**The effects of thermal stress during the early developmental stages of freshwater fish**

by

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# Abstract

Fishes experience thermal stress in their natural environment that directly affects their physiology, especially during the developing stages. This is more concerning considering climate change and rising temperatures in aquatic ecosystems. This thesis investigated the short- and long-term effects of acute and chronic thermal challenges on developing freshwater fishes. I designed a multispecies OpenArray™ qPCR 'chip' to measure the mRNA response of *Acipenser* sturgeons to thermal stress. Next, I used the chip to quantify the effects of acute thermal stress on 60 days post-fertilization (dpf) lake sturgeon, *Acipenser fulvescens*, and 2-year-old shortnose sturgeon, *Acipenser brevirostrum*, and Atlantic sturgeon, *Acipenser oxyrinchus oxyrinchus.* Common and species- or population-specific expression patterns were detected, with acclimation temperature playing an important role. Next, I investigated the long-term effects of early-life daily thermal fluctuations happening during embryonic and larval stages of zebrafish, *Danio rerio*. The effects of early-life thermal fluctuations on mRNA expression of cellular stress genes were clearer in the juvenile and adult stages, compared to early-life stages. The $CT_{max}$ significantly increased at 60 dpf in thermally-challenged fish, but not at 90 dpf. Also, fish experiencing thermal fluctuations had a shorter forklength at 60 and 90 dpf. The mRNA expression of neurodevelopmental genes and the proliferation of radial-glial cells and the neuron density in the telencephalic pallial niche of the forebrain were stage-dependent. Although more severe at the embryonic and larval stages, the differences in mRNA expression and neuron proliferation and density diminished toward adult stages. In males, PCNA and HuC/D staining showed persistent effects on cell proliferation and neuronal density in adults. Thermally-challenged fish showed downregulation of muscle genes at embryonic and larval stages and an upregulation at later stages. Early-life thermal fluctuations increased the activity of fish measured by a dark/light locomotion test at all life stages. This thesis contributes to a better understanding of freshwater fish biology by introducing and successfully using a new tool for the health assessment of *Acipenser* species and conducting one of the first investigations on the long-term effects of early-life thermal conditions on the thermotolerance, development of the brain and muscle, and swimming behavior of zebrafish.

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# Contribution of Authors

For Chapter 2, Ken M. Jeffries and Nicholas J. Bernier were involved in funding acquisition, contributed to gene selection and the development of the *Acipenser* OpenArray™ chip, and supervised the research. Will S. Bugg designed and performed experiments on lake sturgeon. Faith M. Penny and Scott A. Pavey designed and performed experiments on Atlantic and shortnose sturgeon. Hossein Haghighi and Kyle Madden designed and tested the primers and probes for the OpenArray™ chip. Hossein Haghighi ran the OpenArray assays, analyzed the data, and wrote the first draft. Ken M. Jeffries, Nicholas J. Bernier, Will S. Bugg, Faith M. Penny, Scott A. Pavey, and Kyle Madden commented on the first draft, and the chapter was revised by Hossein Haghighi.

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The results from all the data chapters were put together and concluded by Hossein Haghighi, supervised by Ken M. Jeffries.

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# Chapter 1 Introduction

## 1.1 Thermal stress in aquatic ecosystems

Increased consumption of fossil fuels in the last few centuries has increased the concentration of greenhouse gases in the atmosphere (Ledley et al., 1999). This increase in greenhouse gases can increase the heat retention and temperature of the atmosphere (Bilgili et al., 2024). This increase in temperature is predicted to change the climate in different regions of the world (Bajoria et al., 2024). Climate change can have detrimental effects on natural habitats, influencing the organisms living there (Jeltsch et al., 2011). Changes in temperature regime are one of the important effects of climate change on the natural habitat (van Vliet et al., 2013; Kraemer et al., 2021). These changes in temperature can happen in different forms, such as moving the maximum and minimum temperatures in an environment or affecting the spatial and temporal arrangement or location of temperatures in aquatic ecosystems (Waldock et al., 2018; Brkic, 2023). All these changes are not without consequences on the life of organisms living in these habitats (Eissa and Zaki, 2011; Grimm et al., 2013).

Temperature is one of the abiotic factors affecting the physiology, behavior, and distribution of aquatic organisms (López-Olmeda and Sánchez-Vázquez, 2011). Temperature changes affect physiological processes in animals by influencing reaction rates and the equilibria that determine the non-covalent interactions responsible for macromolecular and membrane stabilization (Guderley and Pierre, 1996). Animals can be divided into several thermal strategy categories: Animals that maintain a constant body temperature by heat generation and physiological thermoregulatory mechanisms, which require energy, are called ‘homeotherms’; while animals that do not actively regulate their body temperature and it changes with environmental temperature are called ‘poikilotherms’ (Handler et al., 1994; Guschina and Harwood, 2006; Ivanov, 2006). Based on the source of body temperature, animals that produce and retain most of their internal body heat through metabolism are called ‘endotherms’; however, animals in which physiological mechanisms for heat production and retention are weak are called ‘ectotherms’ (Davenport, 1992; Nespolo et al., 2011). In addition, the term ‘eurytherm’ is used to describe animals that can tolerate wide ranges of temperature, yet ‘stenotherm’ is used for animals that can only live in a narrow range of temperature (Giomi and Pörtner, 2013; Kristiansen et al.,

2020). Most fishes are ectotherms; however, in terms of thermal tolerance, they can be either eurythermal or stenothermal (Wieser and Medgyesy, 1990; Logan and Buckley, 2015; Haesemeyer, 2020; Radtke and Bernaś, 2025). Because of their ectothermic nature, exposure to temperatures higher or lower than their tolerance range can result in thermal stress in fishes, which affects them in different ways, such as increasing production of reactive oxygen species (ROS) and oxidative stress, affecting their immune response and their ability to cope with pathogens, and influencing growth (Nakano et al., 2014; Rossi et al., 2017; Velmurugan et al., 2019; Rodgers and Gomez Isaza, 2022; Liu et al., 2025).

One of the effects of thermal stress is triggering the general stress response in fish (Hanke et al., 2019). Increased blood cortisol concentrations have been reported in several fishes exposed to high temperatures (e.g., Pérez-Casanova et al., 2008; Yang et al., 2020). The main effect of a cortisol increase is the mobilization of energy, which is reflected by increased glucose levels (Iwama et al., 1999). Catecholamines also play a similar role in releasing glucose, but more rapidly, which is important for the short-term stress response (Bonga, 2011; Reid, 2011). In addition, stress hormones can promote immediate survival through improving the absorption of oxygen in gills and its transportation through the blood, increasing the heart rate and blood pressure (McEwen, 2007; Reid, 2011). Different environmental cues are known to trigger stress hormone release, and stress hormones often lead to movement in animals (Goossens et al., 2020). In terms of response to thermal challenges, this stress response may play an important role in behavioral thermoregulation as an attempt to flee from uncomfortable temperatures to find a place with more favorable temperatures, as increased activity is a common behavior in fishes exposed to temperatures below or higher than their comfort zone (Shvartsburd and Vijayan, 2025). However, the increase in stress hormones is not without drawbacks, and in the case of prolonged stress conditions, it can put pressure on the mechanisms involved in the general and cellular physiology of animals necessary for survival (Sloman, 2011; Cool and Zappetti, 2019; Alfonso et al., 2021).

Changes in metabolism are very common in animals exposed to thermal stress (Rossi et al., 2017; Guzzo et al., 2019; Pilakouta et al., 2019; Alfonso et al., 2020). In most cases, exposure to high temperatures increases the metabolic rate of fishes (Hanna et al., 2008; Yang et al., 2020; Alfonso et al., 2021). At the organismal level, this increase in the metabolic rate is usually

reflected in the increased oxygen consumption rate (Pérez-Casanova et al., 2008). At the cellular level, these changes in energy use can be seen as increased glycolysis and oxidative phosphorylation to match the elevated energy demand caused by increased metabolism (Zhao et al., 2022). Sometimes this increased metabolic rate may overwhelm the oxygen delivery system and cause complete or partial shifting to anaerobic metabolism (Christjansen et al., 2025). Because increased temperature usually increases energy demand in the organism, it can also deplete organs such as the liver of energy-storage molecules, such as glycogen and lipids, and reduce the hepatosomatic index, which is the ratio of liver mass to body mass in fish (Rossi et al., 2017). Shifting to the metabolism of lipids and proteins is also reported in fish exposed to high temperatures (Liu et al., 2019). This is usually followed by depletion of more convenient energy resources, such as glycogen, as a result of constant release of stress hormones during chronic stress (Olsen et al., 2021; Kaur et al, 2025; Bai et al., 2025). This is sometimes accompanied by gluconeogenesis, which is the production of glucose from noncarbohydrate substrates during glucose scarcity, and is also common during thermal stress and can increase the breakdown of proteins for energy production (Liu et al., 2019; Zhang et al., 2019). As a result, thermal stress can change the chemical composition in the body by depleting biomolecules used for the storage of energy (Van Weerd and Komen, 1998; Pankhurst and Munday, 2011; Li et al., 2023; Carrillo-Longoria et al., 2023).

Increased temperature also results in cellular stress response in animals (Somero, 2019). Increased mRNA and protein expression of heat shock proteins is one of the common indicators of cellular response to thermal stress (Liu et al., 2019; Yang et al., 2020; Zhao et al., 2022). Heat shock proteins have important roles in protein maturation and denatured protein management, and their upregulation can be a sign of increased instability in the structure of proteins caused by increased temperature (Moseley, 1997; Sharp et al., 1999; Sørensen et al., 2003; Somero, 2019). An increased oxidative stress response is another cellular stress response observed in animals under thermal stress, especially in organs with a detoxification role, such as the liver (Madeira et al., 2013; Wang et al., 2019; Roychowdhury et al., 2021). This increased oxidative stress response is usually a mechanism to deal with increased reactive oxygen species (ROS), which may be produced in a cell after a stressful situation (Schieber and Chandel, 2014). An increased oxidative stress response results in increased expression and activity of antioxidant proteins, such as SOD, CAT, and GPX (Forgati et al., 2017; Chen et al., 2021; Zhao et al., 2022). Antioxidant

proteins detoxify the ROS caused by thermal stress at the cellular level and help with their safe excretion from the body (Limón-Pacheco and Gonsebatt, 2009; Kurutas, 2016). Sometimes, even despite increased activity of the antioxidant proteins and detoxification system in fish, increased oxidation of lipids and carbonylation of proteins happen during thermal stress, especially if the stress is acute or chronic (Forgati et al., 2017; Zhao et al., 2022). If not detoxified, increased damage caused by ROS due to thermal stress to structural proteins can increase cell death (Burlacu et al., 2001). Apoptosis, or programmed cell death, is a safe method for removing malfunctioning or damaged cells and has been reported to increase during thermal stress (Cheng et al., 2015; Liu et al., 2022). However, chronic thermal stress can cause more serious damage, such as necrosis of the tissue, disrupting the normal function of organs (Liu et al., 2016; Abdel-Tawwab et al., 2025).

At the organismal level, thermal stress is known to affect the immune system and the ability of fish to respond to pathogens (Abram et al., 2017). Exposure to thermal stress is known to affect the expression of many genes coding proteins involved in the immune system in fish, such as interleukins, interferon regulatory factor 2, tumor necrosis factor-alpha, and major histocompatibility complex (Perez-Casanova et al., 2008; Yang et al., 2022; Elbahnaswy et al., 2024; Liu et al., 2025). Increased levels of interleukins and immunoglobulin G in the blood have also been reported in fish experiencing thermal stress (Shaalan et al., 2024; Elbahnaswy et al., 2024). Increased lysozyme activity, phagocytic activity, respiratory burst activity, and myeloperoxidase activity are some other effects of thermal stress on the immune system detected in fish exposed to thermal stress (Elbahnaswy et al., 2024; Hao et al., 2024; Singh et al., 2026). Exposure to thermal stress has also been shown to affect the immune response to pathogens or immunostimulants (Larsen et al., 2018; Elkatatny et al., 2020; Saravia et al., 2022). If thermal stress continues, all these changes may decrease the ability of the immune system to defend against different pathogens, which may result in several kinds of diseases (Snieszko, 1973; Ojolicka et al., 1994; Grant et al., 2003; Schade et al., 2014).

In general, thermal tolerance and thermal performance are two concepts used to describe how temperature affects animals (Ern et al., 2023). The thermal performance of animals is usually measured in terms of instantaneous physiological rates across temperatures, where it increases slowly by increasing temperature up to the maximum performance level, but drops

rapidly after passing this temperature (Sinclair et al., 2016). The hypothetical Thermal Performance Curve (TPC) is usually used to describe this pattern, in which the temperature where the highest performance occurs is called the optimum temperature ($T_{opt}$), and the lower and upper limits are called critical thermal minimum ($CT_{min}$) and critical thermal maximum ($CT_{max}$), respectively (Schulte et al., 2011). These two concepts, $CT_{min}$ and $CT_{max,}$ indicate the lowest and the highest temperature tolerable for the fish and are frequently used to define the thermotolerance (De Bonville et al., 2025). Both thermal performance and thermal tolerance in one species can be affected by acclimation temperature (Kır, 2020; Larios-Soriano et al., 2019; Scheuffele et al., 2022; Garcia-Villarreal et al., 2025). Local adaptation also plays an important role, and the same species from different geographical regions are reported to have different thermal performance and thermal tolerance (Fangue et al., 2006; McKenzie et al., 2013; Yu et al., 2018; Bugg et al., 2020). Fishes usually develop adaptations over time to cope with environmental stressors such as temperature (Schulte, 2007; Atkins and Travis, 2010; Schulte, 2014; Fé-Gonçalves et al., 2020).

One of the important factors determining the thermotolerance and thermal performance of animals is their physiological capacity to deal with the adverse effects of increased temperature (Ern et al., 2023). These physiological responses to environmental conditions are known to be adjustable through acclimation, which involves flexible changes in physiological pathways, cellular machinery, and tissue structure (Eldridge et al., 2015; Morley et al., 2019). However, the acclimation capacity of animals to temperature can be different and factors such as species and local adaptation play an important role in it (Ruthsatz et al., 2024). Species adapted to more thermally stable environments may have a difficult time acclimating to thermal changes, but it may be easier for eurytherms that live in an environment with more variable temperatures (Brett, 1956). In addition, temperature changes happening at lower rates provide the internal physiology of the fish with enough time to slowly manage the adverse effects of increased temperature and acclimate to the new temperature (Volkoff and Rønnestad, 2020). This may happen through mechanisms such as increasing food intake to deal with the shortage of energy caused by increased metabolic rate, improving hypoxia tolerance, and upregulation of antioxidant proteins to deal with increased ROS (Nadermann et al., 2019; Jensen and Benfey, 2022; Carrillo-Longoria et al., 2024). In contrast, more sudden changes in temperature happening in a short

time can make it difficult for the animal to respond to the consequences of fast physiological changes and result in a lower thermotolerance (Moyano et al., 2017).

Long-term survival of animals in an environment is usually determined by adaptation via directional selection of genes (Young et al., 1989; Logan et al., 2014). Climate change is predicted to change the temperature regime in the aquatic environment, which can affect the physiology of animals living in aquatic habitats (Sunde et al., 2019). Fishes usually develop adaptations over time to cope with environmental stressors such as temperature (Schulte, 2007; Atkins and Travis, 2010; Schulte, 2014; Fé-Gonçalves et al., 2020). However, climate change is predicted to change the temperature regime in the aquatic environment, which can affect the physiology of animals living in these habitats (Sunde et al., 2019). Given the slow adaptation rate for upper thermal tolerance of physiological systems, they may not be able to adapt to the new thermal regime in their environment, which may result in extinction of species (Morgan et al., 2020).

## 1.2 Environmental pressures and development

The effects of environmental variables may be more significant on the early-life stages of aquatic animals (Moyano et al., 2017). Fish in the embryonic and larval stages do not benefit from fully developed organs and systems for dealing with changes in physiology to maintain homeostasis, which makes it more difficult for the developing fish to acclimate to a changing temperature compared to adults (Vasconcelos et al., 2010; Ramos et al., 2015; Madeira et al., 2020; Dahlke et al., 2020; Cowan et al., 2024). In addition, smaller animals usually have a higher surface-to-volume ratio, which makes retention of internal temperature and consequently thermoregulation more challenging (Favilla et al., 2024). Furthermore, the lack of proper mechanisms for movement makes it difficult for them to escape from unfavorable situations and find a more suitable niche (Louhi et al., 2023). This is of even more importance as fish are ectothermic and depend on their movement to behaviorally control their internal temperature (Haesemeyer, 2020).

The results of temperature change on developing fish depend on many factors, such as developmental stage, rate of change, the amplitude of change, and the natural thermal tolerance

of species (Mora and Maya, 2006; Logan and Buckley, 2015; Réalis-Doyelle et al., 2016; Moyano et al., 2017; Nati et al., 2021). Mild changes within a species' thermotolerance and at a natural rate might be manageable by species through acclimation (Wen et al., 2013; Sandblom et al., 2014). However, extreme changes are known to have severe effects on developing fish and often result in mortality (Réalis-Doyelle et al., 2016). Even in situations where some developing fish are capable of surviving drastic changes in temperature, detrimental effects such as increased deformity and changes in growth rate are possible outcomes (Réalis-Doyelle et al., 2016; Dubey et al., 2023).

Exposure to nonlethal environmental challenges during early-life stages may cause long-term effects by altering development of specific organs, age at maturity, fecundity, egg size, and lifespan (Jonsson and Jonsson, 2014). These long-term effects might be beneficial and make the animals more resistant to similar stressors, or have negative effects on their coping mechanisms (Langenhof and Komdeur, 2018). The changes caused by the effects of environmental stressors are regulated by many physiological and signaling pathways, which involve upregulation of genes and proteins, structural changes in tissue along with behavioral modifications (Burggren and Blank, 2009; Vindas et al., 2018; Fogelman and Canli, 2019; Mazzelli et al., 2020; Canosa and Bertucci, 2023). Many of these changes might be reversible in many cases after removing the environmental pressure, returning the animal to a developmental pathway that results in a normal range of adult phenotype suited for typical environmental conditions (Burggren, 2020). This plasticity allows animals to make appropriate modifications to survive or prepare for new environmental conditions (Stager et al., 2025).

## 1.3 *Acipenser* sturgeons

*Acipenser* sturgeons are an ancient group of fishes with widespread distribution in the temperate zones of the northern hemisphere (Bemis and Kynard, 1997). Taxonomically, they belong to the family Acipenseridae, with genus *Acipenser* containing 17 species (Kuhajda, 2014). These fishes have a long lifespan, and it may take them 10-20 years to reach sexual maturity (Auer, 1995). All sturgeons reproduce in freshwater at highly variable times during the year, and spawning sites are usually areas with hard substrate of gravel to boulder-sized rocks

(Rochard et al., 1990; Bemis and Kynard, 1997). In terms of habitat and life history, sturgeons can be divided into three groups: anadromous species, which live in the sea but enter rivers for spawning, semi-anadromous species, which spend most of their lives in the river and sometimes migrate to estuarine regions but do not enter the sea, and freshwater residents, which spend their entire life span in the river and lakes (Dettlaff et al., 1993). These species are commercially important fish in many countries, but their population has decreased over the past century due to habitat fragmentation, overfishing, and pollution (Williot et al., 2002).

In North America, six species of *Acipenser* have been recorded: Atlantic sturgeon, *Acipenser oxyrinchus oxyrinchus*; lake sturgeon, *Acipenser fulvescens*; shortnose sturgeon, *Acipenser brevirostrum*; white sturgeon, *Acipenser transmontanus*; and green sturgeon, *Acipenser medirostris* (Haxton et al., 2016). These species have a wide distribution from Canada to Florida, USA, and have played an important role as a food source in the lives of Indigenous people and European settlers (Hannibal-Paci and George, 1998; Dadswell, 2006; Haxton et al., 2016; Bruch et al., 2016). However, since the 1800's, populations of these species have been declining (Lenhardt et al., 2006). Construction of dams that block access to spawning areas, low water quality, dredging, water withdrawals from rivers, commercial fisheries (i.e., bycatch), and changes in the environmental conditions are some of the current significant threats to these species (Kynard, 1996; Bruch et al., 2016). As a result, several species of sturgeons are listed as endangered or critically endangered in the International Union for Conservation of Nature (IUCN) red list (IUCN, 2025). In Canada, Atlantic sturgeon, *Acipenser oxyrinchus oxyrinchus*, of the St. Lawrence and Maritimes populations are listed as threatened species by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) (COSEWIC, 2026). In addition, the effects of thermal stress caused by climate change on these species, and their ability to compensate for this stressor through acclimation, are one of the rising concerns about the survival of these species (Dichiera et al., 2024).

## 1.4 Zebrafish

Zebrafish, *Danio rerio*, is a tropical freshwater fish of the family Cyprinidae, endemic to South Asia (Steele et al., 2014; Parichy, 2015). These fish are relatively small and can grow to

25-35 mm (Spence et al., 2008). In their natural habitat, they usually live in slow-moving or stagnant, shallow water with high transparency, often in shaded locations (López-Olmeda and Sánchez-Vázquez, 2011). These animals can tolerate a wide range of temperatures that can fluctuate from 10 to 40 °C (Aleström et al., 2019). Zebrafish are omnivorous and mostly consume zooplankton and insects, although phytoplankton, algae, invertebrate eggs, arachnids, detritus, sand, and mud have also been detected in their gastrointestinal tract (Spence et al., 2008). Sexual maturation usually happens 3-4 months post-hatch (Liew and Orbán, 2013). The average lifespan of this fish is up to 44 months, but living up to 66 months has also been reported in rare cases (Lawrence, 2007; Spence et al., 2008). Strong sexual dimorphism does not exist in zebrafish; however, there are some morphological differences in the shape of the body and pigmentation pattern between sexes (Nasiadka and Clark, 2012).

In the last century, zebrafish have been widely used as a model organism in genetic and developmental studies (Lawrence, 2007). This wide usage is because of the technical and practical advantages that make it a suitable animal for scientific investigations (Segner, 2009). Small size, short generation time, and robustness to environmental influences are some of the characteristics that make this animal a suitable vertebrate to grow in the lab (Meunier, 2012). In addition, these fish produce transparent eggs, which allow researchers to visually analyze the developmental processes (Dooley and Zon, 2000). Similarities in cellular functions, and also function and morphology of organs, between zebrafish and humans are some of the other factors making them a great model for human-related conditions (Bugel et al., 2014; Shin and Fishman, 2002).

Similarity between zebrafish and terrestrial vertebrates in terms of general organization and behavioral and physiological systems, such as stress-regulating systems, makes them suitable non-rodent alternatives for early-life studies (Brittijn et al., 2009). Transparency of these fish in the early-life stages makes them suitable for live imaging of the nervous system (Fetcho, 2006). Multiple studies on this model in the last few decades have provided us with a plethora of genomic, transcriptomic, and proteomic data that make working with these animals convenient (Howe et al., 2013; Palmblad et al., 2013; Kelkar et al., 2014; Alli Shaik et al., 2014; Woods et al., 2026). Using this information, several transgenic strains for studying the nervous system of this model have been produced and are commonly used by researchers worldwide (Udvadia and

Linney, 2003; Yoshida and Macklin, 2005; Jung et al., 2010; Harrison et al., 2014). This also helped scientists to develop green fluorescent protein (GFP) labeling of many functional proteins in the brain in several strains (Udvadia and Linney, 2003). Other histological methods, such as immunohistochemistry and *in situ* hybridization, have also been developed and are widely available to researchers (Doganli et al., 2016). In addition, a variety of protocols and methodologies have been developed to study the behavior of zebrafish in different research contexts (Orger et al., 2004; Orger and Polavieja, 2017; Basnet et al., 2019; Maeda et al., 2021).

## 1.5 Quantitative PCR and thermal stress response

As gene transcription that is then modified into mRNA is the first step in gene expression, the mRNA can be quantified and used to study the cellular response to acute and chronic stressors (Jeffries et al., 2021). Many transcriptomic studies investigate the effects of an environmental factor on the expression of one or several genes involved in cellular function and pathways, often by using targeted quantitative PCR (qPCR) (Gonzalez and Pierron, 2015). Relative gene expression analysis often uses qPCR in which the mRNA expression of one or several genes in an individual or group is measured relative to a control group or individual (Schmittgen and Livak, 2008). To do so, the total RNA of the sample is extracted and converted into complementary DNA (cDNA). In this process, random primers are used for the initiation of amplification by reverse transcriptase enzymes that use free nucleotides as building blocks to produce a complementary sequence from the template RNA strand. The cDNA is used to amplify the gene of interest through qPCR using cycling conditions and primers specifically designed for that gene (Valasek and Repa, 2005). The sample with higher mRNA levels of that gene, which results in a higher initial template in the cDNA, exceeds a certain threshold called cycle of threshold (Ct) in PCR sooner than those with lower mRNA expression (Kubista et al., 2006). After normalizing the Ct of the target gene with Ct of one or several endogenous reference genes, the relative mRNA expression of that gene can be measured and compared between different groups (Wong et al., 2005).

Although the basic principles of relative mRNA expression using qPCR are similar in the different methods, several innovations have improved the method's efficiency. OpenArray™

technology is one of these high-throughput innovations. Besides using specific primers, OpenArray™ chips use the TaqMan probe detection method, which binds specifically to the target gene and does not detect nonspecific amplifications or primer-dimers (Islam et al., 2026). In addition, the number of genes that can be run by using OpenArray™ chips is also high (56 genes for 24 samples in duplicate), which allows us to analyze the expression of a larger number of genes in more samples in a shorter time compared with traditional qPCR approaches. Analyzing the mRNA expression of more genes in several samples is important in transcription profiling. In other words, studying several genes involved in different pathways with different biological functions at the same time and comparing them between different populations can help us in having a better systemic view of the changes happening in physiology, which has been practiced before using other methods such as cDNA microarrays and RNA sequencing (Goetz and MacKenzie, 2008; Bugg et al., 2021). This systemic view can provide a better understanding of the physiological response of animals to environmental changes caused by a variety of factors, such as climate change (Mazurais et al., 2011).

## 1.6 Thesis objectives

The focus of this thesis was on the effects of thermal stress on freshwater fish during early life stages. These effects were studied both in exposure to acute thermal stress (Chapter 2) and long-term chronic thermal challenges (Chapters 3, 4, and 5). To study the effects of acute thermal stress, I chose *Acipenser* sturgeons, as these species are native to temperate regions of North America, and these regions are expected to experience greater changes in thermal regime as a result of climate change (Ficke et al., 2007). Additionally, these regions usually experience heatwaves, which have been predicted to increase in intensity, frequency, and duration due to climate change (Perkins-Kirkpatrick and Lewis, 2020; Polazzo et al., 2021). This is important because heatwaves are climate extremities that can increase the temperature of the environment more than the physiological tolerance of animals (Salwadkar, 2025). Therefore, studying the effects of exposure to temperatures equal to or higher than the upper thermal limit of these species using techniques such as the $CT_{max}$ trial can improve our insight into physiological responses to probable exposures to similar stressors. To study the effects of acute thermal stress

on freshwater fish, I designed a tool (Chapter 2) that can help researchers to investigate the cellular effects of thermal stress from different perspectives. It was also important to produce the chip in a way that could be used for multiple species of sturgeons in the *Acipenser* genus. In addition, the OpenArray™ chip was used to see how exposure to early-life acute thermal stress, which exposes animals to their upper thermal limit, can affect cellular processes. To do so, I used the multispecies OpenArray™ chip to quantify the mRNA expression of 56 genes in three *Acipenser* sturgeons: lake sturgeon (*Acipenser fulvescens*), shortnose sturgeon (*Acipenser brevirostrum*), and Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*), which have experienced acute thermal stress in terms of $CT_{max}$. The experimental design imitated acute thermal stress happening in natural habitats, especially shallow waters, during heatwaves.

Many freshwater fish spawn in the spring, and their offspring experience thermal fluctuations and heatwaves during early life stages, which may not necessarily result in the death of the entire cohort. Because temperature has been observed to affect developing fish from different aspects (Martell et al., 2005; Long et al., 2012; Ashaf-Ud-Doulah et al., 2021; Zeb et al., 2026; Adzijovski et al., 2026), it is possible that some of these changes, even if they do not cause mortality, may continue and have long-term effects. Studying the long-term effects of early-life exposures to higher temperatures can improve our insight into the long-lasting effects of these kinds of exposures on the survival of fish populations. To study the long-term effects of thermal stress on freshwater fish, I chose zebrafish, *Danio rerio*, because it has a shorter lifespan and reaches maturity in a few months, which makes it easier to study the long-term effects of chronic thermal challenges in adult fish. In addition, there is a great plethora of knowledge on zebrafish biology, and established techniques for breeding, rearing, and experimenting with this species. In Chapters 3, 4, and 5, the goal was to focus on the long-term effects of repeated thermal fluctuations, as a type of chronic thermal challenge, that happens during early-life stages. In Chapter 3, the goal was to study the long-term effects of early-life thermal fluctuations on cellular stress response, body size, and thermotolerance as estimated by $CT_{max}$. In Chapters 4 and 5, these effects were studied on the development of the brain and muscle, respectively.

Four objectives were followed to answer these questions:

1. Design a multispecies OpenArray™ chip to quantify the mRNA expression of genes involved in general heat shock response, oxidative stress response, apoptosis, hypoxia,

immune system, metabolism, and osmoregulation, and use it to quantify the transcriptomic response to acute thermal stress in three *Acipenser* sturgeon: lake sturgeon, Atlantic sturgeon, and shortnose sturgeon (Chapter 2).

**Hypothesis:** Acute thermal stress triggers a common and species-, population-specific cellular response, which is detectable at the transcriptomic level.

**Prediction:** Acute thermal stress will change the mRNA expression pattern of genes involved in general heat shock response, oxidative stress response, apoptosis, hypoxia, immune system, metabolism, and osmoregulation. This change in the mRNA expression pattern will vary between species and populations.

2. To quantify the mRNA expression of cellular stress response genes in the zebrafish that have experienced early-life daily thermal fluctuations, in the larval stage and in the juvenile and adult stages, along with measuring their thermotolerance (Chapter 3).

   **Hypothesis 1:** Exposure to thermal fluctuations at early-life stages increases the cellular stress response, which can result in higher basal expression levels of genes coding for cellular stress responses that can be detectable at later life stages.

   **Prediction 1:** Daily thermal fluctuation during the embryonic and larval stages will upregulate the mRNA expression of genes coding heat shock proteins and oxidative stress response genes in zebrafish. This upregulation will continue and will be detectable in the adult stage.

   **Hypothesis 2:** Exposure to thermal fluctuation at the early-life stages increases the thermotolerance of fish measured by the $CT_{max}$ test, and this increased thermotolerance will continue to the juvenile and adult fish.

   **Prediction 2:** Experiencing daily thermal fluctuations during embryonic and larval stages will increase the $CT_{max}$ in the juvenile and adult fish.

3. To investigate the effects of early-life daily thermal fluctuations on the development of the brain using mRNA expression and histology (Chapter 4).

   **Hypothesis:** Early-life daily thermal fluctuations affect the mRNA expression of neurodevelopmental genes, proliferation of brain stem cells, and neuron density in the forebrain of zebrafish, and these effects continue even after removing the thermal challenge.

**Prediction:** Exposure to daily thermal fluctuations during the embryonic and larval stages will change the mRNA expression pattern of key genes involved in the development of the brain. In addition, early-life exposure will change the quantity of proliferating radial-glial cells in the forebrain of zebrafish, which will change the population of neurons in this region.

4. To investigate the effects of early-life daily thermal fluctuations on the development of muscle using mRNA expression of genes coding for structural proteins (Chapter 5).
   **Hypothesis:** Early-life thermal fluctuations affect the mRNA expression of genes coding for proteins involved in the structure of skeletal muscle, and these changes will be detectable in later life stages.
   **Prediction:** Exposure to early-life daily thermal fluctuations will increase the mRNA expression of genes coding structural proteins in the skeletal muscle in zebrafish, and this expression pattern continues even after removing the thermal fluctuation at the juvenile and adult stages.

# Chapter 2 High-throughput multispecies qPCR assays to study the effects of acute thermal stress in three species of *Acipenser* sturgeons[1]

## 2.1 Abstract

Fishes experience thermal stress in their natural environment that can be caused by natural or anthropogenic factors and can directly affect their physiology. In this study, we developed a multispecies OpenArray™ qPCR "chip" to measure the effects of acute thermal stress on the mRNA response of *Acipenser* sturgeons. The qPCR chips were then tested on three species: *Acipenser fulvescens*, *Acipenser oxyrinchus oxyrinchus*, and *Acipenser brevirostrum.* The *A. fulvescens* were from two genetically distinct populations (Burntwood River and Winnipeg River, Manitoba, Canada) that were acclimated to three different temperatures (16, 20, and 24 °C) to quantify the effects of genetic background and acclimation temperatures on the transcriptomic response to acute thermal stress. Additionally, juvenile *A. oxyrinchus oxyrinchus* and *A. brevirostrum* were acclimated to 10 °C and then experienced acute thermal stress. All three species were sampled for gill and liver before and after experiencing acute thermal stress, which was applied using a $CT_{max}$ test. The mRNA expression of 52 genes of interest was quantified using the multispecies qPCR chip for all three species, along with 4 reference genes. We showed that acute thermal stress affected the mRNA expression of 17-26 of the thermal metabolic stress genes that were assayed using the chip, depending on the species. However, the mRNA patterns varied between species and genetically distinct populations, suggesting differences in cellular responses to thermal stress that can lead to different susceptibilities to thermal stress in their natural habitats.

[1] A modified version of this chapter has been published in open-access format in the *Journal of Fish Biology*. Ken M. Jeffries and Nicholas J. Bernier were involved in funding acquisition, contributed to gene selection and developing the *Acipenser* OpenArray™ chip, and supervised the research. Will S. Bugg designed and performed experiments on lake sturgeon. Faith M. Penny and Scott A. Pavey designed and performed experiments on Atlantic and shortnose sturgeon. Hossein Haghighi and Kyle Madden designed and tested the primers and probes for the OpenArray™ chip. Hossein Haghighi ran the OpenArray assays, analyzed the data, and wrote the first draft. All the authors read and provided comments on the manuscript. Found in: Haghighi, H., Madden, K., Bugg, W. S., Penny, F. M., Pavey, S. A., Bernier, N. J., & Jeffries, K. M. (2026). High-throughput multispecies quantitative polymerase chain reaction assays to study the effects of acute thermal stress in three species of *Acipenser* sturgeon. *Journal of Fish Biology*, in press.

## 2.2 Introduction

Average water temperatures are increasing due to climate change (Houghton, 2001), which can have profound effects on aquatic environments globally (Fernández, 2022; Ryan and Ryan, 2006). Ectotherms, such as fishes, are sensitive to changes in water temperature (Burraco et al., 2020) because their internal temperature is highly dependent on ambient temperature, and temperature directly influences their metabolic rate (Lear et al., 2020). Fishes have evolved to tolerate fluctuations in temperature in their natural distribution (Cuculescu et al., 1998); however, with rapid episodic changes in temperature, such as heat waves, full thermal acclimation is not always possible (Volkoff and Rønnestad, 2020). Thus, rapid temperature increases caused by heat waves have the potential to result in population-level impacts on fishes (Morgan et al., 2020). Consequently, there is a need to understand the effects of temperature on the physiological performance and thermotolerance of fishes (Lefevre et al., 2021). Transcriptomic-based approaches can help us to investigate the physiological status of wild animals, which can connect the transcriptomic profile of animals to fitness-related characteristics (Jeffries et al., 2021).

The metabolic rate of animals represents the energy needed for maintenance, growth, and reproduction (Riemer et al., 2018). Because temperature determines ectotherms' metabolic rate, high temperature can increase the cellular energy demand (Sun et al., 2019). In the presence of sufficient oxygen supply to tissues, glucose is metabolized to pyruvate through glycolysis, which is then shuttled into mitochondria where it goes through the tricarboxylic acid (TCA) cycle and is used to produce ATP through oxidative phosphorylation (Bertram et al., 2006). However, in the absence of oxygen, anaerobic glycolysis is used to produce ATP, which has a lower efficiency for producing ATP (Vazquez et al., 2010). In this process, after glycolysis, pyruvate is converted to lactate by lactate dehydrogenase, and the by-product of this reaction, nicotinamide adenine dinucleotide ($NAD^+$), is used to produce ATP (Chandel, 2021). In addition, stress hormones such as catecholamines and cortisol are important regulators of energy flux in adipose tissue (Peckett et al., 2011). Changes in the expression of genes involved in the metabolism of lipids have also been reported in several species in response to thermal stress (Ma et al., 2015; Balbuena-Pecino et al., 2019; Zhao et al., 2021). Moreover, increased cortisol levels are often associated with situations that result in anaerobic metabolism (De Boeck et al., 2001). Thermal

stress has been reported to increase expression and activity of enzymes involved in anaerobic metabolism or gluconeogenesis, such as lactate dehydrogenase and phosphoenolpyruvate carboxykinase 1 (Windisch et al., 2011; Dalvi et al., 2017; Sun et al., 2019). If not enough ATP is produced in the cell, important activities such as protein production and ion regulation may fail, eventually resulting in cell death (Staples and Buck, 2009).

Acute thermal stress leads to physiological responses in the fish (Morgan et al., 2019), which include changes at the cellular level (Somero, 2020). Upregulation of heat shock proteins (HSP), important in the folding of proteins (Craig et al., 1993; Whitley et al., 1999), is a common cellular response observed in aquatic animals exposed to increased temperature (Zheng et al., 2019). Thermal stress has also been observed to increase the production of reactive oxygen species (ROS) (Belhadj Slimen et al., 2014). Typically, antioxidant proteins such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) detoxify ROS and minimize the adverse effects of these compounds (Matès, 2000). When the production of ROS is more than the antioxidant capacity of cells, ROS can cause damage to cells by lipid peroxidation, and alteration of proteins and DNA, which can lead to cell death (Fleury et al., 2002). Programmed cell death, or apoptosis, is a type of cell death that can be caused by intracellular stress such as increased levels of ROS, especially through the activation of intrinsic apoptotic pathways (Villalpando-Rodriguez and Gibson, 2021). In this phenomenon, the release of cytochrome c from mitochondria results in the activation of apoptotic protease-activating factor-1 (Apaf-1), which aids in the formation of an apoptosome complex that eventually causes the activation of executioner caspases by initiator caspases (Green and Llambi, 2015). Executioner caspases, such as caspase-3, activate cytoplasmic endonucleases and proteases that degrade nucleic acids in the nucleus and proteins (Elmore, 2007), respectively, leading to controlled cell death.

Sturgeons are an ancient group of fishes mostly dwelling in the river systems of the northern hemisphere (Carmona et al., 2009). Since the 1800's, populations of these species have been threatened by factors such as overfishing and habitat destruction (Lenhardt et al., 2006). As a result, several species of sturgeons are listed as endangered or critically endangered in the International Union for Conservation of Nature (IUCN) red list (IUCN, 2025). In addition, thermal stress can affect the physiology of sturgeons, impacting the survival of these species

(Sardella and Kultz, 2014; Wassink et al., 2019; Rodgers et al., 2019). Quantitative PCR (qPCR) has been used in several studies on *Acipenser* sturgeons to investigate the cellular and systemic responses to increased temperature (Bugg et al., 2020; Chen et al., 2022; Bugg et al., 2023a; Penny et al., 2023; Wang et al., 2023). The objective of this study was to develop a multispecies *Acipenser* OpenArray™ qPCR "chip" to estimate the expression of 56 genes coding proteins with important functions in the immune system, metabolism, osmoregulation, response to hypoxia, apoptosis, general stress response, and oxidative stress response. We then used this *Acipenser* chip to quantify the effects of acute thermal stress after exposure to a critical thermal maximum trial ($CT_{max}$) protocol during early life stages in three species of *Acipenser* sturgeon: lake sturgeon (*Acipenser fulvescens*), shortnose sturgeon (*Acipenser brevirostrum*), and Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*). These species are endemic to North America, and their populations have decreased in recent decades because of anthropogenic activities (Pollock et al., 2015; Fernandes et al., 2010; Kynard and Horgan, 2002; Gilligan-Lunda et al., 2024). Despite being morphologically similar, and for shortnose and Atlantic sturgeon, existing in similar geographical areas, these three focal species diverged millions of years ago and have different life histories and ploidies (Trifonov et al., 2016; Penny et al., 2023). Studying the resilience of these species in response to climate change and thermal stress can increase our insight into their biology and help with future management decisions. In this study, we used the custom-designed OpenArray™ chip to examine how similar species with different genetic, geographical, and acclimation histories respond to acute thermal stress at the transcriptomic level. In addition, we aim to identify the common and species-specific genes that can work as biomarkers of thermal stress in *Acipenser* species.

## 2.3 Materials and Methods

### 2.3.1 Primer and probe optimization for the OpenArray™ chip

We designed a multispecies *Acipenser* OpenArray™ qPCR chip that contains primers and probes for amplifying and detecting 56 genes in *Acipenser* sturgeons. The chip comprises genes involved in the immune system (6 genes), metabolism (15 genes), osmoregulation (4 genes), response to hypoxia (7 genes), apoptosis (4 genes), oxidative stress response (7 genes), general

stress response (9 genes), and endogenous controls (4 genes). Genes were selected based on markers of thermal and metabolic stress from other species (e.g., salmonids; Islam et al., 2026) and previous *Acipenser* sturgeon studies, and would be relevant physiological processes for monitoring sturgeons in the wild and in captivity. Each chip can quantify the mRNA expression of the 56 genes in 24 samples in duplicate (56 genes × 24 samples × 2 technical replicates = 2688 reactions). The produced cDNA from each sample is loaded on each chip using QuantStudio™ 12K Flex Accufill System (Thermo Fisher Scientific, USA). The chips are run on a QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific, USA), which can run four chips at a time, increasing the number of samples measured each time to 96 (10752 reactions).

To design the universal OpenArray™ chip for *Acipenser* sturgeons, the available genomic and transcriptomic information of different *Acipenser* species was acquired from several sources such as RNA sequencing data of lake sturgeon (Bugg et al., 2023), white sturgeon (*Acipenser transmontanus*) (Doering et al., 2016), Siberian sturgeon (*Acipenser baerii*) (Klopp et al., 2020), and online DNA and RNA sequences belonging to different *Acipenser* species available on National Center for Biotechnology Information (NCBI) (www. https://www.ncbi.nlm.nih.gov/). After the alignment of sequences from different species using Geneious Prime (2023.0.1), primers and probes for each gene were designed based on the consensus sequence at the conserved region of each gene using Primer Express (3.0.1), following a similar protocol used for designing multispecies chips for salmonids (Islam et al., 2024).

The designed primers were tested on lake sturgeon, Atlantic sturgeon, shortnose sturgeon, and white sturgeon (tissues were provided by a collaborator for the purpose of testing the primers; the white sturgeon were originally collected from the Nechako River, British Columbia, Canada) with the SYBR Green® detection on a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, USA). The quality of the amplification and the specificity of primers were checked by monitoring amplification curves and melt curves, respectively. After generating standard curves, the efficiency of PCR reactions was calculated for each primer by the formula $E=[10^{(1/\text{-slope})}]-1$(Wong and Medrano, 2005). Primers with efficiencies between 80 and 120% were used for developing the OpenArray™ chip following established protocols for optimal assay design for the OpenArray™ technology. The chips were then tested on three species of

*Acipenser* sturgeon that underwent $CT_{max}$ protocols outlined below. The sequence of primers and probes and the calculated efficiency for primers are listed in the **Supplementary Table 1**.

## 2.3.2 Experimental design

### 2.3.2.1 Lake sturgeon

The tissue samples of *A. fulvescens* used in this study come from a previous study investigating the effects of acclimation temperature on the thermal physiology of two distinct populations from Burntwood River and Winnipeg River in northern and southern Manitoba, respectively (Bugg et al., 2020). These two populations live in habitats 650 km apart, and studies show that they are genetically distinct (Bugg et al., 2023). Juvenile lake sturgeon were produced by fertilizing gametes harvested from wild-caught females and males from the two populations at the University of Manitoba, Canada. Beginning at 30 days post-fertilization (DPF), the juveniles from these two populations were acclimated to 16, 20, and 24 °C for 30 days. After 30 days (60 DPF), and before the ($CT_{max}$) trial (Pre-$CT_{max}$), eight fish from each acclimation temperature of both Winnipeg River (1.02 ± 0.41 g; 67.71 ± 10 mm $L_T$) and Burntwood River (1.14 ± 0.51 g; 71.51 ± 10 mm $L_T$) were euthanized by immersion in an overdose of tricaine methane sulfonate solution buffered with an equal volume of sodium bicarbonate. The gill tissue was collected and preserved in RNA*later*® (Thermo Fisher Scientific, Waltham, USA), until transferred to -80 °C for storage. Another eight fish from each acclimation temperature were netted and placed individually in well-aerated experimental units (~200 ml of water volume and 9.5 cm long × 5 cm across) at the acclimation temperature one hour before the $CT_{max}$ trial. The $CT_{max}$ experiment was conducted by increasing the water temperature in the tank by 0.3 °C $min^{-1}$ with constant aeration until the fish could not flip themselves back after being flipped on their back within 3 seconds. At this moment, the fish was euthanized by immersing in tricaine methanesulfonate solution (250 $mg.L^{-1}$) buffered with an equal volume of sodium bicarbonate, and the gill tissue was removed and preserved. This experiment was approved by the animal care committees at the University of Manitoba (AUP#F15-007) in accordance with the guidelines of the Canadian Council on Animal Care.

### 2.3.2.2 Atlantic and shortnose sturgeon

Samples of Atlantic and shortnose sturgeon came from another study investigating the physiological response to acute thermal stress (Penny et al., 2023). The experiment was approved by the animal care committees at the University of New Brunswick (AUP#2018-1S-02) in accordance with the guidelines of the Canadian Council on Animal Care. In that study, two-year-old juvenile Atlantic sturgeon (122 ± 10.3 g; 33.8 ± 0.84 cm total length ($L_T$)) and shortnose sturgeon (136 ± 8.6 g; 32.9 ± 1.0 cm $L_T$) were obtained from Acadian Sturgeon and Caviar (Carter's Point, Kingston, New Brunswick, Canada) and brought to the University of New Brunswick, Canada. These were the descendants of broodstocks originally collected from the Saint John River, New Brunswick, Canada. Fish from both species were separated into two groups: the $CT_{max}$ group and the Control group. They were then acclimated to 10 °C for 30 days. The $CT_{max}$ was performed on the $CT_{max}$ groups by increasing temperature at a rate of 0.1 °C $min^{-1}$ until the fish was not able to maintain an upright orientation. At this timepoint, the fish was removed from the tank, and after anesthetizing in a tricaine methanesulphonate solution (MS-222; 1 $g \cdot l^{-1}$) buffered in sodium bicarbonate (2 $g \cdot l^{-1}$) and euthanizing by cervical dislocation, the gill and liver tissues were collected. Eight fish from the Control group, which experienced the same handling process except for no increase in temperature, were collected and sampled the same way. Samples from both groups were preserved in RNA*later*® and stored at -80 °C.

## 2.3.3 RNA extraction, cDNA synthesis

Total RNA from liver and gill samples of Atlantic and shortnose sturgeon, and liver tissue of white sturgeon, was extracted using RNeasy® Plus Micro Kit (QIAGEN, Germany), and PureLink RNA Mini Kits (Invitrogen; Ambion Life Technologies) were used for the lake sturgeon gill samples. Samples were homogenized separately using a TissueLyser (QIAGEN) at 50 Hz for 5 min in the lysis buffer available in each specific kit, and RNA purification was performed following the manufacturer's instructions. NanoDrop One (Thermo Fisher Scientific) was used to measure RNA concentration and purity, and RNA with a 260/230 ratio between 1.7-

2, and a 260/280 ratio higher than 1.8 was selected to be used for cDNA synthesis. To measure the integrity of RNA, 400 ng of RNA from each sample was loaded on 1% agarose gel to visually check 18S rRNA and 28S rRNA bands. Before cDNA synthesis, genomic DNA was eliminated by treating 1000 ng of total RNA with Invitrogen™ *ezDNase* enzyme (Thermo Fisher Scientific) at 37 °C for 2 minutes. The DNase-treated RNA was used directly for cDNA synthesis using QuantiTect® Reverse Transcription Kit (QIAGEN) following the manufacturer's instructions.

### 2.3.4 Relative mRNA expression analysis

To quantify the mRNA expression of genes under study using the universal *Acipenser* OpenArray™ chip, the synthesized cDNA was diluted 1:4 with nuclease-free water. Then, 2.5 µl of the diluted cDNA was mixed with 2.5 µl of 2X TaqMan Real-Time PCR Master Mix (Thermo Fisher Scientific, USA). The samples were loaded onto the chip in duplicate using QuantStudio™ 12K Flex Accufill System and real-time PCR was done using QuantStudio 12K Flex Real-Time PCR System following the manufacturer's instructions. The quality of amplification was assessed using the QuantStudio 12K Flex Real-Time PCR System software, and samples with sigmoidal amplification curves and amplification scores higher than 0.125 were selected for further analysis. To calculate the efficiency of PCR for each gene, baseline-corrected fluorescence data were exported from the software, and the efficiency of each reaction was calculated by LinRegPCR (Version 2021.2). For each gene, the mean value of all the efficiencies from single reactions was calculated for each species and listed in **Supplementary Table 2**. The average efficiency value was used to calculate the relative gene expression by applying the Pfaffl Method (Pfaffl, 2001). To measure relative mRNA expression in lake sturgeon, all the treatments for each population were normalized to the samples collected from treatment acclimated to 16 °C before the $CT_{max}$ trial (i.e., Pre-16). For Atlantic and shortnose sturgeon, the mRNA expression of each gene in the $CT_{max}$ group was calculated relative to the individual species' Control group. Some mRNA expression data were excluded from the relative mRNA expression analysis due to low PCR efficiency. Specifically, data for *cam1*, *mhc1a*, and *atp1a* were removed for all species. Also, *casp3* was excluded for lake sturgeons, the liver of

Atlantic sturgeon, and the gills of shortnose sturgeon. In Atlantic sturgeon, *tp53*, *cox1*, *lipe*, and *ca15* were removed from liver data, while *lipe*, *tp53*, and *cox1* were also excluded from gill data. Additionally, *ca15* was excluded from the liver data of shortnose sturgeon.

### 2.3.5 Statistical analysis

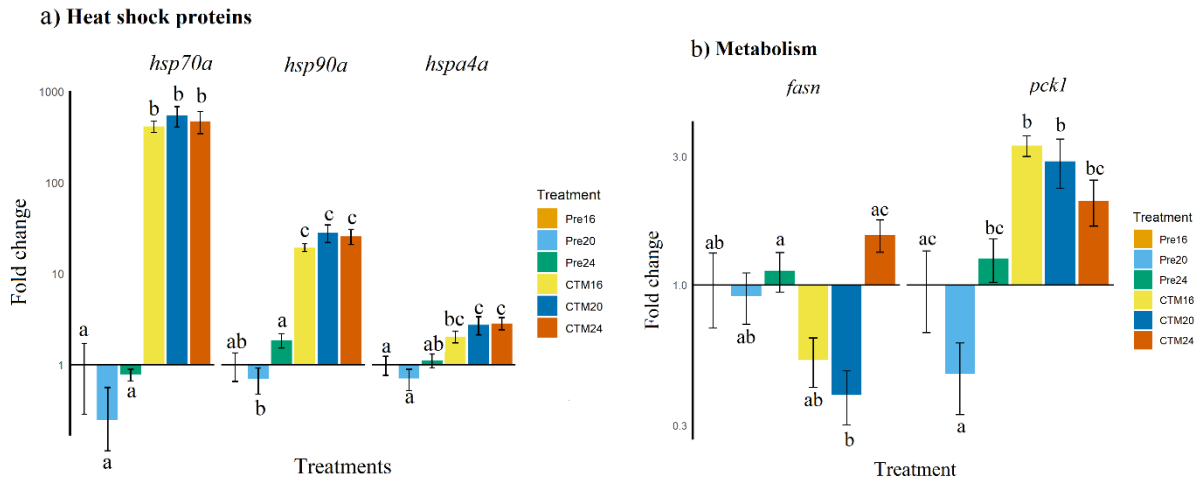
All the statistical analyses were conducted using R 4.3.2 (R Core Team, 2024). The Shapiro–Wilk test was used to assess the normality of data distribution in treatment groups for all three species. Because the relative mRNA expression for individuals was not normally distributed, data from the lake sturgeon experiment were log2-transformed, and Two-way ANOVA was used to measure the significant difference (p-value <0.05) between groups, considering acclimation temperatures and $CT_{max}$ as two factors that affect the expression of each gene. The p-values from the Two-way ANOVA testing the significance of acclimation temperatures, $CT_{max}$, and their interactions are provided in **Supplementary Table 3**. The Bonferroni correction was used to adjust the p-value and control for false positives. For Atlantic and shortnose sturgeon, the comparison between groups was conducted using Mann–Whitney U tests. To check the overall transcriptomic change in response to thermal stress, unsupervised Principal Component Analysis (PCA) was conducted for each species using $C_t$ values normalized to the four internal control genes.

## 2.4 Results

### 2.4.1 Lake sturgeon

The $CT_{max}$ measured for the two populations of lake sturgeon reported by Bugg et al. (2020) was slightly higher in the Winnipeg River fish than in the northern Burntwood River fish (0.12, 0.72, and 0.74 °C in the fish acclimated to 16, 20, and 24 °C, respectively) (**Supplementary Table 4**). In the gill samples collected from this experiment after $CT_{max}$ and analyzed in the present study, the *hsp* genes exhibited the highest upregulation in both lake sturgeon populations (**Figure 2.1a; 2.2a**). The *hsp70a* gene showed marked upregulation following $CT_{max}$ across all

acclimation temperatures in both populations (from 323 to 637-fold change). Similarly, *hsp90a* mRNA expression increased significantly in the gill tissue of Burntwood River sturgeons acclimated to 16 (19-fold), 20 (28-fold), and 24 °C (25-fold). Lake sturgeon samples from the Winnipeg River population also showed significant upregulation of *hsp90a* at 16 (9.5-fold), 20 (7.6-fold), and 24 °C (8.8-fold) after $CT_{max}$. In the Burntwood River population, the mRNA expression of *hspa4a* was significantly upregulated in fish acclimated to 16 (2-fold), 20 (2.7-fold), and 24 °C (2.8-fold) after $CT_{max}$. In the Winnipeg River population, however, *hspa4a* was only significantly upregulated in the fish acclimated to 24 °C (2.87-fold) in response to $CT_{max}$. In contrast to other HSPs, the mRNA expression of *hspa8* was significantly downregulated after $CT_{max}$ in the Winnipeg River population acclimated to 20 °C (0.43-fold). Finally, in the Winnipeg River population, the gene coding for cold-inducible RNA binding protein (CIRBP) showed significant downregulation in all three acclimation temperatures (0.35, 0.36, and 0.45-fold at 16, 20, and 24 °C, respectively) after $CT_{max}$, but no significant change was observed in the Burntwood River population.



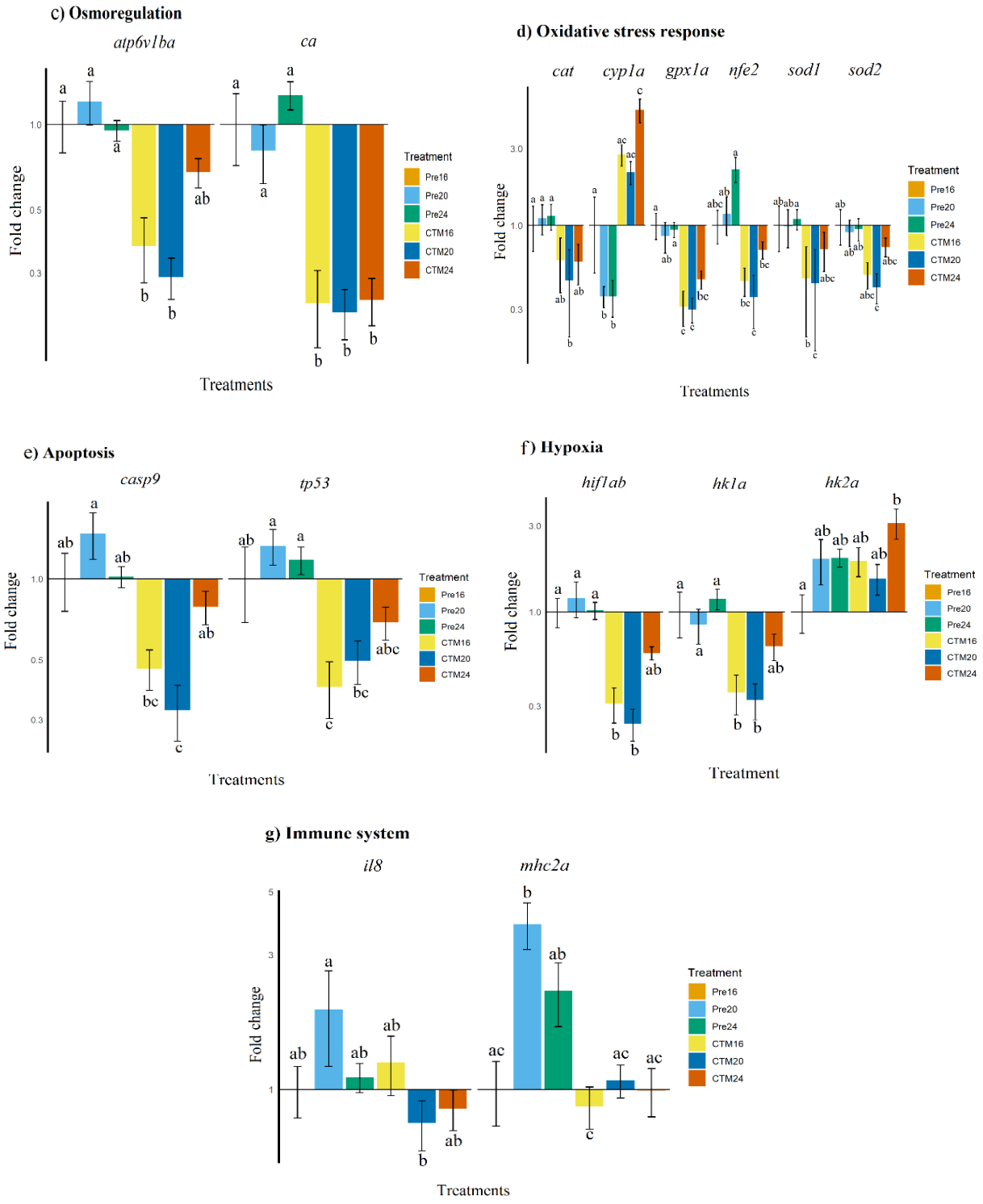
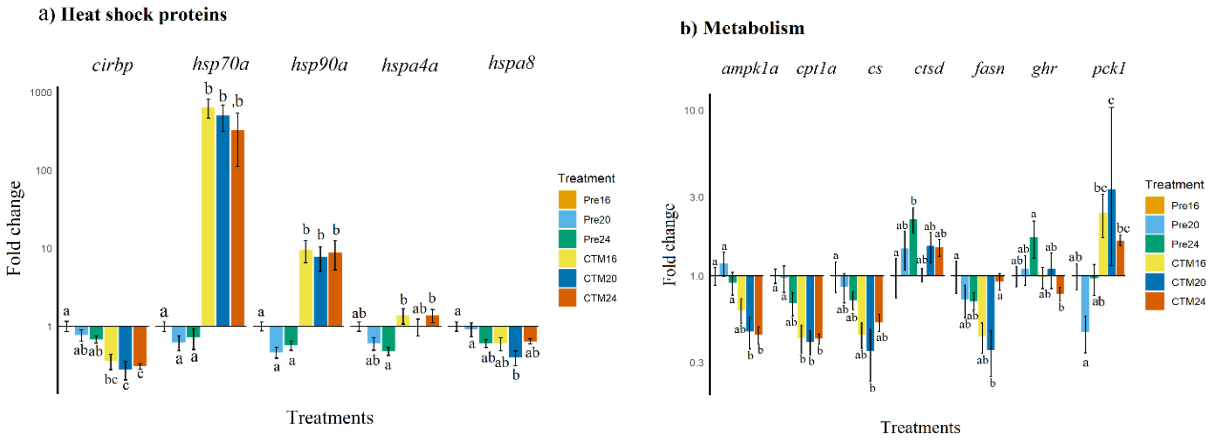



**Figure 2.1** The relative mRNA expression of genes with significant differences between groups in the Burntwood River lake sturgeon (*Acipenser fulvescens*) population that were acclimated to 16, 20, and 24 °C. A subset of those fish was then exposed to a $CT_{max}$ trial. Genes involved in different biological processes are presented in different panels. The mRNA expression of each

gene is relative to the 16 °C acclimation temperature, and groups that do not share a common letter are significantly different from each other. A significant difference between groups was measured by the two-way ANOVA. For better representation, a logarithmic scale has been used on the y-axis. Error bars show standard errors.

Genes involved in energy metabolism were also affected by acute thermal stress, but the response varied by population and acclimation temperature (**Figure 2.1b; 2.2b**). In the Burntwood River population, after $CT_{max}$, the *pck1* gene was upregulated in all three acclimation temperatures (3.2, 6.1, and 1.64-fold in 16, 20, and 24 °C, respectively) in response to $CT_{max}$, but this upregulation was only statistically significant at 16 and 20 °C acclimation temperatures. The upregulation of this gene in the Winnipeg River population after $CT_{max}$ was only significant in fish acclimated to 20 °C (7.3-fold). In contrast, $CT_{max}$ significantly downregulated the mRNA expression of *ampk1a* (0.38 and 0.48 folds in 20 and 24 °C, respectively), *cpt1a* (0.42 and 0.41 folds in 16 and 20°C, respectively), and *ghr* (0.45-fold in 24 °C) in the Winnipeg River population. The mRNA expression of *ctsd* did not show a significant change in expression after $CT_{max}$, but acclimation to 24 °C significantly increased (2.1-fold) the mRNA expression of this gene in the Winnipeg River population compared to 16 °C acclimation temperature.



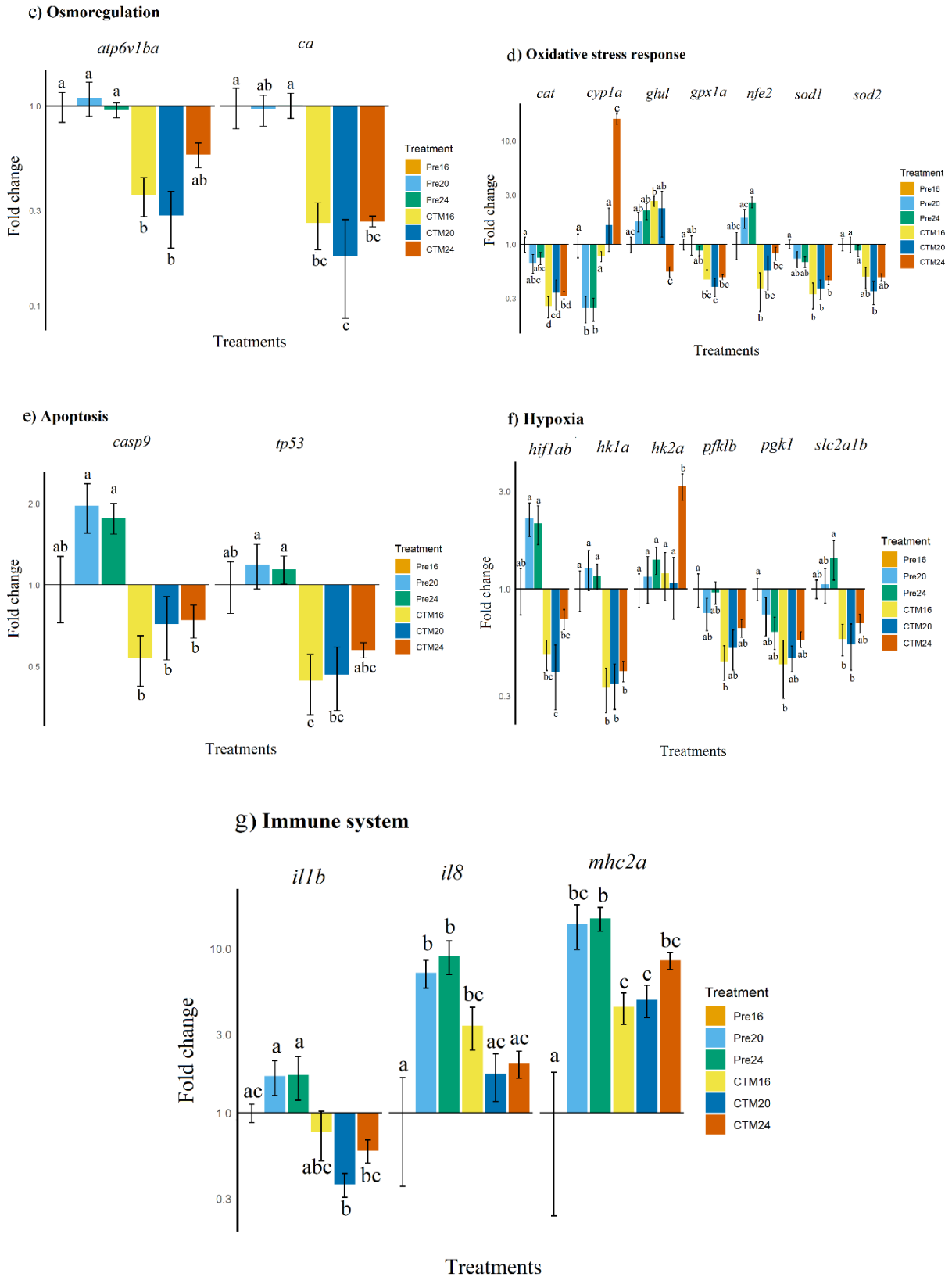

c) Osmoregulation
atp6v1ba
ca
d) Oxidative stress response
cat
cyp1a
glul
gpx1a
nfe2
sod1
sod2
e) Apoptosis
casp9
tp53
f) Hypoxia
hif1ab
hk1a
hk2a
pfklb
pgk1
slc2a1b
g) Immune system
il1b
il8
mhc2a
Fold change
Treatments
Treatment
Pre16
Pre20
Pre24
CTM16
CTM20
CTM24

**Figure 2.2** The relative mRNA expression of genes with significant differences between groups in the Winnipeg River lake sturgeon (*Acipenser fulvescens*) population that were acclimated to 16, 20, and 24 °C. A subset of those fish was then exposed to a $CT_{max}$ trial. Genes involved in different biological processes are presented in different panels. The mRNA expression of each gene is relative to the 16 °C acclimation temperature, and groups that do not share a common letter are significantly different from each other. A significant difference between groups was measured by the two-way ANOVA. For better representation, a logarithmic scale has been used on the y-axis. Error bars show standard errors.

Genes involved in osmoregulation also showed significant change in mRNA expression in both populations of lake sturgeon following $CT_{max}$ (**Figure 2.1c; 2.2c**). The mRNA expression of *ca* after $CT_{max}$ was significantly downregulated in both populations of lake sturgeons acclimated to 16, 20, and 24 °C. This downregulation was between 0.18 and 0.26-fold in both populations, with no significant difference between acclimation temperatures. Similarly, *atp6v1ba* mRNA expression after $CT_{max}$ was significantly downregulated in both populations of lake sturgeons, but the downregulation was only statistically significant in fish acclimated to 16 and 20 °C. This downregulation was 0.39 and 0.24-fold in the Burntwood population, and 0.35 and 0.25-fold in the Winnipeg River population acclimated to 16 and 20 °C, respectively.

Acute thermal stress affected the mRNA expression of genes involved in oxidative stress response and xenobiotic metabolism (**Figure 2.1d; 2.2d**). Acclimation to 20 and 24 °C for 30 days significantly decreased the mRNA expression of *cyp1a* in both populations of lake sturgeon compared to 16 °C acclimation temperature (0.36-fold in the Burntwood River population and 0.24-fold in the Winnipeg population). However, $CT_{max}$ significantly increased the mRNA expression of this gene in fish acclimated to 20 °C (5-fold in Burntwood River and 6.3-fold in Winnipeg River) and 24 °C (14-fold in Burntwood River and 68-fold in Winnipeg River). The mRNA expression of *nfe2*, a master regulator of antioxidant gene expression, was downregulated after $CT_{max}$ in both populations of lake sturgeons in this study, but this downregulation was only statistically significant in the lake sturgeon from the Burntwood River population acclimated to 20 (0.3-fold) and 24 °C (0.34-fold), and the Winnipeg River population acclimated to 24 °C (0.31-fold). Among the antioxidant genes, *gpx1a* was significantly downregulated after $CT_{max}$ in the Burntwood River population acclimated to 16 (0.31-fold), 20 °C (0.34-fold), and 24 °C (0.48-fold). This gene was also downregulated in the Winnipeg River population acclimated to

16 °C (0.45-fold) and 20 °C (0.39-fold). In the genes coding for superoxide dismutase, the mRNA expression of *sod1* was significantly downregulated after $CT_{max}$ in the Burntwood River acclimated to 16 °C (0.46-fold) and 20 °C (0.44-fold), and in the Winnipeg River populations acclimated to 16 °C (0.32-fold). The mRNA expression of *sod2* was also downregulated after $CT_{max}$ in the Burntwood River (0.45-fold) and Winnipeg River fish (0.34-fold) acclimated to 20 °C. The mRNA expression of *cat* was downregulated in both populations after $CT_{max}$, but it was only statistically significant in the Burntwood River population acclimated to 20 °C (0.40-fold) and the Winnipeg River population acclimated to 16 °C (0.25-fold).

Genes involved in the regulation of apoptosis were also downregulated in response to acute thermal stress. The mRNA expression of *casp9* was significantly downregulated in the Burntwood River population acclimated to 20 °C (0.22-fold), and the Winnipeg River population acclimated to 20 °C (0.36-fold) and 24 °C (0.41-fold). Acclimation to 16 and 20 °C also significantly downregulated the mRNA expression of *tp53* in both sturgeon populations (0.37-0.39-fold in Burntwood River and 0.39-0.44-fold in Winnipeg River).

The mRNA expression of *hif1ab* was significantly downregulated in the Burntwood River population acclimated to 16 °C (0.3-fold) and 20 °C (0.2-fold), and the Winnipeg River population acclimated to 20 °C (0.17-fold). In addition, genes involved in glycolysis were mostly downregulated following acute thermal stress (**Figure 2.3; 2.4**). The only gene that was upregulated after $CT_{max}$ was *hk2a*; however, the upregulation was only significant in the Winnipeg River population acclimated to 24 °C (2.3-fold). In contrast, the mRNA expression of *hk1a* was significantly downregulated in the Burntwood River population acclimated to 16 °C (0.35-fold) and 20 °C (0.38-fold), and Winnipeg River acclimated to 16 °C (0.32-fold), 20 °C (0.26-fold), and 24 °C (0.34-fold). In the Winnipeg River population, the mRNA expression of *pgk1* and *pfklb* was significantly downregulated in fish acclimated to 20 °C (0.61 and 0.67-fold in *pgk1* and *pfklb*, respectively).

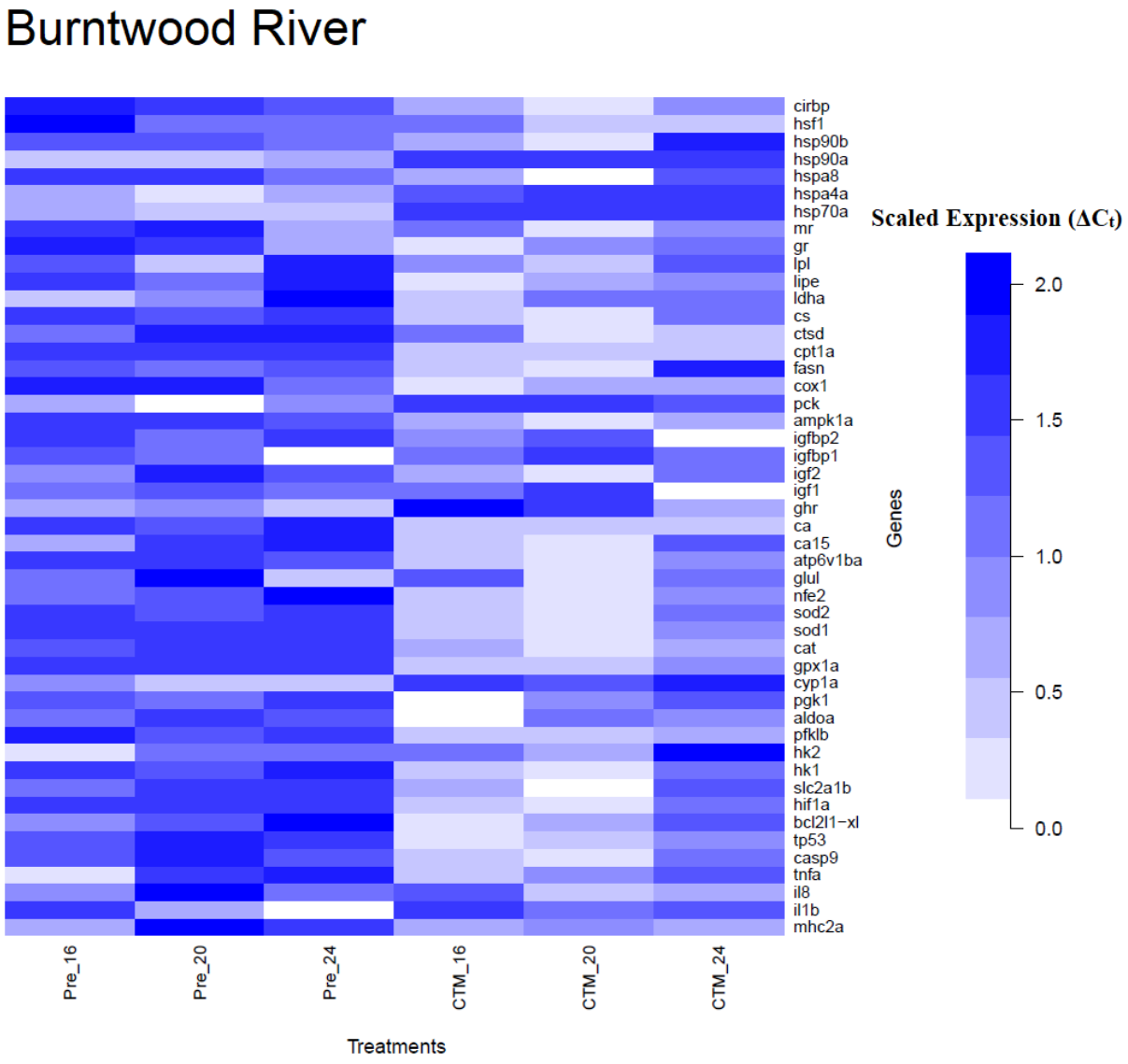



**Figure 2.3** The mRNA expression pattern of 52 genes measured in lake sturgeons (*Acipenser fulvescens*) from the Burntwood River population in response to acclimation temperatures and acute thermal stress. Juvenile lake sturgeons were acclimated to 16, 20, and 24 °C for 30 days, and then a subset of them experienced $CT_{max}$. To generate this plot, the mRNA expression of each gene in the gill is measured using qPCR, and $C_t$ values are normalized to the mean $C_t$ value of four control genes and scaled for better representation.

The effects of acute thermal stress on the immune system were more pronounced in the mRNA expression of proinflammatory genes. In the Burntwood River population, *il8* mRNA expression was significantly decreased (0.39 fold) after $CT_{max}$ in the fish acclimated to 20 °C. Acclimation to 20 and 24 °C for 30 days significantly increased the mRNA expression of *il8* in the Winnipeg River population compared to 16 °C (7-9 fold), but the mRNA expression of this gene was downregulated (0.22-0.23 fold) in these two acclimation temperatures after $CT_{max}$, in contrast with 16 °C acclimation temperature, which showed 3.3 fold upregulation. The mRNA expression of *il1b* also showed significant downregulation in the Winnipeg River sturgeons acclimated to 20 °C (0.21-fold) and 24 °C (0.34-fold). Additionally, *mhc2a* mRNA expression pattern was different between the two populations. The mRNA expression of this gene increased by increasing the acclimation temperature from 16 °C to 20 °C (3.84-fold) in the Burntwood River population, and from 16 °C to 20 °C (14-fold) and 24 °C (15-fold) in the Winnipeg River population. However, the mRNA expression of *mhc2a* had a different pattern after $CT_{max}$. It showed significant upregulation in the Winnipeg River population acclimated to 16 °C (4.4-fold), but significant downregulation in the Burntwood River population acclimated to 20 °C (0.27-fold).

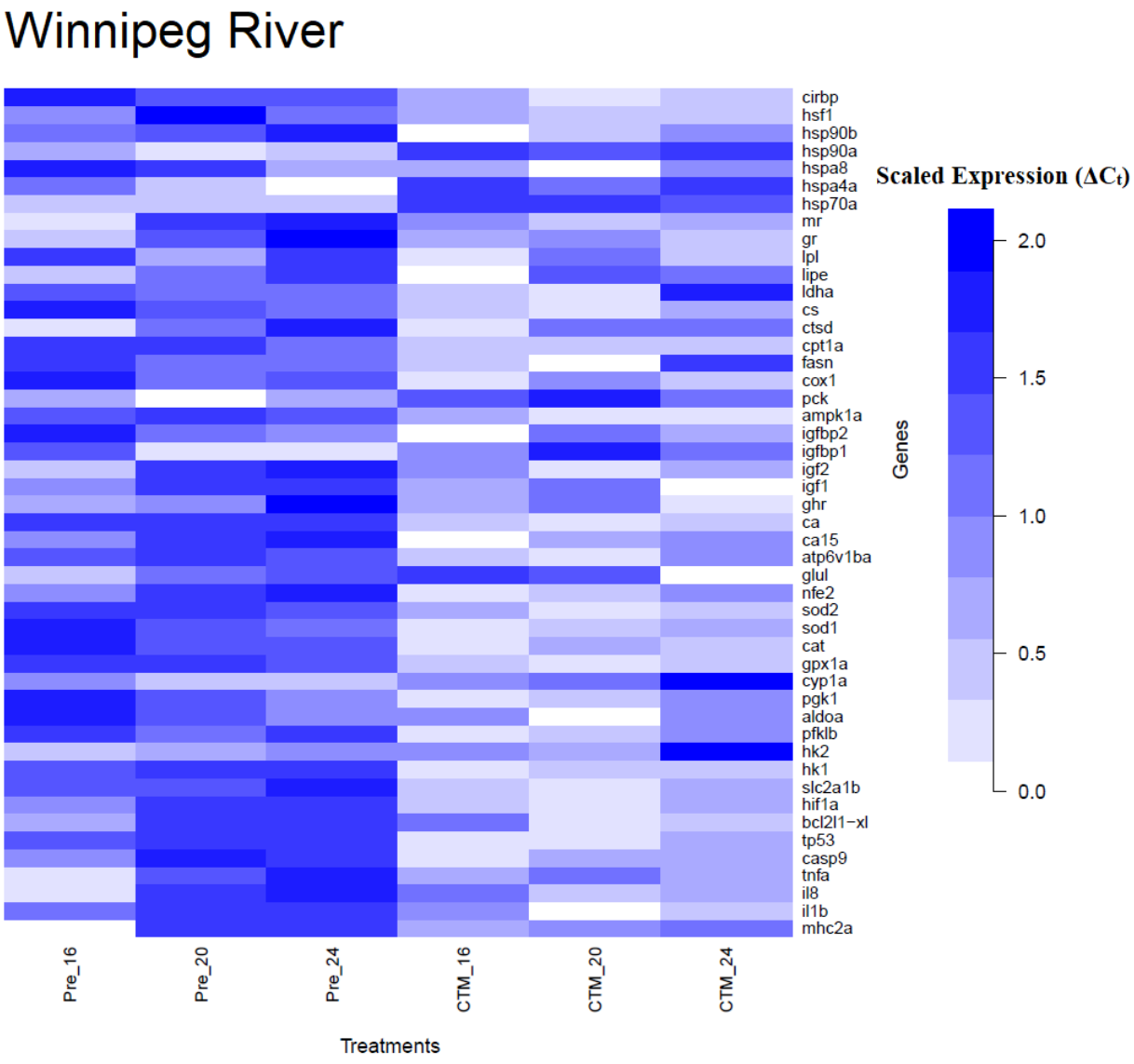


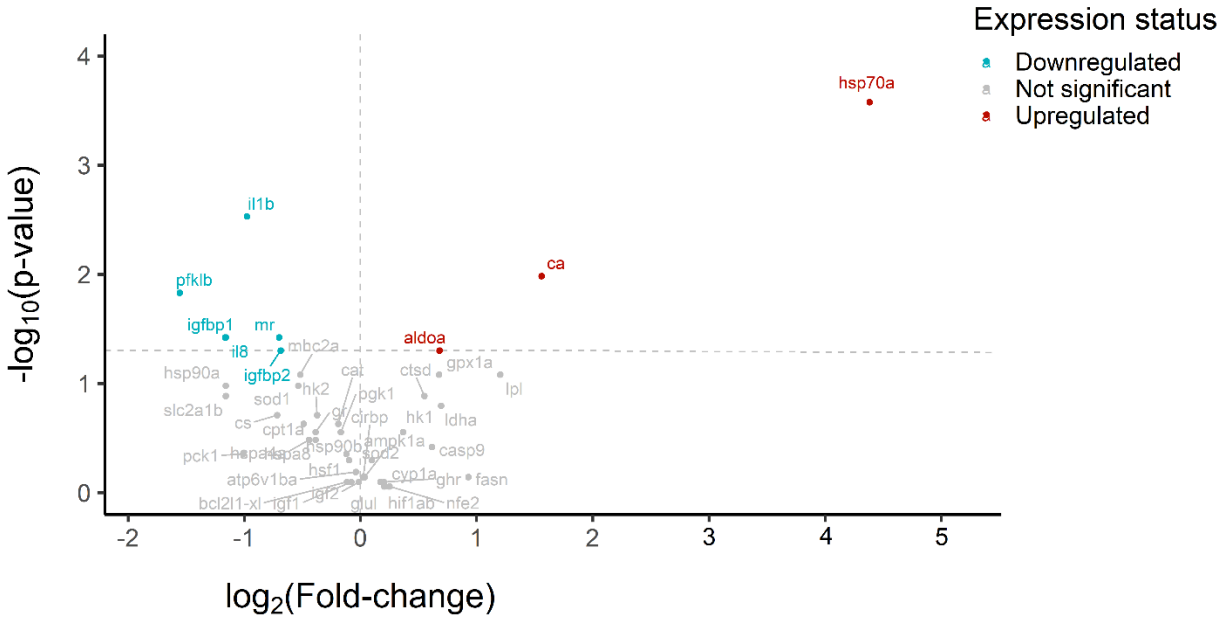

**Figure 2.4** The mRNA expression pattern of 52 genes measured in lake sturgeon (*Acipenser fulvescens*) from the Winnipeg River population in response to acclimation temperatures and acute thermal stress. Juvenile lake sturgeons were acclimated to 16, 20, and 24 °C for 30 days, and then a subset of them experienced $CT_{max}$. To generate this plot, the mRNA expression of each gene in the gill is measured using qPCR, and $C_t$ values are normalized to the mean $C_t$ value of four control genes and scaled for better representation.

## 2.4.2. Atlantic and shortnose sturgeon

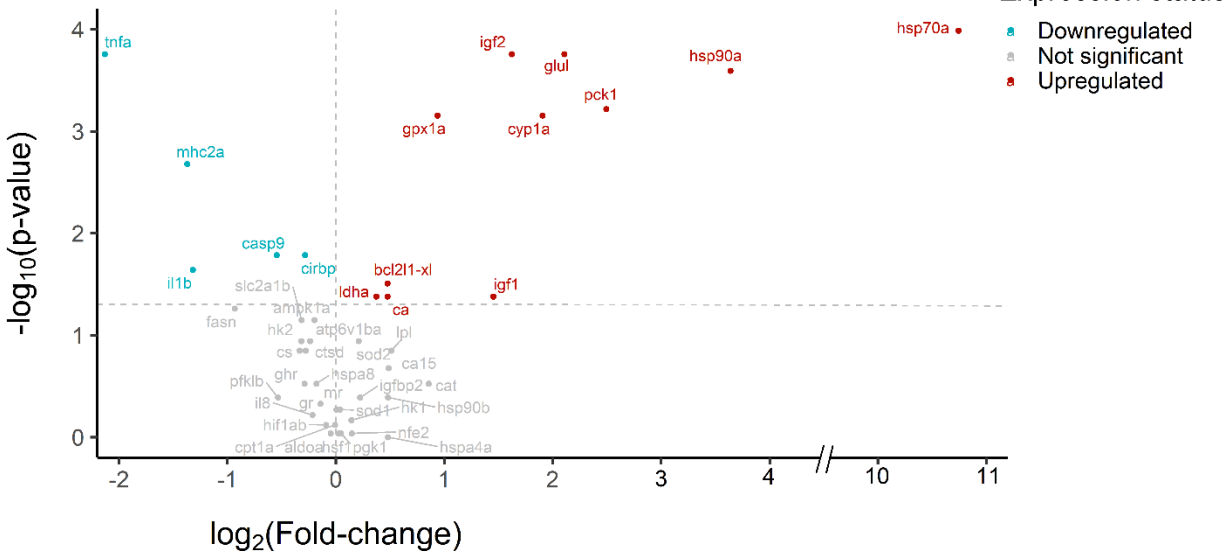
The $CT_{max}$ values measured by Penny et al. (2023) in Atlantic and shortnose sturgeon showed slightly higher (0.65 °C) thermotolerance in Atlantic sturgeon (**Supplementary Table 4**). mRNA expression analysis in the samples collected from this experiment before and after $CT_{max}$ showed that acute thermal stress significantly upregulated the mRNA expression of *hsp70a* in both gill (1476 to 321-fold-changes, respectively) and liver (17 to 73-fold-changes, respectively) tissue of Atlantic and shortnose sturgeon (**Figure 2.5**). Similarly, *hsp90a* was upregulated in the gill tissue of both species (12.5-12-fold) and the liver tissue of shortnose sturgeon (73-fold). In addition, *hspa4a* expression increased in the gill tissue of shortnose sturgeon (2-fold). Following $CT_{max}$, *cirbp* expression was downregulated in the gill tissue of Atlantic sturgeon (0.8-fold), but upregulated in the liver tissue of the shortnose sturgeon (1.3-fold).

In the Atlantic sturgeon, acute thermal stress significantly downregulated *igfbp1* (0.44-fold) and *igfbp2* (0.62-fold) in the liver, while upregulating *igf1* (2.7-fold), *igf2* (3-fold), *ldha* (1.29-fold), and *pck1* (6.9-fold) in the gill (**Figure 2.5a; 2.5b**). In shortnose sturgeon, acute thermal stress significantly increased the mRNA expression of *cpt1a* (1.29-fold), *pck1* (8-fold), *igf2* (1.74-fold), and *igfbp2* (1.38-fold) in the gill, as well as *ampk1a* (1.91-fold), *ctsd* (1.92-fold) in the liver (**Figure 2.5c; 2.5d**). In Atlantic sturgeon, *ca* was significantly upregulated in both gill (1.39-fold) and liver (2.95-fold) tissue. In shortnose sturgeon, *atp6v1ba* expression was significantly increased (1.66-fold) in the liver (**Figure 2.5c**). No osmoregulatory genes showed significant changes in mRNA expression in the gill tissue of shortnose sturgeon.

a) Atlantic sturgeon, liver




b) Atlantic sturgeon, gill



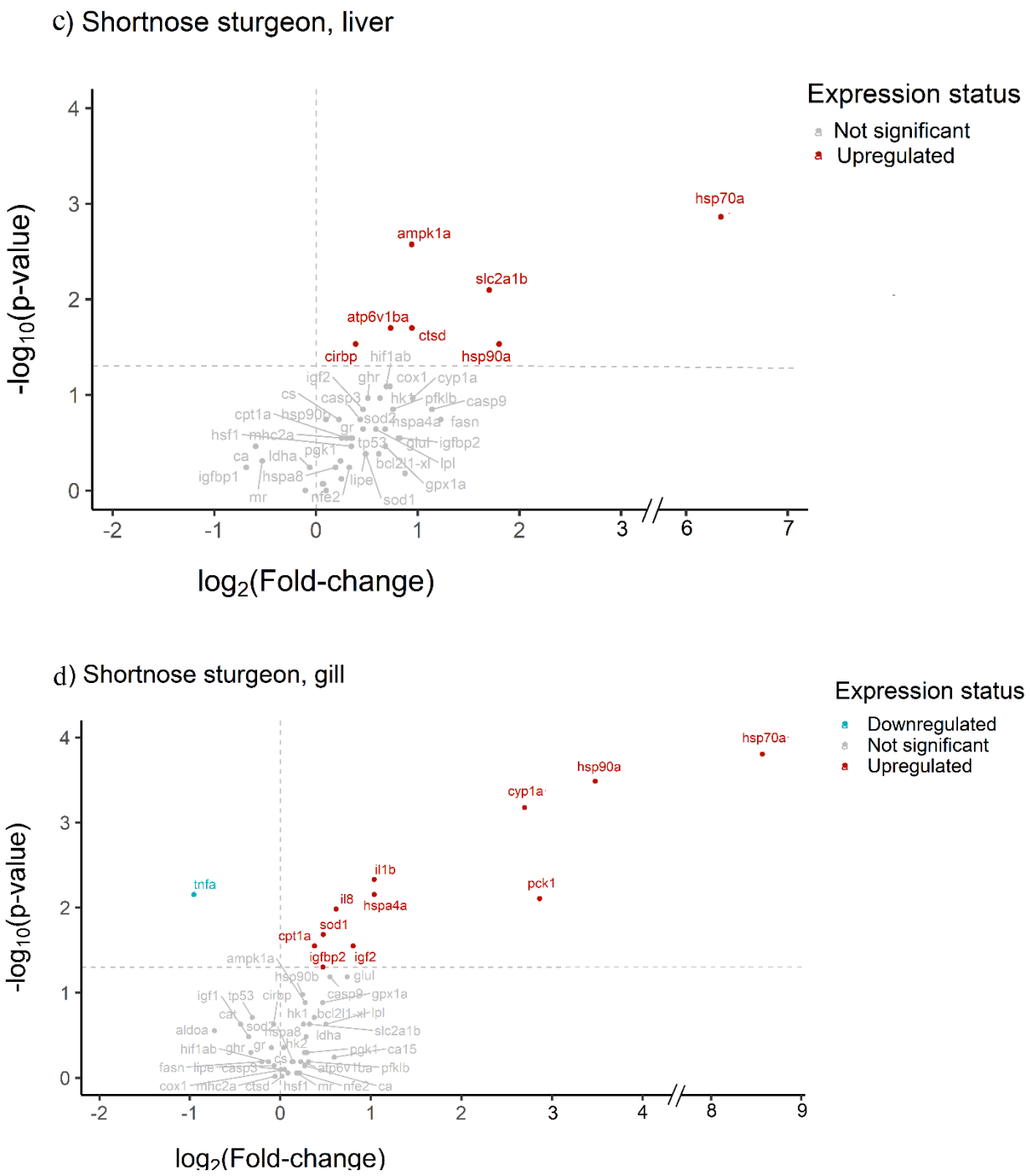



**Figure 2.5** Relative mRNA expression of 52 target genes in the liver and gill of Atlantic and shortnose sturgeon, *Acipenser oxyrinchus oxyrinchus,* and *Acipenser brevirostrum*, in response to $CT_{max}$. Each species was separately acclimated to 10 °C and then experienced $CT_{max}$. The mRNA expression of each gene after $CT_{max}$ is calculated relative to a subset of fish before $CT_{max}$, ie., the Control group. The statistical significance between the Control group and $CT_{max}$ group was determined by the Mann–Whitney U test. For better representation, p-values and fold-

change mRNA expression have been transformed to log10 and log2, respectively. The horizontal dotted line shows a p-value of 0.05, and the vertical dotted line shows a one-time fold-change in mRNA expression.

The effects of acute thermal stress on the mRNA expression of genes associated with xenobiotic metabolism and oxidative stress response were more pronounced in the gill than in the liver (**Figure 2.5**). Similar to observations in lake sturgeon, acute thermal stress increased the mRNA expression of *cyp1a* in the gill tissue of Atlantic (3.75-fold) and shortnose sturgeons (7.26-fold) (**Figure 2.5b; 2.5d**). In Atlantic sturgeon, acute thermal stress significantly upregulated *glul* (4.3-fold) and *gpx1a* (1.9-fold) in the gill, whereas in shortnose sturgeon, only *sod1* (1.38-fold) was upregulated following exposure to $CT_{max}$. In contrast, acute thermal stress did not affect the mRNA expression of genes involved in xenobiotic metabolism and oxidative stress response in the liver tissue of either species (**Figure 2.5a; 2.5c**).

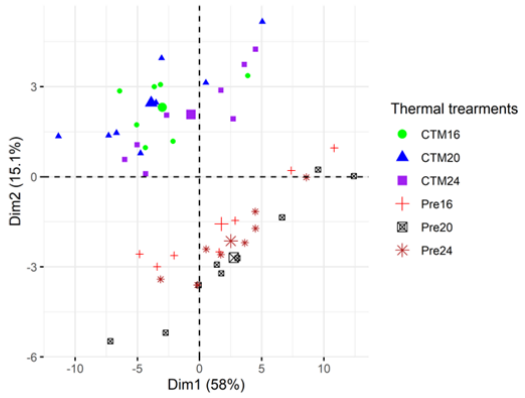
In Atlantic sturgeon, *casp9* mRNA expression in the gill was significantly downregulated after $CT_{max}$ (0.68-fold) (**Figure 2.5b**). Conversely, *bcl2l1-xl* was significantly (1.39-fold) upregulated in the gill in response to acute thermal stress. The mRNA expression of apoptosis-related genes did not show any significant change in the liver of Atlantic sturgeon or in either the gill or liver tissue of shortnose sturgeon (**Figure 2.5a; 2.5c; 2.5d**).

Genes involved in glycolysis and anaerobic metabolism were exclusively upregulated in the liver of Atlantic and shortnose sturgeons. In the Atlantic sturgeon, acute thermal stress significantly upregulated (1.6-fold) the mRNA expression of *aldoa*, whereas in shortnose sturgeon, *slc2a1b* was significantly (3.25-fold) upregulated (**Figure 2.5a; 2.5c**).

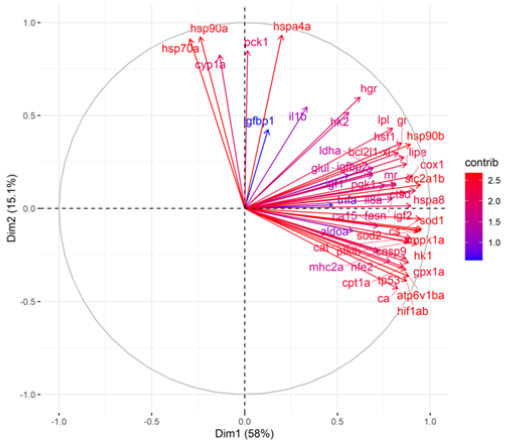
In the gill tissue of Atlantic sturgeon, acute thermal stress significantly downregulated the mRNA expression of the immune genes *tnfa* (0.22-fold), *mhc2a* (0.38-fold), and *il1b* (0.4-fold), while in the liver, *il1b* (0.5-fold) and *il8* (0.44-fold) were downregulated (**Figure 2.5a; 2.5b**). In the shortnose sturgeon, the only immune gene significantly affected by $CT_{max}$ was *tnfa,* which was downregulated (0.51-fold) in the gill (**Figure 2.5c; 2.5d**).

## a) Lake sturgeon, Burntwood River

1) PCA plot

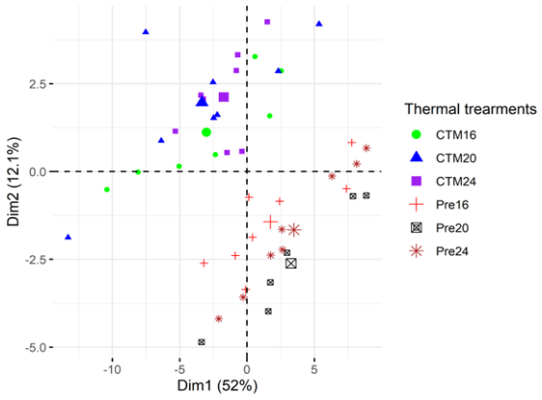



2) PCA loading plot

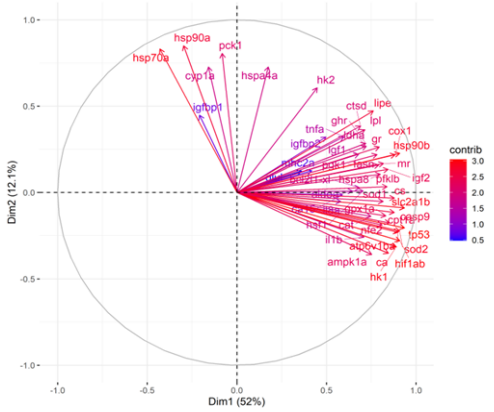



## b) Lake sturgeon, Winnipeg River

1) PCA plot

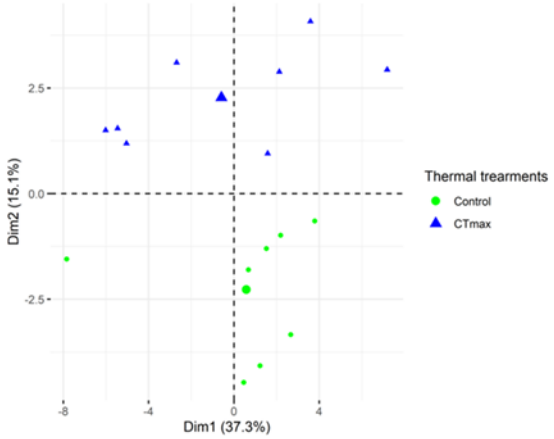



2) PCA loading plot

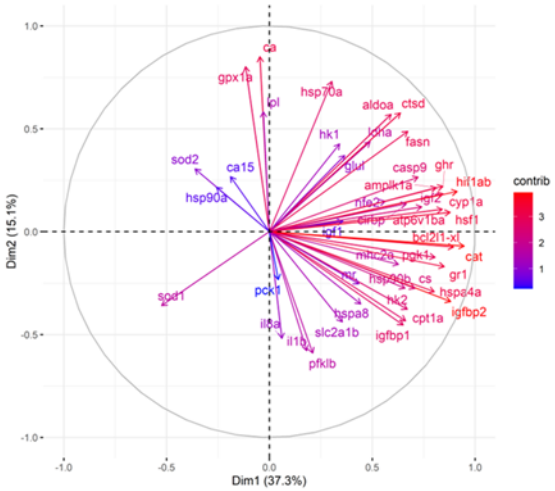


## c) Atlantic sturgeon, Liver

1) PCA plot

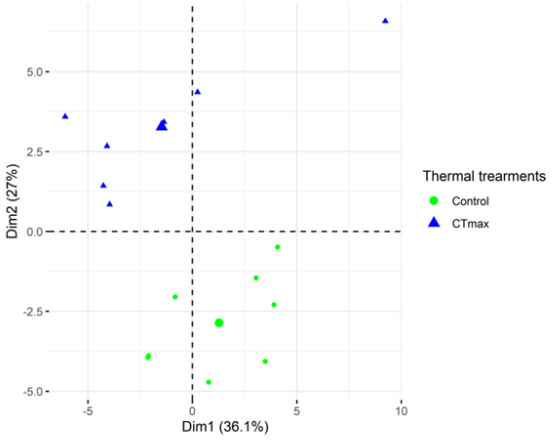



2) PCA loading plot

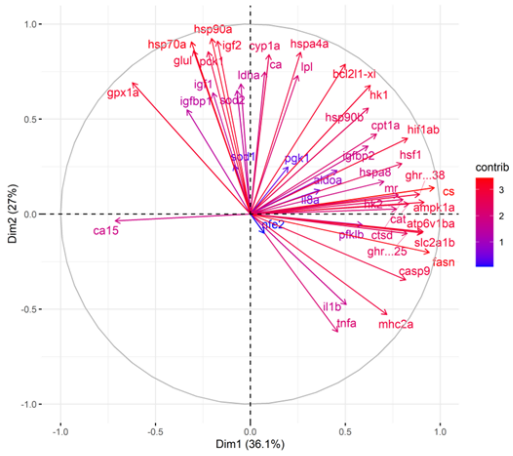



## d) Atlantic sturgeon, Gill

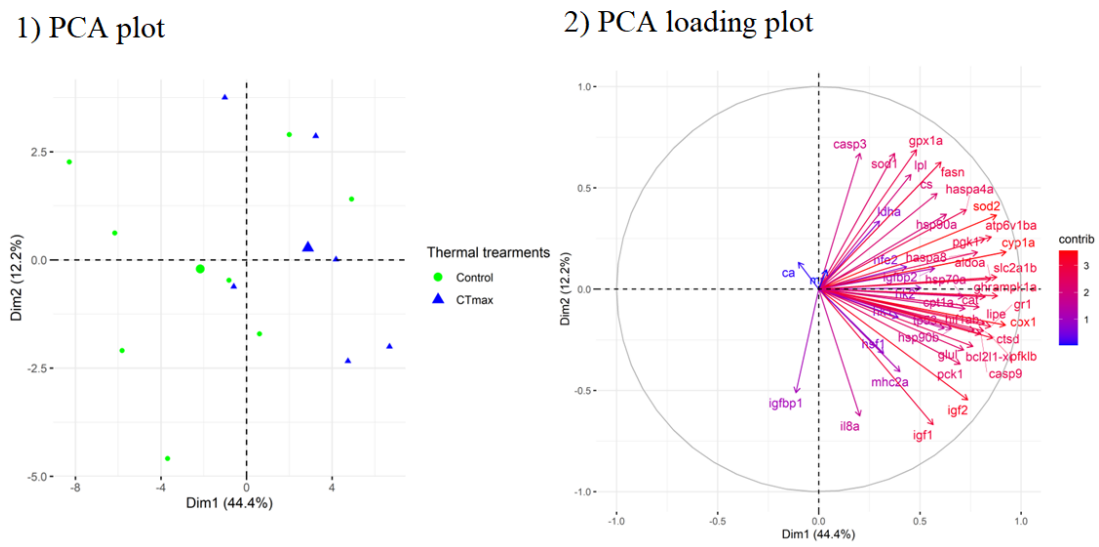
1) PCA plot




2) PCA loading plot



e) Shortnose sturgeon, Liver




f) Shortnose sturgeon, Gill

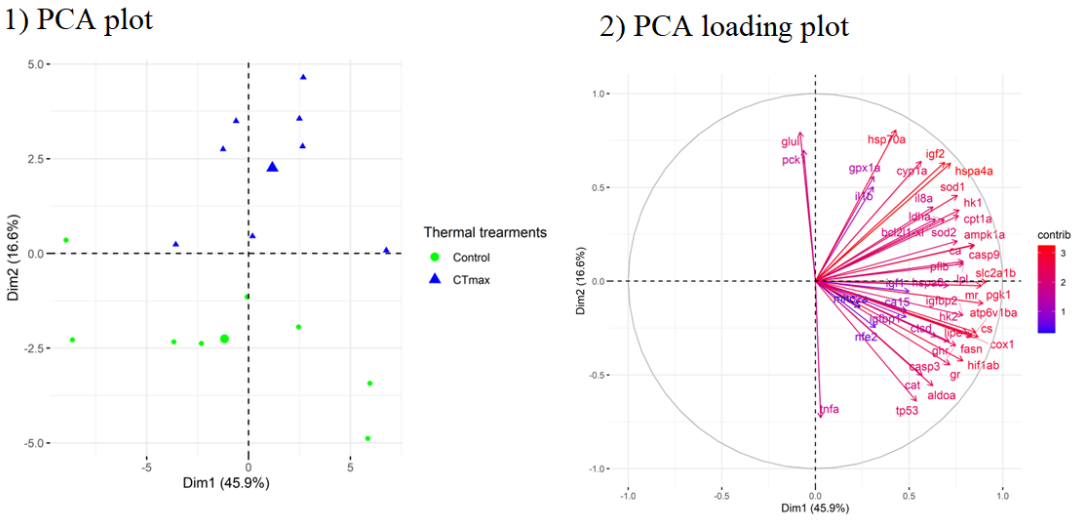



**Figure 2.6** Plots from Principal Component Analysis showing the overall similarity in the mRNA expression pattern of 52 genes studied in three *Acipenser* species, and Loading score plots showing the contribution and direction of each gene in PC1 (Dim1) and PC2 (Dim2) in each species. Lake sturgeon (*Acipenser fulvescens*) were acclimated to 16, 20, and 24 °C and then experienced $CT_{max}$. Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*) and shortnose

sturgeon (*Acipenser brevirostrum*) were acclimated to 10 °C before $CT_{max}$. The mRNA expression of each gene was measured in the samples collected before and after $CT_{max}$ using qPCR. To generate the PCA plots, the $C_t$ value of each gene was normalized to the mean $C_t$ value of four control genes to generate unsupervised PCA plots.

### 2.4.3 Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was used to investigate the overall changes in all the mRNA expression assayed in response to acute thermal stress and determine the main drivers of differences in mRNA expression patterns. Principal Component Analysis revealed that acute thermal stress altered overall mRNA expression patterns across all three species and both tissues examined (**Figure 2.6**). In both lake sturgeon populations, acclimating to different temperatures changed the mRNA expression pattern of 48 genes before and after acute thermal stress (**Figure 2.6a1; 2.6b1**). Dimension 1 accounted for about 36.1-58 % variation among samples in all PCA plots (**Figure 2.6**). The contribution of genes in the second dimension was aligned more closely with the differential mRNA expression between temperature treatments in the gill tissues of lake sturgeon, the liver and gill tissue of Atlantic sturgeon, and the gill tissue of shortnose sturgeon (**Figure 2.6a2; 2.6b2; 2.6c2; 2.6d2; 2.6f2**). In contrast, in the liver tissue of shortnose sturgeon, the contribution of genes in Dimension 1 more closely reflected the differential mRNA expression pattern between treatments (**Figure 2.6e**).

## 2.5 Discussion

The multispecies *Acipenser* OpenArray™ chip developed and presented in this study was able to amplify a wide selection of genes across multiple functional categories in three sturgeon species. Relative mRNA expression analysis showed that acute thermal stress changed the mRNA expression pattern of genes in the immune system, metabolism, osmoregulation, response to hypoxia, apoptosis, general stress response, and oxidative stress response. This mRNA expression pattern was affected by acclimation temperature, species, and tissues under study. Acute thermal stress affected the mRNA expression of 17-26 of the thermal metabolic stress

genes that were assayed using the chip, depending on the species. Based on assessments of the assay efficiency, 52 genes in the gill tissue of lake sturgeon, 48 genes in the gill and liver of Atlantic sturgeon, 53 genes in the gill, and 52 genes in the liver of shortnose sturgeon were suitable for relative expression analysis. Across tissues and species, 46 of these genes were consistently suitable for analysis and can be readily applied to assess stressors for these sturgeon species. Future versions of this *Acipenser* chip will then need to redesign the assays with poor efficiency until there is a full suite of 56 assays that work across each species and tissue.

### 2.5.1 Heat shock response and aerobic metabolism genes

Heat shock proteins are well-described markers of cellular stress, and their production is induced by a variety of factors, including acute thermal stress (Sharp et al., 1999). Among the HSPs studied, *hsp70a* and *hsp90a* were significantly upregulated in response to acute thermal stress in all the species and tissues, except in the liver of the Atlantic sturgeon, where *hsp90a* was downregulated. Upregulation of *hsp70a* and *hsp90a* is a common response to temperature and has been reported in several species (Shin et al., 2018; Sun et al., 2021; Yan et al., 2017). These two protein chaperones have been known to cooperate with the assistance of another protein called Hsp70/Hsp90 organizing protein, HOP, to regulate the folding of proteins such as hormone receptors (Odunuga et al., 2004). The upregulation of these two proteins has also been reported in response to increased temperature in sturgeons (Luo et al., 2022; Chen et al., 2023; Simide et al., 2016; Bugg et al., 2020; Penny et al., 2023); however, this upregulation was not significant in the muscle of shortnose sturgeon (Penny et al., 2023). Both *hsp70* and *hsp90* are inducible heat shock proteins, and their rapid upregulation has been reported in response to protein denaturation under thermal stress (Shin et al., 2018; Sun et al., 2021; Yan et al., 2017; Chen et al., 2005; Nguyen et al., 2021). Thus, the upregulation of these genes after $CT_{max}$ may be an indicator of increased misfolded or damaged proteins in the liver and gill tissue of sturgeons in our study. However, the consistency in upregulation of *hsp70* and *hsp90a* in response to acute thermal stress across species and tissues makes them useful biomarkers for the detection of thermal stress in *Acipenser* sturgeons.

Genes such as *hk1, pgk1*, and *slc2a1b,* which are involved in the transportation of glucose into the cell and glycolysis (Augustin, 2010; Chandel, 2021), were downregulated in the gill tissue of the lake sturgeon in response to acute thermal stress. Downregulation of these genes may be a sign of decreased glycolysis. In addition, *pck1*, which plays a critical regulatory role in gluconeogenesis (Yu et al., 2021), was upregulated in both populations of lake sturgeons. This upregulation in *pck1* is also detected in the gill tissue of Atlantic and shortnose sturgeon, although genes involved in glycolysis were not downregulated. In the gill tissue of Atlantic sturgeon, the mRNA expression of *ldha* was also upregulated. The upregulation of *ldha* has been reported in other fish species in response to thermal stress (Cheng et al., 2018). Upregulation of *ldha* along with *pck1* may suggest that anaerobic glycolysis and gluconeogenesis are mechanisms induced simultaneously in response to acute thermal stress. However, in the gill tissue of shortnose sturgeon, *cpt1a* was upregulated. The protein coded by *cpt1a* is a key rate-limiting enzyme in fatty-acid oxidation. (Schlaepfer and Joshi, 2020). Increased mRNA expression of *cpt1a* can be an indicator of the higher mitochondrial oxidation of long-chain fatty acids. Overall, changes in the mRNA expression pattern of genes involved in metabolism show that acute thermal stress can cause depletion of energy resources, such as cellular glucose, by shifting to less-efficient ATP production pathways and anaerobic metabolism. This depletion of glucose can eventually result in the usage of alternative energy resources, such as lipids, and the production of glucose from non-carbohydrate precursors.

The mRNA expression of *ampk1a* was upregulated in the liver of shortnose sturgeon. Because *ampk1a* works as an energy sensor in the cell and is activated by an increase in the ratio of AMP/ATP and/or ADP/ATP (Ke et al., 2018), its upregulation might be a sign of decreased production, or increased demand, for ATP during acute thermal stress. This upregulation was paralleled by an upregulation of *slc2a1b* and *ctsd,* which might be a cellular strategy to increase the import of glucose and the breakdown of proteins for energy production. Glucose is often stored as glycogen in the liver and can be released (Adeva-Andany et al., 2016) by glycogenolysis following hormonal stimulation under stressful conditions (Rabasa and Dickson, 2016). A decrease in the glycogen level in the liver, along with an increase in ATPase level, has been reported in aquatic species exposed to thermal stress (Han et al., 2023). In this study, these responses to acute thermal stress suggest an increase in the demand for ATP in shortnose

sturgeon's liver, which upregulates *ampk1a* to provide cells with energy-storing molecules by breaking down proteins, transporting glucose, and glycogenolysis.

### 2.5.2 Oxidative stress response and apoptosis

An increase in the mRNA expression and activity of genes with antioxidant properties, such as *gpx*, *sod*, and *cat*, has been observed in several fishes following thermal stress (Roychowdhury et al., 2021; Liu et al., 2024). In the present study, the mRNA expression of *cyp1a* was upregulated in the gill tissue of all three species after $CT_{max}$. This gene is important in the metabolism of polyaromatic hydrocarbons and is usually upregulated by activating the Aryl hydrocarbon receptors (Ah-R) (Collier and Pritsos, 2003); however, it is also involved in the production of anti-inflammatory molecules such as epoxyeicosatrienoic acid and helping to maintain normal physiological function through cell signaling and immunity (Stading et al., 2020). Upregulation of pro-inflammatory cytokines, such as interleukins, was observed in this research and also in similar studies (Liu et al., 2022; Zhao et al., 2024; Pérez-Casanova et al., 2008; Guo et al., 2023), which might be triggered by upregulation of *cyp1a*.

Although *glul* was upregulated in the gill tissue of Atlantic sturgeon, it showed significant downregulation in the gill tissue of Winnipeg River lake sturgeon acclimated to 24 °C. Glutamate-ammonia ligase, the protein coded by *glul*, is a glutamine synthetase important in detoxifying ammonia (Marginean et al., 2013; Ip and Chew, 2010). The upregulation of glutamine synthetases has been reported in response to increased ammonia concentrations in aquatic animals (Liu et al., 2014; Banerjee et al., 2018). Because increased temperature can increase the production and excretion of ammonia in fish (Zheng et al., 2008; Kieffer and Wakefield, 2009), upregulation of this gene might be a detoxification response to increased production and release of ammonia in Atlantic sturgeon. Downregulation of other oxidative stress response genes, such as *cat*, *gpx1a*, *nfe2*, *sod1*, and *sod2,* in the gill tissue of the Winnipeg River lake sturgeon may be an indicator of decreased detoxification potential of those cells under acute thermal stress. However, the mRNA expression of *gpx1a* and *sod1* was upregulated in the gill tissue of the Atlantic sturgeon and shortnose sturgeon, respectively. This increase might be a response to an increase in the production of hydrogen peroxide or superoxide radicals, which

have been reported in a variety of fish species (Chen et al., 2021; Liu et al., 2022; Yu et al., 2017; Dash et al., 2023). The mRNA expression of oxidative stress response genes did not show significant changes in the liver, showing the lower sensitivity of the oxidative stress response of this organ to acute thermal stress or lower production of ROS.

In lake sturgeon, acute thermal stress significantly downregulated the mRNA expression of pro-apoptotic genes *casp9* and *tp53,* although acclimation to higher temperatures resulted in a moderate upregulation of these genes after 30 days. Significant downregulation of *casp9* and upregulation of *bcl2l1-xl* were also observed in the gill tissue of Atlantic sturgeon. The protein coded by *casp9* has an important role in activating *tp53*-dependent apoptosis (Wu and Ding, 2002). However, *bcl2l1-xl* is an antiapoptotic protein that aids in cellular survival by regulating reactive oxygen species production (Loo et al., 2020). The downregulation of *casp9* and *tp53*, and the upregulation of *bcl2l1-xl* may be signs of decreased apoptosis during acute thermal stress.

### 2.5.3 Immune response genes

Acclimation to higher temperatures has been reported to reduce the immune system response to pathogens in lake sturgeon (Bugg et al., 2023). However, acclimation to higher temperatures increased the mRNA expression of *mhc2a* in both populations of lake sturgeon, although this increase only happened at 20 °C acclimation temperature for the Burntwood River population. MHC class II is usually produced in the thymic epithelial cells, or antigen-presenting cells, and other cells do not produce this protein unless they are stimulated by *ifng* caused by infection, inflammation, or trauma (Reith et al., 2005). Similarly, *il8* also showed an upregulation pattern similar to *mhc2a* in both populations. The upregulation of immune response genes such as *myd88*, *c3,* and *lysozyme c* caused by acclimation to the higher temperature (20 °C) has been reported in lake sturgeon; however, fish acclimated to higher temperatures also had lower mRNA expression of *il1b* following exposure to bacterial lipopolysaccharides (LPS) (Bugg et al., 2023b). Additionally, *il1b* showed significant downregulation in the Burntwood River population in response to acclimation to 24 °C. Interleukins are proteins with a pro-inflammatory role in animals (Secombes and Bird, 2011). The significant change in the mRNA

expression of these genes, along with the upregulation of *mhc2a* in the gill, might be a sign of an inflammatory response in gill tissue to acute thermal stress.

Lake sturgeon exposed to acute thermal stress had a downregulation of *il1b* in the Burntwood River population and *il8* in the Winnipeg River population. Population-specific immune responses, especially in the expression of cytokines such as *il1b* have been observed previously in species like the European perch, *Perca fluviatilis*, upon exposure to pathogens (Gebauer et al., 2026). In humans, such differences are believed to be caused by ancestral differences in immune response, especially in susceptibility to inflammation (Nédélec et al., 2016). In addition, the composition and abundance of pathogens can be different in different aquatic ecosystems that may work as a selective force, and this co-evolutionary dynamic between pathogens and hosts can result in local adaptations (Rajkov et al., 2020). Because lake sturgeon from Burntwood River and the Winnipeg River come from two genetically distinct populations, this local adaptation may be responsible for different immune responses to acute thermal stress. Atlantic sturgeon showed significant downregulation of *il1b, tnfa,* and *mhc2a* in the gill, and *il1b* and *il8* in the liver. In shortnose sturgeon, although *tnfa* was significantly downregulated, *il1b* and *il8* were upregulated in the gill, and liver tissue did not show any significant change in the mRNA expression of genes involved in the immune system. Genes such as *il1b*, *il8*, and *tnfa* code for cytokines with important roles in the immune system, especially inflammation (Demir, 2022). These proteins are usually produced by white blood cells or other non-immune cells during inflammation and play their role by mechanisms such as activation of other white blood cells, induction of other cytokines, inhibition of neutrophil adhesion to the vascular endothelium, and stimulation of angiogenesis (Koper-Lenkiewicz et al., 2022). Differential mRNA expression patterns of these genes in sturgeons in this study can be because of different sensitivities of species to inflammation caused by thermal stress.

## 2.6 Conclusion

Together, these molecular responses demonstrate some differentiation in the response to thermal stress across sturgeon species, but also conserved mechanisms employed by diverse sturgeon species and populations to respond to this key stressor. The assays used to measure

these responses are widely applicable across the species assessed here and can be used to evaluate the responses to thermal stress across different biological mechanisms. Particularly, upregulation of HSPs and downregulation of most genes involved in oxidative stress response and apoptosis were observed in all the sturgeons investigated in this study and may potentially be used as biomarkers of thermal stress, especially in gill tissue. Gill-based assays can be used to non-lethally assess the response of sturgeons to acute thermal stress and provide new insights into the effects of this key stressor in future studies or the monitoring of threatened wild populations. In addition, species-specific mRNA expression patterns of genes involved in immune response or metabolism in this study show different physiological responses to thermal stress across different species and populations that can potentially be indicators of different susceptibilities to acute temperature stress, suggesting the need for species-specific considerations when making management decisions.

## 2.8 Acknowledgments

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# Chapter 3 Long-term effects of early-life thermal fluctuations on the cellular stress response and $CT_{max}$ of zebrafish, *Danio rerio*[2]

## 3.1 Abstract

Temperatures in aquatic ecosystems have been affected by anthropogenic activities such as agricultural and industrial water use, and climate change caused by greenhouse gas emissions. Changes in water temperature directly affect the cellular and organismal physiology of fishes because most fishes are ectotherms. These effects can take different forms, such as increased cellular stress and reactive oxygen species (ROS) production. However, the type and magnitude of response depend on the duration and frequency of exposure to elevated water temperature. In this study, we investigated the long-term effects of exposure to daily thermal fluctuation occurring during early-life stages of zebrafish, *Danio rerio,* on mRNA expression and $CT_{max}$ in later developmental stages. To do so, wild-type zebrafish were exposed daily to a + 5 °C fluctuation in temperature from ambient (28 °C) to 33 °C over the first 30 days post fertilization (dpf), before being held until 90 dpf at ambient temperature. The fish that experienced daily thermal fluctuation were compared to a control group that was kept at 28 °C throughout the experiment and sampled at the same timepoints. Samples were collected at 18 (larval), 30, 60 (juvenile), and 90 (adult) dpf to study the mRNA expression of heat shock proteins and oxidative stress genes. The thermotolerance of fish was tested using $CT_{max}$ trials at 60 and 90 dpf. Daily thermal fluctuation over the first 30 dpf led to a significant increase in the mRNA expression of *hsp47*, *gstp1a*, *sod1*, and *sod2* genes at 60 dpf, and *hsp47*, *hsp90aa1*, *hsp90ab1*, *cat*, *glulb*, *gstp1a*, *sod1*, and *sod2* genes at 90 dpf. The only significant increase detected during the larval stage was *glulb* at 18 dpf. Fish that experienced thermal fluctuation also had a higher $CT_{max}$ at 60 dpf, but this increased thermotolerance significantly decreased from 60 to 90 dpf; there was no difference between treatments. Overall, our study demonstrates that early-life thermal fluctuations increased cellular stress responses and thermotolerance in zebrafish as they aged.

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## 3.2 Introduction

Water temperature in freshwater ecosystems often fluctuates on diurnal, seasonal, and annual time scales (Komatsu, 1985; Zhang et al., 2016; Żelazny et al., 2018). Aquatic ectotherms have adapted to tolerate natural fluctuations in water temperature (Goldspink, 1995). However, the temperature of aquatic ecosystems has been influenced by human activities (McBryan et al., 2013). For example, the discharge of warm water from power plants, and agricultural and farming activities have altered the natural thermal regime of aquatic ecosystems, causing fluctuations in water temperature (Bukola et al., 2015; Guimarães, 2023). Further, climate change has also affected the magnitude and intensity of heatwaves in aquatic ecosystems (Woolway et al., 2021; Woolway et al., 2022). High temperatures can directly affect fish populations and, in extreme cases, can result in mortality (Ern et al., 2023).

Most fishes are ectotherms and environmental temperature can directly affect their physiology, metabolism, and behavior (Alfonso et al., 2020). Thermal stress has been reported to trigger an organismal stress response through increased cortisol and glucose levels in the blood (Kim et al., 2019). At the cellular level, the upregulation of genes such as heat shock proteins (HSPs), which code proteins that function as protein chaperones, has also been observed in numerous different species (Komoroske et al., 2015; Jeffries et al., 2018; Kim et al., 2019; Bugg et al., 2020; Mackey et al., 2021; Gavarikar and Craig, 2025). Additionally, thermal stress has also been reported to increase oxidative stress in fishes, which causes oxidation in lipids and other structural molecules (Lushchak and Bagnyukova, 2006). This increase in oxidation of biomolecules can result in the upregulation of the expression of antioxidant proteins that are involved in an oxidative stress response (Madeira et al., 2013). Increased temperature can also affect the susceptibility of fish to xenobiotics and reduce the immune system response to pathogens (Dietrich et al., 2014; Bugg et al., 2023). Increased upregulation of genes coding for apoptosis and proinflammatory proteins, such as IL-7, IL-8, IL-10, and IL-1β, has also been reported following thermal shock (Liu et al., 2022). Collectively, changes in temperature affect the cellular stress response in fish, which can reduce their performance and potentially affect fitness (Jeffries et al., 2018).

In fishes, thermal stress during the early larval life stages can impact metabolism, growth rate, and survival (Pankhurst and Munday, 2011). For instance, increasing temperature in the embryonic stage has been shown to increase the number of abnormal embryos in *Danio rerio,* decrease hatching success in pearl gourami, *Trichogaster leeri,* and accelerate the beginning of endogenous feeding in *blue discus, Symphysodon aequifasciatus* (Schirone and Gross, 1968; Pereira et al., 2016; Mattos et al., 2024). Moreover, exposure to increased temperature in the larval stage has been shown to result in developmental deformities in Indian major carp rohu, *Labeo rohita*, changes in body composition in devil stinger, *Inimicus japonicus,* increased activity of larvae in olive flounder, *Paralichthys olivacous*, and increases in the pelagic larval period and swimming ability in fire clownfish, *Amphiprion melanopus* (Fukuhara, 1990; Green and Fisher, 2004; Wen et al., 2013; Ashaf-Ud-Doulah et al., 2021). Overall, exposure to temperatures higher or lower than the species-specific ideal range can have adverse effects on growth in fish in different life stages and increase morphological abnormalities and mortality (Steinarsson and Björnsson, 1999; Abdel et al., 2004; Le et al., 2011; Tsuji et al., 2014; Shahjahan et al., 2018; Islam et al., 2020). Additionally, temperature strongly influences the developmental rate of fish during early life stages (Kimmel et al., 1995), and their limited thermal tolerance at this sensitive developmental window makes them especially vulnerable to fluctuations in temperature (Dahlke et al., 2025).

Early-life exposure to stress has been reported to have long-lasting effects on animals (Lux, 2018). Although some of these effects can be beneficial and improve animals' resilience to subsequent stressors, they can also have detrimental effects (Cramer et al., 2019). For instance, non-lethal thermal stress during early life has been reported to cause long-lasting epigenetic changes and increase the oxidative stress response in adult animals (Costantini et al., 2012; Zhou et al., 2018). The long-term effects of constant thermal conditions have been studied in several species; however, literature on the long-term effects of fluctuating temperatures remains limited (Blanchard et al., 2025). Because thermal stress has been reported to affect the physiology of animals from different perspectives such as oxidative stress response, energy use, and protein metabolism (Kumar et al., 2015; Madeira et al., 2016; Resende et al., 2022; Khieokhajonkhet et al., 2023), studying the possible long-term effects of temperature on the cellular stress response and thermotolerance across different life stages in fishes can increase our understanding of thermal plasticity in animals.

In this study, we investigated the long-term effects of exposure to daily thermal fluctuation at the embryonic and larval stages of zebrafish. We hypothesized that early-life thermal fluctuations would affect the mRNA expression of genes involved in general stress and oxidative stress responses, and also modify thermotolerance in fish. To test this hypothesis, we exposed zebrafish to daily thermal fluctuations during the embryonic to early larval stage (0-30 days post-fertilization; dpf), and by using qPCR, we measured the mRNA expression of genes involved in a cellular stress response from the larval to adult stage (90 dpf). To determine if daily thermal fluctuations affect the thermotolerance of fish in later life stages, Critical Thermal Maximum ($CT_{max}$) trials were further conducted on juvenile and adult fish at 60 and 90 dpf, respectively. Additionally, we employed transcriptomic markers to estimate the sex of individuals based on gene expression to analyze relative mRNA expression for males and females separately at 60 and 90 dpf. This work contributes to a growing number of studies that demonstrate how different thermal treatments during early life stages can lead to delayed effects at later life stages, affecting growth, thermotolerance, and stress responses in fish.

## 3.3 Materials and Methods:

### 3.3.1 Fish breeding and husbandry

The experiment was approved by the animal care committees at the University of Manitoba (22-030 (AC11760)) in accordance with the guidelines of the Canadian Council on Animal Care. Wild-type AB (WT-AB) zebrafish were bred and reared in the Rady Biomedical Fish Facility at the University of Manitoba, Winnipeg, Manitoba, Canada, on a 12 light (7 am):12 dark (7 pm) light cycle unless stated otherwise. Fish were housed on a recirculating Tecniplast housing system (Tecniplast Inc. USA), equipped with automatic dosing, UV and carbon filters, with a stable pH and conductivity of 7.4 and 900 µS, respectively. Facility water was maintained at 28 $^{o}$C.

For breeding, two females and three males were introduced together in the evening into a zebrafish breeding tank with a meshed bottom to separate the fish from the eggs and facilitate embryo collection the following day. To mimic the natural habitat, artificial plants were placed in the tank. Breeding occurred the following morning with initiation of the light cycle, stimulated

by the gradual increase in light intensity. Fertilized eggs were collected and transferred to Petri dishes with embryo medium (0.01 $mg.L^{-1}$ methylene blue dissolved in fish facility water) at a maximum density of 25 embryos $dish^{-1}$. Petri dishes were then transferred to a Fisherbrand 18L Low Temp Incubator (Thermo Fisher Scientific, Pittsburgh, USA). To reduce the potential for vertical transmission of pathogens and fungi from parents to offspring, embryos were bleached at 1 dpf by them in 7 $mg.L^{-1}$ chlorine for 5 min, followed by three washes with embryo medium, each for 3 min in three separate containers, as described by Kütter et al. (2023), with minor modifications to the bleach concentration. Incubation of eggs in the incubator continued until 5 dpf, when almost all the eggs were hatched.

Upon depletion of the yolk sac at 5 dpf, larvae were fed two portions of GEMMA Micro 75 (Skretting, Maine, USA) and one portion of live rotifer daily. Juvenile fish (> 30 dpf) were fed two portions of GEMMA Micro 150 and one portion of brine shrimp nauplii (Brine Shrimp Direct, Ogden, USA) daily, as described by Varga (2016), with minor modifications in the dry food brand. Temperature, oxygen, and ammonia in the tanks were measured throughout the experiment.

### 3.3.2 Temperature treatment

Immediately following embryo collection at 0 dpf, zebrafish were separated into two groups: (1) temperature treatment, and (2) control. From 0 to 6 dpf, the fish were held in a Low Temp incubator, where the temperature regimes were maintained for both groups. At 6 dpf, larvae were transferred to 18 (L) × 14 (W) × 10 (H) cm aerated containers filled with fish facility water up to 3 cm until 30 dpf. Then, the containers were placed in 56 (L) × 30 (W) × 30 (H) cm coolers. Four coolers were dedicated to each group, and each of them contained three containers. Each container in a cooler was treated as one tank replicate. The coolers were filled with distilled water, and the temperature was maintained at 28 °C by using electric heaters placed at the bottom of each cooler. To maintain homogeneous temperature in the coolers and subsequently in the containers, the water in the coolers was constantly mixed with a pump. Fish were kept in these containers until the end of the larval stage (30 dpf), after which fish were transferred to standard 3.5 L Tecniplast Inc. zebrafish tanks (27 (L) × 11 (W) × 16 (H) cm) and reared on a

Tecniplast recirculating aquatic housing system at 28 °C until the end of the experiment (90 dpf) as described above. The initial number of tanks for each group differed from 10 to 12, and the density of fish in each tank was kept at 8-12 fish per tank. However, the number of tanks decreased as fish were haphazardly sampled from tanks throughout the experiment, and the remainders were combined to maintain a density of 8-12 fish per tank.

In the temperature treatment group, at 12:00 pm daily, prior to feeding, the temperature was raised to 33 °C from ambient 28 ºC water at the rate of ~0.8 °C $min^{-1}$ and then decreased at the same rate back to 28 °C over a 2-hour period during the embryonic and larval stages (0-30 dpf). The control group experienced similar handling but did not experience any thermal fluctuations during embryonic and larval stages, and the temperature for this group was kept constant at 28 °C throughout. In addition, to study growth, 15 fish from each group were haphazardly collected across containers and coolers at 15 30, 60 and 90 dpf and their size was measured and compared between the two groups using the Mann-Whitney U test (p-value < 0.05). At 15 and 30 dpf, because of the small size, the total length was measured, but in the juvenile and adult fish, the fork length, which is a better criterion for growth because it is less affected by potential damage in the tail (Fishbio, 2023), was measured. In addition, thecondition and mortality of fish were also visually monitored on a daily basis.

### 3.3.3 Critical Thermal Maximum ($CT_{max}$) trials

Acclimation to higher temperatures has been shown to increase the $CT_{max}$ in fishes (Bugg et al., 2020; Waterbury et al., 2024). To test if daily thermal fluctuations had an effect on zebrafish $CT_{max}$, we conducted $CT_{max}$ on 60 and 90 dpf zebrafish that experienced daily thermal fluctuations during embryonic and larval stages compared to the control group. The trials were conducted in an 18 (L) × 14 (W) × 10 (H) cm container filled with fish facility water up to 8 centimeters. The containers were placed in coolers filled with distilled water, and the temperature was maintained at 28 °C. Three hours before the $CT_{max}$ trials, nine to 15 fish were haphazardly chosen from tanks and introduced into separate well-aerated containers. To conduct the $CT_{max}$, we increased the temperature in the containers at a target rate of 0.3 °C $min^{-1}$ using an electric heater in the cooler. The temperature was monitored using digital thermometers and

recorded using temperature probes (Witrox Oxygen probe, Loligo Systems; Viborg, Denmark). The behavior of the fish was constantly monitored (approximately 1 h) until they lost equilibrium and could not re-orientate themselves. When fish lost equilibrium and could no longer re-orient, the temperature was recorded, and the fish was netted and euthanized in a separate tank using tricaine (0.4%, pH 7.4). A two-way ANOVA followed by a Bonferroni correction was used to test the significant difference (p-value < 0.008) between groups.

### 3.3.4 mRNA expression

We collected 10 fish from each group at each timepoint to study changes in mRNA expression: 1 fish from each container at 18 and 30 dpf (1 individual × 10 containers × 2 groups × 2 timepoints), 1 fish from each tank at 60 dpf (1 individuals × 10 tanks × 2 groups × 1 timepoints) and 2 fish from each tank at 90 dpf (2 individuals × 5 tanks × 2 groups × 1 timepoints). Samples were collected in the morning after euthanizing the fish with tricaine (0.4%, pH 7.4), and were preserved in RNA*later* (Thermo Fisher Scientific, Waltham, USA). After 24 h of preservation at 4 °C, samples were transferred to a -80 °C freezer for storage. Total RNA was extracted using MagMax mirVana™ Total RNA Isolation Kit (Applied Biosystem, Germany) on a KingFisher Duo Prime (ThermoFisher Scientific, USA) machine. Briefly, the whole fish was homogenized using a TissueLyser (QIAGEN, Germany) at 50 Hz for 5 min in the lysis buffer provided by the kit. The homogenized tissue was centrifuged and transferred to an extraction plate, and RNA purification was conducted following the manufacturer's protocol. The quantity and purity of extracted RNA were measured using a NanoDrop One spectrophotometer (ThermoFisher Scientific). For RNA integrity, we loaded 400 ng of RNA on a 1% agarose gel and checked the 18S and 28S rRNA bands. Before cDNA synthesis, 300 ng of total RNA from each sample was treated with Invitrogen™ *ezDNase* enzyme (Thermo Fisher Scientific, USA) at 37 °C for 2 min to eliminate genomic DNA. Then, the DNase-treated RNA was used for cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) following the manufacturer's instructions.

We used qPCR to measure and compare the mRNA expression of four genes involved in cellular heat shock response and seven oxidative stress response genes (**Table 3.1**). Sequences of

genes were downloaded from the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/), and primers were designed using Primer Express (3.0.1) (Thermo Fisher Scientific) (size: 17-30 bp; Melting temperature (Tm): 58-60 °C; GC%: 30-80%). For qPCR, the synthesized cDNA was diluted 1:4 with nuclease-free water. Then, 5 µl of the diluted cDNA was mixed with 6 µl of PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, USA), 0.1 µl of forward primer (10 mM), 0.1 µl of reverse primer (10 mM), and 0.8 µl of nuclease-free water in a 384-well plate. The qPCR was performed using a QuantStudio 5 (Applied Biosystems, USA) machine, and the thermal conditions for qPCR included initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 20 s. Melt-curve analysis was also included in the analysis to confirm that there was no non-specific amplification or primer-dimer formation. The efficiency of primers was measured by generating standard curves using serial dilution. The method described by Pfaffl (2001) was used to analyze mRNA expression data using three internal control genes for normalization. Because the distribution of data was not normal as determined by Shapiro–Wilks's tests, a Mann-Whitney U test was used to determine significant differences (p-value < 0.05) between the temperature treatment and control group. The same test was also used to compare the difference in the mRNA expression of each gene between males and females.

**Table 3.1**. List of primers used for amplification of oxidative stress and heat shock response genes in zebrafish, *Danio rerio*, along with internal control genes.

| **Gene symbol** | **Primer sequence** | **PCR Efficiency (%)** | **Function** |
|---|---|---|---|
| *gstp1a* | F: CGACTTGAAAGCCACCTGTGT<br>R: TCGACCCAGATGTCTCAGCAT | 100 | Oxidative stress response |
| *cat* | F: TCGTGGTTTCGCAGTGAAGTT<br>R: CGGATTGCGCTTCTGAGAGT | 100 | Oxidative stress response |
| *gpx1a* | F: CCAGGAGAACTGCAAGAATGAA<br>R: AAACAGAGGGTGGGCGTTTT | 100 | Oxidative stress response |

| | | | |
|---|---|---|---|
| *sod1* | F: GTGCAGGTCCGCACTTCAA<br>R: CTGGCATCAGCGGTCACAT | 85 | Oxidative stress response |
| *sod2* | F: AAGCGTGACTTTGGCTCATTTC<br>R: CACACGCTGCAATCCTCAAT | 110 | Oxidative stress response |
| *glulc* | F: GCTCAAGCACACCCGAGAAC<br>R: TATTCCTGCTCCATGCCAAAC | 130 | Oxidative stress response |
| *glulb* | F: GACCCCAACAAACTGGTTCTG<br>R: GACCATCTGTGCCGAGAAGAG | 100 | Oxidative stress response |
| *hsp70* | F: TCAAGCGCAACACAACCATC<br>R: ATTTGCCCAGCAGGTTGTTG | 85 | Heat shock response |
| *hsp90aa1* | F: AGCTGGCGGATCGTTCACTGTC<br>R: AAAACTCGCCGTACTCCTCATTGG | 100 | Heat shock response |
| *hsp47* | F: ACGGGGCTATGATCGTCAA<br>R: AGAGCGGGTCACCAGGAAA | 100 | Heat shock response |
| *hsp90ab1* | F: TGTCAGAACTGCTGCGTTAC<br>R: ACAAATGCAGAGTGGGCAAC | 100 | Heat shock response |
| *gapdh* | F: CTGGTGACCCGTGCTGCTT<br>R: TTTGCCGCCTTCTGCCTTA | 96 | Internal control gene |
| *rpl13* | F: CGGACGTGGCTTCACCTT<br>R: TCTGTTGCGACGACGTGAGT | 110 | Internal control gene |
| *ef1a* | F: GGTGGAATCGACAAGAGAACCA<br>R: GTCCAACACCCAGGCGTACT | 86 | Internal control gene |

### 3.3.5 Sex identification

We attempted to identify the sex of each fish before mRNA expression analysis to avoid misinterpretation of results caused by potential sex-biased expression of oxidative stress and

HSP responses detected in fishes (Li et al., 2014; Błońska et al., 2021). Due to difficulties in distinguishing males from females through visual inspection, especially in younger fish, and also the lack of genetic sex markers, we used transcriptomic methods for sex determination. To identify the sex of each fish based on mRNA expression, 12 genes that were previously reported to have sex-biased expression were chosen (Wong et al., 2014; King et al., 2020; **Table 3.2**). To test the accuracy of these genes for sex determination, the mRNA expression patterns were tested on 5 adult male and 5 adult female zebrafish (10-month-old) with clear external sex-specific characteristics that were visible under a microscope. Since the candidate genes used for the determination of sex may be expressed in different organs, such as the brain and the ovary, RNA was extracted from a homogenized sample of each fish. To do so, the entire fish was frozen in liquid nitrogen and ground in a mortar and pestle. Total RNA extraction, cDNA synthesis, and qPCR were conducted as before. Then, the mRNA expression of these 12 genes in females was measured relative to males using Pfaffl's method (Pfaffl, 2001). The sequence of primers used for amplifying these genes in qPCR, along with the mRNA expression of each gene in females relative to males, is listed in **Table 3.2**. Among the 12 genes tested, *bmp15*, *ccnb1*, *gyg1a*, *kpna2*, *rdh10b*, and *zp3* showed a higher difference in mRNA expression between males and females and were chosen to be used in this study.

We used a Random Forest technique to determine the sex of individuals from the thermal exposure trials using the mRNA expression of genes with sexually biased patterns. To do so, the mRNA expression of *bmp15*, *ccnb1*, *gyg1a*, *kpna2*, *rdh10b*, and *zp3* was measured for each individual using qPCR, and the $C_t$ (cycle of threshold) value was normalized to the geometric mean of internal control genes *ef1a*, *gapdh*, and *rpl13* ($Ct_{(target\ gene)}$-$Ct_{(control\ gene)}$). Then, the normalized $C_t$ values from adult fish with known sex were used to train a Random Forest model using the Random Forest package in R (Liaw and Wiener, 2002). The performance of the model was evaluated using the classification accuracy test, which is defined as the proportion of correctly predicted instances among the total number of instances. After training the model, the normalized $C_t$ of the six sexually biased genes checked in the individuals from the temperature treatment group and control group was used to estimate the sex of that individual using the trained model.

**Table 3.2**. List of primers and probes for amplification of genes used for sex determination in zebrafish, *Danio rerio*, along with their fold-change expression in females relative to males.

| **Gene name** | **Primer sequence** | **PCR efficiency (%)** | **Fold-change expression in females** |
|---|---|---|---|
| *gyg1a* | F: AGTATGCCAAAGGAGCAATGGTA<br>R: GCACATCCACCAGCCTGACT | 94 | 20.6 |
| *rdh10b* | F: TGCACCCCTCGGGTCAT<br>R: TGCACTTATCAGCCCCAATAAA | 91 | 73.9 |
| *cyp19a1b* | F: GCCTGTGTTGGGAAGCACAT<br>R: ACTGGCTGCATGGAGAGGTT | 96 | 0.66 |
| *sox9a* | F: CGACGCATATTAAAACCGAACA<br>R: TACTGCGCTCTGGTGATGGA | 94 | 0.55 |
| *dio2* | F: GCCTGGCTTCTTCTCCAACTG<br>R: GCCGGCTGTGGTTAACATG | 92 | 0.69 |
| *bmp15* | F: GAGGCGAGTGTCACGTCTCTT<br>R: TGTGCACCAATAACGAGCAAA | 110 | 507.1 |
| *ccnb1* | F: TGTTGGTGTAACGGCCATGT<br>R: GGTGGTGTATGCACGGTCTGT | 97 | 98.6 |
| *kpna2* | F: CCCATGTTCCCAGCTCTGTT<br>R: TCCACCAGGTACGGCACAAT | 100 | 45.5 |
| *zp3* | F: TGTGCCTCTGCGTGTGTATGT<br>R: AAGTGGGAGCTGGAACCTGTAG | 100 | 11806.6 |
| *hsd3b1* | F: AGGCTGGTTCGACTGTTGCT<br>R: GTCTCTCCCCGGCAATCAT | 100 | 0.92 |
| *hsd17b3* | F: GCCAAAATACCATGTCCCATTT<br>R: TCCTGTCATTGCGGTTGAAA | 98 | 0.33 |
| *igf1* | F: CAGCAAACCGACAGGATATGG<br>R: ATCGTGGAGATTTGCCTGTCTT | 92 | 0.4 |

## 3.4 Results

Highest mortality was observed at the embryonic and adult stages in both groups, but it decreased at the juvenile and adult stages, with no difference between the groups based on our visual monitoring. In addition, the temperature treatment group and the control group did not show a significant difference (p-value > 0.05) in length at 15 and 30 dpf; however, fork length was significantly smaller in the temperature treatment group (p-value < 0.05) at 60 and 90 dpf (**Figure 3.1**). We found that $CT_{max}$ was significantly higher in the temperature treatment group at 60 (p-value < 0.008), but not at 90 dpf (**Figure 3.2**). There was a 0.62 °C difference in average $CT_{max}$ between the temperature treatment group and control group at 60 dpf, but this difference was decreased to 0.27 °C at 90 dpf. Additionally, the $CT_{max}$ significantly decreased from 60 dpf to 90 dpf by 0.68 °C in the temperature treatment group (p-value < 0.05), but there was no significant difference in $CT_{max}$ between the control groups at 60 and 90 dpf (p-value > 0.008).

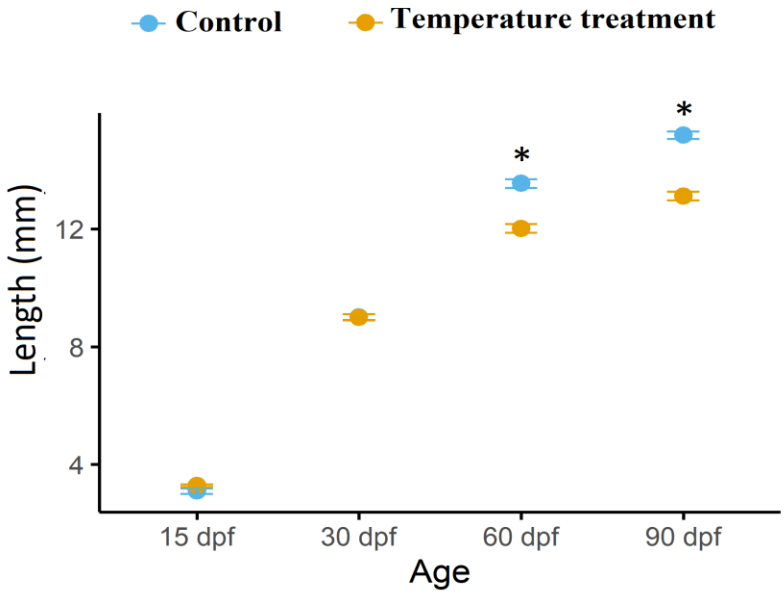



**Figure 3.1**. Length of *Danio rerio* measured at 15, 30, 60, and 90 dpf in the temperature treatment group and control group. Fish in the temperature treatment group experienced daily thermal shock (+ 5 °C) during the larval stage (1-30 dpf) and then were kept at 28 °C until 90 dpf to study the long-term effects of early-life thermal fluctuations. Fish in the control group did not experience any thermal fluctuations and were kept at 28 °C throughout the experiment. At 15 and 30 dpf, total length was measured, but at 60 and 90 dpf, fork length was measured. At 30 dpf, the length data of the control group and temperature treatment group overlap. The statistically

significant difference (p-value < 0.05) between groups at each timepoint was measured using Mann-Whitney U tests and shown by using asterisks (*). Error bars show standard error (SE).

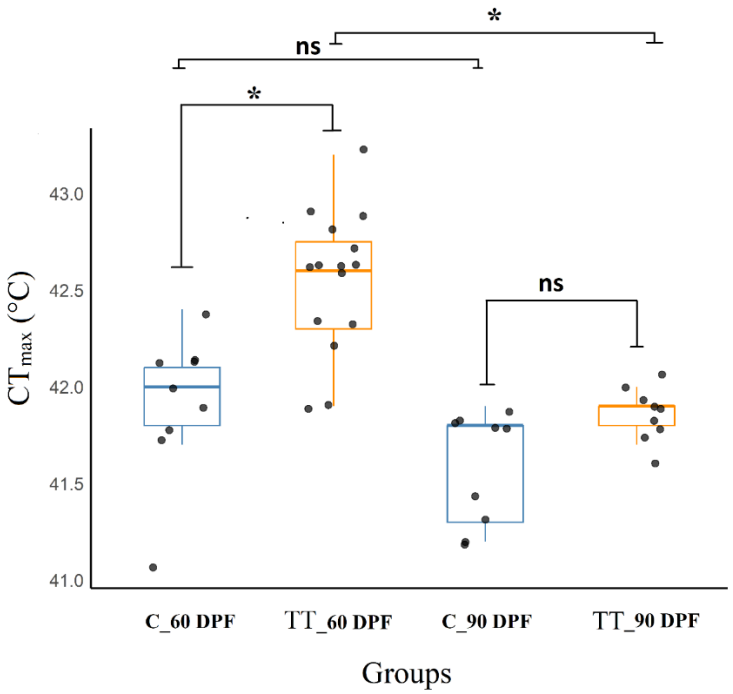


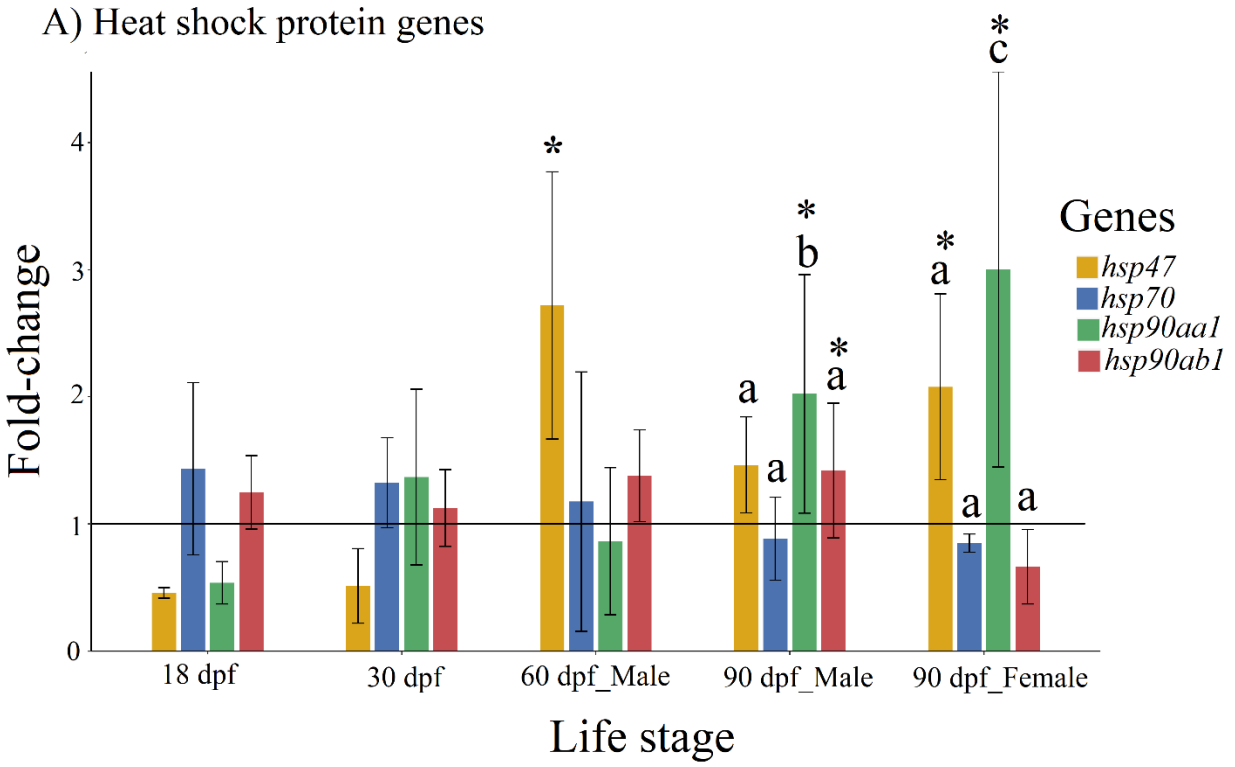

**Figure 3.2** Critical thermal maximum ($CT_{max}$) of *Denio rerio* was measured at 60 and 90 dpf in the temperature treatment (TT) and control (C) group. In the temperature treatment group, fish experience daily thermal fluctuation (+ 5 °C) every day during the larval stage and then are kept at 28 °C during the juvenile stage until the adult stage (90 dpf). The control group was kept at 28 °C throughout the experiment (1-90 dpf) and did not experience any thermal fluctuations. The significant difference (p-value < 0.008) between groups and also between different timepoints tested using the Mann-Whitney U test is shown using asterisks (*).

The classification accuracy test for the Random Forest model showed 100% accuracy of the model in distinguishing males from females using mRNA expression patterns measured in the fish with known sex. However, when we used the mRNA expression of *bmp15*, *ccnb1*, *gyg1a*, *kpna2*, *rdh10b*, and *zp3* to distinguish males from females in the temperature treatment group and control group, it was only feasible at 60 and 90 dpf. At 60 dpf, the sex ratio of males:females was 80:20 in the temperature treatment group, and 67:33 in the control group. At 90 dpf, the sex ratio of male:female was 70:30 in the temperature treatment group, and 40:60 in the control

group. Due to a low number of females in the temperature treatment group at 60 dpf, female samples were excluded, and only samples from males were used for mRNA expression analysis at 60 dpf. However, at 90 dpf, both sexes were included in the analysis.

Although the temperature treatment group did not exhibit a significant change (p-value > 0.05) in the mRNA expression of the four *hsp* genes during the larval stage, there was a significant difference (p-value < 0.05) in mRNA expression at 60 and 90 dpf (**Figure 3.3A**). At 60 dpf juvenile animals, *hsp47* was significantly upregulated (2.71-fold) (p-value < 0.05), although the other *hsp* genes did not show a significant change. At 90 dpf, *hsp90aa1* was significantly upregulated (p-value < 0.05) in both males (2-fold) and females (3-fold), and this upregulation was significantly higher (p-value < 0.05) in females compared to males. The mRNA expression of *hsp90ab1* was significantly upregulated (p-value < 0.05) in males (1.42-fold) at 90 dpf, while slightly downregulated in females (0.66-fold). Additionally, *hsp47* showed significant (p-value < 0.05) upregulation (2.07-fold) in the females measured at 90 dpf.

During the larval stage, only *glulb* was significantly upregulated (p-value < 0.05) (1.71-fold) at 18 dpf in the temperature treatment group (**Figure 3.3B**). At 60 dpf, the mRNA expression of *gstp1a* (1.49-fold), *sod1* (1.68-fold), and *sod2* (2.15-fold) were significantly upregulated (p-value < 0.05). At 90 dpf, *cat* (1.96-fold), *glulb* (1.93-fold), *gstp1a* (1.9-fold), *sod1* (1.59-fold), and *sod2* (1.42-fold) showed significant upregulation (p-value < 0.05) in males, but none of the oxidative stress genes showed significant upregulation in females. Notably, however, *sod2* was the only gene with a significant difference (p-value < 0.05) in the mRNA expression between males and females at 90 dpf.


A) Heat shock protein genes
Fold-change
Life stage
18 dpf
30 dpf
60 dpf_Male
90 dpf_Male
90 dpf_Female
Genes
hsp47
hsp70
hsp90aa1
hsp90ab1

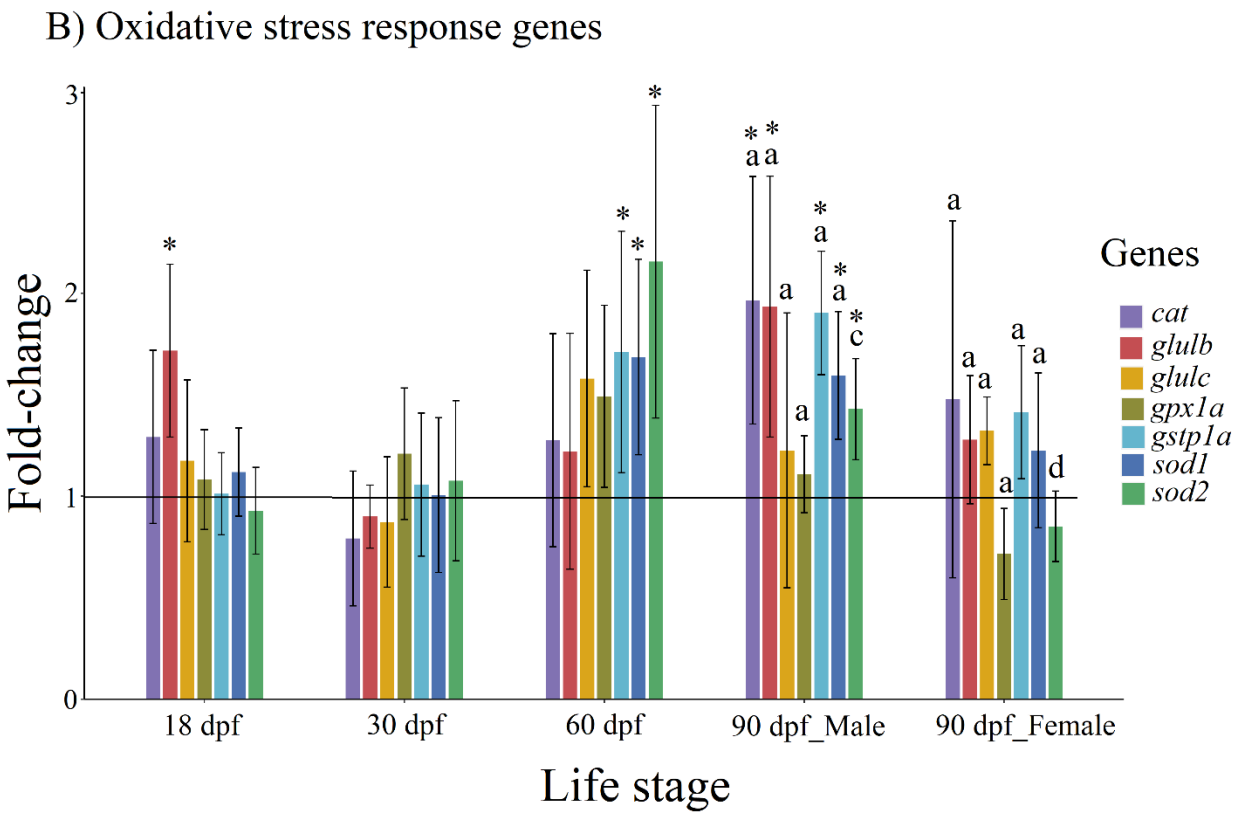

B) Oxidative stress response genes
Fold-change
Life stage
18 dpf
30 dpf
60 dpf
90 dpf_Male
90 dpf_Female
Genes
cat
glulb
glulc
gpx1a
gstp1a
sod1
sod2

**Figure 3.3** Relative mRNA expression of genes coding HSPs (A) and proteins involved in oxidative stress response (B) in response to early-life thermal fluctuations in the *Danio rerio*. Fish experienced daily thermal fluctuations (+ 5 °C) during the larval stage (1-30 dpf) and were then kept at 28 °C until 90 dpf to study the long-term effects of early-life thermal fluctuations. The mRNA expression of genes at each timepoint was measured compared to a control group that was kept at 28 °C during the experiment. In these graphs, the changes in the mRNA expression of each gene are shown as fold-change upregulation or downregulation relative to the control group (1-fold); the mRNA expression level of these genes in the control group is shown with a horizontal line parallel to the x-axis. The asterisks (*) show the significant differences (P-value < 0.05) between the mRNA expression of each gene in the temperature treatment group and the control group at each timepoint. At 60 dpf, only the relative mRNA expression in the males is shown, and females were omitted from statistical analysis due to a low number. At 90 dpf, relative mRNA expression was measured separately for males and females, and significant differences (P-value < 0.05) between sexes are shown by different letters for each gene.

## 3.5 Discussion

Fluctuations in the temperature of aquatic environments can result in stress at cellular and physiological levels in aquatic animals (Podrabsky and Somero, 2004; Dong et al., 2006; Folkedal et al., 2012; Terrazas et al., 2017; Sherif et al., 2024); however, the long-term effects of thermal stressors on fish during early development are not well-known. In the present study, after treating fish with daily thermal fluctuations from 0 to 30 dpf, most of the *hsp* genes we measured exhibited significant upregulation in juvenile and adult fish, suggesting delayed cellular responses caused by the thermal stress during the larval stage. This increase might be because of the role of *hsp* genes in the reassembly of structures damaged by heat shock (Lindquist and Craig, 1988). In our study, *hsp90aa1, hsp90ab1,* and *hsp47* were upregulated in the juvenile and adult stages. Heat shock protein 90 (HSP90) plays an important role in the final function of proteins (Li and Buchner, 2012), such as in the configuration of steroid hormone receptors (Prodromou, 2016). The amount and type of steroid receptors expressed in males and females are typically different (Voigt et al., 2009), which might partially explain the sex-biased mRNA expression of these proteins in adults in response to early-life temperature fluctuations in the present study. Heat shock protein 47 (HSP47) is involved in chaperone-assisted folding of collagen (Tasab et al., 2000). The upregulation of *hsp47* is also reported in injured tissue, for example, in fibrotic liver (Nagata, 2003). Thermal stress can increase oxidative stress and cause

damage at the cellular level of tissues (Lushchak and Bagnyukova, 2006; Roychowdhury et al., 2021), which may partially explain the upregulation of *hsp47* in zebrafish.

The mRNA expression of genes involved in an oxidative stress response was also affected by temperature treatment during the embryonic and larval stages. The effects of temperature on oxidative stress genes are a phenomenon that has been reported in several species, such as large-scale shovel-jawed fish, *Onychostoma macrolepis*, lake sturgeon*, Acipenser fulvescens*, brook trout, *Salvelinus fontinalis,* and zander, *Sander lucioperca* (Yu et al., 2017; Bugg et al., 2020; Mackey et al., 2021; Chen et al., 2021). An upregulation of antioxidant proteins has been reported along with increased oxidation of biomolecules after exposure to higher temperatures (Musa et al., 2021). Antioxidant proteins can help cells detoxify free radicals and reactive species (Pamplona and Costantini, 2011). In our study, most oxidative stress response genes were upregulated at 60 and 90 dpf, especially in males. Male-biased oxidation of biomolecules, such as lipids, and increased expression of antioxidant proteins have been reported in adult round goby, *Neogobius melanostomus* exposed to thermal stress (Błońska et al., 2021), which is consistent with the male-biased mRNA expression patterns in our study.

The effects of early-life exposure to thermal fluctuations on the mRNA expression of genes included in our study were more pronounced in the juvenile and adult stages. Except for *glulb*, all the other genes quantified in this study did not show any significant change in mRNA expression during the larval stage due to the temperature treatment. Rather, most genes showed significant upregulations in mRNA expression in juvenile and adult fish. The similarity in the mRNA expression pattern of oxidative stress response genes in the temperature treatment group and control group might be because of higher expression of these proteins in the early-life stage of animals, a pattern that has been suggested to make individuals more resilient toward thermal fluctuations (Hsu et al., 2008; Almroth et al., 2010; Del Vesco et al., 2017). The expression of HSPs is known to change as animals age, and preconditioning animals with higher temperature in their early-life stage has been observed to affect the expression of these proteins in response to future thermal stressors (Tower, 2009; Hoffman et al., 2024). In addition, these proteins play an important role during early development and are expressed at high basal levels during early developmental stages (Krone et al., 1997; Loones et al., 1997; Krone et al., 2003). It is possible that naturally higher basal expression of oxidative stress response and heat shock protein genes

might also help with coping with the harmful effects of thermal fluctuations, making the upregulation of these genes unnecessary. The upregulation of HSPs in the temperature treatment group in our study shows the delayed effects of exposure to higher temperatures at the cellular level, which also coincided with higher thermotolerance in adult fish.

Exposure to early-life thermal fluctuations significantly increased the $CT_{max}$ of juvenile zebrafish in the present study, but this increase was not significant in adult fish. This increase in $CT_{max}$ was detectable 30 days after the conclusion of the thermal fluctuations at 60 dpf, but it declined at 90 dpf. This suggests a delayed effect of early-life thermal experience on later thermotolerance that could be reversed by acclimation to stable 28 °C. The $CT_{max}$ of fish has been shown to be plastic (Blair and Glover, 2019). Further, exposure to higher acclimation temperatures has been reported to increase the thermotolerance of species, such as yellowtail amberjack, *Seriola lalandi,* brook trout*, Salvelinus fontinalis,* lake sturgeon*, Acipenser fulvescens,* bull trout, *Salvelinus confluentus,* tidepool sculpin*, Oligocottus maculosus,* and zebrafish*, Danio rerio* (Fangue et al, 2011; Morrison et al., 2019; Bugg et al., 2020; Larios-Soriano et al., 2021; Best et al., 2025; Silva-Garay et al., 2025). Thermal preconditioning, in terms of repeated thermal shock or $CT_{max}$, has not been reported to have a significant effect on the thermotolerance of some species, such as mummichog, *Fundulus heteroclitus,* and rainbow trout, *Oncorhynchus mykiss* (Healy and Schulte, 2012; Guo et al., 2023). However, experiencing thermal treatments such as repeated $CT_{max}$, increased acclimation temperature, and previous thermal challenge has been reported to increase $CT_{max}$ in *Danio rerio* (Morgan et al., 2018; Metz et al., 2025). Interestingly, Gavarikar and Craig (2025) did not observe a significant difference in the $CT_{max}$ of fish that experienced diurnal thermal fluctuations during the larval stage after five months of acclimation to 28 °C, when compared to those raised at 28 °C their entire life. In our study, acclimating the zebrafish back to 28 °C significantly decreased the $CT_{max}$ from 60 to 90 dpf, but there was no difference in $CT_{max}$ between the temperature treatment and control group at 90 dpf. The difference in $CT_{max}$ between our study and the Gavarikar and Craig (2025) results might be because in their study, the fish were acclimated to 28 °C for five months, compared to the 2-month acclimation to 28 °C in the present study. This longer acclimation time before conducting $CT_{max}$ might be the reason for the difference in $CT_{max}$ between our study and the results from Gavarikar and Craig (2025).

Experiencing different thermal conditions during the larval stage also affected the growth and size of the fish. Although no difference was found in the size of fish in the larval stage, fish that experienced thermal fluctuations were significantly smaller at the juvenile and adult stages. Exposure to high temperatures has been reported to increase metabolism in ectotherms (Resende et al., 2022; Aguilar et al., 2022). Although the relationship between metabolic rate and growth is complex and sometimes contradictory, there are studies showing increased metabolism along with decreased growth in other species exposed to high temperatures (Álvarez and Nicieza, 2004; Roychowdhury et al., 2019; Jourdain-Bonneau et al., 2023; Hinchcliffe et al., 2024). Decreased growth, along with increased mRNA expression of cellular stress genes detected in our research, could be the result of increased metabolism in the temperature treatment group, and the impacts of that remained even after ending thermal fluctuations.

In conclusion, our study shows the importance of early-life thermal treatments on cellular mechanisms, growth, and thermotolerance of zebrafish. Fishes have evolved adaptations for natural fluctuations in their environment, but changes in the thermal regime of ecosystems caused by anthropogenic activities can challenge their physiological limits. Our study shows that chronic thermal fluctuations during early life stages can increase thermotolerance measured via $CT_{max}$ trials. However, this increase in thermotolerance coincides with a long-lasting increase in cellular stress and decreased growth. The effects of early-life temperature treatments on fishes may not be limited to the parameters measured in this study and may also involve other fitness-related traits, such as reproductive success and survival, that should be investigated in future studies over the entire life cycle of the species. In addition, the difference in the mRNA expression of cellular stress response genes between males and females suggests the importance of sex-specific differences in aquatic animals for dealing with environmental challenges, which is worth further investigation.

## 3.6 Acknowledgment

We appreciate the help and support provided by the Animal Care Staff at the Rady Biomedical Fish Facility and the Lindsey lab. This work was funded by the University of Manitoba Collaborative Research Program Grant and NSERC Discovery Grants to BWL and KMJ.

# Chapter 4. Long-term effects of early-life thermal fluctuations on brain development and behavior of zebrafish, *Danio rerio*

## 4.1 Abstract

In this study, we investigate the long-term effects of early-life daily thermal fluctuations on the development of the zebrafish (*Danio rerio*) brain and its swimming behavior. To address this, we bred wild-type *D. rerio* in the laboratory and divided them into two groups: a temperature treatment group (TT) and a control group (C). Fish in the TT group were kept at 28 °C and experienced daily thermal fluctuations at noon, by increasing the temperature from 28 °C to 33 °C, followed by a decrease to 28 °C over a period of two hours. After 30 days post fertilization (dpf), daily thermal fluctuations were terminated, and the fish were acclimated to 28 °C until the end of the experiment (90 dpf). Controls were kept at 28 °C throughout the experiment but underwent the same handling conditions. The mRNA levels of nine neurodevelopmental genes, *id1*, *nrd1*, *nrd2*, *ngn1*, *ngn3*, *otpa*, *otpb*, *bdnf*, and *ngfb,* were quantified at 12, 24, 50 hours post-fertilization (hpf), and 6, 10, 15, 18, 21, 24, 27, 30, 35, 40, 45, 50, 60 dpf using qPCR. The proliferation of radial-glial cells within the telencephalic pallial niche and overall neuronal density of the pallium were further measured at 10, 27, 60, and 90 dpf using Proliferating Cell Nuclear Antigen (PCNA) and HuC/D immunohistochemistry, respectively. In addition, thigmotaxis and light/dark locomotion tests were used at 15, 30, 60, and 90 dpf to study behavioral changes resulting from the thermal fluctuations. Our results showed that early-life thermal fluctuations changed the mRNA expression pattern of neurodevelopmental genes such as *bdnf*, *nrd1*, *nrd2*, *ngn1*, *ngn3*, *otpa*, and *otpb* in the brain in a stage-dependent manner. Proliferation of radial-glial cells within the telencephalic pallial niche of the TT group was significantly lower at 10 dpf, but significantly higher at 27 dpf, compared to control tissue. The proliferation of radial-glial cells did not show significant changes at 60 dpf, but it was significantly lower in males at 90 dpf. The neuron density was only significantly higher in the TT group at 27 dpf, and in males at 90 dpf. Behavioral analysis did not show any sign of increased thigmotaxis at any timepoint, but the dark/light locomotion test was significantly affected by early-life thermal treatment, showing increased activity in dark and light conditions. Overall, our results show that early-life thermal treatment can affect the mRNA expression of neurodevelopmental genes, influencing long-term neural stem cell proliferative activity with increasing stress-like behavior in *D. rerio*.

## 4.2 Introduction

Water temperature is not constant in aquatic environments and fluctuates daily and seasonally (Żelazny et al., 2018; Komatsu, 1985; Zhang et al., 2016). Changes in water temperature can affect the biochemical and physiological activities of animals living in these environments (Ahmad et al., 2011). Adaptation of animals to their environment has enabled them to tolerate natural fluctuations in water temperature (Goldspink, 1995). However, exposure to temperatures higher than the normal physiological range for a species or population can put the animal under thermal stress (Hossain Aunkor et al., 2025). Most fishes are ectotherms, and their internal body temperature is at or near the surrounding water temperature, which makes them susceptible to fluctuations in temperature (Roychowdhury et al., 2021). Extreme changes in water temperature, which may be caused by anthropogenic activities such as climate change and warm water discharge from industrial facilities, can commonly result in thermal stress for fish species (Guimarães et al., 2023; Dettleff et al., 2025).

Early-life stress is known to impact brain development and behavior (Smith and Pollak, 2020). In fishes, a study on Atlantic salmon, *Salmo salar*, showed that exposure to different kinds of unpredictable stress at the parr stage can result in lower hypothalamic catecholaminergic and brainstem serotonergic responses to stress in later life stages (Vindas et al., 2016). Additionally, gilthead seabream, *Sparus aurata*, exposed to early-life stress showed downregulation of gene coding for brain-derived neurotrophic factor *(bdnf)* in the amygdala homolog (Dm3), and higher neuronal activity in the hippocampus homolog (Dlv) and the Dm3 (Vindas et al., 2018). In addition, early-life chronic stress has been reported to increase anxiety and reduce novel environment exploration in zebrafish, *Danio rerio* (Golla et al., 2020). However, zebrafish that experienced early-life stress in terms of chasing with a net in their late-larval and early juvenile stage have shown enhanced performance in finding the exit in the maze test, and less anxiety in the juvenile stage (DePasquale et al., 2016).

In this study, we investigated the effects of early-life daily thermal fluctuations on the neurodevelopmental gene expression, neurogenesis, and behavior of zebrafish. The hypothesis was that early-life thermal stress can have long-term effects on the mRNA expression of neurodevelopmental genes, proliferation of radial-glial stem cells, neuron density, and behavior

of zebrafish. Zebrafish were bred, and the offspring experienced early-life daily thermal fluctuations (±5 °C fluctuation in temperature) during the embryonic and larval stages. Then, the daily thermal fluctuations were removed, and the fish were acclimated to a stable temperature (28 °C). Then, the mRNA expression level of nine genes involved in the development of the central nervous system was measured using qPCR. In addition, the proliferation of radial-glial cells and neuron density was measured. To investigate the effects of early-life thermal fluctuations on the behavior of zebrafish, we measured thigmotaxis, which is the tendency of an animal to remain close to the wall and is measured as an index of anxiety, in addition to measuring the activity of fish in a light-dark transitioning condition, which is often used as an index of increased stress (Simon et al., 1994; Basnet et al., 2019). The results showed that early-life thermal treatment can affect the mRNA expression of neurodevelopmental genes, with long-term effects on the brain development and behavior of zebrafish.

## 4.3 Materials and Methods

### 4.3.1 Fish breeding and husbandry

The experiment was approved by the animal care committees at the University of Manitoba (22-030 (AC11760)) in accordance with the guidelines of the Canadian Council on Animal Care. Wild-type AB (WT-AB) zebrafish were bred and reared in the Rady Biomedical Fish Facility at the University of Manitoba, Winnipeg, Manitoba, Canada, on a 12 light:12 dark light cycle. In the evening, two females and three males were introduced into a zebrafish breeding tank with a meshed bottom separating the fish from the eggs. To mimic the natural habitat, artificial plants were placed in the tank. Breeding took place the next morning, stimulated by the gradual increase in light intensity. Fertilized eggs were collected and transferred to Petri dishes with embryo medium (0.01 $mg.L^{-1}$ methylene blue dissolved in fish facility water) at a maximum density of 25 embryos/dish. Petri dishes were then transferred to a Fisherbrand 18L Low Temp Incubator until 5dpf (Thermo Fisher Scientific, Pittsburgh, USA). At 1 dpf, embryos were bleached by submerging them in 7 $mg.L^{-1}$ chlorine for 5 min, followed by three washes with embryo medium, each for 3 min in three separate containers, as described by Kütter et al. (2023), with minor modifications to the bleach concentration.

Upon depletion of the yolk sac at 5dpf, larvae were fed two portions of GEMMA Micro 75 (Skretting, Maine, USA) and one portion of live rotifer daily. Juvenile fish (> 30 dpf) were fed two portions of GEMMA Micro 150 and one portion of brine shrimp nauplii (Brine Shrimp Direct, Ogden, USA) daily, as described by Varga (2016) with minor modifications in dry food brand. Temperature, oxygen, and ammonia in the tanks were measured throughout the experiment.

### 4.3.2 Temperature treatment

Beginning at the embryonic stage (0 dpf), zebrafish were separated into two groups: temperature treatment (TT) and control (C). In the temperature treatment group, fish experienced daily thermal fluctuations during the embryonic and larval stages (0-30 dpf). In this group, fish were maintained at 28 °C, but daily at 12:00, before feeding, the temperature was raised to 33 °C at the rate of ~0.8 °C $min^{-1}$ and then decreased at the same rate back to 28 °C over a 2-hour period. The control group experienced similar handling but did not experience any thermal fluctuations during embryonic and larval stages, and the temperature for this group was kept constant at 28 °C throughout. From 0 to 6 dpf, the fish were held in incubators where the temperature regimes were maintained for the two groups. At 6 dpf, larvae were transferred to 18 (L) × 14 (W) × 10 (H) cm aerated containers filled with fish facility water up to 3 cm. Then, the containers were placed in 56 (L) × 30 (W) × 30 (H) cm coolers. Four coolers were dedicated to each treatment, and each of them contained three containers. Each container in a cooler was treated as one tank replicate. The coolers were filled with distilled water, and the temperature was maintained at 28 °C by using electric heaters placed at the bottom of each cooler. To maintain homogeneous temperature in the coolers and subsequently in the containers, the water in the coolers was constantly mixed with a pump. Fish were kept in these containers until the end of the larval stage (30 dpf). Upon reaching juvenile stage at 30 dpf, fish were transferred to standard 3.5 L Tecniplast Inc. zebrafish tanks (27 (L) × 11 (W) × 16 (H) cm) and reared on a Tecniplast recirculating aquatic housing system at 28 °C until the end of the experiment (90 dpf). The initial number of tanks for each group differed from 10 to 12, and the density of fish in each tank was kept at 8-12 fish per tank. However, the number of tanks decreased as fish were

haphazardly sampled from tanks throughout the experiment, and the remainders were combined to maintain a density of 8-12 fish per tank.

### 4.3.3 Relative mRNA expression

We collected 10-15 fish from each group at each timepoint to study changes in mRNA expression: 1 embryo from each Petri dish at embryonic stage (1 individual × 15 Petri dish × 2 groups × 3 timepoints), 1 fish from each container at larval stages (1 individual × 10 containers × 2 groups × 8 timepoints), 1 fish from each tank at juvenile stage (1 individuals × 10 tanks × 2 groups × 5 timepoints) and 2 fish from each tank at 90 dpf (2 individuals × 5 tanks × 2 groups × 1 timepoints). Samples were collected in the morning after euthanizing the fish with tricaine (0.4%, pH 7.4), and were preserved in RNA*later* (Thermo Fisher Scientific, Waltham, USA). After 24 h of preservation at 4 °C, samples were transferred to a -80 °C freezer for storage. Total RNA was extracted using MagMax mirVana™ Total RNA Isolation Kit (Applied Biosystem, Germany) on a KingFisher Duo Prime (ThermoFisher Scientific, USA) machine. Briefly, the whole fish was homogenized using a TissueLyser (QIAGEN, Germany) at 50 Hz for 5 min in the lysis buffer provided by the kit. The homogenized tissue was centrifuged and transferred to an extraction plate, and RNA purification was conducted following the manufacturer's protocol. The quantity and purity of extracted RNA was measured using a NanoDrop One spectrophotometer (ThermoFisher Scientific). For RNA integrity, we loaded 400 ng of RNA on a 1% agarose gel and checked the 18S and 28S rRNA bands. Before cDNA synthesis, 300 ng of total RNA from each sample was treated with Invitrogen™ ezDNase enzyme (Thermo Fisher Scientific, USA) at 37 ˚C for 2 min to eliminate genomic DNA. Then, the DNase-treated RNA was used for the cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) following the manufacturer's instructions.

We used qPCR to measure and compare the mRNA levels of nine genes involved in neurogenesis and the development of the brain (**Table 4.1**). These genes were chosen for their important roles in brain development, neurogenesis, and neural fate determination (Lyden et al., 1999; Acampora et al., 2000; Sun et al., 2001; Mie et al., 2003; Del Giacco et al., 2006; Choi et al., 2009; Bath et al., 2012; Tutukova et al., 2021). Sequences of genes were downloaded from the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/), and

primers were designed using Primer Express (3.0.1) (Thermo Fisher Scientific) (size: 17-30 bp; Melting temperature (Tm): 58-60 °C; GC%: 30-80%). For qPCR, the synthesized cDNA was diluted 1:4 with nuclease-free water. Then, 5 µl of the diluted cDNA was mixed with 6 µl of PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, USA), 0.1 µl of forward primer (10 mM), 0.1 µl of reverse primer (10 mM), and 0.8 µl of nuclease-free water in a 384-well plate. The qPCR was performed using a QuantStudio 5 (Applied Biosystems, USA) machine, and the thermal conditions for qPCR included initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 20 s. Melt-curve analysis was also included in the analysis to confirm that there was no non-specific amplification or primer-dimer formation. The efficiency of primers was measured by generating standard curves using serial dilution. The method described by Pfaffl (2001) was used to analyze mRNA expression data using three internal control genes for normalization. Because the distribution of data was not normal, as determined by Shapiro–Wilks's tests, a Mann-Whitney U test was used to determine significant differences between the temperature treatment and control group. In order to decrease the chance of a false-positive significant result caused by testing the mRNA expression of several genes, a Bonferroni correction was used, and p-values were adjusted to 0.005.

**Table 4.1** List of primers used for amplification of genes coding for proteins involved in neurogenesis in zebrafish, *Danio rerio*, along with internal control genes *gapdh, rpl13,* and *ef1a*.

| **Gene symbol** | **Protein** | **PCR Efficiency (%)** | **Primer sequence** |
|---|---|---|---|
| *otpa* | Orthopedia homeobox a | 1.85 | F: TCCAGCCAGCAGAGTCAGAA<br>R: CTCCTCGCGCATGAAGATGT |
| *otpb* | Orthopedia homeobox a | 2.1 | F: CGGAGTCACGGGTTCAGGTA<br>R: CCGGAAAACATTGGTCGTCTT |
| *bdnf* | Brain-derived neurotrophic factor | 2 | F: CACTTTCGAGCAGGTCATTGAG<br>R: AGGCATACAGGTCAACGTCCTT |
| *id1* | Inhibitor of DNA binding 1 | 2.1 | F: AGAAAGCCAGCAAGATGGAGAT<br>R: AGCTCCGCGTTGAGTGTTGT |

| | | | |
|---|---|---|---|
| *ngfb* | Nerve growth factor b | 2.2 | F: CGGAGGCTCTGGGTGTTTAG<br>R: TCCACGCCACCAGGTTCT |
| *ngn1* | Neurogenin 1 | 2.2 | F: ACCGCGAGAGGAACAGGAT<br>R: TGTGAGCGAAGCGCAGAGT |
| *ngn3* | Neurogenin 3 | 2.1 | F: CAGAAGCGTCCTGCCAACTT<br>R: CGAACGTGATCTGCAATTCTCA |
| *nrd1* | Neurogenic differentiation factor 1 | 1.97 | F: CGCAAAGTTGTTCCGTGCTA<br>R: TTGCCCGACCTCAAGATTTC |
| *nrd2* | Neurogenic differentiation factor 2 | 1.97 | F: ACCTTCACTGCTTCCGGACAT<br>R: TCCTGCTCTTCGTCCTTGGT |
| *gapdh* | glyceraldehyde-3-phosphate dehydrogenase | 96 | F: CTGGTGACCCGTGCTGCTT<br>R: TTTGCCGCCTTCTGCCTTA |
| *rpl13* | Ribosomal protein L13 | 110 | F: CGGACGTGGCTTCACCTT<br>R: TCTGTTGCGACGACGTGAGT |
| *ef1a* | elongation factor 1a | 86 | F: GGTGGAATCGACAAGAGAACCA<br>R: GTCCAACACCCAGGCGTACT |

### 4.3.4 Sex identification

We tried to determine the sex of each fish before mRNA expression analysis and histological analysis to avoid any misinterpretation of results caused by probable sex-biased expression of genes involved in neurogenesis. To determine the sex of each fish based on mRNA expression, 12 genes that were previously reported to have sex-biased expression were chosen (Wong et al., 2014; King et al., 2020). To test the accuracy of those genes for sex determination, the mRNA expression patterns were examined on 5 adult male and 5 adult female zebrafish (10-month-old) with clear external sex-specific characteristics that were visible under a microscope. Because the candidate genes used for the determination of sex may be expressed in different organs, such as the brain and the ovary, RNA was extracted from a homogenized sample of each fish. To do so, the entire fish was frozen in liquid nitrogen and ground in a mortar using a pestle. Total RNA extraction, cDNA synthesis, and qPCR were conducted as before. Then, the mRNA

levels of these 12 genes in females were measured relative to males using Pfaffl's method (Pfaffl, 2001). The sequence of primers used for amplifying these genes in qPCR, along with the mRNA expression of each gene in females relative to males, is listed in **Table 4.2**. Among the 12 genes tested, *bmp15*, *ccnb1*, *gyg1a*, *kpna2*, *rdh10b*, and *zp3* showed a higher difference in mRNA expression between males and females and were chosen to be used in this study.

We used the Random Forest technique to determine the sex of individuals from the thermal exposure trials using the mRNA expression of genes with sexually biased patterns. To do so, the mRNA expression of *bmp15*, *ccnb1*, *gyg1a*, *kpna2*, *rdh10b*, and *zp3* was measured for each individual using qPCR, and the Ct (cycle of threshold) value was normalized to the geometric mean of internal control genes *ef1a*, *gapdh*, and *rpl13* (Ct (target gene)-Ct (control gene)). Then, the normalized Ct values from adult fish with known sex were used to train a Random Forest model using the Random Forest package in R (Liaw and Wiener, 2002). The performance of the model was evaluated using the classification accuracy test, which is defined as the proportion of correctly predicted instances among the total number of instances. After training the model, the normalized Ct of the six sexually biased genes checked in the individuals from the temperature treatment group and control group was used to estimate the sex of that individual using the trained model.

**Table 4.2** List of primers and probes for amplification of genes used for sex determination in zebrafish, *Danio rerio*, along with their fold-change mRNA expression in females relative to males.

| **Gene name** | **Primer sequence** | **PCR efficiency (%)** | **Fold-change expression in females** |
|---|---|---|---|
| *gyg1a* | F: AGTATGCCAAAGGAGCAATGGTA<br>R: GCACATCCACCAGCCTGACT | 94 | 20.6 |
| *rdh10b* | F: TGCACCCCTCGGGTCAT<br>R: TGCACTTATCAGCCCCAATAAA | 91 | 73.9 |
| *cyp19a1b* | F: GCCTGTGTTGGGAAGCACAT<br>R: ACTGGCTGCATGGAGAGGTT | 96 | 0.66 |
| *sox9a* | F: CGACGCATATTAAAACCGAACA<br>R: TACTGCGCTCTGGTGATGGA | 94 | 0.55 |

| | | | |
|---|---|---|---|
| *dio2* | F: GCCTGGCTTCTTCTCCAACTG<br>R: GCCGGCTGTGGTTAACATG | 92 | 0.69 |
| *bmp15* | F: GAGGCGAGTGTCACGTCTCTT<br>R: TGTGCACCAATAACGAGCAAA | 110 | 507.1 |
| *ccnb1* | F: TGTTGGTGTAACGGCCATGT<br>R: GGTGGTGTATGCACGGTCTGT | 97 | 98.6 |
| *kpna2* | F: CCCATGTTCCCAGCTCTGTT<br>R: TCCACCAGGTACGGCACAAT | 100 | 45.5 |
| *zp3* | F: TGTGCCTCTGCGTGTGTATGT<br>R: AAGTGGGAGCTGGAACCTGTAG | 100 | 11806.6 |
| *hsd3b1* | F: AGGCTGGTTCGACTGTTGCT<br>R: GTCTCTCCCCGGCAATCAT | 100 | 0.92 |
| *hsd17b3* | F: GCCAAAATACCATGTCCCATTT<br>R: TCCTGTCATTGCGGTTGAAA | 98 | 0.33 |
| *igf1* | F: CAGCAAACCGACAGGATATGG<br>R: ATCGTGGAGATTTGCCTGTCTT | 92 | 0.4 |

### 4.3.5 Brain histology

Sampling for brain histology was performed at 10-, 27-, 60-, and 90 dpf. Sampling days were selected based on mRNA expression data, when the difference in mRNA expression between the temperature treatment group and the control group was more pronounced. Ten fish at 10, 27, and 60 dpf, and 20 fish at 90 dpf were collected from each treatment and euthanized with tricaine (0.4%, pH 7.4). Sampling from 10 and 27 dpf was performed by fixing the whole body of the fish, but for 60 and 90 dpf fish, the dorsal cranium was removed for better penetration of fixatives, and the head was separated from the rest of the body. The internal body organs were collected and preserved in RNA*later* for qPCR-based sex determination of 90 dpf fish. After sampling, different fixation procedures were used depending on life stages. For fish collected at 10 dpf, the entire fish was fixed in ice-cold 4% paraformaldehyde (PFA) (dissolved in 1X PBS) for 4 hours before embedding. Fish collected at 27 and 60 dpf were first fixed in 2%

PFA for three hours and then transferred to the EDTA (ethylenediaminetetraacetic acid) solution for three hours. Fish collected at 90 dpf were fixed in 2% PFA for four hours and then transferred to EDTA solution for 24 hours. After fixation, the fish were embedded in plastic molds using a sucrose gelatine solution (20g sucrose + 8g fish gelatine powder + 100mL 1X-PBS) on crushed dry ice, and blocks were stored in the – 80 °C freezer until cryosectioning. Cryosectioning was performed using a Leica CM3050 S Cryostat (Leica Biosystems, Germany) at -30 °C to obtain 20 µm cross-sections of tissue in proximity to level 71 of the zebrafish brain atlas (**Figure 4.1**; Wullimann et al., 1996). Sections were collected on Superfrost glass slides, and, after drying at room temperature for 1 hour, were stored at – 80 °C until immunohistochemistry.

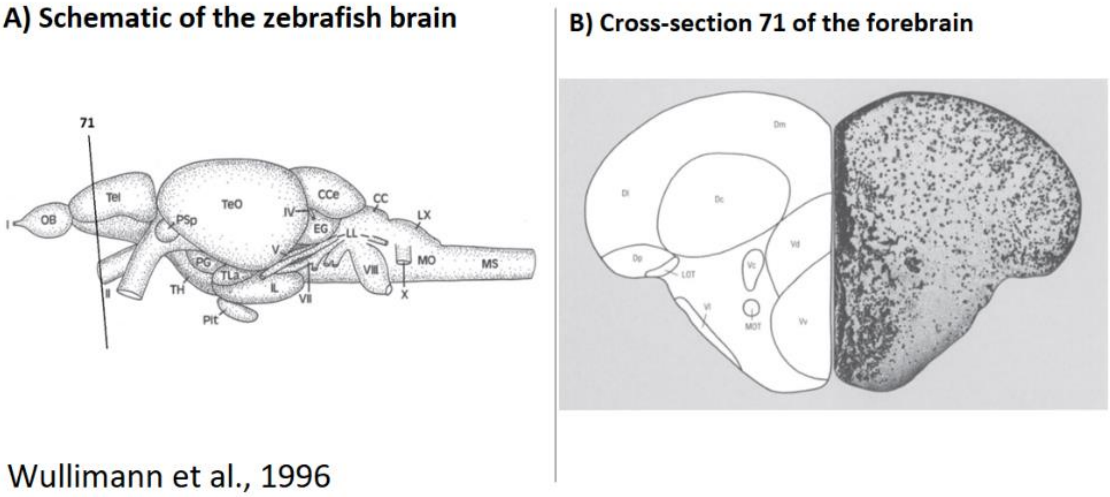



**Figure 4.1** Schematic of the zebrafish brain (A) and the cross-section 71 of the forebrain (B) show the region of the brain from which the slides were prepared for histological analysis.

## 4.3.6 Immunohistochemistry

Sections were stained for the immature neuronal marker HuC/D and Proliferating Cell Nuclear Antigen (PCNA) to quantify the number of proliferating radial-glial cells and the density of neurons, respectively. Mouse-anti-HuC/D (IgG2b; Molecular Probes) and mouse-anti-PCNA (IgG2b; Molecular Probes) were used as primary antibodies. Goat-anti-mouse IgG Alexa488 was

used as a secondary antibody for both markers. Staining for each marker was done on separate slides, which permitted the same secondary antibody for both primary antibodies.

Before staining, the sections were dried at room temperature and in the dark for 1 hour. Then, the sections were washed with 1X phosphate-buffered saline (PBS) with 5% Triton-X (1X-PBS-TX) and then blocked with blocking solution (0.1 g bovine serum albumin and 0.2 mL normal goat serum in PDT [2 mL 10× PBS, 0.02 mL DMSO, 0.02 mL Triton, brought to 20 mL with Milli-Q water], final volume 10 mL) for 1 h at room temperature. Antigen retrieval occurred at 88 °C for HuC/D and 92 °C for PCNA staining, while slides were submerged in 50 mM Tris buffer (pH 8.0 to 8.52). Sections were then incubated with the primary antibody dissolved in blocking solution at 4 °C for 12 h overnight. The next morning, excessive primary antibody was washed by three rinses of the slides with 1X-PBS-TX. The sections were incubated with secondary antibody dissolved in 1X-PBS-TX with DAPI (4′,6-diamidino-2-phenylindole) for 1 h at room temperature. Because DAPI binds to the double-stranded DNA in every cell, it was used for counterstaining purposes, making a background staining, which makes it easier to pinpoint the location of HuC/D and PCNA-stained cells in the section. Slides were washed by 10 repetitions of quick rinse with 1X PBS-TX in 10-minute intervals. The slides were sealed using Immu-Mount (Epredia, USA) and coverslipped and stored at 4 °C for imaging. Imaging was done using a Leica Stellaris 5 confocal microscope (Leica, Germany). Z-stacks were obtained at 1 µm intervals through the 20 µm depth of sections using a 20x dry objective at a minimum resolution of 1024 x 1024.

Different strategies were used for the quantification of neurons due to differences in the abundance of HuC/D and PCNA-positive cells. The higher abundance of HuC/D-positive cells made it difficult to count individual cells. Therefore, to quantify the number of neurons marked with the mouse-anti-Hu antibody, the total volume of pixels in the 3D space (i.e., voxels) (um3) of all the HuC/D-positive neurons in the pallial region of cross-section 71 of the forebrain was measured in the 20 µm section using a Leica Application Suite X (4.8.2.29567) after removing background noise and filtering objects smaller than 100 µm. The number of PCNA-positive cells was less abundant; therefore, manual counting through z-stacks was completed for this proliferative marker. Because the distribution of data was not normal, as determined by Shapiro–Wilks's tests, a Mann-Whitney U test was used to determine significant differences (p-value <

0.05) in the number of $PCNA^+$ cells and the volume of HuC/D-stained cells between the temperature treatment and control group. The same test was also used to compare the differences between males and females.

### 4.3.7 Behavioral analysis

Behavioral analysis was conducted at 15, 30, 60, and 90 dpf, in the morning of each day. Twelve fish from each group were used for behavioral analysis at each timepoint. For 15-dpf fish, all 12 fish were tested on one 12-well plate (~3.5 $cm^3$ (surface area), ~1 cm (depth)), but 30, 60, and 90-dpf fish were tested in two 6-well plates (~9 $cm^3$ (surface area), ~1 cm (depth)) due to their larger size. The plates containing fish were transferred to the DanioVision™ system (Noldus, The Netherlands) 30 minutes before to reduce the effects of handling stress on the results. The temperature was maintained at 28 °C throughout the analysis using a temperature control unit. The activity of the fish was measured using a GenlCam camera with 1280x960 resolution at 30 frames per second (fps), and the data was collected using EthoVision XT 15 software. Care was taken to do the behavior analysis in a quiet room with the lowest amount of disturbance.

To measure thigmotaxis, each well in the plate was divided into two equal zones in the software: inner and outer zones. The analysis was carried out over 30 minutes in the dark condition, and the distance moved and the time spent by the fish in each zone were measured. To reduce the effect of size on the movement data, the movement data (distance moved and time) for each fish were divided by the size of each fish before comparison between the temperature treatment group and the control group at each timepoint. Higher movement and time spent in the outer zone were considered as higher thigmotaxis. A Mann-Whitney U test was used to test the statistical significance ($p$-value $< 0.05$) between two groups.

To perform the light-dark locomotion test, the machine was programmed to alternate the light condition between dark and light every 7.5 minutes for 1 hour. The distances moved in each condition were measured using EthoVision XT 15 software and compared between groups at each timepoint. To reduce the effect of size on the movement data, the distance moved by each

fish was divided by the size of each fish before comparison. A linear mixed-effects model was used to check if the movement of fish in the temperature treatment was significantly different (p-value < 0.05) from that of the control group over the 1-hour alternating light condition. In this model, the thermal treatment (temperature treatment or control), light condition (dark or light), and the interaction of these two were considered as fixed effects. Each fish and the interaction between the fish and the thermal treatment were considered as random effects.

## 4.5 Results

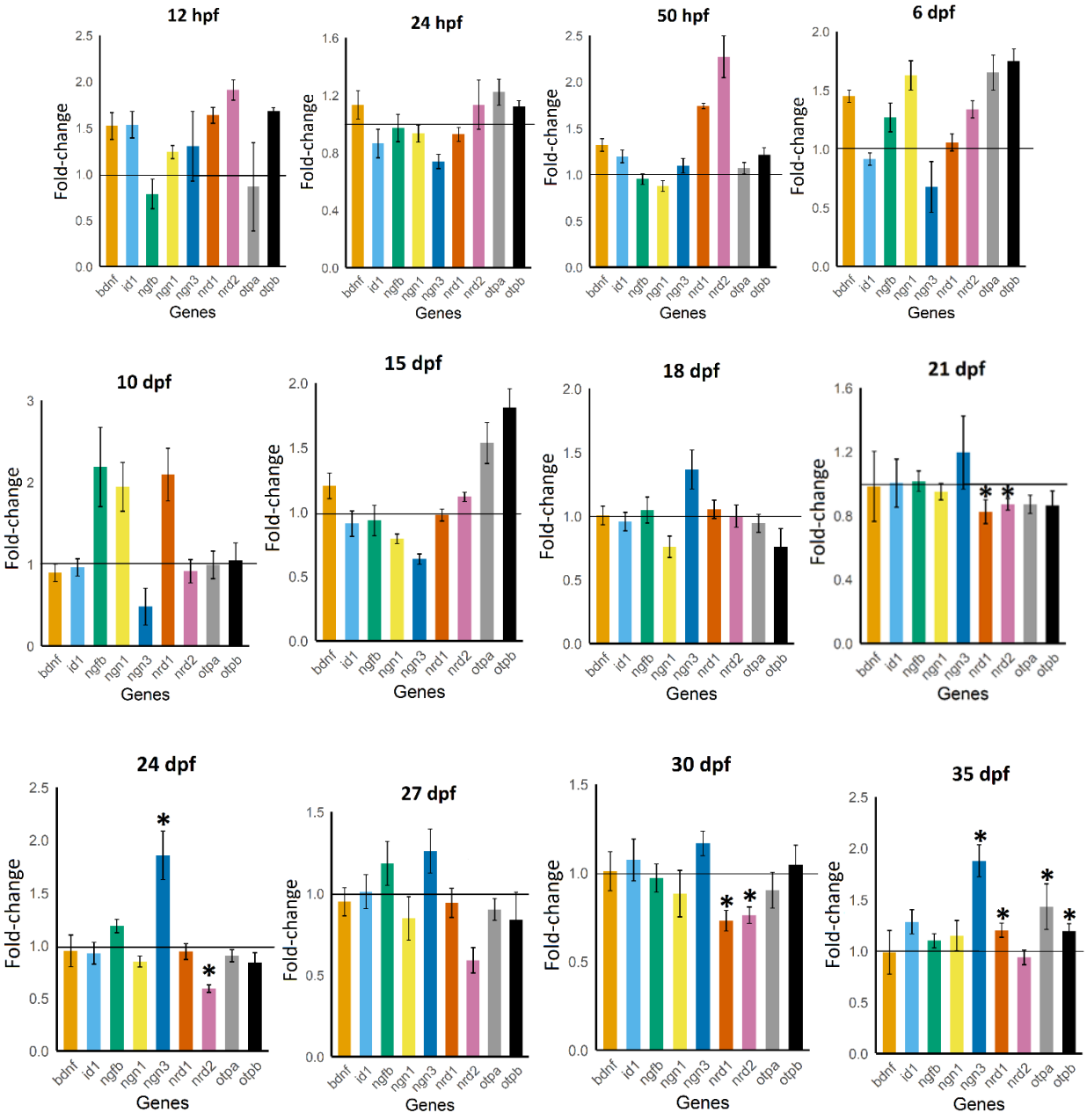
### 4.5.1 mRNA expression analysis

We used the mRNA expression of genes previously known for their sex-biased expression, and a machine learning technique to sex fish used in this study. The classification accuracy test used for testing the Random Forest model showed 100% accuracy in the model for distinguishing males from females using mRNA expression patterns measured in the fish with known sex. However, when we used the mRNA expression of *bmp15*, *ccnb1*, *gyg1a*, *kpna2*, *rdh10b*, and *zp3* to distinguish males from females in the temperature treatment group and control group, it was only feasible at 60 and 90 dpf. At 60 dpf, the sex ratio of males: females was 80:20 in the temperature-treated group, and 67:33 in the control group, as indicated by the mRNA patterns. At 90 dpf, the sex ratio of males:females was 70:30 in the temperature treatment group, and 40:60 in the control group. Due to a low number of females in the temperature treatment group at 60 dpf, the samples that were predicted to be females were excluded, and only samples predicted to be males were used for mRNA expression analysis at 60 dpf. However, at 90 dpf, both sexes were included in the analysis.

The mRNA expression was used to measure the effects of early-life thermal treatments on the mRNA expression of genes involved in brain development. The results showed that early-life thermal fluctuations had different effects on the mRNA expression of nine genes measured at the embryonic, larval, juvenile, and adult stages (**Figure 4.2**). The mRNA expression pattern of genes measured at ≤18 dpf did not show any statistically significant (p-value > 0.005) changes in the mRNA expression pattern in response to early-life thermal fluctuations, despite some genes

showing large fold-change differences in the relative mRNA expression pattern measured. For instance, *nrd2* at 12 (1.9-fold) and 50 hpf (2.2-fold), and *ngfb* (2.1-fold), *ngn1* (1.9-fold), and *nrd1* (2.09-fold) at 10 dpf had higher mRNA expression, but the differences were not statistically significant (p-value > 0.005).

After 18 dpf, statistically significant changes were observed in the mRNA expression levels of several genes. The mRNA expression of *bdnf* (0.6-fold) and *ngn1* (0.54-fold) was downregulated at 40 and 45 dpf, respectively. In contrast, *ngn3* mRNA expression was upregulated at 24 (1.85-fold) and 35 dpf (1.87-fold) but downregulated at 40 dpf (0.6-fold). The mRNA expression of *nrd1* showed both upregulation and downregulation across developmental stages, with increased mRNA expression at 35 dpf (1.2-fold) and 50 dpf (1.25-fold), and decreased mRNA expression at 21 dpf (0.82-fold) and 30 dpf (0.72). The significant change in *nrd2* mRNA expression was downregulation at 21 (0.87-fold), 24 (0.52-fold), and 30 dpf (0.76-fold). In addition, *otpa* mRNA expression was upregulated at 35 (1.43-fold) and 60 dpf (1.28-fold), but it was downregulated by 40 dpf (0.7-fold). Similarly, the mRNA expression of *otpb* was upregulated at 35 dpf (1.19-fold).


12 hpf
24 hpf
50 hpf
6 dpf
10 dpf
15 dpf
18 dpf
21 dpf
24 dpf
27 dpf
30 dpf
35 dpf
Fold-change
Genes
bdnf
id1
ngfb
ngn1
ngn3
nrd1
nrd2
otpa
otpb

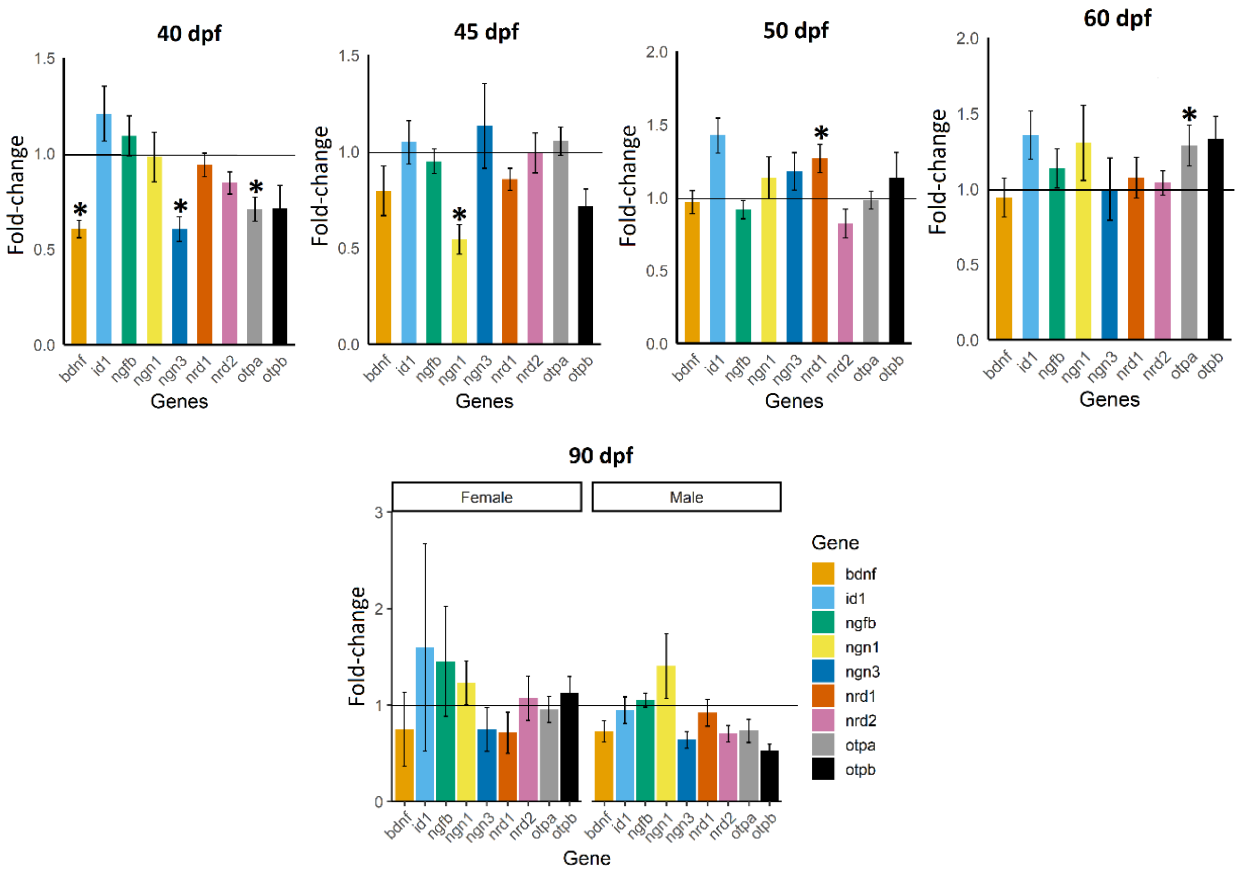
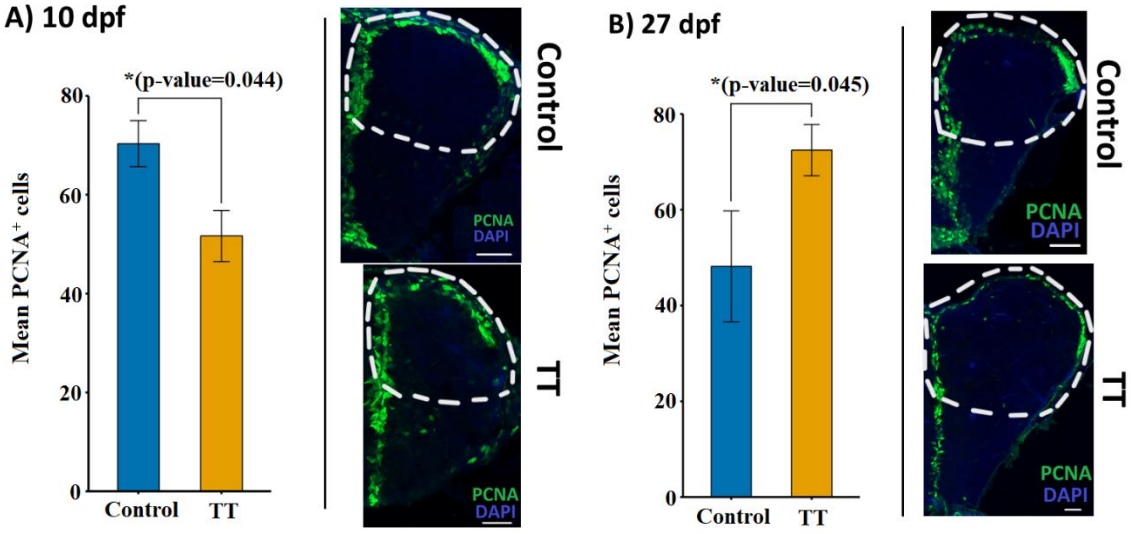



**Figure 4.2** Relative mRNA expression analysis showing the effects of daily thermal fluctuation during embryonic and larval stages on the mRNA expression of genes involved in neurogenesis of zebrafish, *Danio rerio*. Samples were collected from fish throughout the experiment, and the mRNA expression of each gene in the temperature treatment group was measured compared to a control group that did not experience temperature fluctuations. Due to low RNA concentrations, five larvae from fish younger than were pooled for RNA extraction, but 18 dpf fish and older were measured individually. The mRNA expression of each gene in plots is shown as fold-change downregulations and upregulations compared to the control group (1-fold); the mRNA expression level of these genes in the control group is shown with a horizontal line parallel to the x-axis. The statistical significance of the mRNA expression of each gene between experimental groups and also between males and females at 90 dpf was measured using a Mann-Whitney U test. To reduce the false positives, the p-value of the tests was adjusted to 0.005, and statistical differences are shown using an asterisk (*). No significant difference in the mRNA expression of neurodevelopmental genes was observed between males and females.

### 4.5.2 Radial-glial cells proliferation in the forebrain

The PCNA staining method was used to look at changes in the proliferation of radial-glial cells that sit in the niche, which borders the periphery of the dorsal pallium over development (Lindsey and Tropepe, 2006; Caron et al., 2022). Using PCNA staining, there were different radial-glial cell proliferation patterns in the pallium region of the forebrain at the four time points (i.e., 10, 27, 60, and 90 dpf; **Figure 4.3; Figure 4.4**). At 10 dpf, the proliferation of radial-glial cells in the pallium was significantly lower (p-value < 0.05) in the temperature treatment group compared to the control group. At 27 dpf, however, the result was opposite, and proliferation of cells was significantly higher (p-value < 0.05) in the temperature treatment group. Proliferation of radial-glial cells at 60 dpf did not show significant changes (p-value > 0.05) in response to early-life daily thermal fluctuations. Cell proliferation at 90 dpf, 60 days after terminating daily thermal fluctuations, was significantly lower (p-value < 0.05) in male zebrafish exposed to early-life daily fluctuations, but no significant difference was measured in females. In addition, the population of proliferating cells experiencing early-life thermal fluctuation did not show a significant difference between individuals predicted to be males and females (p-value > 0.05).



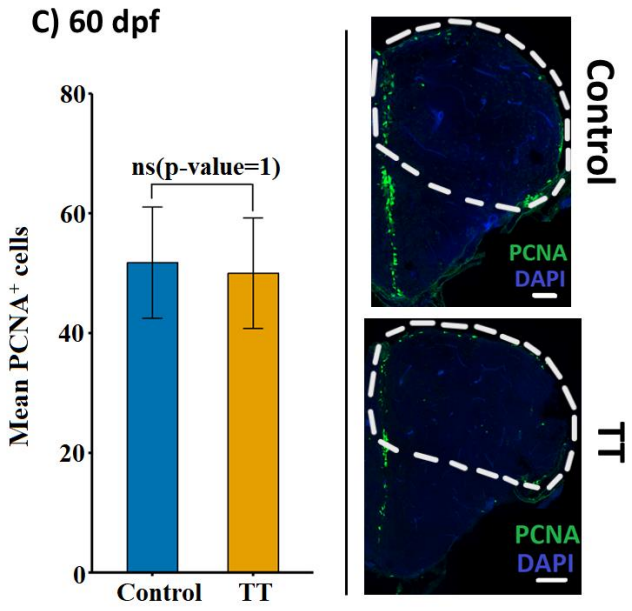



**Figure 4.3.** Histological results from PCNA staining showing the effects of daily thermal fluctuation during embryonic and larval stages on the proliferation of radial-glial cells in the forebrain in zebrafish, *Danio rerio*, during larval and juvenile stages. Samples were collected from fish at 10, 27, and 60 dpf, and the population of PCNA-positive cells was measured using immunohistochemistry on cross-section 71 of the forebrain. The mean PCNA-positive cells in the pallium region (marked by the dotted line) of the cross-section 71 and a chosen micrograph of stained tissue are presented in panels A (10 dpf), B (27 dpf), and C (60 dpf) for the temperature treatment (TT) and the control group. The PCNA-positive cells in the micrographs are labeled in green, and DAPI staining is shown in blue. The statistical significance (p-value < 0.05) in the mean PCNA-positive population between the temperature treatment and the control group was measured using Mann-Whitney U tests and shown with asterisks (*). The scale bars show 25 µm.

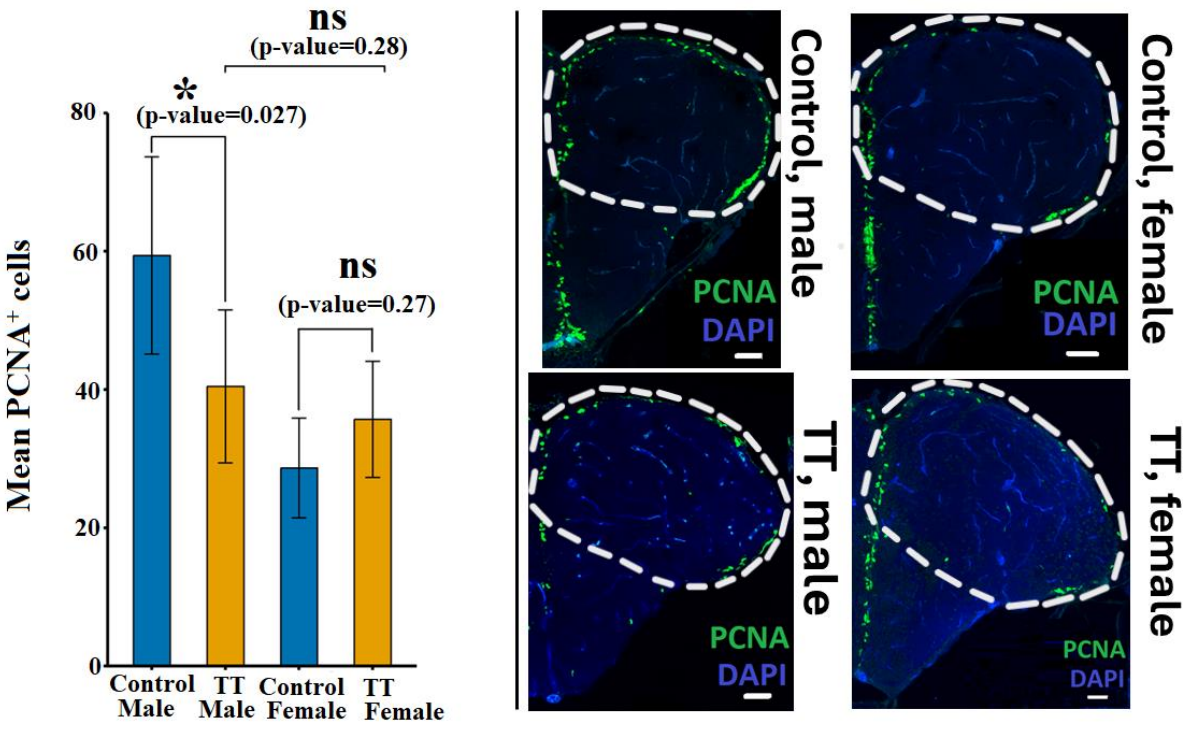



**Figure 4.4** Histological results from PCNA staining showing the effects of daily thermal fluctuation during embryonic and larval stages on the proliferation of radial-glial cells in the forebrain in adult zebrafish, *Danio rerio*. Samples were collected from fish at 90 dpf, and the population of PCNA-positive cells was measured using immunohistochemistry on cross-section 71 of the forebrain. The mean PCNA-positive cells in the pallium region (marked by the dotted line) of the cross-section 71 and a chosen micrograph of stained tissue are presented for the temperature treatment (TT) and the control group. The PCNA-positive cells in the micrographs are labeled in green, and DAPI staining is shown in blue. The statistical significance (p-value < 0.05) in the mean PCNA-positive population between the temperature treatment and the control group was measured using Mann-Whitney U tests and shown with asterisks (*). The scale bars show 25 µm.

### 4.5.3 Neuron density in the forebrain

The HuC/D is a protein often used as a marker of all neurons (Desmet et al., 2014). In our study, the density of neurons measured via HuC/D staining showed different responses to early-life thermal fluctuations depending on life stage (**Figure 4.5; Figure 4.6**). There was no significant difference in the neuron density at 10 dpf. However, at 27 dpf, the density of HuC/D-stained neurons was significantly higher in the temperature treatment group (p-value < 0.05). At

60 dpf, the difference in the neuron density between the temperature treatment and the control group was not statistically significant (p-value > 0.05). At 90 dpf, the density of neurons in the pallium region of the forebrain of males exposed to early-life daily temperature treatment was significantly higher than that of the control group (p-value < 0.05). However, the density of neurons in the females in the temperature treatment group did not show a significant difference from the control group (p-value > 0.05). In addition, the difference in the population of neurons in the temperature treatment group did not show a significant difference between males and females at 90 dpf (p-value < 0.05).

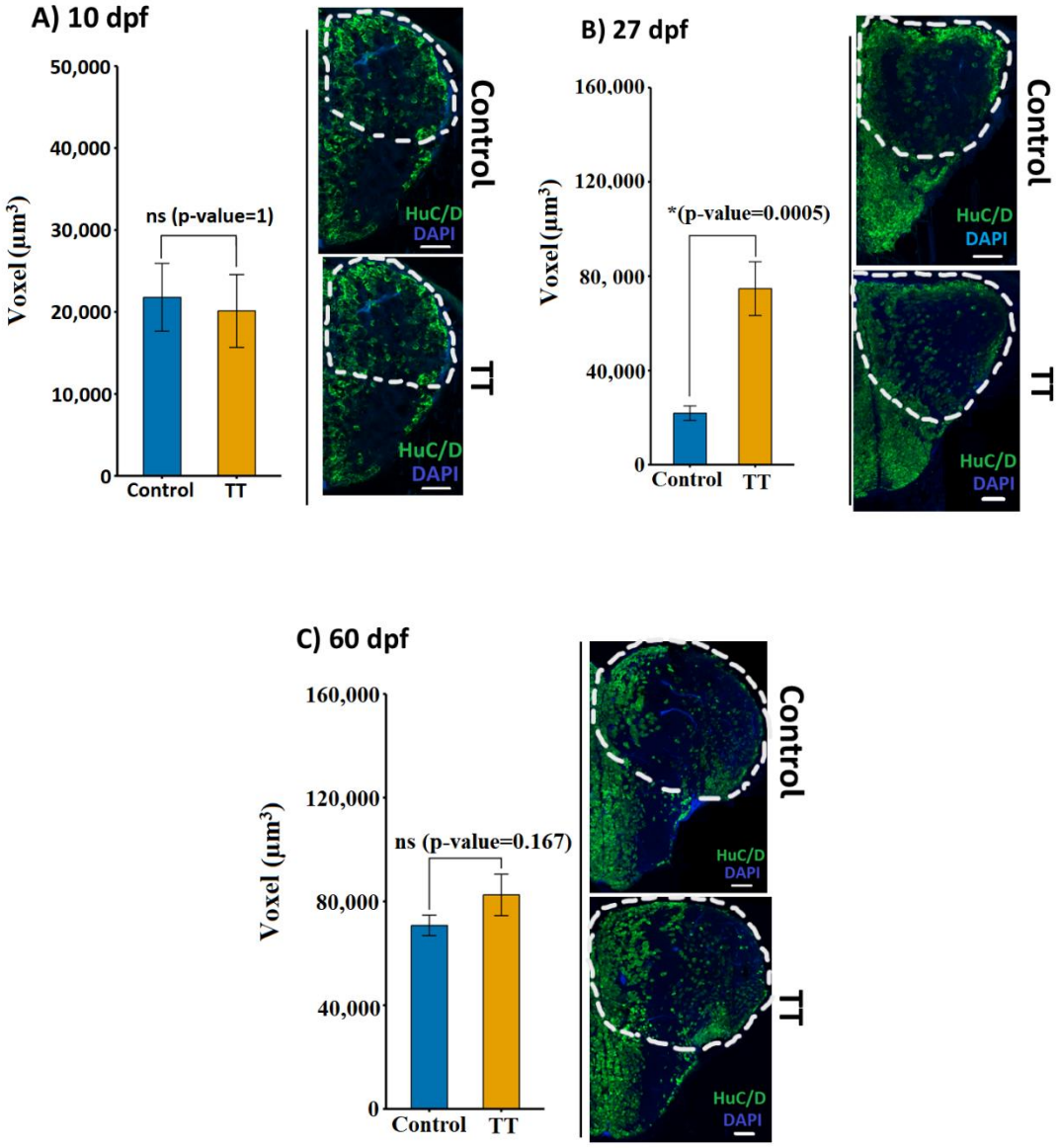


**Figure 4.5** Histological results from HuC/D staining showing the effects of daily thermal fluctuation during embryonic and larval stages on the neuron density in the forebrain of zebrafish, *Danio rerio*, during larval and juvenile stages. Samples were collected from fish at 10, 27, and 60 dpf, and the density of HuC/D-positive cells was measured using immunohistochemistry on cross-section 71 of the forebrain. The mean HuC/D-positive cells in the pallium region (marked by the dotted line) of the cross-section 71 and a selected micrograph of stained tissue are presented in panels A (10 dpf), B (27 dpf), and C (60 dpf) for the temperature treatment (TT) and the control group. The HuC/D-positive cells in the micrographs are labeled in green, and DAPI staining is shown in blue. The statistical significance (p-value < 0.05) in the mean HuC/D-positive population between the temperature treatment and the control group was measured using the Mann-Whitney U test and shown with asterisks (*). The scale bars show 25 µm.

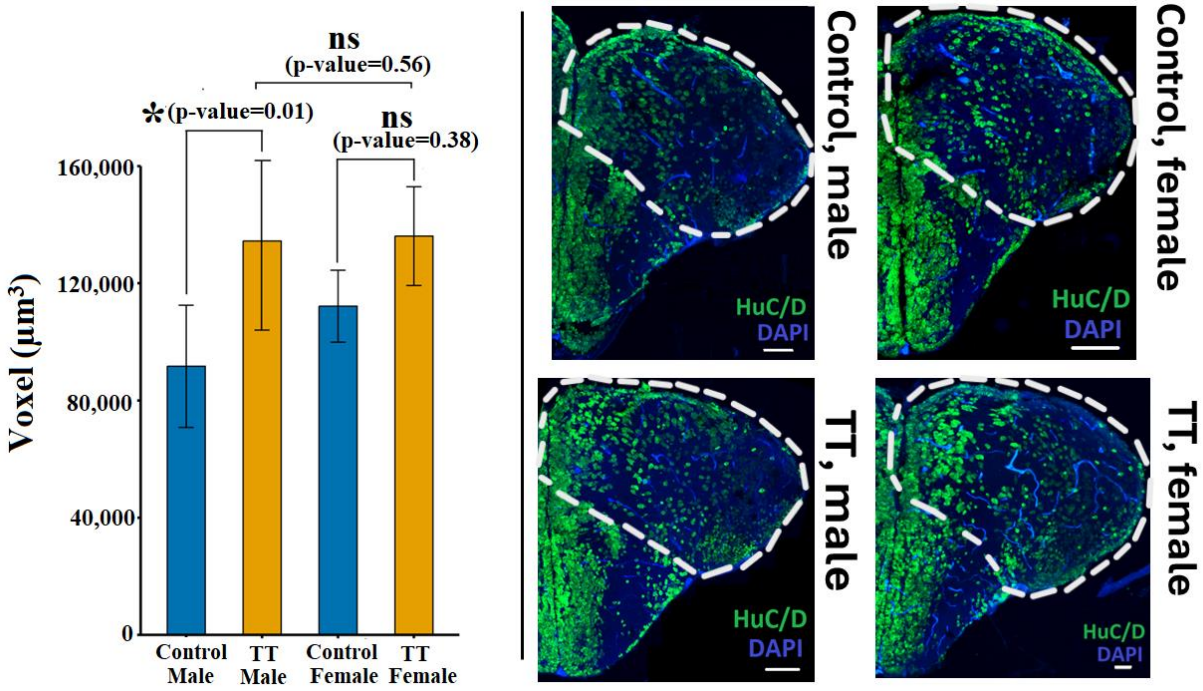



**Figure 4.6** Histological results from HuC/D staining showing the effects of daily thermal fluctuation during embryonic and larval stages on the neuron density in the forebrain of adult zebrafish, *Danio rerio*. Samples were collected from fish at 90 dpf, and the density of HuC/D-positive cells was measured using immunohistochemistry on cross-section 71 of the forebrain. The mean HuC/D-positive cells in the pallium region (marked by the dotted line) of the cross-section 71 and a selected micrograph of stained tissue are presented for the temperature treatment (TT) and the control group. The HuC/D-positive cells in the micrographs are labeled as green, and DAPI staining is shown in blue. The statistical significance (p-value < 0.05) in the mean HuC/D-positive population between the temperature treatment and the control group was measured using the Mann-Whitney U test and shown with asterisks (*). The scale bars show 50 µm

### 4.5.4 Behavior analysis

The movement data collected from the thigmotaxis test showed that the fish aged 15, 30, 60, and 90 dpf had 66-67%, 76-75, 90%, and 78-87% of their movement in the outer zone (**Figure 4.7**). In terms of time, fish at 15, 30, 60, and 90 dpf spent 71-79%, 82-83%, 93%, and 82-90% of their time in the outer zone. However, there was no significant difference (p-value > 0.05) in the total movement and time spent in the outer zone in the fish exposed to early-life daily thermal fluctuation, suggesting no increased thigmotaxis.

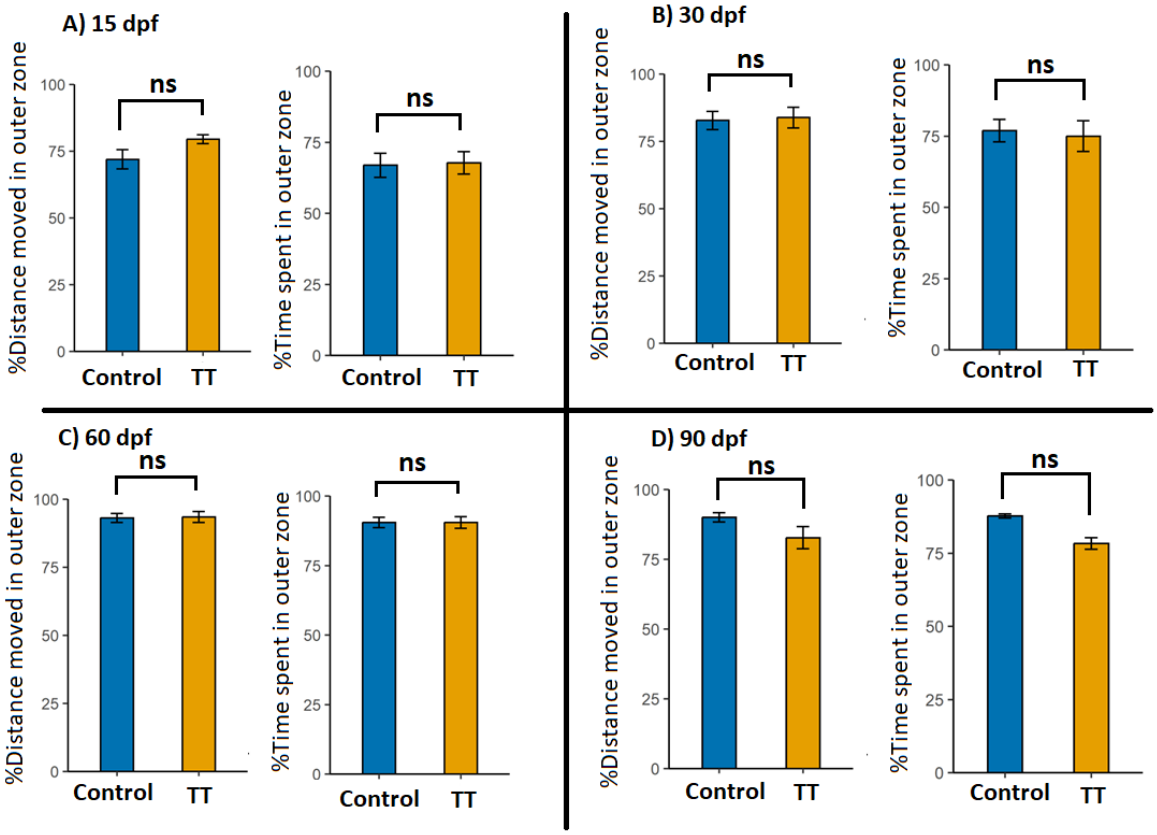



**Figure 4.7** Impact of early-life daily thermal fluctuations on the thigmotaxis in zebrafish, *Danio rerio,* was measured at A) 15, B) 30, C) 60, and D) 90 dpf. Thigmotaxis in the temperature treatment (TT) and control group was measured by placing the fish in an arena and tracking the movement of the fish in the outer and inner zones of each arena in a dark condition for 30 minutes. The percentage of total distance moved in the outer zone and the percentage of total time spent in the outer zone were measured and compared between the control and temperature treatment group using the Mann-Whitney U test. No statistical significance was measured between the two experimental groups, indicated by ns on the plots.

The dark/light locomotion test showed different results at different life stages (**Figure 4.8**). Overall, the mixed effect model showed that temperature treatment significantly affected the movement of fish measured at 15 (Estimate = 44.6, SE = 15, t = 2.97), 30 (Estimate = 26.8, SE = 10.98, t = 2.44), 60 (Estimate = 14, SE = 6.73, t = 2.09), and 90 (Estimate = 13.18, SE = 6.76, t = 1.94) dpf. At 15 dpf, the distance moved in both dark (Estimate = -44.7, SE = 15, t = -2.97, p-value = 0.0068) and light (Estimate = -49.1, SE = 15, t = -3.26, p-value =0.0034) conditions was significantly higher in the fish exposed to daily thermal fluctuations. At 30 dpf, the distance moved in the dark condition was statistically higher in the temperature treatment group (Estimate = -26.8, SE = 11, t = -2.44, p-value = 0.022) but did not show statistical significance in the light condition (Estimate = -16.8, SE = 11, t = -1.52, p-value = 0.14). At 60 dpf, the statistical test result was similar to 30 dpf, showing significantly higher movement in the dark condition (Estimate = -14.09, SE = 6.73, t = -2.09, p-value =0.047), but similar movement in the light (Estimate = 1.91, SE = 6.73, t = 0.28, p-value =0.77). In 90 dpf, the movement of fish in light conditions was significantly higher in the temperature treatment group (Estimate = -15.8, SE = 6.76, t = -2.33, p-value = 0.028), but this difference was not significant in the dark condition (Estimate = -13.2, SE = 6.76, t = -1.94, p-value = 0.063).

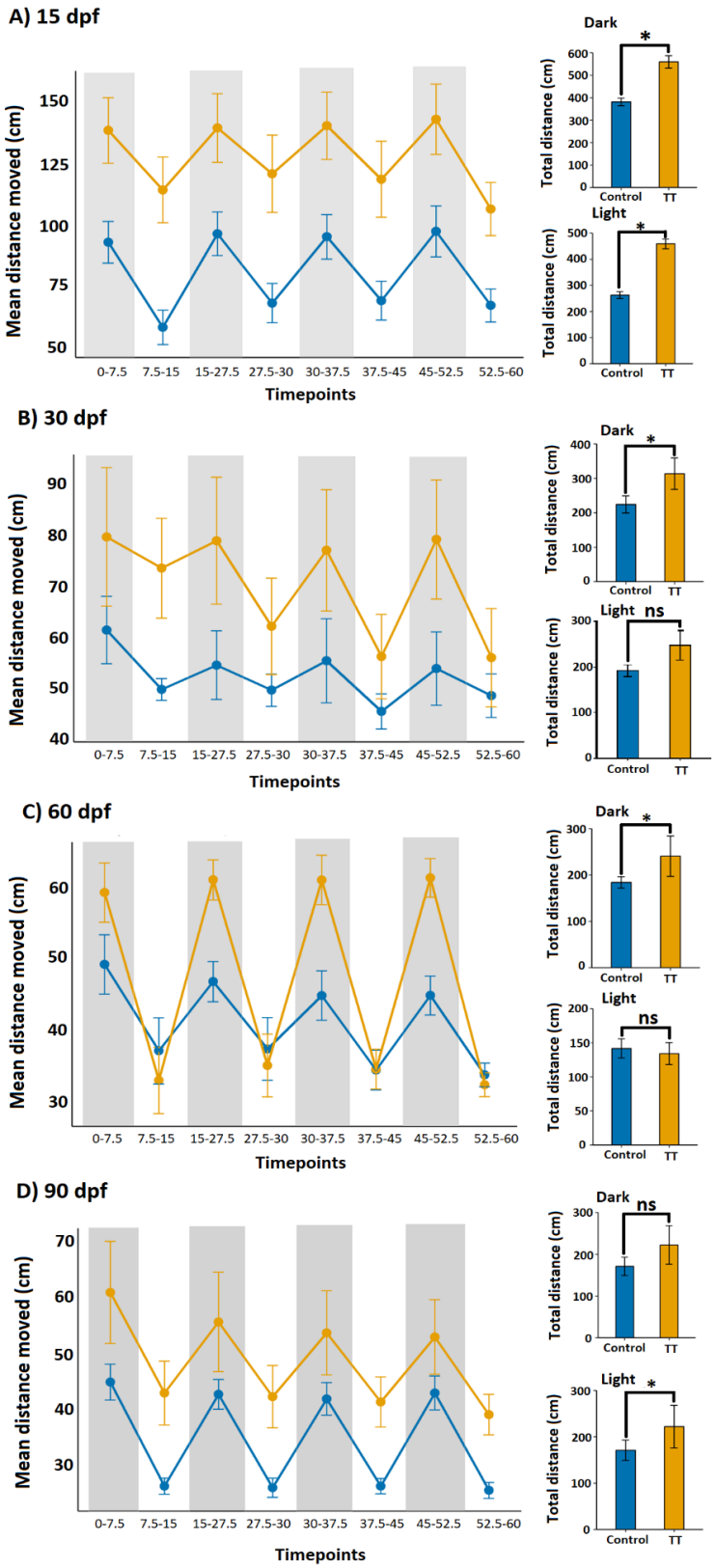



**Figure 4.8.** Light/Dark locomotion test performed at A) 15, B) 30, C) 60, and D) 90 dpf. The test was performed on both experimental groups: The temperature treatment group (TT), in which zebrafish were kept at 28 °C and experienced a thermal fluctuation from 28 to 33 °C every day for 2 hours during their embryonic and larval stage (0-30 dpf), then kept at 28 °C until 90 dpf, and the control group, in which the temperature was maintained at 28 °C throughout the experiment. To conduct this test, individual fish were placed in each arena for 1 hour, and the lighting condition alternated between dark and light every 7.5 minutes. Fish activity, measured as the mean distance moved at each time point, was compared between two experimental groups using a linear mixed-effects model. The statistical significance in the mean distance moved by fish between two groups (P-value < 0.05) is shown using asterisks (*); those without statistical significance (P-value<0.05) are indicated as not significant (ns) on the plot.

## 4.6 Discussion

The brain is an important organ involved in perceiving, regulating, and acclimating to new temperature fluctuations (Guan et al., 2025). The dorsal part of the forebrain, the pallium, is known to be involved in many vital functions, such as receiving sensory information, learning, memory, and avoidance response in ray-finned fishes (Kaplan et al., 1967; Rodriguez et al., 2002; Broglio et al., 2005; Vargas et al., 2009; Butler, 2024). This part of the brain might play a role in the perception and response to heat in fishes. For example, in Arctic sculpin, *Myoxocephalus scorpioides*, changes in forebrain temperature have been reported to increase the activity of fish in terms of behavioral thermoregulatory response to avoid unfavorable temperatures (Stromme et al., 1971). In our study, we investigated the effects of early-life thermal treatment on the proliferation of radial-glial cells and neuron density in the telencephalic pallial niche, along with the mRNA expression of genes involved in neurogenesis and brain development. Our results showed that exposure to daily thermal fluctuations during the embryonic and larval stages changed the mRNA expression of seven out of nine genes studied in zebrafish, with a variable mRNA expression pattern throughout life stages. Histological data also showed a significant decrease (10 dpf) and then an increase (27 dpf) in the proliferation of radial-glial cells in the forebrain of larvae and adult zebrafish, but not in juvenile fish. In addition, the neuron density in the forebrain showed significant changes in the later stages of larval development and adult fish, but the early larval stage and juvenile fish did not show significant changes. In addition, fish that experienced daily thermal fluctuations in their embryonic and larval stages showed higher activity in response to changing light conditions.

Neurogenesis in zebrafish starts a few hours after fertilization with the formation of the neural plates, which will then chronologically transform to the neural keel, neural rod, neural tube, and eventually the central nervous system (Chapouton and Godinho, 2010). The first 24 h of neurogenesis are usually called primary neurogenesis, during which different types of motor neurons and sensory neurons are produced, and are followed by secondary neurogenesis (Alunni et al., 2020). The process of neurogenesis is usually controlled by the production and secretion of a variety of different signaling molecules and growth factors (Schmidt et al., 2013). Transcription factors of the neural basic helix-loop-helix class, such as Neurogenin, and their downstream mediators, such as Neurod, play important roles in the regulation of vertebrate

neurogenesis and are required for neural commitment (Seo et al., 2007). In our study, the mRNA expression patterns of most neurodevelopmental genes showed an increase in fold-change upregulation in the larval stage, but a downregulation trend at later larval stages. After removing daily thermal fluctuation, the mRNA expression pattern of most genes showed an upregulation at 35 dpf, but then the trend diminished by the end of the juvenile stage, and no difference was detected in the adult fish. It seems that early-life thermal fluctuations can affect the mRNA expression of regulatory factors, but these changes are reversible through acclimation.

The histological data in our study showed that early-life daily thermal fluctuation decreased the proliferation of radial-glial cells at 10 dpf, but the density of neurons was not different between treatments. This discrepancy between cell proliferation and neuron density in our results can be caused by different factors. We used PCNA staining as a marker for measuring the proliferation of brain cells. This staining method stains all the dividing cells in G1 and S phases, some of which may differentiate into neurons or glia (Raucci et al., 2006). Therefore, it is possible that the reduced proliferation detected in our study might be due to the reduced proliferation of glial cell progenitors caused by thermal treatment. However, some of this reduced proliferation might have resulted in reduced neurogenesis as environmental stressors, such as temperature, have been known to affect mRNA expression of neurodevelopmental genes and neurogenesis in animals (Penafiel et al., 2001; Kanagawa et al., 2006; Koyama et al., 2018; Tang et al., 2022; Mete et al., 2023). Animals' nervous systems are known to compensate for dead or damaged neurons via synaptogenesis and dendritic remodeling, which can result in increased neural density (Cotman and Scheff, 1979; Fuchs et al., 2006). In our study, the decrease in the number of proliferating cells at 10 dpf might have been compensated for by increased neuronal connectivity. At 27 dpf, both proliferation and differentiation increased significantly. This increase in the proliferation of radial-glial cells and neuron density at 27 dpf, following the decreased proliferation at 10 dpf, might be compensatory growth after removing daily thermal fluctuations.

Furthermore, gonadal maturation has been reported to increase the proliferation of radial-glial cells in blackhead seabream, *Acanthopagrus schlegelii* (Lin et al., 2015). In *D. rerio*, ovaries and testes start differentiating at 15 and 30 dpf, respectively, and by 60 dpf, they are fully differentiated (Wang et al., 2022). In addition, early-life temperature is known to be important in

sex determination in the zebrafish and influences the sex ratio toward male-biased sex determination (Sfakianakis et al., 2011; Villamizar et al., 2012; Santos et al., 2017). Although we did not extensively investigate the effects of thermal fluctuation on gonadal differentiation and sex ratio, the sex ratio in the temperature treatment group might have been affected by daily thermal fluctuation, influencing the neurogenesis and neuron density measured in the brain at 27 dpf. Furthermore, sexual maturation in zebrafish is known to be influenced by size rather than age (Chen and Ge, 2013). Therefore, the timing of gonadal differentiation or maturation could be influenced by daily thermal fluctuation, influencing neurogenesis at 27 dpf.

The neurogenesis and structural changes in the brain can continue in adult animals to some degree, especially in response to environmental changes (Aoki and Siekevitz, 1988; Radley and Morrison, 2005; Johansen-Berg, 2007; Kolb and Gibb, 2011; Kizil et al., 2011). In our study, at 60 dpf, 30 days after stopping daily thermal fluctuation, the proliferation and density of neurons were not significantly different between the two experimental groups. Considering that the proliferation and density of neurons were increasing at the end of the larval stage, it seems that the increase did not continue after the termination of thermal fluctuation. The mRNA expression of neurodevelopmental genes is consistent with these changes, as there was an upregulation of *ngn3*, *nrd1*, *otpa,* and *otpb* until 35 dpf, but this pattern was more variable after 35 dpf. The proliferation and differentiation of radial-glial cells in the brain are usually a dynamic process and are highly regulated (Hardwick et al., 2015). Mechanisms such as asymmetric neurogenic divisions and apoptosis control the number of cells in the brain (Homem et al., 2015). In our study, it seems that after removing the daily thermal fluctuations and acclimation to 28 °C, the proliferation of radial-glial cells and neural density in the temperature treatment group decreased, and toward 90 dpf, there was no difference between the females. In addition, no significant changes in the mRNA expression of neurodevelopmental genes were observed at 90 dpf. It seems that the proliferation of radial-glial cells in the brain and neurogenesis is a dynamic process during development, influenced by environmental parameters such as temperature.

Although no significant changes between the two sexes that experienced temperature treatment were observed in our study, the changes in the proliferation and population of neurons were significant in the males. Sex hormones are known to affect the proliferation of cells in the brain, which might be responsible for differences in the proliferation of radial-glial cells detected

in our study (Diotel et al., 2013). In addition, there are studies that show the proliferation of cells in the zebrafish forebrain is sexually dimorphic (Ampatzis et al., 2012). This dimorphic pattern in the proliferation of neurons in response to environmental stressors has been reported before in fish experiencing social stress; Specifically, when fish were kept with dominant conspecifics, male fish experiencing the stress had higher cortisol levels and lower cell proliferation in the brain (Tea et al., 2019). This difference in the proliferation of radial-glial cells and neuron density suggests sex-specific influence of early-life temperature treatment on neurogenesis in *D. rerio*.

Changes in the environmental factors can affect cell proliferation and cause structural changes (Von Krogh et al., 2010; Sørensen et al., 2012; Kolb et al., 2014; Takada et al., 2016; Zhang et al., 2019). These structural changes in the brain caused by early-life treatments are known to be long-lasting and detectable in adult animals, sometimes accompanied by behavioral changes (Hanson et al., 1969; Eachus et al., 2021). Early-life thermal fluctuations increased total zebrafish activity in both dark and light, and these effects persisted into 60 and 90 dpf. Increased dark/light locomotion is reported to be influenced by stress hormones such as epinephrine and cortisol (Basnet et al., 2019). This might support the hypothesis that early-life thermal fluctuations served as a stressor, increasing the release of stress hormones, as reported in several species (Ryan, 1990; LeBlanc et al., 2012; Hanke et al., 2019). Fish reared at higher temperatures often exhibit higher basal cortisol levels (Alfonso et al., 2022). Studies on *D. rerio* have shown that animals that experience early-life stress have higher basal levels of stress hormones, even months after removing the stressor (Chin et al., 2022). The stress response caused by stress hormones often triggers fight-or-flight response in animals to help with survival (McEwen, 2006). Because of their ectothermic nature, fish can only change their internal temperature through behavioral mechanisms such as moving in the environment in order to find a place with a favorable temperature (Haesemeyer, 2020). Increased activity in response to increased temperature has been reported in barred killifish, *Pseudoxiphophorus jonesii* (Culumber, 2020). In addition, larval zebrafish are known to be able to navigate heat gradients in the laboratory via behavioral strategies such as increasing their vigorous swimming and increasing the frequency and speed of swim bouts (Haesemeyer, 2020). In our study, it is possible that, during thermal shock, there was a heightened stress response to the temperature

treatment that increased the search for a more favorable temperature to behaviorally regulate temperature.

Physical activities and exercise are also known to affect neurogenesis in animals (Van Praag, 2008). In zebrafish, for instance, restrained movement has been observed to promote cell differentiation over self-renewal in the neural precursor population of the forebrain (Hall et al., 2018). It is possible that, other than physiological changes caused by increased temperature, increased movement has also affected the proliferation of radial-glial cells and neuron density in the forebrain of thermally challenged zebrafish. Increased neurogenesis in the brain in response to increased physical activity has also been observed in adult mammals (Fabel and Kempermann, 2008; Yau et al., 2014). In our study, the increased activity that continued to the adult stage in the group that experienced thermal fluctuations might be responsible for the changes in proliferation and population of neurons in the forebrain of juvenile and adult zebrafish.

In conclusion, the mRNA expression, histological, and behavioral analyses suggested changes in the development of the brain and behavior of zebrafish reared under thermal fluctuations during embryonic and larval stages. These effects were detected mostly in the embryonic and larval stages, but were traceable in the adult animals even after acclimation to a stable temperature. These results indicate the importance of temperature during early critical stages of development and its consequences in the long term. We found that early-life thermal treatment can affect the proliferation of cells and the density of neurons in the brain, but details about the population of specific neurons or glia were not studied in this research, which can be investigated in the future. In addition, we speculate that significant increases (27 dpf) and decreases (90 dpf) of radial-glial cells, followed by increases (27 and 90 dpf) in the neuronal density in the brain, might be the result of thermal stress, and it is worth investigating in future studies measuring physiological indicators of stress, such as stress hormones.

## 4.7 Acknowledgment

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# Chapter 5. Long-term effects of early-life thermal stress on the mRNA expression of skeletal muscle structural proteins of zebrafish, *Danio rerio*

## 5.1 Abstract

Temperature in aquatic environments often fluctuates depending on the time of day or season. Aquatic animals living in these environments have adopted mechanisms to regulate their metabolism to cope with these routine temperature changes. However, changes in the average temperature of aquatic ecosystems caused by climate change increase the amplitude of these thermal fluctuations and affect the life of animals exposed to this condition, especially ectotherms. Because of higher vulnerability to environmental stressors, the effects of thermal fluctuations are more severe during early-life stages. In this study, we investigated the effects of early-life daily thermal fluctuations on the genes coding structural proteins of muscle in the zebrafish, *Danio rerio*. Wild-type zebrafish were bred in the lab and divided into two groups: a temperature treatment group and a control group. In the temperature treatment group, offspring were kept at 28 °C but experienced a +5 °C increase in temperature over a 2 h period every day during the embryonic and larval stages, followed by a decrease to 28°C until 90 days post fertilization (dpf). Zebrafish in the control group did not experience any thermal fluctuations, and the temperature was kept constant at 28 °C throughout the experiment. Samples were collected from each group at 12, 24, and 50 hours post fertilization (hpf), and 6, 10, 15, 18, 21, 24, 27, 30, 35, 40, 45, 50, 60, and 90 dpf to measure the mRNA expression of *act1a*, *act1b*, *fmyhc1.1, fmyhc1.2, fmyhc1.3, fmyhc2.1, fmyhc2.2, smyhc1,* and *smyhc2* in the temperature treatment group relative to the control group. Based on our results, early-life thermal fluctuations downregulated the mRNA expression of most fast myosin heavy chain isoforms and actin measured in this study in the larval stages; However, the mRNA expression pattern of these genes showed a greater upregulation trend in juvenile and larval stages after removing daily thermal fluctuations. Overall, early-life thermal fluctuations affected the mRNA expression of genes coding structural muscle proteins that were even detectable after terminating thermal fluctuation in the juvenile and adult fish. Our results highlight the importance of early-life thermal treatments on the mRNA expression of genes coding for proteins involved in the structure of muscle.

## 5.2 Introduction

Temperature is one of the most important abiotic factors influencing the lives of fish (Bartolini et al., 2015). The temperature in aquatic ecosystems is not always stable and can increase due to natural and anthropogenic factors (Akbarzadeh et al., 2018). Animals living in these ecosystems can tolerate natural changes in the water temperature (Goldspink, 1995). However, extreme changes in temperature can have detrimental effects on aquatic animals, such as impacting their immune response or reproductive abilities (Abram et al., 2017; Pankhurst and Munday, 2011). The effects of temperature changes are particularly significant for ectothermic animals, such as fishes, whose body temperature is equal to or near the temperature of the surrounding water, and temperature changes can directly affect their physiological and behavioral activities (Alfonso et al., 2020; Fatma and Ahmed, 2020).

Temperature changes can affect the metabolism and production of proteins in fishes (McCarthy and Houlihan, 1996). If food is not limited, increased temperature within a particular range for each species can increase the production of proteins (Hidalgo et al., 1987; Mathers et al., 1993; Huang et al., 2016). In addition, increased food intake, which usually happens with a moderate temperature increase, can help with the increased production of proteins (Volkoff and Rønnestad, 2020). As a result, fish reared at slightly higher temperatures usually have higher growth rates (Mathers et al., 1993; Jobling, 1995; Burel et al., 1996; Person-Le Ruyet et al., 2004). However, growing under temperatures higher than the natural temperature range can have negative effects on the metabolism of proteins in animals, especially increasing proteolysis and autophagy in the protein-dense tissues such as muscle (Ma et al., 2020; Molina et al., 2023; Becerra et al., 2025).

Myofibrillar proteins, such as myosin and actin, account for 66 to 77 % of the total proteins in fish muscle (Phillips and Williams, 2011). Vertebrates usually have several isoforms of each myofibrillar protein in the structure of their muscle (Schiaffino and Reggiani, 1996). Several studies have demonstrated that temperature influences the expression of myofibrillar proteins in a range of fishes in embryonic, larval, and adult stages (Xie et al., 2001; Wilkes et al., 2001; Buckley and Somero, 2009; Campos et al., 2013; Ikeda et al., 2017; Coughlin et al., 2025). In

addition, changes in the quantity and size of muscle fibers are observed in fish species that are grown at different temperatures (Stickland et al., 1988).

In this study, we investigated the long-term effects of early-life thermal fluctuations on zebrafish, *Danio rerio*. We tested the hypothesis that exposure to early-life thermal stress can affect the mRNA patterns of structural proteins in the skeletal muscle of adult zebrafish. To test this hypothesis, we exposed newborn zebrafish to daily thermal shock (+ 5 °C increase from 28 °C) at the embryonic and larval stages (0-30 days post-fertilization (dpf)), then acclimated them to 28 °C until adult stage (90 dpf). Then, fish were sampled at embryonic, larval, juvenile, and adult stages, and the mRNA expression of nine structural proteins was measured using qPCR. Our results showed that early-life thermal fluctuations changed the mRNA expression of genes coding for proteins involved in skeletal muscle structure, but these effects were stage-dependent and highly variable between individuals.

## 5.3 Materials and Methods:

### 5.3.1 Fish breeding and husbandry

The experiment was approved by the animal care committees at the University of Manitoba (22-030 (AC11760)) in accordance with the guidelines of the Canadian Council on Animal Care. Wild-type AB (WT-AB) zebrafish were bred and reared in the Rady Biomedical Fish Facility at the University of Manitoba, Winnipeg, Manitoba, Canada, on a 12 light (7 am):12 dark (7 pm) light cycle unless stated otherwise. Fish were housed on a recirculating Tecniplast housing system (Tecniplast Inc. USA), equipped with automatic dosing, UV and carbon filters, with a stable pH and conductivity of 7.4 and 900 µS, respectively. Facility water was maintained at 28ºC.

For breeding, two female and three male zebrafish were placed into a breeding tank in the evening, with a meshed bottom to separate the fish from the eggs and facilitate embryo collection the following day. To mimic the natural habitat, artificial plants were placed in the breeding tank. Breeding occurred the following morning with initiation of the light cycle, stimulated by the gradual increase in light intensity. Fertilized eggs were collected and transferred to Petri dishes

with embryo medium (0.01 mg. $L^{-1}$ methylene blue dissolved in fish facility water) at a maximum density of 25 embryos $dish^{-1}$ Petri dishes were then transferred to a Fisherbrand 18L Low Temp Incubator (Thermo Fisher Scientific, Pittsburgh, USA). To reduce the potential for vertical transmission of pathogens and fungi from parents to offspring, embryos were bleached at 1 dpf by them in 7 $mg.L^{-1}$ chlorine for 5 min, followed by three washes with embryo medium, each for 3 min in three separate containers, as described by Kütter et al. (2023), with minor modifications to the bleach concentration. Embryos were maintained in the incubator until 5 dpf, when almost all the larvae were hatched and their nutrient-rich yolk sacs depleted.

Upon depletion of the yolk sac at 5dpf, larvae were fed two portions of GEMMA Micro 75 (Skretting, Maine, USA) and one portion of live rotifer daily. Juvenile fish (> 30 dpf) were fed two portions of GEMMA Micro 150 and one portion of brine shrimp nauplii (Brine Shrimp Direct, Ogden, USA) daily, as described by Varga (2016), with minor modifications in the dry food brand. Temperature, oxygen, and ammonia in the tanks were measured throughout the experiment. In addition, total length at 15 and 30 dpf and fork length at 60 and 90 dpf were measured and compared between the two groups using the Mann-Whitney U test ($p$-value < 0.05).

### 5.3.2 Temperature treatment

Immediately following embryo collection at 0 dpf, zebrafish were separated into two groups: (1) temperature treatment (TT), and (2) control (C). From 0 to 6 dpf, the fish were held in incubators where the temperature regimes were maintained for the two groups. At 6 dpf, larvae were transferred to 18 (L) × 14 (W) × 10 (H) cm aerated containers filled with fish facility water up to 3 cm until 30 dpf. Then, the containers were placed in 56 (L) × 30 (W) × 30 (H) cm coolers. Four coolers were dedicated to each group, and each of them contained three containers. Each container in a cooler was treated as one tank replicate. The coolers were filled with distilled water, and the temperature was maintained at 28 °C by using electric heaters placed at the bottom of each cooler. To maintain a homogeneous temperature in the coolers and, subsequently, in the containers, the water in the coolers was constantly circulated by a pump. Fish were kept in these containers until the end of the larval stage (30 dpf), after which fish were transferred to

standard 3.5 L Tecniplast Inc. zebrafish tanks (27 (L) × 11 (W) × 16 (H) cm) and reared on a Tecniplast recirculating aquatic housing system at 28 °C until the end of the experiment (90 dpf) as described above. The initial number of tanks for each group differed from 10 to 12, and the density of fish in each tank was kept at 8-12 fish per tank. However, the number of tanks decreased as fish were haphazardly sampled from tanks throughout the experiment, and the remainders were combined to maintain a density of 8-12 fish per tank.

In the temperature treatment group, at 12:00 pm daily, prior to feeding, the temperature was raised to 33 °C from ambient 28 ºC water at the rate of ~0.8 °C $min^{-1}$ and then decreased at the same rate back to 28 °C over a 2-h period during the embryonic and larval stages (0-30 dpf). The control group experienced similar handling but did not experience any thermal fluctuations during embryonic and larval stages, and the temperature for this group was kept constant at 28 °C throughout.

### 5.3.3 Sex identification

We attempted to determine the sex of each fish before mRNA expression analysis to avoid misinterpretation of results caused by sex-biased mRNA expression of genes coding for muscle proteins. Due to difficulties in distinguishing males from females through visual inspection, especially in younger fish, and also the lack of genetic sex markers, we used transcriptomic methods for sex determination. To determine the sex of each fish based on mRNA expression, 12 genes that were previously reported to have sex-biased expression were chosen (Wong et al., 2014; King et al., 2020; **Table 5.1**). To test the accuracy of these genes for sex determination, the mRNA expression patterns were tested on 5 adult male and 5 adult female zebrafish (10-month-old) with clear external sex-specific characteristics that were visible under a microscope. Since the candidate genes used for the determination of sex may be expressed in different organs, such as the brain and the ovary, RNA was extracted from a homogenized sample of each fish. To do so, the entire fish was frozen in liquid nitrogen and ground in a mortar using a pestle. Total RNA extraction, cDNA synthesis, and qPCR were conducted as before. Then, the mRNA expression of these 12 genes in females was measured relative to males using Pfaffl's method (Pfaffl, 2001). The sequence of primers used for amplifying these genes in qPCR, along with the mRNA

expression of each gene in females relative to males, is listed in **Table 5.1**. Among the 12 genes tested, *bmp15*, *ccnb1*, *gyg1a*, *kpna2*, *rdh10b*, and *zp3* showed a higher difference in mRNA expression between males and females and were chosen to be used in this study.

We used a Random Forest technique to determine the sex of individuals from the thermal exposure trials using the mRNA expression of genes with sexually biased patterns. To do so, the mRNA expression of *bmp15*, *ccnb1*, *gyg1a*, *kpna2*, *rdh10b*, and *zp3* was measured for each individual using qPCR, and the $C_t$ (cycle of threshold) value was normalized to the geometric mean of internal control genes *ef1a*, *gapdh*, and *rpl13* ($Ct_{(target\ gene)}$-$Ct_{(control\ gene)}$). Then, the normalized $C_t$ values from adult fish with known sex were used to train a Random Forest model using the Random Forest package in R (Liaw and Wiener, 2002). The performance of the model was evaluated using the classification accuracy test, which is defined as the proportion of correctly predicted instances among the total number of instances. After training the model, the normalized $C_t$ of the six sexually biased genes checked in the individuals from the temperature treatment group and control group was used to estimate the sex of that individual using the trained model.

**Table 5.1** List of primers and probes for amplification of genes used for sex determination in zebrafish, *Danio rerio*, along with their fold-change mRNA expression in females relative to males.

| Gene name | Primer sequence | PCR efficiency (%) | Fold-change expression in females |
|---|---|---|---|
| *gyg1a* | F: AGTATGCCAAAGGAGCAATGGTA<br>R: GCACATCCACCAGCCTGACT | 94 | 20.6 |
| *rdh10b* | F: TGCACCCCTCGGGTCAT<br>R: TGCACTTATCAGCCCCAATAAA | 91 | 73.9 |
| *cyp19a1b* | F: GCCTGTGTTGGGAAGCACAT<br>R: ACTGGCTGCATGGAGAGGTT | 96 | 0.66 |
| *sox9a* | F: CGACGCATATTAAAACCGAACA<br>R: TACTGCGCTCTGGTGATGGA | 94 | 0.55 |

| | | | |
|---|---|---|---|
| *dio2* | F: GCCTGGCTTCTTCTCCAACTG<br>R: GCCGGCTGTGGTTAACATG | 92 | 0.69 |
| *bmp15* | F: GAGGCGAGTGTCACGTCTCTT<br>R: TGTGCACCAATAACGAGCAAA | 110 | 507.1 |
| *ccnb1* | F: TGTTGGTGTAACGGCCATGT<br>R: GGTGGTGTATGCACGGTCTGT | 97 | 98.6 |
| *kpna2* | F: CCCATGTTCCCAGCTCTGTT<br>R: TCCACCAGGTACGGCACAAT | 100 | 45.5 |
| *zp3* | F: TGTGCCTCTGCGTGTGTATGT<br>R: AAGTGGGAGCTGGAACCTGTAG | 100 | 11806.6 |
| *hsd3b1* | F: AGGCTGGTTCGACTGTTGCT<br>R: GTCTCTCCCCGGCAATCAT | 100 | 0.92 |
| *hsd17b3* | F: GCCAAAATACCATGTCCCATTT<br>R: TCCTGTCATTGCGGTTGAAA | 98 | 0.33 |
| *igf1* | F: CAGCAAACCGACAGGATATGG<br>R: ATCGTGGAGATTTGCCTGTCTT | 92 | 0.4 |

### 5.3.4 Relative mRNA expression

We collected 10-15 fish from each group at each timepoint to study changes in mRNA expression: 1 embryo from each Petri dish at embryonic stage (1 individual × 15 Petri dish × 2 groups × 3 timepoints), 1 fish from each container at larval stages (1 individual × 10 containers × 2 groups × 8 timepoints), 1 fish from each tank at juvenile stage (1 individuals × 10 tanks × 2 groups × 5 timepoints) and 2 fish from each tank at 90 dpf (2 individuals × 5 tanks × 2 groups × 1 timepoints). Samples were collected in the morning after euthanizing the fish with tricaine (0.4%, pH 7.4), and were preserved in RNA*later* (Thermo Fisher Scientific, Waltham, USA). After 24 h of preservation at 4 °C, samples were transferred to a -80 °C freezer for storage. Total RNA was extracted using MagMax mirVana™ Total RNA Isolation Kit (Applied Biosystem, Germany) on a KingFisher Duo Prime (ThermoFisher Scientific, USA) machine. Briefly, samples were homogenized using a TissueLyser (QIAGEN, Germany) at 50 Hz for 5 min in the

lysis buffer provided by the kit. The homogenized tissue was centrifuged and transferred to an extraction plate, and RNA purification was conducted following the manufacturer's protocol. Because of low concentration in the RNA extracted from the embryos and larvae younger than 18 dpf, RNA extraction for these fish was conducted from a pool of five individual eggs or larvae. However, for 18 dpf fish and older, RNA was extracted from each fish separately. The quantity and purity of extracted RNA were measured using a NanoDrop One spectrophotometer (ThermoFisher Scientific). For RNA integrity, we loaded 400 ng of RNA on a 1% agarose gel and checked the 18S and 28S rRNA bands. Before cDNA synthesis, 300 ng of total RNA from each sample was treated with Invitrogen™ *ezDNase* enzyme (Thermo Fisher Scientific, USA) at 37 °C for 2 min to eliminate genomic DNA. Then, the DNase-treated RNA was used for the cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) following the manufacturer's instructions.

We used qPCR to measure and compare the mRNA expression of nine genes coding skeletal muscle structural proteins (**Table 5.2**). Gene sequences were downloaded from the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/), and primers were designed using Primer Express (3.0.1) (Thermo Fisher Scientific) (size: 17-30 bp; Melting temperature (Tm): 58-60 °C; GC%: 30-80%). For the qPCR, the synthesized cDNA was diluted 1:4 with nuclease-free water. Then, 5 µl of the diluted cDNA was mixed with 6 µl of PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, USA), 0.1 µl of forward primer (10 mM), 0.1 µl of reverse primer (10 mM), and 0.8 µl of nuclease-free water in a 384-well plate. The qPCR was performed using a QuantStudio 5 (Applied Biosystems, USA) machine, and the thermal conditions for qPCR included initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 20 s. Melt-curve analysis was also included in the analysis to confirm that there was no non-specific amplification or primer-dimer formation. The efficiency of primers was measured by generating standard curves using serial dilution. The method described by Pfaffl (2001) was used to analyze mRNA expression data using three internal control genes for normalization. Because the data did not fit a normal distribution, as determined by Shapiro–Wilks's tests, Mann-Whitney U tests were used to determine significant differences between the temperature treatment and control group. The same test was also used to compare the difference in the mRNA expression of each

gene between males and females. To reduce the false positives, the p-value of the tests was adjusted to 0.005 using a Bonferroni correction.

**Table 5.2**. List of primers used for amplification of genes coding skeletal muscle functional proteins in zebrafish, *Danio rerio*, along with internal control genes *gapdh, rpl13,* and *ef1a*.

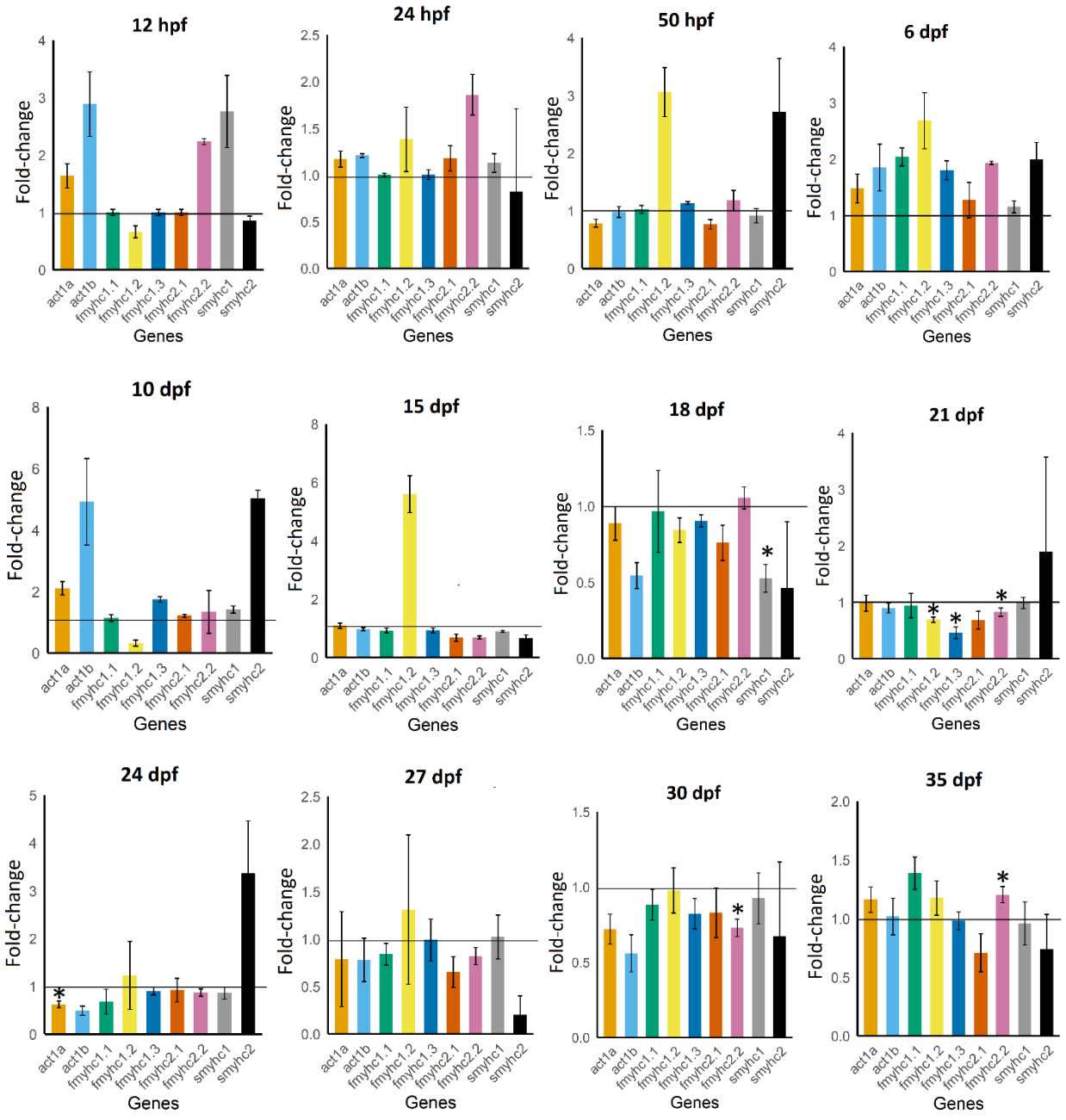
| **Gene symbol** | **Protein** | **PCR Efficiency (%)** | **Primer sequence** |
|---|---|---|---|
| *act1a* | actin 1a | 92 | F: TGACGATGATGAGACCACAGCTT<br>R: ATGGATGGGAAGACGGCTCTA |
| *act1b* | actin 1b | 103 | F: CATCGAGCACGGCATCATT<br>R: GTCAGCAGAGTTGGGTGCTCT |
| *fmyhc1.1* | Myosin heavy chain 1.1, fast skeletal muscle | 90 | F: TGAGAAGCAGAGAGCCGATCTT<br>R: GCTTCACGCTTCTTATTCATCTCA |
| *fmyhc1.2* | Myosin heavy chain 1.2, fast skeletal muscle | 100 | F: CCCAGAGGGACAGTTCATTGAT<br>R: AACTTGCTGCTCAAGCTTTGTCTTA |
| *fmyhc1.3* | Myosin heavy chain 1.3, fast skeletal muscle | 94 | F: CCTCCAGCGTGTCAAGCAA<br>R: TGCGGCACATCTTCTCTAGGTTA |
| *fmyhc2.1* | Myosin heavy chain 2.1, fast skeletal muscle | 87 | F: CTAAGAACGCACTGGCCCAC<br>R: CGCTGAGCAAGCTTCTTCTTA |
| *fmyhc2.2* | Myosin heavy chain 2.2, fast skeletal muscle | 98 | F: GGAGGCTGACAAAATCTCTTACCTAT<br>R: GTACACCTGTGGCACAGTCTGG |
| *smyhc1* | Myosin heavy chain 1, slow skeletal muscle | 110 | F: GCTGCCAGTGTACGATTCCTCT<br>R: AGTGATGAGGACGGACTGGTTC |
| *smyhc2* | Myosin heavy chain 2, slow skeletal muscle | 103 | F: TGAGCAGCTGTGCATCAACTTT<br>R: ACTCCCACTCGATCCCTTCC |
| *gapdh* | glyceraldehyde-3-phosphate dehydrogenase | 96 | F: CTGGTGACCCGTGCTGCTT<br>R: TTTGCCGCCTTCTGCCTTA |
| *rpl13* | Ribosomal protein L13 | 110 | F: CGGACGTGGCTTCACCTT<br>R: TCTGTTGCGACGACGTGAGT |
| *ef1a* | elongation factor 1a | 86 | F: GGTGGAATCGACAAGAGAACCA<br>R: GTCCAACACCCAGGCGTACT |

## 5.4 Results

The classification accuracy test for the Random Forest model showed 100% accuracy of the model in distinguishing males from females using mRNA expression patterns measured in the fish with known sex. However, when we used the mRNA expression of *bmp15*, *ccnb1*, *gyg1a*, *kpna2*, *rdh10b*, and *zp3* to distinguish males from females in the temperature treatment group and control group, it was only feasible at 60 and 90 dpf. At 60 dpf, the sex ratio of males: females was 80:20 in the temperature treatment group, and 67:33 in the control group. At 90 dpf, the sex ratio of males: females was 70:30 in the temperature treatment group, and 40:60 in the control group. Due to a low number of females in the temperature treatment group at 60 dpf, female samples were excluded, and only samples from males were used for mRNA expression analysis at 60 dpf. However, at 90 dpf, both sexes were included in the analysis.

Relative mRNA expression analysis of skeletal muscle showed different mRNA expression patterns in the embryonic, larval, juvenile, and adult stages of zebrafish in response to early-life daily thermal fluctuations. Although almost all nine genes coding skeletal muscle proteins exhibited fold-change upregulation or downregulation in the temperature treatment group relative to the control group at each time point, no statistically significant difference was observed in the fish younger than 18 dpf (**Figure 5.1**).

The effects of early-life thermal fluctuations on the mRNA expression of structural skeletal muscle genes in zebrafish were smaller during the embryonic and larval stages than in juvenile and adult stages. All significant mRNA expression changes during early stages of development were downregulations (**Figure 5.1**). Specifically, *smyhc1* was significantly (p-value < 0.005) downregulated (0.5-fold) at 18 dpf. At 21 dpf, the mRNA expression of *fmyhc1.2* (0.3-fold), *fmyhc1.3* (0.54-fold), and *fmyhc2.2* (0.20-fold) was also downregulated (p-value < 0.005). Additionally, *ac1a* and *fmyhc2.2* were significantly (p-value < 0.005) downregulated at 24 (0.40-fold) and 30 (0.27-fold) dpf, respectively.


12 hpf
24 hpf
50 hpf
6 dpf
10 dpf
15 dpf
18 dpf
21 dpf
24 dpf
27 dpf
30 dpf
35 dpf
Fold-change
Genes
act1a
act1b
fmyhc1.1
fmyhc1.2
fmyhc1.3
fmyhc2.1
fmyhc2.2
smyhc1
smyhc2

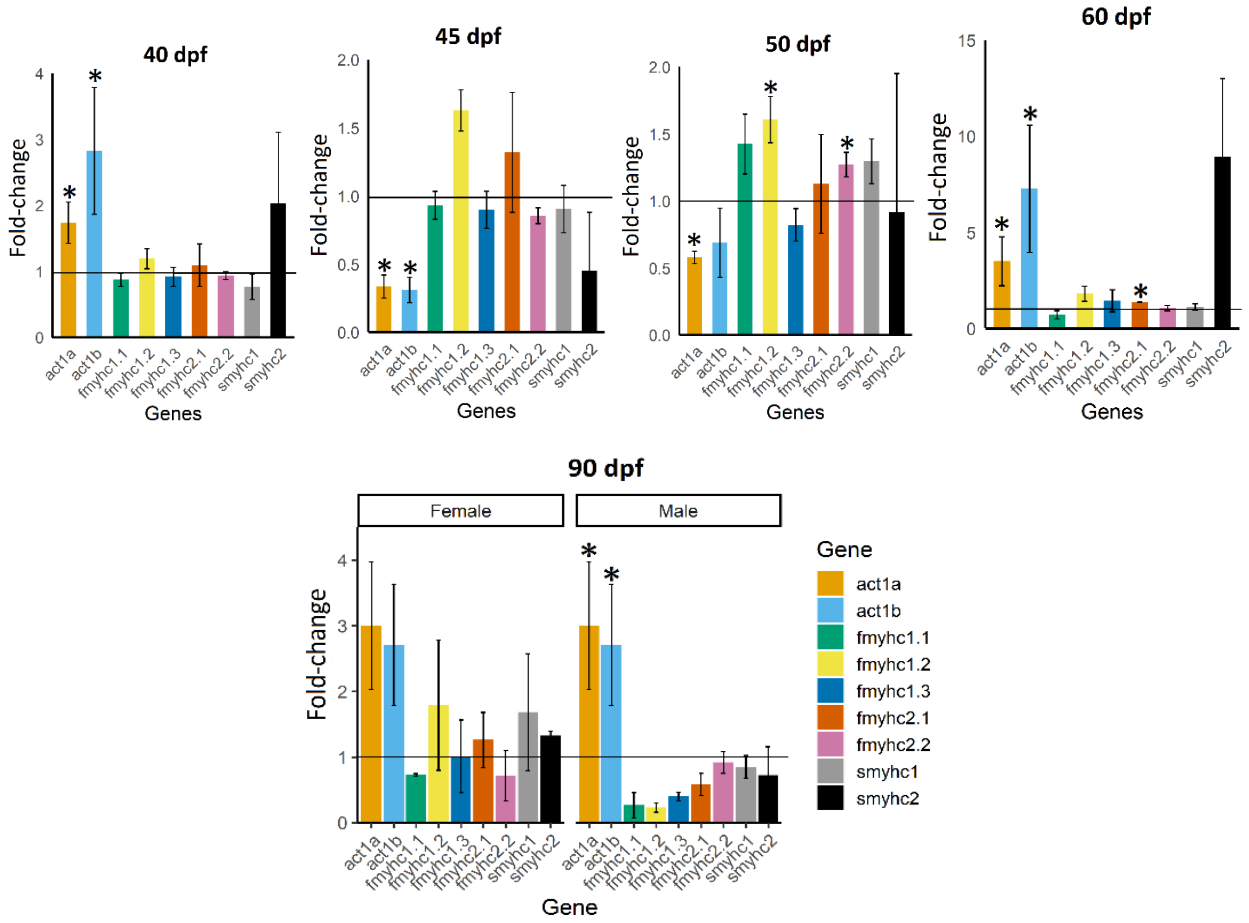



**Figure 5.1** Relative mRNA expression analysis showing the effects of daily thermal fluctuation during embryonic and larval stages on the mRNA expression of genes coding structural proteins in skeletal muscle of zebrafish, *Danio rerio*. The zebrafish were kept at 28 °C until 90 dpf and experienced daily +5 thermal fluctuation during their embryonic and larval stage. The mRNA expression of genes coding structural proteins in the skeletal muscle was measured in different life stages and compared to a control group reared at 28 °C. The mRNA expression of each gene in plots is shown as fold-change relative to the control group, whose expression level is 1. The bars showing the mRNA expression level of each gene in the control groups are not shown in this plot, and the expression level of these genes is shown with a horizontal line parallel to the x-axis. The statistical significance of the mRNA expression of each gene between experimental groups and also between males and females at 90 dpf is measured using the Mann-Whitney U test. To reduce the false positives, the p-value of the tests was adjusted to 0.005. The statistical significance (P-value <0.005) between the temperature treatment group and the control group is shown using an asterisk (*). No statistically significant changes in the mRNA expression of muscle genes were observed between males and females.

The effects of exposure to daily thermal fluctuations on the mRNA expression of skeletal muscle genes were more pronounced in juvenile and adult zebrafish. Skeletal muscle fast myosin heavy chains isoforms (*fmyhc*) were among the genes showing significant upregulation. Specifically, the mRNA expression of *fmyhc2.2* was upregulated at 35 dpf (1.2-fold) and 50 dpf (1.27-fold), while *fmyhc1.2* (1.6-fold) showed increased mRNA expression at 50 dpf. In addition, *fmyhc2.1* was significantly upregulated at 60 dpf (1.37-fold).

The mRNA expression of two isoforms of *act1* measured in this study also showed notable changes in expression in the juvenile and adult fish (**Figure 5.1**). The mRNA expression of *act1a* showed downregulation at 40 (0.73-fold), 45 (0.50-fold), and 50 dpf (0.35-fold), but upregulation in males measured at 60 (3.49-fold) and 90 dpf (1.63-fold). Similarly, *act1b* was significantly upregulated at 40 (2.28-fold), and in males measured at 60 (7.26-fold) and 90 dpf (2-fold).

## 5.6 Discussion

Early-life thermal treatment is known to affect the development of muscle in fishes, such as affecting the fast muscle area in Siberian sturgeon, *Acipenser baerii***,** larvae, or muscle growth in zebrafish, *Danio rerio* (Johnston and Hall, 2004; Aidos et al., 2017; Lin et al., 2022). These changes in development can be seen as differences in the mRNA expression of genes involved in the development of muscle, such as the expression of muscle growth regulatory and structural proteins in Senegalese sole, *Solea senegalensis,* rainbow trout, *Oncorhynchus mykiss,* sea bass, *Dicentrarchus labrax*, and changes in fast muscle area in Siberian sturgeon, *Acipenser baerii* (Wilkes et al., 2001; Campos et al., 2013; Aidos et al., 2017). In our study, we investigated the effects of temperature fluctuations in the embryonic and larval stages on the mRNA expression of genes involved in the development of skeletal muscles in zebrafish. Our results showed that exposure to early-life daily thermal fluctuations influenced the mRNA expression of genes coding structural proteins of skeletal muscles in zebrafish. However, the direction of expression changes caused by exposure to early-life thermal fluctuation was not consistent throughout life stages. The effects on mRNA expression were more significant in the juvenile and adult stages than in the embryonic and larval stages. Moreover, high variability was detected in the mRNA

expression of individuals within experimental groups at each timepoint, which affected the statistical inference.

The mRNA expression of most muscle genes studied here did not show statistically significant changes in the embryonic and larval stages, especially in the fish younger than 18 dpf, where samples from five fish were pooled due to low RNA concentrations. This could be because the small sample size and high variation in data points reduce the statistical power (Columb and Atkinson, 2016; Mascha and Vetter, 2018). However, there were fold-change differences in the mRNA expression pattern of genes in the embryonic and early larval stages, which show the effects of thermal fluctuations on the muscle gene mRNA expression. Embryonic and larval stages are important periods in development, and exposure to temperatures outside the optimal temperature range of the species has been recorded to have severe effects on the normal development of fishes (Réalis-Doyelle et al., 2016; Dubey et al., 2023). Determination and differentiation of skeletal muscle are usually controlled by basic helix-loop-helix (bHLH) transcription factors such as MyoD, myogenin, *myf-5,* and *myf-6* (Watabe, 1999; Johnston et al., 2001). Studies on juvenile pacu, *Piaractus mesopotamicus*, larval rainbow trout, *Oncorhynchus mykiss*, and sea bass, *Dicentrarchus labrax,* have shown that there is a species-specific temperature range at which the mRNA expression of these transcription factors and structural proteins, such as myosin heavy chain, increases along with muscle growth, but temperatures higher or lower than this range affect the structure of the muscle and sometimes delay its development (Wilkes et al., 2001; Gutierrez de Paula et al., 2014). In addition, the mRNA expression of myogenic genes changes through development (Johansen et al., 2005). In our study, the stage-dependent changes observed in the mRNA expression of structural proteins might be caused by the interaction between natural changes in the expression of genes during development and the changes caused by thermal fluctuation.

The effects of daily thermal fluctuations on the mRNA expression of muscle genes continued through juvenile and adult stages. Most significant changes were an upregulation in the genes measured in juvenile and adult fish, potentially due to an increase in muscle growth after removing thermal fluctuations. Hyperplasia and hypertrophy are observed in several teleost species during myogenesis in both larval and adult stages (Watabe, 1999). In studies investigating the effects of thermal treatments during early-life stages on muscle, exposure to

both low and high temperatures at the early-life stage has been reported to have effects on the structure of muscle that can last even after acclimating to a more favorable temperature (Carey et al., 2009; Johnston et al., 2009). Effects of temperature on growth are known to act through changes in food intake, protein synthesis, metabolism, and the expression of growth factors (Burel et al., 1996; McCarthy and Houlihan, 1997; Person-Le Ruyet et al., 2004; Reinecke, 2010). However, delayed growth can sometimes be compensated for through compensatory growth after removing environmental stressors such as temperature (Huang et al., 2008; Kim et al., 2018). This compensatory growth usually results in the upregulation of genes coding proteins involved in organ structure (He et al., 2015; Yang et al., 2019; Liu et al., 2020). The upregulation in the mRNA expression of genes coding structural proteins in the skeletal muscle might be a sign of compensatory growth for increasing the number of muscle fibers or their size after acclimation to 28 °C for 60 days. Although compensatory growth usually increases the growth rate of animals after removing environmental pressure, full recovery depends on the duration of the stressor and may not occur in a short time, resulting in animals with lower weight and length (Santiago et al., 2004; Peng et al., 2017). This might suggest that the effects of early-life thermal treatment on the developing animals are reversible after acclimation to a more favorable temperature for the species, however the effects persist even months after ending exposure.

Red and white muscles compose the majority of muscle mass in fishes (Kiessling et al., 2006). Myosin and actin play an important role in the structure and function of both these muscle types, and several isoforms of these proteins are known to exist in these tissues (Cooper, 2000; Watabe, 2002; McGuigan et al., 2004; Okagaki et al., 2005; Watabe and Ikeda, 2006; Bertola et al., 2008; Alves-Costa et al., 2015; Lee et al., 2017; Chen et al., 2023). Environmental factors such as food availability, diet, and exercise are reported to affect the expression of these proteins (Hevrøy et al., 2005; Baldwin and Haddad, 2010; Fiaz et al., 2014; Wang et al., 2022). Temperature treatments have been shown to affect the expression and abundance of these proteins in the skeletal muscle of fish; however, this pattern may be species or study-specific (Crockford and Johnston, 1990; Imai et al., 1997; Watabe, 2002; Hall et al., 2003; Weber and Bosworth, 2005). In our study, the mRNA expression patterns of these proteins were stage-dependent and did not differ significantly between males and females. The expression of genes coding muscle structural proteins, and also the structure of muscle, is known to change throughout development in growing animals (Veggetti et al., 1993; Mascarello et al., 1995; Chen

et al., 2023). The stage-dependent response in our study might be an indicator of a complex interaction between natural growth and temperature treatment.

Many biological activities of fishes, such as finding food, avoiding predators, and migration, depend on the fish's ability to swim (Hinch and Bratty, 2000; Fu et al., 2013). Both slow and fast muscles are involved in generating the thrust needed to swim at slow and fast velocities (Fernández and Calvo, 2009). Because of its importance in swimming behavior, structural changes in muscle can reduce the fitness and survival of fish (Cano-Barbacil et al., 2020). Many temperate freshwater fish species spawn in the spring and summer, and their offspring experience warm temperatures in early-life stages before their first winter (Koenigbauer et al., 2024). Many natural aquatic ecosystems also experience heatwaves in summer, and the intensity and frequency of these heatwaves are expected to increase as the trend of climate change continues (Woolway et al., 2021; Jiang et al., 2025). In our study, the mRNA expression of proteins involved in both slow and fast muscle showed significant changes in response to daily thermal treatment, which can potentially affect the swimming ability of fish, considering the role of these proteins in the structure and proper function of slow and fast muscle. Considering the stage-dependent upregulations and downregulations in the mRNA expression of genes coding for muscle proteins in the fish that experienced thermal fluctuations in the early-life stages, experiencing unnatural thermal fluctuations caused by climate change raises concerns about the development of muscle in fish.

In conclusion, our results show that early-life thermal treatment affects the mRNA expression of structural genes in the muscle of zebrafish, and these effects can persist even in adulthood. These changes in the mRNA expression pattern of muscle genes can potentially affect the development of muscle in fish and influence the swimming performance of fish, which might affect the survival of fishes. Transcriptional profiling combined with responses across levels of biological organization, such as histological approaches for measuring the effects at the structural level and measuring the swimming performance of fish, should be applied in future research to provide better insight into the effects of early-life thermal treatment on the development of muscle. We observed variability in the mRNA expression pattern of some genes in both treatments in our experiments, suggesting individual-specific expression response to the temperature treatment. This variation might be a sign of diversity in the response to thermal

stressors caused by either genetic or epigenetic factors, which is worth investigating in future research projects.

## 5.7. Acknowledgement

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## Chapter 6. Thesis conclusion

There is a great amount of homology in the sequence of genes in organisms because of being descended from a common ancestor. This homology is even larger in animals within a family or genus. I took advantage of this biological characteristic and used OpenArray™ technology and designed a multispecies chip that enabled us to quantify the mRNA expression of 52 genes associated with cellular stress and general health, along with four control genes, in *Acipenser* sturgeons (Chapter 2). Despite challenges in designing universal primers and probes that work across the sturgeon species, I managed to design primers and probes applicable to mRNA expression analysis of several *Acipenser* species. The universality of the primers and probes on this chip makes it a flexible tool for researchers worldwide to monitor the health condition of several *Acipenser* species. In addition, OpenArray™ technology makes it possible for researchers to perform 2,688 reactions on each chip, and given the ability of each Quant Studio 12 K to run 4 chips per run, it can increase the number of reactions up to 10,752 per run. This number is significantly higher than traditional qPCR methods, in which 96-384 reactions are done at each run of the machine. The higher reaction number allows researchers to quantify the mRNA expression of 52 biomarker genes in a larger number of samples.

The designed chip was used for mRNA expression analysis of three *Acipenser* species exposed to acute thermal stress, and quality control showed high performance in the amplification of most genes in these species (Chapter 2). The high-efficiency amplification allowed relative mRNA expression analysis of target genes in response to acute thermal stress. The mRNA expression analysis showed species-specific and population-specific responses to acute thermal stress between species. These differences in mRNA patterns between species can reflect a bigger difference in the physiological response and coping mechanisms for dealing with thermal fluctuations, caused by long-term adaptation to different environments. Physiological differences in response to different temperatures signify the importance of specific considerations for each species, or different populations of the same species, when making management decisions, such as selecting breeders for stock enhancement programs. This requires extensive studies of population structure using molecular and morphological techniques to understand their distribution pattern. In the case of stock enhancement programs, it can reduce the risk of swapping genotypes between populations, which may result in increased outbreeding

depression and reduced fitness (Ward, 2006). Despite specific mRNA expression patterns in populations and species, some common mRNA expression patterns in the cellular stress response were also detected among *Acipenser* species. These similarities in expression can be used as a powerful biomarker for health assessment across species. Non-lethal sampling is a great technique for health monitoring of endangered species, and the practicality of non-invasive transcriptomic analysis has been successfully tested in several species, such as Japanese eel, *Anguilla japonica* and Atlantic salmon *Salmo salar,* using mucus and gill tissue (Rees et al., 2005; Yasuike et al., 2026; Méndez et al., 2026). Using non-lethal techniques for mRNA expression of genes with common expression responses to thermal stress can be used as a powerful tool for health monitoring of *Acipenser* species.

The long-term effects of early-life thermal conditions on the developing freshwater fish were also studied in this thesis. My results showed that early-life experience with temperature can have long-term effects on developing freshwater fish (Chapter 3). These long-term effects can include acclimation to thermal fluctuations or increasing their ability to withstand a more severe form of thermal challenge in the future. However, the acquired acclimation or tolerance to a changing environment was observed to be caused by plasticity, and it diminished after acclimation to a more stable environment. As physiological changes made during acclimation are likely costly, the lost tolerance for higher temperatures might be a strategy to reduce energy spent on mechanisms and pathways that are no longer needed (Moyen et al., 2020). Similar to many other physiological adjustments, acclimation to fluctuating temperatures was observed to be consequential at the cellular level, changing the mRNA expression of genes involved in cellular maintenance and oxidative stress response, such as *hsp70*, *hsp90aa1*, *cat*, *glul*, g*stp1a*, and *sod1*. However, these upregulations in the mRNA expression were more pronounced in the later life stages, with different expression patterns between sexes. This increase in the expression of genes involved in cellular stress response might be a sign of physiological changes, such as changes in growth and metabolic rates, that increase the cellular stress response in fish, with sex-specific effects. This increased expression of cellular stress response genes as a result of experiencing early-life thermal fluctuation might be responsible for the increased thermotolerance via increasing the cellular capacity of fish for dealing with adverse effects of acute thermal stress, such as increased misfolded proteins or oxidative stress.

The long-term effects of early-life thermal treatment on the development of the brain and behavior showed significant changes in the proliferation of brain stem cells and neuron density (Chapter 4). My results suggest that the proliferation of cells in the brain and neurogenesis can be influenced by thermal fluctuations. These changes might be the result of an accelerated development rate in the fish experiencing daily thermal fluctuations. It is also possible that because thermal fluctuation has happened in a fairly short period every day, it has worked as a chronic stressor, increasing the basal level of stress hormones. Stress hormones can affect brain cell proliferation and neurogenesis, probably as a coping mechanism. In my thesis, early-life thermal fluctuations affected the proliferation of brain cells at the embryonic and larval stages, followed by significant changes in the neuron density. However, after acclimation to 28 °C, these changes in the proliferation and density of neurons showed major plasticity toward juvenile and adult stages, reducing the difference between groups. These findings suggest the importance of early-life thermal treatments on the development of the central nervous system in zebrafish and the high capacity of this system for acclimation to new environments.

In addition, behavioral changes, in terms of increased activity in dark and light conditions, were observed in the fish that were kept under thermal fluctuation (Chapter 4). This increased activity started in the larval stage, during exposure to higher temperatures, which might have been an attempt to find an environment with an ideal temperature. This behavior might have started with stress hormones and continued even after the larval stages because of the increased basal level of stress hormones. In addition, increased fish activity might have affected the proliferation and differentiation of neurons in the forebrain, as increased activity is known to increase neurogenesis in the brain (Van Praag, 2008). Regardless of the mechanisms causing the changes in the brain and behavior, their long-term continuation is concerning and worth more investigations. Increased activity may raise the fish's metabolic rate, deplete energy reserves, or alter lipid and protein composition, which can reduce growth or reproductive efficiency. In my study, reduced growth was observed in the zebrafish exposed to early-life thermal fluctuations, which can be caused by increased metabolic rate. On the other hand, increased activity may improve foraging behavior in a natural environment and have positive effects on growth and reproduction. All these effects may influence the survival of species in the long term.

The mRNA expression of genes involved in the structure of muscle was also affected in *D. rerio* experiencing early-life thermal fluctuation (Chapter 5). This mRNA expression pattern was very variable and stage-dependent, with continuation into the adult stage. These changes in the mRNA expression of genes coding structural proteins in muscle might have happened because of several reasons, such as increased metabolism and its effects on the metabolism of macromolecules, such as lipids and proteins, for the production of energy, or changes in the development rate caused by increased temperature. Considering the increased activity in the fish experiencing early-life thermal fluctuations, it might be caused by increased workload on the muscle, which can affect muscle growth physiology and protein turnover. No matter if these changes in mRNA expression are caused by direct effects of increased temperature on development, or by increased activity resulting from increased stress hormones, their consequences on swimming behavior are important. This importance stems from the fact that fishes depend on their skeletal muscle for migration, predation, and escape. Significant changes in the structure of the muscle can decrease the ability of fish to meet their needs and lower their chance of survival. These changes in the mRNA expression of muscle genes in response to early-life thermal treatment are worth studying using histological techniques to measure the quantity and quality of muscle fibers, along with measuring the critical swimming speed ($U_{crit}$), which shows the maximum swimming capacity in a fish.

Overall, my study answered questions regarding the short-term and long-term effects of chronic and acute changes in water temperature on the physiology of freshwater fishes using molecular, histological, and behavioral analysis. The results showed that the transcriptomic response of sturgeons to acute thermal stress is variable depending on species, acclimation history, and local adaptations. This variability is in the form of differences in the activation or suppression of pathways essential for maintaining homeostasis. In addition, I demonstrated that early-life exposure to daily thermal fluctuations affects thermotolerance and long-term cellular stress response, along with changing the development of the brain and muscle, and influencing the activity of fish, which continues into the adult stage even after acclimation to a stable temperature.

That said, there is so much left to learn about the mechanisms controlling these physiological responses, and their short-term and long-term effects on different aspects of

freshwater fish life. For instance, the mechanisms driving different cellular responses to acute thermal stress between different *Acipenser* species, or the same species adapted to different environments, are worth investigating. This could be done by studying the effects of acute thermal stress on the expression of proteins coded by these genes, and their systemic relationship in the cell. Investigating the metabolites and products of different pathways in which these proteins are involved can also improve our understanding of the capacity of these pathways for maintaining cells under thermal challenges. In the zebrafish study, early-life experience with thermal fluctuations increased the thermotolerance of fish, which was accompanied by increased expression of some cellular stress genes. It is possible that increased expression of the cellular stress genes has improved the ability of fish to cope with acute thermal stress. However, the expression pattern of these genes was studied in the whole body of zebrafish, and studying the detailed expression pattern of these genes in separate organs such as the heart and liver can increase our insight into the mechanisms by which they increase thermotolerance in fish.

In addition, sex-biased expression of cellular stress genes such as *hsp90aa1* and *sod2* was also detected in the zebrafish exposed to daily thermal fluctuations at the early-life stages. Although I did not measure the effects of early-life thermal fluctuations on sex determination of zebrafish, most samples collected at the juvenile and adult stages were identified as male using transcriptomic markers. Early-life thermal experiences are known to affect sex determination in zebrafish and result in a male-skewed sex ratio at higher temperatures (Sfakianakis et al., 2012; Valdivieso et al., 2020). The effects of exposure to daily thermal fluctuations on sex determination and sex-biased expression of cellular stress genes are worth investigating in future studies.

Investigating the mechanisms causing the changes detected in the development of the brain of *Danio rerio* is another interesting topic for research. This could be studied by investigating the effects of early-life thermal treatments on the mRNA expression of genes and proteins involved in regulatory pathways important for the formation of different parts of the brain, along with studying the population of different types of neurons and glia. In the muscle, the mRNA expression of proteins can be measured using proteomics and histology techniques to see if the mRNA expression changes actually result in structural changes in muscle fibers. In addition, the importance of each protein in the zebrafish can be studied by selective downregulation using

techniques such as RNA Interference (RNAi) and CRISPR Interference (CRISPRi) (Lee et al., 2009; Cai et al., 2018; Wang et al., 2023) and studying its effects on the swimming capacity of fish. Studying the effects of these changes on metabolism, reproduction, and swimming behavior of freshwater fish can also help us to understand their long-term consequences regarding their survival.

# Appendix

**Supplementary Table 2.1.** The efficiency of primers designed for the OpenArray™ chip. Before being used on the OpenArray™ chip, the primers were tested on four *Acipenser* species: lake sturgeon (*Acipenser fulvescens*), white sturgeon (*Acipenser transmontanus*), Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*), and shortnose sturgeons (*Acipenser brevirostrum*). Each primer was used to perform SYBR Green-based qPCR on a serial dilution of pooled cDNA (five points) from several individuals of each species, separately. Efficiency was calculated using the slope of the standard curve in formula E = 10^(-1/slope). The primers and probes were designed and tested in two separate labs. Because only one lab had access to the tissues from all the species, some primers were not tested on all the species.

| Gene | Protein | Primer/Probe sequence | Efficiency (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | ***A. fulvescens*, liver** | ***A. fulvescens*, gill** | ***A. brevirostrum*, liver** | ***A. brevirostrum*, gill** | ***A. oxyrinchus oxyrinchus*, liver** | ***A. oxyrinchus oxyrinchus*, gill** | ***A. transmontanus*, whole body** |
| *cam1* | Calmodulin 1 | F: ACAAGGACGGCGATGGC<br>R: TCAAGAATTCAGGGAAGTCAATTGT<br>P: AGGTCACTGGGTCAGAA | 97 | 99 | 100 | 98 | 99 | 103 | 100 |
| *mhc1a* | Major histocompatibility complex class 1 | F: GGCTGCAGAAGTACGTCCAGTA<br>R: TCTGCAGATCCACGAGCTTTC<br>P: TCCTCCTGCAGTGACT | 98 | 102 | 91 | 112 | 96 | 96 | 98 |
| *mhc2a* | Major histocompatibility complex class 2 | F: GAAGTCCTGTGTGTTCCTGGC<br>R: CAAGTGTGTCACGGTCTCAGC<br>P: CTGCTGCTGACTGTGT | 104 | 105 | 96 | 105 | 95 | 103 | 107 |
| *il1b* | Interleukin 1β | F: TTTGAGCCTCTCCACCTACATACC<br>R: ATTATTGCCCCCGATGCC<br>P: ACCCAGCCCGGATT | 101 | 105 | 108 | 100 | 99 | 109 | 97 |
| *il8* | Interleukin 8 | F: ACTGCAAGGATGCTGAAGTCATT<br>R: GTTGGTTCCAAGCAAATCTGATC<br>P: CCACTTTGAAATCTG | 92 | 100 | 117 | 100 | 121 | 101 | 102 |
| *tnfa* | Tumor necrosis factor α | F: TGTACTCGCAGGTGTCGTTCA<br>R: CTTTGAGGCGCTCAGGAGAT | 100 | 117 | 112 | 112 | 90 | 97 | 111 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | P: CACAACATCGAGCGACTT | | | | | | | |
| *casp9* | Caspase 9 | F: GCCTTCGACTGCTGTGTTGTAA<br>R: GCCATCAACACCATGGATAGC<br>P: AACCACAACAGGTTTC | 120 | 94 | 103 | 103 | 108 | 112 | 95 |
| *casp3* | Caspase 3 | F: ATTGTTTTTCATACAGGCTTGTAGAGG<br>R: TGGAGTACGCGTACAAGAAATCA<br>P: TGACAGCTCTCCCCAGAA | 114 | 99 | 101 | 88 | 86 | 99 | 96 |
| *tp53* | Tumor protein p53 | F: CGATTCCAGCAGTCCAGCA<br>R: GCATCTGGACCGGGCAG<br>P: TACTCTCCGGGCCTCAA | 107 | 102 | 103 | 96 | 96 | 81 | 99 |
| *bcl2l1-xl* | BCL2 apoptosis regulator | F: AAGATTTTATAAGCTACAAACTGTCCCA<br>R: ATTGTCCGTCCCGTTTATGGA<br>P: CAGGTTAATGGGGATGGT | 106 | 113 | 96 | 111 | 118 | 117 | 117 |
| *hif1ab* | Hypoxia Inducible Factor 1 | F: GGTGTTGATCTGCGATCCAA<br>R: GCTGGTATCCCATCAGCTCTGT<br>P: CCACACCCGTCAAA | 108 | 107 | 114 | 106 | 108 | 107 | 115 |
| *slc2a1b* | Solute carrier family 2 member 1a | F: TTGGGAGGCGAAACTCCAT<br>R: ATGAGAGCAGCAGCGATGAA<br>P: TGTTTTCCAACATTTTGG | 106 | 114 | 115 | 94 | 107 | 96 | 93 |
| *hk1* | Hexokinase 1 | F: AAGCAGACGGTTCAAATGGAAA<br>R: CATGGTCAAACAGCCGTGTT<br>P: TCCAGAGGACATCATG | 92 | 97 | 85 | 108 | 100 | 109 | 91 |
| *hk2* | Hexokinase 2 | F: GCCCATGGGATTCACCTTCT<br>R: TTTGAAACCCTTCGTCCACTTC<br>P: CCCCTGTCAGCAGAA | 89 | 101 | 113 | 109 | 113 | 106 | 103 |
| *pfklb* | Phosphofructokinase liver b | F: CGCCCTGCACAGGATCAT<br>R: GCGCCAGGTACCCACAGT<br>P: CTCAGAGTCACCAGAGAA | 89 | 101 | 113 | 109 | 113 | 106 | 103 |
| *aldoa* | Aldolase | F: GCCCGACGGAGATCATGAT<br>R: AGGAGAGTCCCCTCGAGATAGAC<br>P: TCACGGAGAAGGTTTT | 103 | 98 | 96 | 95 | 87 | 95 | 86 |
| *pgk1* | Phosphoglycerate kinase 1 | F: CCGGGCCAAGCAGATTG<br>R: GTCCATCATGTTCTTGGTTCCAT<br>P: CCAGTGGGTGTGTTTG | 99 | 116 | 117 | 109 | 117 | 116 | 99 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *cyp1a* | Cytochrome P450, family 1, subfamily A | F: ACTGCTCTCTCCTGGTGCTTAATG<br>R: CCTGCCCACATGCTCATCTA<br>P: TCCCACCCTGATATC | 103 | 102 | 119 | 111 | 105 | 107 | 188 |
| *gpx1a* | Glutathione peroxidase 1a | F: AGTTGATGTGAACGGGAAGGA<br>R: AGACGGGGCTCCACACAATA<br>P: AGACCCCATGTTCACTTT | 105 | 94 | 100 | 96 | 115 | 98 | 92 |
| *cat* | Catalase | F: TGGGGAAACTCGTGCTGAAC<br>R: AAGCGTCCCTGCAGCATCT<br>P: TTCGACCCCAGCAACA | 105 | 94 | 100 | 96 | 115 | 98 | 92 |
| *sod1* | Superoxide dismutase 1 | F: CTCCTGGAGAGCATGGCTTT<br>R: CCATGGGTTTTGCCAAGTG<br>P: CCATGCATTTGGAGACAA | 82 | 88 | 98 | 90 | 116 | 101 | 93 |
| *sod2* | Superoxide dismutase 1 | F: GTTGGTGTCCAGGGATCAGG<br>R: TAGGCATGCTCCCAAACATCA<br>P: AATCAGGACCCCCTACAA | 105 | 101 | 110 | 102 | 96 | 105 | 107 |
| *nfe2* | Nuclear factor, erythroid 2 | F: GGTAGGAGACTGCAGGGTCG<br>R: GATGCCTCACCTGCCTCACT<br>P: CAGGTGAACTCCATGTCA | 92 | 93 | 98 | 99 | 84 | 109 | 94 |
| *glul* | Glutamate-ammonia ligase (glutamine synthase) | F: GTCGCCATGAAACCTCCAAC<br>R: GTCGCAGTTGGCAGAAGGA<br>P: TGGGGCTAGTATCCGAAT | 107 | 104 | 100 | 99 | 117 | 109 | 104 |
| *atpa1a* | Sodium-potassium ATPase subunit alpha 1a (freshwater isoform) | F: GTTCCTCAGCAAGCCCTAGTTATC<br>R: CCTTCACTTCCACTAGGTCTCCAA<br>P: TATTAATGCTGAGGAAGTTGT | 107 | 104 | 100 | 99 | 117 | 109 | 104 |
| *atp6v1ba* | ATPase, H+ transporting, lysosomal, V1 subunit B, member a | F: GAGGACATGCTTGGTCGAGTATT<br>R: CCCATGATATCCAGAAAGTCTTCAG<br>P: TGGCTCTGGTAAACCTA | 103 | 98 | 108 | 103 | 102 | 101 | 111 |
| *ca15* | Carbonic anhydrase 15a (membrane-bound) | F: AGGCGGTTATCTGGACTGTGTT<br>R: CTGCCCACTGGCTGAGAAAT<br>P: CCATCCCCATTAGCA | 94 | 99 | 87 | 107 | 102 | 98 | 108 |
| *ca* | Carbonic anhydrase | F: CACTCCTTCCAGGTTGACTTCG<br>R: CGGAGGATCCCCAGTGAAA<br>P: ACTGCGTCAGTTTCA | 104 | 100 | 112 | 107 | 101 | 110 | 118 |
| *rpl7* | 60S ribosomal protein L7 | F: GCAGCGTATTCCTCTGGTTGA<br>R: GACAGTTTGAAGGGCCACAAG | 98 | 100 | 102 | 98 | 112 | 109 | 100 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | P: TTGGCAAGAGTGGTATC | | | | | | | |
| *rpl13a* | Ribosomal protein L13a | F: GGCGGACCGGTTCAATAAG<br>R: TCGCATCTCACAATCACCACTT<br>P: TTGTCGGCCATAGTTG | 97 | 96 | 108 | 102 | 106 | 104 | 102 |
| *ef1a* | Elongation factor 1 alpha | F: AGAACATGATCACCGGGACC<br>R: TGATGAGCTGCTTCACACCC<br>P: TGGAGGTGTTGGTGAGTT | 107 | 109 | 95 | 112 | 108 | 98 | 94 |
| *rps9* | 40S ribosomal protein S9 | F: GACGCCCCTTCGAGAAGTC<br>R: TGAGCAGGTCTCTGGCAGC<br>P: AGTTCACTCTGGCCAAGA | 117 | 109 | 105 | 103 | 82 | 117 | 104 |
| *ghr* | Growth hormone receptor | F: TCACAAACTGCTTGGCTCAGA<br>R: GAGTCGTCGTCCTTGATGTTCA<br>P: CTGAATGGCAACTGC | 101 | 114 | | | | | |
| *igf1* | Insulin-like growth factor 1 | F: TGCTGCTTCCAGAGCTGTGA<br>R: GGCTTAACAGGCGCACAGTAC<br>P: CTGAGGCGACTGGAG | 102 | 116 | | | | | |
| *igf2* | Insulin-like growth factor 2 | F: TGGTGACAGAGGCTTTTATTTCAG<br>R: CTTTTTGTGACCTTCGGATGTTT<br>P: AAGCCACCAAGCAGAT | 95 | 111 | | | | | |
| *igfbp1* | Insulin-like growth factor binding protein 1 | F: CAGCCGAACCTATCCGTTGT<br>R: CCGCCGGACATCCAAGT<br>P: CTGTACAGCGGAGAAGT | 97 | 101 | | | | | |
| *igfbp2* | Insulin-like growth factor binding protein 2 | F: GAGGGCCGCAAGACGAA<br>R: GAGTCGGATCACAGCCAAATC<br>P: TCAGCGGAATGAAG | 95 | 105 | | | | | |
| *ampk1a* | AMP-activated protein kinase catalytic subunit alpha-1 | F: GCTGGTCCAGAGGTGGATATCT<br>R: CCCACACAGCAAGGCATACA<br>P: AGCAGCGGGGTGAT | 96 | 111 | | | | | |
| *pck1* | Phosphoenolpyruvate carboxykinase | F: CAATCCAGAGAACGGCTTTTTC<br>R: ATGGCATTGGGGTTGGT<br>P: CCCCTGGAACCTCAGT | 92 | 112 | | | | | |
| *cox1* | Cytochrome c oxidase subunit 1 | F: ACCCGGTGCCCTGCTT<br>R: GGCGTGGGCTGTAACGATA<br>P: CGATGATCAGATCTAC | 104 | 100 | | | | | |
| *fasn* | Fatty acid synthase | F: GCCGCCATGCCCTACAG | 98 | 108 | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | R: GTGCCCATACCTGAGCAGATG<br>P: ATCCAGCAAAGCCAG | | | | | | | |
| *cpt1a* | Carnitine palmytoltransferase 1A | F: GCCAATGCCCGTCAGAACT<br>R: CAACTGCATCCAAACTCTGCTT<br>P: CTTCAGCAAAGGGAAG | 89 | 105 | | | | | |
| *ctsd* | Cathepsin D | F: TCTTCGCTGAAGCCGTGAA<br>R: GAAGCGAGCCACCAGGAA<br>P: CAGCCGGGTATCAC | 94 | 114 | | | | | |
| *cs* | Citrate Synthase | F: GAGCCATCGACCCCAGTCT<br>R: CGCTGTATCCCAGCATGTTG<br>P: TGGTCTCACAACTTC | 95 | 108 | | | | | |
| *ldha* | Lactate Dehydrogenase A4 | F: TGGAGCGGGGTGAATGTC<br>R: GTGCCCATATCAGGGTTCAGA<br>P: CTGGTGTCTCCCTCAAG | 90 | 86 | | | | | |
| *lipe* | Hormone-sensitive lipase | F: GGCGAAAATGGCATCAAAA<br>R: CGCCACCCTTCTTCTTAATCAA<br>P: ACGAATTGGAAAGTAAAC | 103 | 84 | | | | | |
| *lpl* | Adipose triglyceride lipase/lipoprotein lipase | F: CGGAGTGGGTTGTCGACTACA<br>R: GGCGTGCGCAGAGAGAA<br>P: CAGCATCGAATCCAA | 102 | 95 | | | | | |
| *gr* | Glucocorticoid receptor | F: GGAGCCCTTGGAACTTAAATGC<br>R: CAGCGGGGAAAGCAAAGA<br>P: ATGGCGAGGACGATGA | 105 | 85 | | | | | |
| *mr* | Mineralocorticoid receptor | F: GGACAGACGTGGCAGAGGTT<br>R: CCAGATCATGCATGGCATCT<br>P: TACCAGCTGACTAAACTG | 98 | 85 | | | | | |
| *hsp70a* | Heat shock protein 70a | F: GCACTGGCCGTTCATGGT<br>R: CCCCTTTGTATTCCACTTGCA<br>P: AAGCAGGGAAACCCAAA | 94 | 109 | | | | | |
| *hspa4a* | Heat shock 70 kDa protein 4a | F: TGAACACAGCTTACAGTGGCAACT<br>R: CATCTTACCCTCGTTTTCAACAAA<br>P: AGCAACGATACGCTGAA | 93 | 82 | | | | | |
| *hspa8* | Heat shock cognate 71 kDa protein (hsc70) | F: CAGTGGTCCAGTCCGATATGAA<br>R: GGGACGGCCACCATCAC<br>P: CACTGGCCATTCAA | 89 | 87 | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *hsp90a* | Heat shock protein 90ba | F: GACCCGCAGACTCATTCCA<br>R: CAATACCCAGCCCCAGCTT<br>P: CCGCATCTACAGGATGA | 91 | 92 | | | | | |
| *hsp90b* | Heat shock protein 90bb | F: GGTTGTCAAGAAACACTCCCAGTT<br>R: TCCTTTTCACGCTCTTTTTCCA<br>P: ACCCCATCACCCTATAT | 102 | 101 | | | | | |
| *hsf1* | Heat shock transcription factor 1 | F: GATCAGGGCCACTTTGCAAA<br>R: AAGCTGGCCATGTTGTTATGC<br>P: TGCTTCCCAAATACTT | 89 | 85 | | | | | |
| *cirbp* | Cold inducible RNA binding protein | F: CCAACGAGCAAGGTTTGGA<br>R: TGATGACAACAACCTCAGAGATCTG<br>P: TGTTCGCCAAGTATG | 95 | 90 | | | | | |

**Supplementary Table 2.2** The efficiency of qPCR conducted using the Openarray™ chip for three *Acipenser* species: Atlantic sturgeon, *Acipenser oxyrinchus oxyrinchus* (liver and gill), shortnose sturgeon, *Acipenser brevirostrum* (liver and gill), and lake sturgeon, *Acipenser fulvescens*. To measure efficiency for each gene in each tissue, the raw fluorescence data were exported from QuantStudio™ 12K Flex Software for each reaction, and the efficiency of PCR was measured using LinRegPCR software. Then, the mean efficiency and standard deviation (SD) of the efficiencies from all the individuals were calculated.

| **Gene symbol** | **Mean efficiency (%) ± SD** | | | | |
|---|---|---|---|---|---|
| | **Atlantic sturgeon (Liver)** | **Atlantic sturgeon (Gill)** | **Shortnose sturgeon (Liver)** | **Shortnose sturgeon (Gill)** | **Lake sturgeon (Gill)** |
| *cam1* | 38 ± 14 | 40 ± 12 | 41 ± 28 | 41 ± 27 | 33 ± 20 |
| *mhc1a* | 33 ± 58 | 50 ± 72 | 30 ± 23 | 39 ± 52 | 36 ± 26 |
| *mhc2a* | 106 ± 30 | 96 ± 27 | 116 ± 35 | 119 ± 40 | 103 ± 33 |
| *il1b* | 70 ± 24 | 59 ± 13 | 40 ± 45 | 67 ± 64 | 68 ± 20 |
| *il8* | 102 ± 25 | 105 ± 16 | 103 ± 14 | 107 ± 15 | 93 ± 24 |
| *tnfa* | 70 ± 47 | 102 ± 21 | 70 ± 15 | 98 ± 32 | 84 ± 31 |
| *casp9* | 98 ± 15 | 98 ± 16 | 82 ± 21 | 95 ± 17 | 86 ± 25 |
| *casp3* | 54 ± 26 | 44 ± 26 | 63 ± 17 | 62 ± 16 | 53 ± 18 |
| *tp53* | 0 ± 0 | 0 ± 0 | 60 ± 17 | 77 ± 67 | 73 ± 52 |

| | | | | | |
|---|---|---|---|---|---|
| *bcl2l1-xl* | 84 ± 42 | 92 ± 43 | 80 ± 36 | 91 ± 39 | 80 ± 36 |
| *hif1ab* | 91 ± 26 | 90 ± 16 | 90 ± 15 | 92 ± 18 | 84 ± 25 |
| *slc2a1b* | 84 ± 30 | 76 ± 31 | 84 ± 28 | 67 ± 39 | 73 ± 28 |
| *hk1* | 96 ± 40 | 107 ± 35 | 89 ± 33 | 106 ± 22 | 96 ± 28 |
| *hk2* | 67 ± 29 | 50 ± 37 | 70 ± 28 | 94 ± 28 | 75 ± 27 |
| *pfklb* | 100 ± 33 | 85 ± 23 | 109 ± 30 | 91 ± 29 | 80 ± 27 |
| *aldoa* | 116 ± 33 | 116 ± 19 | 114 ± 23 | 112 ± 19 | 121 ± 34 |
| *pgk1* | 97 ± 27 | 92 ±15 | 105 ± 21 | 110 ± 18 | 93 ± 29 |
| *cyp1a* | 125 ± 36 | 93 ± 32 | 112 ± 22 | 86 ± 26 | 86 ± 25 |
| *gpx1a* | 109 ± 35 | 95 ± 19 | 110 ± 14 | 98 ± 15 | 96 ± 28 |
| *cat* | 86 ± 22 | 95 ± 11 | 88 ± 17 | 94 ± 11 | 87 ± 22 |
| *sod1* | 84 ± 39 | 83 ± 40 | 103 ± 23 | 105 ± 21 | 96 ± 28 |
| *sod2* | 85 ± 58 | 72 ± 40 | 76 ± 28 | 100 ± 17 | 72 ± 25 |
| *nfe2* | 84 ± 32 | 83 ± 26 | 75 ± 23 | 87 ± 27 | 78 ± 33 |
| *glul* | 89 ± 27 | 112 ± 14 | 84 ± 41 | 92 ± 20 | 84 ± 30 |
| *atpa1a* | 28 ± 22 | 8 ± 12 | 20 ± 24 | 8 ± 12 | 0 ± 0 |
| *atp6v1ba* | 88 ± 46 | 83 ± 20 | 99 ± 15 | 100 ± 14 | 90 ± 26 |
| *ca15* | 0 ± 0 | 97 ± 51 | 0 ± 0 | 90 ± 19 | 90 ± 41 |
| *ca* | 96 ± 26 | 129 ± 12 | 94 ± 21 | 104 ± 28 | 103 ± 28 |
| *rpl7* | 105 ± 41 | 118 ± 31 | 117 ± 38 | 117 ± 47 | 100 ± 36 |
| *rpl13a* | 100 ± 30 | 91 ± 20 | 75 ± 29 | 95 ± 28 | 79 ± 32 |
| *ef1a* | 80 ± 37 | 90 ± 28 | 76 ± 48 | 89 ± 28 | 60 ± 39 |
| *rps9* | 90 ± 34 | 92 ± 28 | 70 ± 32 | 88 ± 33 | 62 ± 31 |
| *ghr* | 93 ± 57 | 97 ± 16 | 87 ± 13 | 92 ± 12 | 83 ± 25 |
| *igf1* | 100 ± 27 | 98 ± 15 | 65 ± 24 | 102 ± 16 | 87 ± 25 |
| *igf2* | 87 ± 22 | 96 ± 13 | 80 ± 18 | 93 ± 16 | 82 ± 21 |
| *igfbp1* | 88 ± 30 | 80 ± 46 | 84 ± 40 | 74 ± 16 | 80 ± 27 |
| *igfbp2* | 108 ± 34 | 83 ± 23 | 108 ± 28 | 88 ± 20 | 96 ± 25 |
| *ampk1a* | 89 ± 40 | 96 ± 40 | 94 ± 19 | 102 ± 18 | 84 ± 30 |
| *pck1* | 102 ± 39 | 87 ± 40 | 100 ± 36 | 89 ± 35 | 88 ± 32 |
| *cox1* | 0 ± 0 | 0 ± 29 | 119 ± 23 | 128 ± 24 | 120 ± 52 |
| *fasn* | 96 ± 25 | 99 ± 14 | 104 ± 16 | 92 ± 14 | 95 ± 24 |
| *cpt1a* | 85 ± 32 | 100 ± 26 | 75 ± 29 | 90 ± 33 | 81 ± 29 |
| *ctsd* | 90 ± 29 | 101 ± 24 | 94 ± 12 | 94 ± 10 | 89 ± 21 |
| *cs* | 98 ± 32 | 102 ± 25 | 93 ± 18 | 100 ± 9 | 96 ± 26 |
| *ldha* | 96 ± 37 | 103 ± 29 | 93 ± 20 | 97 ± 20 | 91 ± 29 |
| *lipe* | 9 ± 32 | 37 ± 12 | 85 ± 14 | 98 ± 37 | 84 ± 36 |
| *lpl* | 103 ± 36 | 96 ± 32 | 100 ± 14 | 86 ± 14 | 92 ± 29 |
| *gr* | 75 ± 34 | 87 ± 30 | 75 ± 32 | 82 ± 31 | 80 ± 26 |
| *mr* | 96 ± 22 | 97 ± 10 | 99 ± 9 | 108 ± 9 | 91 ± 22 |

| | | | | | |
|---|---|---|---|---|---|
| *hsp70a* | 102 ± 36 | 103 ± 30 | 112 ± 25 | 105 ± 13 | 108 ± 36 |
| *hspa4a* | 68 ± 28 | 85 ± 21 | 73 ± 25 | 92 ± 24 | 80 ± 24 |
| *hspa8* | 106 ± 30 | 113 ± 18 | 108 ± 19 | 116 ± 18 | 104 ± 35 |
| *hsp90a* | 83 ± 31 | 71 ± 28 | 80 ± 20 | 74 ± 15 | 82 ± 29 |
| *hsp90b* | 88 ± 43 | 85 ± 30 | 84 ± 34 | 79 ± 39 | 85 ± 34 |
| *hsf1* | 94 ± 26 | 88 ± 18 | 93 ± 17 | 94 ± 18 | 86 ± 24 |
| *cirbp* | 82 ± 24 | 93 ± 13 | 95 ± 14 | 93 ± 10 | 87 ± 26 |

**Supplementary Table 2.3** The p-values from the two-way ANOVA test for each gene in two populations of lake sturgeon, *Acipenser fulvescens*. The two-way ANOVA was used to assess the effects of acclimation temperatures (16, 20, 24 °C), acute thermal stress (as measured by $CT_{max}$), and their interaction on the expression of each gene. The expression data of *cam1*, *mhc1a*, *casp3*, and *atpa1a* were excluded from the two-way ANOVA due to low PCR efficiencies and are shown as NA in this table.

| **Gene symbol** | **P-value** | | | | | |
|---|---|---|---|---|---|---|
| | **Burntwood River population** | | | **Winnipeg River population** | | |
| | **Acclimation** | **$CT_{max}$** | **Acclimation-$CT_{max}$ interaction** | **Acclimation** | **$CT_{max}$** | **Acclimation-$CT_{max}$ interaction** |
| *cam1* | NA | NA | NA | NA | NA | NA |
| *mhc1a* | NA | NA | NA | NA | NA | NA |
| *mhc2a* | 0.0009 | 2.29e-05 | 0.02 | 1.01e-07 | 0.84 | 3.98e-05 |
| *il1b* | 0.06 | 0.11 | 0.15 | 0.27 | 5.32e-06 | 0.02 |
| *il8* | 0.51 | 0.04 | 0.03 | 0.008 | 0.01 | 6.62e-06 |
| *tnfa* | 0.01 | 0.24 | 0.37 | 0.26 | 0.40 | 0.37 |
| *casp9* | 0.29 | 6.55e-07 | 0.003 | 0.05 | 5.42e-06 | 0.61 |
| *casp3* | NA | NA | NA | NA | NA | NA |
| *tp53* | 0.13 | 1.77e-06 | 0.39 | 0.58 | 3.74e-06 | 0.79 |
| *bcl2l1-xl* | 0.55 | 0.34 | 0.99 | 0.86 | 0.05 | 0.15 |
| *hif1ab* | 1.68e-09 | 0.08 | 0.13 | 0.03 | 5.08e-08 | 0.07 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *slc2a1b* | 0.34 | 0.02 | 0.34 | 0.17 | 3.95e-05 | 0.87 |
| *hk1* | 0.03 | 2.88e-06 | 0.51 | 0.75 | 1.61e-08 | 0.81 |
| *hk2* | 0.007 | 0.06 | 0.04 | 0.002 | 0.04 | 0.06 |
| *pfklb* | 0.78 | 5.87e-05 | 0.97 | 0.37 | 0.0008 | 0.42 |
| *aldoa* | 0.15 | 0.03 | 0.54 | 0.29 | 0.04 | 0.48 |
| *pgk1* | 0.13 | 0.69 | 0.39 | 0.66 | 0.005 | 0.15 |
| *cyp1a* | 0.01 | 5.78e-13 | 0.002 | 0.0002 | 3.30e-12 | 1.15e-10 |
| *gpx1a* | 0.33 | 5.51e-09 | 0.36 | 0.58 | 2.02e-06 | 0.58 |
| *cat* | 0.77 | 1.74e-05 | 0.53 | 0.7 | 7.32e-07 | 0.19 |
| *sod1* | 0.26 | 6.13e-05 | 0.52 | 0.59 | 1.47e-05 | 0.13 |
| *sod2* | 0.26 | 0.0002 | 0.27 | 0.37 | 5.94e-06 | 0.44 |
| *nfe2* | 0.02 | 5.82e-06 | 0.69 | 0.008 | 5.94e-06 | 0.92 |
| *glul* | 0.68 | 0.35 | 0.02 | 0.01 | 0.63 | 1.12e-06 |
| *atpa1a* | NA | NA | NA | NA | NA | NA |
| *atp6v1ba* | 0.27 | 8.14e-09 | 0.007 | 0.15 | 4.41e-07 | 0.09 |
| *ca15* | 0.003 | 0.002 | 0.09 | 0.11 | 0.35 | 0.99 |
| *ca* | 0.53 | 1.89e-10 | 0.70 | 0.35 | 1.83e-07 | 0.79 |
| *ghr* | 0.39 | 0.65 | 0.79 | 0.66 | 0.05 | 0.04 |
| *igf1* | 0.37 | 0.16 | 0.09 | 0.70 | 0.04 | 0.37 |
| *igf2* | 0.45 | 0.02 | 0.15 | 0.40 | 0.03 | 0.08 |
| *igfbp1* | 0.02 | 0.007 | 0.34 | 0.70 | 0.16 | 0.19 |
| *igfbp2* | 0.53 | 0.04 | 0.05 | 0.86 | 0.11 | 0.23 |
| *ampk1a* | 0.67 | 4.84e-05 | 0.45 | 0.32 | 3.35e-06 | 0.36 |
| *pck1* | 0.7 | 8.09e-08 | 0.01 | 0.78 | 1.63e-05 | 0.03 |
| *cox1* | 0.95 | 0.01 | 0.63 | 0.83 | 0.0005 | 0.47 |
| *fasn* | 0.001 | 0.02 | 0.01 | 0.04 | 0.02 | 0.02 |
| *cpt1a* | 0.87 | 2.42e-08 | 0.99 | 0.33 | 7.61e-07 | 0.35 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *ctsd* | 0.96 | 0.007 | 0.23 | 0.008 | 0.40 | 0.42 |
| *cs* | 0.19 | 0.0002 | 0.27 | 0.46 | 0.0009 | 0.36 |
| *ldha* | 0.07 | 0.36 | 0.37 | 0.26 | 0.27 | 0.17 |
| *lipe* | 0.61 | 0.06 | 0.73 | 0.61 | 0.69 | 0.98 |
| *lpl* | 0.21 | 0.63 | 0.93 | 0.94 | 0.05 | 0.28 |
| *gr* | 0.92 | 0.41 | 0.56 | 0.66 | 0.05 | 0.04 |
| *mr* | 0.15 | 0.002 | 0.46 | 0.27 | 0.08 | 0.10 |
| *hsp70a* | 0.03 | 2e-16 | 0.03 | 0.28 | 2e-16 | 0.75 |
| *hspa4a* | 0.23 | 1.64e-08 | 0.19 | 0.12 | 0.0003 | 0.15 |
| *hspa8* | 0.45 | 0.009 | 0.02 | 0.11 | 0.003 | 0.02 |
| *hsp90a* | 0.01 | 2e-16 | 0.01 | 0.62 | 2.68e-14 | 0.53 |
| *hsp90b* | 0.52 | 0.33 | 0.52 | 0.16 | 0.008 | 0.93 |
| *hsf1* | 0.29 | 0.11 | 0.97 | 0.89 | 0.04 | 0.39 |
| *cirbp* | 3.47e-06 | 0.19 | 0.07 | 0.08 | 5.4e-08 | 0.74 |

**Supplementary Table 2.4** $CT_{max}$ data of lake sturgeon from Burntwood River and Winnipeg River, Atlantic sturgeon, and shortnose sturgeon. Sixty-day-old lake sturgeon from Burntwood River and Winnipeg River were acclimated to 16, 20, and 24 °C for 30 days and then experienced $CT_{max}$. Two-year-old Atlantic and shortnose sturgeons were acclimated to 10 °C for 30 days, and then experienced $CT_{max}$. Lake sturgeon $CT_{max}$ data are from Bugg et al. (2020). Atlantic sturgeon and shortnose sturgeon $CT_{max}$ data are from Penny et al. (2023). SE: Standard Error

| **Species, population** | **Age** | **Acclimation temperature** | **Mean $CT_{max}$(°C) + SE** |
|---|---|---|---|
| Lake Sturgeon, Burntwood River | 60 days | 16 °C | 32.21 + 0.13 |
| Lake Sturgeon, Burntwood River | 60 days | 20 °C | 33.90 + 0.15 |
| Lake Sturgeon, Burntwood River | 60 days | 24 °C | 34.74 + 0.24 |
| Lake Sturgeon, Winnipeg River | 60 days | 16 °C | 32.33 + 0.07 |
| Lake Sturgeon, Winnipeg River | 60 days | 20 °C | 34.62 + 0.1 |
| Lake Sturgeon, Winnipeg River | 60 days | 24 °C | 35.48 + 0.24 |
| Atlantic Sturgeon | 2 years | 10 °C | 28.56 + 0.14 |
| Shortnose Sturgeon | 2 years | 10 °C | 27.91 + 0.76 |

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