Basic and Applied Aspects of Thermal Acclimation in Juvenile Brook Trout (*Salvelinus fontinalis*)

By

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Chapter 1: General Introduction

Most fishes are poikilotherms, which means their body temperature fluctuates with the surrounding environment (Schulte, 2011). As such, temperature is the master abiotic factor for fish (Fry, 1947) and, ultimately, temperature influences most of their life processes. Temperature has a significant impact on phenology, i.e. the study of the timing of seasonal activities, which can include activities such as reproduction and migration (Sydeman and Bograd, 2009). Additionally, temperature controls the rate of development, oxygen consumption rates, and influences locomotor actions and behaviours (Beitinger et al., 2000). Due to the control of temperature on phenology and other processes, temperature is the major driver of a species' distribution and biogeographic patterns (Rahel, 2002). The magnitude at which temperature dictates life processes in fishes varies by species and is shaped by its evolutionary history (Hochachka and Somero, 2002). It is suffice to say that temperature changes in the surrounding environment can have significant consequences for fish.

The most consequential change to the aquatic thermal environment in the past thousands of years is currently occurring. Global climate change is altering thermal habitats of all freshwater ecosystems and altering species distributions (Parmesan, 2006), causing disease outbreaks (Hermoso, 2017), influencing phenology (Krabbenhoft et al., 2017), and decreasing survival (Bassar et al., 2016). Additionally, species are also adapted to specific hydrologic conditions and changes in these conditions, especially due to climate change, can result in extirpation of locally specialized species (Cross and Moss, 1987; Mion et al., 1998; Modde et al., 2001). Moreover, fluctuations in environmental conditions can further increase the success of invasive species as the environmental conditions become suitable for their proliferation (Baltz and Moyle, 1993; Ross et al., 2001; Annear et al., 2004), which may ultimately result in loss of

local native species to an area as well. If species are unable to adapt to direct and indirect effects of climate change, there will be impacts on the survival and proliferation of freshwater fishes.

Thermal acclimation

The potential for fishes to survive in warming water will depend on their ability to adapt, acclimate, and relocate (Nogués-Bravo et al., 2018). At short time scales, the initial cellular response includes modifying activities of biochemical systems to reprogram gene and protein expression and other processes to compensate for thermal disturbances (Somero, 2020). In contrast, acclimation (i.e., phenotypic plasticity) occurs on the order of weeks to months, and cells begin to alter the expression of genes and biochemical processes (Hochachka and Somero, 2002). This acclimation process works towards maintaining homeostasis, or relative constancy of the internal environment of the organism despite the change (Cannon 1926, 1939), to help the organism adjust and thrive in the altered conditions. Moreover, as organisms acclimate to a temperature change in their environment, factors such as heart rate, ventilation rate, metabolic rate, neural conduction persistence, membrane fluidity, and enzymatic reaction rates shift (Willmer et al., 2000). Because of the profound effect temperature has on physiological function, thermal acclimation ultimately dictates adaptive responses to environmental change in populations (Crozier and Hutchings, 2014). Additionally, thermal acclimation demonstrates when sublethal thresholds begin to have adverse effects on fish (Jeffries et al., 2018). Fish that can acclimate to changing thermal conditions, are likely to contribute most to future generations (Schulte, 2014), therefore, if fish can modify their physiological processes in response to warming temperatures, their populations may withstand climate change-related temperature increases in their current distribution.

Despite being able to thermally acclimate, a fish may still be negatively impacted by elevated temperatures due to the interactive nature of temperature and other stressors (Schulte, 2014). Therefore, it is imperative to study thermal acclimation paired with other acute stressors (Gingerich et al., 2007). Other environmental stressors can come in the form of infectious diseases, altered flow regimes, fragmentation of habitat, contaminants, and other abiotic factors such as dissolved oxygen and salinity (Reid et al., 2019). Additionally, anthropogenic sources, such as fisheries capture, can pose an added stress to individuals (Gingerich et al., 2007). In fact, air exposure during angling and water temperature has been found to interact and increase mortality in fishes (Gingerich et al., 2007; Cooke et al., 2013). As catch-and-release angling consists of events with multiple stressors that can be influenced by temperature and lead to sublethal effects (e.g., Gale et al., 2013), it becomes necessary to understand the interactive implications of temperature acclimation and acute stress.

Fishes differ greatly in their abilities to tolerate temperatures outside their thermal envelopes (Rahel, 2002). Species that function within a narrow range of temperatures are termed stenothermal, while species that can function across a wider range of temperatures are termed eurythermal (Hochachka and Somero, 2002). More specifically, there are physiological, biochemical, and molecular mechanisms that differ between stenotherms and eurytherms and these mechanisms establish biogeographical patterning and susceptibility to shifts in ambient temperature (Somero et al., 1996; Rahel, 2002). For example, phenotypic plasticity is incredibly important for eurytherms in terms of protein isoform systems and membrane-systems, where rebuilding of biochemical machinery and cellular reorganization is accomplished seasonally and sometimes diurnally (Carey and Hazel, 1989). In contrast, phenotypic plasticity may be limited in stenothermal species, as they may have lost the ability of expressing certain genes during

evolution in thermally stable environments (Bosch et al., 1988; Dietz and Somero, 1993; Hofmann et al., 2000). Overall, whether a species has a narrow or wider tolerance is dictated by homologous proteins and genetically fixed differences that establish thermal tolerance ranges (Somero et al., 1996). Through these cellular and evolutionary mechanisms, temperature dictates where a fish can live and ultimately where they can thrive.

There is a range of temperatures where a species can optimally function, termed the thermal niche (Hochachka and Somero, 2002). A way to ensure that a species stays within their thermal niche is for the organisms to disperse to locations with preferred temperatures (Nogués-Bravo et al., 2018). In fish, dispersion can include seasonal migrations and diurnal vertical migrations (Martins et al., 2011; Cott et al., 2015; Raby et al., 2018). Additionally, fish can thermoregulate behaviourally by selecting thermally heterogenous microhabitats (Brett, 1971; Nevermann and Wurtsbough, 1994; Nielsen et al., 1994; Biro, 1998; Newell and Quinn, 2005). However, if organisms are unable to relocate sufficiently and reside outside their thermal niche, they may exhibit abnormal behaviours such as burst swimming and rapid ventilation (Gale et al., 2013). Ultimately, if a fish is unable to leave or acclimate to the temperature of the environment it is in, mortality can occur. When mortality occurs widely in a population, extirpation from an area is possible.

Thermal biology of salmonids and climate change

A family of fishes that is potentially at risk to climate change is the Salmonidae. Salmonidae are ray-finned, freshwater fishes that includes the salmons and trouts (*Salmo, Oncorhynchus*), chars (*Salvelinus*), whitefishes (*Coregonus, Prosopium, Stenodus*), and graylings (*Thymallus*). Collectively known as salmonids, they are found in the northern waters of North America, Europe, and Asia (Scott and Crossman, 1973). Over their history, salmonids have been found to

be well-suited for newly ice-free areas (Northcote, 1997) and as such, recolonized recently de-glaciated regions (Behnke, 1972). Preference for cooler temperatures is also reflected in salmonid life history strategies as many species migrate or live in headwater streams that are ideal for spawning and rearing of young (Northcote, 1997). In fact, lower overwinter temperatures have been found to be essential for spawning success in salmonids (Langford, 1983; Gerdaux, 1998). Furthermore, in temperate stream ecosystems, salmonids may rely on groundwater discharges for cool water refugia (Meisner, 1990b). Preference for cold water systems particularly puts salmonids at risk from future warming events.

Salmonids are already being threatened by global climate change, as rising temperatures are beginning to reach the thermal tolerances for some populations (Gunn and Snucins, 2010; Hasler et al., 2012; Chadwick et al., 2015). While there are differences in thermal sensitivity among the salmonids, most species generally have a low optimal temperature for growth [i.e., 12–16℃] (Kovach et al., 2019), which makes the family especially sensitive to higher temperatures. Exposure to temperatures beyond thermal niches of salmonids can induce physiological changes as well as ecological responses. In terms of physiology, at sub-lethal levels, warm temperatures can lead to reduced growth, stress, and increase susceptibility to disease (Carter, 2005). With respect to ecology, changes in temperature regimes influence competitive dominance (Carter, 2005), age at smoltification, sexual maturation, spawning activity, and migration (Finstad and Hein, 2012; Morita et al., 2014). For example, in partially migratory masu salmon (*Oncorhynchus masou*), the proportion of males exhibiting migration behaviours decreased with increasing temperatures (Morita et al., 2014). Additionally, salmonids begin to die when there is not enough oxygen to sustain their maintenance requirement due to hypoxia sensitivity (Holt and Jørgenson, 2015). Hypoxia will be exacerbated as climate change

continues due to the tight relationship between dissolved oxygen levels and temperature. Periods of ice cover are also becoming shorter with climate change (Benson et al., 2012), leaving systems without the thermal buffer that prolonged ice cover brings (Helland et al., 2011; Watz et al., 2013; Watz et al., 2015). With loss in ice cover and ultimate increase in water temperatures, metabolic activities of salmonids will be altered as tissue oxygen demand is a positive function of water temperature (Brett, 1964; Beamish, 1978). Ultimately, variable environmental conditions as a result of climate change can influence the overall energy budget of salmonids (Hedger et al., 2013) and estimates suggest that an increase of 3°C in water temperature will result in 20% decrease in the range and abundance of cold-water salmonid populations (Casselman, 2002; Stitt et al., 2014). However, if salmonids have the capacity to acclimate to the changes in temperature, it is possible salmonids could survive and thrive in their current distribution.

Among the salmonids, brook trout (*Salvelinus fontinalis*) is a species that is native to Eastern and Central North America (Scott and Crossman, 1973). Thermal habitat loss has already led to mortality in wild brook trout populations (Gunn and Snucins, 2010) and due to cooler temperature preferences, it is predicted that their populations will continue to struggle (Chu et al., 2005; Stitt et al., 2014). Brook trout have been studied for over 70 years (Smith et al., 2020) and have been the focus of many thermal preference studies (see Smith and Ridgway, 2019). In their study, Smith and Ridgway (2019) reviewed brook trout literature related to defining their thermal habitat. Through their compilation they concluded a preferred temperature (temperature at which fish congregate when placed within a thermal gradient) of 15°C with a 10–20°C realized thermal niche for brook trout in nature with an upper threshold of 21–23.5°C (Smith and Ridgway, 2019). Similarly, other thermal tolerance studies on brook trout have

observed a decline in growth rate as temperature increases above 16°C with an upper limit for positive growth at 23.5°C (Chadwick and McCormick, 2017). Furthermore, at temperatures above 16°C plasma cortisol levels significantly increased and the cellular stress response induced, as exhibited by elevated gill Hsp70 levels (Chadwick and McCormick, 2017). While some studies have documented local adaptation for heat tolerance across a set of streams in Newfoundland, Canada (Wells et al., 2016), the spatial scale of local adaptation for brook trout remains unknown. Unfortunately, climate projections continue to show that range losses for brook trout along their southern boundary could reach up to 49% by 2050 (Meisner, 1990a; Chu et al., 2005; Flebbe et al., 2006). As brook trout are a highly sought-after sportfish (Gale et al., 2013; Brownscombe et al., 2014; Lizée et al., 2017) and an environmentally important predator, it is imperative to understand how brook trout populations will respond as temperatures continue to rise.

Thermal stress physiology

If fish are exposed to a thermal disturbance or stressor, a generalized stress response may be induced. The stress response begins when the neuroendocrine system releases catecholamines (e.g., norepinephrine, epinephrine) that trigger a physiological cascade, resulting in the activation of the hypothalamic-pituitary-inter-renal (HPI) axis (Wendelaar Bonga, 1997; Barton, 2002). To measure the stress response, it is necessary to use physiological tools to assess the concentrations of organic substances in tissues that indicate activation of the HPI axis. Appropriate metrics include concentrations of glucocorticoids, like cortisol, which in fish is a hormone released from the interrenal cells of the head kidney in response to activation by ACTH (Adrenocorticotropic hormone; Wendelaar Bonga, 1997; Barton, 2002). Elevated levels of cortisol and catecholamines in the blood ultimately lead to gluconeogenesis, thereby elevating levels of plasma glucose

throughout the body (Wendelaar Bonga, 1997). Metabolites, like glucose are useful secondary indicators of the activation of the HPI axis, as they represent essential energy processes in fish that change in response to release of cortisol into the circulatory system (Barton, 2002). Whole-organism measurements, such as the rate of oxygen consumption, can also indicate that the HPI-axis has been activated and energy demands on the organism have changed (Burton et al., 2011). At critical high or low temperatures, mitochondrial anaerobic metabolism can be induced because of low tissue oxygen levels due to a mismatch of oxygen supply and demand (Pörtner, 2002). This concept is further explained by the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis that states that the temperature dependent performance curve of animals is shaped by the capacity for oxygen delivery to the body in relation to oxygen demand (Pörtner, 2001, 2002; Pörtner et al., 2017). For example, this includes the mechanisms of oxygen delivery (ventilatory oxygen uptake and cardiovascular oxygen transport) as well as oxygen utilization at the mitochondrial level to maintain aerobic metabolism (Pörtner et al., 2017). If a mismatch between oxygen demand and delivery continues, general organism performance declines and can ultimately cause long-term constraints in fitness and plays a role in defining the thermal niche of the animal (Verberk et al., 2016). Lactate, another metabolite, also gives insight into the response to acute stress as it is an end product of anaerobic metabolism and increases concentration in the blood and white muscle of fish after exposure to events such as extensive exercise (Wood et al., 1983). Additionally, changes in osmolality and ion concentrations can indicate acute stress and give insight into osmotic and acid-base regulation (McDonald and Milligan, 1997), which can change in response to changes in metabolic processes. The physiological indices mentioned above are important for indicating the presence of a stress response in fishes, however,

incorporating molecular strategies, such as the abundance of specific mRNA transcripts, also provides key information in the response at the cellular level.

Tools that examine the levels of mRNA transcribed within a cell or tissue, known as the transcriptome, such as high-throughput qPCR, microarrays, and RNA-sequencing, have been incorporated into ongoing studies within a conservation physiology framework (Connon et al., 2018). Transcriptomics, the study of transcriptome-wide processes, has been highly successful in determining thermal tolerance thresholds of individuals (Connon et al., 2018) and contributed to advances in understanding the cellular processes behind whole-organismal physiology (Miller et al., 2014; Evans, 2015). Furthermore, how long a fish maintains a cellular stress response is a factor in determining how a species will cope with high temperatures (Jeffries et al., 2014). To understand the transcriptomic response, it is important to differentiate between measuring mRNA abundance and gene expression. Gene expression (i.e., the level of expression of a specific protein) is useful to identify the functional response to a stressor. Whereas the transcriptomic response (i.e., mRNA abundance) is often more rapid and is an intermediate step between the genotype of an animal and protein function, and therefore can be used to estimate changes in gene expression (Connon et al., 2018). Proteins and mRNA transcripts may be expressed continuously to maintain critical cellular processes (constitutive) or they may be induced as a response to stressors (inducible) in order to enhance survival (Iwama et al., 1998). In terms of mRNA abundance, changes in the expression of transcripts that are constitutive would be expected to respond to chronic events such as thermal acclimation, in contrast to inducible genes which respond to acute change and could be expected to gradually decrease abundance throughout acclimation. Thus, by monitoring mRNA abundance and changes in the

expression of transcripts, one can determine the impacts of changing temperatures on an individual and particularly the stress response that follows exposure to warming temperatures.

To adequately understand the stress response, it is necessary to study constitutive and inducible genes that are involved in various essential processes in fish including response to temperature and stress as well as genes related to osmoregulation, metabolism, and other regulatory processes. Among those genes involved in the stress response, constitutively expressed heat shock proteins such as Hsp90ab1 (heat shock protein-90 alpha beta-1) and Hspa8 (heat shock protein alpha-8), are important to measure as they are molecular chaperones that can help prevent denaturation of proteins and have a regulatory role in autophagy (I.e., cell regulation that removes unnecessary and/or dysfunctional components; Stricher et al., 2013; Akbarzadeh et al., 2018). In terms of constitutive expression, these heat shock proteins have been observed in studies as early as 1993 to have more constant presence at lower temperatures with a significant increase at higher temperatures (*Carassius auratus* 30°C, Kikuchi et al., 1993). The constitutive pattern whereby heat shock proteins remain relatively constant until a critical higher temperature is reached, is expected. Additionally, Nupr1 (nuclear protein-1) is a nuclear protein that plays a regulatory role in cell growth and apoptosis (Momoda et al., 2007). Nupr1 acts as a transcription factor whose upregulation suggests that cellular and physiological pathways are being affected (Momoda et al., 2007), whereby, Nupr1 transcription can aid in continued cell growth and control of cell death despite the disturbance. Serpinh1 (Serpin Family H Member 1) is another gene involved in the response to stress and is key in the restoration of homeostasis during heat stress as it aids in the biosynthesis and stabilization of collagen (Jeffries et al., 2014; Akbarzadeh et al., 2018; Swirplies et al., 2019). A member of the glutathione peroxidases, Gpx1 (glutathione perioxidase-1), plays a key role in the oxidative stress response and cell immunity

(Sattin et al., 2015). Other genes of importance include those involved in the osmoregulatory processes that help maintain ion and acid-base homeostasis. Key genes in this area including Na+/K+-ATPase (Atp1a3) that help to create electrochemical gradients and provide energy for cells during active transport (Michael et al., 2016). Similarly, ion channels and co-transporters (ATP-sensitive inward rectifier K^+ channel "Irk", V-type-H-ATPase "Vat", Cystic fibrosis transmembrane conductance regulator "Cftr", and $Na^+/K^+/2Cl^-$ co-transporter "Nkcc") play significant roles in ion regulation across the gill (Evans et al., 2008; Dymowska et al., 2012; Hiroi and McCormick, 2012). Finally, metabolically related genes such as glucose-6-phosphatase (G6pc) that help to facilitate glucose into the blood may indicate the onset of recovery post stress as this is related to energy metabolism (Momoda et al.,2007; Wiseman et al., 2007). Using transcriptomics and examining genes that are involved in a suite of responses, especially to temperature, I will be able to understand from a cellular level the impacts of temperature and acute stress on juvenile brook trout.

Thesis Objectives and Hypotheses

Fish are inherently impacted by changes in their thermal environment and climate change is threatening many salmonid populations with extirpation (Meisner, 1990b; Friedland, 1998; Flebbe et al., 2006; Waples and Hendry, 2008). Climate change introduces several additional stressors to fish environments such as fluctuating temperatures, increased susceptibility to disease, and altered habitat (Reid et al., 2019). By pairing physiological indices with mRNA abundance, and comprehensively studying both temperature acclimation and acute stress events, we can obtain a holistic picture of the influence that warming temperatures have on salmonids, specifically brook trout. Furthermore, understanding the length of time that brook trout maintain a stress response indicates how they will cope with higher temperatures, making it useful to

determine if species or populations will successfully respond to climate warming (Jeffries et al., 2014). The objective of my theses was to understand the effects that global climate change will potentially have on brook trout populations. I aimed to examine 1) how thermal acclimation influences cellular and physiological processes and 2) how brook trout recover after thermal acclimation and exposure to paired acute stress events. Within these objectives, I predicted that mRNA abundance of genes associated with the cellular stress response would change when fish were exposed to temperatures beyond the 20°C thermal threshold identified by past studies on brook trout (e.g., Meisner, 1990a; Benfey et al., 1997; DeWeber and Wagner, 2015; Chadwick et al., 2015; Chadwick and McCormick, 2017). Additionally, I predicted that exposure to the acute stress events would result in an elevated stress response and be exacerbated at higher temperatures, with longer recovery time in those individuals acclimated to temperatures higher than the preferred range for brook trout.

<u>Chapter 2: Thermal Acclimation in Juvenile Brook Trout (*Salvelinus fontinalis*)</u>

Abstract

Rising water temperatures are affecting the health and distribution of aquatic organisms. Brook trout (*Salvelinus fontinalis*), a popular freshwater sport fish adapted to cooler environments, is at risk to high temperature due to its habitat preferences. Using six acclimation temperatures that span the thermal distribution of the species (5°C, 10°C, 15°C, 20°C, 23°C, and 25°C), I quantified the stress response of juvenile brook trout by measuring physiological and molecular parameters to understand the processes that occur when fish are thermally stressed. Evidence of a stress response was apparent *via* exposure to a range of temperatures outside their preferred range (i.e., >20°C) and by exhaustive exercise using a chase and air exposure protocol. Using qPCR, I quantified mRNA abundance of genes associated with stress and temperature-induced cellular mechanisms to aid in the identification of a sub-lethal temperature threshold for this species. Furthermore, I sought to understand the effect of acclimation temperature on their recovery when exposed to acute stress events that simulate the paired stressors of exhaustive exercise and air exposure; stressors that may be potentially associated with catch-and-release angling. I predicted that exposure to acclimation temperatures beyond those to be preferred by brook trout caused individuals to exhibit an increased stress response and increased recovery duration. My results indicate that brook trout are negatively affected by temperatures above 20°C and a sub-lethal threshold exists between 20°C and 23°C. Brook trout exhibited signs of cellular impairment with upregulation of genes at 20°C and above (Hsp90ab1, Hspa8, Gpx1) or downregulation at 20°C and above (Nkcc and Nupr1). After exposure to paired stress events there was increased activation of the HPI axis at 20°C and above as exhibited by elevated levels

of cortisol, lactate, and glucose. Additionally, metabolic results suggested that energy expenditure is higher at elevated temperatures. These findings are imperative to understanding the implication of global climate change on brook trout populations and will be useful for managing fish habitat and regulating recreational angling.

Introduction

Global climate change is a persistent threat to biodiversity. Many terrestrial, freshwater, and marine species have shifted their geographical ranges, seasonal activities, and migration patterns due to warming environmental temperatures (Nogués-Bravo et al., 2018). Freshwater ecosystems are especially threatened because of their convergence of biological niches with human freshwater exploitation (Reid et al., 2019). It is estimated that 50% of freshwater species are threatened by climate change (Darwall and Freyhof, 2016; Reid et al., 2019), and among the many environmental factors impacting freshwater species, warming temperatures are the most significant threat. Freshwater species can either adapt, disperse, acclimate, or die in response to changing temperatures (e.g., Addo-Bediako et al., 2000; Root et al., 2003; Fuller et al., 2010; Sunday et al., 2010), therefore understanding how freshwater fishes currently respond to warming temperatures is a research and management priority.

Warming temperatures are a major threat for freshwater fishes because temperature is the master abiotic factor for fish. Temperature controls most (if not all) major physiological and ecological processes in fish (Fry, 1947; Beitinger and Bennett, 2000; Somero, 2005). When fish are exposed to temperatures beyond their species-specific thermal range, the magnitude of change and the length of time of their exposure dictates the type of biological responses that will occur (Hofmann and Somero, 1996). With climate change, it is assumed that annual exposure to increased temperatures may surpass the physiological limits of some species, which could lead to extirpation or extinction (Nogués-Bravo et al., 2018). Where possible, movement away from warming water can reduce thermal stress (Neill and Magnuson, 1974; Coutant, 1975; Richards et al., 1977) and therefore prevent extinction but lead to extirpation. Often, however, behavioral and physiological responses to temperature are acute in nature, and therefore unavoidable. Acute

responses to temperature serve to protect cells, tissues, and organ systems and occur after seconds to minutes of exposure to a thermal stress (Sopinka et al., 2016; Chadwick and McCormick, 2017). Acute responses can include the release of the glucocorticoid cortisol, which helps to mobilize energy stores (Sapolsky et al., 2000; Barton, 2002). Metabolites such as glucose are also acute responses that help fish survive and recover from thermal stress by providing energy for demanding tissues (Brett, 1964; Beamish, 1978; Wendelaar Bonga, 1997). Ultimately, should thermal responses fail to protect the organism, mortality will occur, which if widespread can lead to population extirpation and species extinction.

Animals, however, can thermally acclimate, and this adaptive response to environmental change can prevent or mitigate against extirpation and extinction (Crozier and Hutchings, 2014). Thermal acclimation occurs when organisms are exposed to a change in temperature for weeks to months (Hochachka and Somero, 2002). Through acclimation, organisms attempt to maintain homeostasis, whereby the body undergoes physiological changes to keep the biological processes operating at the same rate throughout the organism's exposure to environmental change (Schreck and Tort, 2016). Additionally, changes at the transcript- and protein-level occur to re-organize and regulate cellular machinery, which involves increases in the expression of genes involved in the oxidative stress response, collagen stabilization, immune function, and protein folding (Jeffries et al., 2012; Komoroske, 2015). In addition to alterations in gene regulation, factors such as heart rate, ventilation rate, metabolic rate, and enzymatic reactions shift in response to thermal acclimation (Willmer et al., 2009). Acclimation ability allows a fish to alter their phenotype and potentially successfully respond to environmental change (Hochachka and Somero, 2002). By measuring the integration of transcriptomics with other physiological parameters, we can identify physiological thresholds that are predictive of compensatory

responses and detrimental outcomes (Connon et al., 2018). Moreover, research on aspects of thermal acclimation will aid in identifying if fish are able to acclimate to higher temperatures or if climate change will cause extirpation or extinction.

Brook trout (*Salvelinus fontinalis*) populations in North America are facing the threat of thermal stress and possible extirpation (Chadwick et al. 2015). Brook trout are distributed throughout formerly glaciated and non-glaciated regions of central and eastern North America (Scott and Crossman, 1973; Stitt et al., 2014), and their habitat generally consists mainly of clear, cool, and well-oxygenated streams, with temperatures that are generally below 20°C (Scott and Crossman, 1973). Brook trout rely on cold-water seeps for multiple life stages and developmental events (Ridgway and Blanchfield, 1998; Ridgway, 2008; McDermid et al., 2012), making cool thermal refugia crucial for some brook trout populations. In fact, temperature is more predictive of brook trout occurrence than geomorphological features in their natural range (Rashleigh et al., 2005). Brook trout are a cool water species (Cherry et al., 1975; Power, 1980; Stitt et al., 2014), meaning that they can withstand only a semi-narrow range of temperature change in their environment. The preferred temperature for brook trout is predicted to be approximately 15°C with a lab-derived fundamental niche of 13–17°C (e.g., Graham, 1949; Fry, 1971; Cherry et al., 1977; Stitt et al. 2014) and an upper threshold of 21–23.5°C that induced thermal avoidance and physiological stress (Meisner, 1990a; Benfey et al., 1997; DeWeber and Wagner, 2015; Chadwick et al., 2015; Chadwick and McCormick, 2017). Studies have indicated that brook trout are limited by temperatures above 20°C and are not present in locations above a 24-day mean maximum of 22°C (Wehrly et al., 2007; Robinson et al., 2010; Morrison et al., 2020). Moreover, brook trout are usually absent in locations where the daily mean temperature exceeds 23°C (Ricker, 1934; MacCrimmon and Campbell, 1969; Meisner, 1990b; Wehrly et al.,

2007), which could be partly due to a physiological stress response (heightened levels of plasma glucose and cortisol and Hsp70 expression) occurring above 21°C (Chadwick et al., 2015). In their native range, brook trout populations are decreasing in the southern end of their distribution, presumably because of increased exposure to warm temperatures (Gunn and Snucins, 2010; Budy et al., 2019). Clearly, brook trout are selected for cold hardiness (Chu et al., 2005; Stitt et al., 2014) and not for warmer temperatures, therefore future increases in temperatures will create difficult environmental conditions for these fish to thrive, making it ever more critical to study the relationship between temperature and their physiological stress response.

To predict the effects of climate change on brook trout, understanding the physiological systems that dictate their thermal limits, and their acclimation capacity is necessary (Somero, 2010). Therefore, my first objective was to examine the effects of temperature acclimation on cellular and physiological processes in juvenile brook trout. Brook trout were thermally acclimated across six different temperatures (5–25°C) for a period of 21 days. Within these acclimation temperatures, I predict that there is a sub-lethal threshold between 20–23°C for these juvenile brook trout as supported by findings in Morrison et al. (2020), that showed reduced physiological performance between 20°C and 23°C. Post-acclimation, brook trout gill and liver tissues were sampled to measure mRNA transcript abundance patterns. I hypothesized that mRNA transcript levels of genes associated with the cellular stress response would change when fish were acclimated to temperatures beyond their preferred range. Specifically, I expected genes involved in a cellular stress response (HSPs, Serpinh1, Gpx1, Nupr1) to be upregulated at 20°C and at higher temperatures. Conversely, at sub-lethal temperatures, cellular responses may differ and may peak prior or near detrimental physiological changes (Jeffries et al., 2014; Jeffries et al.,

2018). For this reason, I predicted that those genes involved in osmoregulation (Atp1a3, Atp1a3a, Irk, Cftr, Nkcc, and Vat) would be relatively stable at temperatures below 20°C, but would decline once the sublethal threshold is reached. Furthermore, activation of the hypothalamic-pituitary-inter-renal (HPI) axis and the associated changes in hormonal and metabolic indicators would also change when fish were exposed to temperatures that approach brook trout specific minimum (0°C, Hynes, 1970) and maximum (25.4°C, Fry et al., 1947). For indicators such as cortisol and glucose, I expected relatively similar levels across all temperature groups, as these fish are not being handled or exposed to any targeted stress. Similarly, I expected muscle lactate concentrations to remain largely unchanged across temperatures compared to concentrations in the following acute stress groups.

My second objective was to observe the effects of 21-d temperature exposures on the physiological recovery of a different subset of brook trout following paired acute stress events (i.e., chasing and air exposure). I hypothesized that chasing and air exposing the fish would result in varied levels of physiological changes, and that physiological recovery would be different depending on the treatment temperature. Specifically, I predicted that the HPI axis activity would increase significantly at temperatures near 20–23°C post-acute stressor. Therefore, I expected elevated cortisol and glucose levels at temperatures 20°C and above, with the higher temperature groups experiencing the longest recovery time due to increased energetic demands (Suski et al., 2003; Gingerich et al., 2007; Gale et al., 2013). Additionally, I predicted that muscle lactate would be elevated across all temperature groups after exposure to the stress events (Suski et al., 2006; Louison et al., 2017). Lastly, as the fundamental thermal niche of brook trout has been estimated to be 13–17°C (Smith and Ridgway, 2019), I expected the

physiological indicators described above to be decreased at 10°C, however, as 5°C approaches brook trout minima, the indicators would be elevated.

Methods

Study Animals

Sampled juvenile brook trout were first generation (F1) brook trout originally obtained from the Whiteshell Fish Hatchery in eastern Manitoba, Canada. The strain originated from Gods Lake and Gods River in northern Manitoba, and was brought to the Whiteshell Hatchery, Manitoba in the 1970s. The fish were then stocked into the South Duck River on the east slope of the Duck Mountains, Manitoba. After the stocking, a new brood stock was established at the hatchery from fish recaptured from the South Duck River (Kevin Dyck, personal comm., 2018). In 2016, brood stock brook trout were obtained from the Whiteshell Hatchery and bred at the Department of Fisheries and Oceans Canada (DFO) Freshwater Institute in Winnipeg, Manitoba. Hatching occurred in January 2017, and fish were at "swim-up stage" on February 15, 2018. All fish were held in one of two aerated 600 L circular flow-through tanks at approximately 10°C. Fish were fed *ad libitum* with commercial pellet fish food (EWOS Pacific: Complete Fish Feed for Salmonids, Cargill) throughout the 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 collected, placed into 200 L aerated flow-through tanks, and exposed to one of six temperatures representing a thermal gradient consistent with brook trout distribution and known preferenda based on lab studies, for 21 d (n = 26 per temperature group, 5, 10, 15, 20, 23, and 25°C [n=50 per temperature tank, 24 fish used in other

study]). Temperature exposures were staggered across four months in 2018–19: 10°C beginning on October 11, 25°C on October 23, 23°C on November 2, 20°C on November 16, 15°C on December 17, and 5 °C on January 1. Fish were first transferred to a 200 L acclimation tank at 10–11°C and were allowed 1 d to recover from the handling stress. Then, the water was gradually changed to the assigned treatment temperature at a rate of 1.5–2°C day^{-1} using heating and 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 3 wk 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, Massachusetts, USA) and controlled with WitroxCTRL software (Loligo® Systems, Tjele Denmark), where it fluctuated daily (± 1.5 °C of the treatment temperature) to simulate diurnal temperature changes in the water (Durhack et al., 2020). A 12:12 hour day-night cycle was used throughout the experiment (65 min of dawn and dusk, full-light starting at 07:05, and full dark at 19:05). Dissolved oxygen was kept constant throughout the experiment. Previous studies have shown that the minimum oxygen requirement for brook trout is 5mg/L (Graham,1949; Raleigh, 1982; Spoor, 1990), our experimental setup kept the dissolved oxygen above 7mg/L as we aimed to maintain the oxygen levels near saturation. The 25°C group was not exposed to the full 3-wk treatment period, as fish exhibited potential skin infections, reduced feeding, and mortality. The 25°C treatment group was sampled after 11 d.

Tissue Sampling

After the 3-wk temperature acclimation period, fish were divided into two groups. The first group was lethally sampled upon removal from the acclimation tank (n = 10 per temperature). Fish were euthanized separately with a dose of buffered tricaine methanesulfonate solution [MS-

222] (300mg·l^{-1}; buffered with 600mg·l^{-1} of sodium bicarbonate $NaHCO_3$) and then measured for length (total and fork length ± 0.1 cm) and body mass (g) prior to tissue sampling. Blood was collected by severing of the caudal fin and using ammonium-heparinized capillary tubes (Fisherbrand®, Fisher Scientific, Pittsburgh, Pennsylvania, USA) to collect blood (Lawrence et al., 2020). Whole-blood glucose was immediately measured using a UltraMini® Glucose Meter (OneTouch®, LifeScan Canada, Burnaby, British Columbia, Canada) then blood samples were centrifuged at 3.0 × 1000 *g* for 6 min. Plasma was removed, placed in a cryovial, and flash frozen in liquid nitrogen. The whole second gill arch with filaments from the left side of each fish was excised and placed in RNALater™ (Invitrogen™, Carlsbad, CA, USA). The entire liver was removed, weighed (wet mass ± 0.01 g), halved, and placed in RNALater. The liver mass was used to calculate the hepatosomatic index (HSI), which serves as an indicator of aggregate condition of the fish (Barton, 2002). HSI was calculated as liver mass / (body mass – liver mass) × 100. A filet of white muscle was taken from the fish's left side, sectioned, placed in aluminum foil, and stored at -20°C. Then a small section was taken from the fish's right side, stored in a cryovial and placed in liquid nitrogen. All the collected tissues were stored at -80°C, though tissues stored in RNALater were first stored at 3°C for 24 h.

Acute Stress and Recovery

The second group of fish from the temperature exposure tanks (n = 16) underwent an acute stress and recovery trial prior to being sampled. All fish were put through a 2 min chase test (e.g., Suski et al., 2006) and then air exposed for 5 min (e.g.,Gingerich et al., 2007). The chase test consisted of placing the fish into an annular swim chamber that consisted of a bucket with 15–20 L of water in it with a cone in the centre to force the fish to swim around the perimeter of the container. The fish was then taken out of the water for the air exposure. During the air exposure,

fish were measured for length and weight following the same protocol as described above. Eight of the fish were then placed into a holding tank for 30 min before being euthanized (Cook et al., 2011). Blood and white muscle samples were then collected as described above. The other eight fish were placed in an intermittent-flow respirometer for 24 h to record oxygen consumption (Chabot et al., 2016). After 24 h, fish were euthanized and sampled for blood and white muscle as described above.

Intermittent flow respirometry was used to quantify various metabolic metrics (Norin and Clark, 2016). Oxygen was measured by in-line probes connected to respirometry chambers (Presence, Regensburg, Germany) and oxygen consumption was 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 standard metabolic rate (SMR) and maximum metabolic rate (MMR). Standard metabolic rate (SMR) is defined in an ectotherm as the minimal metabolic costs required to maintain physiological functions, including homeostasis (Beamish, 1964; Brett, 1964; Fry, 1971). In contrast, maximum metabolic rate (MMR) is an organism's maximum aerobic metabolic rate that is usually achieved during exhaustive exercise (Fry, 1971; Brett and Groves, 1979). For SMR calculations, the lowest 20th quantile of MO_2 estimates were used after removing the first 10 hours of measurements to only include estimates from when the fish was returned to a resting state (Norin and Clark, 2016). Following this, the 'FishMO2' package (Chabot et al., 2016) was used to further analyze MO_2 estimates. Three measurements of maximum metabolic rate (MMR) estimates were taken at the onset for each fish (Norin and Clark, 2016). Aerobic Scope (AS) was calculated by subtracting SMR estimates from the MMR for each fish, which allows one to understand the ability of that organism to allocate energy to

other demanding processes (e.g., growth, reproduction; Eliason and Farrell, 2016). Finally, excess post-exercise oxygen consumption (EPOC) was determined by calculating the area bounded between the recovery curve and SMR value, between the time where MMR and SMR were reached. Measuring excess post-exercise oxygen consumption (EPOC) clarifies the capacity for fish to recover from high intensity exercise (Scarabello et al., 1991; Lee et al., 2003; Svendsen et al., 2010) and the total energetic cost for aerobic and anaerobic metabolism (Svendsen et al., 2010).

Physiological Assays

Blood plasma samples were used to measure plasma cortisol, plasma glucose, and plasma osmolality. Plasma cortisol levels were quantified using an enzyme-linked immunosorbent assay (ELISA; 1:50 dilution; Neogen Corporation, Lexington, Kentucky, USA), previously validated for use in fishes (Sink et al., 2008). The osmolality was determined using a VAPRO vapour pressure osmometer (Wescor Inc., Logan, Utah, USA). Briefly, plasma samples of 10 µL were placed onto a solute-free paper disc into the sample holder of the instrument and osmolality readings were displayed based on the solution vapor pressure. Plasma glucose was quantified using a hexokinase kinetic glucose assay that was adapted for a 96-well plate (Treberg et al., 2007). For the glucose assay, a dilution factor of 1:29 was used. This assay was conducted to provide a correction factor for the glucose values obtained from the UltraMini® Glucose Meter.

The white muscle was used to determine the amount of muscle lactate. The protocol outlined by Booth et al. (1995) was used to extract tissue metabolites and an enzymatic assay was used to determine the final amounts of muscle lactate in the samples (Lowry and Passonneau, 1972; Gutman and Wahlefeld, 1974). Water content of the muscles was determined by drying pre-weighed frozen tissues in 65°C for 48 h until a constant mass was obtained. To

calculate water content, the wet mass (before baking) of the muscle was compared to that of dry weight (after 48 h).

Quantitative real-time RT-PCR

Total RNA was extracted from the gill and liver tissues using a Qiagen RNeasy Plus Mini Kit (Qiagen, Toronto, ON, CA) following manufacturer's protocols. For each gill sample, the filaments were cut out from the arch of gill (0.5–1 cm^3). Similarly, the liver samples were divided into pieces, approximately 0.1–0.3 cm^3. Each sample was then placed in homogenization tubes containing 600 μL of Buffer RLT Plus and a metal bead before being disrupted for 6 min at 50 rps. The lysate was then centrifuged for 3 min at 13,000 RPM. The supernatant (600μL) was transferred to gDNA eliminator spin columns nested in 2 mL collection tubes. Tubes were centrifuged for 30 s at 13,000 RPM, the column was removed, and 600 μL of ethanol (70 % for gill tissue and 50 % for liver tissue) was added to the flow-through and mixed by pipetting. Next, 700 μL of the solution was transferred to an RNeasy spin column that was placed in a 2 mL collection tube, and centrifuged for 15 s at 13,000 RPM. The flow through was then discarded and 700 μL of Buffer RW1 was added and the tubes were centrifuged for another 15 s. The flow-through was, again, discarded and 500 μL of Buffer RPE was added to the column and centrifuged again for 15 s. Flow through was, again, discarded, 500 μL of Buffer RPE was added, and then centrifuged for 2 min. The RNeasy spin column was placed in a new 2 mL collection tube and centrifuged again for 1 min to aid in drying the membrane. Finally, the RNeasy column was placed in a new 1.5 mL collection tube, 30 μL of RNase-free water was added to the spin column membrane, and centrifuged for 1 min, to elute the RNA of the sample.

The RNA was eluted using 30 µL of RNase- free water and all the samples were checked for purity (A260/A280 & A260/A230) and concentration (ng/µL) using a NanoDrop One Spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA) For purposes of cDNA synthesis, RNA concentrations were chosen to be in the 100–500 ng/µL range and diluted with RNase free water to that concentration if they were initially higher. The absorbance ratios (A260/A280 & A260/A230) had to be at least 1.8 or above, as ratios lower than these values indicate that there are impurities such as reagents present in the samples. If samples had ratios lower than 1.8 or low concentrations, the extraction process was repeated for that sample until a proper yield was quantified.

Gel electrophoresis on a 1% w/v agarose gel was used to check the integrity of the RNA. The solution was microwaved for 105 s and then poured into the gel apparatus with combs inserted to create wells. Once the gel hardened, tris-acetate (TAE) buffer was poured over the gel and into the apparatus. Into each well, approximately 100 ng of RNA isolate was mixed with 4.5–5.5 µL of DNase/RNase-free distilled water (UltraPure, ThermoFisher Scientific, Wilmington, DE, USA) and 1µL of gel loading buffer (BlueJuice, ThermoFisher Scientific, Wilmington, DE, USA). Additionally, 1Kb Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA) was loaded into the gel to act as a reference band. Gels were then visualized using a benchtop UV transilluminator (Endress & Hauser, Upland, California). For cDNA synthesis, 1 µg of total RNA was reverse transcribed into cDNA using the Qiagen QuantiTect Reverse Transcription Kit (Qiagen, Valencia, California, USA). For this method, UltraPure water was added to each sample to bring the total volume to 8 µL (the plate was kept on ice throughout the entire procedure). All samples were randomized for loading into the plate. Additionally, RNA samples (two from each temperature) were pooled together and added into seven additional wells, in order to create pools

to use for generating a standard curve in qPCR (~2 µL of sample and ~14 µL of RNAse free water, depending on sample concentration). Master mix, consisting of gDNA Wipeout Buffer (7x, 4 µL) and 8 µL of RNase-free water, was added to each well, to yield a total volume of 14 µL and the plate was incubated for 2 min at 42°C. Six µL of reverse transcription master mix that included Quantiscript Reverse Transcriptase, Quantiscript RT Buffer (5x), and RT Primer Mix, was added to each sample to yield a total volume of 20 µL. The mastermix was also added to 14 µL of water to serve as negative controls for the qPCR. The plate was incubated for 15min at 42°C and then for 3 min at 95°C, then stored at -20°C.

Primers were designed for three reference genes, 60s ribosomal protein L7 and L8 (Rpl7 and Rpl8) and 40s ribosomal protein S9 (Rps9). Additionally, primers were designed for fourteen target genes: Na^+/K^+-transporting ATPase subunit alpha-3 and alpha-3a (Atp1a3 and Atp1a3a), cystic fibrosis transmembrane conductance regulator (Cftr), cold-inducible RNA-binding protein (Cirbp), glucose-6-phosphatase (G6pc), glutathione peroxidase-like peroxiredoxin (Gpx1), ATP-sensitive inward rectifier K^+ channel 8 (Irk8), heat shock cognate 71 kDA protein (Hspa8), heat shock protein HSP 90-beta (Hsp90ab1), $Na^+/K^+/2Cl^-$ co-transporter (Nkcc1), nuclear protein 1 (Nupr1), serpin h1 (Serpinh1), and B and E 1 subunits of V-type H-ATPase (VatB and VatE1) [Table 2.1]. All forward and reverse primers were designed using Primer Express 3.0.1 (Applied Biosystems, ThermoFisher Scientific), Wilmington, DE, USA [Table 2.1] and purchased from Integrated DNA Technologies (Coralville, Iowa, USA). Primers were generated using the brook trout transcriptome data from Sutherland et al. (2019). Primer efficiencies were tested using pooled RNA samples from the treatment fish in a 1:4 standard curve. For this to be accomplished, original primers were first diluted with water (based on operating instructions) and then further diluted and separated to create a 60 µM concentration.

Master mixes of each primer were created using the forward and reverse primers (60 µM), water (UltraPure, ThermoFisher Scientific, Wilmington, DE, USA) and PowerUP SYBR Green Master Mix (Applied Biosystems, ThermoFisher Scientific, Wilmington, DE, USA), to create a 0.5µM concentration. After this, the plate was sealed and placed in the QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific, Life Technologies Corporation, Carlsbad, California, USA) and qPCR was carried out according to manufacturer's instructions. Initial results from the system were analyzed using QuantStudio™ Design and Analysis Software v1.5.1 (ThermoFisher Scientific, Life Technologies Corporation, Carlsbad, California, USA). If a primer was deemed unsuitable (not binding properly, insufficient efficiency [< 85 %], insufficient R^2 [< 0.98]), new primer sequences were generated, ordered, and tried again using the same method, until a proper primer pair was validated. For each qPCR reaction, a mastermix was added consisting of forward and reverse primers (60µM, 0.1 µL of each primer), 0.8 µL water (UltraPure, ThermoFisher Scientific, Wilmington, DE, USA), and 6 µL of PowerUP SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Wilmington, DE, USA), to create a 0.5 µM concentration (5 µL of cDNA), which was run on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Life Technologies Corporation, Carlsbad, California USA) in 384 well plates for all treatment fish on the gill and liver tissues, for a total of 12 µL per well. Target mRNA levels were normalized to the three reference genes using the $2^{\wedge -\Delta Ct}$ method (Livak and Schmittgen, 2001). The average of the mRNA levels per treatment group of the three reference genes was plotted to confirm that there were not any differences between the groups and ensure that the reference genes were not changing with the experimental treatment. Reference genes were also confirmed using a pair-wise comparison with BestKeeper Version 1 (Pfaffl et al., 2004). These

comparisons confirmed that the three reference genes used were stable and suitable for the experiment.

Statistical Analysis

For the temperature acclimation groups, one-way ANOVAs were used to determine if temperature significantly predicted mRNA levels. Tukey's HSD tests were run to determine significant differences of mRNA abundance between temperatures. Assumptions of normality and variance were assessed using a Shapiro-Wilk normality test and a Levene's test, respectively. As mass can factor in some of the parameters (i.e., glucose, osmolality, water content, HSI) an ANCOVA was initially run with variables, but was shown to have no effect. For the acute stress group, two-way ANOVAs were used to determine if holding temperature and treatment group (unhandled, acute stress, acute recovery) predicted the physiological response variables (plasma cortisol, plasma glucose, tissue lactate, osmolality). Again, a Tukey's HSD test was run to determine if significant differences existed at each temperature between the three treatment groups. If assumptions of a two-way ANOVA were not met, a generalized linear mixed effects model (glmm) was used. Additionally, for the respirometry parameters (SMR, MMR, time recovery, aerobic scope, and EPOC), values were compared using a one-way ANOVA. When data did not meet the assumptions of a one-way ANOVA, a Kruskal-Wallis test was used, followed by a Dunn's post-hoc test. For all tests, the level of significance (α) was 0.05. All statistical analyses were run in R v.1.2.5033 (R Core Team, 2019)

Results

Chronic Temperature Exposure

Gill mRNA abundance

Gill mRNA abundance differed across temperatures for four of the twelve genes (Figures 2.1, 2.2, 2.3 and Table 2.2). Abundance of Nkcc mRNA at 23 and 25°C was three-fold lower than abundance at 5 and 10 °C (Figure 2.2; TukeyHSD, $p < 0.01$). Abundance of Gpx1 mRNA was two-fold higher at the highest temperatures of 23 and 25°C compared with 5, 10, and 15°C (TukeyHSD, $p < 0.05$). Abundance of Hsp90ab1 mRNA was two-fold higher at 25°C in comparison to the three coldest temperature treatments (TukeyHSD, $p < 0.05$). Lastly, for Atp1a3, mRNA was 4.2-fold greater in the 10 °C group compared to the 15°C (Figure 2.1; TukeyHSD, $p > 0.05$). For mRNA abundances of Atp1a3a, Cftr, Cirbp, Hspa8, Irk8, Serpinh1, VatB, and VatE1 there were no significant relationships with temperature exposure (TukeyHSD, $p > 0.05$, Table 2.2).

Liver mRNA abundance

In the liver tissue, almost all the genes displayed significant responses (Figure 2.4, Table 2.3). Heat shock proteins Hspa8 (Hsc70) and Hsp90ab1 were significantly upregulated at 23 and 25°C, with a 4-fold and 6-fold increase, respectively, compared to the lower temperatures (TukeyHSD, $p < 0.05$). Gpx1, had the highest mRNA abundance at 5°C and was significantly elevated when compared to 20, 23, and 25°C with a 4-fold increase in expression (TukeyHSD, $p < 0.05$). Abundance of G6pc was highest at 20°C and 3-fold higher than abundances at 5°C (TukeyHSD, $p = 0.003$). Lastly, Nupr1 showed varied abundance with the 20°C having the highest mRNA abundance, significantly higher than 25°C by 2-fold (TukeyHSD, $p = 0.002$). For

Serpinh1, there were no significant changes in mRNA abundance between temperature groups (TukeyHSD, $p > 0.05$).

Other indicators

Across the six temperatures, HSI values were equal across temperatures except at the highest temperature (one-way ANOVA; Df = 5, 59, SS = 4941, MS = 988.1, F = 4.088, $p = 0.003$; Figure 2.5). The 25°C temperature group exhibited the lowest index values at a mean of 1.05 ± 0.04 S.E. (TukeyHSD, $p < 0.05$). Percent water content of the white muscle showed similar patterns to the HSI values (one-way ANOVA; Df = 5, 59, SS = 28.59, MS = 5.719, F = 4.89, $p < 0.001$; Figure 2.6). No differences were exhibited between fish in the 5, 10, 15, 20 and 23°C temperatures. However, the highest temperature (25°C) had the highest water content percentage (76.6% ± 0.41).

Acute Stress Exposure

Stress Indicators

Chasing followed by an air exposure had an effect on physiological variables associated with the stress response in juvenile brook trout. Muscle lactate significantly increased on average 1.8 times across all five temperatures 30 min after the application of the stressor compared to fish sampled directly out of the acclimation tanks (TukeyHSD, $p < 0.001$) (Figure 2.7). Muscle lactate levels significantly decreased following the 24 h recovery period across all temperatures, except at 5°C, as lactate levels remained equal between unhandled and acute stress groups at that temperature. At 23°C, muscle lactate was lowest after 24 h of recovery (6.2 µmol/g ± 0.798). Plasma cortisol also varied with temperature, those exposed to colder temperatures (5°C and 10°C) had equal plasma cortisol across all three groups. However, fish exposed to warmer temperatures (15, 20, and 23°C) had significantly elevated plasma cortisol in both the acutely

stressed and 24 h recovery fish (TukeyHSD, $p < 0.001$, Table 2.5; Figure 2.8). Plasma glucose levels were also equal for fish exposed to colder temperatures (5 and 10°C) across the three groups, and juvenile brook trout exposed to warmer temperatures (15, 20, and 23°C) had significantly elevated plasma glucose following the chase and air exposure stressors (Table 2.6). At 20°C and 23°C, plasma glucose in the acutely stressed sampling group were 1.8-fold and 1.7-fold higher than the unhandled group (Figure 2.9) and after a 24 h recovery period, glucose levels were equal to that of the temperature exposure only group (Figure 2.9). Plasma osmolality was equal across groups for those fish exposed to the colder temperatures (5°C and 10°C). Osmolality in fish exposed to 15°C and 20°C and were chased and exposed to air increased significantly by 1.1-fold and 1.2-fold, respectively (Figure 2.10, Table 2.7). The 23°C group had significantly lower osmolality post-24 h, but the unhandled group and acute stress group had equal levels of osmolality.

Acute Stress Recovery

Standard metabolic rate increased with temperature (Figure 2.11 [A]) and SMR of individuals exposed to 20 and 23°C were significantly higher than those exposed to 5,10, and 15°C (glmm, $p < 0.001$, Table 2.8). The highest SMR occurred at 23°C, which was 1.4 times higher than at 20°C. There were no differences between the SMR of individuals exposed to 5, 10, and 15°C. Maximum metabolic rate was highest in juvenile brook trout at 23°C and MMR was equal for fish exposed to 10, 15, and 20°C (TukeyHSD, $p > 0.05$). Fish exposed to 5°C had the lowest MMR and it was 1.6-fold lower when compared to fish exposed to warmer temperatures (TukeyHSD, $p < 0.001$; (Table 2.8; Figure 2.11 [B]). EPOC values were significantly elevated for fish exposed to 15 and 20°C (72.46 mg/g ± 5.57 oxygen consumed) compared to those at 10 and 23°C (35.61 mg/g ± 4.77; Figure 2.11 [E], Table 2.8). Fish at 10°C exhibited the lowest

EPOC (23.95 mg/g ± 5.93 oxygen consumed). Similarly, the longest recovery times were in fish exposed to 5, 15, and 20°C (Figure 2.11 [C]), which where 2-fold higher compared to fish exposed to 10 and 23 °C (12.84 h ± 0.5). Aerobic scope values did not differ across temperature (Figure 2.11 [D], TukeyHSD $p > 0.05$, Table 2.8).

Discussion

Juvenile brook trout showed evidence of chronic thermal stress at higher acclimation temperatures and the acute stress response was more pronounced at warmer acclimation temperatures. Transcripts involved in heat stress and regulatory responses exhibited signs of cellular activity, as genes tended to be up or downregulated at 20°C and higher (Hsp90ab1, Hspa8, Gpx1; Nkcc and Nupr1). At a tissue level, elevated levels of cortisol, lactate, and glucose after a paired stress event, indicate increased activation of the HPI axis at 20°C and higher. Additionally, energy expenditure was higher at elevated temperatures, and fish experienced extended recovery times at high acclimation temperatures. The data suggests that a sub-lethal threshold for brook trout lies between 20 and 23°C and that there is a significant interaction between these high temperatures and acute stress, preventing full recovery after stress-inducing activities. Overall, brook trout may be unable to cope if water temperatures in their natural habitat rise above 20°C, and this may lead to extirpation from some systems.

Brook trout are negatively affected at temperatures above 20°C as first exhibited by the heat shock response in gill and liver tissues. Heat-shock proteins are part of a ‘classic’ temperature-induced cellular stress response (Iwama et al., 1998; Somero, 2010; Currie, 2011; Jeffries et al., 2014) and play a critical role in reducing and repairing damage to cellular proteins that arises from physical or chemical stress (Somero, 2010). In the liver tissue, both *hsp90ab1* and *hspa8* (*hsc70*) were significantly elevated by a 4 to 6-fold increase at 23 and 25°C. Similarly, in the gill tissue there was a significant 2-fold increase in abundance for *hsp90ab1* at 25°C. Numerous studies on fish have exhibited elevated heat shock protein transcript abundance with chronic high temperature exposure (Podrabsky and Somero, 2004; Quinn et al., 2011; Jeffries et al., 2012; Jeffries et al., 2014; Stitt et al., 2014; Akbarzadeh at el., 2018). Specifically, in brook

trout, although not significant, Chadwick and McCormick (2017) showed increased inducible *hsp70* abundance in gill tissue across a chronic exposure (16–24°C, 24 days) at 22 and 24°C. In other salmonids, arctic char (*Salvelinus alpinus*), sockeye salmon (*Oncorhynchus nerka*), and pink salmon (*Oncorhynchus gorbuscha*), *hsp90ab1* and *hsc70* have higher expression at temperatures ≥ 19°C (Quinn et al., 2011; Jeffries et al., 2012; Jeffries et al., 2014; Akbarzadeh et al., 2018). When temperatures are near a species' thermal limit, heat shock protein expression is activated (Currie, 2011). Changes in the levels of these proteins indicate an organism's short- and long-term ability to cope with thermal stress (Kassahn et al., 2009). Therefore, increased heat shock protein abundance at temperatures above 20°C suggest that brook trout are not able to acclimate to high temperatures. However, others have found that heat shock proteins can return to basal level or be downregulated during acclimation to chronically high temperatures (Meyer et al., 2011), indicating possibly successful acclimation. Therefore, it appears that brook trout, at the gill tissue level, may have acclimated to the elevated temperatures as exhibited by reduced activity expression of *hspa8*. While this contradicts the elevated patterns of heat shock proteins that I observed in the liver tissue, the variation may be in part due to the different tissues sampled and the organization of those tissues at gene level. Overall, at a cellular level, the observed increased in heat shock protein abundance during chronic acclimation at high temperatures indicates that brook trout are activating a cellular stress response above 20°C.

As temperature surpassed 20°C, an observable threshold was reached across other genes expressed in gill and liver tissues. Specifically, genes involved in the oxidative stress response, cell growth, and apoptosis demonstrated a change in expression. *Glutathione peroxidase 1* (Gpx1) is responsible for catalyzing 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). I detected a

significant 2-fold increase of *gpx1* mRNA abundance above 20°C in the gill tissue. As temperatures increase, there is greater oxygen demand and water uptake at the gill, due to increased gill ventilation and perfusion (Hunn, 1982). Additionally, as the metabolic rate increases, there is an increase in oxygen uptake by mitochondria, potentially resulting in elevated production of ROS (Davidson and Shiestl, 2001). As shown by the metabolic rate results, standard metabolic rate increased significantly with temperature and this increased metabolism at higher temperatures may be linked with elevated ROS and supports the increased abundance of *gpx1* observed in the gill. In contrast, in the liver tissue I observed significant 4-fold increase in abundance at cooler temperatures (i.e., 5 and 10°C). In Antarctic species (*Champsocephalus gunnari, Chaenocephalus aceratus, Pseudchaenichthys georgianus, Dissostichus eleginoides,* and *Notothenia* rossi), Ansaldo et al. (2000) observed an increase of glutathione perioxidase in the heart and liver tissues compared to the gills and muscle tissues. This increase of glutathione perioxidase in the liver may be explained by the fact that aerobic metabolism is highly dependent on the oxidation of unsaturated fats in the liver and these fats are susceptible to oxygen radical attack (Roberfroid and Calderon, 1995). The relationship between higher fat content in the liver and higher ROS attack may aid in explaining why the liver tissue exhibited increased abundance of *gpx1*, however, the cause of the *gpx1* increase is unclear.

I also measured the abundance of *nuclear protein 1* (Nupr1) in the liver tissue. *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). In my study, *nupr1* abundance was highest at 20°C, significantly higher than 25°C by 2-fold, which had the lowest expression. Although not significant, lower abundance was also observed at temperatures lower than 20°C. A U-shaped pattern or inverted U-shaped response have been observed in other studies. For example, Jeffries

et al. (2018) observed the inverted U-shaped pattern for genes in delta smelt (*Hypomesus transpacificus*) exposed to temperatures ranging from 14–27°C, where several genes exhibited up- or downregulation at 20 and 25°C, but not at 27°C. Furthermore, it has been observed with sub-lethal stressors, there can be cellular responses that exhibit a peak near or prior to detrimental physiological changes (Jeffries et al., 2015; Jeffries et al., 2018). These non-linear responses have also been found in various toxicology studies that indicate sub-lethal exposure does not always show a dose dependent curve (Calabrese and Baldwin, 1999; Vandenberg et al., 2012; Lagarde et al., 2015). The inverted-U pattern seen in Nupr1 abundance may indicate that there are cellular feedback mechanisms involved in biological pathways to be impacted, resulting in downregulation at 25°C (Conolly and Lutz, 2004).

At higher water temperatures, brook trout also exhibited elevated activity of the HPI axis and endocrine stress response. While the effects of elevated cortisol at low severity of stress may be positive on routine life functions, effects can become negative at more prolonged elevation in cortisol concentration (Schreck, 1992). In my study, cortisol levels were significantly elevated post-stress in the acute stress group at 15, 20, and 23°C. Cortisol levels remained elevated 24-h later, indicating that together the high temperature and presence of an acute stressor, initiated an increased HPI axis response. This trend of significantly elevated cortisol at higher temperatures post-acute stress event has been displayed in several other studies (Jain and Farrell, 2003; Suski et al., 2003; Meka and McCormick, 2005; Suski et al., 2006; McLean et al., 2016). Conversely, at cooler temperatures, the cortisol response was depressed across all three groups. Colder temperatures beyond a species range can be a major constraint that limits aerobic scope and cellular function in individuals (Pörtner, 2002). In this study, at the lower temperatures (5 and 10°C) cortisol levels remained the same across all three groups (unhandled, acute-stress, acute-

recovery). The decreased response of cortisol may be because the aerobic processes are highly limited at these cooler temperatures. Moreover, at low temperatures, the temperature coefficient (Q10) values can become much larger due to the increase of the energy barriers in the reaction, specifically at temperatures outside the species normal range (Hochachka and Somero, 2002). Therefore, the lower cortisol levels at 5°C are also potentially due to a higher Q10 value, as this temperature is outside the normal range for brook trout (Smith and Ridgeway, 2019), resulting in a slower enzymatic rate. In other studies, peak plasma cortisol levels for fish exposed to lower temperatures were also significantly delayed, possibly due to reduced enzymatic activity in cold conditions (Van Ham et al., 2003; Louison et al., 2017). For example, in northern pike (*Esox lucius*) captured during ice angling, plasma cortisol levels did not reach peak values until 4 h post-capture (Louison et al., 2017). Therefore, it is likely that the timing of blood sampling and measurement, specifically in the acute-stress group (sampled 30-min post paired stress events) did not allow all temperature groups (especially 5°C) to reach peak values, explaining the lower levels of cortisol expressed in the acute-stress group results. While reactivity was limited at lower temperatures, brook trout still exhibited an increased HPI axis response at higher temperatures, indicating an interactive effect of temperature and acute stress, which can also be further seen in other energy indicators in our study. Glucose showed a similar response pattern to stress and temperature as cortisol. Glucose increases throughout the body to meet energetic demand during a stress response (Barton and Iwama, 1991; Mommsen et al., 1999), potentially explaining why it was significantly increased after the stressful event at 15, 20, and 23°C. An increase in blood glucose with temperature and exercise has been displayed across species (Suski et al., 2006; Landsman et al. 2011) as well as in brook trout (Kieffer et al., 2011).

Lactate is a by-product of anaerobic metabolism and involved in the energy response. During anaerobic metabolism, which can be brought about by extensive exercise and activity, there is a buildup of concentrations of lactate in the white muscle of fish (Wood et al., 1983). The positive relationship between exercise and lactate concentration was observed in this study where, across all temperatures, the acute stress events induced anaerobic metabolism as exhibited by significantly elevated lactate in the white muscle. Several studies on a range of fishes including brook trout have demonstrated similar findings in either muscle lactate or plasma lactate concentrations when fish were exposed 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). When increasing temperatures are paired with exhaustive exercise and air exposure, muscle lactate (Suski et al., 2006) and plasma lactate (Meka and McCormick, 2005) concentrations were found to increase significantly in largemouth bass (*Micropterus salmoides*) and rainbow trout (*Oncorhynchus mykiss*), respectively. Our results, however, showed a significant decrease in muscle lactate after 24 h recovery in the 23°C group, potentially because of a release of lactate into the blood, as rainbow trout have been observed to release 10 to 20% of total muscle lactate produced into the blood at high temperatures (Milligan and Wood, 1986; Milligan and Girard, 1993; Kieffer et al., 1994). Fish may also be retaining the lactate in the muscle and recycle it *in situ* for glycogenesis (Milligan, 1996). Therefore, the observed decrease in muscle lactate after 24 h recovery in the 23°C group may be because the fish released it into the blood stream, or, because the lactate was recycled through glycogenesis to help the fish return its energy stores to pre-stress levels.

Standard metabolic rate increased significantly with temperature up to 23°C and metabolic rate recovery time from an acute stressor increased up to 20°C. I observed that SMR

increased with temperature, where at 20 and 23°C, SMR was significantly higher than the lower temperatures. There is a strong influence of temperature on SMR in ectotherms (Fry, 1971; Hulbert and Else, 2004) and fish respiration is a positive function of water temperature (Brett, 1964; Beamish, 1978), therefore higher SMR at higher temperatures was expected. However, SMR may decline once the upper thermal threshold is reached for a species and this has been observed in other brook trout acclimation studies (Stitt et al. 2014; Durhack et al., 2020). Brook trout that were acclimated across a range of temperatures from 5–23°C displayed increasing SMR up until 15°C where it was significantly elevated but then decreased at 20°C and further (Durhack et al., 2020). The difference in SMR values between this study and Durhack et al. (2020) may be due to a difference in methods as their study incorporated just the chase protocol to a point of exhaustion, while the current study had a paired stress event with a shorter chase protocol and subsequent air exposure. However, another study using west slope cutthroat trout (*Oncorhynchus clarkii lewisi*) observed a similar trend to my study where SMR continued to increase at temperatures above 20°C to a max SMR value at 22°C (Macnaughton et al., 2020). I measured HSI as the liver is the major reservoir for glycogen in fish and important for gluconeogenesis from lactate, amino acids, and glycerol (Walton and Cowey, 1982; Foster and Moon, 1986). In our study, 25°C fish exhibited the lowest HSI which is supported by the fact that at elevated temperatures there is an increase in metabolic rate which leads to the consumption of fats and carbohydrates from the liver, resulting in decreased HSI (Hochachka and Somero, 2002; Mellery et al., 2016). Aerobic scope remained constant across temperatures. Constant aerobic scope values were also observed in Nile perch (*Lates niloticus*) that were acclimated for three weeks at 27, 29, and 31°C, but exhibited no effect of temperature treatment on AS (Nyboer and Chapman, 2017). A similar trend of constant AS has also been observed in

chinook salmon (*Oncorhynchus tshawytscha*, Poletto et al., 2017) and pink salmon (*Oncorhynchus* gorbuscha, Clark et al., 2011). The maintenance of this constant AS in the acclimated fish may be evidence for metabolic compensation (Donelson and Munday, 2012).

Time to recovery and EPOC had similar patterns with 15 and 20°C having the highest values, where 23°C displayed the lowest recovery time of all groups. Regarding recovery time, those fish acclimated to 23°C exhibited the shortest recovery time, despite having the highest SMR and other elevated cellular and whole-body impairment. It is possible that the short recovery time can be attributed to metabolic suppression. Metabolic rate depression has been a strategy used by other animals to combat adverse environmental conditions (Hochachka and Guppy, 1987) and has been documented in several fish species including: *Oncorhynchus mykiss* (Rissanen et al., 2006), *Carassius auratus* (Stangl and Wegener, 1996; Van Waversveld et al., 1988; van Ginneken et al., 2004), *Carassius carassius* (Johansson et al., 1995) and *Brachydanio rerio* (Stangl and Wegener, 1996). For example, goldfish (*Carassius auratus*) have suppressed their metabolism to 30% of the aerobic standard metabolic rate at an environmental temperature of 20°C (Van Waversveld et al., 1988). The relationship between adverse conditions (temperature) and metabolic suppression may explain the quick recovery of the 23°C fish. By supressing their metabolism, fish are able to limit further carbon dioxide build up in the blood (acidosis) and regulate their blood pH by excreting protons (Claiborne, 1998). Therefore, suppression allows fish to keep their blood pH in a safe range, promoting survival, in the adverse conditions. Overall, if the quick recovery of the 23°C is due to metabolic suppression, then our findings that brook trout are limited above 20°C is further supported, as it is apparent that the 23°C are resorting to alternative methods to prevent acidosis from occurring, and possibly ultimately death.

Brook trout osmoregulation was impacted at elevated temperatures. Osmolality signifies the status of osmoregulation in fishes as it is an indicator of ion balance, particularly the circulating concentrations of Na^+ and Cl^- (McDonald and Milligan, 1997). Osmolality is a useful indicator for acute stress, but it is difficult to interpret for chronic stress exposure due to multiple internal and external factors (McDonald and Milligan, 1997). For the brook trout that were exposed to the acute stress event, I observed that plasma osmolality significantly increased by 1.2-fold at 15 and 20°C. A study by Jeffries et al. (2012) showed similar results in wild pink (*Oncorhynchus gorbuscha*) and sockeye salmon (*Oncorhynchus nerka*) whose levels of plasma osmolality significantly increased at higher temperatures (13°C vs. 19°C). Increased plasma osmolality with acclimation temperature has also been found in several other fish studies (Allanson et al., 1971; Wagner et al., 1997; Cataldi et al., 1998; Masroor et al., 2018). Post-stress, those fish acclimated to 23°C showed a significant decrease in plasma osmolality concentration. The drop in plasma osmolality may be explained by interactive effects between the chronic acclimation temperature exposure with the acute stress event. Prolonged exposure to 23°C and the addition of the acute stress event may have caused a net loss of ions. Net loss of ions during chronic stress occurs across sites (gills, kidney) and can cause a significant drop in plasma concentration (McDonald and Milligan, 1992). I found that the water content of the muscle was highest at 25°C, which can occur because of increased plasma ions and volume, leading to a reduction in muscle water content (Randall and Brauner, 1991). Additional evidence of osmoregulation interference was exhibited by unhandled fish increased abundance levels in Nkcc at low temperatures, with decreased abundance at higher temperatures, and elevation in post-stress plasma osmolality. *Na^+-K-Cl^- cotransporter* (Nkcc) is involved in active ion absorption or secretion on the apical or basolateral plasma membrane (Hiroi et al., 2008). It is a

transporter and uptakes ions in freshwater and plays a role in acid-base regulation and ammonia secretion (Hiroi and McCormick, 2012). In my study, abundance of *nkcc* significantly increased by 3- to 5-fold respectively at 5 and 10°C compared to abundance at 23 and 25°C in the unhandled group. At low temperatures, plasma Cl^- has been found to increase, in order to maintain blood pH at these lower temperatures (Evans, 2008). Additionally, the *Na^+-K-Cl^- pump* has been observed to inactivate at lower temperatures, causing *nkcc* to be upregulated to combat the inactivation of the sodium-potassium pump (Metz et al., 2003). Therefore, it could be that there was increased Cl^- regulation and the inactivation of the *Na^+-K-Cl^- pump*, explaining why I observed increases of *nkcc* at lower temperatures. Conversely at higher temperatures, I observed a significant downregulation of *nkcc*. *Na^+-K-Cl^- pump* activity increases with increasing acclimation temperatures (Murphy and Houston, 1974). Due to the increase in *Na^+-K-Cl^- pump* activity, *nkcc* is not relied upon or needed, as the pump performs similar functions to *nkcc*. This upregulation of *Na^+-K-Cl^- pump* activity may thereby explain the downregulation of *nkcc* at 23 and 25°C. Brook trout osmoregulatory capacity is impacted by increased temperatures as exhibited by abundance levels of Nkcc and plasma osmolality.

My findings are supported by 53 other publications on brook trout that Smith and Ridgway (2019) reviewed, indicating that the preferred temperature for this species is ~ 15°C with an upper thermal tolerance threshold of 21–23.5°C. Various biomarkers have shown that at temperatures above 20°C, brook trout will be adversely impacted, this will particularly be a concern as temperatures continue to rise due to global climate change. As highlighted by Morrison et al. (2020), brook trout exhibited a sub-lethal threshold between 20 and 23°C. Furthermore, Chadwick and McCormick (2017) demonstrated that brook trout growth is limited by higher temperatures especially those above 23°C, and that may play a role in driving their

distribution. Together, the evidence presented from these studies, along with my own, emphasize that above 20°C brook trout are significantly impaired and threatened by global climate change.

Chapter 2: Tables and Figures

Table 2.1: Primer sequences used to target selected genes in transcriptome of brook trout. (Some genes were analyzed in both tissues, [L] denotes Liver, [G] denotes Gill).

Gene	Primer	PCR Efficiency (%)	Sequence (5'-3')	Primer length (base pairs)	Product length (base pairs)	Function/Role
Na^+/K^+-ATPase alpha-3	*atp1a3* forward	96	TCCTGGCCTACGGAATCCA	19	74	Na^+/K^+ regulation
	atp1a3 reverse		GAGCACAACACCCAGGTACAAA	22	74	
Na^+/K^+-ATPase alpha-3-alpha	*atp1a3a* forward	104.1	TGTTCCTGGGCATCACCTTC	20	89	Na^+/K^+ regulation
	atp1a3a reverse		CACGATGATGCCGATGAGGA	20	89	
Cystic fibrosis regulator	*CFTR* forward	95.8	TCAAACAACGCCCCGATAC	19	75	Chloride transporter
	CFTR reverse		CAACCTGACCACCACTGAGGTA	22	75	
Cold-inducible RNA-	*cirbp* forward	100.6	AGGTATGGGCAGGCAATCTG	20	73	RNA stabilization involved in osmotic stress and cold shock response

binding protein	*cirbp* reverse		AAGAGGGAGGGCAAGACAAAA	21	73	
Glucose-6-phosphatase	*g6pc* forward	110.8	CACTTCCCTCACCAGGTTGT	20	76	Glycogen metabolism
	g6pc reverse		TCCATTGGACCCGGTCAAAG	20	76	
Glutathione peroxidase	*gpx1* forward	90.1^{G} & 91.6^{L}	CGTTCTTGCAGTTCTCCTGATG	22	70	Oxidative stress response, involved in cell immunity
	gpx1 reverse		ACCGACAAGGGTCTCGTGAT	20	70	
Heat shock cognate 71	*hspa8* forward	91.2^{G} & 93.7^{L}	GGGTTCATGGCAACCTGATT	20	67	Heat shock protein, regulatory role in autophagy
	hspa8 reverse		ACGTTGCCTTCACTGACTCTGA	22	67	
Heat shock protein 90 beta	*hsp90ab1* forward	92.5^{G} & 97.0^{L}	CAACATGGAGCGCATCATG	19	79	Heat shock protein, role in cell response to stress and buffer against cell mutation
	hsp90ab1 reverse		CAGGTGTTTCTTGGCCATCA	20	79	
ATP-sensitive inward rectifier K^{+} channel 8	*irk8* forward	86.5	CCCTGTTCTCGGATGTTCTTG	21	72	ATP-sensitive inward rectifier K^{+} channel 8
	irk8 reverse		GGTGAACAAAGCACGCTTCA	20	72	

$Na^+/K^+/2Cl^-$ co-transporter	*nkcc1* forward	101.2	CGGGAATTGTTCTCTCCTGTGT	22	82	$Na^+/K^+/2Cl^-$ co-transporter (solute carrier family 12 member 2)
	nkcc1 reverse		GCAATCGCTGAGGTCGAAA	19	82	
Nuclear protein 1	*nupr1* forward	95.5	TGGCCTTCTTTTCAGTGTTCTG	22	89	Cell growth and apoptosis regulator
	nupr1 reverse		GGAAGCCAGCGACAATACCA	20	89	
60s ribosomal protein L7	*rpl7* forward	87.5^G & 95.0^L	TCTGACGCAGACGCATGAG	19	86	Reference gene ribosomal protein L7
	rpl7 reverse		CGAAACTGGCCTTCGTCATC	22	86	
60s ribosomal protein L8	*rpl8* forward	96.8^G & 91.0^L	GCCACAGTCATCTCCCACAA	20	63	Reference gene ribosomal protein L8
	rpl8 reverse		GGAGCCAGAGGGAAGCTTAAC	21	63	
40s ribosomal protein S9	*rps9* forward	85.0^G & 99.6^L	GAGTTGGGTTTGTCGCAAGAC	21	68	Reference gene ribosomal protein S9
	rps9 reverse		CCTGGTCGAGACGAGACTTCTC	22	68	
Serpin h1	*serpinh1* forward	92.1^G & 92.4^L	CCCAAGCTGTTCTACGCTGA	20	83	Biosynthesis of collagen and role in restoration of homeostasis during heat stress

	serpinh1 reverse		AGTCTGCCGAGGAAGAGGAT	20	83	
V-type H-ATPase B	*vatB* forward	104.0	GCTTCAGCATTTCTTTGGGAAA	22	89	Regulation of H^+ gradient
	vatB reverse		TCAGGGCCCTTATGACAACAG	21	89	
V-type H-ATPase E1	*vatE1* forward	96.5	GGCTGGGTCCTTGGCTATGT	20	85	Regulation of H^+ gradient
	vatE1 reverse		GGTGTTAAAGGCTCGCGACG	20	85	

Table 2.2. Results of one-way ANOVA and Kruskal-Wallis test for twelve genes in gill tissue. Significant interactions were found in Atp1a3, Nkcc, Gpx1, Hspa8, and Hsp90ab1, with an $\alpha<0.05$.

Gene	Df	Sum of Squares	Mean of Squares	F values/ Chi-squared	P value
Atp1a3	5, 59	24.39	4.878	3.674	0.006
Atp1a3a	5, 59	7.30	1.460	0.836	0.53
Irk8	5, 59	14.60	2.920	1.739	0.142
Nkcc	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

Table 2.3. Results of one-way ANOVA for six genes in liver tissue. Significant interactions were found in all genes with an $\alpha < 0.05$, except for SerpinH1.

Gene	Df	Sum of Squares	Mean of Squares	F value	P value
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	$5.39e^{-06}$
Hsp90ab1	5, 59	54.31	10.86	15.14	$6.45e^{-09}$
Nupr1	5, 59	4444	888.8	4.918	0.001
SerpinH1	5, 59	4.63	0.9258	0.573	0.72

Table 2.4. Results of the two-way ANOVA values for muscle lactate. All groups display significant interactions with an $\alpha < 0.05$. Treatment group denotes the differences within the unhandled, acute stress, and recovery groups. The temperature group represents differences within the five different temperatures, while the treatment × temperature group represents interactions between the three treatment groups and the five different temperatures.

Group	Df	Sum of Squares	Mean of Squares	F value	P value
Treatment	2	13.135	6.568	125.415	$2.0e^{-16}$
Temperature	4	4.107	1.027	19.606	$3.53e^{-12}$
Treatment × Temperature	8	4.106	0.513	0.513	$3.53e^{-10}$

Table 2.5. Results of the two-way ANOVA values for plasma cortisol. All groups display significant interactions with an $\alpha < 0.05$. Treatment group denotes the differences within the unhandled, acute stress, and recovery groups. The temperature group represents differences within the five different temperatures, while the treatment × temperature group represents interactions between the three treatment groups and the five different temperatures.

Group	Df	Sum of Squares	Mean of Squares	F value	P value
Treatment	2	37274	18637	46.15	$1.77e^{-14}$
Temperature	4	6593	1648	4.08	0.004
Treatment × Temperature	8	13919	1740	4.31	0.0002

Table 2.6. Results of the two-way ANOVA values for plasma glucose. All groups display significant interactions with an α<0.05. Treatment group denotes the differences within the unhandled, acute stress, and recovery groups. The temperature group represents differences within the five different temperatures, while the treatment × temperature group represents interactions between the three treatment groups and the five different temperatures.

Group	Df	Sum of Squares	Mean of Squares	F value	P value
Treatment	2	2.9573	1.4787	64.803	$2e^{-16}$
Temperature	4	0.7413	0.1853	8.122	$8.92e^{-06}$
Treatment × Temperature	8	1.2648	0.1581	6.929	$2.31e^{-07}$

Table 2.7. Results of the generalized linear model values for plasma osmolality. Coefficients denote the groups being compared (i.e., treatment group × temperature).

Coefficients	Estimate	Std. Error	T value	P value
TreatmentAcute-Recovery	$8.645e^{-05}$	$7.199e^{-05}$	1.201	0.232
TreatmentAcute-Stress	$3.502e^{-05}$	$7.133e^{-05}$	0.491	0.624
Temperature10	$-6.094e^{-05}$	$6.616e^{-05}$	-0.921	0.359
Temperature15	$2.104e^{-04}$	$6.915e^{-05}$	3.043	0.003
Temperature20	$2.507e^{-04}$	$6.961e^{-05}$	3.601	<0.001
Temperature23	$1.705e^{-05}$	$6.701e^{-05}$	0.254	0.799
TreatmentAcute-Recovery × Temperature10	$1.388e^{-04}$	$1.021e^{-04}$	1.359	0.177
TreatmentAcute-Stress × Temperature10	$-8.267e^{-05}$	$9.914e^{-05}$	-0.834	0.406
TreatmentAcute-Recovery × Temperature15	$-6.588e^{-05}$	$1.047e^{-04}$	-0.629	0.530
TreatmentAcute-Stress × Temperature15	$-3.649e^{-04}$	$1.011e^{-04}$	-3.611	<0.001
TreatmentAcute-Recovery × Temperature20	$-1.134e^{-04}$	$1.032e^{-04}$	-1.099	0.274
TreatmentAcute-Stress × Temperature20	$-5.081e^{-04}$	$1.005e^{-04}$	-5.055	$1.62e^{-06}$
TreatmentAcute-Recovery × Temperature23	$2.754e^{-04}$	$1.047e^{-04}$	2.630	0.01
TreatmentAcute-Stress × Temperature23	$-7.266e^{-05}$	$1.005e^{-04}$	-0.723	0.471

Table 2.8. Results of the Kruskal-Wallis test for SMR and one-way ANOVA values for MMR, recovery time, aerobic scope, and EPOC. All parameters, except aerobic scope, displayed significant interactions with an $\alpha<0.05$.

	Df	Sum of Squares	Mean of Squares	F value/ Chi-Squared	P value
SMR	4, 40	-	-	30.43	$4.009e^{-06}$
MMR	4, 40	1.272	0.318	8.84	$4.45e^{-05}$
Recovery Time	4, 40	506.7	126.7	27.69	$1.51e^{-10}$
Aerobic Scope	4, 40	0.598	0.149	1.29	0.292
EPOC	4, 38	11931	2929	8.594	$7.23e^{-05}$

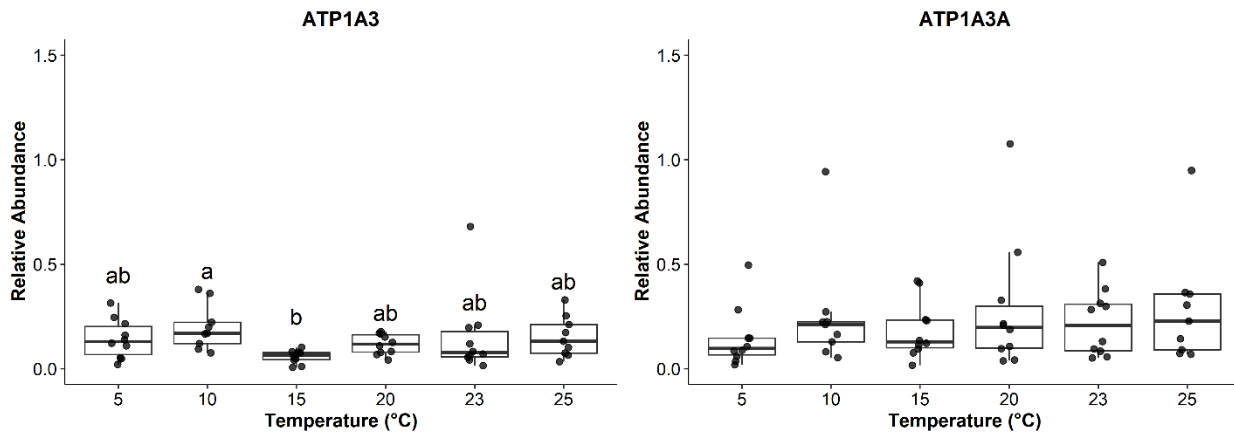

Figure 2.1. Gene expression box plots of Atp1a3 and Atp1a3a in gill tissue for juvenile brook (*Salvelinus fontinalis*) trout held at various temperatures (n = 60). Statistical significance was accepted at α=0.05. Letters denote statistical differences between temperature.

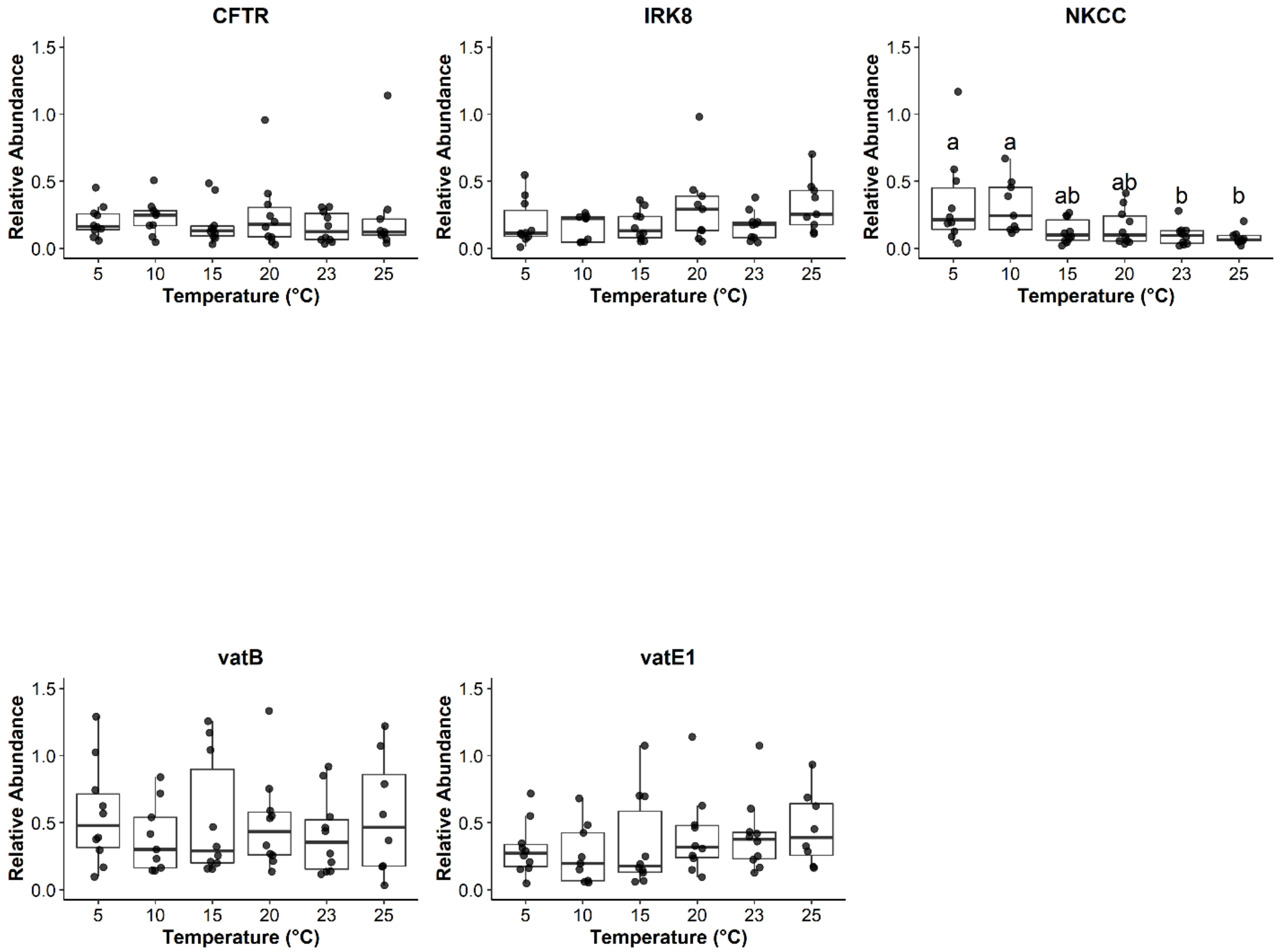

Figure 2.2. Gene expression box plots of biomarkers (ion regulation, metabolism) in gill tissue for juvenile brook trout (*Salvelinus fontinalis*) held at various temperatures (n = 60). Statistical significance was accepted at α=0.05. Letters denote statistical differences between temperature.

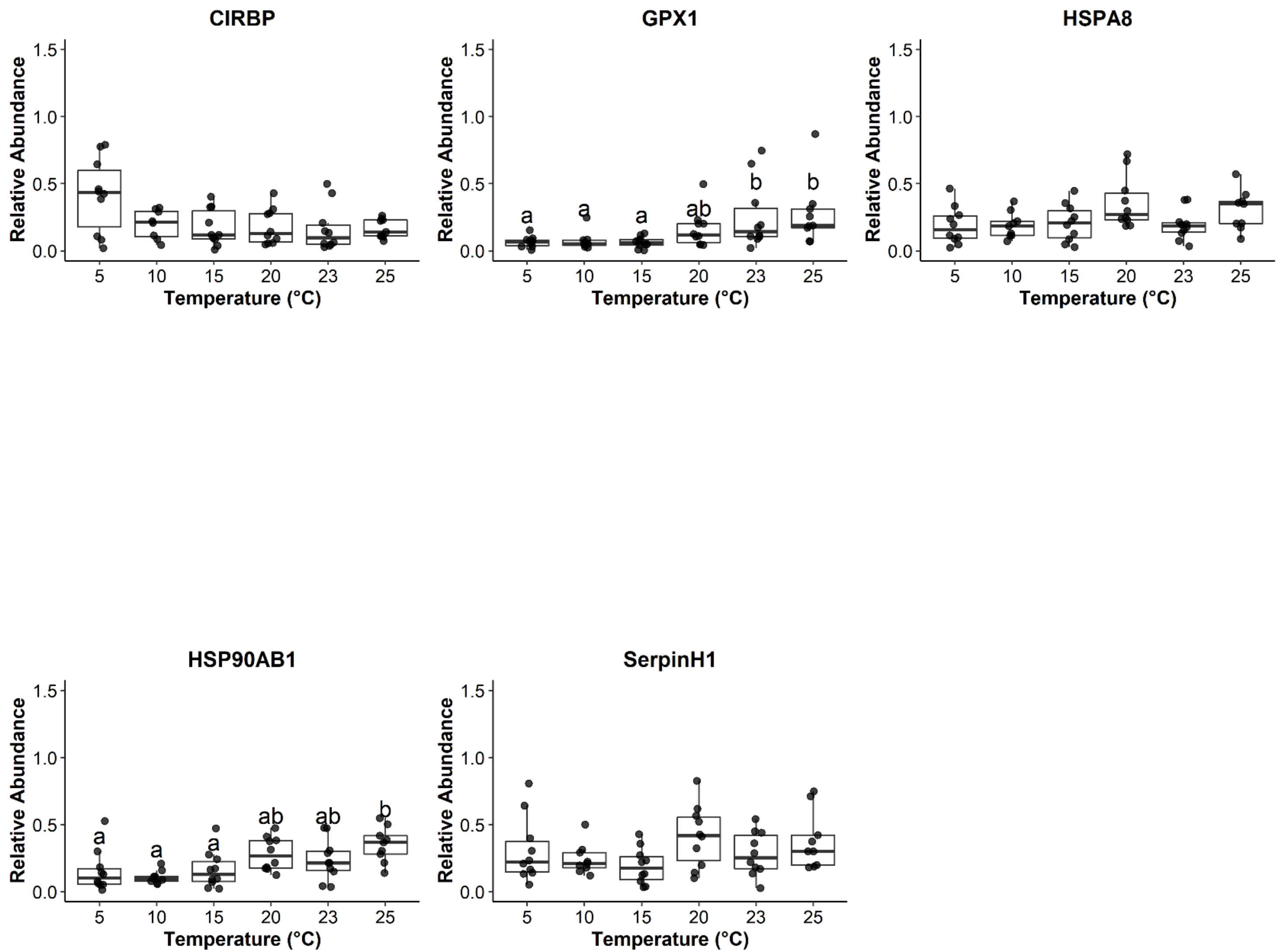

Figure 2.3. Gene expression box plots of thermal stress biomarkers in gill tissue for juvenile brook trout (*Salvelinus fontinalis*) held at various temperatures (n = 60). Statistical significance was accepted at α=0.05. Letters denote statistical differences between temperature.

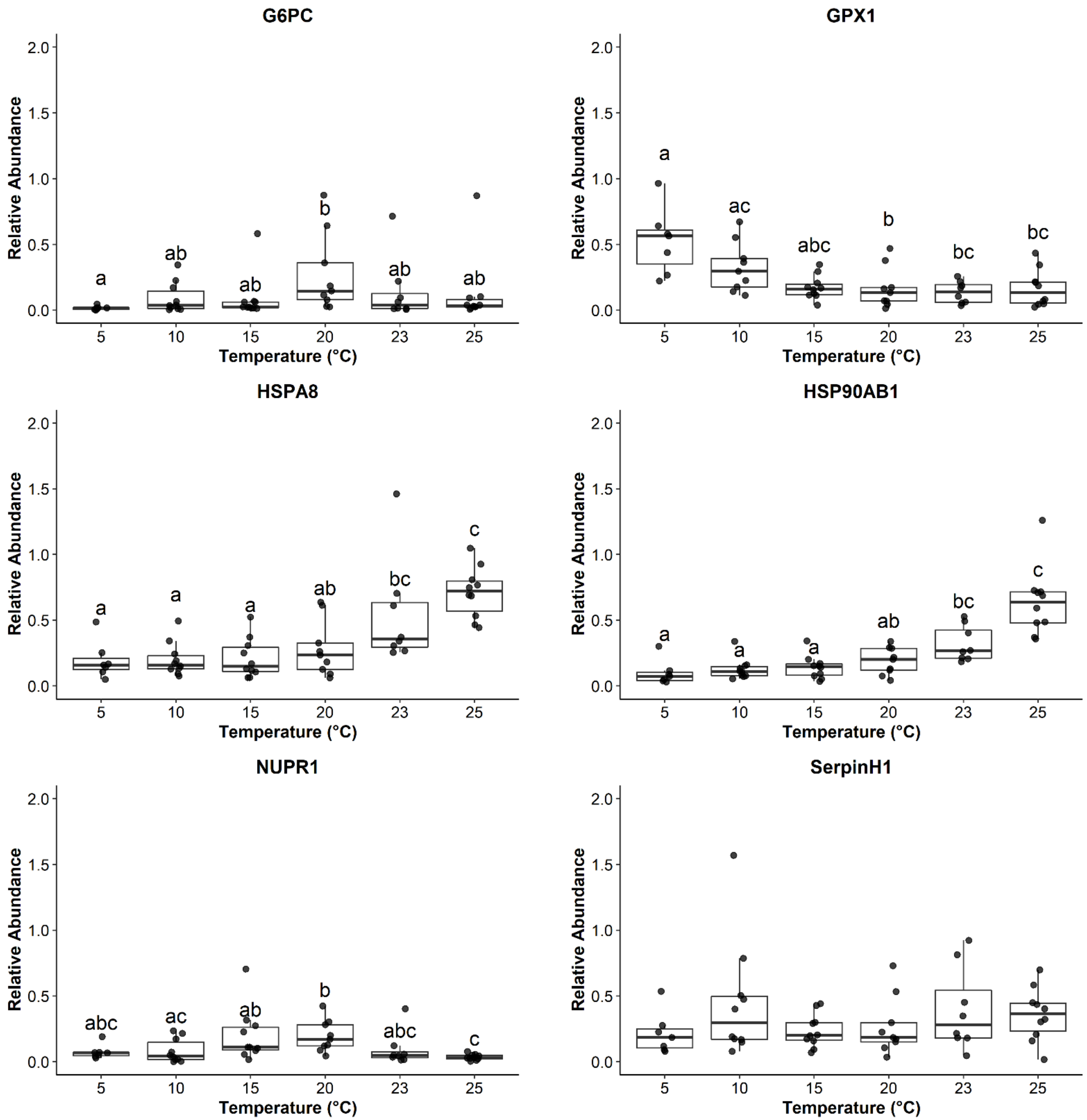

Figure 2.4. Gene expression box plots of biomarkers (thermal stress, metabolism, oxidative stress) in liver tissue for juvenile brook trout (*Salvelinus fontinalis*) held at temperature within and outside their thermal range (n = 60). Statistical significance was accepted at α = 0.05. Letters denote statistical differences between temperature.

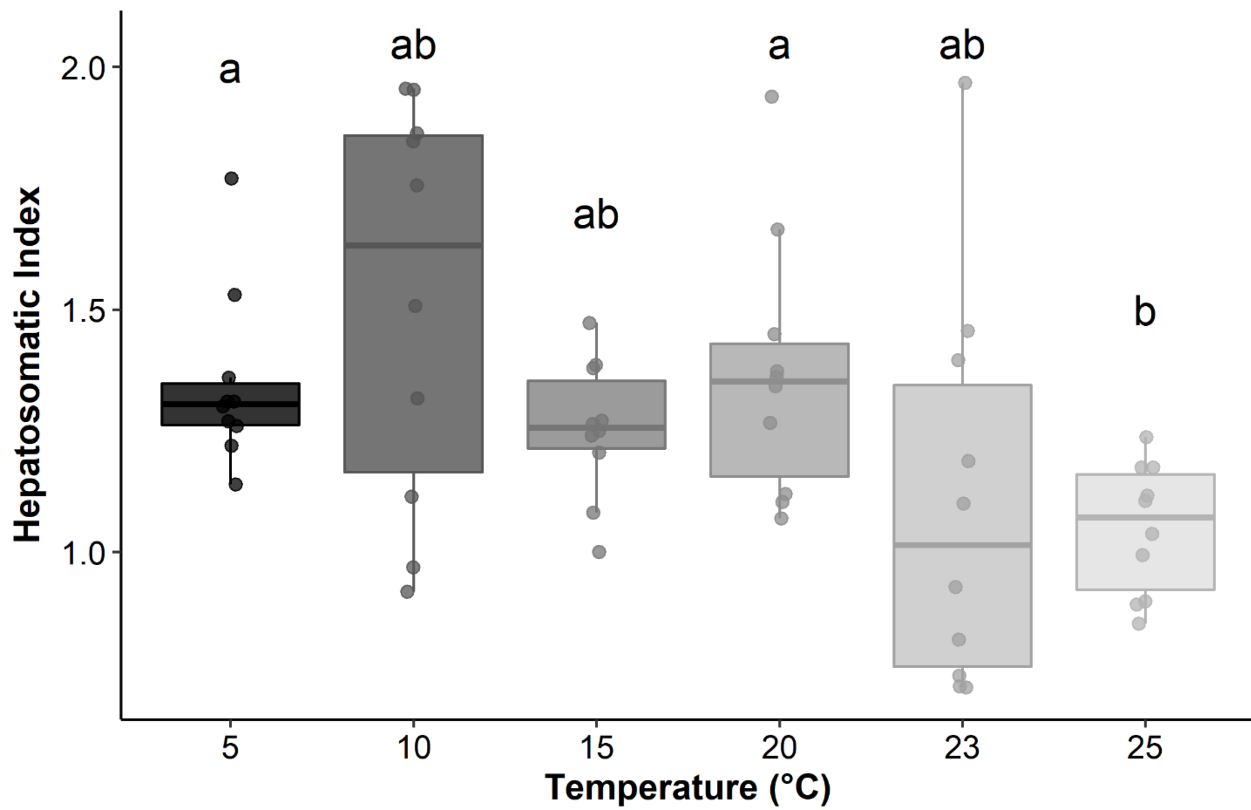

Figure 2.5. Hepatosomatic index values across six temperatures for the unhandled group of juvenile brook trout (*Salvelinus fontinalis*) (n = 60). Using a one-way ANOVA ($p = 0.003$), statistical significance was accepted at $\alpha = 0.05$. Letters denote statistical differences between temperatures.

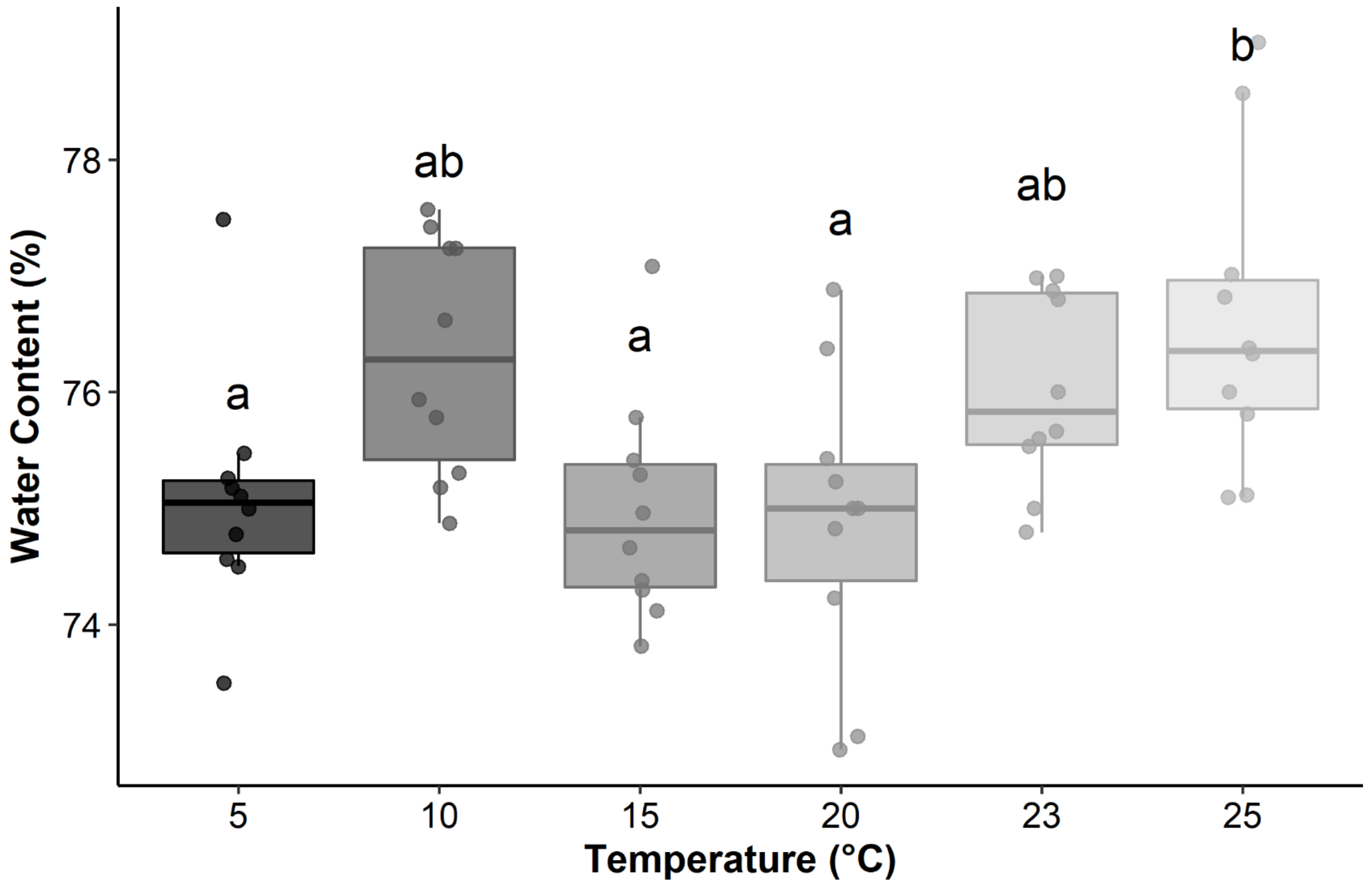

Figure 2.6. Percent Water Content values in white muscle across six temperatures for the unhandled group of juvenile brook trout (*Salvelinus fontinalis*) (n = 60). Using a one-way ANOVA ($p < 0.001$), statistical significance was accepted at $\alpha = 0.05$. Letters denote statistical differences between temperatures.

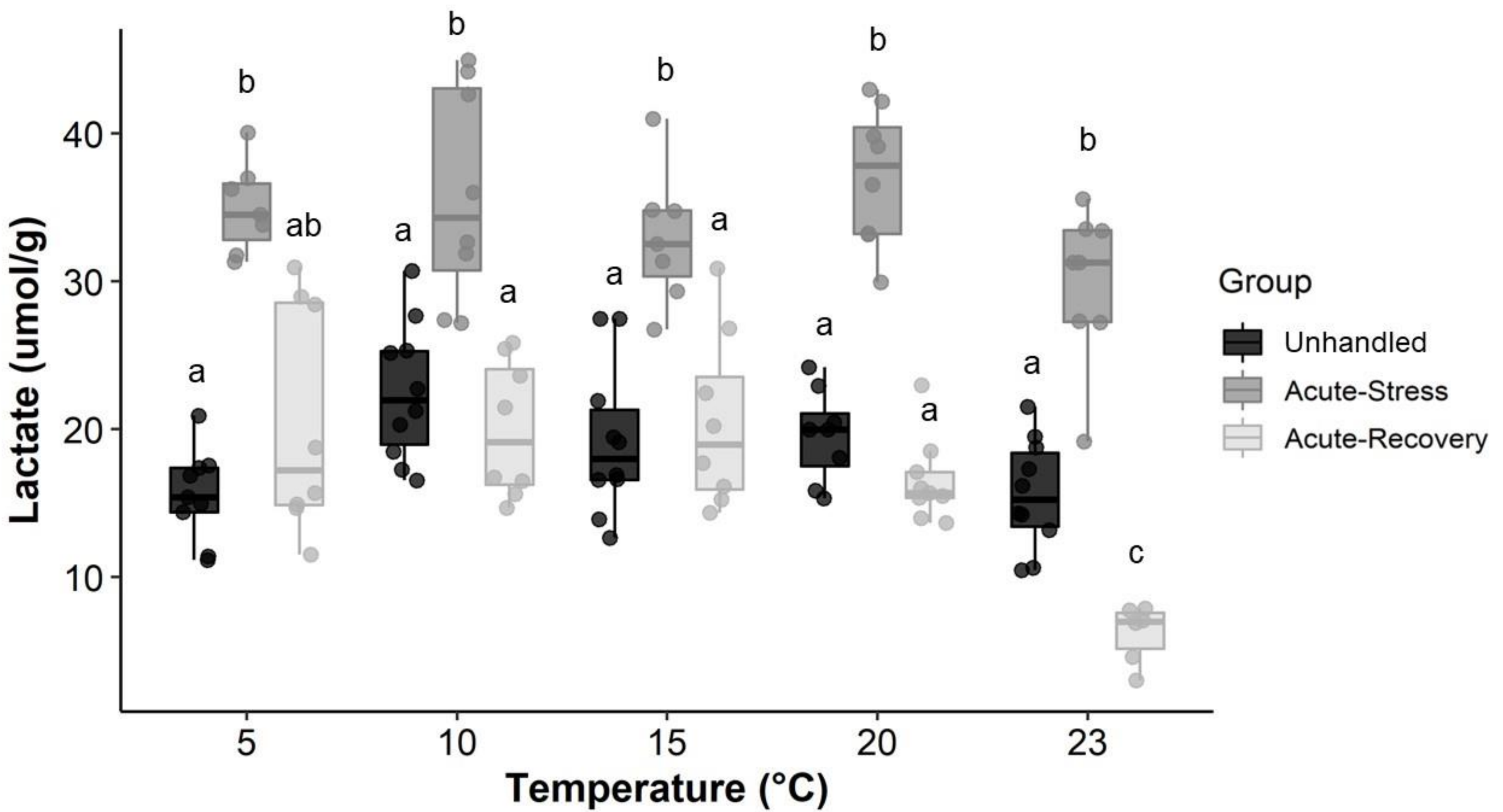

Figure 2.7. Muscle lactate of juvenile brook trout (*Salvelinus fontinalis*) unhandled group (n = 47), acutely stressed group (n = 38), and acute stress and recovery group (n = 38) across five acclimation temperatures. Using a two-way ANOVA (treatment × temperature, $p = 3.53e^{-10}$), statistical significance was accepted at $\alpha = 0.05$ where letters denote statistical differences within temperature treatments.

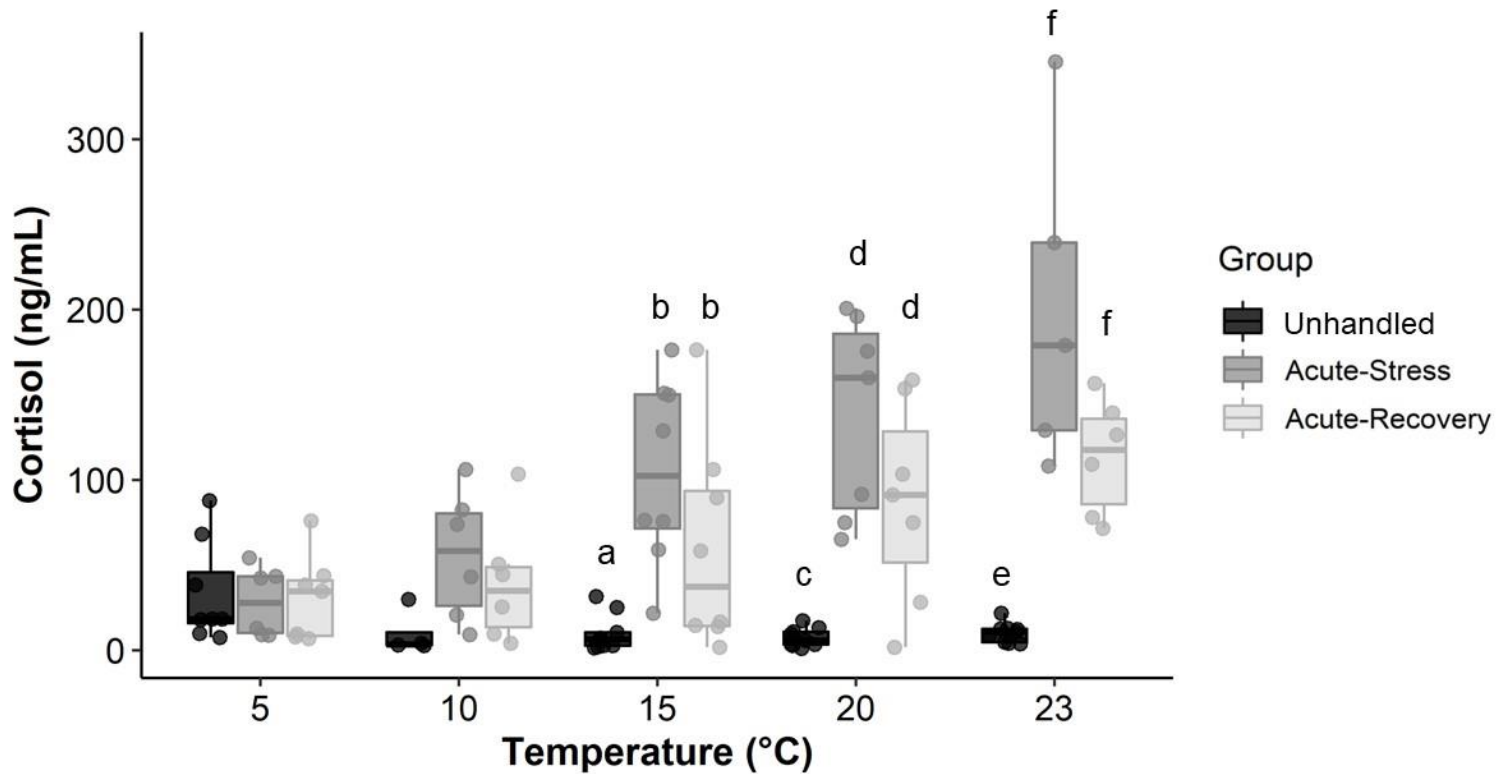

Figure 2.8. Plasma cortisol of juvenile brook trout (*Salvelinus fontinalis*) unhandled group (n = 38), acutely stressed group (n = 32), and acute recovery group (n = 34) across five acclimation temperatures. Using a two-way ANOVA (treatment × temperature, p = 0.0002), statistical significance was accepted at α = 0.05 where letters denote statistical differences within temperatures (15°C = ab, 20°C = cd, 23°C = ef).

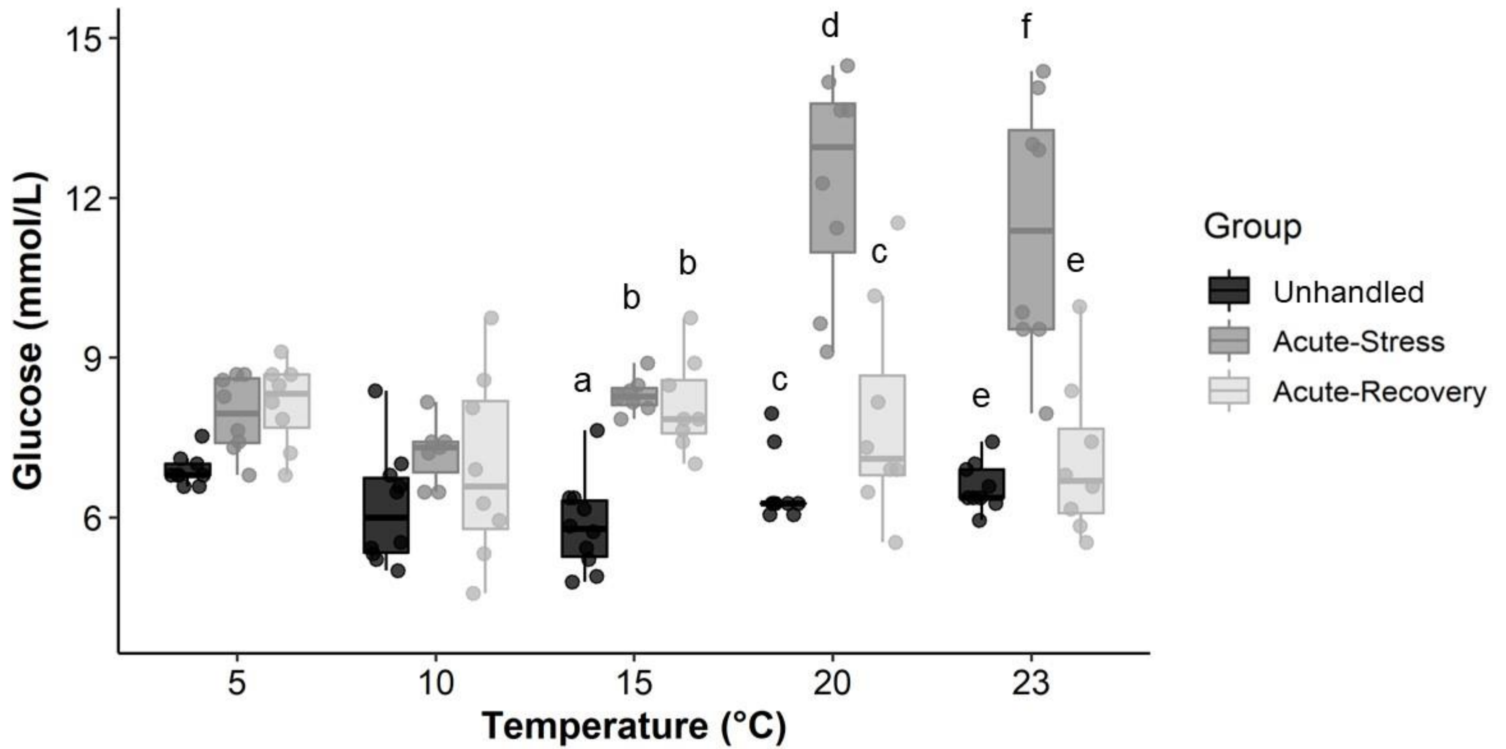

Figure 2.9. Plasma glucose of juvenile brook trout (*Salvelinus fontinalis*) unhandled group (n = 50), acutely stressed group (n = 40), and acute recovery group (n = 40) across five acclimation temperatures. Using a two-way ANOVA (treatment × temperature, $p = 2.31e^{-7}$), statistical significance was accepted at $\alpha = 0.05$ where letters denote statistical differences within temperatures (15℃ = ab, 20℃ = cd, 23℃ = ef).

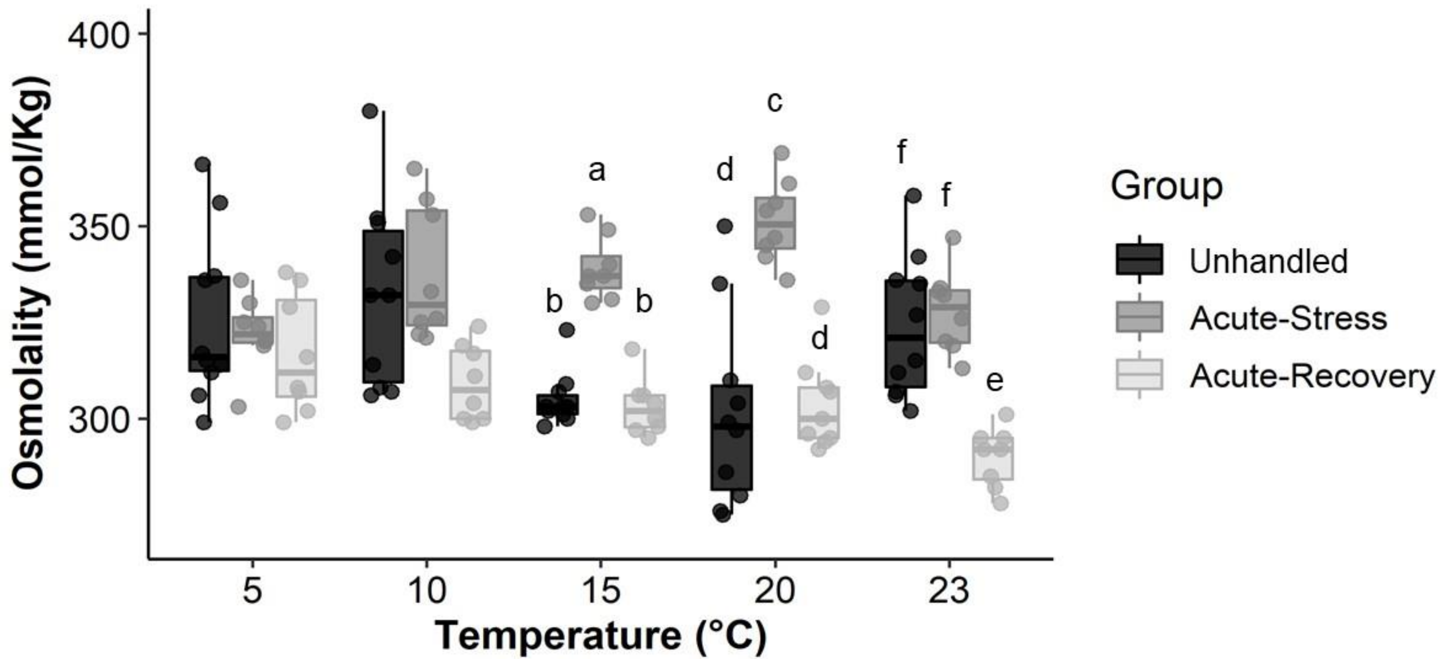

Figure 2.10. Plasma Osmolality of juvenile brook trout (*Salvelinus fontinalis*) unhandled group (n = 49), acutely stressed group (n = 40), and acute recovery group (n = 40) across five acclimation temperatures. Using a generalized linear mixed effects model (glmm, treatment × temperature), statistical significance was accepted at α = 0.05 where letters denote statistical differences within temperatures (15℃ = ab, 20℃ = cd, 23℃ = ef).

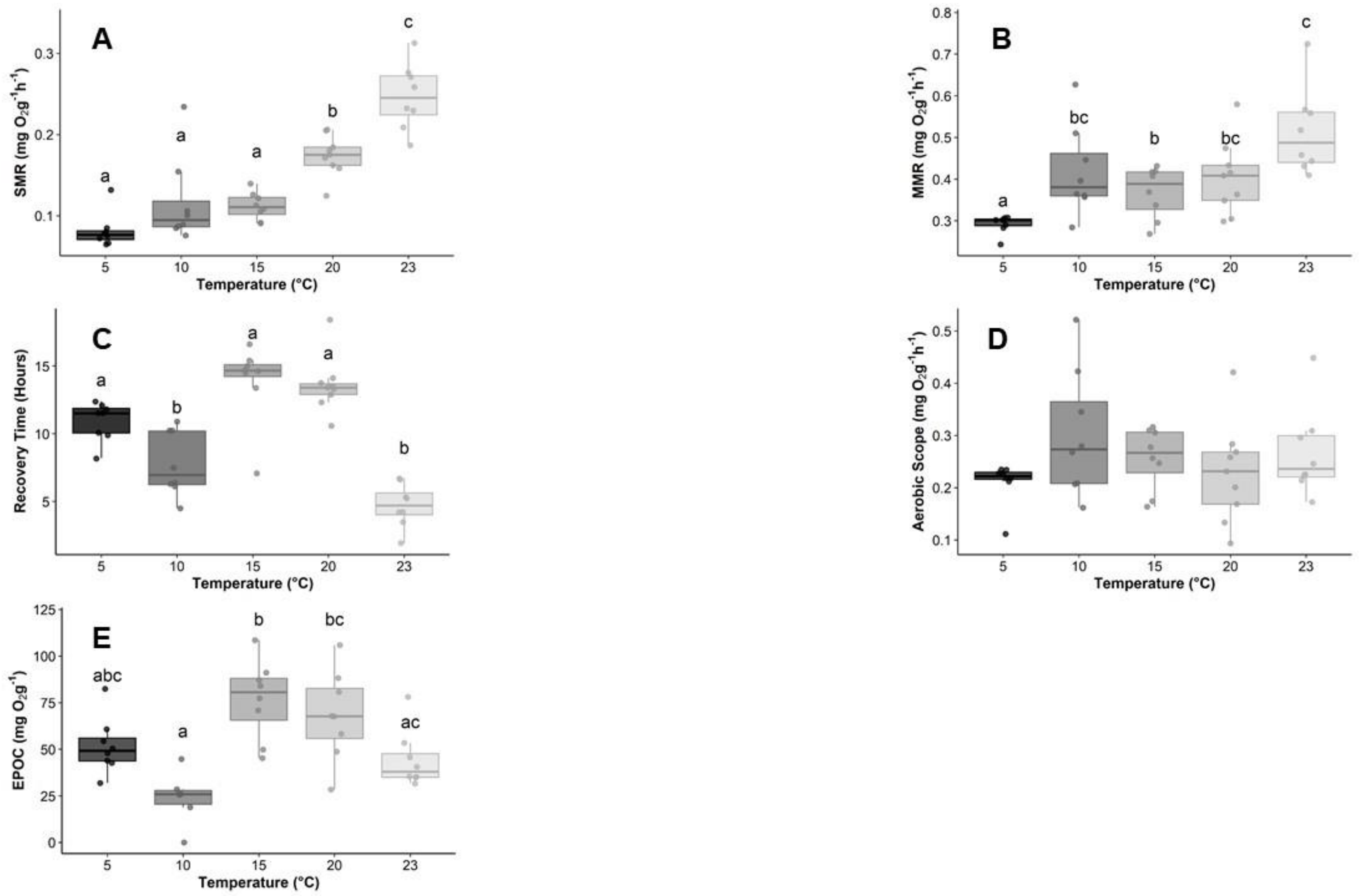

Figure 2.11. (A) Standard metabolic rate (n = 41) (B) Max metabolic rate (n = 41) (C) Recovery time in Hours post stress events (n = 41) (D) Aerobic scope (n = 41) (E) Excess post-exercise oxygen consumption values (n = 39). Statistical significance was accepted at α=0.05. Letters denote statistical differences between temperatures for juvenile brook trout (*Salvelinus fontinalis*).

Chapter 3: General Discussion

Brook trout are an environmentally important predator and valued sportfish, and as my study indicates, they may be adversely impacted as global climate change continues and temperatures rise. My study has highlighted that there exists a sub-lethal threshold above 20°C where brook trout exhibit impaired physiological functioning. This threshold is further supported by a suite of thermal studies on brook trout highlighted by Smith and Ridgway (2019) and a more recent study by Morrison et al. (2020). As it is apparent that global climate change is not slowing down, it becomes imperative that actions are taken to protect brook trout, especially as there are other factors that may interact with these warming temperatures and further impair brook trout populations.

Altered Flow Regimes

To successfully protect a species such as brook trout, it is important to conserve the habitat where they live. Unfortunately, global climate change is not the only force creating unstable thermal habitat for this species. Altered flow regimes that can occur due to the damming of rivers significantly alters the environment and results in changes in water temperatures. The changes in water temperature mainly result in significant increases in temperature in parts of the river system. For example, studies have found that when water is released by the dam from outlets near the top of a reservoir, that water will be warmer than the rest of the river all year round (McCully, 1996). Furthermore, one study by Dare and Hubert (2003) reported that increased water temperatures due to river regulation by damming, resulted in an effect on species distribution, behavior, and abundance (Dare and Hubert, 2003; Murchie et al., 2008). This change in temperature can also occur in the opposite direction where areas are much colder compared to normal conditions. These conditions are especially true with high-pulsed flows that

result in lower than normal downstream water temperatures (Murchie et al., 2008). Moreover, the creation of dams in river systems has a suite of effects in addition to changes in temperature, this includes extirpation of species, closures of fisheries, groundwater depletion, declines in water quality and availability, and more frequent and intense flooding (Poff et al., 1997). Additionally, changes in flow regime can affect the evolution of mode of adaptation including life history, behavioral, or morphological (Lytle and Poff, 2004). As brook trout are most common in river systems, altered flow regimes, and the resulting change in water conditions, is a significant problem that needs to be addressed. To protect brook trout and other fish species residing in river environments, damming methods need to be adjusted, monitored, and changed to ensure healthy fish populations. For the dams to allow for the river systems to remain as natural as possible, collaboration must occur between scientists, engineers, and Indigenous communities, as many indigenous communities' livelihoods are negatively affected by dams. Furthermore, it may be necessary to appeal to organizations like Manitoba Hydro to change their policy in relation to the use of dams for energy. However, infrastructure is among the many threats that exist in the protection of brook trout.

Catch-and-Release Angling Regulations

Catch-and-release (C&R) angling is done as a recreational sport, but is also managed to conserve fish populations as fish are released and only certain species and sizes can be harvested. By combining physiological studies such as this one with C&R methods, we can inform anglers on how to minimize impacts of C&R angling events to ensure proper recovery of fish populations (Cooke et al., 2013). Additionally, as indicated by previous studies, if we determine the temperature tolerances of popular C&R species, like brook trout, we can better understand at what temperatures these fish should be angled. For example, there is a general trend that warm

temperatures exacerbate mortality and impairment, and this information can aid managers in deciding on fishery regulations (e.g., closures) during high temperatures (Gale et al., 2013). In fact, closures have occurred before with other salmonids, specifically Atlantic Salmon, in Newfoundland rivers (Gale et al., 2013). Currently, regulations are set to protect fish from being angled during sensitive spawning periods. This includes regulations and alerts sent out by Fisheries and Oceans Canada as well as specific date ranges set out in the Manitoba fishing regulations manual (Manitoba Fisheries, 2018). In the Manitoba fishing regulations manual, dates from about April to May are set out as dates that certain areas cannot be angled due to spawning events. This approach can be used for high temperatures that induce stress and possibly mortality in brook trout based on this experiment. For example, Fisheries and Oceans Canada can send out alerts when temperatures hit 20°C and above to let anglers know that brook trout cannot be angled currently. Similarly, date ranges during periods of high temperatures in the summer can be set aside as periods where brook trout cannot be angled based on past climate data. Over time, if brook trout populations begin to decline due to angling practices in combination with global climate change, it may be necessary to list designatable units of brook trout as an endangered species under SARA and it might be necessary to further limit angling practices. To lead this effort, scientists must continue to conduct research and appeal to Fisheries and Oceans Canada.

Future Studies

While my study encompassed a range of six different temperatures spanning brook trout preferred and non-preferred conditions and incorporates a suite of physiological components ranging from molecular to whole body measurements, there is still more research to be done in this area. Brook trout have been studied, especially their thermal preference, for over 70 years

(Smith et al., 2020), but the data on transcriptomic responses of this species is still necessary to do into the future. My study incorporates transcriptomics but only that of a specific population that originated from God's Lake in Manitoba, Canada. To further understand the fate of this species as climate change continues, it is necessary to have a diverse knowledge spanning multiple populations and generations of brook trout. Therefore, I think that is it necessary to continue to study genes and transcriptomics in brook trout as it is key information that will aid us in determining organismal responses to environmental stressors and identifying response pathways that better aid us in protecting the species (Connon et al., 2018).

Additionally, it is also important to acknowledge that my study was limited in the age of the species that we could use and the length and type of sampling conducted, due to it being conducted in a lab setting. My study looked at juvenile brook trout (approximately 1 year of age), therefore the results found are representative of the species at that age specifically and could differ in older individuals (Schreck and Tort, 2016). To obtain a holistic understanding, future studies could incorporate a range of ages of the species. My study also used hatchery fish and previous studies have shown that wild populations are able to handle higher temperatures than hatchery individuals (Carline and Machung, 2001; Cooke and Suski, 2005; Robinson et al., 2008), therefore it is important to continue to conduct studies both on wild and lab-reared populations. Further, as described earlier in the cortisol response, it is likely that we did not allow enough time for cortisol to hit peak values, before sampling, at the cooler temperature of 5°C as supported by Louison et al. (2017). For future studies, to obtain a more rounded understanding, looking at parameters such as cortisol at different time scales would help us understand the stress response in greater detail.

Summary and Conclusions

1. **Brook trout are negatively affected by temperatures above 20°C.** This is supported by the heat shock response in gill and liver tissues, oxidative and cell growth response seen in Gpx1 and Nupr, and osmoregulatory response seen in Nkcc.
2. **Brook trout's ability to mount an neuroendocrine stress response is impacted at higher water temperatures.** After exposure to the paired stress event, elevated levels of cortisol, lactate, and glucose were exhibited at 20°C and above.
3. **Brook trout energy expenditure is higher at elevated temperatures and recovery is impacted**. Metabolically (standard metabolic rate, maximum metabolic rate, time to recovery, aerobic scope, and excess post exercise oxygen consumption) brook trout increased energy expenditure as temperatures increased, as indicated by an increased SMR and time to recovery with increasing temperatures.
4. **Brook trout will be significantly impacted by climate change as it appears there is a sub-lethal threshold for them between 20°C and 23°C.** In summary, my findings suggest that as water temperatures rise above 20°C, brook trout will be impacted and possibly unable to thermally acclimate.

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Appendix

Table A.1: Further transcript information for the fourteen target genes of the study and three reference genes.

Subject	Length	Species	Database	Acc #	Score	Evalue	% 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_ATP1A3.1.1	3087	*Danio rerio*	Refseq_protein	NP_571759.2	4867	0	91.3
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_LO C101477634.1.1	681	*Maylandia zebra*	Refseq_rna	XM_004548350.1	259	$1e^{-148}$	84.8