to E3 (primer sdY). Presence of a fluorescence band indicates successful DNA amplification for the target gene (for sdY, male), whereas absence of a visible band indicates no DNA amplification for that gene (for sdY, female).

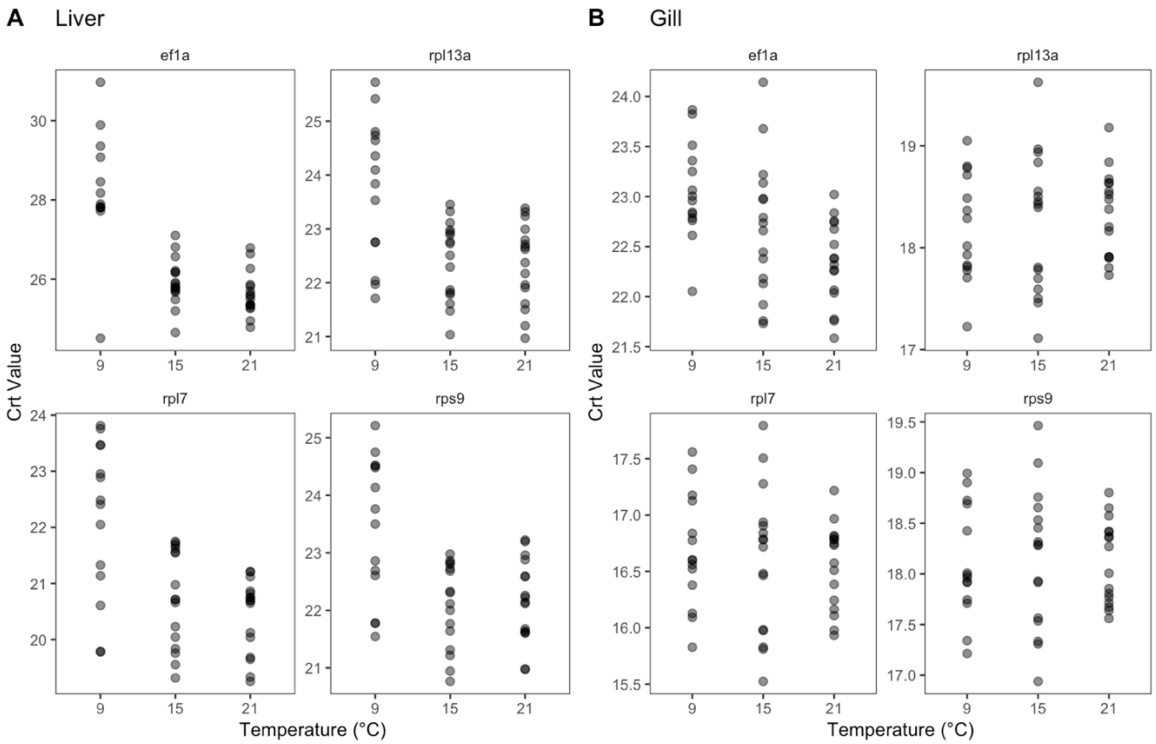

Figure 8. Raw Crt values for reference genes from liver (A) and gill (B) tissue samples to assess stability and thus application as normalization parameters in gene expression analysis for juvenile bull trout (*S. confluentus*) held at three acclimation temperatures (n = 7 for 9°C; n = 8 for 15°C and 21°C) for 60 d.

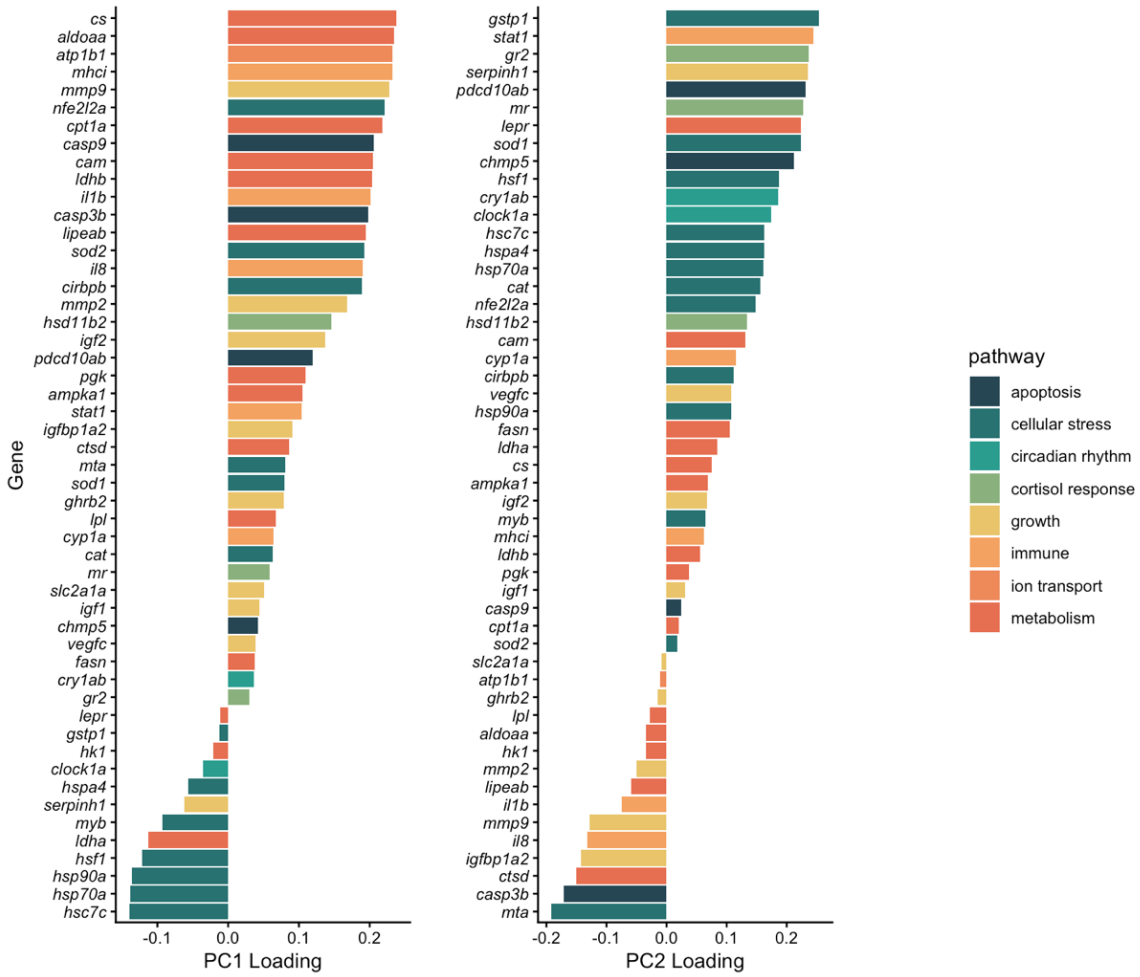

Figure 9. Transcript loading coefficients (x-axis) that contributed to the variance in each principal component of the gill tissue PCA plot (Figure 4A). Transcripts (y-axis) are categorized according to broad functional groups; see Appendix A, Table 2 for the full name and description of each gene.

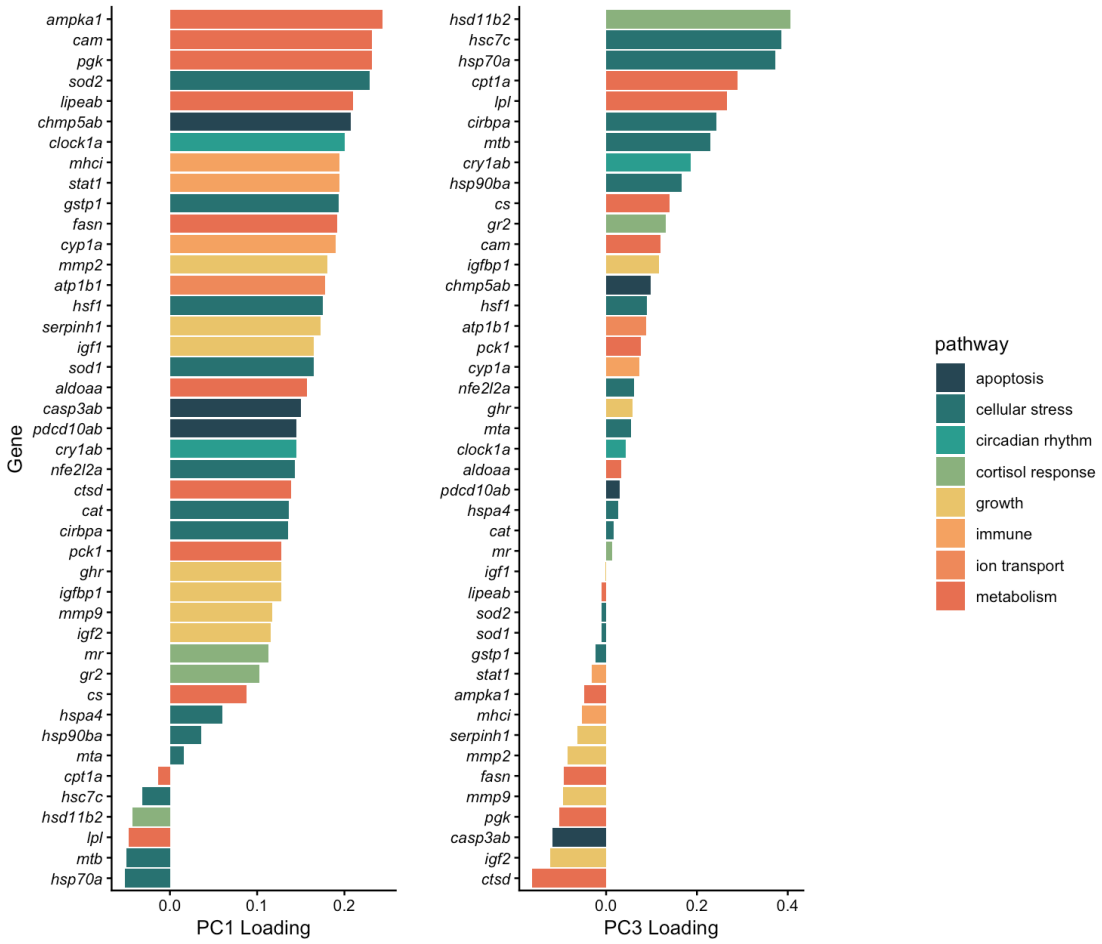

Figure 10. Transcript loading coefficients (x-axis) that contributed to the variance in each principal component of the liver tissue PCA plot (Figure 5A). Transcripts (y-axis) are categorized according to broad functional groups; see Appendix A, Table 2 for the full name and description of each gene.