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RESEARCH ARTICLE

Molecular and physiological responses predict acclimation limits in juvenile brook trout (*Salvelinus fontinalis*)

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ABSTRACT

Understanding the resilience of ectotherms to high temperatures is essential because of the influence of climate change on aquatic ecosystems. The ability of species to acclimate to high temperatures may determine whether populations can persist in their native ranges. We examined physiological and molecular responses of juvenile brook trout (*Salvelinus fontinalis*) to six acclimation temperatures (5, 10, 15, 20, 23 and 25°C) that span the thermal distribution of the species to predict acclimation limits. Brook trout exhibited an upregulation of stress-related mRNA transcripts (*heat shock protein 90-beta*, *heat shock cognate 71 kDa protein*, *glutathione peroxidase 1*) and downregulation of transcription factors and osmoregulation-related transcripts (*nuclear protein 1*, *Na$^+$/K$^+$/2Cl$^-$ co-transporter-1-a*) at temperatures ≥20°C. We then examined the effects of acclimation temperature on metabolic rate (MR) and physiological parameters in fish exposed to an acute exhaustive exercise and air exposure stress. Fish acclimated to temperatures ≥20°C exhibited elevated plasma cortisol and glucose, and muscle lactate after exposure to the acute stress. Fish exhibited longer MR recovery times at 15 and 20°C compared with the 5 and 10°C groups; however, cortisol levels remained elevated at temperatures ≥20°C after 24 h. Oxygen consumption in fish acclimated to 23°C recovered quickest after exposure to acute stress. Standard MR was highest and factorial aerobic scope was lowest for fish held at temperatures ≥20°C. Our findings demonstrate how molecular and physiological responses predict acclimation limits in a freshwater fish as the brook trout in the present study had a limited ability to acclimate to temperatures beyond 20°C.

KEY WORDS: Transcriptomics, Stress response, Metabolic rate, Temperature, Ectotherm, Freshwater fish

INTRODUCTION

In ectotherms, temperature is a 'master' abiotic factor as it affects most major physiological and ecological processes (Fry, 1947; Beitinger and Bennett, 2000; Somero, 2005). For this reason, shifts in the thermal characteristics of a species' habitat that go beyond their specific thermal limits can lead to numerous changes including: altered species distribution, disease outbreaks, phenological modifications and decreased survival (Bassar et al., 2016; Hermoso, 2017; Krabbenhoft et al., 2014). Ultimately, temperatures that surpass the physiological limits of a species will lead to extirpation or extinction (Nogués-Bravo et al., 2018); however, adaptive responses can reduce climate-mediated mortality (Kingsolver and Buckley, 2017).

Acclimation is an adaptive response whereby a reversible phenotypic change occurs because of exposure to an environmental condition (i.e. temperature) for a period of days to months (Hochachka and Somero, 2002; Crozier and Hutchings, 2014; Havird et al., 2020). During acclimation, ectotherms undergo changes in biological processes to maintain homeostasis (Schreck and Tort, 2016). Changes at a cellular level can alter an ectotherm's phenotype and allow for 'normal' processes to occur despite environmental change (e.g. altered expression of protein isoforms; Hochachka and Somero, 2002). Individuals that can acclimate to changing thermal conditions are more likely to contribute to future generations because of increased survival (Somero, 2010). Further, the capacity of an individual to acclimate predicts sublethal responses to thermal stress that could influence performance and reproduction (Komoroske et al., 2015). Therefore, understanding acclimation in ectotherms can predict population-level responses to warming climates (Schulte, 2014).

An organism's ability to acclimate to warm temperatures can be investigated with an approach that integrates whole-organism physiology with molecular techniques to reveal the underlying mechanisms of tolerance (Connon et al., 2018). This approach often uses whole-organism oxygen consumption rate as an indicator of performance because oxygen consumption varies with temperature, suggesting changes in energetic demands being placed on the organism (Pörtner, 2001, 2002; Pörtner et al., 2017). Additionally, when ectotherms are exposed to a thermal disturbance, the glucocorticoid cortisol is released as an end product of the hypothalamic-pituitary–interrenal (HPI) axis (Wendelaar Bonga, 1997; Barton, 2002). Increases in circulating levels of cortisol can result in the increased mobilization of energy resources, such as glucose, which contributes to the increased tissue metabolic rates at higher temperatures (Wendelaar Bonga, 1997; Barton, 2002). Cortisol is also often used to indicate an organism's ability to respond to a stressor (Barton, 2002; Sopinka et al., 2016). Cellular-level responses include changes in the expression of genes responsible for various processes that help ectotherms maintain performance. Therefore, transcriptomics has contributed to advances in understanding the cellular processes behind whole-organism physiological responses (Miller et al., 2014; Evans, 2015). For example, some isoforms of heat shock proteins may be continuously expressed over time (i.e. constitutive), which can indicate that thermal acclimation is occurring in an organism (Iwama et al., 1998). Additionally, inducible isoforms can indicate an acute response to elevated temperatures (Iwama et al., 1998).

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Some cellular responses may also be altered before detrimental physiological or whole-organism changes, which can help to identify sub-lethal thresholds (Jeffries et al., 2014, 2018). Therefore, changes in the expression of some genes, as estimated by the abundance of mRNA transcripts, also provide information about the acute and chronic effects of temperature at the cellular level. Overall, by using molecular approaches to quantify acute and chronic effects of temperature at a cellular level and pairing it with whole-body parameters (HPI response, oxygen consumption), a more complete understanding of an organism's tolerance to changing temperatures and climate can be obtained.

Temperate freshwater fishes are a common ectotherm model for studies on climate change because their distributions are changing (Comte et al., 2013) and extensive work has been done to characterize their ecophysiology (e.g. Costa and Sinervo, 2004; Eliason et al., 2011; Chadwick et al., 2015). Additionally, freshwater fishes are among the most at-risk animals on the planet (WWF, 2020) and it is estimated that 50% of freshwater species are threatened by climate change and associated warming temperatures (Darwall and Freyhof, 2016; Reid et al., 2019). Fishes differ greatly in their abilities to tolerate temperatures outside their thermal distribution (Rahel, 2002) and sometimes individual fish thermoregulate behaviourally to remain in waters with 'preferred' temperatures (Martins et al., 2011; Cott et al., 2015; Raby et al., 2018), or select thermally heterogeneous habitats (Brett, 1971; Neverman and Wurtsbaugh, 1994; Nielsen et al., 1994; Biro, 1998; Newell and Quinn, 2005). However, if a fish is unable to leave or acclimate to the temperature of the environment, exposure may lead to mortality (e.g. Hasler et al., 2012). Therefore, acclimation is a critical process to study to better understand how freshwater fish will respond to climate change.

In our study, we aimed to address how multiple scales of biological organization respond during acclimation to a range of temperatures to predict sub-lethal and lethal thermal limits in a temperate freshwater fish. We used brook trout, *Salvelinus fontinalis* (Mitchill 1814), a predominantly freshwater salmonid species in North America that is under threat from warming temperatures in its native range (Chadwick et al., 2015). The preferred temperature (i.e. temperature at which fish congregate when placed within a thermal gradient in the laboratory) for brook trout is approximately 15°C (Graham, 1949; Fry, 1971; Cherry et al., 1977; Stitt et al., 2014; Smith and Ridgway, 2019) and habitat use in the wild becomes limited at temperatures ranging from 21 to 23.5°C (Meisner, 1990; Benfey et al., 1997; DeWeber and Wagner, 2015; Chadwick et al., 2015; Chadwick and McCormick, 2017), suggesting a sub-lethal thermal limit. Climate projections show 49% contraction in the southern limit of brook trout distribution by 2050 (Meisner, 1990; Chu et al., 2005; Flebbe et al., 2006). Because of the risk of rising temperatures and the habitat requirements of brook trout, it is important to understand how thermal acclimation occurs across multiple scales of biological organization for this species.

Our first objective was to examine the effects of temperature acclimation on cellular and physiological processes in juvenile brook trout to identify sub-lethal thresholds and acclimation limits for this species. Our second objective was to examine the effects of acclimation temperature on blood stress indices and metabolic rate following exposure to acute exhaustive exercise and air exposure stressors, as well as metabolic rate recovery following the stressor. Previous studies have suggested that temperatures between 20 and 23°C lead to reduced physiological performance in brook trout (Smith and Ridgway, 2019; Morrison et al., 2020). Brook trout in the present study were acclimated for a minimum of 21 days to one of six different temperatures (5, 10, 15, 20, 23 and 25°C) that span their thermal distribution. We predicted that mRNA abundance of constitutive transcripts associated with the cellular stress response (i.e. reducing damage to cellular proteins due to heat stress, preventing damage from reactive oxygen species, and regulating cell growth) would be elevated when fish are acclimated to temperatures beyond a sub-lethal threshold (i.e. >20°C in brook trout). Additionally, we predicted that an exhaustive exercise and air exposure treatment would increase levels of physiological indices of stress (i.e. plasma cortisol, plasma glucose, plasma osmolality and muscle lactate) at temperatures ≥20°C. Further, we predicted that metabolic rate would increase with temperature (i.e. standard metabolic rate, SMR), where those fish exposed to the higher temperature groups (≥20°C) would experience the longest metabolic recovery from acute exhaustive exercise and air exposure stressor. In this study, we found that brook trout have a reduced ability to acclimate to temperatures ≥20°C as supported by changes in mRNA transcript abundance, SMR and the ability to recover from exercise stress.

MATERIALS AND METHODS

Study animals

The juvenile brook trout (mass 38.3±1.7 g, fork length 14.5 ±0.2 cm) used in this study were first generation (F1) brook trout originally obtained from the Whiteshell Fish Hatchery in eastern Manitoba, Canada. In 2016, brood stock brook trout were bred at the Fisheries and Oceans Canada (DFO) Freshwater Institute in Winnipeg, MB, Canada. After hatching (January 2017) and when past the swim-up stage, fish were moved to one of two aerated 600 l circular flow-through tanks held at approximately 10°C. Fish were fed *ad libitum* with commercial pellet fish food (EWOS Pacific, Complete Fish Feed for Salmonids, Cargill, Winnipeg, MB, Canada) for a 35 week rearing period. All methods were approved by the Freshwater Institute Animal Care Committee (FWI-ACC-AUP-2018-02/2019-02).

Temperature treatment

Juvenile brook trout (n=140) were haphazardly netted from the general population tank and placed into 200 l aerated, flow-through tanks and exposed to one of six temperatures (5, 10, 15, 20, 23 and 25°C; n=50 per temperature tank) for 21–30 days. Because of logistic constraints, temperature exposures were staggered across four months in 2018–19: 10°C beginning on 11 October, 25°C on 23 October, 23°C on 2 November, 20°C on 16 November, 15°C on 17 December and 5°C on 1 January. The treatments were conducted in random order (i.e. starting with 10°C and ending with 5°C) to try to minimize growth or timing effects. On the first day of each temperature treatment, fish were transferred to a 200 l acclimation tank at ∼10°C and were given 1 day to recover from the handling stress. The water temperature was then gradually adjusted to the assigned treatment temperature at a rate of 1.5–2°C day^{-1} using heating or cooling coils that were placed in an auxiliary tank plumbed to the holding tank. Once the treatment temperature was reached, fish remained at the temperature for a minimum 21 day acclimation period (Beitinger et al., 2000). Throughout the treatment period, the water temperature of the holding tank was measured using a HOBO Tidbit v2 Sensor (ONSET Computer Corporation, Bourne, MA, USA) and controlled with WitroxCTRL software (Loligo® Systems, Tjele, Denmark), where it fluctuated daily by ±1.5°C of the treatment temperature to simulate diurnal temperature changes (Durhack et al., 2021). A 12 h:12 h day:night cycle was used throughout the experiment for all treatments (65 min of dawn and dusk, full-light starting at 07:05 h, and full dark at

19:05 h) and there was no natural light entering the room. Dissolved oxygen was kept above 7 mg l^{-1} throughout the experiment. After the 21 day acclimation period, fish were assigned to one of three groups: unhandled, acute stress or acute recovery. Those in the unhandled group were immediately sampled (see 'Tissue sampling of unhandled group', below; n=10). Those fish in the acute groups were exposed to a 2 min chase test and 5 min air exposure, and were either sampled 30 min after the stressor exposure to allow physiological parameters to reach elevated values (the acute stress group; n=8; Biron and Benfey, 1994; Benfey and Biron, 2000) or placed in a respirometry chamber for 24 h and sampled afterwards (the acute recovery group; n=8), as detailed below. Fish from the 25°C group were not exposed to the full 21 day treatment period, as they exhibited potential fungal infections, reduced feeding and mortality. Therefore, the 25°C treatment group was sampled for tissues after 11 days (see below) and these fish were not subjected to the acute stress experiments. Additionally, there were two mortalities after assessment of maximum metabolic rate (MMR) by intermittent-flow respirometry (see below) in the fish from the 20°C temperature group. These values were not used in any of the calculations for the study.

Tissue sampling of unhandled group

Fish in the unhandled group (n=60; n=10 per treatment group) were individually euthanized in a buffered tricaine methanesulfonate solution (300 mg l^{-1} MS-222; buffered with 600 mg l^{-1} $NaHCO_3$) with water at the same temperature as for their treatment. Fish were measured for length and body mass prior to tissue sampling. Blood was collected by severing the caudal fin and using ammonium-heparinized capillary tubes (Fisherbrand®, Thermo Fisher Scientific, Pittsburgh, PA, USA). Whole-blood glucose was immediately measured using a UltraMini® Glucose Meter (OneTouch®, LifeScan Canada, Burnaby, BC, Canada), after which blood samples were centrifuged at 3000 ***g*** for 6 min. Plasma was removed, flash frozen in liquid nitrogen and stored at −80°C until analysis. The second two gill arches from the left side (looking anteriorly) of each fish and the liver were sampled and placed in RNA*later*™ (Invitrogen™, Carlsbad, CA, USA) and stored at 4°C overnight prior to storage at −80°C. White muscle was taken from the fish's right side (looking anteriorly), placed in liquid nitrogen and stored at −80°C measurement of muscle lactate.

Acute stress and recovery

For the acute stress and acute recovery groups, another subset of fish from each acclimation temperature, with the exception of the 25°C group (see above), underwent an acute 2 min chase (e.g. Suski et al., 2006) and 5 min air exposure (e.g. Gingerich et al., 2007) and/or a recovery trial (n=80; n=8 per temperature and acute stress group group). The chase test consisted of placing individual fish into a bucket (diameter ∼43 cm) with 15–20 l of water at the acclimation temperature. There was a pipe in the centre of the bucket to force the fish to swim around the perimeter of the container. Once the water was added to the bucket, the bucket was plugged, and the fish was manually chased using a small net. After 2 min, the fish was netted out of the water and air exposed for 5 min, during which time it was measured for length and mass. Following the acute stressor exposure, eight fish were placed into a holding tank at their acclimation temperature for 30 min to allow plasma cortisol and glucose to reach the putative peak values (Biron and Benfey, 1994; Benfey and Biron, 2000) before being sampled for blood and white muscle as described above for unhandled individuals.

The remaining eight fish from the acute stressor exposure (i.e. those fish not placed into holding tanks following the stressor) were placed in an intermittent-flow respirometry system, in a water bath held at the same temperature as the acclimation treatment, for 24 h to record oxygen consumption. After the 24 h concluded fish were sampled for blood and white muscle as described previously. Intermittent-flow respirometry was used to quantify oxygen consumption as an estimate of metabolic rate. Oxygen consumption was measured by in-line probes connected to respirometry chambers (Presence, Regensburg, Germany) and automatically calculated by AutoResp software (Loligo Systems, Viborg, Denmark). To validate the quality of measurements, r^2 values for rates of oxygen decline were also automatically generated. Only r^2 values above 0.9 were used for final analysis of SMR and MMR. For SMR calculations, the lowest 20th quantile of oxygen consumption rate ($\dot{M}_{O_2}$ estimates) were used after removing the first 10 h of measurements to ensure fish were at minimum oxygen consumption levels; Chabot et al., 2016; Norin and Clark, 2016). Following this, the 'FishMO2' package in R (Chabot et al., 2016) was used to analyse $\dot{M}_{O_2}$ estimates over time including the calculation of SMR and plotting of r^2 values for each individual at each temperature to include those with r^2>0.9. Background respiration from microbial respiration (biochemical oxygen demand, BOD) was also estimated by including an empty respirometry chamber in each trial and oxygen consumption values from these chambers were subtracted from the SMR measurements. MMR was estimated from the acute recovery treatment fish. Three measurements of oxygen consumption were taken to estimate MMR when each fish was first placed into the respirometry chamber post-exercise and air exposure event (Norin and Clark, 2016). Time to recovery was determined as the elapsed time from when the fish was placed into the respirometer to the time when oxygen consumption first began to stabilize (Cooke et al., 2014). This allowed us to capture the progressive decline in oxygen consumption to more baseline levels where presumably recovery of metabolites and stress hormones is possible, too (Brett, 1971; Eliason et al., 2013; Zhang et al., 2018). Aerobic scope (AS) was calculated by subtracting SMR estimates from MMR for each fish. Factorial aerobic scope (FAS) was also calculated by comparing MMR with SMR (MMR/SMR; Eliason and Farrell, 2016).

Physiological assays

Blood plasma samples were used to measure cortisol, glucose and osmolality. Plasma cortisol levels were quantified using an enzyme-linked immunosorbent assay (ELISA; 1:50 dilution; Neogen Corporation, Lexington, KY, USA), previously validated for use in other salmonids (e.g. Jeffries et al., 2012b; Sopinka et al., 2017; Durhack et al., 2020). The plasma osmolality was determined using a VAPRO vapour pressure osmometer (Wescor Inc., Logan, UT, USA). Plasma glucose was quantified using a hexokinase kinetic assay that was adapted for a 96-well plate (Treberg et al., 2007) where plasma samples were diluted 1:30. Results from the hexokinase kinetic assay were used to develop a correction factor for the whole-blood glucose levels measured using the UltraMini® Glucose Meter (see 'Tissue sampling of unhandled group' above). Glucose values from the hexokinase kinetic assay and the glucose meter were compared using linear regression and the resulting equation of the line was used to correct whole-blood values measured with the handheld meter (Fig. S1).

White muscle was used to determine the amount of muscle lactate. White muscle samples were first powdered in liquid nitrogen using a mortar and pestle, and tissue metabolites were extracted

using an 8% perchloric acid solution mixed with EDTA, which was later neutralized using a base solution (mixture of sodium hydroxide, sodium chloride and imidazole) to pH 7–8 (Booth et al., 1995). After metabolite extraction, lactate concentration was determined using an enzymatic assay that utilized the reaction of converting lactate to pyruvate using NAD^+ (nicotinamide adenine dinucleotide) and lactate dehydrogenase (Lowry and Passonneau, 1972; Gutman and Wahlefeld, 1974).

Quantitative PCR

Total RNA was extracted from the gill and liver tissues using a Qiagen RNeasy Plus Mini Kit (Qiagen, Toronto, ON, Canada) following the manufacturer's protocols. The RNA samples were checked for purity (A_{260}/A_{280}, A_{260}/A_{230}) and concentration using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The integrity of the RNA was assessed by electrophoresis on a 1% agarose gel. For cDNA preparation, 1 µg of total RNA was reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocols, with the exception that the total volume was scaled to 32 µl.

All forward and reverse quantitative PCR (qPCR) primers (Table 1) were designed using Primer Express 3.0.1 (Applied Biosystems, Thermo Fisher Scientific). Primers were designed using sequences from the brook trout transcriptome from Sutherland et al. (2019) (Table S1). Primers were designed for 13 target genes (Table 1) that represented transcriptomic responses consistent with high temperature [*cold-inducible RNA-binding protein* (*cirbp*), *heat shock cognate 71 kDa protein* (*hspa8*), *heat shock protein 90-beta-1* (*hsp90ab1*), *serpin h1* (*serpinh1*)], osmoregulatory function [Na^+/K^+-*transporting ATPase subunit alpha-3* (*atp1a3*), *cystic fibrosis transmembrane conductance regulator* (*cftr*), *ATP-sensitive inward rectifier* K^+ *channel 8* (*irk8*), $Na^+/K^+/2Cl^-$ *co-transporter-1-a* (*nkcc1a*), *V-type H-ATPase* B and E1 subunits (*vatb* and *vate1*)] and general cellular function [*glucose-6-phosphatase* (*g6pc*), *glutathione peroxidase-like peroxiredoxin* (*gpx1*), *nuclear protein 1* (*nupr1*)]. Target genes were selected based on previous literature that identified these genes as being expressed during times of stress or exposure to high temperatures in salmonids (Momoda et al., 2007; Jeffries et al., 2012a,b; Jeffries et al., 2014; Akbarzadeh et al., 2018; Swirplies et al., 2019). Primers were designed for three reference genes, *60s ribosomal protein L7* and *L8* (*rpl7* and *rpl8*) and *40s ribosomal protein S9* (*rps9*) (Table 1). Primer efficiency was tested by generating standard curves using cDNA synthesized from the RNA pooled from 6 individuals from the treatment groups. Each 12 µl qPCR reaction consisted of 1 µl of a 1:10 dilution of cDNA, 500 nmol l^{-1} forward and reverse primer, 6 µl of PowerUP SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific) and 4.8 µl RNase-free water. The qPCR reactions were run on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Life Technologies Corporation, Carlsbad, CA, USA) in

Table 1. Primer sequences for qPCR in brook trout (*Salvelinus fontinalis*)

Gene	Function	Primer sequence (5′–3′)	Product size (bp)	Efficiency (%)
atp1a3	Na^+/K^+ regulation	F: TCCTGGCCTACGGAATCCA R: GAGCACAACACCCAGGTACAAA	74	96[G]
cftr	Chloride transporter	F: TCAAACAACGCCCCGATAC R: CAACCTGACCACCACTGAGGTA	75	96[G]
cirbp	RNA stabilization involved in osmotic stress and cold shock response	F: AGGTATGGGCAGGCAATCTG R: AAGAGGGAGGGCAAGACAAAA	73	101[G]
g6pc	Glycogen metabolism	F: CACTTCCCTCACCAGGTTGT R: TCCATTGGACCCGGTCAAAG	76	111[L]
gpx1	Oxidative stress response, involved in cell immunity	F: CGTTCTTGCAGTTCTCCTGATG R: ACCGACAAGGGTCTCGTGAT	70	90[G]; 91[L]
hspa8	Heat shock protein, regulatory role in autophagy	F: GGGTTCATGGCAACCTGATT R: ACGTTGCCTTCACTGACTCTGA	67	91[G]; 94[L]
hsp90ab1	Heat shock protein, role in cell response to stress and buffer against cell mutation	F: CAACATGGAGCGCATCATG R: CAGGTGTTTCTTGGCCATCA	79	93[G]; 97[L]
irk8	ATP-sensitive inward rectifier K^+ channel 8	F: CCCTGTTCTCGGATGTTCTTG R: GGTGAACAAAGCACGCTTCA	72	87[G]
nkcc1a	Ion regulation	F: CGGGAATTGTTCTCTCCTGTGT R: GCAATCGCTGAGGTCGAAA	82	101[G]
nupr1	Cell growth and apoptosis regulator	F: TGGCCTTCTTTTCAGTGTTCTG R: GGAAGCCAGCGACAATACCA	89	96[L]
rpl7	Reference gene ribosomal protein L7	F: TCTGACGCAGACGCATGAG R: CGAAACTGGCCTTCGTCATC	86	88[G]; 95[L]
rpl8	Reference gene ribosomal protein L8	F: GCCACAGTCATCTCCCACAA R: GGAGCCAGAGGGAAGCTTAAC	63	96[G]; 91[L]
rps9	Reference gene ribosomal protein S9	F: GAGTTGGGTTTGTCGCAAGAC R: CCTGGTCGAGACGAGACTTCTC	68	85[G]; 100[L]
serpinh1	Biosynthesis of collagen and role in restoration of homeostasis	F: CCCAAGCTGTTCTACGCTGA R: AGTCTGCCGAGGAAGAGGAT	83	92[G]; 92[L]
vatb	Regulation of H^+ gradient	F: GCTTCAGCATTTCTTTGGGAAA R: TCAGGGCCCTTATGACAACAG	89	104[G]
vate1	Regulation of H^+ gradient	F: GGCTGGGTCCTTGGCTATGT R: GGTGTTAAAGGCTCGCGACG	85	97[G]

F, forward primer; R, reverse primer. For efficiency values, superscript L denotes liver, superscript G denotes gill.
atp1a3, Na^+/K^+-*transporting ATPase subunit alpha-3*; *cftr*, *cystic fibrosis transmembrane conductance regulator*; *cirbp*, *cold-inducible RNA-binding protein*; *g6pc*, *glucose-6-phosphatase*; *gpx1*, *glutathione peroxidase-like peroxiredoxin*; *irk8*, *ATP-sensitive inward rectifier* K^+ *channel 8*; *hspa8*, *heat shock cognate 71 kDa protein*; *hsp90ab1*, *heat shock protein 90-beta-1*; *nkcc1a*, $Na^+/K^+/2Cl^-$ *co-transporter-1-a*; *nupr1*, *nuclear protein 1*; *vatb*, *V-type H-ATPase B*; *vate1*, *V-type H-ATPase E1*; *rpl7*, *60 s ribosomal protein L7*; *rpl8*, *60 s ribosomal protein L8*; *rps9*, *40 s ribosomal protein S9*.

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384-well plates. Target mRNA levels were normalized to the three reference genes using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). The stability of the reference genes across treatments was confirmed using a pair-wise comparison with BestKeeper Version 1 (Pfaffl et al., 2004).

Statistical analysis

To determine whether mass was a significant factor contributing to physiological response variables (plasma cortisol, plasma glucose, tissue lactate, osmolality), a two-way analysis of covariance (ANCOVA) was initially run, but mass was shown to have no significant effect. Therefore, the effect of acclimation temperature and treatment (unhandled, acute stress, acute recovery) on the physiological response variables (plasma cortisol, plasma glucose, tissue lactate, osmolality) was examined using two-way analyses of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) *post hoc* tests. Assumptions of normality and equal variance were assessed using a Shapiro–Wilk normality test and a Levene's test, respectively. If assumptions of normality and equal variance were not met, a generalized linear mixed effects model (glmm; http://www.R-project.org/) was used. We focused on the effects of the exhaustive exercise and air exposure stressor within an acclimation temperature and across acclimation temperatures within the three treatment groups for the *post hoc* tests (Tukey HSD or glmm).

To determine the effect of acclimation temperature on the mRNA abundance and oxygen consumption parameters (SMR, MMR, time to recovery and AS), ANOVA were used followed by Tukey's HSD *post hoc* tests. If data failed to meet the assumptions of the ANOVA (see above), a Kruskal–Wallis test was run, followed by Dunn's *post hoc* test. For metabolic estimates (SMR and MMR), mass was used in the calculation of these metrics and therefore mass-specific effects are not a factor and there was no significant difference in mass between treatments. All statistical analyses were run in R v.1.2.5033 (http://www.R-project.org/). The level of significance (α) was 0.05 when one variable was analysed (i.e. mRNA abundance) and for multiple comparisons (i.e. treatment and temperature). Only qPCR results that were statistically different between acclimation groups are presented.

RESULTS

Chronic temperature exposure effects on mRNA abundance

Gill mRNA abundance differed across acclimation temperatures for genes associated with thermal and oxidative stress (Fig. 1). The abundance of *gpx1* mRNA in the gill was 2-fold higher in fish acclimated to 23 and 25°C than in those at 15°C and below (Fig. 1A; one-way ANOVA, $F_{5,59}$=5.735, P<0.001). Conversely, the abundance of *hsp90ab1* mRNA in the fish gill was not significantly elevated until the acclimation temperature reached 25°C, with a 2-fold increase in comparison to the three coldest temperature treatments (Fig. 1B; one-way ANOVA, $F_{5,59}$=4.321, P=0.002).The abundance of *nkcc1a* mRNA in the gill of fish held at 23 and 25°C was 3-fold lower than at 5 and 10°C (Fig. 1C; one-way ANOVA, $F_{5,59}$=5.069, P<0.001).

In liver tissue, the mRNA abundance of fives genes displayed significant responses (Fig. 2). The mRNA abundance of heat shock proteins *hspa8* (*hsc70*) and *hsp90ab1* in the liver was significantly elevated in fish acclimated to 23 and 25°C, with a 4-fold (Fig. 2C; one-way ANOVA, $F_{5,59}$=8.828, P<0.001) and 6-fold increase (Fig. 2D; one-way ANOVA, $F_{5,59}$=15.14, P<0.001), respectively, than at 15°C and below. The mRNA abundance of *gpx1* in the liver was elevated 4-fold in fish acclimated to 5°C compared with those at 20°C and above (Fig. 2B; one-way ANOVA, $F_{5,59}$=4.924, P=0.001). The mRNA abundance of *g6pc* in the liver was highest in fish acclimated to 20°C and 3-fold higher than in those at 5°C (Fig. 2A; one-way ANOVA, $F_{5,59}$=3.123, P=0.016). Lastly, *nupr1*

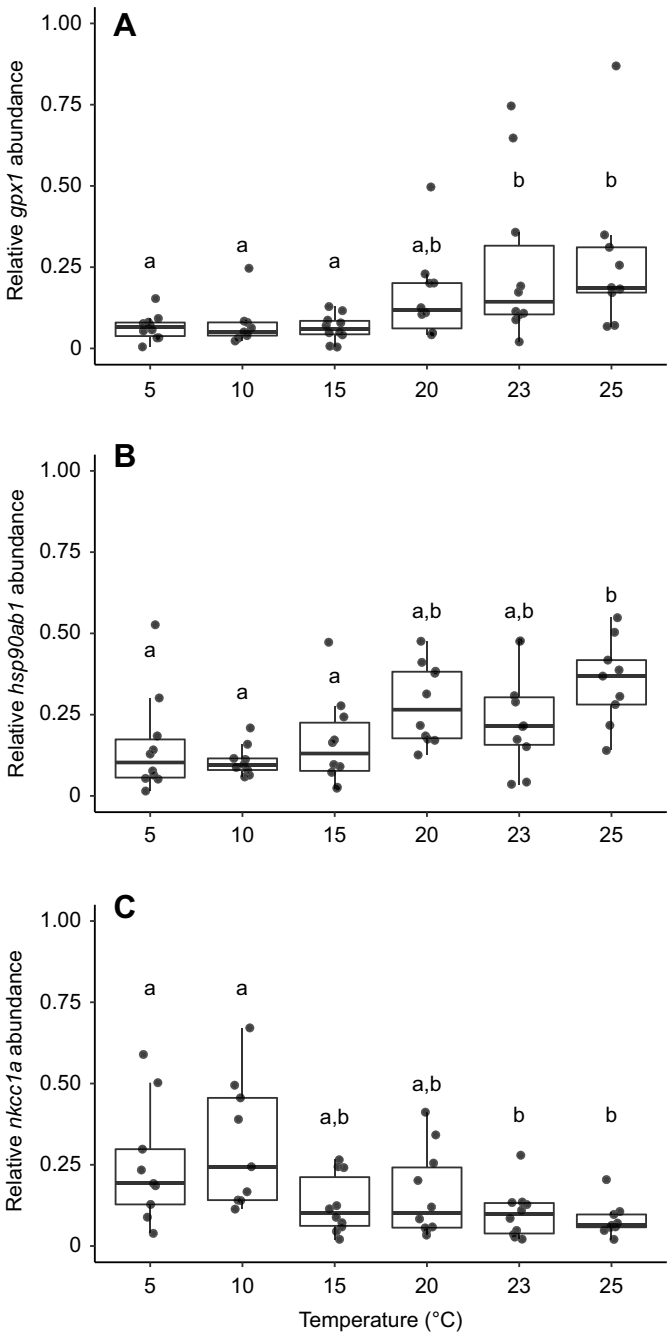

Fig. 1. Transcript abundance of thermal stress biomarkers in gill tissue for juvenile brook trout (*Salvelinus fontinalis*) acclimated to temperatures spanning their thermal distribution (*n*=10). Relative mRNA abundance of (A) *glutathione peroxidase-like peroxiredoxin*, *gpx1*; (B) *heat shock protein 90-beta-1*, *hsp90ab1*; and (C) $Na^+/K^+/2Cl^-$ *co-transporter-1-a*, *nckcc1a*. Fish were held for 21 days at the respective acclimation temperature, with the exception of those from the 25°C treatment, which were sampled after 11 days (see Materials and Methods for details). Groups that do not share a letter are significantly different from one another (one-way ANOVA, P<0.05; see Table S2). Horizontal bars in the boxplot represent the median response value and the 75% and 25% quartiles. Whiskers represent ±1.5 times the interquartile range, and each dot represents an individual response value.

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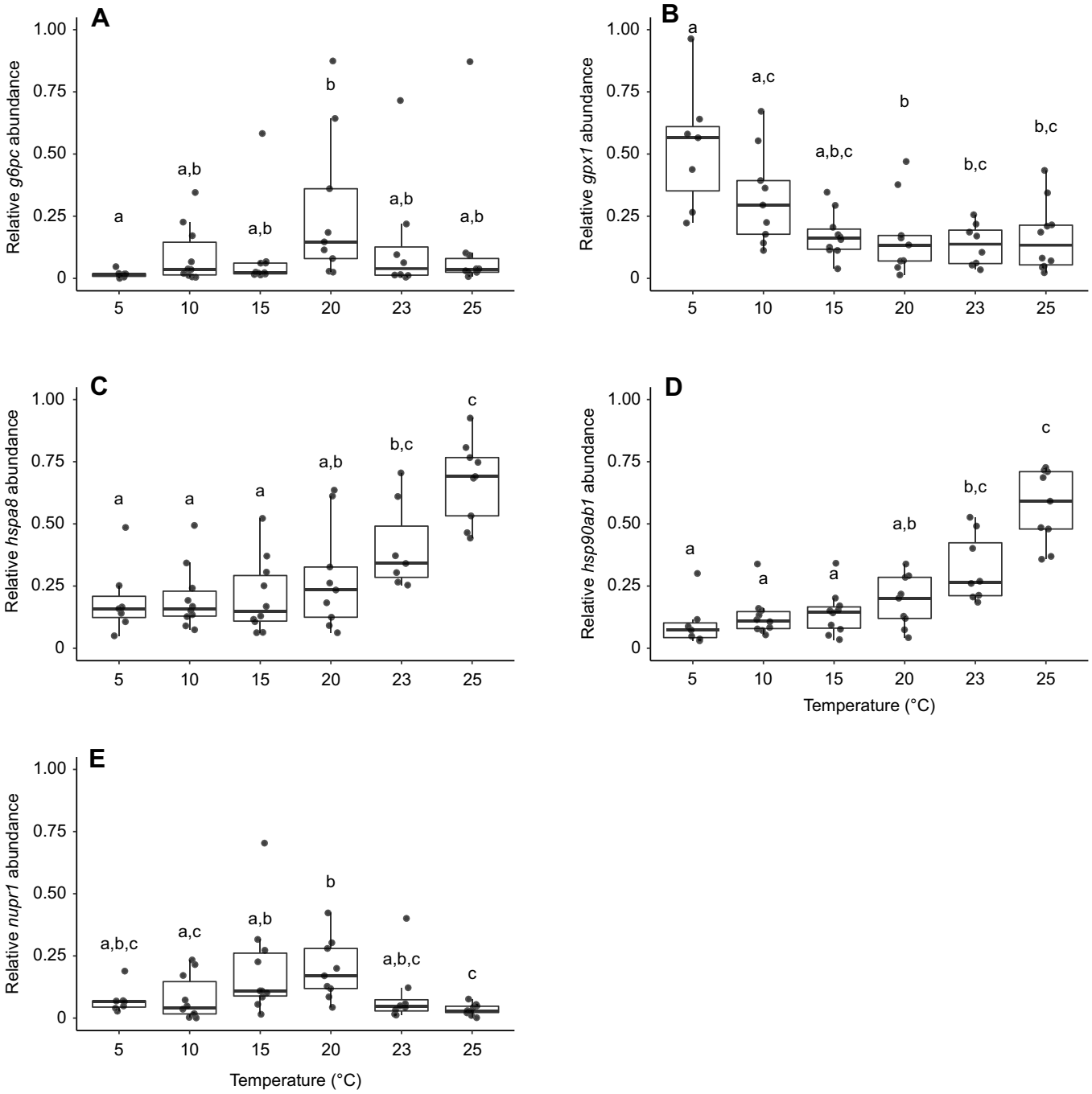

Fig. 2. Transcript abundance of thermal stress biomarkers in liver tissue for juvenile brook trout acclimated to temperatures spanning their thermal distribution (*n*=10). Relative mRNA abundance of (A) *glucose-6-phosphatase*, *gcpc*; (B) *glutathione peroxidase-like peroxiredoxin*, *gpx1*; (C) *heat shock cognate 71 kDa protein*, *hspa8*; (D) *heat shock protein 90-beta-1*, *hsp90ab1*; and (E) *nuclear protein 1*, *nupr1*. Fish were held for 21 days at the respective acclimation temperature, with the exception those from the 25°C treatment, which were sampled after 11 days (see Materials and Methods for details). Groups that do not share a letter are significantly different from one another (one-way ANOVA, $P<0.05$; see Table S2). Horizontal bars in the boxplot represent the median response value and the 75% and 25% quartiles. Whiskers represent ±1.5 times the interquartile range, and each dot represents an individual response value.

mRNA abundance in the liver tissue was significantly elevated by 2-fold in fish acclimated to 20°C versus 25°C (Fig. 2E; one-way ANOVA; $F_{5,59}=4.918$; $P=0.001$).

Chronic temperature exposure effects on the acute stress response

Chasing followed by air exposure had a significant effect on physiological variables associated with the stress response in juvenile brook trout. Regardless of the acclimation temperature, muscle lactate was elevated 30 min following stressor exposure by an average of 1.8 times compared with that of fish sampled directly out of the acclimation tanks (i.e. unhandled) (Fig. 3A; two-way ANOVA, treatment×temperature, $F_8=0.513$, $P<0.001$; see Table S3 for further statistical details). Muscle lactate levels returned to or fell below (for 23°C) pre-stressed levels following 24 h of recovery across all acclimation temperatures. Fish held at 23°C exhibited the

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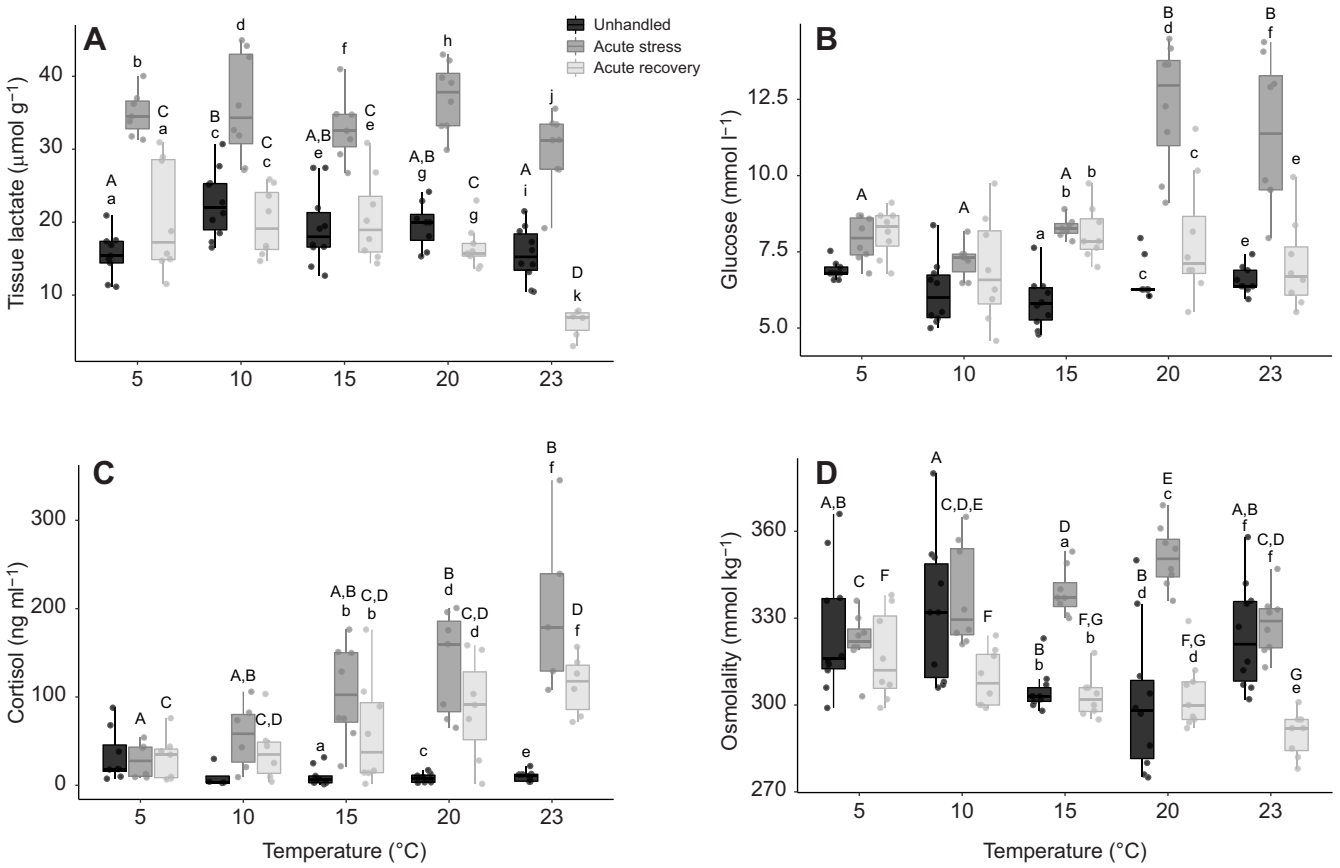

Fig. 3. Physiological parameters collected from juvenile brook trout acclimated to temperatures spanning their thermal distribution for 21 days. (A) Muscle lactate, (B) plasma glucose, (C) plasma cortisol and (D) plasma osmolality were measured in fish directly sampled from the acclimation tank (unhandled, n=10), and sampled 30 min (acute stress, n=8) and 24 h (acute recovery, n=8) after exposure to an acute stressor, consisting of 3 min of chasing and 5 min of air exposure. Within an acclimation temperature, groups that do not share a lowercase letter are significantly different from one another. Within a group, acclimation temperatures that do not share an uppercase letter are significantly different from one another. For muscle lactate, plasma glucose and plasma cortisol, data were analysed using a two-way ANOVA (P<0.05; see Table S3). Plasma osmolality was analysed using a glmm (P<0.05; see Table 2). Horizontal bars in the boxplot represent the median response value and the 75% and 25% quartiles. Whiskers represent ±1.5 times the interquartile range, and each dot represents an individual response value.

lowest muscle lactate levels after 24 h of recovery from the stressor (6.2±0.8 μmol g^{-1}) compared with those in the recovery groups at the other temperatures.

Acclimation temperature also had a significant effect on both the plasma cortisol and glucose response to an acute stressor. For fish exposed to colder temperatures (5 and 10°C), exposure to the acute stressor had no significant effect on either plasma cortisol (Fig. 3C) or glucose (Fig. 3B) levels 30 min post-stressor exposure or following 24 h of recovery. However, fish exposed to warmer temperatures (15, 20 and 23°C) exhibited significantly elevated plasma cortisol and glucose levels following stressor exposure. Plasma cortisol levels remained elevated 24 h post-stressor exposure in fish exposed to warmer temperatures, whereas comparisons across temperatures showed that following recovery, fish at 23°C exhibited significantly higher cortisol levels than those at 5°C (two-way ANOVA, treatment×temperature, F_8=4.31, P=0.001; see Table S3 for further statistical details). Plasma glucose levels returned to pre-stress levels (i.e. unhandled) following 24 h of recovery for fish held at 20 and 23°C, but not for those held at 15°C (two-way ANOVA, treatment×temperature, F_8=6.929, P<0.001; see Table S3 for further statistical details). Comparisons across acclimation temperatures within the acute stress group showed significant elevation of plasma glucose in fish acclimated to 20 and 23°C versus 5, 10 and 15°C.

Similarly, plasma osmolality did not differ significantly among groups (i.e. unhandled, acute stress, acute recovery) for fish held at colder acclimation temperatures (5 and 10°C; Fig. 3D, Table 2). Plasma osmolality increased by approximately 1.1- and 1.2-fold in response to stressor exposure for fish acclimated to 15 and 20°C, respectively (Table 2). At 23°C, fish had significantly lower plasma osmolality 24 h post-stressor exposure, but levels did not differ significantly between the unhandled fish and fish exposed to the acute stressor (Table 2).

Chronic temperature exposure effects on metabolic rate

Mean SMR of juvenile brook trout was not different between temperature treatments (0.0817, 0.1164 and 0.1119 mg O_2 g^{-1} h^{-1} for 5, 10 and 15°C acclimation, respectively) until acclimation temperature reached 20°C (Fig. 4A; Kruskal–Wallis, $\chi^2_{4,40}$=30.43, P<0.001; see Table S4 for further statistical details). Mean SMR increased to 0.174±0.875 mg O_2 g^{-1} h^{-1} for fish acclimated to 20°C and increased 1.4 times further to 0.247±0.538 mg O_2 g^{-1} h^{-1} in fish held at 23°C. Mean MMR was highest in juvenile brook trout acclimated to 23°C (0.514±0.916 mg O_2 g^{-1} h^{-1}) and lowest for fish held at 5°C (0.292±2.311 mg O_2 g^{-1} h^{-1}; Fig. 4B; one-way ANOVA, $F_{4,40}$=8.84, P<0.001; see Table S4 for further statistical details). Mean AS was not significantly affected by acclimation temperature, being lowest at 5°C (0.210±0.014 mg O_2 g^{-1} h^{-1}) and

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Table 2. Results of the generalized linear model for plasma osmolality of juvenile brook trout acclimated to different temperatures and exposed to acute stress

Coefficients	Estimate	s.e.	*t*-value	*P*-value
Intercept	0.0030	0.0000	64.96	**<0.001**
Treatment Acute-Recovery	0.0000	0.0000	1.201	0.232
Treatment Acute-Stress	0.0000	0.0000	0.491	0.624
Temperature 10	−0.0000	0.0000	−0.921	0.359
Temperature 15	0.0002	0.0000	3.043	**0.003**
Temperature 20	0.0002	0.0000	3.601	**<0.001**
Temperature 23	0.0000	0.0000	0.254	0.799
Treatment Acute-Recovery ×Temperature 10	0.0001	0.0001	1.359	0.177
Treatment Acute-Stress ×Temperature 10	−0.0000	0.0000	−0.834	0.406
Treatment Acute-Recovery ×Temperature 15	−0.0000	0.0001	−0.629	0.530
Treatment Acute-Stress ×Temperature 15	−0.0004	0.0001	−3.611	**<0.001**
Treatment Acute-Recovery ×Temperature 20	−0.0001	0.0001	−1.099	0.274
Treatment Acute-Stress ×Temperature 20	−0.0005	0.0001	−5.055	**<0.001**
Treatment Acute-Recovery ×Temperature 23	0.0003	0.0001	2.630	**0.010**
Treatment Acute-Stress ×Temperature 23	−0.0000	0.0001	−0.723	0.471

Fish were acclimated to one of five temperatures (5, 10, 15, 20 and 23°C) and exposed to one of three treatments (unhandled, acute stress and acute stress recovery). Coefficients denote the groups being compared (i.e. treatment group×temperature); significant *P*-values are in bold.

highest at 10°C (0.301±0.043 mg O_2 g^{-1} h^{-1}; Fig. 4D; one-way ANOVA, $F_{4,40}$=1.29, P=0.292; see Table S4 for further statistical details). Mean FAS was highest for juvenile brook trout acclimated to 5 and 10°C (3.8±0.3 and 4.0±0.5 mg O_2 g^{-1} h^{-1}, respectively) compared with those held at 20 and 23°C (2.4±0.2 and 2.1±0.1 mg O_2 g^{-1} h^{-1}, respectively; Fig. 4E; one-way ANOVA, $F_{4,40}$=7.6, P<0.001; see Table S4 for further statistical details). Recovery time was approximately twice as long for fish acclimated to 5, 15 and 20°C (10.9, 13.9 and 13.6 h respectively) compared with those at 10 and 23°C (7.8 and 4.7 h respectively; Fig. 4C; one-way ANOVA, $F_{4,40}$=27.69, P<0.001; see Table S4 for further statistical details).

DISCUSSION

Our study assessed the ability of juvenile brook trout to acclimate to temperatures that span the thermal distribution of the species. We found that the physiology of juvenile brook trout was impacted at acclimation temperatures ≥20°C as indicated by the suite of indices and variables assessed in this study (Fig. 5). Several markers of thermal stress increased (i.e. mRNA abundance of *gpx1* and *hsp90ab1* in gill, along with *hsp90ab1* and *hspa8* in liver, and glucose and cortisol responses post-acute stress event) or decreased (i.e. mRNA abundance of *nkcc1a* in gill and *gpx1* in liver) with increasing temperature. Further, we found a non-linear response pattern with mRNA abundance of *g6pc* and *nupr1* in liver tissue and the osmolality response, where the peak level at 20°C was higher than that at 23 and 25°C. We also generally found significantly higher SMR and significantly lower FAS at acclimation temperatures ≥20°C when compared with the 5°C and 10°C treatments, indicating a metabolic effect at the higher acclimation temperatures.

Transcripts involved in heat stress and regulatory responses (i.e. *hsp90ab1*, *hspa8*, *gpx1*, *nkcc1a* and *nupr1*) exhibited differential abundance at 20°C and higher, suggesting a thermal threshold at the transcript level. Elevated levels of plasma cortisol and glucose after the exhaustive exercise and air exposure stressor suggest increased activation of the HPI axis at 20°C and higher. Increases in muscle lactate post-exercise also suggest increased reliance on anaerobic metabolism at 20°C and higher, though this may be due to slower metabolism at cooler temperatures. It is important to note that regardless of acclimation temperature, all blood parameters were sampled 30 min post-stressor or 24 h following recovery; therefore, we did not necessarily observe the peak change in these variables. Finally, SMR was higher at elevated temperatures (≥20°C), and fish experienced extended recovery times at high acclimation temperatures (15 and 20°C), indicating a more pronounced response to the acute stressor at elevated temperatures. Overall, the ability of juvenile brook trout to acclimate to temperatures beyond 20°C appears to be reduced.

Cellular-level response to chronic temperature exposure

Juvenile brook trout at temperatures above 20°C showed a cellular heat shock response in gill and liver tissues. Heat shock proteins are part of a ‘classic’ temperature-induced cellular stress response and play a critical role in reducing and repairing damage to cellular proteins that arise from physical or chemical stress (Iwama et al., 1998; Somero, 2010; Currie, 2011). When temperatures begin to approach a species’ thermal limit [brook trout upper thermal limit (ULT)=25.3°C; Fry et al., 1946; Fry, 1951; Wehrly et al., 2007], heat shock protein expression is often increased (Currie, 2011). In the present study, mRNA levels of both *hsp90ab1* and *hspa8* (*hsc70*) were significantly elevated at 23 and 25°C compared with those at ≤15°C. Similarly, in the gill tissue there was a significant increase in abundance of *hsp90ab1* mRNA at 25°C compared with that at ≤15°C. Other salmonid species, such as arctic char (*Salvelinus alpinus*; ULT= 21.5–22.7°C; Baroudy and Elliott, 1994), sockeye salmon (*Oncorhynchus nerka*; ULT=24.9–25.1°C; Orsi, 1971; Brett, 1952) and pink salmon (*Oncorhynchus gorbuscha*; ULT=23.9°C; Brett, 1952), have elevated mRNA levels of *hsp90ab1* and *hspa8* at temperatures ≥19°C (Quinn et al., 2011; Jeffries et al., 2012a, 2014; Akbarzadeh et al., 2018). As both *hsp90ab1* and *hspa8* are constitutive isoforms (Iwama et al., 1998), changes in their expression would be expected in chronic events such as thermal acclimation, and our results further suggest that brook trout are activating a chronic cellular stress response above 20°C, as evidenced by increased mRNA abundance at these temperatures.

As temperature surpassed 20°C, an apparent thermal threshold was reached for genes involved in the oxidative stress response in gill and liver tissues. Glutathione peroxidase 1 is responsible for catalysing the reduction of H_2O_2 into H_2O or alcohol and thereby prevents damage caused by reactive oxygen species (ROS; Sattin et al., 2015). As metabolic rate increases with temperature, there is an increase in oxidative phosphorylation in the mitochondria, potentially resulting in the elevated production of ROS (Davidson and Schiestl, 2001). The SMR of brook trout increased significantly at higher temperatures in the present study, potentially resulting in elevated ROS production, and thus may be responsible for the increase in abundance of *gpx1* mRNA observed in the gill at temperatures above 20°C. In contrast, a significant increase in the abundance of *gpx1* mRNA in the liver was observed at cooler temperatures (i.e. 5 and 10°C) compared with that in fish held at 20°C. Multiple Antarctic fishes (e.g. *Champsocephalus gunnari*, *Chaenocephalus aceratus*, *Pseudchaenichthys georgianus*, *Dissostichus eleginoides* and *Notothenia rossi*) exhibit higher levels of glutathione peroxidase in the heart and liver tissues

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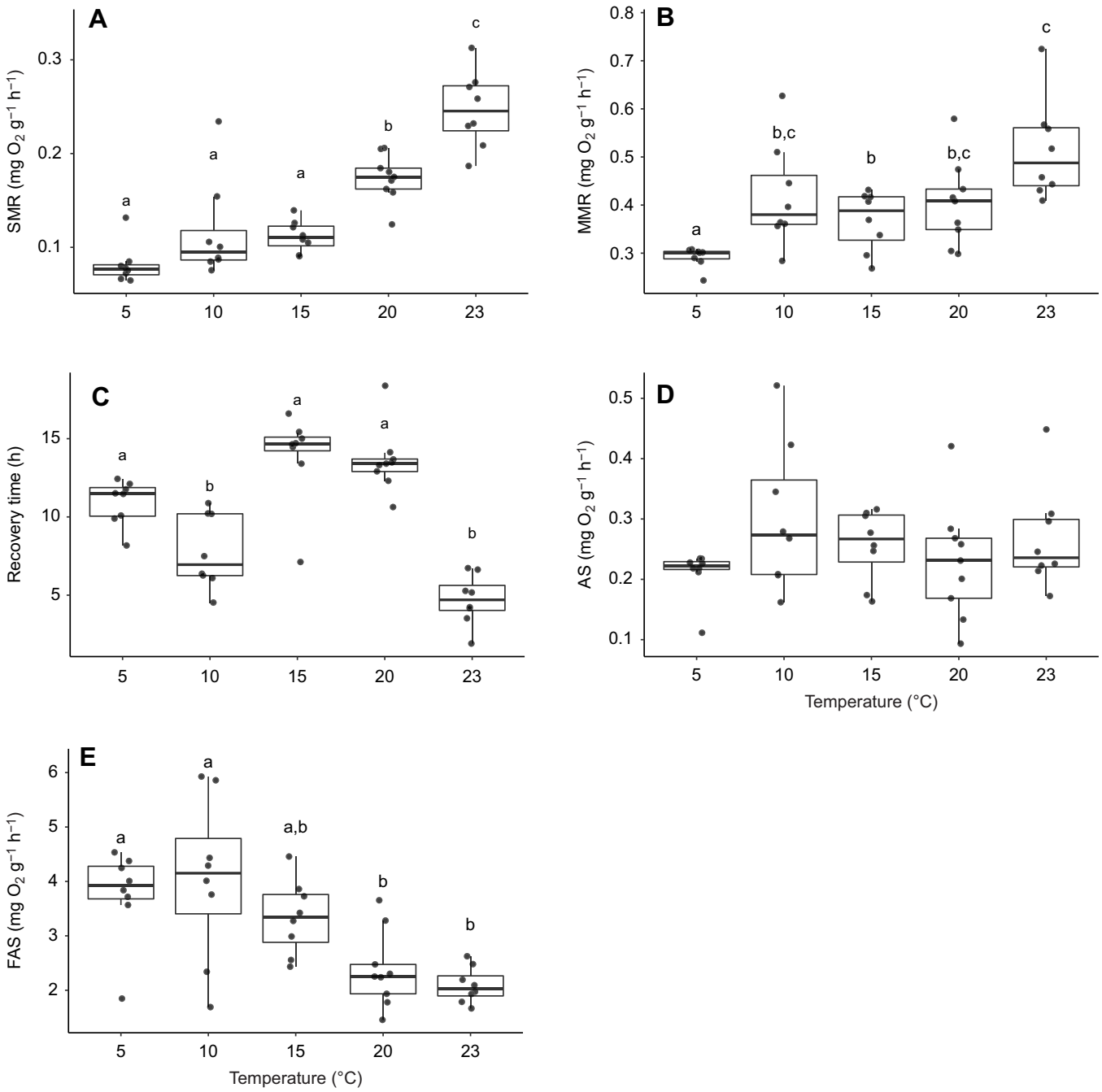

Fig. 4. Metabolic and recovery parameters collected from juvenile brook trout acclimated to temperatures spanning their thermal distribution (*n*=8 per treatment). (A) Standard metabolic rate (SMR), (B) maximum metabolic rate (MMR), (C) recovery time (hours post-stress event), (D) aerobic scope (AS) and (E) factorial aerobic scope (FAS). Groups that do not share a letter are significantly different from one another [Kruskal–Wallis (SMR only), one-way ANOVA, $P<0.05$; see Table S4]. Horizontal bars in the boxplot represent the median response value and the 75% and 25% quartiles. Whiskers represent ±1.5 times the interquartile range, and each dot represents an individual response value.

compared with the gills and muscle tissues (Ansaldo et al., 2000). The increased glutathione peroxidase response in the liver of brook trout in the present study suggests that *gpx1* is important for tissue-specific cold acclimation. An increase of glutathione peroxidase in the liver may also be due to the dependence of aerobic metabolism on the oxidation of unsaturated fats in the liver, fats that are susceptible to oxygen radical attack (Roberfroid and Calderon, 1995). Previous work on this same group of brook trout found that hepatosomatic index (ratio of liver mass to body mass) was lowest at 23 and 25°C and higher at cooler temperatures (5°C), indicating a higher fat content at cooler temperatures (Morrison et al., 2020). The relationship between higher fat content in the liver and higher ROS attack is supported by results from Morrison et al. (2020) and may explain why liver tissue exhibited increased abundance of *gpx1* mRNA in our study. Overall, *gpx1* mRNA abundance differed across gill and liver tissue, where increased abundance was observed at warmer temperatures for the gill tissue in contrast to increased abundance at cooler temperatures for the liver tissue.

The mRNA abundance of genes involved in cellular processes (e.g. cell growth) and metabolic processes (e.g. glycogen production)

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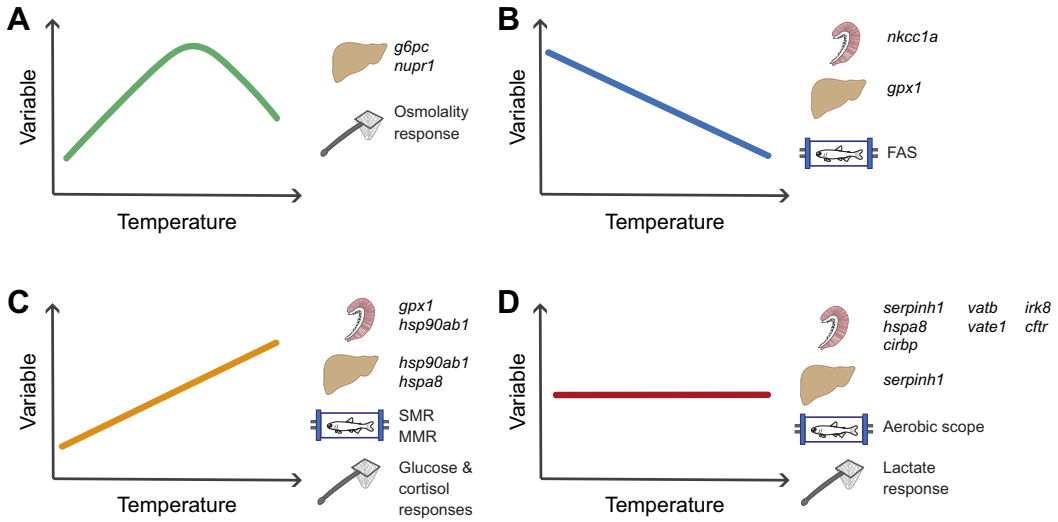

Fig. 5. Response curves highlighting the major trends for mRNA abundance, physiological parameters and metabolic results observed with increasing temperature after 3 weeks of acclimation in juvenile brook trout. (A) The mRNA abundance of *g6pc* and *nupr1* in liver and osmolality response post-acute stress event had peak values at 20°C. (B) The mRNA abundance of *nkcc1a* in gill and *gpx1* in liver and FAS exhibited a progressive decrease with increasing temperature. (C) The mRNA abundance of *gpx1* and *hsp90ab1* in gill, along with *hsp90ab1* and *hspa8* in liver, SMR and MMR, and glucose and cortisol responses post-acute stress event showed a progressive increase with temperature. (D) The mRNA abundance of *serpinh1*, *vatb*, *irk8*, *hspa8*, *vate1*, *cftr* and *cirbp* in gill and *serpinh1* in liver, AS, and lactate response post-acute stress event showed no change with increasing temperature. Note that the mRNA abundance for *atp1a3* did not have a clear pattern; therefore, it is not included here.

exhibited peak levels at 20°C that subsequently decreased at higher temperatures. Because some cellular responses can exhibit a peak near or prior to detrimental physiological changes (Jeffries et al., 2014, 2018; Schulte, 2015), the peak mRNA transcript levels at 20°C may suggest that it is near a sub-lethal threshold. Nuclear protein 1 is involved in the regulation of cell growth and apoptosis (Mallo et al., 1997) and plays a role as a transcription factor (Momoda et al., 2007). *nupr1* mRNA abundance in the liver was highest in juvenile brook trout held at 20°C, and then decreased by 2-fold in fish held at 25°C in the present study. This decline in *nupr1* mRNA abundance in fish held at 25°C compared with 15 and 20°C suggests a potential sub-lethal threshold for *nupr1* at temperatures above 20°C. Additionally, higher mortality rates were observed in fish held at 25°C, further supporting the shutdown of certain cellular processes at temperatures beyond 23°C. This increased abundance of mRNA at 20°C was also observed for liver *g6pc*, which plays a role in glycogen metabolism, where levels peaked at 20°C. Therefore, elevation of *nupr1* and *g6pc* abundance at 20°C and decreased abundance at 25°C is consistent with a shift in the cellular processes being activated at the highest acclimation treatments.

Whole-animal responses to chronic temperature exposure

Acclimation temperature may have affected the ability of the juvenile brook trout to mount a stress response to an acute stressor. Plasma cortisol and glucose levels typically increase in fish 30–60 min following exposure to an acute stressor (Biron and Benfey, 1994; Wendelaar Bonga, 1997; Benfey and Biron, 2000), though this is dependent on environmental temperature, where higher temperatures result in a faster increase than at colder temperatures (Milligan, 1996; Van Ham et al., 2003; Chadwick and McCormick, 2017; Louison et al., 2017). In our study, the cortisol and glucose response to an acute stressor was impaired or delayed in fish exposed to lower temperatures (5 and 10°C), as there was no apparent increase in plasma cortisol and glucose levels 30 min following stressor exposure (acute stress group) or after 24 h of recovery (acute recovery group). In other studies, peak plasma cortisol levels for fish exposed to lower temperatures were also significantly delayed, possibly due to reduced enzymatic activity at cold temperatures (Van Ham et al., 2003; Louison et al., 2017). Additionally, as cortisol is synthesized *de novo* and not stored in the body like catecholamines, its release into the bloodstream results in increased levels throughout the body and is slightly delayed compared with other endocrine responses (Pickering, 1981; Milligan, 1996). Conversely, plasma cortisol levels were significantly elevated 30 min after exhaustive exercise and air exposure in fish held at temperatures ≥15°C. Notably, plasma cortisol levels remained above 100% of pre-stressed levels (i.e. unhandled group) 24 h post-stressor exposure (20 and 23°C), suggesting that fish had not fully recovered from the stressor at higher temperatures. A doubling of cortisol to the observed values of 100–300 ng ml^{-1} could result in decreased growth and loss of mass for organisms (Chadwick and McCormick, 2017). Plasma glucose levels also showed similar increases post-stressor exposure in fish held at temperatures ≥15°C (33–100% increase), with the exception that glucose levels returned to pre-stress levels for fish acclimated to 20 and 23°C. Elevated circulating cortisol levels at higher temperatures post-exposure to an exhaustive exercise stressor have been found in several other studies (e.g. Jain and Farrell, 2003; Suski et al., 2003, 2006; Meka and McCormick, 2005; McLean et al., 2016). For example, rainbow trout (*Oncorhynchus mykiss*) in southwest Alaska that were angled during a warmer year compared with a cooler year (13.2 versus 9.8°C) exhibited significantly increased plasma cortisol concentrations post-angling event (Meka and McCormick, 2005). Our data suggest that at higher temperatures, especially post-stressor exposure, there was an elevated stress response in brook trout, demonstrated by increased plasma cortisol and glucose. However, possibly because of enzyme kinetics, it is unclear whether the 30 min sampling time was sufficient for plasma cortisol and glucose to reach peak values at the cooler temperatures (5 and 10°C).

Lactate is a by-product of anaerobic metabolism and therefore increased concentrations of lactate in white muscle in fish can be

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caused by extensive exercise and activity (Wood et al., 1983). Across all acclimation temperatures, the exhaustive exercise and air exposure stressor led to a significant transient increase (100–150% greater) in lactate in the white muscle 30 min post-stressor exposure, that returned to or below pre-stress (i.e. unhandled) levels 24 h later. Several studies on a range of fishes, including brook trout, have demonstrated increased muscle lactate or plasma lactate concentrations when fish were subjected to exercise and air exposure (Beggs et al., 1980; Ferguson and Tufts, 1992; Booth et al., 1995; Milligan, 1996; Farrell et al., 2001; Kieffer et al., 2011; Landsman et al., 2011). Interestingly, the fish held at 23°C showed a 70% decrease in muscle lactate below pre-stress levels after 24 h recovery. A decrease in muscle lactate can be caused by a release of lactate into the blood or because the lactate in the muscle is recycled *in situ* for glycogenesis (Milligan and Wood, 1986; Milligan and Girard, 1993; Kieffer et al., 1994; Milligan, 1996). Therefore, the observed decrease in muscle lactate after 24 h recovery in the 23°C group may be a result of its release into the bloodstream or recycling through glycogenesis to help the fish return its energy stores to pre-stress levels. Our results suggest that the exhaustive exercise and air exposure induced anaerobic metabolism, as exhibited by the increase in muscle lactate across all temperature groups.

We found some evidence that osmoregulation in brook trout was impacted at elevated temperatures, as shown by increased levels of plasma osmolality and changes in mRNA abundance of *nkcc1a*. Plasma osmolality can be used to estimate the osmoregulatory ability of fishes as an indicator of ion balance, particularly for circulating concentrations of Na^+ and Cl^- (McDonald and Milligan, 1997). For the brook trout that were subjected to the exhaustive exercise and air exposure stressor, we observed a significant transient increase (13–16% higher) in plasma osmolality 30 min post-stressor exposure that returned to pre-stress levels (i.e. unhandled) in fish held at 15 and 20°C. After exposure to an acute stressor, increased cardiac output would lead to increased blood perfusion at the gill (Mazeaud and Mazeaud, 1981; Sopinka et al., 2016). However, there is a trade-off between increasing oxygen uptake at the expense of increasing gill permeability to water and ions, which is termed the osmorespiratory compromise (Randall et al., 1972; Nilsson, 2007; Onukwufor and Wood, 2018) and may contribute to alterations in plasma osmolality. This compromise may lead to haemodilution and aid in explaining why there was no peak in osmolality after 1 h in the acute stress group at 23°C and there were significantly lower osmolality levels after 24 h of recovery. At a molecular level, *nkcc1a*, a gene involved in ion regulation, showed increased mRNA abundance in the gill at low temperatures, with decreased abundance at higher temperatures. As the $Na^+/K/Cl^-$ cotransporter is involved in active ion absorption or secretion across cellular membranes in gills (Hiroi et al., 2008), this may further suggest that brook trout osmoregulatory ability was potentially impacted by increased temperatures.

Both SMR and MMR increased with acclimation temperature in juvenile brook trout, with the highest levels evident in fish held at 23°C. There is a strong relationship between water temperature and metabolic rate in ectotherms (Fry, 1971; Hulbert and Else, 2004; Brett, 1964; Beamish, 1978) and therefore increased SMR at higher temperatures was expected in brook trout. AS was not significantly affected by acclimation temperature, likely because of the significant increase in SMR with rising temperature and a similar trend in MMR. Our finding of a stable AS across temperatures is similar to results seen in a study run concurrently on the same batch of fish by Durhack et al. (2021) that found maximum MMR and AS to occur at 15°C. A major difference between the two studies was the chase protocol to estimate MMR; while our study used a standardized 2 min chase followed by 5 min air exposure, Durhack et al. (2021) used an exhaustive chase protocol with minimal air exposure. The main difference between the results of the two studies was the temperature when peak MMR and AS occurred, with Durhack et al. (2021) finding highest MMR and AS estimates at 15°C, while our study found highest MMR at 23°C. A maximum AS at or near 15°C would be expected based on previous literature that found this to be the optimal growth and preferred temperature for brook trout (Smith and Ridgway, 2019). A possible reason why our MMR and AS estimates did not agree with those of other studies may be that our air exposure was longer, which could have led to increased oxygen consumption as a response to increased oxygen demand during recovery at the higher temperatures. Stable AS values were also observed in Nile perch (*Lates niloticus*) that were acclimated for 3 weeks at 27, 29 and 31°C (Nyboer and Chapman, 2017). A similar trend of stable AS across temperatures has also been observed in chinook salmon (*Oncorhynchus tshawytscha*; Poletto et al., 2017) and pink salmon (*O. gorbuscha*; Clark et al., 2011). The maintenance of similar AS in fish acclimated to different temperatures may be evidence for metabolic compensation (Donelson and Munday, 2012). If the energy available to allocate to other processes (AS) remains constant, the body may have to adjust and metabolically compensate to maintain energy, oxygen, heart rate and other vital processes (Eliason and Farrell, 2016). Conversely, there was a change in FAS, where we observed highest FAS at 5 and 10°C and a significant decrease at 20 and 23°C, a similar decrease at high temperatures to that seen by Poletto et al. (2017). FAS is a useful tool to indicate how energy allocation may be affected by environmental conditions, such as temperature (Eliason and Farrell, 2016). Therefore, our results suggest that energy allocation is affected by temperatures greater than 15°C, further suggesting that 15°C is where metabolic capacity is optimized for brook trout (Smith and Ridgway, 2019) and energy allocation is decreased at these higher temperatures. Overall, our metabolic data reflect an increase in metabolic activity at higher temperatures with possible metabolic compensation as indicated by constant AS across all temperatures.

The fish acclimated to 23°C exhibited the shortest recovery time, despite having the highest SMR estimates and evidence of cellular impairment. In contrast, time to recovery was highest at 15 and 20°C. The short recovery time at 23°C does not seem to be due to this group having a higher SMR, as the AS between all groups across the study was not significantly different. Considering the constant AS across groups, it is possible that this quick recovery time in the 23°C group may be due to respiratory alkalosis and/or active suppression of metabolic rate. Metabolic rate suppression is a strategy used by other animals to combat adverse environmental conditions (Hochachka and Guppy, 1987). For example, goldfish (*Carassius auratus*) can suppress their metabolism to up to 30% of the aerobic metabolic rate at elevated environmental temperatures (Van Waversveld et al., 1988). Post-exhaustive exercise and air exposure, there may be elevated blood carbon dioxide levels and lower blood pH (Wang et al., 1994; Milligan, 1996) and this can be exacerbated at high temperatures. Suppression of metabolic rate may also be used to limit further carbon dioxide build up in the blood (i.e. acidosis) to regulate the blood pH of fishes (Claiborne, 1998). Therefore, one possible explanation for the short recovery time in the fish from the 23°C acclimation group could be metabolic suppression.

Conclusion

We demonstrated that the acclimation ability of brook trout to temperatures ≥20°C is impaired as shown by changes in mRNA

transcript abundance, metabolic processes and responses to exhaustive exercise and air exposure. Our findings are consistent with previous work that suggested that upper temperatures limiting habitat use in brook trout are around 21–23.5°C (reviewed in Smith and Ridgway, 2019). Our study indicated that there is a sub-lethal threshold between 20 and 23°C when chronic temperatures may begin to adversely impact the physiological performance of brook trout. Previous work on this same group of brook trout also showed that between 20 and 23°C, fish were no longer able to increase their critical thermal maxima with acclimation temperature and there were increased plasma lactate levels indicating anaerobic metabolism (Morrison et al., 2020). Furthermore, Chadwick and McCormick (2017) demonstrated that brook trout growth is limited by higher temperatures, especially those above 23°C, and this may play a role in driving their distribution. Collectively, these studies in combination with the present study suggest that there is a limitation to the ability of brook trout to cope with chronic temperatures ≥20°C, which can potentially provide a benchmark for understanding the ability of some brook trout populations to persist in the wild in the future. In summary, our study demonstrates how brook trout respond during acclimation to higher temperatures at multiple scales of biological organization and how integration of whole-organism physiology and molecular techniques can aid in understanding the sublethal temperature thresholds of a species.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.T.H., T.D., C.J.M., E.C.E., K.M.J.; Methodology: T.E.M., C.T.H., T.D., J.D.J., C.J.M., E.C.E., K.M.J.; Software: T.D., C.J.M.; Validation: T.E.M., C.T.H., J.D.J., K.M.J.; Formal analysis: T.E.M., T.D., J.D.J., C.J.M., K.T., C.T.H.; Investigation: T.E.M., T.D., J.D.J., C.J.M., K.T., K.M.J.; Resources: C.T.H., T.D., E.C.E., K.M.J.; Data curation: T.E.M.; Writing - original draft: T.E.M., C.T.H., K.M.J.; Writing - review & editing: T.E.M., C.T.H., T.D., J.D.J., C.J.M., K.T., E.C.E., K.M.J.; Visualization: T.E.M.; Supervision: C.T.H., J.D.J., K.M.J.; Project administration: C.T.H., E.C.E., K.M.J.; Funding acquisition: C.T.H., E.C.E., K.M.J.

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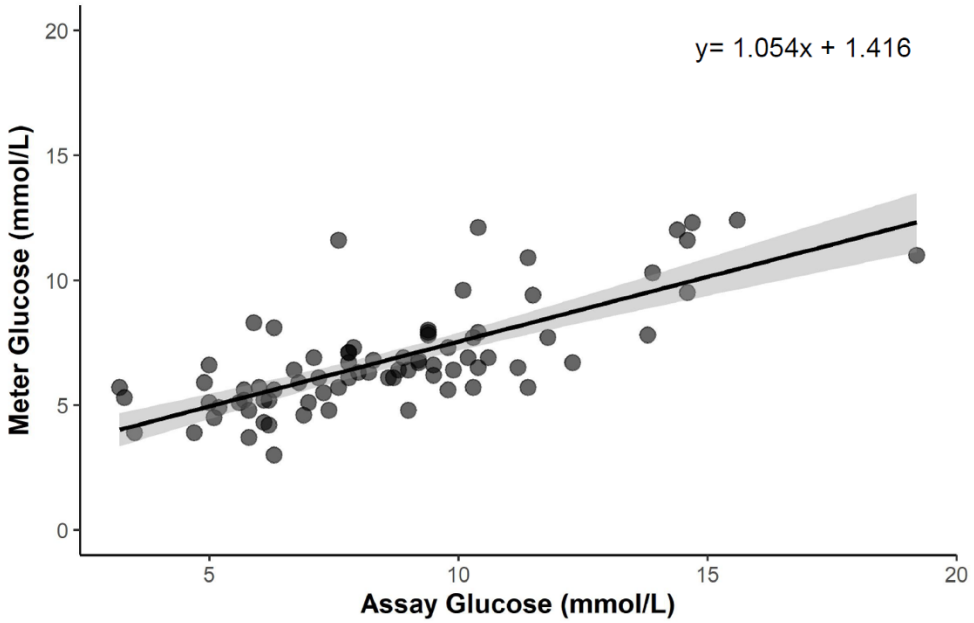

Fig. S1. Comparison of glucose values from the hexokinase kinetic assay and the glucose meter using a linear regression (y = 1.054x + 1.416, $P < 0.001$, $r^2 = 0.54$) for juvenile brook trout (*Salvelinus fontinalis*) held at temperatures spanning their thermal distribution (n = 10 per temperature treatment).

Table S1. Transcript information for the thirteen target and three reference genes retrieved from a previously published brook trout transcriptome in Sutherland et al., (2019).

Subject	Length (bp)	Species	Database	Accession no.	Score	E. value	% Identity
QSF_HSP70.6.8	1932	*Oncorhynchus mykiss*	Swissprot	HSP70_ONCMY	2732	0	86.5
QSF_SERPH.3.3	1233	*Salmo salar*	Refseq_rna	NM_001139968.1	840	0	92.6
QSF_ATP1A3.7.8	972	*Oncorhynchus mykiss*	Refseq_rna	NM_001124630.1	458	0	84.8
QSF_G6PC.1.2	1059	*Felis catus*	Swissprot	G6PC_FELCA	1059	$1e^{-25}$	49.9
QSF_CIRBP.4.24	468	*Salmo salar*	Refseq_rna	NM_001139676.1	152	$7e^{-79}$	88
QSF_GPX1.1.4	255	*Oncorhynchus mykiss*	Refseq_rna	NM_001124525.1	418	0	91.2
QSF_HSP90B.2.6	1161	*Salmo salar*	Refseq_rna	NM_001123532.1	818	0	95.5
QSF_LOC10117185.1.1	228	*Oryzias latipes*	Refseq_protein	XP_004086462.1	312	$1e^{-31}$	76.4
QSF_RL7.4.13	942	*Salmo salar*	Refseq_rna	NM_001140480.1	523	0	93.6
QSF_RS9.1.4	267	*Rattus norvegicus*	Swissprot	RS9_RAT	200	$1e^{-16}$	97.4
QSF_RL8.1.6	774	*Danio rerio*	Swissprot	RL8_DANRE	1267	$1e^{-177}$	93.8
QSF_IRK8.1.1	1266	*Salmo salar*	Refseq_rna	NM_001140360.1	1828	0	97.2
QSF_NKCC1A.1.2	3453	*Salmo salar*	Refseq_rna	NM_001123683.1	479	0	94.1
QSF_LOC100136366.1.1	4557	*Salmo salar*	Refseq_rna	NM_001123534.1	4750	0	96.9
QSF_LOC100136607.1.1	561	*Oncorhynchus mykiss*	Refseq_rna	NM_001124597.1	633	0	97.6
QSF_LOC101477634.1.1	681	*Maylandia zebra*	Refseq_rna	XM_004548350.1	259	$1e^{-148}$	84.8

Table S2. Results of one-way analysis of variance (ANOVA) and Kruskal-Wallis test for the mRNA abundance of genes measured in gill and liver tissue of juvenile brook trout (*Salvelinus fontinalis*) acclimated to 5, 10, 15, 20, 23, and 25˚C.

Tissue	Gene	Df	Sum of Squares	Mean of Squares	*F* values/ Chi-squared	*P*-value
Gill	*atp1a3*	5, 59	24.39	4.878	3.674	**0.006**
	irk8	5, 59	14.60	2.920	1.739	0.142
	nkcc1a	5, 59	35.33	7.066	5.069	< **0.001**
	vatb	5, 59	-	-	2.268	0.811
	vate1	5, 59	10.28	2.056	1.475	0.213
	cftr	5, 59	2.96	0.591	0.4	0.846
	cirbp	5, 59	14.83	2.966	1.469	0.216
	gpx1	5, 59	6007	1201.4	5.735	< **0.001**
	serpinh1	5, 59	10.55	2.111	1.933	0.104
	hspa8	5, 59	11.94	2.387	2.252	0.063
	hsp90ab1	5, 59	25.33	5.066	4.321	**0.002**
Liver	*g6pc*	5, 59	67.56	13.51	3.123	**0.016**
	gpx1	5, 59	36.22	7.245	4.924	**0.001**
	hspa8	5, 59	36.59	7.318	8.828	< **0.001**
	hsp90ab1	5, 59	54.31	10.86	15.14	< **0.001**
	nupr1	5, 59	4444	888.8	4.918	**0.001**
	serpinh1	5, 59	4.63	0.9258	0.573	0.720

atp1a3, Na^+/K^+-transporting ATPase subunit alpha-3; *irk8, ATP-sensitive inward rectifier K^+ channel 8*; *nkcc1a, $Na^+/K^+/2Cl^-$ co-transporter-1-a*; *vatb, B subunit of V-type H-ATPase*; *vate1, E 1 subunits of V-type H-ATPase*; *cftr, cystic fibrosis transmembrane conductance regulator*; *cirbp, cold-inducible RNA-binding protein*; *gpx1, glutathione peroxidase-like peroxiredoxin*;

serpinh1, *serpinh1*; *hspa8*, *heat shock cognate 71 kDA protein*; *hsp90ab1*, *heat shock protein 90-beta*; *g6pc*, *glucose-6-phosphatase*; *nupr1*, *nuclear protein-1*.

Data were analyzed using a one-way ANOVA except if data were not equally variable or normally distributed, in which case a Kruskal-Wallis test was used; significant *P*-values are represented as bolded text

Table S3. Results of the two-way analysis of variance (ANOVA) for muscle lactate, as well as plasma cortisol and glucose of juvenile brook trout (*Salvelinus fontinalis*) acclimated to five temperatures (5, 10, 15, 20, and 23˚C) and exposed to one of three treatments (unhandled, acute stress, and acute-stress recovery).

Variable	**Group**	**Df**	**Sum of Squares**	**Mean of Squares**	***F* value**	***P*-value**
Muscle lactate	Treatment	2	13.135	6.568	125.415	< **0.001**
	Temperature	4	4.107	1.027	19.606	< **0.001**
	Treatment × Temperature	8	4.106	0.513	9.801	< **0.001**
Plasma cortisol	Treatment	2	37274	18637	46.15	< **0.001**
	Temperature	4	6593	1648	4.08	**0.004**
	Treatment × Temperature	8	13919	1740	4.31	< **0.001**
Plasma glucose	Treatment	2	2.9573	1.4787	64.803	< **0.001**
	Temperature	4	0.7413	0.1853	8.122	< **0.001**
	Treatment × Temperature	8	1.2648	0.1581	6.929	< **0.001**

Significant *P*-values are represented as bolded text

Table S4. Results of the one-way ANOVA and Kruskal-Wallis test for standard metabolic rate (SMR) and maximum MR (MMR), recovery time, aerobic scope (AS), and factorial aerobic scope (FAS) of juvenile brook trout (*Salvelinus fontinalis*) acclimated to 5, 10, 15, 20, and 23˚C.

Variable	**Df**	**Sum of Squares**	**Mean of Squares**	***F* value/ Chi-Squared**	***P*-value**
SMR	4, 40	-	-	30.43	< **0.001**
MMR	4, 40	1.272	0.318	8.84	< **0.001**
Recovery Time	4, 40	506.7	126.7	27.69	< **0.001**
AS	4, 40	0.598	0.149	1.29	0.292
FAS	4,40	23.91	5.978	7.57	<**0.001**

Significant *P*-values are represented as bolded text