Population-specific transcriptional plasticity and sub-lethal thermal thresholds in developing lake sturgeon, *Acipenser fulvescens*

by

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Abstract

Changing temperatures elevate threats to physiological function in endangered freshwater species such as the lake sturgeon, *Acipenser fulvescens*, especially throughout vulnerable periods of early development. If temperatures breach sub-lethal thresholds, transcriptomic plasticity and acclimation capacity and a reduction of protein level responses may be diminished, increasing the vulnerability of lake sturgeon to additional environmental stressors. My thesis research investigated the effects of changing temperatures on the physiology of developing lake sturgeon from Manitoba, Canada. A common garden strategy was employed, where lake sturgeon from northern and southern populations within Manitoba were reared at equivalent and environmentally-relevant temperatures. Lake sturgeon demonstrated acclimation-specific effects on thermal tolerance related phenotypes including morphology, metabolic rate, critical thermal maximum, transcriptional responses, mortality and transcriptional responses to cold stress later in life with relative performance in many traits declining as acclimatory temperatures increased. Many of the above phenotypic responses were population-specific, with lower thermal maxima and sub-lethal thermal thresholds in the northern population of lake sturgeon. Next, I used mRNA sequencing of gill tissue and found enhanced transcriptional plasticity in the southern population relative to the less thermally tolerant northern counterparts. Pathway-specific functional analysis implicated mitochondrial function, oxidative damage, and immunocompetence as key mechanisms modulated by increasing acclimation temperatures with functional analysis indicating additional population-specific biological processes. Last, we specifically investigated the effects of sub-lethal thermal thresholds on stress and innate immune capacity through transcriptional profiling using qPCR. Acclimation temperature influenced the endocrine stress response and impaired the activation of molecular pathways involved in the immune, stress, and fatty acid responses of pathogen-challenged lake sturgeon in early development. Collective results suggest that, as environmental temperatures intensify, transcriptional plasticity, sub-lethal thermal tolerance thresholds, and overall physiological plasticity are diminished, likely resulting in the increased susceptibility of developing lake sturgeon to the effects of compounding environmental stressors.

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I would finally like to thank the University of Manitoba for allowing me to conduct this research, NSERC for funding provided, and the sturgeon that gave their lives so that we could not only learn more about their biology but help preserve their brethren.

Dedication

For the preservation of the global environment, and the biodiversity which it supports.

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Chapter 1. Introduction

1.1 Temperature effects on Biological Function

Temperature is fundamental to the development and biological function of ectotherms, with influence spanning transcriptional processes and enzymatic kinetics to range distributions, survival, and population level selection processes (Wendelaar Bonga, 1997; Somero, 2010; Schulte, 2015). The effects of temperature are pervasive across biological levels of organization (cellular, tissue, individual, population, and community level outcomes) and thus influence whole organism fitness traits including growth, metabolic rate, immune response, tolerance to environmental stressors, and reproductive capacity (Johnston and Dunn 1987; Donelson et al., 2010; Dittmar et al., 2013; Schulte, 2015; Makrinos and Bowden, 2016; McBryan et al., 2016; Alfonso et al., 2020). Generally, as environmental temperatures begin to change, these physiological traits may be stimulated to increase short-term fitness, however once temperatures exceed an organism's tolerance thresholds, stress responses may be further induced with detriments to overall physiological performance (Jeffries et al., 2018; Alfonso et al., 2020). For acute exposures, this may mean the induction of cellular mechanisms to preserve internal homeostasis, including activation of transcriptomic and hormonal signaling networks, mobilization of energy reserves, and, if possible, movement to a more thermally suitable habitat (Schreck and Tort, 2016; Fabbri & Moon, 2016). However, if temperatures increasingly deviate outside of an organism's thermal scope or the organism cannot move to a less stressful environment, there may be additional chronic effects of thermal stress, which may impair the overall normal function of the organism, with the potential for negative effects on growth, osmoregulation, immunity, reproduction, and survival (Alfonso et al., 2020). In this scenario, the organism must exhibit plasticity to alter their physiology and acclimate to environmental conditions, enabling them to persist in their thermal environment. Globally, environmental temperatures are expected to increase in mean and variability, reducing suitable habitat for many species, and likely increasing the thermal stress burden on ectothermic organisms (Ficke et al., 2007; Miller et al., 2014; Thornton et al., 2014; Vasseur et al., 2014; Vollset et al., 2020). Thus, it will be increasingly important to understand how thermal plasticity can promote acclimation and survival for these organisms as their environmental conditions intensify.

1.2 Thermal Plasticity

In order to survive in environments with physiologically stressful temperatures, species often must make changes to alter their physiology. This ability to alter a given expressed genotype to produce a range of phenotypes within a population or species in response to environmental change is known as phenotypic plasticity (Stearns, 1989). These plasticly induced phenotypic changes at an individual level can alter organismal physiology and buffer against acute environmental changes (McKenzie et al., 2021). Physiological flexibility can be an adaptive response stimulated in stressful environments, and is a reversible phenotypic change, that may be persistent for days to months, deemed acclimation (Hockachka and Somero, 2002; Crozier and Hutchings 2014; Havird et al., 2020; Mackey et al., 2021). Acclimation can induce physiological changes which arise from the differential transcription of genes into messenger RNA's (mRNA) which ultimately are translated into proteins with phenotypic effects (Smith et al., 2013; Connon et al., 2018; Jeffries et al., 2019; Jeffries et al., 2021). As populations may have unique genetic backgrounds, mRNA sequences, and protein characteristics; their plasticity to environmental change may also vary (Fangue et al, 2006; Dittmar et al., 2013; Bolnick et al., 2015; Geerts et al, 2014; Yampolsky et al, 2014; Pereira et al, 2017; Earhart et al., 2022). In species with widespread latitudinal ranges, leading edge populations may demonstrate increased thermal sensitivities across broad physiological processes as temperatures change, due to differences in their thermal habitat, when compared to center populations (Bennet et al., 2019). Thus plasticity, as well as underlying transcriptional and physiological responses to temperature alteration, may differ resulting in altered tolerance thresholds. While organisms may exhibit high levels of acclimatory plasticity to changes in their thermal environment, there is a finite capacity for phenotypic alteration (Gunderson and Stillman, 2015; Jeffries et al., 2018; McKenzie et al., 2021). Once this capacity has been breached, chronic temperatures that exceed sub-lethal thresholds may result in decreased immunocompetence, thermal tolerance, physiological consequences to overall organismal performance and ultimately mortality (Alfonso et al., 2020).

1.3 Sub-lethal Thresholds

As environmental temperatures surpass an organisms acclimatory capacity (the ability to make and maintain phenotypic changes induced by environmental alterations; Donelson and Munday, 2012), fitness-related traits often decline limiting organismal performance prior to

mortality (Williams et al., 2016). These sub-lethal thresholds are represented by non-linear response patterns (response trajectories in a U or inverted U shape which deviate from linearity) that can be used to predict the severity of a physiological response to thermal stress (Jeffries et al., 2018). As sub-lethal limits are approached, there is an activation of cellular and protective mechanisms (Kassahn et al., 2009). If sub-lethal limits are breached and physiological mechanisms promoting acclimation are not adequate to protect the organism, accumulation of reactive oxygen species can cause cellular damage with impacts on protein and membrane stability as well as enzymatic function as energy stores are depleted and antioxidant defense systems fail (Heise et al., 2006; Williams et al., 2016; Jeffries et al., 2018; Gio et al., 2019; Alfonso et al., 2020). Thus, a decline in physiological performance indicates strong fitness-related consequences if environmental conditions continue to intensify. The chronic induction of these molecular responses can have significant impacts on development, immunity, and a broad range of physiological traits, as resources are diverted from these essential biological processes to enhance immediate survival (Alfonso et al., 2020). Molecular characterization of these acclimatory limits and resultant sub-lethal effects on fitness can indicate whether species or populations will be able to plasticly respond to further increases in environmental temperature (Somero, 2010; Connon et al., 2018).

1.4 Methods for determining Thermal Limits

An organism's thermal limits can be assessed by a variety of criteria, spanning classic assessments of whole organism fitness (critical thermal maxima, metabolic rates, aerobic scope, abundance of energy stores etc.) down to the abundance of mRNA's responsible for the activation of cellular protective mechanisms and responses to thermal stress. These thermal limits can be affected by the duration of the stressor and performed rapidly in a matter of hours to assess an organisms' acute tolerances, or last for longer periods of time allowing an organism to acclimate to their surrounding environment to highlight chronic effects. Critical thermal maxima (CT_{max}) approach is an experimental procedure which examines the acute thermal limits of an organism acclimated to a particular temperature or thermal regime, warming their environment at a consistent rate until the organism is unable to maintain their dorsal lateral orientation and loses equilibrium (LOE) (Beitinger et al., 2000). In contrast, measurements of metabolic rates examine the amount of oxygen an organism consumes in different environments

or following stressors over a time course, shedding light on the impacts on bioenergetic performance and respiratory physiology (Chabot et al., 2016). Together these metrics provide insight into effects of temperature on whole-organism physiology, but struggle to highlight causative physiological impediments.

Assessments of an organism's overall morphology can also indicate limitations in growth and development as temperatures increase. As growth potential is influenced by temperature, an organism's fastest growth rates typically occur at relatively high temperatures, near their thermal limits (Neubauer and Anderson, 2019). Provided an adequate food supply in captivity, at these temperatures fish will often grow larger and accrue energy stores up until their thermal limits have been reached, energy stores are utilized, and growth plateaus, as well as important physiological indices (i.b.i.d.). Measurements of body condition can be calculated from weight and length data to provide a mass length relationship for comparison (Fulton, 1911) and insight into the circumstances interacting between feeding conditions, infections, and physiological factors that may affect growth (Le Cren, 1951). Additionally, the relationship between the relative weights of organs and body weight can provide insights into the utilization of resources for growth, storage, or other developmental process. For example, the multifunctional liver processes and stores glycogen and fats as energy reserves. By calculating the weight of the liver in comparison to the body weight of an organism (i.e., hepatosomatic index; HSI), researchers can provide a quantitative relationship for comparison across study groups, which may provide an indication of the energy reserves stored versus expended (Rossi et al., 2017; Morrison et al., 2020). While these measurements provide some indication of relative condition, stress, or energy reserves, they are limited in their scope and can be supplemented with deeper analysis underlying biological compounds (e.g. stress hormones, energy reserves, and immune components) which may be impart responsible for changes in morphology.

Direct observation of stress hormones, energy reserves, and immune components provides a more complete view of physiological factors affected by organismal stress and thermal limitations. Many of the physiological mechanisms triggered by the stress response rely on the production and release of the hormone cortisol, which has a multifaceted role impacting many processes including oxygen uptake, mobilization of energy reserves, growth, reproduction and suppression of immune function (Wendelaar Bonga, 1997). Quantification of energy-related compounds such as fatty acids, glycogen, and glucose can provide insight into the energetic state

of an organism (Lermen et al., 2004; Rossi et al., 2017), which may influence whole-organism metabolic and thermal limits. Further, determining the activity of immune-related components like C3, lysozyme, and cytokines highlights the potential suppressive effects of cortisol on the immune system as thermal stress increases (Dittmar et al., 2013; Schreck and Tort, 2016).

Quantification of mRNA transcript abundance can highlight the magnitude and type of cellular processes modulated by changes in the thermal environment (Jeffries et al., 2018). Using real-time quantitative reverse transcription polymerase chain reactions (i.e., qPCR) allows for the targeted measurement of genes involved in processes underlying the generation of signaling cascades, hormonal fluctuations, utilization of energy stores, stress associated protein abundance, and induction of immune responses. Further, quantification of transcriptome-wide mRNA abundance via mRNA sequencing (i.e., RNAseq), can provide an integrative assessment of the expression of all transcripts currently expressed by an organism providing an overarching view of whole-transcriptome responses to environmental change. These mRNA level processes represent the precursors to modifications of the proteome, and phenotypic alteration which may be used to acclimate to thermally stressful environments. Ultimately, modifications of these processes may be indicative of long-term fitness consequences for organisms and highlight the functional processes they use to persist in changing environments (Connon et al., 2018).

As the temperature and time spent during acclimation can have strong impacts on sub-lethal thresholds imposed by thermal stress (Peck et al., 2014; Tomanek and, Somero, 2000), taking samples through a timeseries are important to provide a more holistic understanding of organismal plasticity in an environment with increased temperatures. Ideally, the above methods can be used in combination to demonstrate the physiological effects of sub-lethal thermal thresholds, highlighting whole-organism tolerances and thermal limits (Connon et al., 2018).

1.5 Plasticity of North American Sturgeon to Elevated Temperatures

Sturgeons, one of the most endangered collective group of animals globally (Bemis et al., 1997; IUCN, 2021), make their homes in freshwater ecosystems throughout the northern hemisphere. Across the North American continent, 8 species of sturgeon face a variety of anthropogenic threats that may be exacerbated by increasing environmental temperatures. To investigate the plasticity of these species to temperature, data was collected from a variety of literature and experimental sources detailing the effects of acclimation on critical thermal

maximum (Ziegeweid et al., 2007; Wilkes, 2011; Zhang and Kieffer, 2014; Rodgers et al., 2019; Bugg et al., 2020, Penman et al., 2021). This sturgeon-specific data was then compared to publicly available data on the acclimation response ratios of marine and freshwater fishes (Gunderson and Stillman, 2015). Acclimation response ratios provide a quantitative means to compare the change in CT_{max} or other thermal performance metrics across a variety of acclimation temperatures, indicating the thermal plasticity of an organism. This can be calculated by subtracting the performance at lower temperatures (CT_{max1}) from performance at higher temperatures (CT_{max2}) and dividing by the change in acclimation temperature between the treatments (Δ^oC):

$$Acclimation\ Response\ Ratio = \frac{CT_{max2} - CT_{max1}}{\Delta^o C}$$

Overall, there is limited data on the thermal tolerances and acclimation response ratios of North American sturgeons, with information available for only three species in the genus *Acipenser*: white sturgeon, *Acipenser transmontanus*, lake sturgeon, *A. fulvescens*, and shortnose sturgeon, *A. brevirostrum* (Figure 1.1A). These data suggest that sturgeon species have a higher average acclimatory capacity than other measured fish species (Figure 1.1B), fish that inhabit a variety of other environments (Figure 1.1C), and other well represented orders of fish in the dataset (Figure 1.1D). Additionally, data indicate that, in general, as sturgeon develop, their overall thermal plasticity is decreased (Figure 1.2A) and that at higher acclimatory temperatures their acclimatory capacity is diminished (Figure 1.2B, C). Together, the sturgeon-specific results available in the literature highlight increased thermal plasticity when compared to other fish species, high levels of thermal plasticity in early development, and diminishing plasticity as temperatures increase over 20°C, with cross-species trends for sturgeon in development plasticity and thermal limits.

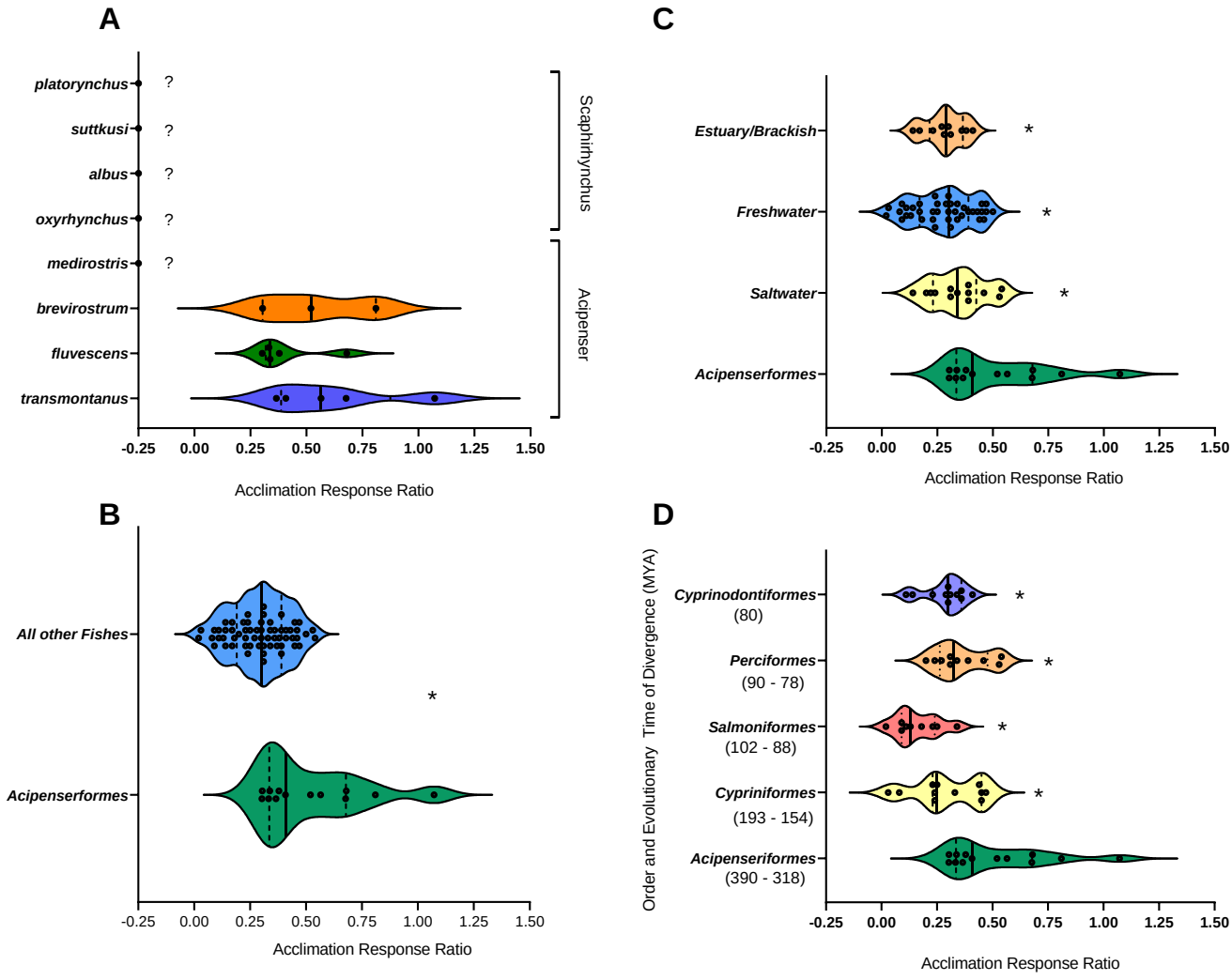

Figure 1.1 Thermal Plasticity of North American Sturgeons as compared A) across sturgeon species, B) to all other fishes, C) across aquatic habitats, and D) across strongly represented orders of fishes. Data are expressed as acclimation response ratio as described above and compared to the findings of acclimation response ratios of aquatic organisms as presented by Gunderson and Stillman (2015). Information on habitat type and taxonomy were collected from FishBase (Froese and Pauly, 2022). For Figure D, only orders with > 5 representative studies were included. Statistical significance for Figure B was determined by a Welch's T test ($P < 0.005$) while for C-D significance was determined by a one-factor ANOVA with a Holm-Sidak correction following a square root transformation ($P < 0.05$). For plots A-D the middle line represents the median of the data, while dotted lines represent the upper and lower quartiles. Each individual point represents the acclimation response ratio of a population, age range, or specific grouping of fish from the

cited literature with each individual point representing the total acclimation response ratio for a given study or data set. All sturgeon related data was collected on fish in freshwater and can be originally found in: Ziegeweid et al., 2007; Wilkes, 2011; Zhang and Kieffer, 2014; Rodgers et al., 2019; Bugg et al., 2020, Penman et al., 2021; Kieffer and Bard, *In Review*; Earhart et al., *In prep*; Bugg et al., *In prep*.

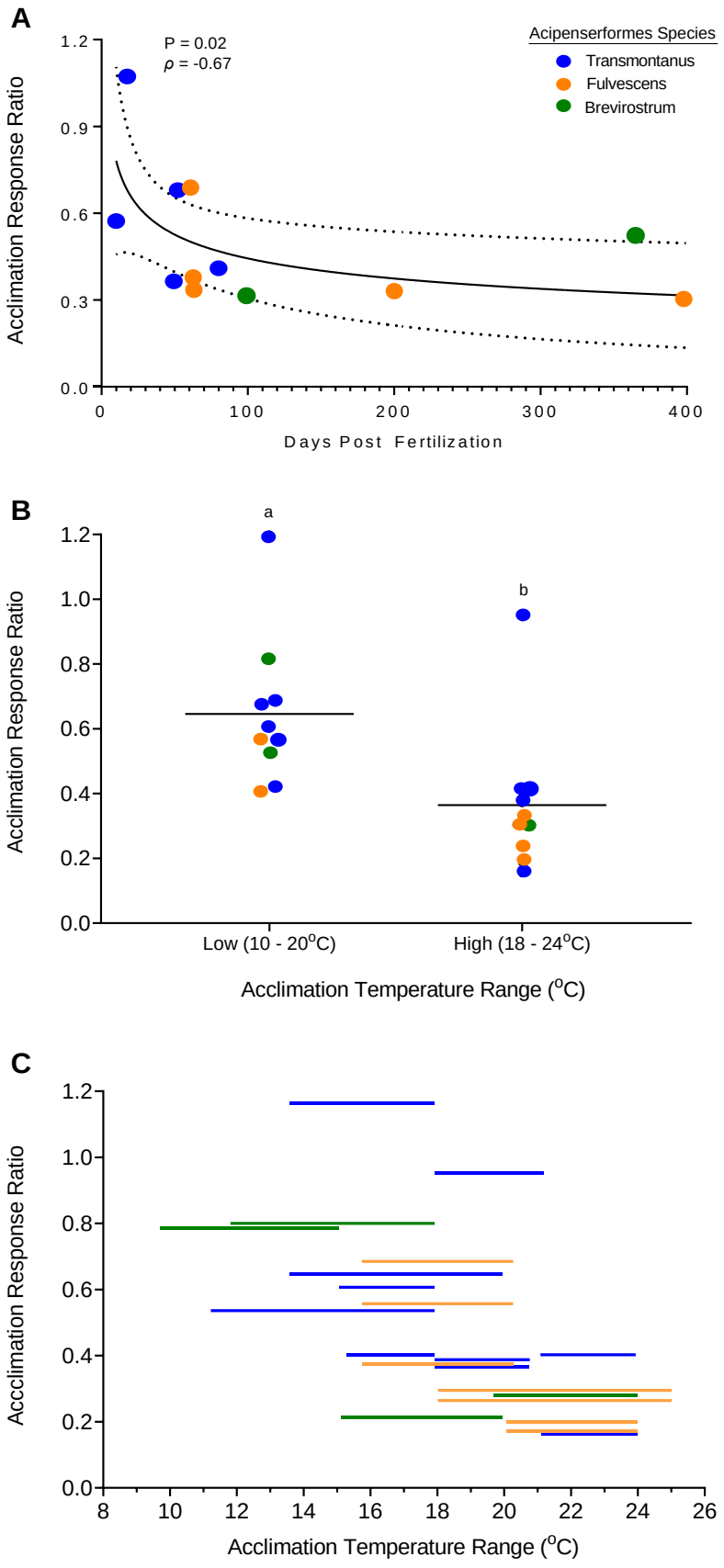
A
P = 0.02
ρ = -0.67
Acipenserformes Species
Transmontanus
Fulvescens
Brevirostrum
Acclimation Response Ratio
Days Post Fertilization
B
a
b
Acclimation Response Ratio
Low (10 - 20°C)
High (18 - 24°C)
Acclimation Temperature Range (°C)
C
Accclimation Response Ratio
Acclimation Temperature Range (°C)

Figure 1.2 Thermal Plasticity of North American Sturgeons as compared A) throughout development and B-C) across acclimation temperature ranges. Data are expressed as acclimation response ratio as described above. For Figure A, a spearman's correlation was employed with a non-linear fit (dotted lines represent the 95% confidence interval). Statistical significance for Figure B was determined by an unpaired T-test ($P < 0.05$) with the middle line representing the median of the data. Figure C demonstrates the range of thermal acclimations represented by the studies and acclimation response ratios in Figure B. Each individual point represents the acclimation response ratio of a population, age range, or specific grouping of fish from the cited literature. All sturgeon related data can be found in: Ziegeweid et al., 2007; Wilkes, 2011; Zhang and Kieffer, 2014; Rodgers et al., 2019; Bugg et al., 2020, Penman et al., 2021; Kieffer and Bard, *In Review*; Earhart et al., *In prep*; Bugg et al., *In prep*.

1.6 Lake Sturgeon in Manitoba

Lake sturgeon are a wide-ranging species inhabiting the rivers of major watersheds in North America including the St. Lawrence, Hudson Bay, Great Lakes and Mississippi throughout 24 states in the US and 5 Canadian provinces (Figure 1.3A; Bruch et al., 2016; Baril et al., 2018). In Manitoba, endangered lake sturgeon populations inhabit the province's largest river systems with natural impediments and hydroelectric dams limiting gene flow and contributing to genetically distinct northern and southern sub-populations (McDougall et al., 2017; Figure 1.3). Southern populations, such as those in the Winnipeg River, are generally stable, while those to the northern extremes of the province, like the sub-arctic Nelson and Burntwood Rivers, have faced largescale population declines (> 90%) since the 1960s (COSEWIC 2006). Across these Manitoba river systems, water temperatures are projected to increase 2.1-3.4^{o}C by 2050 (Manitoba Hydro, 2015), which may induce thermal stress and further threaten these populations, especially in the northernmost reaches of the province. Additionally, in the first year of life, Manitoba lake sturgeon must undergo a lengthy overwintering period, especially so for northern populations, with long term exposure to near freezing temperatures which may induce cold stress and contribute to high levels of mortality in early development (Deslauriers et al., 2018). These changes in temperature may have developmental effects, limit physiological plasticity, and compound the impacts of other stressors like environmentally pervasive pathogens.

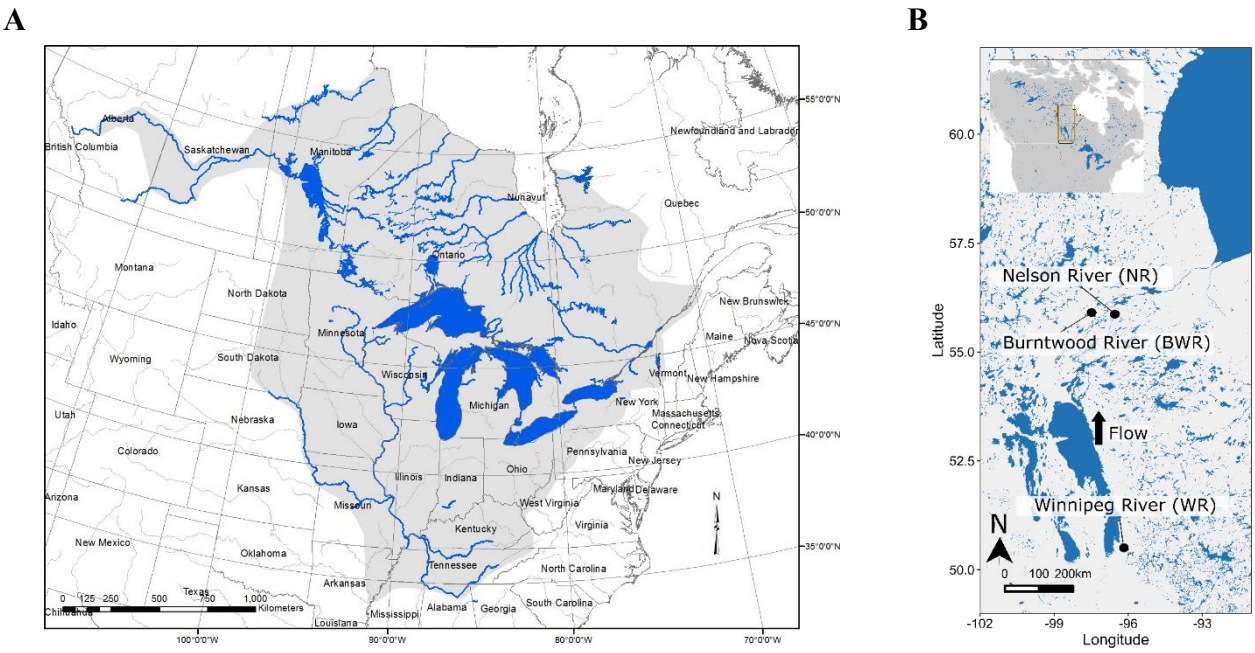

Figure 1.3 A) Lake sturgeon historic range (shaded area; adapted from Harkness and Dymond, 1961) and present distribution (blue colored waters) in North America (included and modified with author permissions; Bruch et al., 2016) and B) Geographic localities of the three studied lake sturgeon, *Acipenser fulvescens*, populations within Manitoba, Canada, highlighting the northern Nelson River (NR) and Burntwood River (BWR) populations, the more southernly Winnipeg River (WR) sturgeon population, as well as the direction of flow throughout the watershed. Between the southern WR and northern BWR and NR populations there are numerous migratory barriers which keep these populations isolated despite a northward flow.

Since the mid 1990's stakeholders have collected gametes from the wild spawning adults of these northern populations, reared the offspring in a hatchery environment throughout early development, and then released them back into their native rivers in order to bolster natural populations (McDougall et al., 2014). Throughout this process sturgeon may face a variety of environmental stressors related to increased temperature environments and elevated pathogen levels in hatcheries, the effects of abrupt cold shock and novel pathogen encounters during stocking, as well as the long-term effects of temperature alteration in their natural environment. Prior to the assembly of this thesis, little was known about the thermal limits, acclimatory capacity, or population-specific responses of lake sturgeon, and in particular as they relate to lake sturgeon populations within Manitoba. As lake sturgeon are a long lived slow growing fish, the individuals being reared in hatcheries currently and developing in the wild will likely encounter increasing thermal variability and live long enough to experience the projected elevated temperatures thresholds of 20 and 24ºC. Therefore, in order to ensure the persistence of these leading edge (northern) lake sturgeon populations, it is paramount that we understand the impacts of thermal stress on the development, plasticity and physiology of endangered populations of lake sturgeon in Manitoba.

1.7 Thesis Hypotheses & Objectives

The analysis of the thermal physiology of developing lake sturgeon, their acclimatory capacity, sub-lethal thermal thresholds and how these concepts apply to the conservation of latitudinally distributed populations within Manitoba, Canada, connect the studies assembled for this thesis. Each chapter uses measurements of relative mRNA abundance, as well as whole-organism performance metrics, to assess the effects of increased environmental temperatures on the plasticity and physiology of developing lake sturgeon. Chapter 2 investigated population- and acclimation-specific responses of lake sturgeon for application in hatchery rearing and release protocols. Chapter 3 and 4 assess the effects of elevated rearing temperatures during early development on whole-organism physiological and transcriptional responses, respectively. Chapter 5 employs a targeted gene-suite qPCR approach to evaluate the effects of elevated temperatures and chronic thermal stress on lake sturgeon immunocompetence.

This thesis asks the question, *what are the physiological effects of increasing temperature on Manitoba populations of lake sturgeon?* These questions are investigated in sturgeon to provide

insight into their hatchery rearing practices and to evaluate the effects of increasing temperatures on wild sturgeon populations. Additionally, sturgeon, Acipenseriformes, represent an ancestral clade, thus insights into their physiological responses may provide context for more newly derived fish species. The fish studied here are latitudinally distributed populations of lake sturgeon throughout Manitoba and represent the progeny of wild-caught individuals from their respective populations.

Four objectives were proposed to answer this question:

1) Investigate the effects of early thermal environment and population on the growth, condition, and cold responsive mRNA transcript abundance of young of year lake sturgeon (Chapter 2)
   Hypothesis: Early acclimation to elevated temperatures has long-term implications for growth and response to cold which may be encountered during late season stocking.
   Prediction: These changes would be apparent in both population- and acclimation-specific responses of growth and condition in young of year sturgeon throughout development and in the cold induced hepatic expression of thermally responsive genes. I expected these physiological alterations, induced by cold shock, would be greater in the southern Winnipeg River population and in warm acclimated Nelson River sturgeon, due to their historical or early developmental exposures to increased environmental temperatures, respectively.

2) Measure key physiological and molecular traits to evaluate the thermal plasticity of latitudinally distributed populations of developing lake sturgeon following acclimation to elevated temperatures (Chapter 3)
   Hypothesis: There will be population-specific responses to acclimation and acute thermal stress.
   Prediction: The southern Winnipeg River population would have increased thermal plasticity which would be apparent in the critical thermal maximum (CT_{max}) and the differential expression of mRNA transcripts important in the response to heat shock, hypoxia, and osmoregulatory disruption, when compared to the northern Burntwood

River population. Further, I predicted temperature-dependent population-specific responses in body size, hepatosomatic index, metabolic rate, and glutathione peroxidase activity.

3) Evaluate transcriptome-wide responses to elevated acclimation temperatures in latitudinally distributed populations of developing lake sturgeon (Chapter 4)
   Hypothesis: Acclimation to elevated temperatures would elicit transcriptome-wide responses associated with energy mobilization, pathogenic infection, oxidative stress, cellular repair, and heat shock proteins, indicative of chronic thermal stress in developing lake sturgeon.
   Prediction: The northern Burntwood River population of lake sturgeon would show greater transcriptional signatures of thermal stress as temperatures increased, relative to the southern Winnipeg River population, based on their lower sub-lethal thresholds for thermal performance (Chapter 3).

4) Determine the impact of temperature on the innate immune response of developing lake sturgeon (Chapters 5 and 6)
   Hypothesis: Immune and stress responses of developing lake sturgeon are interconnected (Chapter 5) and chronic thermal stress compromises innate immunity (Chapter 6)
   Prediction: The measurement of gene expression responses would indicate a simultaneous activation of both stress and innate immune responses when exposed to bacterial lipopolysaccharides in early development (Chapter 5), and that following acclimation to elevated temperatures this transcriptional induction would be diminished in 20ºC when to compared to 16ºC acclimated sturgeon (Chapter 6).

Chapter 1 demonstrated that sturgeon have high levels of thermal plasticity when compared to other fishes. The following chapters explore this thermal plasticity as it applies to lake sturgeon populations in Manitoba, by investigating the effects of population and elevated temperature exposures in early development (Chapter 2), sub-lethal thresholds for populations as temperatures increase (Chapters 3 and 4), the innate immune response of lake sturgeon in early development (Chapter 5) and impacts of increasing temperatures on innate immunity (Chapter

6).

Chapter 2. The effects of population and thermal acclimation on the growth, condition and cold responsive mRNA transcript abundance of age-0 lake sturgeon (*Acipenser fulvescens*)

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2.1 Abstract

In Manitoba, Canada, wild lake sturgeon (*Acipenser fulvescens*) populations exist along a latitudinal gradient and are reared in hatcheries to bolster threatened populations. We reared two populations of lake sturgeon, one from each of the northern and southern ends of Manitoba and examined the effects of typical hatchery temperatures (16ºC) as well as 60-day acclimation to elevated rearing temperatures (20ºC) on mortality, growth and condition throughout early development. Additionally, we examined the cold shock response, which may be induced during stocking, through the hepatic mRNA transcript abundance of genes involved in the response to cold stress and homeoviscous adaptation (*HSP70, HSP90a, HSP90b, CIRP* and *SCD*). Sturgeon were sampled after 1 day and 1 week following stocking into temperatures of 8, 6 and 4ºC in a controlled laboratory environment. The southern population showed lower condition and higher mortality during early life than the northern population while increased rearing temperature impacted the growth and condition of developing northern sturgeon. During the cold shock, *HSP70* and *HSP90a* mRNA transcript abundance increased in all sturgeon treatments as stocking temperature decreased, with higher expression observed in the southern population. Expression of *HSP90b*, *CIRP* and *SCD* increased as stocking temperature decreased in northern sturgeon with early acclimation to 20ºC. Correlation analyses indicated the strongest molecular relationships were in the expression of *HSP90b*, *CIRP* and *SCD*, across all treatments, with a correlation between *HSP90b* and body condition in northern sturgeon with early acclimation to 20ºC. Together, these observations highlight the importance of population and rearing environment throughout early development and on later cellular responses induced by cold stocking temperatures.

2.2 Introduction

Both genotype and early rearing environment have strong impacts on the development of fishes and will shape long-term individual responses to environmental stressors (Fangue et al., 2006; Green & Fisher, 2004; Selong et al., 2001; Whitehead et al., 2012). Specifically, increased environmental temperatures during development may have long-lasting effects on a fish's phenotype later in life, including changes in body size, condition and cellular responses to environmental stressors (Georgakopoulou et al., 2007; Jonsson & Jonsson, 2014; Loughland et al., 2021). At the population level, differences in selective pressures across geographic localities can influence the development of population-specific phenotypes and ultimately whole organism responses (Bugg et al., 2020; Fangue et al., 2006; Jeffries et al., 2019; Whitehead et al., 2012). For species with extensive ranges, latitudinal gradients across populations and the differences in early thermal environment which accompany them will ultimately impact the formation of these early developmental phenotypes (Jonsson & Jonsson, 2019; Pigliucci et al., 2006), and potentially impact the long-term growth, cellular responses and survival of the organism.

Lake sturgeon, *A. fulvescens*, are a long-lived and widely distributed species of conservation concern with populations existing along a latitudinal gradient throughout North America. Hence, lake sturgeon populations are exposed to an array of environmental conditions. In Manitoba, Canada, northern and southern populations of lake sturgeon are separated by historical barriers which limit gene flow (Figure 2.1a; McDougall et al., 2017). These different populations also experience distinct environments in early development and exhibit differential physiological and cellular responses to increased environmental temperatures (Bugg et al., 2020). Northern populations likely experience the greatest impacts of increasing temperatures resultant from global climate change (Sharma et al., 2007; Vincent et al., 2015; Zhang et al., 2019). Thus, hatcheries within the province collect gametes from northern parents for conservation purposes during the reproductive season (late May to mid-July) and rear juvenile lake sturgeon for release to bolster these threatened wild populations. Lake sturgeon that are used to enhance northern populations are typically reared at the Grand Rapids Fish Hatchery at 16^{o}C with the potential for increasing rearing temperatures in the first year of life to increase growth rates and achieve larger sturgeon prior to release (Bugg et al., 2020). Recent studies have demonstrated that subtle

changes in rearing temperature during early development can influence the growth and condition of lake sturgeon prior to a simulated overwintering event, which may influence survival rates (Brandt et al., 2022; Yoon et al., 2019, 2020). However, there is little data available concerning the effects of exposures to elevated temperatures during early development on adaptive responses to cold during the first winter of life. Additionally, young-of-the-year (YOY) lake sturgeon may be released in late season (October and November), when river temperatures begin to decrease (McDougall et al., 2020). As it is difficult to examine the effects of release temperature in a natural setting, laboratory studies can provide useful insights into the effects of stocking temperature post-release to better inform hatchery management and stocking practices.

While many studies investigate the physiological responses of fishes to high temperature thermal stress, there is less focus on the effects of low temperature stress, or cold stress (Donaldson et al., 2008). Cold stress can lead to detrimental sublethal effects on feeding, growth, behaviour, survival and cellular responses of fishes (Cheng et al., 2017; Donaldson et al., 2008; Nikoskelainen et al., 2004; Ward & Bonar, 2003). At the cellular level, cold stress may compromise protein integrity, resulting in the binding of chaperones to proteins and cellular structures to stabilize them from the denaturing effects of environmental stress (Teigen et al., 2015). Heat shock proteins (HSP70, HSP90a and HSP90b) are a family of chaperone proteins that are responsive to both high and low temperature stress, and can be acutely (HSP70, HSP90a) or constitutively (HSP90b) expressed in fishes depending on the severity and duration of the stressor (Buckley et al., 2006; Feder & Hofmann, 1999; Hori et al., 2010; Somero, 2020). Additionally, cold inducible RNA binding protein (CIRP) acts to increase mRNA stability and facilitate translation under thermally stressful conditions (Zhong & Huang, 2017). Across an array of marine and freshwater fishes, the mRNA transcript abundance of CIRP is downregulated under high temperature thermal stress but upregulated at low temperatures across a variety of tissues (Akbarzadeh et al., 2018; Gracey et al., 2004; Jeffries et al., 2012, 2014; Liu et al., 2020; Rebl et al., 2013; Verleih et al., 2015). With its prominent role in response to RNA processing and protein synthesis under cold stress conditions, CIRP expression represents a strong marker for acute cold shock with similar cold-induced chaperone activity apparent from bacteria to humans (Gracey et al., 2004; Jiang et al., 1997; Verleih et al., 2015).

Changes in environmental temperature are also associated with alterations of cellular membrane fluidity in fishes (Cossins & Prosser, 1978; Donaldson et al., 2008; Malekar et al.,

2018). As membrane fluidity plays a key role in cell function (Hazel, 1997), temperature decreases typically lead to changes in the fatty acids of phospholipids through decreasing the levels of saturated fatty acids but increasing unsaturated fatty acids, resulting in an overall increased membrane fluidity (Farkas et al., 2001). The increase of mono- and polyunsaturated fatty acids in phospholipids as a response to temperature decline has been widely observed as an adaptive mechanism termed homeoviscous adaptation, which strongly influences tolerance to cold environments and ultimately survival (Bowden et al., 1996; Hsieh & Kuo, 2005; Kelly & Kohler, 1999; Tiku et al., 1996; Trueman et al., 2000; Wodtke & Cossins, 1991). Synthesis of monounsaturated fatty acids is catalysed by stearoyl-CoA desaturase (SCD), an enzyme involved in a rate-limiting step of the insertion of a double bond into the fatty acid chain, functioning to increase membrane flexibility (Enoch et al., 1976; Hsieh et al., 2007; Jeffcoat et al., 1977). Thus, changes in SCD mRNA transcript abundance may represent altered capacity for homeoviscous adaptation, helping to regulate unsaturated fatty acid levels in cellular membranes and maintain membrane fluidity in response to cold stress. Thus, transcriptional responses of SCD as well as chaperone proteins may provide insight into the cellular plasticity and levels of stress experienced under cold temperatures, which may be encountered during late season stocking, particularly relevant in the northern extent of lake sturgeon distribution.

The aim of this study was to evaluate the effect of population and acclimation temperature on the growth, condition and cold responsive mRNA transcript abundance of YOY lake sturgeon in Manitoba, Canada. With distinct northern and southern populations of lake sturgeon demonstrating population and acclimation specific growth and cellular responses at elevated temperatures (Bugg et al., 2020), we hypothesized that these populations would exhibit divergent responses when reared under common conditions and then exposed to cold stress. Additionally, as early rearing environment can have long-term impacts on a fish's phenotype (Johnson et al., 2014; Jonsson & Jonsson, 2014), we hypothesized that early acclimation, which may resemble future warming scenarios, could have long-term implications for both growth and response to cold. Furthermore, we predicted that these changes would be apparent in both population- and acclimation-specific responses of growth and condition in YOY lake sturgeon throughout development, and in the cold induced hepatic expression of thermally responsive genes (*HSP70, HSP90a, HSP90b, CIRP* and *SCD*), which may be upregulated during fall stocking. Specifically, we first predicted that these physiological alterations to cold shock would

be greater in the southern Winnipeg River (WR) population of lake sturgeon that naturally experience higher annual temperatures, when compared to their northern Nelson River (NR) counterparts. Second, we predicted that NR lake sturgeon acclimated to 20ºC during early development would be more affected by cold stocking than individuals from the same population reared at 16ºC. Finally, we predicted that individual sturgeon with lower overall condition would be the most greatly affected by acute cold shock, regardless of population or early acclimation.

2.3 Methods

2.3.1 Ethical Statement

All animals in this study were reared and sampled under the guidelines established by the Canadian Council for Animal Care and approved by the Animal Care Committee at the University of Manitoba under Protocol #F15-007.

2.3.2 River Temperatures

River water temperatures were measured in both the WR and NR from 2013 to 2016 (Figure 2.1b) by a DigiTemp SDI-12 submersible temperature sensor (Forest Technology Systems, Victoria, British Columbia, Canada) and a series 500 SDI-12 transducer (TE Connectivity, Schaffhausen, CH), respectively. Measurements for water temperatures in the WR were recorded downstream of the generating station at Pointe du Bois where spawning lake sturgeon were caught for this study (50º17’52’’N, 95º32’5’’W), while water temperatures in the NR were recorded 2 km below the Hudson Bay Railway bridge (55º49’43’’N, 96º36’9’’W). Water temperatures were recorded at midnight (0:00:00am) for both rivers and therefore likely represent the lower range of expected daily temperatures. Temperature measurements from 2013 to 2016 were then averaged for each day of the year to create a list of average daily temperatures. These daily temperatures were also averaged for seasonal and yearly temperatures.

2.3.3 Lake Sturgeon Husbandry

In late May and early June of 2018, gametes from wild-caught female and male lake sturgeon were harvested from individuals at the Pointe du Bois Generating Station on the WR (50º17′52’’N, 95º32′51’’W) and near the confluence of the Landing and Nelson Rivers,

respectively (55º19′36’’N, 96º54′16’’W). Gametes from both populations were transported to the Animal Holding Facility at the University of Manitoba where fertilization occurred. Sturgeon from the WR population were the product of fertilization of eggs from three females with the sperm from six males (three maternal families) while sturgeon from the NR were the product of the fertilization of eggs from one female with the sperm from two males (one maternal family). After fertilization, embryos from both populations were de-adhesed by submersion in a clay solution of Fuller’s earth. Then, embryos were rinsed with de-chlorinated fresh tap water and incubated in tumbling jars at 12ºC, however, they re-adhesed in the tumbling jars within 24 h and were moved to aquaria to complete their incubation prior to hatching.

Post-hatch, larval sturgeon were reared in 9 L flow-through aquaria with aeration and bioballs as substrate (Earhart et al., 2020a). Starting at 13 days post fertilization (dpf) rearing temperature was increased at 1ºC day^{-1} until 16ºC was achieved prior to the onset of exogenous feeding (Figure 2.1c). Larval sturgeon were first offered freshly hatched artemia (Artemia International LLC; Texas, USA) three times daily starting at 19 dpf to promote recognition of prey and were first observed feeding at 21 dpf. Sturgeon from each population were then transferred from early rearing tanks to duplicate 9 L experimental tanks $treatment^{-1}$ at 28 dpf, with a density of approximately 150 sturgeon larvae $tank^{-1}$. Rearing density was adjusted with equal numbers of sturgeon from the families representing each population (e.g., 50 fish from each WR maternal family). Starting at 50 dpf, sturgeon from all treatments were slowly transitioned to a primarily bloodworm diet (Hikari Bloodworms, Hikari, California, USA) at an approximate rate of 10% every three days, with feedings reduced to twice day^{-1} starting at 68 dpf, resulting in a total of 27 days to transition to a 100% blood worm diet by 77 dpf. After the feeding transition to bloodworms was completed at 78 dpf, sturgeon were transferred to two duplicate 230 L aquaria $treatment^{-1}$ at an approximate density of 140 sturgeon $tank^{-1}$ and a total of 280 sturgeon $treatment^{-1}$. During the entirety of the experiment, fish were fed to satiation offering food until there was no more consumed.

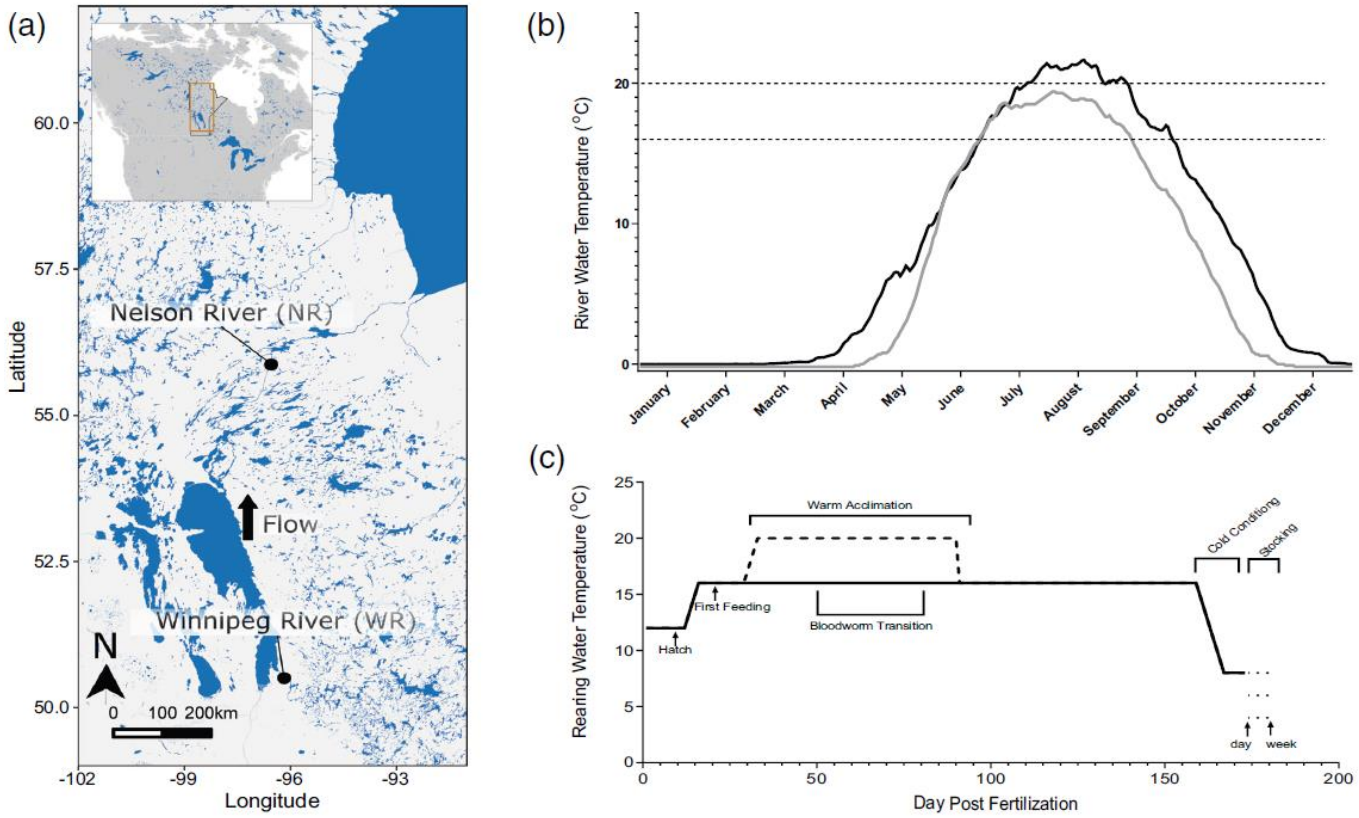

Figure 2.1 a) Geographic localities, b) river temperatures, and c) rearing temperatures for Winnipeg River (WR) and Nelson River (NR) populations of lake sturgeon reared under experimental acclimation conditions. River temperatures were measured at midnight and tick marks on the x-axis indicate the middle of each given month. Dashed lines indicate different acclimation temperatures used in the current study, with 16ºC representing typical hatchery rearing temperatures and 20ºC depicting elevated temperatures that may be expected in natural environments in the future or in hatcheries with elevated temperature rearing protocols. Rearing water temperatures remained at 12ºC until post-hatch, after which they were raised to 16ºC prior to first feeding. An additional NR treatment was acclimated to 20ºC for 60 days between 30 and 90 days post fertilization (dpf). At 160 dpf cold conditioning began, reducing rearing temperatures down to 8ºC, prior to cold shock. At 173 dpf sturgeon ere acutely stocked into temperatures of 8,6 and 4ºC with liver samples taken 1 day (24 h) and 1 week (168 h) for investigation of hepatic mRNA transcript abundance in response to cold temperature exposure, represented by arrows in the above figure. The full black line represented temperatures in the 16ºC acclimation treatment, while the dotted line represents the period of early acclimation to 20ºC for the warm acclimated NR treatment.

2.3.4 Body Measurements

Every 30 days throughout development, five sturgeon were selected from each replicate tank for a total of 10 sturgeon per treatment. Selected sturgeon were then euthanized by immersion in an overdose of tricaine methanesulfonate solution (250 mg l^{-1}; MS-222, Syndel Laboratory, Vancouver, Canada) buffered with an equal mass of sodium bicarbonate. Body mass (weighed to 0.001 g) and total length (measured to 1 mm) were recorded for each sampled sturgeon and Fulton's condition factor (K; Fulton, 1911) was subsequently calculated as:

$$K = \frac{mass\ (g)}{total\ length\ (cm)^3}\ x\ 100$$

2.3.5 Acclimation and Cold Shock

Beginning at 30 dpf, a subset of lake sturgeon from both the WR and NR populations was acclimated to a 20ºC treatment. Water temperatures were increased from 16ºC at a rate of 1ºC per day for 4 days until 20ºC was reached. Lake sturgeon then remained at this acclimation temperature for 60 days before returning to 16ºC. After this 60-day warm acclimation period, temperatures in the 20ºC treatments were reduced back to 16ºC to match temperatures in the 16ºC treatments, where they remained in all treatments until 160 dpf. A WR 20ºC treatment was included in the original experimental design but nearing the end of warm acclimation showed signs of pathogenic infection and elevated mortality, and thus was removed from the study. Mortality and temperature were monitored at least two times daily across all treatments during this acclimation period. At 160 dpf, cold conditioning began, reducing the rearing temperatures by 1ºC per day from 16ºC until they reached 8ºC. Tank temperatures were then held at 8ºC for 1 week prior to the beginning of cold shock trials. This temperature transition is reflective of standard hatchery operations for stocking of juvenile lake sturgeon in October and November in Manitoba. YOY lake sturgeon are acclimated to 8ºC in the hatchery for 1 week prior to release, but this may result in acute cold shock and increased post-release mortality if river temperatures are below this temperature.

At 173 dpf, following cold conditioning to 8ºC for 1 week, 10 lake sturgeon from each treatment group (WR 16, NR 16 and NR 20ºC), five sturgeon per replicate rearing tank, were selected and a simulated stocking event was performed by placing fish into one of six 9 l aquaria

per treatment in a Multi-Stressor unit (Aquabiotech, Coaticook, Quebec, Canada). For each treatment, two of the six tanks 4 were held at 8, 6 or 4°C, which represent stocking temperatures in the late fall. For each stocking temperature, individual tanks were then subsequently sampled 1 day (24 h) and 1 week (168 h) post-stocking. In addition to euthanizing sturgeon and taking body measurements as described above, liver tissue was dissected and preserved in RNAlater (Thermo Fisher Scientific, Waltham, USA), held at 4°C for 24 h, and then stored at -80°C prior to analysis. Throughout the current experiment, including both development and cold stocking, temperatures in each treatment were recorded every 15 min by HOBO Water Temperature Pro v2 Data Loggers (Onset Computer Corporation, Bourne, MA, USA).

2.3.6 RNA Extraction, cDNA synthesis, and qPCR

Total RNA was extracted from the livers of all YOY lake sturgeon treatment groups using a PureLink RNA mini Kit (Invitrogen; Ambion Life Technologies) following the manufacturer's instructions. Liver tissue was homogenized in 500 µl of lysis buffer, for 5 min at 50 Hz using a TissueLyser II (Qiagen, Germantown, MD, USA). Total RNA concentration, purity, and integrity was assessed using a Nanodrop One (Thermo Fischer Scientific) and gel electrophoresis, respectively, for each sample. Synthesis of cDNA was conducted with a qScript cDNA synthesis kit (Quantbio; Beverly, Massachusetts) following the manufacturer's instructions from 1 µg of DNAse treated total RNA. A SimpliAmp Thermal Cycler (ThermoFisher; Waltham, Massachusetts) with cycling conditions of 1 cycle of 22°C for 5 min, 1 cycle of 42°C for 30 min, and 1 cycle of 85°C for 5 min and a final hold at 4°C was used to perform the cDNA synthesis. The cDNA samples were stored at -20°C.

Real-time quantitative polymerase chain reactions (RT-qPCR) for each gene of interest (*HSP70, HSP90a, HSP90b, CIRP,* and *SCD*) and reference gene (*RPS6* and *RPL7*) were conducted using 5 µl of Bio-Rad SsoAdvanced Universal SYBR Green Supermix (Bio-Rad; Hercules, California), 0.1 to 0.05 µl of 100 µM primers, 2 µl of cDNA per sample, and nuclease-free water adjusted for each assay to bring the total volume of each well to 10 µl. For all assays, each well contained 0.025 µl forward and 0.025 µl reverse primer, except *SCD* and reference genes *RPS6* and *RPL7* which used 0.05 µl forward and 0.05 µl reverse primer well^{-1}. The cDNA for all assays was diluted 1:10 with nuclease-free water prior to its inclusion in RT-qPCR assays.

Primers for *SCD* were designed from an annotated white sturgeon, *Acipenser transmontanus*, liver transcriptome (Doering et al., 2016). Primers for *CIRP* were designed based on sequences from the Adriatic sturgeon *Acipenser naccarii*, which were retrieved from Anaccariibase (Sequence ID: CDNA3-4_11_2010_0_rep_c10569; Vidotto et al., 2013) and aligned against publicly available transcripts using NCBI BLAST (Johnson et al., 2008), sharing highly conserved regions with the sterlet sturgeon, *Acipenser ruthensus* (Sequence ID: XM_034911883.1; 95.2% Identity). Primer sequences for *HSP70*, *HSP90a*, *HSP90b*, *RPS6* and *RPL7* were designed based on sequences from an annotated lake sturgeon ovary transcriptome produced through pyrosequencing and are the same as used in other lake sturgeon thermal stress experiments (Hale et al., 2009; Yusishen et al., 2020; Table 2.1). The expression of all genes of interest were normalized to the expression of reference genes *RPS6* and *RLP7*, using the geometric mean (Vandesompele et al., 2002), and analysed following the application of the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001). The expression of all genes of interest were normalized to the WR 16ºC, 8ºC stocking temperature, 1 week post-stocking timepoint.

Table 2.1 Primer sequences used to investigate changes in the mRNA transcript abundance of chaperones, responses to cold stress, and homeoviscous adaptation induced during cold shock trials for young of the year lake sturgeon (*Acipenser fulvescens*). Target genes *HSP70*, *HSP90a, HSP90b, Cold Inducible RNA binding protein* (*CIRP*) and *Stearoyl-CoA Desaturase* (*SCD*) were selected based on their responsiveness as chaperones as well as their roles in cold stress and homeoviscous adaption. *RPS6* and *RPL7* were used as reference genes. Efficiencies are listed as a percentage.

Gene	Forward	Reverse	Efficiency (%)
HSP70	CTGTCACTCGGACTTTAACTTTAATTT	AACTGTCCTAAAGAACTGCCTTATCC	94.0
HSP90a	GATCACACGAGCGGATTTGC	ATGTTGTGCTCTGTCCTGCG	96.4
HSP90b	GGAACCAAGGCTTCATGGA	CCAACACCAAACTGACCAATCA	92.8
CIRP	TTCGACACAAACGAGCAGTC	TCACCACAACTTCCGAGACA	98.1
SCD	AGCCAAGTTGCGTTGAGA	GTCCTCGTGGGTTGGTTACTT	93.1
RPS6	CTGGCTGGATTCTGATTTGGATG	ATCTGATTATGCCAAGCTGCTG	95.6
RPL7	TGCTTAGGATTGCTGAGCCG	GATCTTTCCGTGACCCCGTT	96.0

2.3.7 Statistical Analysis

Throughout development, data collected on the condition of YOY lake sturgeon were analysed with two-factor ANOVAs with either population and developmental timepoint (dpf) or acclimation treatment and developmental timepoint included in the model as fixed effects for comparisons of WR 16ºC with NR 16ºC, and NR 16ºC with NR 20ºC acclimation treatments, respectively. For comparisons of length and weight throughout development, data could not be successfully transformed to pass the assumptions for ANOVAs, so they were analysed with nonparametric Wilcoxon signed ranked tests to compare sequential developmental timepoints as well as across acclimation treatments and populations, comparing either WR 16ºC with NR 16ºC, or NR 16ºC with NR 20ºC.

The mRNA transcript abundance and condition factor data from cold shock trials were analysed using three-factor ANOVAs, with population, stocking temperature and time or acclimation treatment, stocking temperature and time included in the model as fixed effects for comparison of WR 16ºC with NR 16ºC, and NR 16ºC with NR 20ºC acclimation treatments, respectively. For all ANOVAs, the Shapiro–Wilk and Levene tests were used to assess normality of data and homogeneity of variance as well as the visual inspection of fitted residual plots. If the assumptions of either test were violated, a ranked, log or square root transformation was performed on the data set. Following ANOVAs, Tukey's honestly significant difference tests from the 'multcomp' package (Hothorn et al., 2008) were used to assess differences between populations, acclimation treatments, stocking temperatures and time points. Detailed results for all ANOVA analyses are provided in Supplementary Table A.2.1.

Semi-partial Spearman's correlations were used to investigate the relationship between the mRNA transcript abundance of each gene and that of other studied genes, as well as the condition factor of each representative sturgeon from cold shock trials using the 'ppcor' package (Seongho, 2015), while accounting for variance in both stocking temperature and time. This analysis was conducted individually for each of the representative treatments (WR 16ºC, NR 16ºC and NR 20ºC; n = 48, 45 and 50, respectively). Values are reported as estimated Spearman's rho (ρ) with significance indicated by $^{*}P < 0.05$ and $^{**}P < 0.00001$. Additionally, principal component analyses were conducted on the same data, individually for each treatment, graphing variable plots to investigate the relationship between stocking temperature, time, the expression of assayed genes, as well as condition factor.

Differences in mortality between populations and acclimation treatments during the acclimation period (from 30 to 90 dpf) were analysed using Cox proportional hazards models using the 'survival' and 'survminer' packages (Kassambara et al., 2019; Therneau, 2015) with covariates of both population and acclimation temperature included in the model. Next, a pairwise comparison was conducted using the 'pairwise_survdiff' function in the 'survminer' package and a Bonferroni correction to compare mortality across both populations and acclimation treatments. Hazard models were evaluated using the 'cox.zph' function in the 'survival' package to ensure that the assumptions of Cox proportional hazards were met. All statistical analyses were performed using R 4.0.0 with a significance level (α) of 0.05.

2.4 Results

2.4.1 River Temperatures

From 2013 to 2016, under average yearly conditions, the WR exceeded 20^{o}C 14% and 16^{o}C 27.1% of days. In contrast, the NR never exceeded 20^{o}C and only exceeded 16^{o}C on 21.4% of days. Over the years of temperature recording from 21 June to 21 September when larval lake sturgeon are undergoing rapid development and growth, the WR was on average 1.89 ± 1.2^{o}C (mean ± S. D.) warmer than the NR. This discrepancy increased in the fall, 22 September to 21 December, when sturgeon are likely accumulating stores for overwintering, with water temperatures in the WR 3.54 ± 1.8^{o}C warmer than in the NR. When averaging across the total year, 1 January to 31 December, the WR was 1.85 ± 1.8^{o}C warmer than the NR.

2.4.2 Effects of population and acclimation on mortality, growth and condition

2.4.2.1 Mortality

Population, but not acclimation temperature, had a significant effect on the mortality of YOY lake sturgeon in this study, as determined by Cox proportional hazard models. The WR 16^{o}C treatment had a hazard ratio of 3.97 when compared to combined NR 16 and 20^{o}C treatments ($P < 0.0001$) with elevated cumulative mortality of 11.3% in the WR, compared to 4.4% for NR sturgeon. Further pairwise analysis showed that the WR 16C treatment had higher levels of mortality than either individual NR acclimation treatment ($P < 0.0001$ and $P < 0.05$ for 16 and 20^{o}C treatments, respectively).

2.4.2.2 Population comparisons

Throughout development, both WR and NR sturgeon increased their length with each developmental timepoint ($P < 0.05$; Table 2.2), except between 150 and 173 dpf for NR sturgeon, in which cold conditioning began. At 30 dpf, larval sturgeon from the WR were larger than the NR counterparts ($P < 0.05$), but this difference did not persist throughout development. Similarly, lake sturgeon from both populations increased their weight with each developmental timepoint until 150 dpf ($P < 0.05$). However, neither sturgeon from the WR or NR reared at 16C increased their weight between 150 and 173 dpf once cold conditioning began. Overall, the condition factor of YOY lake sturgeon reared at 16^{o}C was affected by population ($P < 0.0001$, $F = 28.02$). There was no significant difference in condition factor prior to bloodworm transition at 30 dpf, after which the NR population had elevated condition relative to their WR counterparts at 60, 120 ($P < 0.05$) and 173 dpf ($P < 0.0001$; 13.3%, 8.8% and 8.3%, respectively).

Table 2.2 Body measurements of Winnipeg River (WR) and Nelson River (NR) populations of lake sturgeon (*Aciepsner fulvescens*) throughout their early development, when reared at 16 and 20°C. Significant differences in length and weight throughout development and across treatments were determined by Wilcoxon signed ranked tests as the data were nonparametric. Significance differences in condition were determined via two-factor ANOVA ($P < 0.05$) followed by Tukey's honestly significant difference post hoc test comparing either populations between WR 16°C and NR 16°C or acclimation treatments between NR 16°C and NR 20°C. Data are represented as a mean ± S.E.M. (n = 10–30 per treatment). *Represents significant differences between WR 16°C and NR 16°C populations, while †indicates significant differences between NR 16°C and NR 20°C acclimation treatments, within a developmental time point. Differences throughout developmental time, within a given treatment, are noted with letters a, b and c. Data are expressed as mean ± S.E.M. with sample number (n) in parentheses.

Days post fertilization	WR 16°C	NR 16°C	NR 20°C
		Length (mm)	
30	26.97 ± 0.35 (10)^{*a}	25.52 ± 0.32 (10)$^{*\dagger a}$	24.6 ± 0.25 (10)$^{\dagger a}$
60	54.29 ± 1.15 (10)^{b}	54.69 ± 1 (10)$^{\dagger b}$	59.7 ± 1.24 (10)$^{\dagger b}$
90	75 ± 3.94 (10)^{c}	74.13 ± 1.65 (10)$^{\dagger c}$	86.4 ± 1.46 (10)$^{\dagger c}$
120	112.9 ± 3.59 (10)^{d}	112.2 ± 3.51 (10)$^{\dagger d}$	124.3 ± 2.34 (10)$^{\dagger d}$
150	135.7 ± 3.54 (10)^{e}	145.4 ± 3.96 (10)$^{\dagger e}$	158.7 ± 4.49 (10)$^{\dagger e}$
173	145.17 ± 3.11 (30)^{f}	146.87 ± 2.18 (30)$^{\dagger e}$	158 ± 2.37 (30)$^{\dagger e}$
		Weight (g)	
30	0.067 ± 0.00 (10)^{*a}	0.059 ± 0.00 (10)$^{*\dagger a}$	0.05 ± 0.00 (10)$^{\dagger a}$
60	0.50 ± 0.04 (10)^{b}	0.57 ± 0.03 (10)^{b}	0.67 ± 0.039 (10)^{b}
90	1.49 ± 0.28 (10)^{c}	1.48 ± 0.11 (10)$^{\dagger c}$	2.02 ± 0.12 (10)$^{\dagger c}$
120	4.64 ± 0.34 (10)^{d}	5.03 ± 0.43 (10)$^{\dagger d}$	6.55 ± 0.38 (10)$^{\dagger d}$
150	8.63 ± 0.73 (10)^{e}	10.4 ± 0.66 (10)^{e}	12.31 ± 1.16 (10)^{e}
173	11.41 ± 0.49 (30)^{e}	10.18 ± 0.56 (30)$^{\dagger e}$	13.19 ± 0.53 (30)$^{\dagger e}$
		Condition factor (*K*)	
30	0.341 ± 0.01 (10)	0.353 ± 0.01 (10)†	0.335 ± 0.01 (10)$^{\dagger ab}$
60	0.309 ± 0.01 (10)*	0.35 ± 0.01 (10)*†	0.313 ± 0.01 (10)$^{\dagger ab}$
90	0.316 ± 0.02 (10)	0.358 ± 0.01 (10)†	0.316 ± 0.02 (10)$^{\dagger ab}$
120	0.32 ± 0.01 (10)*	0.348 ± 0.01 (10)*	0.338 ± 0.01 (10)^{ac}
150	0.338 ± 0.01 (10)	0.337 ± 0.01 (10)†	0.3 ± 0.01 (10)$^{\dagger b}$
173	0.326 ± 0.01 (30)*	0.353 ± 0.00 (30)*†	0.32 ± 0.01 (30)$^{\dagger c}$

2.4.2.3 Acclimation temperature comparisons

Sturgeon from the NR, in both 16 and 20ºC treatments, increased their length and weight throughout development, except from 150 to 173 dpf when cold conditioning began ($P < 0.05$). At 30 dpf, before acclimation began, NR sturgeon in 16ºC treatments were longer and heavier than those in the NR 20C treatment ($P < 0.05$). However, once acclimation started, sturgeon from the 20ºC acclimation treatment became longer and heavier than their 16ºC counterparts, with these differences persisting for the majority of the remaining experiment (60–173 dpf for length and 90, 120 and 173 dpf for mass; $P < 0.05$). The overall condition factor of YOY lake sturgeon from the NR was affected by both acclimation treatment ($P < 0.0001$, $F = 44.34$) and developmental timepoint ($P < 0.001$, $F = 4.52$). Condition factor did not change throughout development in the NR 16ºC treatment, but in the NR 20ºC acclimation treatment there was a significant decrease between 120 and 150 dpf timepoints, followed by an increase at 173 dpf relative to 150 dpf ($P < 0.05$). Between acclimation treatments, the NR 16ºC treatment had a 5% higher condition factor than their NR 20ºC counterparts at 30 dpf ($P < 0.05$), which increased with acclimation, reaching 11.8% and 13.3% by 60 and 90 dpf ($P < 0.05$), respectively, and persisted to 150 and 173 dpf ($P < 0.005$).

2.4.3 Effects of stocking on cold-inducible mRNA transcript abundance and condition

2.4.3.1 Population comparisons

Across populations of sturgeon reared at 16ºC, the mRNA transcript abundance of *HSP70* was affected by an interaction between stocking temperature and time ($P < 0.005$, $F = 6.2$) as well as population and time ($P < 0.0001$, $F = 23.6$; Figure 2.2A). Northern and southern populations expressed different levels of *HSP70* mRNA at several timepoints, with higher expression observed in the NR population after 1 day in 8ºC, but with increased expression in WR sturgeon following 1 week in 8 and 6ºC ($P < 0.05$). Throughout time, the NR sturgeon decreased their expression of *HSP70* in 8ºC over the course of the week 5.2-fold, while WR sturgeon increased their expression in 6 and 4ºC over the same time period ($P < 0.05$). For both populations, expression increased in colder temperatures at the 1 week timepoint, with increased expression in 4ºC relative to 8ºC ($P < 0.05$). However in 6ºC only the WR population increased expression relative to 8ºC stocked sturgeon ($P < 0.01$). The mRNA transcript abundance of *HSP90a* was affected by interactions between stocking temperature and time ($P < 0.0001$, $F =$

11.3) as well as population and time ($P < 0.0001$, $F = 36.6$; Figure 2.2B). After 1 day in 8 and 4°C, the NR population had higher mRNA transcript abundance of *HSP90a* than their southern WR counterparts ($P < 0.05$). However, by 1 week, this trend was reversed, with higher expression in southern WR sturgeon in each stocking temperature ($P < 0.05$). Across timepoints, NR sturgeon decreased expression of *HSP90a* 8.8-fold in 8°C over the course of the week-long exposure ($P < 0.001$), while WR sturgeon increased their expression in temperatures of 6 and 4°C ($P < 0.01$). Both populations increased their expression at 1 week as temperatures decreased, with higher expression in 6 and 4°C when compared to 8°C. In contrast to the changes in expression observed for *HSP90a*, the constitutive form of the gene *HSP90b* showed no effect of population, time or stocking temperature over the week-long exposure (Figure 2.2C).

The mRNA transcript abundance of *CIRP* was only affected by time the WR population stocked into 6°C between a day and a week post stocking ($P < 0.05$; Figure 2.2D). Similarly, there was an overall effect of time on SCD mRNA transcript abundance ($P < 0.005$, $F = 10.2$), with an additional effect of stocking temperature ($P < 0.05$, $F = 4.0$; Figure 2.2E). In the NR sturgeon, expression of *SCD* decreased over the week in 6°C, but increased as temperatures decreased in the WR population with higher expression in 4°C relative to 8°C at the 1 week timepoint ($P < 0.05$). Condition factor was affected by population ($P < 0.0001$, $F = 47.9$) with higher condition in sturgeon from the NR at both 1 day and 1 week timepoints in 8 and 6°C and 1 day in 4°C, when compared to WR sturgeon ($P < 0.05$).

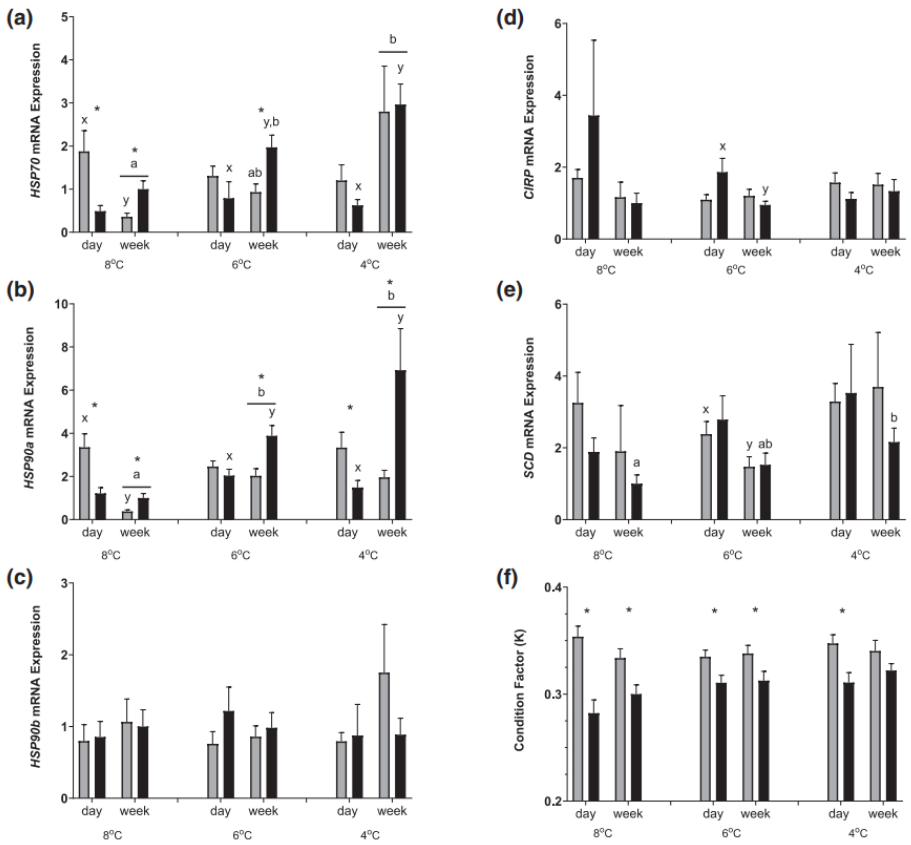

Figure 2.2 Population comparisons of lake sturgeon, *Acipenser fulvescens*, from the Winnipeg (WR) and Nelson Rivers (NR) throughout cold shock trials, in their mRNA transcript abundance of a) *HSP70*, b) *HSP90a*, c) *HSP90b*, d) *CIRP*, e) *SCD*, and f) condition factor, 1 day and 1 week following stocking into 8, 6 and 4ºC water. * Indicates significance between WR and NR populations of lake sturgeon. Letters a, b and c indicate significance between stocking temperatures within a population and at a given timepoint. Letters x and y indicate significant differences between timepoints within a given population and stocking temperature ($P < 0.05$, three-factor ANOVA). The expressions of all genes of interest were normalized to the expression of reference genes RPS6 and RLP7, using the geometric mean (Vandesompele et al., 2002) and analysed following the application of the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001). The expression of all genes of interest were normalized to the mean of the WR 16ºC, 8ºC stocking temperature, 1 week post-stocking timepoint. Data are expressed as mean ± S.E.M. [HSP70 n = 5–10, HSP90a n = 5–10, HSP90b n = 7–10, steryl-CoA desaturase (SCD) n = 6–10, cold inducible RNA binding protein (CIRP) n = 6–10, condition factor (K) n = 10]. Grey bars indicate the NR 16ºC treatment while the black bars represent the WR 16ºC treatment.

2.4.3.2 Acclimation temperature comparisons

Across NR 16 and 20ºC acclimation treatments, the mRNA transcript abundance of *HSP70* was affected by an interaction between stocking temperature and time ($P < 0.0005$, $F = 9.1$; Figure 2.3A). Over the course of the week-long exposure to 8ºC, both NR acclimation treatments decreased expression of *HSP70* ($P < 0.05$). At the 1 week timepoint, however, in the NR 20ºC treatment, mRNA transcript abundance increased with each decrease in stocking temperature, while in the NR 16ºC treatment *HSP70* mRNA transcript abundance was higher in 4ºC relative to 8ºC ($P < 0.05$).

The mRNA transcript abundance of *HSP90a* was affected by interactions of acclimation treatment, stocking temperature and time ($P < 0.05$, $F = 4.0$; Figure 2.3B). In both acclimation treatments *HSP90a* expression decreased over the week-long exposure in 8ºC ($P < 0.0001$), while increasing in colder stocking temperatures relative to 8ºC at the 1 week timepoint ($P < 0.05$). In the 16ºC acclimation treatment after 1 week, expression increased in 6 and 4ºC relative to 8C stocked sturgeon ($P < 0.001$). However in 20ºC acclimated sturgeon after 1 week, expression increased with each decrease in stocking temperature and over the week in 4ºC ($P < 0.005$). This resulted in higher mRNA transcript abundance of *HSP90a* in the 20ºC acclimated sturgeon in 4ºC at the 1 week timepoint, relative to their 16ºC acclimated counterparts ($P < 0.001$). Similarly, expression of the constituitive form *HSP90b* increased in the 1 week timepoint as temperatures decreased, but only in 20ºC acclimated NR sturgeon ($P < 0.05$; Figure 2.3C).

The mRNA transcript abundance of *CIRP* was affected by time ($P < 0.05$, $F = 6.4$) and stocking temperature ($P < 0.05$, $F = 3.6$; Figure 2.3D), and decreased in NR 20ºC acclimated sturgeon over the week-long exposure in 8ºC ($P < 0.005$). In the same acclimation treatment, *CIRP* was elevated in lower temperatures at the 1 week timepoint, 2.4-fold in 4ºC compared to sturgeon stocked into 8ºC ($P < 0.05$). Likewise, *SCD* mRNA transcript abundance was also affected by stocking temperature ($P < 0.0001$, $F = 10.7$) and time ($P < 0.0001$, $F = 20.0$; Figure 2.3E). In the NR 20ºC acclimation treatment in 8ºC, and both acclimation treatments in 6ºC, expression of *SCD* decreased from 1 day to 1 week post-stocking ($P < 0.05$). At the 1 week timepoint in the NR 20ºC acclimation treatment, expression increased as temperatures decreased, with higher expression observed in 4ºC relative to 8ºC stocked sturgeon ($P < 0.005$). Condition factor was affected by acclimation treatment ($P < 0.005$, $F = 10.8$; Figure 2.3F), with higher condition in NR 16ºC acclimated sturgeon in 8ºC 1 day following stocking, relative to 20ºC acclimated sturgeon ($P < 0.05$).

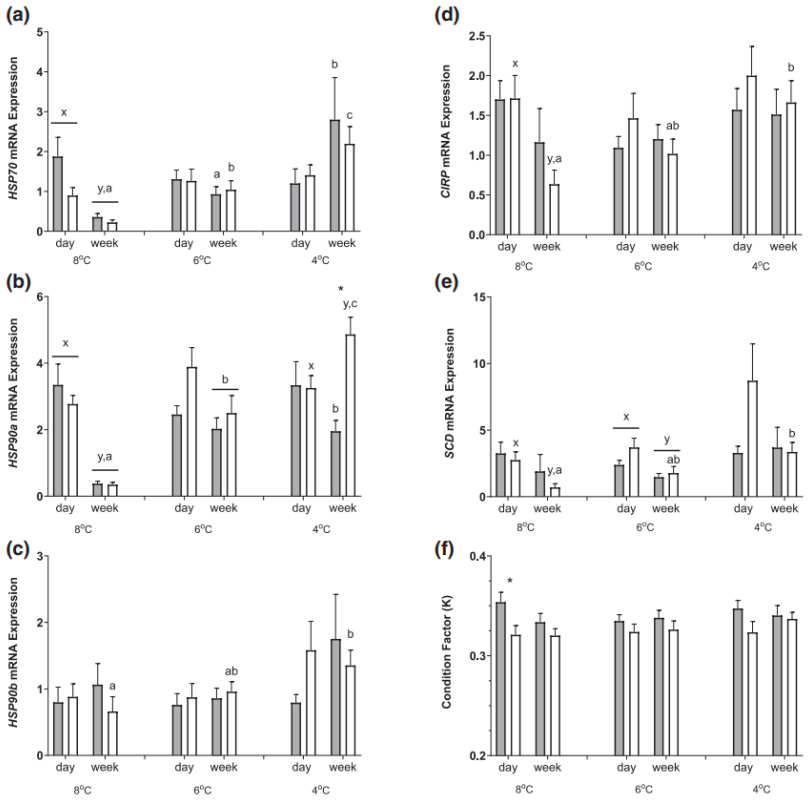

Figure 2.3 Acclimation temperature comparisons of lake sturgeon, *Acipenser fulvescens*, from the Nelson River (NR) 16 and 20ºC acclimation treatments throughout cold shock trials, in their mRNA transcript abundance of a) *HSP70*, b) *HSP90a*, c) *HSP90b*, d) *CIRP*, e) *SCD*, and f) condition factor, 1 day and 1 week following stocking into 8, 6 and 4ºC water. *Indicates significance between NR 16 and 20ºC acclimation treatments of lake sturgeon. Letters a, b and c indicate significance between stocking temperatures within an acclimation treatment at a given timepoint. Letters x and y indicate significant differences between timepoints, within a given acclimation treatment and stocking temperature ($P < 0.05$, three-factor ANOVA). The expression of all genes of interest were normalized to to the mean of the transcript abundance of reference genes RPS6 and RLP7, using the geometric mean (Vandesompele et al., 2002), and analysed following the application of the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001). The expression of all genes of interest were then normalized to the WR 16ºC, 8ºC stocking temperature, 1 week post-stocking timepoint. Data are expressed as mean ± S.E.M. [HSP70 n = 5–10, HSP90a n = 5–10, HSP90b n = 6–10, cold inducible RNA binding protein (CIRP) n = 6–10, steryl-CoA desaturase (SCD) n = 5–10, condition factor (K) n = 10]. Grey bars indicate the NR 16ºC treatment while the white bars represent the NR 20ºC treatment.

2.4.3.3 Gene expression relationships

In the WR 16ºC treatment there were correlated relationships in the mRNA transcript abundance of *HSP70* with *HSP90b, CIRP* and *SCD,* as well as *HSP90b* and *CIRP* ($P < 0.05$, with ρ of 0.40, 0.44, 0.42 and 0.50, respectively). The strongest relationships correlative relationships (ρ) were between *SCD* with *HSP90b* and *CIRP* ($P < 0.00001$, with ρ of 0.6 and 0.74, respectively; Figure 2.4a). Next, in the NR 16ºC treatment there were correlated relationships in the mRNA transcript abundance of *SCD* with *HSP70* and *CIRP* ($P < 0.05$, with ρ of 0.39 and 0.51, respectively). The strongest relationships were between *HSP90b* with *CIRP* and *SCD* ($P < 0.00001$, with ρ of 0.57 and 0.59, respectively; Figure 2.4c). Finally, in the NR 20ºC treatment there were correlated relationships in the mRNA transcript abundance *HSP70* with *HSP90a, HSP90b* and *CIRP,* as well as *HSP90b* and condition factor ($P < 0.05$, with ρ of 0.31, 0.45, 0.43 and 0.35, respectively). The strongest relationships were *HSP70* with *SCD, HSP90b* with *CIRP* and *SCD,* and *CIRP* with *SCD* ($P < 0.00001$, with ρ of 0.56, 0.75, 0.76 and 0.76; Figure 2.4e). Principal component analysis reveal that in both the WR 16ºC and NR 20ºC expression of *HSP70* and *HSP90a* was negatively correlated with stocking temperature (Figure 2.4b,f, respectively), while in the NR 16ºC treatment there was a stronger negative association of these genes with time (Figure 2.4d). Across all treatments *HSP70* and *HSP90a* were grouped together, with the expression of *HSP90b, CIRP* and *SCD* grouping out separately.

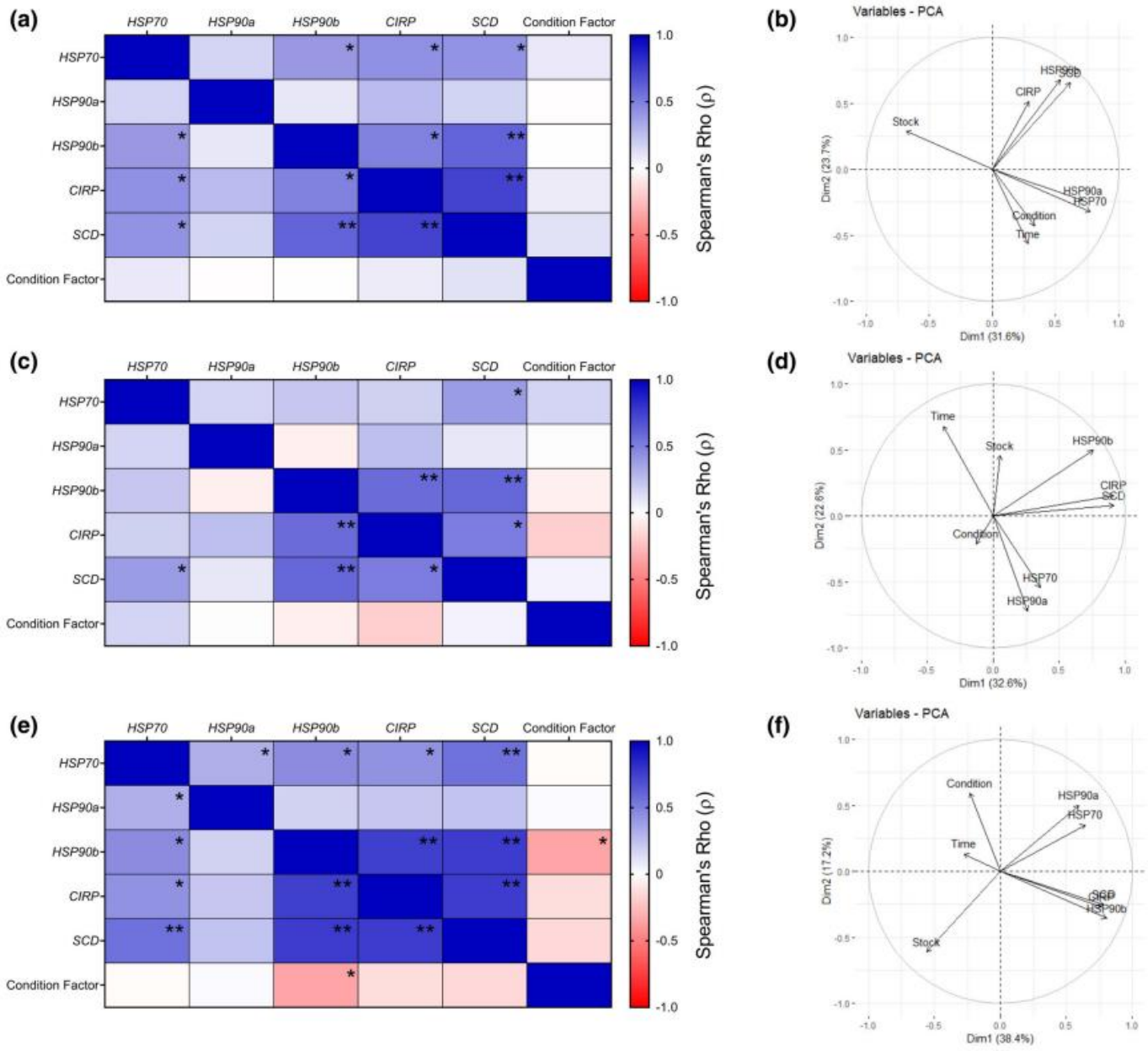

Figure 2.4 Gene expression and condition factor relationships as indicated by semi-partial Spearman's correlations (a, c, e) and PCA variable (b, d, f) plots for young of year lake sturgeon, *Acipenser fulvescens*, from treatments WR 16ºC (a, b), NR 16ºC (c, d), and NR 20ºC (e, f), after stocking into conditions of 8,6, and 4ºC (stock) over the timeseries of 1 day and 1 week (time) (n = 48, 45 and 50, with respect to population and acclimation treatment). Values in correlation matrices represent the ρ from each estimated Spearman's correlation. *Indicates significance at the level $P < 0.05$, while ** indicates significance at the level $P < 0.00001$

2.5 Discussion

Throughout the first 6 months of development leading up to overwintering, we demonstrated changes in the growth, condition factor and mortality of lake sturgeon from northern and southern populations in Manitoba, as well as those acclimated to elevated temperatures from 30 to 90 dpf. Additionally, these same populations and acclimation treatments demonstrate persistent treatment-specific responses at the cellular level when challenged with cold shock, a stressor that may be encountered during late season stocking. These divergent physiological responses between southern and more northern YOY lake sturgeon are likely influenced by genotypes and population-specific thermal environments that lake sturgeon would experience in their respective natural waterways prior to overwintering (Bugg et al., 2020).

2.5.1 Effects of population and early acclimation on mortality, growth and condition

Elevated mortality and lower overall condition were apparent in southern WR sturgeon relative to their northern NR counterparts, suggesting that individuals from the NR population are better suited to the applied hatchery rearing protocols at 16^{o}C. It is possible that WR sturgeon may less effectively transition between hatchery-provided food resources, potentially resultant from differences in gut development or a more specialistic feeding strategy, but further research is required to confirm these speculations. In contrast, the northern NR sturgeon populations experience a shortened feeding and growth season, potentially necessitating faster development or a more generalist strategy to feeding, as observed in Arctic freshwater fishes (Conover & Present, 1990; Laske et al., 2018), and thus resulting in higher condition than their southern counterparts throughout hatchery rearing. Irrespective of cause, these differences in mortality indicate population level differences in early development and should be further investigated. Similarly, when the temperature of the rearing environment was increased, NR sturgeon acclimated to 20^{o}C early in life demonstrated larger body mass and length, but poorer condition than those kept at 16^{o}C throughout development, suggesting a potential thermal threshold for condition factor in northern sturgeon populations if river temperatures continue to increase. Rearing temperatures of 20^{o}C exceed typical riverine temperatures in the northern extent of the lake sturgeons range, and likely the optimum for this population (Bugg et al., 2020). These observed decreases in condition may be due to increased routine metabolic rates at higher temperatures, but after 30 days of acclimation to similar temperatures no increase in routine

metabolic rate was observed in these same populations (Bugg et al., 2020). Alternatively, sturgeon in higher temperatures may regulate their feeding to reduce metabolic costs associated with digestion and other routine activities, limiting their overall condition (Jutfelt et al., 2021). In all treatments, growth continued from 30 to 150 dpf, but ceased in NR sturgeon from both treatments once cold conditioning began. However, growth continued in WR sturgeon, potentially indicating population level differences in their response to environmental cues during the onset of overwintering, with NR sturgeon taking on an energy storage maximization strategy which may aid to enhance lipid stores prior to overwintering (Bugg et al., 2020; Schultz & Conover, 1997; Sogard & Olla, 2000). This resulted in higher condition factor throughout development and after conditioning to colder temperatures, potentially as an adaptation to survive a prolonged overwintering period in their northern environment, similar to observations in latitudinal distributed populations of Atlantic silverside, *Menidia menidia* (Schultz et al., 1998). These key differences between populations and acclimation treatments demonstrate the importance of genetic background and early rearing temperature during development on the growth, condition and survival of juvenile lake sturgeon, with the potential for impacts at the hatchery level.

2.5.2 Effects of stocking on cold inducible mRNA transcript abundance

Cold stocking had effects at both the population and acclimation treatment level on the transcriptional responses of heat shock proteins HSP70 and HSP90a in the liver, with increased expression in southern WR and warm acclimated northern NR sturgeon. Sturgeon from the NR stocked into 8ºC decreased the expression of these genes over the course of the week, while their expression was affected by decreasing stocking temperature and increased in both populations and acclimation treatments in 4ºC, relative to 8ºC at the 1 week timepoint. Overall, WR sturgeon had higher mRNA transcript abundance of both heat shock proteins at multiple temperatures 1 week post-stocking, indicating a higher transcriptional responsiveness and perhaps higher levels of cold induced plasticity to thermal stress in this population. Similarly, NR sturgeon acclimated to 20ºC had higher expression of *HSP90a* after 1 week at 4ºC, demonstrating the potential for long-term effects of early acclimation influencing the cold shock response. Transcript induction of both *HSP70* and *HSP90a* has been observed in multiple tissues of the tiger barb Puntius tetrazona and Cyprinus carpio following cold shock (Ferencz et al., 2012; Liu et al., 2020).

Additionally, experiments with embryonic Atlantic salmon, *Salmo salar*, indicate that *HSP70* confers protection against cold stress during early development (Takle et al., 2005). An upregulation of cortisol during cold exposure could modulate the expression of these genes over short periods of time (Celi et al., 2012; Sathiyaa et al., 2001; Vamvakopoulos, 1993). However, cortisol does not always causally relate to HSP expression in fish tissues (Iwama et al., 1999) and in YOY lake sturgeon exposed to overwintering conditions a significant upregulation of cortisol was not observed until several weeks of exposure to near-freezing temperatures while fasted (Deslauriers et al., 2018). Thus, transcriptional upregulation of heat shock proteins likely indicates a prolonged and cortisol-independent plastic response to cold stress when YOY lake sturgeon are stocked into lower temperatures than they are acclimated to prior to release.

In addition to liver changes in *HSP70* and *HSP90a* expression, sturgeon from the NR with early acclimation to 20^{o}C also decreased expression of *CIRP* and *SCD* in the 8^{o}C treatment over the course of the week but demonstrated increased expression of *CIRP*, *SCD* and *HSP90b* in 4^{o}C relative to 8^{o}C. Like *HSP70*, induction of *CIRP* mRNA transcript abundance was observed in several tissues of the tiger barb and common carp when subjected to cold stress (Gracey et al., 2004; Liu et al., 2020). Additionally, upregulation of *CIRP* mRNA was demonstrated in rainbow trout, Oncorhynchus mykiss, kidney and gill following 2 weeks of low thermally stressful temperatures, and the gills of sockeye salmon, *Oncorhynchus nerka*, and pink salmon, Oncorhynchus gorbuscha, acclimated to cool temperatures (Akbarzadeh et al., 2018, Jeffries et al., 2012, 2014; Rebl et al., 2013; Verleih et al., 2015;). As *CIRP* expression is postulated to be induced under moderate hypothermia (Rebl et al., 2013), upregulation of this gene as well as *HSP90b* with decreasing temperatures in the NR 20^{o}C lake sturgeon but not in fish from other treatments demonstrates a lasting effect of this early life warm acclimation and its ability to disrupt transcriptional processes later in life. It is likely that for sturgeon reared at 16^{o}C, temperatures of 4^{o}C were not low enough or 1 week was not long enough to disrupt these same processes and induce *CIRP* expression.

Cold stocking-induced hepatic expression of *SCD* mRNA was affected by stocking temperature and time for both population and acclimation treatment comparisons with decreases observed in 8^{o}C over time and increases in expression as stocking temperatures decreased, similar to expression patterns of *HSP70*, *HSP90a*, *HSP90b* and *CIRP*. In the milkfish, *Chanos chanos*, and hybrid tilapia, *Oreochromis niloticus x O. aureus*, liver *SCD* mRNA transcript

abundance, enzyme activity, mono- and polyunsaturated fatty acids, and desaturation index all increased over the duration of cold shock (Hsieh et al., 2003, 2007; Hsieh & Kuo, 2005). Interestingly, lower levels of the above traits, including polyunsaturated fatty acids and unsaturation index, have been linked to mortality in freshwater alewives, Alosa pseudoharengus, under cold challenge (Snyder et al., 2012; Snyder & Hennessey, 2003). Similarly, in common carp, *C. carpio*, cooling water temperatures induced latent increases in SCD enzyme activity followed by transcriptional upregulation as temperatures continued to decrease (Tiku et al., 1996; Trueman et al., 2000). Thus, the transcriptional induction of SCD in the present study suggests that in colder temperatures, lake sturgeon in this treatment required increased homeoviscous capacity, passing the threshold for adequate regulation of desaturation processes at the enzymatic level. Instead, sturgeon from the WR 16ºC and NR 20ºC upregulated their mRNA synthesis of *SCD*, demonstrating the need to further alter membrane lipids as temperatures decrease, ultimately indicating possible impacts for survival post-release. A closely patterned response can be observed in the mRNA transcript abundance of *HSP90a* at 1 week, with increases in the WR 16ºC and NR 20ºC relative to NR 16ºC as temperatures decrease. Currently, in the WR, sturgeon are exposed to 20ºC for approximately 50 days with no exposure over this threshold for sturgeon in the NR (Bugg et al., 2020). The convergence of these molecular phenotypes between WR sturgeon and warm acclimated NR sturgeon following induction by cold shock demonstrates that if temperatures increase in the NR for this early developmental window, we may expect these responses, and perhaps other phenotypes of NR sturgeon, to more closely resemble that of WR sturgeon in the future.

Taken together, the upregulation of *HSP70*, *HSP90a*, *CIRP*, *SCD* and *HSP90b* observed in the liver at lower temperatures suggests that in these colder environments transcriptional production was not high enough to protect cells from the effects of cold induced thermal stress. However, cold conditioning to 8ºC diminished these effects over the course of the week-long exposure, with observed decreases in the responses of these transcripts when NR sturgeon were stocked into their pre-conditioned temperature. For NR sturgeon stocked into 8ºC, these observed decreases in expression of *HSP70*, *HSP90a*, *CIRP* and *SCD* over the week following stocking may indicate that this short, hatchery used, conditioning period was not enough to completely diminish the effects of temperature change. Thus, longer cold conditioning periods prior to

stocking may be appropriate, and if sturgeon are acclimated to 8ºC, stocking should be conducted before temperatures drop below this threshold to diminish the effects of cold shock on release.

2.5.3 Cold induced molecular relationships

Investigation of correlations and principal component analysis between chaperone and fatty acid metabolism related hepatic mRNA transcript abundance indicated strong transcriptional relationships between some genes. Across WR and NR populations and both NR acclimation treatments, the strongest molecular relationships were present in the hepatic expression of *HSP90b*, *CIRP* and *SCD*, genes that may be expected to respond to chronic, as opposed to acute, cold stress and group out similarly in the PCA analysis. The consistency of these relationships across populations and acclimation treatments indicates the importance of these genes in response to cold stress, even without strong upregulation. In contrast, *HSP70* and *HSP90a* represent more acutely responsive genes which were more highly induced, grouping out separately opposite of stocking temperature, indicating different response trajectories to cold exposure. *HSP70* mRNA transcript abundance was also associated with the expression of *HSP90b*, *CIRP* and *SCD*, but only in the WR 16ºC and NR 20ºC treatments, demonstrating further convergence in the relationships between molecular responses to cold shock in these two treatments. The strongest overall relationships in expression were present in the NR 20ºC treatment, which also showed a negative correlation between sturgeon body condition and *HSP90b* expression. This suggests a relationship between observed decreases in body condition in this treatment with increases in the constitutive levels of molecular responsiveness to cold shock and to potentially higher levels of chronic stress in lower condition individuals.

2.5.4 Conclusions

Overall, these findings demonstrate that both population and early thermal acclimation can have prolonged effects on the growth and condition of YOY lake sturgeon as well as their transcriptional responses to cold environments. If hatcheries aim to release fish with high condition, lower rearing temperatures in early development may be advantageous. However, if the hatchery's objective is to produce the largest fish, early environmental exposures to increased temperatures can increase the long-term growth trajectory of developing lake sturgeon, with consequences for body condition and future cellular responses. Ultimately, key genes involved in

the stress and cold condition responses were upregulated when sturgeon were stocked into waters at a lower temperature than their pre-release hatchery conditioning temperature. These changes were population and acclimation temperature-specific and could have impacts on survival post release. Therefore, consideration of these and other physiological responses is necessary when optimizing hatchery rearing and release practices for species of conservation concern.

2.6 Acknowledgements

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Credit Author Statement

W.B., W.G.A. and K.J. conceived and designed the experiments. W.B., G.Y., C.B., M.E., W.G.A. and K.J. collected gametes from wild spawning lake sturgeon, while W.B., G.Y., C.B. and M.E. reared juveniles and collected data throughout development. W.B., G.Y. and C.B. conducted cold shock trials. W.B. conducted gene expression and data analysis. W.B., G.Y., C.B., M.E., W.G.A. and K.J. wrote, reviewed and edited the manuscript. W.G.A. and K.J. acquired funding and aided in supervision throughout the experimentation and review processes.

Chapter 2 demonstrated that both population and early thermal environment have long lasting effects on the growth, condition, and stress responses of sturgeon later in development. Chapters 3, 4, and 6 further explore these aspects, evaluating their impacts on whole-organism physiology and transcriptional responses of developing lake sturgeon following acclimation to elevated temperatures.

Chapter 3. Effects of acclimation temperature on the thermal physiology in two geographically distinct populations of lake sturgeon (*Acipenser fulvescens*)

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3.1 Abstract

Temperature is one of the most important abiotic factors regulating development and biological processes in ectotherms. By 2050, climate change may result in temperature increases of 2.1 - 3.4ºC in Manitoba, Canada. Lake Sturgeon, *Acipenser fulvescens*, from both a northern and southern population in Manitoba were acclimated to 16, 20, and 24ºC for 30 days, after which critical thermal maximum (CT_{max}) trials were conducted to investigate their thermal plasticity. We also examined the effects of temperature on morphological and physiological indices. Acclimation temperature significantly influenced the CT_{max}, body mass, hepatosomatic index, metabolic rate, and the mRNA transcript abundance of transcripts involved in the cellular response to heat shock and hypoxia (*hsp70*, *hsp90a*, *hsp90b*, *HIF-1α*) in the gill of Lake Sturgeon. Population significantly affected the above phenotypes, as well as the mRNA transcript abundance of Na^+/K^+ *ATPase*-α1 and the hepatic glutathione peroxidase enzyme activity. The southern population had an average CT_{max} that was 0.71 and 0.45ºC higher than the northern population at 20 and 24ºC, respectively. Immediately following CT_{max} trials, mRNA transcript abundance of *hsp90a* and *HIF-1α* was positively correlated with individual CT_{max} of Lake Sturgeon across acclimation treatments and populations ($r = 0.7$, $r = 0.62$, respectively; $P < 0.0001$). Lake Sturgeon acclimated to 20 and 24ºC had decreased hepatosomatic indices (93 and 244% reduction, respectively; $P < 0.0001$) and metabolic suppression (27.7 and 42.1% reduction, respectively; $P < 0.05$) when compared to sturgeon acclimated to 16ºC, regardless of population. Glutathione peroxidase activity and mRNA transcript abundance Na^+/K^+ *ATPase*-α1 were elevated in the northern relative to the southern population. Acclimation to 24ºC also induced mortality in both populations when compared to sturgeon acclimated to 16 and 20ºC. Thus, increased temperatures have wide ranging population-specific physiological consequences for Lake Sturgeon across biological levels of organization.

3.2 Introduction

Across Canada, mean annual and seasonal temperatures have increased between 1.7 and 2.3ºC from 1948 to 2016, with the largest increases occurring in northern Canada (Canada's Changing Climate Report, 2019; Vincent et al, 2015). By 2050, mean annual water temperatures within Manitoba, where several endangered populations of Lake Sturgeon exist, are projected to increase by 2.1 - 3.4ºC (Manitoba Hydro, 2015). As Lake Sturgeon are a long-lived species, which may require as long as 18 - 28 years to mature (COSEWIC, 2006; Scott and Crossman 1998), individuals in Manitoba today may live to see the effects of increased environmental temperatures, such as those projected for 2050. Furthermore, their progeny will most certainly experience elevated temperatures. As temperature is a critical factor impacting fish development (Aloisi et al, 2019; Piper et al, 1982), it is important to understand how future increased environmental temperatures will affect the development and physiology of this iconic species.

Lake Sturgeon populations in Manitoba range throughout the province, from the Winnipeg River in the south, and northward to the outlet of the Nelson and Churchill Rivers into the Hudson Bay (Manitoba Hydro, 2016). Historical barriers exist in waterways between populations, which have limited gene flow (McDougall et al, 2017) and contributed to genetically distinct populations. These latitudinally separated riverine environments likely have differing temperature profiles which may influence population-specific life history traits like the growth and thermal plasticity of these genetically distinct populations (Pollock et al, 2015). Since the mid-1990s, stocking of hatchery-reared Lake Sturgeon has been conducted to bolster remaining wild populations in Manitoba (McDougall et al, 2014). At the Lake Sturgeon hatchery in Grand Rapids, Manitoba, located at the North end of Lake Winnipeg, northern fish are typically reared at 16ºC due to hatchery heating limitations, but the hatchery intends to increase rearing temperatures in the future with system upgrades. However, we know little regarding the potential effect that increased rearing temperatures may have on thermal physiology of Lake Sturgeon throughout development and post-release.

Temperature is a critical abiotic factor for fishes, influencing their biochemical processes, physiology, development, range distributions, community dynamics, and ultimately survival (Boltana et al, 2017; Crozier et al, 2007; Li et al, 2015; O'Gorman et al, 2016; Pankhurst and Munday, 2011; Schulte, 2015). The effects of increased environmental temperatures on teleosts have been well documented, demonstrating changes in behavior (Forsatkar et al, 2016), swimming

performance (Johnson and Bennet, 1995), reproductive potential (Donelson et al, 2010), metabolism (Johnston and Dunn, 1987), tolerance to environmental stressors (McBryan et al, 2016), immune response (Dittmar et al, 2013; Makrinos and Bowden, 2016), and mRNA transcript abundance of thermally responsive genes (Fangue et al, 2006; Campos et al, 2013; Jeffries et al, 2018). In contrast to teleosts, the effects of increasing environmental temperatures on sturgeon species are poorly understood, especially in the mRNA transcript abundance of key regulatory genes involved in responses to heat shock, hypoxia, and osmoregulatory disruption. However, sturgeon oxygen consumption and transcriptional responses have been shown to increase under thermally stressful conditions (Yusishen et al, 2020; Zhang et al, 2017). Additionally, increasing temperatures affect the early development of White Sturgeon, *Acipenser transmontanus* (Cheung, 2019) and Pallid Sturgeon, *Scaphirhynchus albus*, (Kappenman et al, 2013), hematology and development of Shortnose Sturgeon, *A. brevirostrum* (Kappenman et al, 2013; Zhang and Kieffer, 2014), bioenergetic performance and hematocrit levels of Green Sturgeon, *A. medirostris* (Mayfield and Cech Jr., 2004; Sardella et al, 2008), growth and stress of Siberian sturgeon, *A. baerii* (Aidos et al, 2020), and movement patterns, predation rates, and condition factor in Lake Sturgeon, *A. fulvescens* (Moore et al, 2020; Wassink et al, 2019; Yoon et al, 2019).

Many cellular, physiological, and behavioral changes made by fishes as a result of increased environmental temperatures are assessed by changes in mRNA transcript abundance of genes associated with acute thermal stress, resultant cellular damage, and acclimation such as heat shock proteins (*hsp70, hsp90a, hsp90b*) (Fangue et al. 2006; Komoroske et al, 2015; Lund et al, 2002; Shi et al, 2015). Additionally, changes in genes associated with hypoxia tolerance (*HIF-1α*) and osmoregulation (Na^+/K^+ *ATPase*-α1) have implicit roles in the cellular response to thermal stress (Jeffries et al, 2014; McBryan et al, 2013; Portner, 2010; Vargas-Chacoff et al, 2018). Furthermore, changes in condition factor, hepatosomatic index (HSI), metabolic rate, and glutathione peroxidase (GPx) activity, have been shown to relate to increasing environmental temperatures. Condition factor can fluctuate with temperature changes as well as seasonally due to abiotic and biotic factors (Giosa, et al, 2014; Mazumder et al, 2016). Thus, HSI can be a useful additional indicator of changes in body condition and metabolism as well as glycogen and lipid reserves (Chellappa et al, 1995) which are likely to change in the liver with variation observed in natural populations once temperature thresholds and upper physiological limits have been reached (Purchase and Brown, 2001; Morrison et al., 2020). Similarly, metabolic rates (both routine and

maximum) are additionally affected by environmental temperature in many fishes (Norin and Clark, 2015) including sturgeons (Yoon et al, 2018; Zhang and Kieffer, 2017) and likely play a role in the distribution of a species (Payne et al, 2015). Hepatic glutathione peroxidase (GPx) detoxifies oxidatively damaging peroxides formed as a result of acute and chronic thermal stress, (Halliwell and Gutteridge, 1999) with increased mRNA transcript abundance and enzyme activity demonstrated with increased temperatures (Almroth et al, 2015; Dorts et al, 2012). The ability of Lake Sturgeon to make physiological changes to acclimate to their warming environment in response to thermal stress, i.e. phenotypic plasticity, is crucial in an ever-changing environment and may be a key predictor for a species future success (Rodgers et al, 2018; Somero, 2010; Seebacher et al, 2015; Gabriel et al, 2005). Divergent population-specific responses of these physiological parameters may be anticipated in sturgeon populations from differing thermal environments as have been observed in other geographically separated populations (Fangue et al, 2006; Geerts et al, 2014; Pereira et al, 2017; Yampolsky et al, 2014).

The aim of this study was to use measurements of key physiological and molecular variables to evaluate the thermal plasticity of juveniles from different populations of Lake Sturgeon in Manitoba, Canada (Figure 3.1). We acclimated Lake Sturgeon from both northern (Burntwood River – BR) and southern (Winnipeg River – WR) populations to three environmentally-relevant thermal regimes of 16, 20, and 24ºC. As these two geographically distinct populations of sturgeon in Manitoba have independent environmental and genetic histories, we hypothesized that they would exhibit divergent population-specific responses to acclimation and acute thermal stress. We predicted that the southern WR population of Lake Sturgeon, with greater thermal variation and range, would demonstrate increased thermal plasticity compared to their northern BR counterparts as demonstrated in other species (Fangue et al, 2006; Geerts et al, 2014; Pereira et al, 2017; Yampolsky et al, 2014). Additionally, we predicted that these differences in plasticity would be apparent in the critical thermal maximum (CT_{max}) of Lake Sturgeon, and the differential expression of mRNA transcripts important in the response to heat shock, hypoxia, and osmoregulatory disruption (*hsp70, hsp90a, hsp90b, HIF-1α*, and Na^+/K^+ *ATPase*-α1). Furthermore, we predicted temperature dependent population-specific responses in body size, HSI, metabolic rate, and GPx activity as observed in other species under thermally stressful environmental conditions.

Figure 3.1 Geographic locations of two distinct lake sturgeon, *Acipenser fulvescens*, populations within Manitoba, Canada. Map coordinates span approximately: Lat: 49.5 Lng: −1521.6 SE; Lat: 49.5, Lng: −1551.5 SW; Lat: 60.3 Lng: −1551.5 NW; Lat: 60.3 Lng: −1521.6 NE (National Geographic MapMaker Interactive, 2020). The dotted line on the map indicates the historical barrier of Slave Falls (50º 14' 34'' N 95º36' 31'' W) as well as present-day dams separating populations. The grey and black circles indicate the approximate spawning and sampling locations for lake sturgeon on the Burntwood River (BR) and Winnipeg River (WR), respectively.

3.3 Methods

3.3.1 River Temperatures

River water temperatures were measured in both the WR and BR in 2019 by a digitemp SDI-12 submersible temperature sensor, (Forest Technology Systems; Victoria, British Columbia, Canada) and a series 500 SDI-12 transducer (TE connectivity; Schaffhausen, CH), respectively. Water temperature measurements in the WR were recorded downstream of the Pointe du Bois Generating Station where spawning individuals were caught for this study (50º17′52’’N, 95º32′51’’W). Water temperature measurements in the BR were recorded at the Miles Heart Bridge (55º45′12’’N, 97º50′30’’W) approximately 74 air km southwest of the sturgeon spawning site (56º02′46.5’’N, 96º54′18.6’’W) on the same river. Measurements were taken at midnight (0:00:00 am) for both locations therefore they likely represent the lower range of daily temperatures in both rivers. Temperature comparisons between the WR and BR were based on days where data is available for both populations, May 23rd, 2019 to December 31st, 2019.

3.3.2 Lake Sturgeon Husbandry

In May and June of 2019, gametes from wild-caught female and male Lake Sturgeon were harvested from individuals at both the Pointe du Bois Generating Station on the WR (50º17′52’’N, 95º32′51’’W), and below First Rapids on the BR, (56º02′46.5’’N, 96º54′18.6’’W). Eggs and sperm from the WR were transported to the University of Manitoba animal holding facility where fertilization took place, while those from the BR population were fertilized at the Grand Rapids Fish Hatchery (53º09′25.9’’N, 99º17′21.9’’W). Individuals from the WR population were the product of fertilization of eggs from two females with the sperm from two males (two maternal families). Individuals from the BR population were the product of the fertilization of eggs from one female with the sperm from six males (one maternal family). Spawning individuals, particularly in the northern BR population, were limited due to the remote location of the spawning site and also the declining population, with a total adult population estimated between 112 and 579 fish (Lacho and Hrenchuk, 2018). Additionally, the number of families in the southern WR population was limited due to the number of wild-caught spawning females that were able to be sampled in 2019. Post fertilization, embryos from both populations were de-adhesed by submerging embryos in a clay solution (Fullers Earth; Earhart et al, 2020) and gently stirred by hand for 1 h. Embryos were then rinsed with de-chlorinated fresh water and incubated in tumbling

jars at 12ºC until hatch, which occurred at about 9 days post-fertilization (DPF). Post hatch, larvae of equal numbers from each maternal family, were transferred to a total of four 9 L flow-through aquaria, two tanks population^{-1}, with aeration and bio-balls as substrate. Temperature was maintained at 12ºC until 13 DPF, after which temperature was increased at 1ºC day^{-1} until 16ºC to match hatchery rearing conditions. Freshly hatched artemia (Artemia International LLC; Texas, USA), was provided as a starting diet at 19 DPF, prior to complete yolk-sac absorption, after which tank substrate was removed over a 7 d period. Lake Sturgeon were fed to satiation three times daily on a diet of artemia throughout the experiment. All animals in this study were reared and sampled under guidelines established by the Canadian Council for Animal Care and approved by the Animal Care Committee at the University of Manitoba under Protocol #F15-007.

3.3.3 Acclimation

Beginning at 30 DPF, Lake Sturgeon from each population were acclimated to treatments of 16, 20, and 24ºC at a density of about 70 sturgeon 9 L aquaria^{-1} (Figure 3.2). As there were more families, and thus more individual sturgeon from the WR population, Lake Sturgeon from the WR were reared in four tanks, while BR sturgeon were reared in two tanks for each acclimation treatment, to keep stocking density equal across populations throughout acclimation. In the 20 and 24ºC treatments, water temperature was increased from 16ºC at a rate of 1ºC day^{-1} until the desired acclimation temperature was reached. Lake Sturgeon remained at these acclimation temperatures for 30 days and temperature was recorded every 15 min by HOBO Water Temperature Pro v2 Data Loggers (Onset Computer Corporation; Bourne, MA, USA). Mortality as well as temperature via thermometer were recorded at least three times daily. After 29 days of acclimation, fish were fasted for a 24 h period prior to beginning CT_{max} trials (Downie and Kieffer 2016; Downie et al, 2018; Heller et al, 2015, Lee et al, 2015).

At the end of the acclimation, prior to CT_{max} trials, eight fish from each treatment were haphazardly selected and euthanized by immersion in an overdose of tricane methanesulfonate solution (250 mg L^{-1}; MS-222, Syndel Laboratory, Vancouver, Canada) buffered with an equal volume of sodium bicarbonate. Gill tissue was then extracted, preserved in RNA*later* (Thermo Fisher Scientific, Waltham, USA), and stored at -80ºC prior to quantification of mRNA transcripts. An additional 10 fish were haphazardly selected and euthanized from each acclimation treatment, body mass (weighed to 0.0001 g) and total length (measured to nearest 1 mm) was recorded for

each individual, as well as liver wet mass (weighed to 0.0001 g) which was used to calculate the HSI as the ratio of the wet mass of the liver (W_{liver}) to the wet mass of the body (W_{body}):

$$HSI = \frac{W_{liver}}{W_{body}} \times 100$$

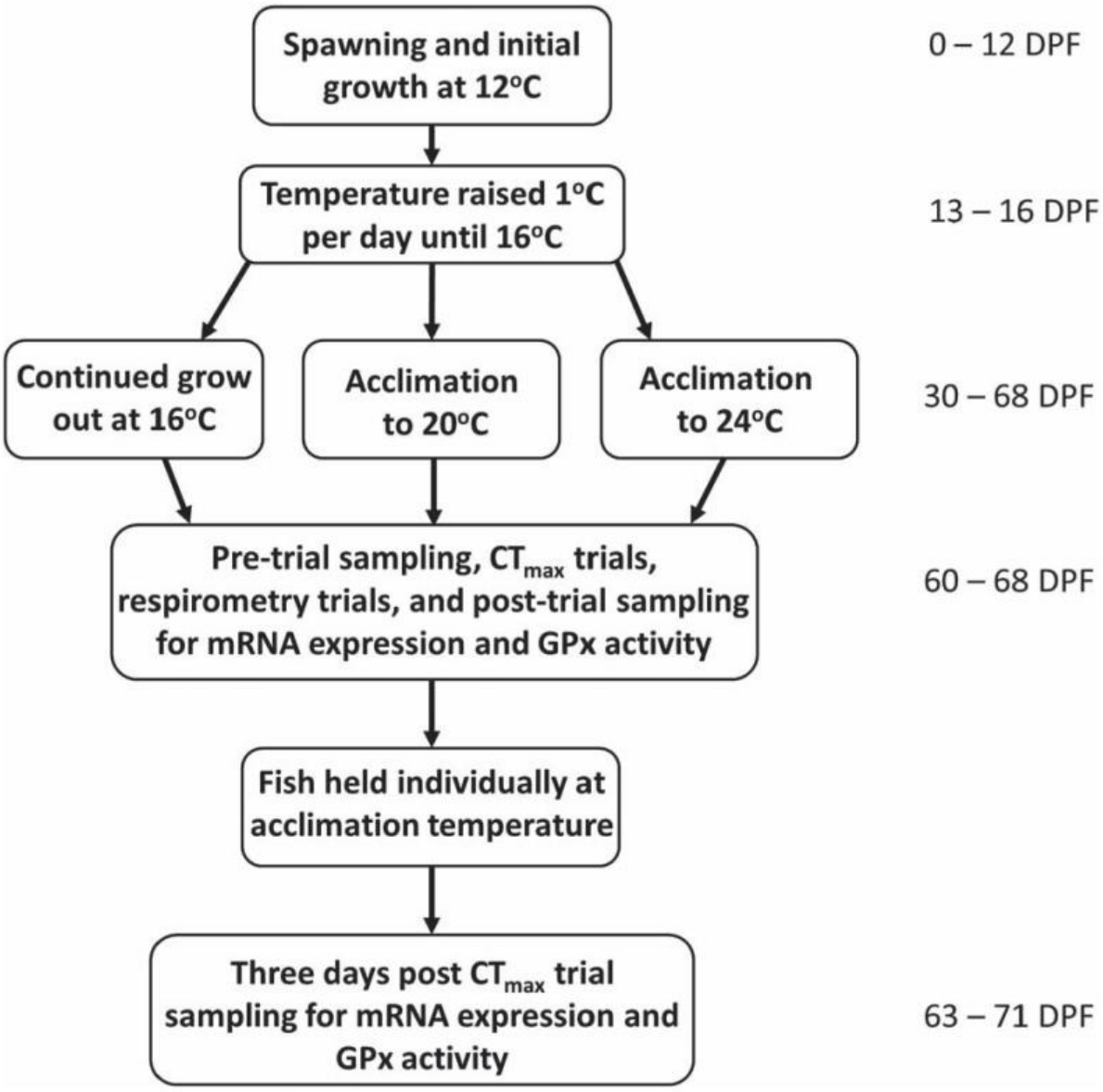


Figure 3.2 Experimental design and timeline for lake sturgeon, *Acipenser fulvescens*, acclimation, CT_{max} (critical thermal maximum) trials, respirometry trials and tissue sampling for mRNA transcript abundance as well as GPx (glutathione peroxidase) activity. All time points are measured as days post fertilization (DPF)

3.3.4 Critical Thermal Maximum Trials

On the day of the CT_{max} trials, eight fish were haphazardly selected from acclimation tanks and placed individually in experimental units (~200 ml water volume and 9.5 cm long x 5 cm across) in an aerated recirculating water bath initially set to the acclimation temperature of the treatment being tested. Experimental units had mesh screened sides to allow for water flow through each unit. Eight fish were tested $trial^{-1}$, with three to four trials conducted over two consecutive days for each experimental treatment. CT_{max} trials were conducted at the same time every day, between 9 am and 12 pm to avoid any confounding effects of diurnal shifts on physiology and gene expression (Lankford et al, 2003; Somero et al, 2020). Temperature of the water bath was regulated by an Isotemp recirculating heater (Fisher Scientific; Hampton, USA) while water temperature was constantly recorded by a temperature probe placed in the center of the experimental setup (Witrox Oxygen probe, Loligo Systems; Viborg, Denmark). Fish were held in these experimental units for 1 h prior to the CT_{max} trials to reduce potential effects of handling stress. After 1 h, trials began by increasing the temperature of the water bath by $0.3^{o}C\ min^{-1}$ until fish were unable to right themselves after a physical disturbance (Bard and Kieffer, 2019; Yoon et al, 2019; Yusishen et al, 2020). When fish were unable to right themselves, the final CT_{max} temperature was recorded, the fish was euthanized, mass and length was recorded, and the gill tissue was removed and preserved as previously described. Liver tissue was then sampled, immediately flash frozen in liquid nitrogen, and stored at -80°C until use for measuring GPx activity. An additional eight fish from a single trial were placed individually into 9 L tanks in a Multi-Stressor unit (Aquabiotech; Coaticook, Quebec, Canada) at their respective acclimation temperature after their CT_{max}, was reached and allowed to recover for three days before tissue sampling. During this 3 d recovery period, Lake Sturgeon were fed and were observed actively feeding on freshly hatched artemia three times daily. From average CT_{max} values, an acclimation response ratio, the rate an organism increases their CT_{max} in response to acclimation, was calculated for each population, subtracting the average CT_{max} of the 16°C acclimation treatment ($CT_{max16^{o}C}$) from that of the 24°C treatment ($CT_{max24^{o}C}$) and dividing by the change in acclimation temperature between treatments (Δ°C):

$$Acclimation\ Response\ Ratio = \frac{CT_{max24^{o}C} - CT_{max16^{o}C}}{\Delta^{o}C}$$

3.3.5 Metabolic Rate

Measurements of whole-body metabolic rate ($\dot{M}O_2$) were taken 3 d after each CT_{max} trial for a given acclimation treatment, using intermittent flow respirometry (Loligo systems, Viborg, Denmark) as previously described (Yoon et al, 2019) with some modifications. In brief, fish were fasted for at least 12 h prior to experimentation. Flow within the chambers (volume: 43.40 +/- 4.32 (mean +/- S.D.) ml) was maintained at a low level to not cause any physical stress, but sufficient for water exchange and accurate measurement of $\dot{M}O_2$. The intermittent respirometry cycle was variable for each temperature treatment to ensure a linear decline in oxygen saturation more than 10%, but not below 70% as metabolic suppression was reported at this point in age 1+ Lake Sturgeon (Svendsen et al. 2014). For 20 and 24°C, the parameters were 360 s flush followed by 60 s wait and 300 s measurement. For 16°C, 360 s flush followed by 60 s wait and 900 s measurement was used. The respirometry chambers were surrounded by a black curtain to minimize any visual disturbance to the fish during each trial. Routine metabolic rate (RMR) was assessed for 2 h following a 4 h period in the metabolic chamber to minimize the effects of transfer. After RMR was assessed, fish were removed from the chambers and a standardized chase protocol was performed for 15 min. Then, fish were immediately returned to the same chambers and $\dot{M}O_2$ was measured for the following two measurement cycles. Biological oxygen demand (BOD) was measured before and after each experiment. Assuming a linear increase, pre and post-experiment BOD data points were used to linearly interpolate BOD over the experiment period and all $\dot{M}O_2$ data was corrected by the corresponding BOD. Only slopes of oxygen decline with $R^2 \geq 0.9$ were used for data analysis. RMR was calculated by averaging $\dot{M}O_2$ measured for 2 h while maximum metabolic rate (MMR) was chosen as the highest $\dot{M}O_2$ after the chase protocol. Factorial aerobic scope (FAS) was calculated by dividing MMR by RMR. There was no 24ºC acclimation treatment available for the BR population for MMR, RMR, or FAS as there were insufficient numbers of Lake Sturgeon remaining to conduct these trials.

3.3.6 RNA Extraction, cDNA synthesis, and qPCR

Gills from Lake Sturgeon were homogenized in 500 µl of lysis buffer, for 10 min at 50 Hz using a TissueLyser II (Qiagen; Germantown, MD, USA). Total RNA was extracted from gill homogenates using a PureLink RNA mini kit (Invitrogen; Ambion Life Technologies) following the manufacturer's instructions. Total RNA purity and concentration was evaluated for all samples

using a Nanodrop One (Thermo Fisher Scientific) followed by gel electrophoresis to asses RNA integrity. Post extraction, RNA samples were stored at -80ºC. cDNA was synthesized from 1 µg of DNAse treated total RNA using a qScript cDNA synthesis kit following the manufacturer's instructions (Quantbio; Beverly, Massachusetts). Synthesis was conducted using a SimpliAmp Thermal Cycler (ThermoFisher; Waltham, Massachusetts) with cycling conditions of 1 cycle of 22ºC for 5 min, 1 cycle of 42ºC for 30 min, and 1 cycle of 85ºC for 5 min and a hold at 4ºC. Following synthesis, cDNA samples were stored at -20ºC.

Real-time quantitative polymerase chain reaction (RT-qPCR) for each gene of interest, *hsp70, hsp90a, hsp90b, HIF-1α*, and *Na^+/K^+ ATPase*-α1, was conducted using 5 µl of Bio-Rad SsoAdvanced Universal SYBR Green Supermix, 0.1 to 0.04 µl of 100 µM primers, 2 µl of diluted cDNA per sample, and nuclease-free water adjusted for each assay to bring the total volume of each well to 10 µl (Table 3.1). For all experimental assays except *HIF-1α*, each well contained 0.025 µl forward and 0.025 µl reverse primer, while this was doubled to 0.05 µl forward and 0.05 µl reverse for each reference gene. For *HIF-1α*, 0.02 µl forward and 0.02 µl reverse primer was used. The cDNA of all samples was diluted 1:10 with nuclease-free water for all RT-qPCR assays. All primers were designed based on sequences from an annotated transcriptome produced by the pyrosequencing of a Lake Sturgeon ovary (Table 3.1; Hale et al, 2009). The expression of the genes of interest was normalized to the relative expression of reference genes *RPS6* and *RPL7* and then analyzed after applying the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001). Expression of all genes was then normalized to that of the WR 16ºC acclimation treatment prior to CT_{max} trials in order to make comparisons between populations. The WR 16ºC acclimation treatment was chosen as a reference based on hatchery rearing conditions and its relatively low levels of mRNA transcript abundance across acclimation treatments, timepoints, and genes of interest.

Table 3.1 Primer sequences used to investigate changes in the mRNA transcript abundance of chaperones as well as ion and oxygen regulation induced during elevated temperature acclimation, critical thermal maximum trials, and following recovery, for young of the year lake sturgeon (*Acipenser fulvescens*). Target genes were chosen based on their roles in the response to heat shock, cellular stress, hypoxia and osmoregulatory disruption. *RPS6* and *RPL7* were used as reference genes and showed stable expression across treatments. Efficiencies are listed as a percentage.

Gene	Forward	Reverse	Efficiency (%)
HSP70	CTGTCACTCGGACTTTAACTTTAATTT	AACTGTCCTAAAGAACTGCCTTATCC	95.1
HSP90a	GATCACACGAGCGGATTTGC	ATGTTGTGCTCTGTCCTGCG	96.8
HSP90b	GGAACCAAGGCTTCATGGA	CCAACACCAAACTGACCAATCA	94.9
HIF-1α	GCAAACAAGTCATGGTGCAT	GGGCCGTTCAGTGTATGAGT	98.2
Na^+/K^+ ATPase-α1	TCGATTGCTTACACCCTGAC	TGCCCAAGTCAATACACAGG	93.8
RPS6	CTGGCTGGATTCTGATTTGGATG	ATCTGATTATGCCAAGCTGCTG	93.0
RPL7	TGCTTAGGATTGCTGAGCCG	GATCTTTCCGTGACCCCGTT	92.7

3.3.7 Glutathione Peroxidase Activity Assays

Initial extraction of GPx from the Lake Sturgeon livers was conducted by homogenizing tissues in ice-cold homogenization buffer (50 mM Tris-HCl, 5 mM EDTA, and 1 mM DTT; pH 7.5). Briefly, 50 µl of homogenization buffer was added to each vial containing tissue, which was then freeze-thawed in liquid nitrogen three consecutive times. Tissues were then centrifuged (Accuspin micro17R, Fisher Scientific) for 10 min at 10,000 RPM at 4°C. The supernatant was removed and stored at -80°C prior to assay measurement, which occurred between 1-3 days post-extraction. Concentrations of GPx were measured using a commercially available enzyme assay kit in 96-well plates (Cayman Chemical; Ann Arbor, Michigan, USA). Samples were diluted based on the manufacturer's instructions, where the concentration of GPx in the sample decreased the absorbance of the well to between 0.02 and 0.135 min^{-1}. Twenty µl of sample was added to each well on the assay plate, followed by 50 µl assay buffer, 50 µl co-substrate mixture, and 50 µl NADPH. Sample wells were compared to the background wells (70 µl assay buffer, 50 µl co-substrate mixture, and 50 µl NADPH) and positive control wells (50 µl assay buffer, 50 µl co-substrate mixture, 50 µl NADPH, and 20 µl GPx control) included on each plate. Absorbance was measured at 340 nm using a plate reader (Power wave XS2, Biotek), and a linear decrease in NADPH was observed. Backgrounds, positive controls, and samples were run in duplicate. The intra and inter-assay variation was 6.05 and 13.83%, respectively. Data was not corrected for inter-assay variability.

3.3.8 Statistical Analysis

Differences in mortality between treatments and populations were analyzed via Cox proportional hazards model in R v3.6.2 (R core Team, 2013) using the "survival" and "survminer" packages (Kassambara et al, 2019; Therneau, 2015). Assumptions for all Cox proportional hazard models were assessed using the "cox.zph" function included in the "survival" package, to ensure that residuals were independent of time. First, a Cox proportional hazards model was run with only the effect of temperature included in the model, to isolate the effect that different thermal environments had on Lake Sturgeon regardless of population. Next, in addition to a Cox proportional hazards model with covariates of temperature and population included, the "pairwise_survdiff" function from the "survminer" package with the same covariates and a Bonferroni correction, was used to compare the mortality across treatments and populations of

Lake Sturgeon.

CT_{max} data was analyzed using nonparametric statistical tests as it could not be transformed to adequately pass the Levene's test. Thus, Kruskal-Wallis multiple comparison tests and a Bonferroni correction were used to determine significance within populations and across acclimation treatments, using the "dunn.test" package (Dinno, 2017). Finally, the Wilcoxon Signed-Rank Test was used to determine significance between populations, within a single acclimation treatment. As CT_{max} data violates assumptions of parametric tests, Spearman's correlations were used to analyze the relationship between expression of the transcripts and GPx activity at each relevant timepoint to the CT_{max} of individual Lake Sturgeon across acclimation treatments and populations. Only significant correlations are reported.

Morphometrics including mass, length, condition factor and HSI, as well as measurements of metabolic rate were analyzed using a two-factor ANOVA with population and acclimation treatment and their interaction included in the model as fixed effects. Three factor ANOVAs were used to analyze GPx activity and mRNA transcript abundance of *hsp70, hsp90a, hsp90b, HIF-1α*, and *Na^+/K^+ ATPase*-α1 with population, acclimation treatment, and time as well as their interactions included in the model as fixed effects.

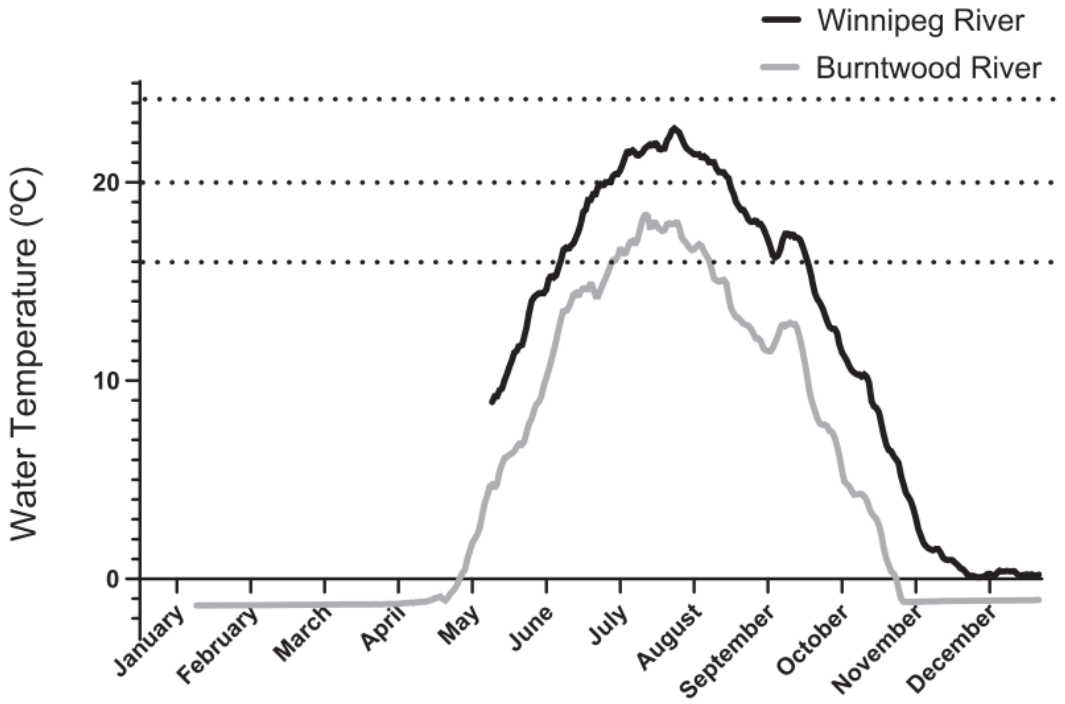
For all ANOVAs, Shapiro-Wilks and Levene's tests were used to assess normality of data and homogeneity of variance respectively. Normality was also visually inspected using fitted residual plots. If assumptions of normality or homogeneity were violated, either a ranked, log, or square root transformation was applied to the data set. Additionally, for each ANOVA the effect of rearing tank was assessed and found to be not significant; therefore, it was not included in final models. Detailed ANOVA results can be found for all ANOVAs in Supplementary Table A.3.1. Following ANOVAs, multiple comparisons tests were performed and corrected with Tukey's honestly significant difference tests from the "multcomp" package (Hothorn et al, 2008). All statistical analyses were performed with a significance level of 0.05.

3.4 Results

3.4.1 River Temperatures

In 2019, the WR temperatures exceeded 20°C 17.6% of measured days, with 36.7% above 16°C, and 63.2% below 16°C. In contrast, the BR was never recorded above 20°C, with temperatures above 16°C recorded on 14.7% of days and 85.3% of days below 16°C (Figure 3.3).

Days exceeding 16ºC were consecutive for both populations, above this threshold in the WR for 100 days from June 20th to September 28th and in the BR for 40 days from July 11th to August 20th. Temperatures in the WR were above 20ºC for 51 days from July 8th to August 28th. Throughout the summer, June 21st to September 21st, when larval Lake Sturgeon are developing, the BR was on average 4.6 ± 0.8ºC colder than the WR.



Temperature Thresholds	Burntwood River (% Time)	Winnipeg River (% Time)
Over 20ºC	0	17.6
Over 16ºC	14.7	36.8
Below 16ºC	85.3	63.2

Figure 3.3 Temperature profiles of two distinct lake sturgeon, *Acipenser fulvescens*, populations as measured in the Winnipeg and Burntwood rivers in 2019. Data for both rivers was measured in 2019 at midnight and % time over-temperature threshold comparisons between the WR and BR populations were based on days where data is available for both populations, 23 May 2019 to 31 December 2019. Tick marks on the x-axis indicate the middle of each given month. Dashed lines indicate the different temperature thresholds and acclimation temperatures used in the current study. The first dotted line at 16ºC represents current hatchery conditions and the first acclimation treatment. The second dotted line at 20ºC indicates potential increased hatchery temperatures, approximate temperatures that populations are currently exposed to and the second acclimation treatment. The third dotted line at 24ºC represents future warming conditions that may be expected in Manitoba, Canada, by 2050.

3.4.2 Mortality

Lake Sturgeon reared at 24ºC, had elevated mortality compared to those at 16 and 20ºC, with a hazard ratio of 5.37 ($P < 0.0001$). The BR and WR Lake Sturgeon had increased mortalities as temperatures increased, with 4.8, 6.9, and 25.5% mortality and 3.3, 3.3, and 15% ($P < 0.05$; n = 145 treatment^{-1}) in 16, 20, and 24ºC, respectively.

3.4.3 Morphometrics

There were significant effects of population and acclimation treatment on mass and length of Lake Sturgeon ($P < 0.001$) as well as an interaction of population and acclimation treatment interaction for mass ($P < 0.05$) and a near interaction for length ($P < 0.06$). Lake Sturgeon from the BR population had a body mass of 0.62 g in 16ºC increased to 0.92 g in 20ºC and were larger when compared to WR fish in both treatments ($P < 0.01$; Table 3.2). The BR Lake Sturgeon had significant increases in mass in 20 (47.1%) and 24ºC (46.9%) when compared to 16ºC ($P < 0.001$). In contrast, Lake Sturgeon from the WR had increased mass with each acclimation treatment, with individuals in 20ºC heavier than the those in 16ºC ($P < 0.05$) and those in 24ºC heavier than both lower treatments, 84.3 and 32.8% respectively ($P < 0.001$).

Sturgeon from the BR population had increased body length of 15.4% in the 20ºC acclimation treatment when compared to WR Lake Sturgeon ($P < 0.001$; Table 3.2). The BR Lake Sturgeon had significant increases in length in 20 and 24ºC treatments when compared to 16ºC ($P < 0.0001$). In contrast, sturgeon from the WR had increased length with each increasing acclimation treatment. Individuals in 20ºC were 8.5% longer than those in 16ºC while those in 24ºC were longer than both lower treatments, 23.4 and 13.7% respectively ($P < 0.005$). There was a significant interaction between population and acclimation treatment on condition factor in Lake Sturgeon (Table 3.2; $P < 0.05$). At 20ºC, WR Lake Sturgeon had condition factors 13.8% greater than BR sturgeon from the same acclimation treatment ($P < 0.05$; Table 3.2).

There was an effect of both population and acclimation treatment on the HSI of Lake Sturgeon (Figure 3.4; $P < 0.001$). Sturgeon from both the WR and BR acclimated to treatments of 16ºC had increased HSI when compared to fish at 20 and 24ºC ($P < 0.0001$). The WR Lake Sturgeon acclimated to 16ºC had hepatosomatic indices 244 and 93% higher than those acclimated to treatments of 20 and 24ºC, respectively ($P < 0.0001$) with similarly results in the BR population ($P < 0.05$). The BR Lake Sturgeon HSI was increased when compared to the WR across all

acclimation treatments ($P < 0.05$).

Table 3.2 Morphometrics of Winnipeg and Burntwood River lake sturgeon, *Acipenser fulvescens*, acclimated to 16, 20 and 24ºC for 30 days in early development. Significance was determined by a two-factor ANOVA ($P < 0.05$) followed by Tukey's honestly significant difference post hoc test. Asterisks represent significant differences between populations at each acclimation treatment. Letters represent significant differences within populations, across acclimation treatments. Morphometric data are expressed as mean +/- SD (n = 10-16 per treatment – indicated by parenthesis).

Acclimation treatment (°C)	Winnipeg River	Burntwood River
	Mass (g)	
16	0.49 ± 0.07^{a} (10)	$0.62 \pm 0.13^{a}*$ (16)
20	0.68 ± 0.14^{b} (10)	$0.92 \pm 0.16^{b}*$ (10)
24	0.90 ± 0.14^{c} (10)	0.91 ± 0.18^{b} (10)
	Length (mm)	
16	53.2 ± 3.2^{a} (10)	55.9 ± 4.6^{a} (16)
20	57.5 ± 4.7^{b} (10)	$66.3 \pm 5.6^{b}*$ (10)
24	65.4 ± 4.6^{c} (10)	66.9 ± 7.2^{b} (10)
	Fulton's condition factor [mg mm³ (100)]	
16	0.33 ± 0.03 (10)	0.35 ± 0.04 (16)
20	0.35 ± 0.05 (10)	$0.31 \pm 0.04*$ (10)
24	0.32 ± 0.03 (10)	0.32 ± 0.09 (10)

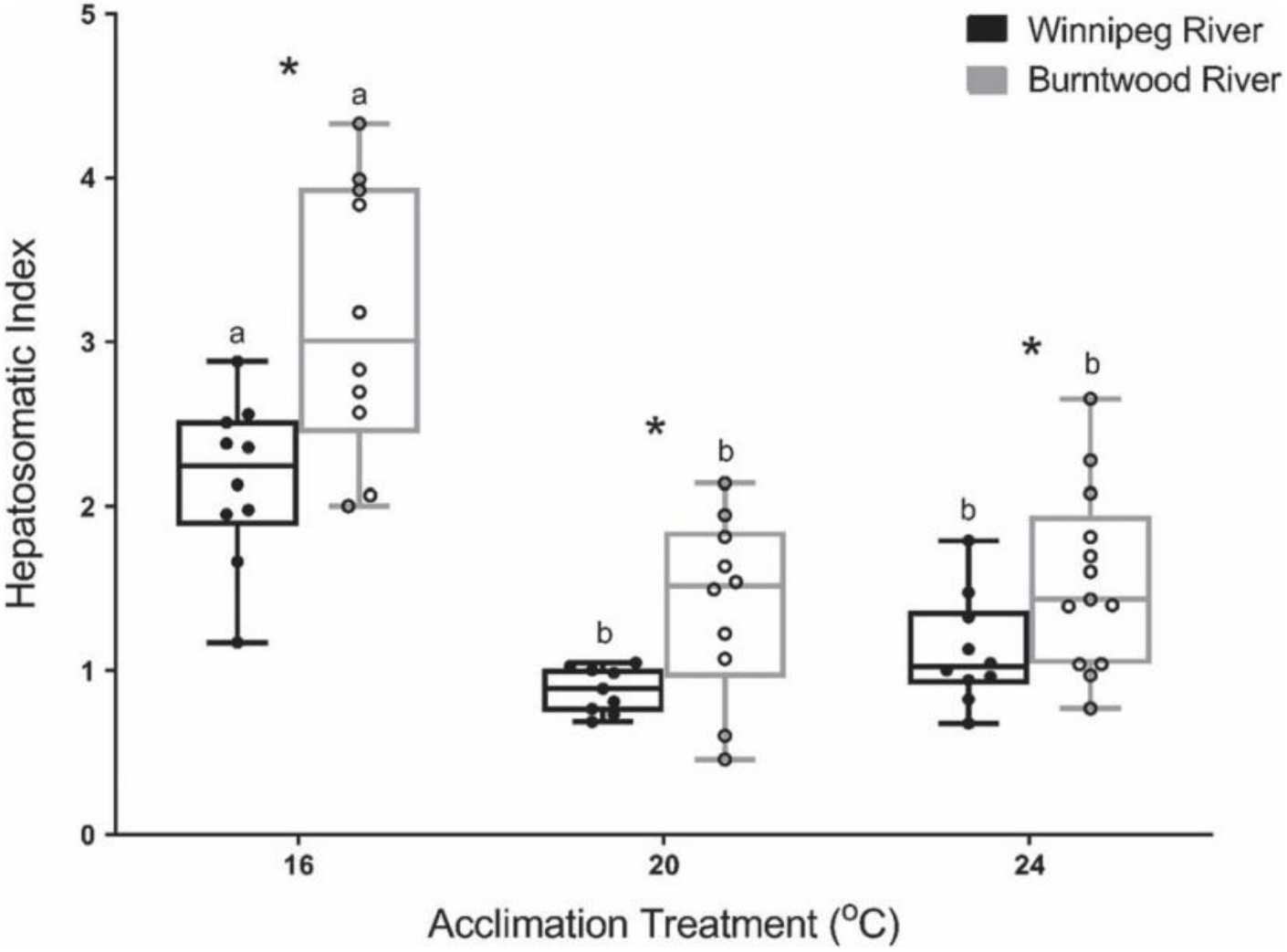


Figure 3.4 Hepatosomatic index of Winnipeg River and Burntwood River lake sturgeon, *Acipenser fulvescens*, after 30 days of acclimation to 16, 20 and 24ºC in early development. Significance was determined by a two-factor ANOVA ($P < 0.05$) followed by Tukey's honestly significant difference post hoc test. Asterisks represent significant differences between populations at each acclimation treatment. Lowercase letters represent significant differences between acclimation treatments, within populations. Box plots represent the mean and 25th and 75th percentiles, whilst whiskers indicate the minimum and maximum values. Dots represent individual data points (n = 10–13 per treatment).

3.4.4 Critical Thermal Maximum

Acclimation treatment and population influenced the CT_{max} with each subsequent acclimation treatment increasing the CT_{max} for each population ($P < 0.05$; Figure 5). At both 20 and 24°C, the WR population had increased CT_{max} compared to the northern BR counterparts by 0.71 and 0.45°C, respectively ($P < 0.05$). Comparisons of CT_{max} show that the two populations of Lake Sturgeon have differing acclimation response ratios. The WR Lake Sturgeon acclimation response ratio was 0.41, while their BR counterparts was 0.34 over the same acclimation treatments.

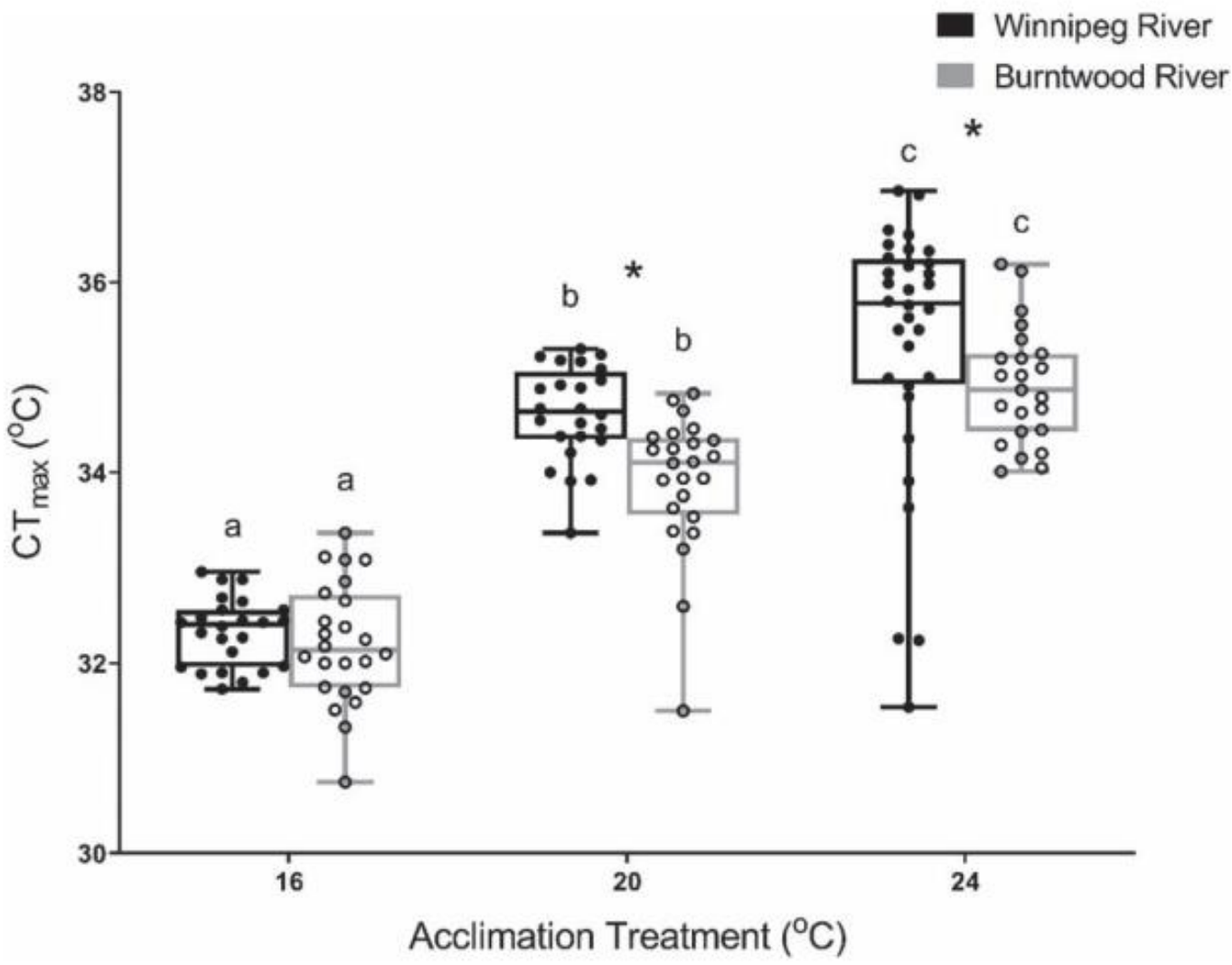


Figure 3.5 Critical thermal maximum (CT_{max}) of Winnipeg River and Burntwood River lake sturgeon, *Acipenser fulvescens*, after 30 days of acclimation to 16, 20, and 24°C in early development. Significance differences within populations, across acclimation treatments, was determined by Kruskal–Wallis multiple comparisons with P-values adjusted with the Bonferroni method. Significant differences across populations, within a single acclimation treatment, was determined by Wilcoxon signed-rank test. Asterisks represent significant differences between populations at each acclimation treatment. Lowercase letters represent significant differences between acclimation treatments, within populations. Box plots represent the mean and 25th and 75th percentiles, whilst whiskers indicate the minimum and maximum values. Dots represent individual data points (n = 24–32 per treatment).

3.4.5 Metabolic Rate

RMR was significantly affected by both population and acclimation temperature ($P < 0.0001$). There were differences in RMR in the WR population between 16 and 24ºC as well as 20 and 24ºC ($P < 0.01$; Figure 3.6A). In the WR 24ºC acclimation treatment, there was a reduction in RMR compared to 16 and 20ºC. In the BR population, there was a 26% decrease in RMR between 16 and 20ºC ($P < 0.05$). Between populations, the WR population had 28 and 70.1% higher RMR than BR in 16 and 20ºC, respectively ($P < 0.01$). The MMR was significantly affected by population and acclimation temperature ($P < 0.01$; Figure 3.6B). There were differences in MMR in the WR population between 16 and 24ºC, as well as 20 and 24ºC ($P < 0.05$). Between populations, the WR population had 29.8% higher MMR than the BR in 20ºC ($P < 0.05$). In the WR population, acclimation to 24ºC led to a reduction of MMR compared to the 16 and 20ºC. There was an effect of acclimation temperature on FAS ($P < 0.05$; Figure 3.6C). In the BR population, FAS was 29.6% higher at 20ºC than 16ºC ($P < 0.01$).

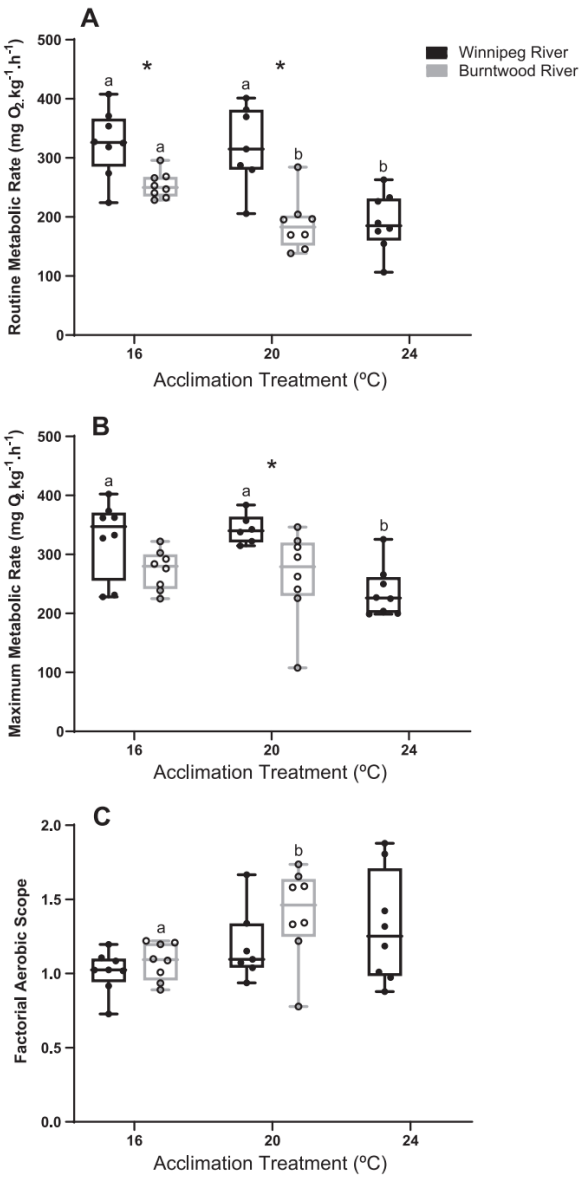


Figure 3.6 A) Resting metabolic rate (RMR), B) maximum metabolic rate (MMR), and C) factorial aerobic scope (FAS), of Winnipeg River and Burntwood River lake sturgeon, *Acipenser fulvescens*, after 30 days of acclimation to 16, 20, and 24ºC in early development. Significance was determined by a two-factor ANOVA ($P < 0.05$) followed by Tukey's honestly significant difference post hoc test. Asterisks represent significant differences between populations at each acclimation treatment. Lowercase letters represent significant differences between acclimation treatments, within populations. Box plots represent the mean and 25th and 75th percentiles, whilst whiskers indicate the minimum and maximum values. Dots represent individual data points (n = 7–8 per treatment).

3.4.6 mRNA Transcript Expression

There was an interactive effect of population, acclimation treatment, and time on the mRNA transcript abundance of *hsp70* ($P < 0.05$; Figure 3.7A). Immediately post CT_{max} trials, mRNA transcript abundance of *hsp70* was elevated in every treatment across populations compared to pre trial levels, and in the WR population 1.7-fold compared to the BR population at 24ºC ($P < 0.05$). Three days post CT_{max}, the BR population expressed *hsp70* mRNA 2.9-fold and 4.6-fold higher at 16ºC than Lake Sturgeon from 20 and 24ºC, respectively ($P < 0.05$).

There was an interactive effect of population, acclimation treatment, and time on the mRNA transcript abundance of *hsp90a* ($P < 0.0001$; Figure 3.7B). Pre CT_{max}, there were significant differences in *hsp90a* mRNA transcript abundance across acclimation treatments for both populations. In the WR population, mRNA transcript abundance of *hsp90a* decreased with increasing acclimation temperature with the 16ºC treatment demonstrating expression 5.3-fold higher than the 24ºC acclimation treatment ($P < 0.05$). In contrast, the BR population increased expression in the 24ºC treatment when compared to 16 and 20ºC. These opposite patterns of *hsp90a* expression resulted in differences between populations within acclimation treatments of 16 and 24ºC ($P < 0.05$). Within the 16ºC acclimation treatment, the WR population had 6.7-fold higher expression compared to that of the BR. In the 24ºC treatment the BR population increased expression 3.3-fold when compared to that of the WR.

Immediately post CT_{max} trials, mRNA transcript abundance of *hsp90a* was elevated in every treatment across populations compared to pre trial levels, and significantly increased at 24ºC for both populations when compared to their respective 16ºC counterparts ($P < 0.05$). The expression of *hsp90a* immediately following the CT_{max} trials was positively correlated with individual CT_{max} of Lake Sturgeon across acclimation treatments and populations ($\rho = 0.7$; $P < 0.0001$).

Three days post CT_{max} trials, mRNA transcript abundance of *hsp90a* was significantly elevated in the BR 16ºC acclimation treatment 3.1 and 2.4-fold compared to 20 and 24ºC treatments, respectively ($P < 0.05$). There were also elevated levels of *hsp90a* expression in the WR 24ºC acclimation treatment ($P < 0.05$), and near significant elevated levels in the 20ºC acclimation treatment ($P < 0.06$), when compared to the BR population.

There was an effect of treatment ($P < 0.0001$) and a trend towards an effect of population ($P < 0.051$) on the expression of *HIF-1α* mRNA (Figure 3.7C). Pre CT_{max}, expression was elevated

in the WR 20ºC acclimation treatment 5.2-fold compared to individuals from the same population in the 16ºC treatment ($P < 0.05$). Immediately following CT_{max}, 24ºC treatments for both populations demonstrated elevated *HIF-1α* mRNA transcript abundance of 5.6 and 3.6-fold relative to WR and BR Lake Sturgeon in 16ºC, and the WR 20ºC treatment had 4.3-fold higher expression than 16ºC. The expression of *HIF-1α* mRNA immediately following CT_{max} was positively correlated with individual CT_{max} of Lake Sturgeon across acclimation treatments and populations ($\rho = 0.62$; $P < 0.0001$).

There was an effect of time ($P < 0.005$) and a near significant effect of population ($P = 0.09$) on the mRNA transcript abundance of *hsp90b* mRNA (Figure 3.7D).

There was an effect of population and time on the expression of *Na^+/K^+ ATPase-α1* mRNA ($P < 0.05$, Figure 3.7E), which demonstrated consistently elevated expression in the BR compared to the WR population across acclimation treatments and timepoints.

3.4.7 Glutathione Peroxidase Activity

There was an interactive effect of population, acclimation treatment, and time on hepatic GPx activity ($P < 0.05$, Figure 3.7F). Pre CT_{max}, the BR population demonstrated a 1.5-fold increase in GPx activity when compared to their WR counterparts at 20ºC ($P < 0.05$). Three days post CT_{max} trials, the BR population showed a 1.8-fold increase in GPx activity compared to their WR counterparts at 16ºC ($P < 0.05$). There was also a significant trend in GPx activity in the BR population 3 days post CT_{max}, where the 24ºC acclimation treatment had activity 2.4-fold higher than the 20ºC acclimated Lake Sturgeon from the same population ($P < 0.05$). The same 20ºC acclimated BR treatment also showed lower GPx activity 3 days post trial compared to pre and post trial levels ($P < 0.05$).

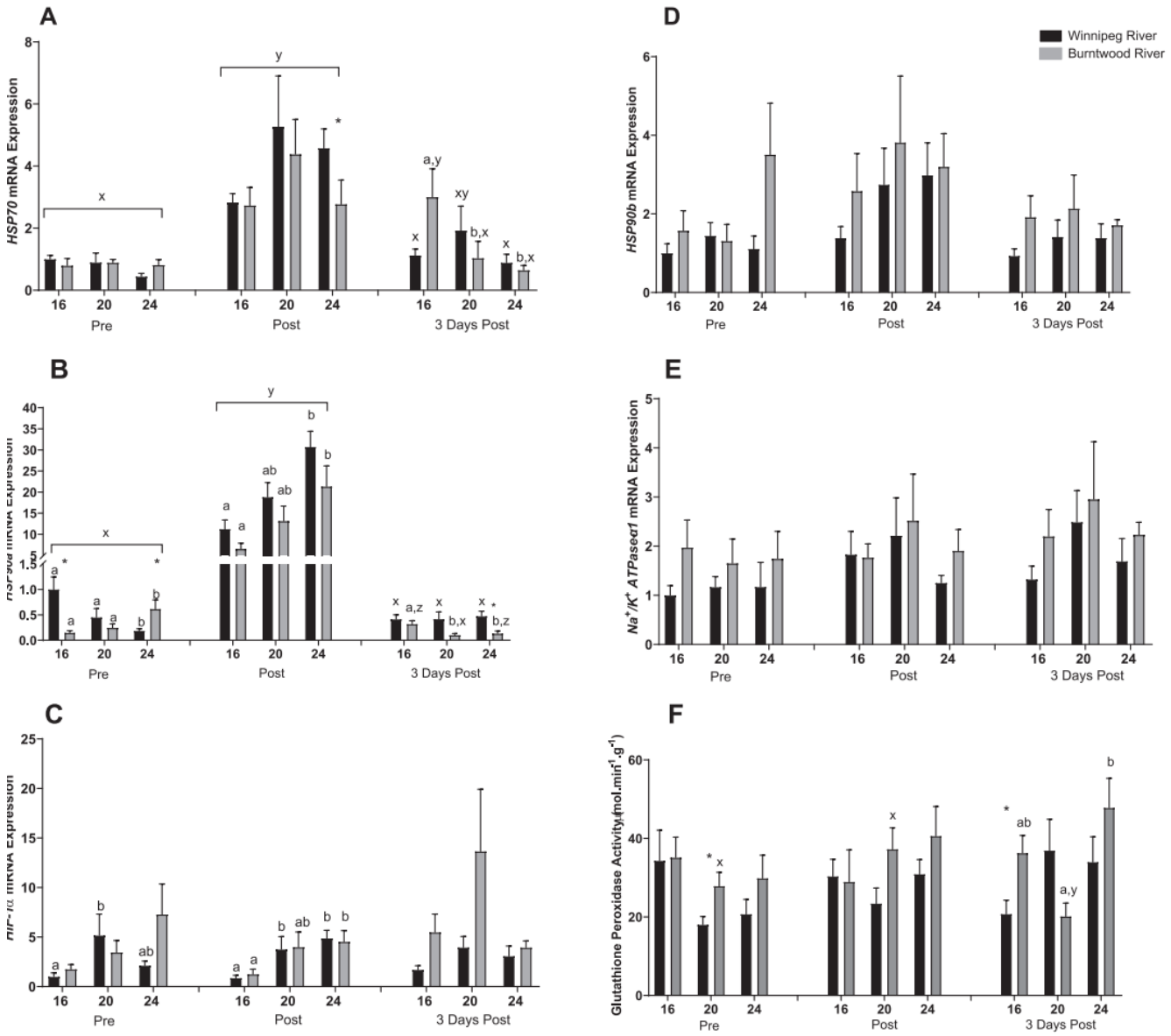


Figure 3.7 Gill mRNA transcript abundance of A) *HSP70*, B) *HSP90a*, C) *HIF-1α*, D) *HSP90b,* E) Na^{+}/K^{+} *ATPase-α1*, and F) liver glutathione peroxidase enzyme activity lake sturgeon, *Acipenser fulvescens*, pre-, immediately post- and 3 days post-CTmax trials after 30 days of acclimation to 16, 20, and 24ºC in early development. Asterisks represent significance between WR and BR populations of lake sturgeon. Lowercase letters a and b represent significance between acclimation treatments. Lowercase letters x, y and z represent significance between time points ($P < 0.05$; three-factor ANOVA). Data are expressed as mean +/− SEM [HSP70 n = 6–8, HSP90a n = 6–8, HIF-1α n = 5–8, HSP90b n=5–8, Na+/K+-α1 n = 5–8, glutathione peroxidase enzyme activity n = 6–8].

3.5 Discussion

In the current study, we demonstrated changes in diverse physiological and molecular phenotypes in response to acclimation temperature in juvenile Lake Sturgeon from a northern and southern population in Manitoba. In addition, Lake Sturgeon populations were affected differently, with alteration of many physiological and molecular characteristics being population dependent. To the best of our knowledge this is the first study to investigate the thermal tolerance of sturgeons across populations, sturgeon thermal tolerance at multiple levels of biological organization, and Lake Sturgeon thermal tolerance at the molecular level.

3.5.1 Physiological Responses to Acclimation

Mortality was elevated in 24ºC acclimation treatments, when compared to 16ºC and 20ºC treatments suggesting that this treatment was stressful to Lake Sturgeon, regardless of population. These increased rates of mortality may be indicative of increased metabolic constraints and cellular stress due to chronic thermal stress potentially leading to decreased pathogen tolerance as observed in Atlantic cod, *Gadus morhau*, and three-spined sticklebacks, *Gasterosteus aculeatus*, during acclimation to thermally stressful conditions (Larsen et al., 2018; Dittmar et al., 2013). Additional signs of metabolic stress are apparent in the decrease in both RMR and MMR at 24ºC in the WR population compared to 16 and 20ºC. In the BR population RMR was lower than WR sturgeon, and decreased between 16 and 20ºC, while in the WR it remained consistent between 16 and 20ºC. These differing thresholds for metabolic suppression between populations may be influenced by their population-specific thermal histories with the BR population experiencing lower yearly temperatures and decreased metabolic rates at lower acclimation temperatures. However, FAS increased with acclimation temperature in both populations, indicating a greater separation between MMR and RMR under increased temperatures. In Green Sturgeon, until a limiting factor such as food availability is reached, growth increases with environmental temperature (Poletto et al, 2018). In the current study, increased mass of BR Lake Sturgeon, when compared to WR sturgeon at 16 and 20ºC, may be indicative of countergradient variation (e.g., Fangue et al, 2009) as Lake Sturgeon from the northern BR population possibly grow faster to take advantage of shorter growing seasons. However, in 24ºC the BR population demonstrated no further increase in either mass or length suggesting an upper thermal limit for growth in this population (e.g., Koskela et al, 1997; Oyugi et al, 2012). In contrast, the WR population had increased in size with each

temperature. These results suggest the presence of population-specific upper thermal thresholds for Lake Sturgeon in Manitoba.

Investigation of HSI indicated an additional influence of elevated temperatures on liver size in Lake Sturgeon with decreased HSI apparent in acclimation temperatures above 16ºC for both populations. Decreases in HSI have been linked to diminishing glycogen reserves in three-spined stickleback *Gasterosteus aculeatus* (Chellappa et al, 1995) and White Sturgeon (Hung et al, 1990). These differences in HSI may be the result of a tradeoff between whole-body RMR and liver function. A reduction in RMR of BR Lake Sturgeon may facilitate decreased energy consumption and result in an increase of hepatic glycogen and lipid reserves that were not evident in WR Lake Sturgeon. In the northern part of their range, Lake Sturgeon must survive through an extensive overwintering period wherein lipid stores likely play a critical role in survival (Byström et al, 2006; Yoon et al, 2019). Cold-adapted populations of fish may be better able to accumulate energy stores, especially glycogen and lipids, as observed in Atlantic cod from different thermal environments (Purchase and Brown, 2001). Thus, lower metabolic rates and higher HSI may be potential evidence of how the northern BR population copes with prolonged sub-Artic winters (Lotka, 1922; Schaefer and Walters 2010), with adoption of an energy storage maximization strategy (Post and Parkinson, 2001). Consequently, rearing Lake Sturgeon for prolonged periods at temperatures above 16ºC may not allow them to accrue these necessary glycogen and lipid reserves, thereby decreasing their ability to survive this overwintering period if released prior to winter. However, further research is necessary to confirm this observation.

3.5.2 Molecular Responses to Acclimation

In addition to physiological responses, there were population differences in the molecular responses of Lake Sturgeon to the acclimation treatments. At 30 d of acclimation and pre-CT_{max} trials, Lake Sturgeon from the WR and BR populations exhibited opposite patterns of *hsp90a* expression with a significant threshold for both populations between 20 and 24ºC. In the BR population, mRNA transcript abundance of *hsp90a* is induced in 24ºC, relative to the 20 and 16ºC, while the WR population demonstrated suppressed mRNA transcript abundance in 24ºC, relative to 16 and 20ºC. These opposite patterns in *hsp90a* mRNA transcript abundance may be indicative of differing mechanisms used to handle chronic thermal stress as observed in redband trout, *Oncorhynchus mykiss gairdnerii*, from different environments (Narum et al, 2013). Additionally,

mRNA transcript abundance of *HIF-1α* in the WR 20ºC acclimation treatment increased, relative to 16ºC, but this increase is not observed at 24ºC, potentially demonstrating a temperature dependent threshold in this population, similar to what has been observed in Crucian carp, *Carassius carassius* (Rissanen et al, 2006). *HIF-1α* expression peaking pre-CT_{max}, in an inverted U-shape may be indicative of sublethal response thresholds that could be predictive of long-term impacts (Jeffries et al, 2015; Jeffries et al, 2018). It is energetically costly to produce and activate HSPs (Heckathorn et al, 1996; Sanchez et al, 1992) and *HIF-1α* likely has metabolic influences (Pelster and Egg, 2003; Richards, 2009). These population-specific metabolic, growth, HSI, and molecular response patterns across acclimation temperatures likely indicate that northern and southern populations differently handle constrained metabolic budgets due to elevated temperatures (Somero, 2020; Tomanek and Somero, 1999). These physiological factors likely play a role in observed behavioral differences in seasonal movement patterns between northern and southern population of Lake Sturgeon across their geographic range, as fish from warmer southern climates move less in the summer than their northern counterparts (Moore et al, 2020).

3.5.3 CT_{max}

In the present study, CT_{max} across treatments and populations ranged from 32.2 to 35.4ºC, which is within the range reported for Lake Sturgeon (Wilkes, 2011; Yusishen et al, 2020). The CT_{max} results demonstrated that Lake Sturgeon from the WR had increased acclimation response ratios when compared to their BR counterparts. However, both populations had acclimation response ratios higher than most species from similar and lower latitudes (Gunderson and Stillman, 2015) which indicates increased thermal plasticity, potentially based on the large genome size of Lake Sturgeon (Ellis et al., 2014; Fontana et al., 2004). Differences in CT_{max} between populations, 0.45 to 0.71ºC, are potentially indicative of population level effects and not just family effects, as families of Lake Sturgeon from the same river systems have exhibited ± 0.18ºC in CT_{max} (95% confidence interval; 6 families; 6 individuals per family; Range 32.4-32.9ºC; Deslauriers et al, *In prep*). As greater climate variability effects thermal plasticity in animals (Rohr et al, 2018; Seebacher et al, 2015; Somero, 2010), the increased acclimation response ratio in the southern population of Lake Sturgeon is likely linked to the more variable thermal environment and greater thermal range which this population would experience.

3.5.4 Molecular and Enzymatic Responses to CT_{max}

Plasticity in mRNA transcript abundance of transcripts involved in response to thermal and hypoxic stressors, *hsp90a* and *HIF-1α*, was observed immediately following CT_{max} trials. Hsp90 is a highly constitutively expressed heat shock protein that also functions to protect cells from thermal stress by aiding in substrate recognition, cellular signaling, and refolding of misfolded proteins (Iwama et al, 2004; Li et al, 2012; Mahanty et al, 2017). In the current study, there was a positive relationship between acclimation temperature and *hsp90a* mRNA transcript abundance post CT_{max} in both populations. The mRNA transcript abundance of *hsp90a* was the most highly induced of genes measured immediately following CT_{max} trials (11.3 to 163-fold increase, relative to pre trial levels). In contrast, expression of the constitutive isoform, *hsp90b*, did not display a similar plastic response following CT_{max} trials. Similar to *hsp90a*, a plastic response is observed in mRNA transcript abundance of *HIF-1α*, a transcription factor involved in the response to hypoxia and regulated by temperature in Crucian carp (Rissanen et al, 2006; Wenger, 2002). In the current study, immediately following CT_{max} trials, Lake Sturgeon from both populations at 24ºC increased mRNA transcript abundance of *HIF-1α* relative to those acclimated to 16ºC, and this expression was also correlated to individual CT_{max} across acclimation treatments and populations. The WR population exhibited an increase in *HIF-1α* mRNA transcript abundance between 16 and 20ºC, and continuing in 24ºC. However, this same increase was delayed in the BR population, only occurring in 24ºC, possibly indicative of differing thermal thresholds for expression induction and the role this protein may play in cross-tolerance to thermal stress (Maloyan et al, 2005), although this was not specifically addressed in this study.

The mRNA transcript abundance of *hsp70* showed elevation in response to CT_{max} trials, with increases in expression ranging from 3 to 10-fold across acclimation treatments and populations. Changes in gill *hsp70* mRNA transcript abundance can subsequently lead to much higher levels of protein expression as observed in the gills of the goby, *Gillichthys mirabilis*, (Buckley et al, 2006; Somero, 2020). Under normal conditions in eukaryotes, *hsp70* functions as a required chaperone for protein assembly (Lindquist, 1992; Roberts et al, 2010). Under times of thermal stress, *hsp70* can act to bind to and stabilize proteins against misfolding and prevent intracellular aggregation, serving as an indicator of stress severity (Logan and Somero, 2011; Tomanek and Somero, 1999; Welch and Feramisco, 1985; Yamashita et al, 2010). Immediately following the CT_{max} trials, there was an increase in mRNA transcript abundance of *hsp70* in the

WR 24ºC treatment, compared to their BR counterparts. This relative increase in expression may be indicative of the WR population's greater ability to acutely upregulate mRNA transcript abundance of *hsp70* after acclimation to thermally stressful temperatures as reported in rainbow trout, *Oncorhynchus mykiss* (Currie et al, 2005). This increased response was not observed in the BR population and may play a role in the increased CT_{max} of the WR population in comparison to the BR in this acclimation treatment. Three days post CT_{max}, expression of *hsp70* returned to near baseline levels in all treatments; except for BR 16ºC, which remained elevated compared to 20 and 24ºC. Similarly, three days post CT_{max}, *hsp90a* was elevated in the BR 16ºC treatment as compared to 20 and 24ºC, and depressed in the 24ºC treatment compared to pretrial levels, but neither of these observations was true for the WR population. Elevated mRNA transcript abundance of *hsp70* and *hsp90a* three days post CT_{max} in the BR 16ºC in comparison to 20 and 24ºC acclimation treatments was possibly due to increased cellular damage, slower rates of cellular repair, and delayed recovery that may impact long term individual fitness (Jeffries et al, 2018; Tomanek and Somero, 2000), while decreases in *hsp90a* in 24ºC could be indicative of further metabolic changes. Additionally, a trend of elevated GPx activity, a family of antioxidant enzymes key in eliminating reactive oxygen species that may form as a result of thermal stress (Do et al, 2019), was observed in the BR population with significantly altered expression pre trials and three days post CT_{max}. Acute exposure to elevated temperatures has been demonstrated to increase GPx activity in the bald notothen, *Pagothenia borchgrevinki*, (Almroth et al, 2015) and the European bullhead, *Cottus gobio* (Dorts et al, 2012). Thus, increased GPx activity in the BR population was most likely a result of higher levels of oxidative stress when compared to WR Lake Sturgeon. Increased mRNA transcript abundance of *hsp70*, *hsp90a*, and enzyme activity three days post CT_{max} in the BR 16ºC treatment, but not at 20 and 24ºC or the WR population, further demonstrate population-specific responses to thermal stress and the ability of acclimation to decrease potentially resultant cellular consequences.

Gill mRNA transcript abundance of *Na^+/K^+ ATPase-α1* revealed further differences between populations. *Na^+/K^+ ATPase-α1* makes up the functional pumping subunit of the heterodimeric protein complex (Hu et al, 2017; Wong et al, 2016) that actively exchanges Na^+ and K^+ ions to maintain cellular ion balance (Ito et al, 2010). At all sampling points, mRNA transcript abundance of *Na^+/K^+ ATPase-α1* was elevated in BR Lake Sturgeon, when compared to their WR counterparts. Expression of *Na^+/K^+ ATPase-α1* mRNA is altered in high temperature acclimation

treatments in three different species of Pacific salmon (Jeffries et al, 2014; Tomalty et al, 2015) as well as in Atlantic salmon (Vargas-Chacoff et al, 2018). This gene has also been implicated as a driver of population differences across different salinities in the semi-anadromous Sacramento splittail, *Pogonichthys macrolepidotus* (Mundy et al, 2020). *Na^+/K^+ ATPase-α1* is under hormonal control (Feraille and Doucet, 2001; Ito et al, 2010; Therien and Blostein, 2000) and isoforms of it can be upregulated in the presence of cortisol and growth hormone (McCormick et al, 2013). Thus, increases in mRNA transcript abundance observed in the current study may have multiple explanations. *Na^+/K^+ ATPase-α1* is not well studied in freshwater fish in response to thermal stressors. In contrast to the above salmon literature, in the current study, there was no evidence of an effect of temperature on the mRNA transcript abundance of *Na^+/K^+ ATPase-α1*. These differences in *Na^+/K^+ ATPase-α1* mRNA transcript abundance may be evidence of further differences between populations, or varying effects of stress and osmotic disruption induced in BR Lake Sturgeon.

3.5.5 Conclusions

Conservation hatcheries continue to rear and release Lake Sturgeon to enhance endangered wild populations throughout their distribution. In order to ensure the success of these stocking programs, and to preserve Lake Sturgeon throughout their natural range, it is necessary to understand the effects of different environmental temperatures on the survival and physiology of sturgeon from diverse populations. This study has demonstrated significant population-specific physiological effects following 30 d acclimation to relevant environmental temperatures and those that may be anticipated within the lifetime of sturgeon currently being released (Manitoba Hydro, 2015). Prolonged temperatures above 16ºC may not be appropriate for rearing Manitoba populations of Lake Sturgeon with decreases in HSI potentially impacting their ability to survive overwintering. While Lake Sturgeon can acclimate to increased environmental temperatures, increases in mortality as well as wide ranging physiological consequences including diminished HSI, metabolic depression, and alteration of gene expression are evident as a result of chronic thermal stress, which are likely related to environmental and possibly genetic differences between populations (Harder et al, 2019). The numbers of individuals captured for spawning and subsequent mix of genetic variability in resultant progeny for this study are restricted due to the endangered status of the species in Manitoba (COSEWIC, 2006; COSEWIC, 2017) and thus limit

our interpretation; however, the data presented suggest population level responses to increased acclimation temperatures and are supported by a number of studies conducted on other geographically separated populations of the same species. This study addressed the effects of increasing environmental temperatures on developing Lake Sturgeon that were fed to satiation; however, natural environments represent a complex set of factors that can interactively affect population and community outcomes (Moe et al, 2012). Thus, future studies should examine interactive effects of multiple stressors on the measured physiological variables to fully understand the consequences on the Lake Sturgeon population health under future warming scenarios.

3.6 Acknowledgements

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Credit Author Statement

W.B., W.G.A. and K.J. conceived and designed the experiments. W.B., G.Y., C.B., W.G.A. and K.J. collected gametes from wild spawning lake sturgeon, while W.B., G.Y., C.B. and A.L. reared juveniles and collected data throughout development. W.B., G.Y., C.B., and A.L. conducted CT_{max} trials. A.S. conducted assessment of glutathione peroxidase activity. W.B. conducted gene expression and data analysis. W.B., G.Y., A.S., A.L., C.B., W.G.A. and K.J. wrote, reviewed and edited the manuscript. W.G.A. and K.J. acquired funding and aided in supervision throughout the experimentation and review processes.

Chapter 3 demonstrated the effects of increased environmental temperatures on whole-organism phenotypes and transcriptional responses, indicating decreased performance and population-specific responses as temperatures increased, with lower sub-lethal thresholds in the northern BWR population, when compared to their southern WR counterparts. Chapters 4, and 6 further investigate these responses using whole-transcriptome analysis and targeted mRNA responses of immune challenged lake sturgeon following thermal acclimation.

Chapter 4. Transcriptome-wide patterns reveal conserved and population-specific responses to increasing acclimation temperatures in developing lake sturgeon (*Acipenser fulvescens*)

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4.1 Abstract

Rising mean and variance in temperatures elevate threats to endangered freshwater species like the lake sturgeon, *Acipenser fulvescens*. Previous research has demonstrated that higher temperatures in early development result in physiological consequences for lake sturgeon populations throughout Manitoba, Canada, with alteration of metabolic rate, thermal tolerance, transcriptional responses, growth, and mortality. In the present study, we acclimated lake sturgeon from northern and southern populations within Manitoba to current and future projected environmental temperatures of 16, 20, and 24˚C for 30 days, and measured gill transcriptional responses using RNAseq. We found population-specific and acclimation-specific responses to the thermal treatments, as well as conserved molecular responses between northern and southern sturgeon populations. Expression profiles revealed a gradient in transcriptional responses consistent with acclimation temperature, with a higher number of differentially expressed transcripts observed in the southern compared to the northern lake sturgeon population as temperatures increase, indicating enhanced transcriptional plasticity. Overall lake sturgeon populations responded to thermal acclimation by upregulating the expression of transcripts involved in transcriptional and translational regulation, mitochondrial function, pathogen responses, and DNA damage, as well as genes associated with pre- and post-transcriptional processes (i.e., methylation, alternative splicing). Further, both populations upregulated transcript expression related to cell structural damage as temperatures increased to 20˚C, but the northern population also responded with increases in damage signaling and recruitment of mitochondrial processes involved in ATP production. Ultimately, these transcriptional responses highlight molecular consequences of increasing temperatures for divergent lake sturgeon populations during vulnerable early developmental periods and the critical influence of transcriptome plasticity on acclimation capacity.

4.2 Introduction

Freshwater fishes have experienced the highest rates of extinction of all vertebrates throughout the 20th century, influenced by the compounding and dynamic climate change-related stressors of elevated temperatures, hypoxic conditions, and pathogens which continue to imperil freshwater fishes and limit suitable habitat (Marcos-López et al., 2010; Burkhead, 2012; Comte et al., 2012; Schade et al., 2014; Saairi et al., 2018; Olusanya and Zyll de Jong, 2018; Krabbenhoft et al., 2020; Vollset et al., 2020; Chapman et al., 2021; Jane et al., 2021). Climate change is increasing both the mean and variance of environmental temperature (Thornton et al., 2014; Dillon et al., 2016). Environmental changes are often occurring faster than organisms can adapt to increasing temperatures, especially in species with long generation times (Crozier and Hutchings, 2014; Morgan et al., 2020; Logan and Cox, 2020). Therefore, phenotypic plasticity will be critical for maintaining fitness in response to climate change and promote survival in these changing environments (Burggren, 2018; Scheiner et al., 2019). While genomic modifications underly transgenerational plasticity, intragenerational plasticity induced to alter an organism's phenotype at the transcriptomic level may act as an adaptive trait (Muschick et al., 2011; Jonsson and Jonsson, 2019). This adaptive role of plasticity may enable modifications to an organism's molecular physiology, thereby increasing an organism's resistance to environmental change, reproductive capacity, and promoting survival (Lafuente and Beldade, 2019; Oomen and Hutchings, 2017; Wellband and Heath, 2017). Further, polyploid organisms with larger genomes may have increased acclimatory capacity at both the transgenerational and intragenerational timescales through transcriptome plasticity (Ellis et al., 2014; Trifonov et al., 2016; Van de Peer, 2017). In populations of polyploid species with different environmental pressures and genetic histories, which may be under genetic constraints (e.g. long generation times) preventing performance traits from evolving rapidly (Logan and Cox, 2020), divergent phenotypes of transcriptomic plasticity can be expected as environmental changes intensify.

During early development, changes in temperature are a critical abiotic factor necessitating prompt phenotypic alteration. Plasticly induced phenotypic changes at an individual level can alter organismal physiology and buffer against acute environmental changes (McKenzie et al., 2021). Physiological flexibility can be an adaptive response stimulated in stressful environments, and is a reversible phenotypic change, that may be persistent for days to months, deemed acclimation (Hockachka and Somero, 2002; Crozier and Hutchings 2014; Havird et al., 2020; Mackey et al.,

2021). Underlying these phenotypic modifications are transcriptional mechanisms that impacting the establishment of long-term phenotypes (e.g., developmental plasticity) (Jonsson and Jonsson, 2019). Exposing developing fish to increasing environmental temperatures will thus alter traits that may have transient as well as long-lasting physiological consequences. The basis for plasticity is variation in phenotypes produced by the same genotype under environmental variation (Lafuente and Beldade, 2019). Acclimatory plasticity is especially important in early life to ensure sufficient phenotypic flexibility, enabling fishes to survive in variable environmental conditions until adulthood when their reproductive potential can be reached (Burggren, 2018). In early developmental stages, different phenotypes and developmental trajectories can emerge from genetically distinct populations exposed to similar environmental change, based, in part, on differences in transcriptome plasticity. Induced molecular responses to stressors often differ between populations, likely as a result of differences in baseline expression or transcriptional plasticity and may be indicative of long-term fitness and survival consequences if phenotypes cannot be adequately altered in early life to allow individuals to persist in changing environments (Whitehead et al., 2012; Bugg et al., 2020; Mundy et al., 2020; Bugg et al., 2021b).

A primary mechanism underlying physiological responses in the face of environmental change are modifications to the abundance of messenger RNA (mRNA) through transcriptional processes (Smith et al., 2013; Connon et al., 2018; Jeffries et al., 2019; Jeffries et al., 2021). Through mRNA sequencing (i.e., RNAseq) and *de novo* transcriptome assembly, transcriptomics approaches can be used when few genomic resources are available for a species (Alvarez et al., 2015; Connon et al., 2018; Oomen & Hutchings 2017; Thorstensen et al., 2020; Komoroske et al., 2020). Examining differential gene expression through transcriptome-wide approaches can illuminate organism's physiologically- and ecologically-relevant responses to environmental change, aid in the identification of sublethal tolerance thresholds, and inform conservation management practices (Connon et al., 2018). As environmental extremes in freshwater systems escalate, transcriptomic techniques provide powerful tools to investigate the acclimatory potential in species of conservation concern, highlighting the genetic mechanisms that promote physiological acclimation to environmental change. If stressors breach sub-lethal thresholds, transcriptomic plasticity and acclimatory capacity may be diminished, suggesting increased vulnerability to additional environmental stressors, and the possibility of severe short-term outcomes, limited recovery, and the potential for impacts on long-term fitness (Smith et al., 2013;

Jeffries et al., 2018).

In fishes, the gill is a multi-purpose tissue reactive to the external environment with roles in respiration, osmoregulation, pathogen defense, acid-base balance, and responses to thermal stress (Lazado and Caipang, 2014; Komoroske et al., 2015; Akbarzadeh et al., 2018; Gilmour and Perry, 2018). Gene transcription producing mRNAs and subsequently the translation into proteins underlies many phenotype modifying processes, contributing to plasticity and physiological change (Schlichting and Smith, 2002). Fishes exposed to increased temperatures alter their physiology through transcriptional mechanisms, enabling them to acclimate to their environment and preserve internal homeostasis in the face of environmental variation. Investigating transcriptional responses in the gill can thus highlight changes at a key interface between these organisms and their environment.

Greater than 50% of the world's river basins face widespread anthropogenic impacts which limit productivity, species richness, and biodiversity, threatening approximately a quarter of freshwater fish species globally (Palmer et al., 2008; IUCN, 2018; Grill et al., 2019; Su et al., 2021; Warkentin et al., 2022). One species of freshwater fish, lake sturgeon, *Acipenser fulvescens,* are widely distributed throughout North America, and within Canada have experienced population declines >90% in the northern extent of their range since the 1960's (COSEWIC, 2006). Historically, lake sturgeon population declines in the 1800s were caused by commercial exploitation for caviar. Since the 1950's however, habitat loss and degradation associated with dams and other barriers have become the most prominent threats to populations and the species' persistence (Cleator et al., 2010; COSEWIC 2017; Van der Lee and Koops, 2021). Temperatures in the northern range for lake sturgeon are also increasing faster than the global average (projected 2.1-3.4°C increase by 2050; Manitoba Hydro, 2015), which may further limit suitable habitat, and imperil these endangered subarctic populations as water temperatures exceed their thermal limits (Vincent et al., 2015; Zhang et al., 2019; Bugg et al., 2021b). Increasing environmental temperatures directly influence the development, metabolism, transcriptomic responses, and survival of northern populations of lake sturgeon under laboratory conditions and may contribute to observed declines in wild stocks (Bugg et al., 2020; Bugg et al., 2021b). Indirectly, elevating temperatures may increase environmental hypoxia and the susceptibility of lake sturgeon to environmentally prevalent bacterial or viral pathogens, which may be transmitted horizontally between individuals or vertically across generations (LaPatra et al., 1994; Georgiadis et al., 2001;

Fujimoto, 2018; Clouthier et al., 2020). Sturgeons may be especially susceptible to the combined effects of increasing temperature, hypoxic environments, and pathogens in early development, when mortality is often high (> 90%; Caroffino et al., 2010a,b) and sturgeon have limited immune responses and ability to escape stressful environments (Webb, 1986; Peake et al., 1997; Schindler, 2001; Breau et al., 2011; Deslauriers and Kieffer, 2012; Gradil et al., 2014 a,b; Verhille et al., 2014). However, despite these vulnerabilities in early development, sturgeon have successfully persisted for hundreds of millions of years in ever-changing environments, evolving elaborate polyploid genetic complexity (4n to 6n common, functional with a maximum of 520 chromosomes, most of any vertebrate; Lebeda et al., 2020), and demonstrating high levels of phenotypic plasticity in early development when compared to other fishes (Chapter 1), with the potential for underlying increased inducible transcriptional plasticity (Braasch and Postlethwait, 2012; Trifonov et al., 2016; Bugg et al., 2020; Bugg et al., 2021b; Penman, 2021; Yoon et al., 2021; Brandt et al., 2021, Brandt et al., 2022). Therefore, early development represents a crucial window to examine lake sturgeon responses to thermal acclimation, when exceptionally plastic responses may be anticipated (Burggren, 2018; Barley et al., 2021).

Here we used RNAseq to investigate transcriptomic responses to increased environmental temperatures in the gills of two latitudinally separated and genetically distinct populations of developing lake sturgeon (McDougall et al., 2017; Bugg et al., 2020). As sturgeons are ancestral polyploid species, with highly variable genomic architecture and phenotypic plasticity (Braasch and Postlethwait, 2012; Trifonov et al., 2016; Bugg et al., 2021b), we expected these populations of lake sturgeon to respond uniquely to the temperature treatments. We examined the gill mRNA transcript abundance in n = 36 individuals from three thermal acclimation treatments in the northern and southern population of lake sturgeon within Manitoba. We then investigated phenotypic plasticity through exploration of 9 total contrasts spanning the molecular mechanisms shared between populations as acclimation temperatures increased, as well as population and acclimation-specific responses to increased environmental temperatures expected to impact these lake sturgeon populations. Previous research showed acclimating lake sturgeon for 30 days to the potentially thermally stressful temperatures of 20°C and 24°C, resulted in population and acclimation-specific changes in mortality, metabolism, thermal tolerance, and transcriptional responses (Bugg et al. 2020). Therefore, we hypothesized that acclimation to these same elevated temperatures during early life history would elicit transcriptome-wide responses associated with

energy mobilization, pathogenic infection, cellular repair, and heat shock proteins indicative of chronic thermal stress. Given the higher mortality and lower thermal tolerance observed in the northern population post-temperature acclimation in a previous study (Bugg et al., 2020), we predicted that the northern population of lake sturgeon would show greater transcriptional signatures of a thermal stress as temperatures increased, relative to the southern population.

4.3 Materials and Methods

4.3.1 Lake Sturgeon Husbandry and Acclimation

Lake sturgeon used in the current study were from the same populations and acclimation treatments described in Bugg et al. (2020). Briefly, gametes were collected from wild male and female lake sturgeon downstream from the Pointe du Boise Generating Station on the southern Winnipeg River (WR; 50°17’52’’N, 95°32’51’’W) and first rapids on the northern Burntwood River (BWR; 56° 02’ 46.5’’ N, 96 °54’18.6’’W) (Figure 4.1). Eggs and sperm from the WR were fertilized at the University of Manitoba animal holding facility while those from the BWR were fertilized at the Grand Rapids Fish Hatchery, and subsequently transferred to the University of Manitoba. Post-fertilization, embryos from each population were submerged in a clay substrate to de-adhease them and then were gently stirred by hand for 1 h. Next, embryos were rinsed with dechlorinated freshwater and kept in tumbling jars to incubate at 12°C until hatch. Water temperature was maintained at 12°C until 13 days post fertilization after which it was increased by 1°C day^{-1} until 16°C, matching typical hatchery rearing conditions in Manitoba. Starting at 19 days post fertilization, and continuing throughout the length of the experiment, developing lake sturgeon were fed freshly hatched artemia (Artemia International LLC; Texas, USA) three times daily. Acclimation began at 30 days post fertilization, by increasing treatments to 20°C and 24°C at a rate of 1°C day^{-1} and keeping one treatment from each population at 16°C. Lake sturgeon from both populations were acclimated to these temperatures for 30 days with water temperatures recorded by HOBO Water Temperature PROv2 Data Loggers (Onset Computer Corporation; Bourne, MA, USA) and checked by thermometer at least three times daily. All animals in this study were reared and sampled under guidelines established by the Canadian Council for Animal Care and approved by the Animal Care Committee at the University of Manitoba under Protocol #F15-007.

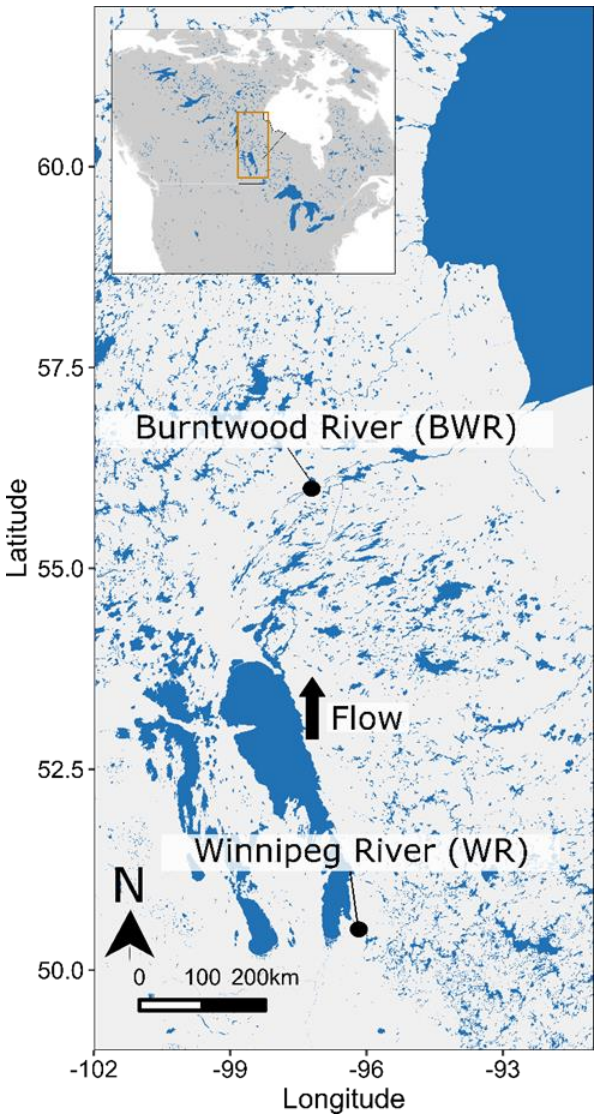

Figure 4.1 Geographic localities of two distinct lake sturgeon, *Acipenser fulvescens*, populations within Manitoba, Canada, highlighting the northern Burntwood River (BWR) and more southernly Winnipeg River (WR) sturgeon populations as well as the direction of flow throughout the watershed.

4.3.2 Sampling and RNA extraction

At the end of the 30 day acclimation, six larval lake sturgeon per treatment were netted and euthanized by immersion in an overdose of tricaine methanesulfonate solution (250 mg L^{-1}; MS-222, Syndel Laboratory, Vancouver, Canada) buffered with an equal volume of sodium bicarbonate. Next, gill tissue was extracted from each larval fish and preserved in RNA*later* (Invitrogen; Carlsbad, CA, USA), held at 4˚C for 24 h and stored at −80˚C prior to RNA extraction. Total RNA was extracted from the gill tissue by homogenization in 500 µl of lysis buffer for 10 min at 50 Hz using a TissueLyser II (Qiagen; Germantown, MD, USA) and then using a PureLink RNA Mini Kit (Invitrogen; Ambion Life Technologies) following manufacturer instructions. Total RNA purity and concentration was evaluated for all samples using a NanoDrop One (Thermo Fisher Scientific) as well as gel electrophoresis to assess RNA integrity. Samples were then stored at -80˚C prior to sequencing.

4.3.3 *De novo* Transcriptome Assembly

Prior to the present study, a lake sturgeon gill reference transcriptome was created by sequencing mRNA from the pooled RNA of 3 individuals. We used a mRNA isolation, stranded library preparation approach to produce 50 million reads of 100 base pair paired-end reads. The transcriptome was *de novo* assembled using Trinity (Grabherr et al., 2011) through the Canadian Centre for Computational Genomics (C3G; https://computationalgenomics.ca/). Assembled transcripts were then annotated with Trinotate (http://trinotate.sourceforge.net/) using a modified approach, relative to using a single source for annotation. First assembled transcripts were blasted against a fish specific database with annotation hits returned. Transcripts that were not annotated from the fish specific database were then blasted against the entire SwissProt library.

4.3.4 Sequencing

Total RNA for each sample was normalized to approximately 15 ng μL^{-1} at a total volume of 10 µL and shipped to the McGill Applied Genomics Innovation Core sequencing facility (https://www.mcgillgenomecentre.ca/) for cDNA library preparation and sequencing. Samples were run on a Bioanalyzer to confirm RNA quality and all samples had a RNA Integrity Number (RIN) between 8.7-10. A mRNA isolation step was performed using NEBNext Poly(A) Magnetic Isolation Modules (New England Biolabs) followed by using NEBNext Ultra II

Directional RNA Library Prep Kit for Illumina (New England Biolabs) to produce stranded cDNA libraries. NEBNext dual adaptors (New England Biolabs) were then individually applied to barcode each library prior to sequencing of 100 base pair paired-end reads. All 36 sturgeon gill samples were sequenced on a single NovaSeq 6000 (Illumina) lane producing 1.53 billion total reads with an average of 42,528,434 (± 17,812,372 SD) reads per sample. Detailed results per sample can be found in Supplementary Table A.4.1.

4.3.5 Quality control and Trimming

Raw read files were uploaded to Galaxy servers (Giardine et al., 2005) and paired reads were joined and checked for quality using FastQC version 0.11.8 (Andrews, 2010). To filter low-quality reads, Trimmomatic version 0.38.0 was applied with the Illuminaclip option set to standard for TruSeq3 adapter sequences, a maximum mismatch count of 2, and a palindrome read alignment threshold of 30. Additionally, a sliding window trimming step was used with a size of 4 base pairs as well as a minimum read length filter set at 20 bases. Ultimately this trimming removed approximately 2.8% (± 0.29% SD) of reads from each file. Full details for quality control and trimming procedures are available in Supplementary File A.4.1, Sheets A-C.

4.3.6 Read Mapping and Differential Expression

After filtering, HISAT2 version 2.1 and StringTie version 2.1.1 (Kim et al., 2015; Pertea et al., 2015) were used to align paired read libraries to the *de novo* assembled gill transcriptome and produce a set of assembled transcripts for each sample, respectively. HISAT2 was run with default parameters and produced an overall alignment rate of approximately 82.6% (± 1.2% SD) per sample. HISAT2 aligned bam files were then input into StringTie to assemble transcripts, using default settings, producing an average of 94,992 (± 11,075 SD) transcripts per sample. StringTie files for each sample were then merged producing a master list of 139,456 transcripts. Total counts for each of these transcripts were then counted in HISAT2 produced aligned read files using featureCounts version 1.6.2 with default settings (Liao et al., 2014; Available in Supplementary File A.4.2). Total counts were then assessed for differential expression using the R v4.0.0 package EdgeR v3.32.1 (Robinson et al., 2010; R Core Team, 2022). Prior to analysis the "filterByExpr" function with default arguments was used to retain transcripts expressed among any individual, removing 6,098 transcripts from analysis due to incomplete data across

individuals, and resulting in a final count of 88,894 transcripts. Full details for read mapping of each individual sample are available in Supplementary File A.4.1, Sheet D.

4.3.7 MDS, Differentially Expressed Transcripts, and Aggregate Heatmapping

A multidimensional scaling (MDS) plot was produced using featureCounts from the above 88,894 transcripts after normalizing the data to the library size using the plotMDS function in the limma package (Ritchie et al., 2015). Next, numbers of differentially expressed genes between the BWR and WR populations of lake sturgeon acclimated to 16˚C, 20˚C, and 24˚C for 30 days in early development were tabulated for upregulated and downregulated genes independently, representing genes with a $\log_2$ fold change > 1 and a *q*-value < 0.05 for each of the 9 individual contrast. The 9 total contrasts compared the populations at each acclimation temperature as well as across acclimation temperatures for each population. Log_2 counts per million (CPM) data for all 88,894 transcripts was then generated and the top 500 genes from each contrast with the highest absolute value $\log_2$ CPM fold change were included in an aggregate list of the most highly differentially expressed genes across all contrasts, resulting in a total of 4,500 transcripts, 2,285 uniquely included (full CPM data is available for all 88,894 transcripts in Supplementary File A.4.3). A heatmap with hierarchical clustering was then produced using the $\log_2$ CPM expression of these aggregate most highly differentially expressed 2,285 unique transcripts using the pheatmap package (Kolde, 2015).

4.3.8 Functional Term Enrichment

Transcripts annotated to the *de novo* lake sturgeon transcriptome were split into groups of upregulated and downregulated genes by treatment based on their $\log_2$ CPM fold change (< -1 or > 1) and were counted for distinct gene names (hereafter referred to as split-contrast). The positively and negatively regulated annotated genes were then analyzed for functional enrichment using gene ontology (GO) terms through enrichR as well as Kegg pathway analysis (Chen et al., 2013; Kuleshov et al., 2016; Xie et al., 2021) with positive hits returned from the "GO_Biological_Processes_2018", "GO_Molecular_Function_2018", "GO_Cellular_Component_2018", and "Kegg_2016" databases and filtered for an adjusted *p*-value < 0.05. The resulting 212 enrichment terms across the upregulated and downregulated spit-contrast were then tabulated into a master file detailing their presence or absence in each split-

contrast (0 or 1; Full presence and absence data across each split-contrast is available in Supplementary File A.4.4). This master file was then used to create UpSet plots of the shared and unique terms both upregulated and downregulated across all split-contrasts, upregulated terms as temperatures increased from 16°C to 20°C, and terms which decreased as temperatures increased from 20°C to 24°C using the 'UpsetR' package v1.4.0 (Conway et al., 2017). Split-contrasts with ≥ 50 functional enrichment terms were then focused on for interpretation of results.

Next, non-redundant enrichment terms in the GO Biological Process category were identified with REVIGO for summary and visualization (Supek et al., 2011) using a list size value of 0.5 to restrict the size of the produced output list. Adjusted *p* values were supplied for all REVIGO analyses to guide the clustering of redundant GO terms by the REVIGO algorithm (Supek et al., 2011). Where GO terms were shared across populations, the geometric mean of the adjusted *p* values from both populations was input into REVIGO. All REVIGO output is available in Supplementary File A.4.5, Sheets A-I.

4.3.9 Aggregate Gene Network Analysis and Enrichment

An aggregate of the most highly differentially expressed transcripts was then investigated, producing a total of 389 annotated most differentially expressed transcripts, with 259 uniquely annotated transcripts, from the aggregate list of 4,500 most differentially expressed transcripts across contrasts used for the heatmap (the full aggregate list of 389 annotated most differentially expressed transcripts is available in Supplementary File A.4.6). These 259 unique gene names were uploaded to OmicsNet (Zhou and Xia, 2018) and input under the genes category using the "official gene symbol" classification and zebrafish *Danio rerio* as the study organism, as this was the most closely related model species option. Next, protein-protein interactions were selected in the network building panel, using STRING (Szklarczyk et al., 2019), comprehensive protein-protein interactions containing both known and predicted interactions, to assembly a network of the 259 unique genes and their potential interactions. The network was then changed to a 2-D model, using a force atlas layout, with seed nodes highlighted with a blue ring around the node, and edge bundling applied. Functional analysis of the produced network was then categorized using the Function Explorer, querying all nodes against the GO: Biological Process database. Processes with a p value < 0.05 and $q < 0.15$ are reported in Supplementary File A.4.7. Discussion of hub transcripts and enriched functional processes focuses on those with at least two pertinent hub

transcripts and inclusion in overarching REVIGO analysis.

4.4 Results

4.4.1 MDS, Gene Regulation, and Differential Expression

Analysis of MDS plots (Figure 4.2A), number of upregulated and downregulated genes (Figure 4.2B), as well as the heatmap displaying the most highly differentially expressed genes across contrasts (Figure 4.2C), demonstrated that the BWR and WR populations of lake sturgeon responded differently to elevated temperatures both in the magnitude and trajectory of transcriptional change. The MDS plot showed divergent response patterns with separation between most treatments, especially so for WR sturgeon in treatments of 20˚C and 24˚C grouping out to the right of the rest of the treatments. Patterns of upregulation and downregulation between the two populations at a given acclimation temperature, and within populations across acclimation temperatures, demonstrated population-specific and acclimation-specific responses to elevated temperatures in developing lake sturgeon. At 16ºC there were a similar number of genes upregulated and downregulated when comparing BWR and WR sturgeon when comparing the two populations. However, at 20˚C and 24˚C there were many more transcripts upregulated in the BWR than decreased when compared to WR sturgeon in the same temperatures, respectively.

When comparing within population across acclimation temperatures, the largest changes in transcript regulation in the BWR and WR sturgeon occurred as acclimation temperatures increased from 16˚C to 20˚C, where the populations differentially expressed a total of 9,334 and 19,216 transcripts, respectively. These changes in transcript expression between 16˚C and 20˚C represent the differential expression of approximately 10.5% and 21.6% of all transcripts for the BWR and WR populations of sturgeon included in the analysis, respectively. Between 16˚C and 24˚C these numbers of differentially expressed transcripts fell drastically (67.8%) in the BWR population, when compared to the 16˚C to 20˚C transition, with just 3,028 total differentially expressed transcripts. However, for WR sturgeon this same transition saw a lesser reduction to 14,850 total transcripts, a much smaller decrease (22.6%) in differential expression over the same acclimatory contrast. A similar decrease in expression, as observed between the BWR 16˚C to 20˚C and 16˚C to 24˚C contrasts, occurred for the WR sturgeon between the WR 16˚C to 24˚C and WR 20˚C to 24˚C contrasts (68%), with a total of 4,753 differentially expressed transcripts between 20˚C and 24˚C. While producing similar qualitative patterns of expression to the BWR,

the number of differentially expressed transcripts was larger in the WR across acclimation treatments.

The heatmap outlined specific patterns of transcript expression of the most highly differentially expressed transcripts, with divergent $\log_2$ CPM expression values as temperatures increased above 16°C for both populations but with differences in these patterns across populations (Figure 4.2C). Differential expression patterns of these transcripts are largely qualitatively similar across the BWR 24°C and WR 16°C treatments, even though they represent highly different acclimation profiles and divergent populations. Further large changes in transcriptional regulation were observed in the WR 20°C and 24°C treatments, when comparing them to patterns of expression observed in the other four treatments, across both populations. Overall, the patterns in the most highly differentially expressed genes align with the MDS analysis in the overlap of the BWR 24°C and WR 16°C treatments, as well as the unique transcriptional profiles of WR sturgeon acclimated to 20°C and 24°C, suggesting that patterns in the most highly differentially expressed transcripts reflect similar transcriptional trajectories as shown in the MDS analysis of all transcripts.

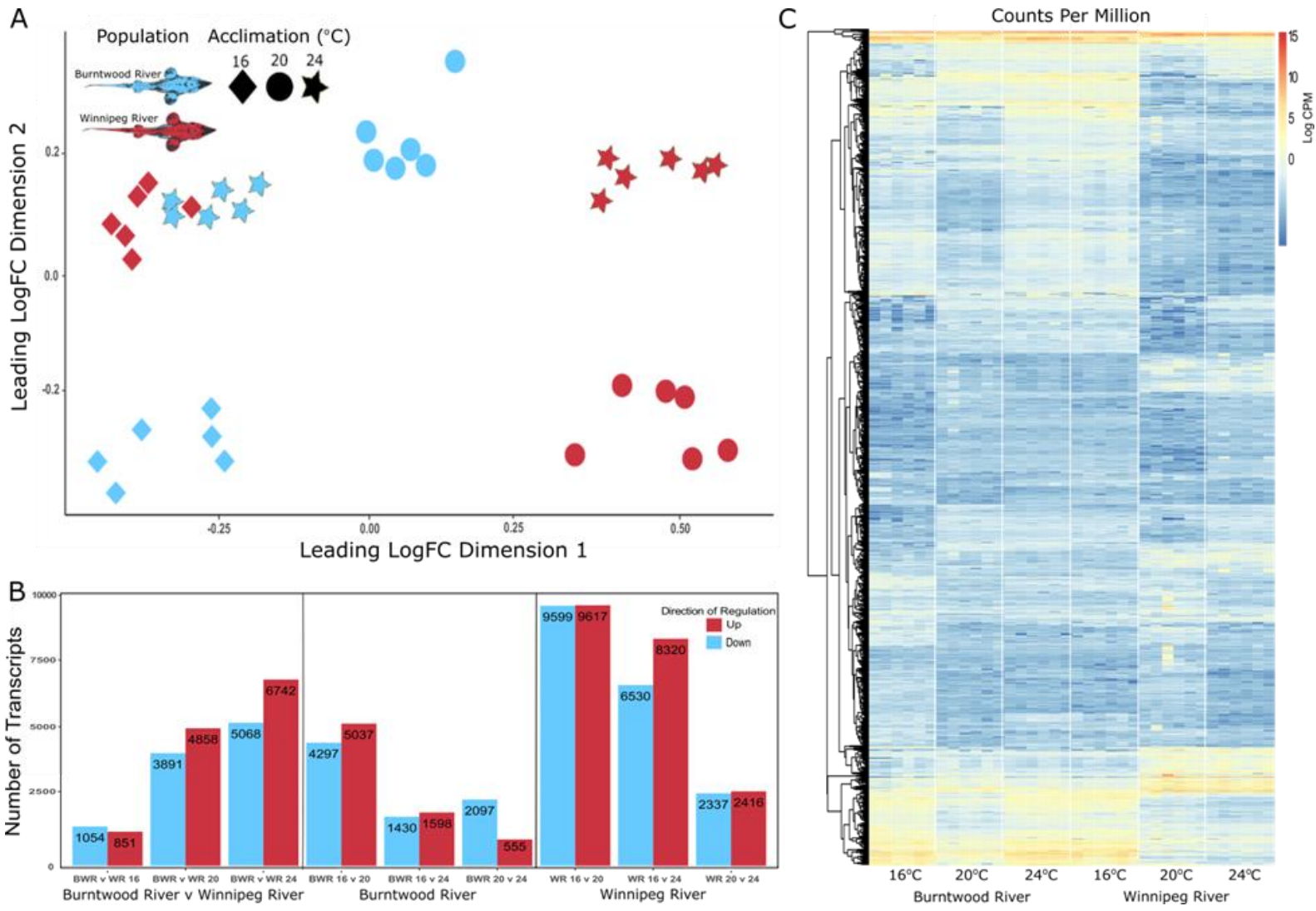

Figure 4.2 A) Multiple dimensional scaling plot, B) numbers of differentially expressed transcripts, and C) aggregate heat map of the top 500 differentially expressed transcripts between each contrast of northern Burntwood River (BWR) and southern Winnipeg River (WR) populations of lake sturgeon in Manitoba, CA, acclimated to 16, 20, and 24˚C for 30 days in early development, measured using RNAseq. Transcript numbers represent upregulation and downregulation with a log2 fold change > 1, respectively, and with a q-value < 0.05. Contrasts are oriented Burntwood River-Winnipeg River at the interpopulation level and lower temperature-higher temperature across acclimation treatments. For the aggregate heat map, only unique transcripts were included for a total of 2,285 transcripts represented. Data are expressed as log2 counts per million (CPM).

4.4.2 Functional Term Enrichment

Term enrichment demonstrated 212 unique GO and Kegg pathway terms distributed across the 17 split-contrasts (Figure 4.3A). Overall, the split-contrasts with the largest number of assigned terms were those underlying processes being upregulated as temperatures increased from 16 to 20°C (WR16v20_neg and BWR16v20_neg; 75 and 85 terms respectively) and those processes that decreased in expression as temperatures continued to increase from 20°C to 24°C (WR20v24_pos and BWR20v24_pos; 62 and 97 terms, respectively). The split-contrasts between the BWR and WR populations as temperatures increase from 16°C to 20°C and again from 20°C to 24°C specifically revealed conserved and population-specific biological processes upregulated as temperatures increase from 16°C to 20°C and those that are decreased with further temperature increase to 24°C. As temperatures increased from 16°C to 20°C there were 50 upregulated functional term responses shared between populations, as well as 39 responses unique to the northern BWR population and 25 distinct to the southern WR population (Figure 4.3C). As acclimation temperatures increased further from 20°C to 24°C there were 26 functional term responses shared between populations, with 71 unique to the BWR population and 38 distinct to the WR population (Figure 4.3D). The largest changes in biological processes for both populations were observed in the upregulation of processes as temperatures increased from 16 to 20°C, and the decrease in processes as temperatures continued to increase from 20 to 24°C. Differentially expressed transcripts which underly these processes can be found in Supplementary File A.4.8.

4.4.3 GO Term and REVIGO Analysis

Overwhelmingly, the majority of all 212 terms were categorized as GO Biological Processes, encompassing 82.55% of terms identified (Figure 4.3B) and highlighting transcriptional mechanisms altered across lake sturgeon populations as temperatures increase, therefore these terms were focused on for further analysis.

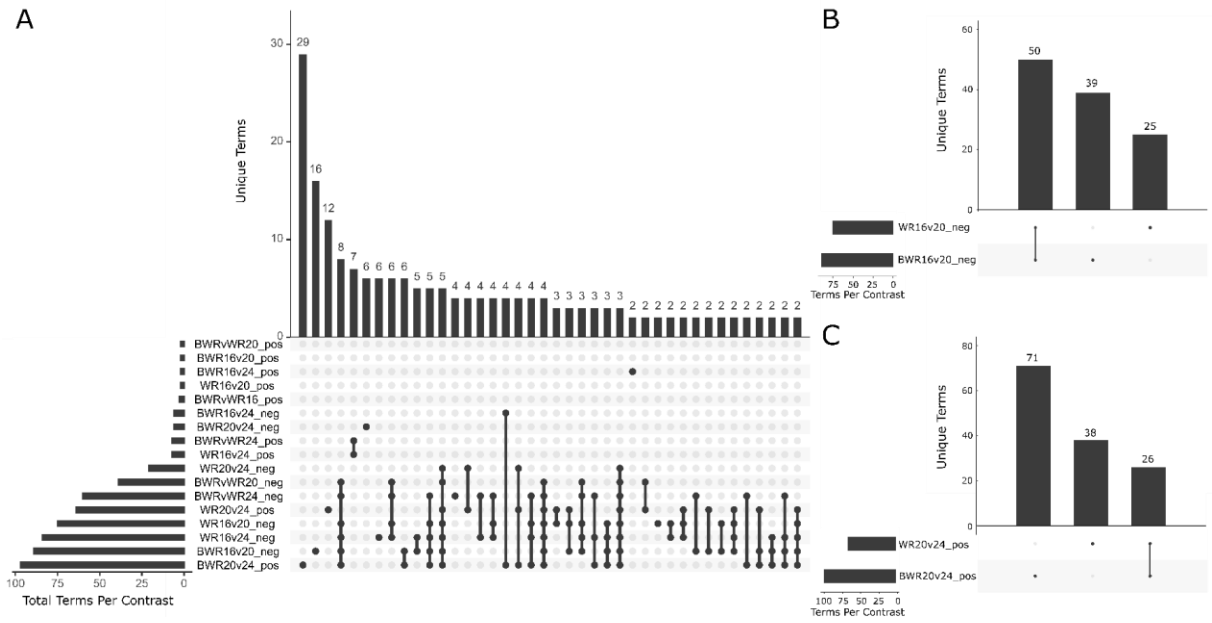

Figure 4.3 GO (gene ontology) and Kegg term distribution illustrated using upset plots demonstrating unique terms across A) all split-contrasts B) upregulated transcripts as temperature increased from 16 to 20˚C, and C) downregulated transcripts as temperatures increased from 20 to 24˚C. These data represent alteration in the transcriptional mechanisms in the gill of developing lake sturgeon from both a northern Burntwood River (BWR) and southern Winnipeg River (WR) population of lake sturgeon (*Acipenser fulvescens*), within Manitoba, Canada, acclimated to temperatures 16˚C, 20˚C, and 24˚C for 30 days in early development, measured using RNAseq. Split-contrasts are oriented BWR-WR at the interpopulation level and lower temperature-higher temperature across acclimation treatments so that "neg" indicates upregulation at higher temperatures, and "pos" indicates downregulation at higher temperatures.

4.4.3.1 Conserved Responses to Increasing Temperature

As the acclimation temperature increased from 16°C to 20°C there were 49 conserved GO Biological Process terms identified as upregulated and shared between the BWR and WR populations of lake sturgeon (REVIGO output displayed in Figure 4.4A). These shared processes were involved in regulation of mRNA processing, ncRNA processing (including mRNA splicing via spliceosome and transesterification), translational regulation (including gene miRNA gene silencing), mitochondrial translation, ribosomal biogenesis (including mitochondrial respiratory chain complex assembly), L-methionine salvage, protein methylation, DNA damage responses, as well as viral gene expression and transcription (Supplementary File A.4.5, Sheet A).

As acclimation temperature further increased from 20°C to 24°C there were 19 conserved GO Biological Process terms that decreased in expression in both the BWR and WR lake sturgeon populations (REVIGO output displayed in Figure 4.4B). These shared processes were involved in RNA polymerase II transcription, ncRNA processing (including mRNA splicing via spliceosome and transesterification), regulation of translational machinery, mitochondrial protein insertion into the inner mitochondrial membrane, mitochondrial organization, and cell cycle regulation (Supplementary File A.4.5, Sheet B).

Across acclimatory treatments, there were 16 GO Biological Process terms identified as upregulated with increasing temperature between 16°C and 20°C but then decreased in expression between 20°C and 24°C. Largely these mechanisms were involved in mRNA processing as well as splicing, mitochondrial organization, ribosome biogenesis, and regulation of the cell cycle (Supplementary File A.4.5, Sheet C).

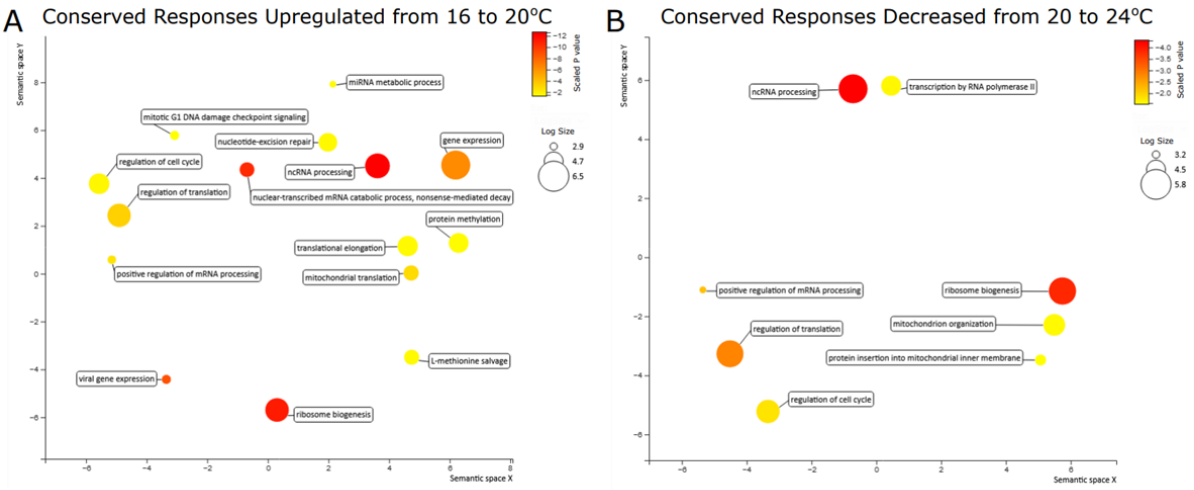

Figure 4.4 REVIGO plots of GO (gene ontology) biological process terms conserved between the Winnipeg River (WR) and Burntwood River (BWR) populations of developing lake sturgeon, *Acipenser fulvescens*, within Manitoba, Canada, which were upregulated as temperatures increased from A) 16 to 20°C, and which decreased as temperatures increased from B) 20 to 24°C during a 30-day acclimation, measured using RNAseq. Bubble color indicates the significance of the adjusted p value at after applying a log10 scale (darker color is a more significant term), while bubble size indicates the frequency of a GO term GO annotation database (larger bubble is a more general term).

4.4.3.2 Population-specific Responses to Increasing Temperature

As temperatures increased from 16°C to 20°C, there were also population-specific increases in transcripts involved in various biological processes. Specific to the WR population, there were 25 unique GO Biological Process terms identified as upregulated (Figure 4.5A) largely characterized by changes in telomerase localization as well as protein targeting, binding, ligation, modification, and removal. There were also biological processes upregulated involved in the assembly of metallo-sulfer clusters, digestive development, mitotic cell cycle phase transitioning, and viral processes (Supplementary File A.4.5, Sheet D). In the BWR population, there were an additional 38 unique GO Biological Process terms upregulated (Figure 4.5B). These BWR specific upregulated terms involved histone methylation, regulation of mRNA stability, mRNA export from the nucleus, mitochondrial function, transcriptional termination, anatomical structure homeostasis, and a variety of DNA damage processes (Supplementary File A.4.5, Sheet E).

As temperatures increased further from 20°C to 24°C there were more population-specific changes in transcripts, however these changes were involved in the decrease of the expression of transcripts involved in these biological processes. Specific to the WR population, there were 34 GO Biological Process terms that decreased between 20°C and 24°C (Figure 4.5C). These processes were largely associated with production of miRNA's involved in gene silencing, mitotic cell cycling/phase transition, monooxygenase activity, responses to UV, DNA repair and damage signaling as well as nuclear membrane disassembly and telomerase localization (Supplementary File 5, Sheet F). Additionally, in the BWR population, there were 53 unique GO Biological Process terms that decreased in expression between 20°C and 24°C (Figure 4.5D). These BWR specific downregulated processes involved DNA, RNA, and protein level modifications, including changes to RNA structure, gene silencing, alternative splicing, protein folding, and protein methylation (including histone methylation). Further there was a decrease in various processes involved in mitochondrial translation, transport, protein targeting, and ATP synthesis. Finally, there were decreases in processes involved in chordate embryonic development, Wnt signaling (conical and planar cell), and DNA damage recognition (Supplementary File A.4.5, Sheet G).

Across acclimatory treatments, there were several population-specific GO Biological Process terms identified as upregulated with increasing temperature between 16 and 20°C but then decreased in expression between 20°C and 24°C. There were 9 processes upregulated in both populations between 16°C and 20°C but then decreased in expression between 20°C and 24°C,

uniquely in the WR population. These processes were involved in regulation of mRNA processing, miRNA production involved in gene silencing, chromosomal organization, as well as DNA damage and repair responses (Supplementary File A.4.5, Sheet H). Additionally, there were 17 GO Biological Process terms upregulated in both the BWR and WR between 16°C and 20°C but then decreased in expression between 20°C and 24°C only in the BWR population. These processes were involved in RNA processing, gene silencing by miRNA, mitochondrial respiration/function, protein synthesis as well as methylation, and DNA damage recognition (Supplementary File A.4.5, Sheet I).

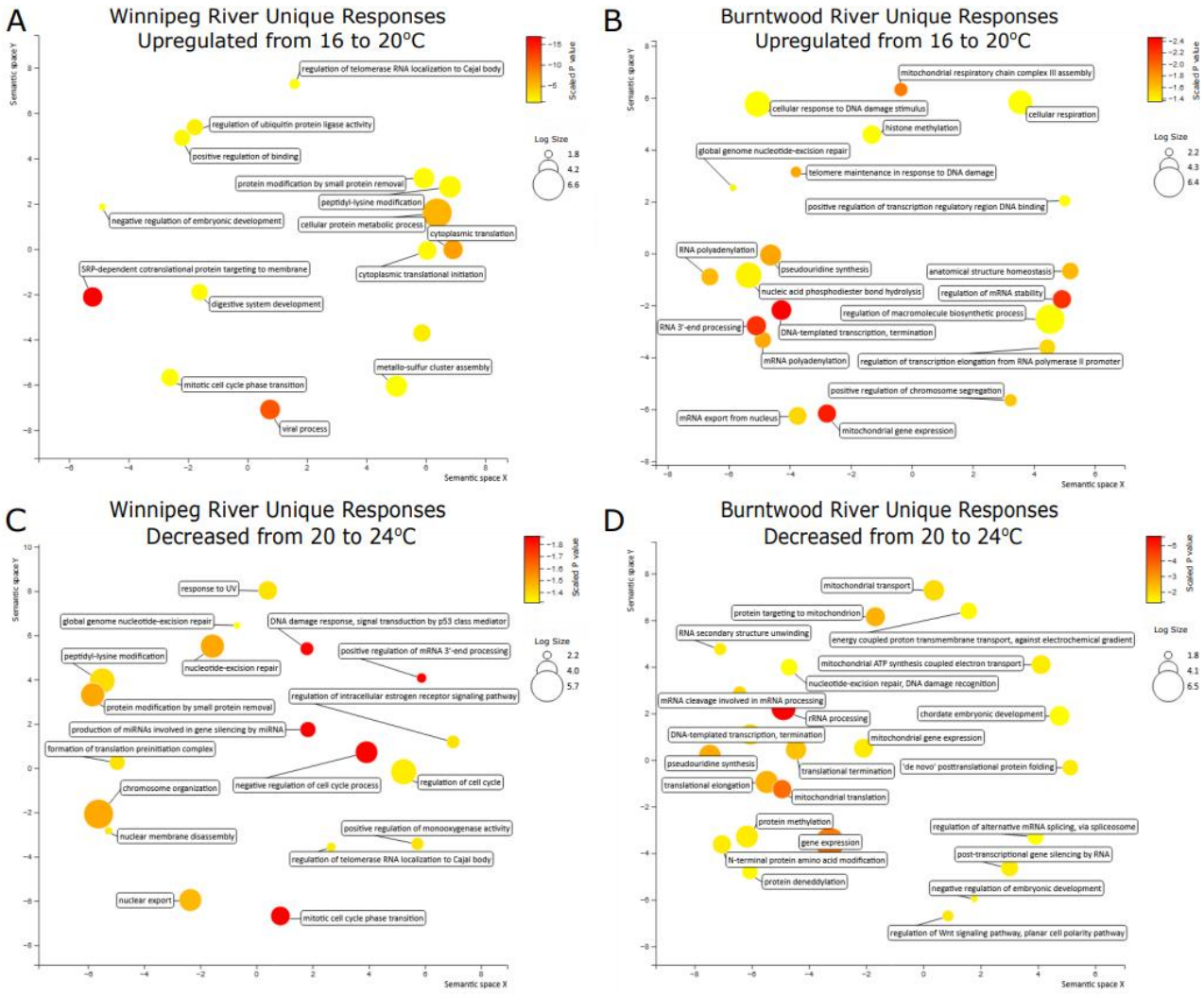

Figure 4.5 REVIGO plots of GO (gene ontology) biological process terms unique to the Winnipeg River (WR) and Burntwood River (BWR) populations of developing lake sturgeon, *Acipenser fulvescens*, within Manitoba, Canada, as acclimation temperatures increase during a 30-day acclimation, measured using RNAseq. Plots represent A) WR unique responses upregulated from 16 to 20ºC B) BWR unique responses upregulated from 16 to 20ºC) WR unique responses decreased 20 to 24ºC and D) BWR unique responses decreased from 20 to 24ºC. Bubble color indicates the significance of the adjusted p value at after applying a log10 scale (darker color is a more significant term), while bubble size indicates the frequency of a GO term GO annotation database (larger bubble is a more general term).

4.4.4 Aggregate Transcript Network Analysis and Functional Enrichment

The aggregate most highly differentially expressed transcript network demonstrated connections across many different functional groups of transcripts that were altered in developing lake sturgeon during acclimation to elevated temperatures. Transcripts identified as hubs (orange circles, highlighted in blue; Figure 4.6) form many responsive network connections and are active in cell cycling (*cdk2*, *src*, *rcc2*), chaperone activity and protein folding (*hspe1*, *hsc70*, *vimp*, *cct4*), pre and post transcriptional modification (*carm1, larp7*) as well as immune and inflammatory activation (*traf6*, *prkcbb*, *egr1*, *egr2b*). Others indicate activation of more general cellular stress responses (*sgk1*) involving maintenance of metabolic processes (*csnk2b*), heart development (*tll1*), and osmotic balance (*cftr*). Many of these processes were also present in the network GO Biological Process term enrichment with enriched pathways including cell cycling, protein folding, responses to heat, immune (general, adaptive, inflammation), heart development, responses to stress, and DNA repair processes (Supplementary File A.4.7).

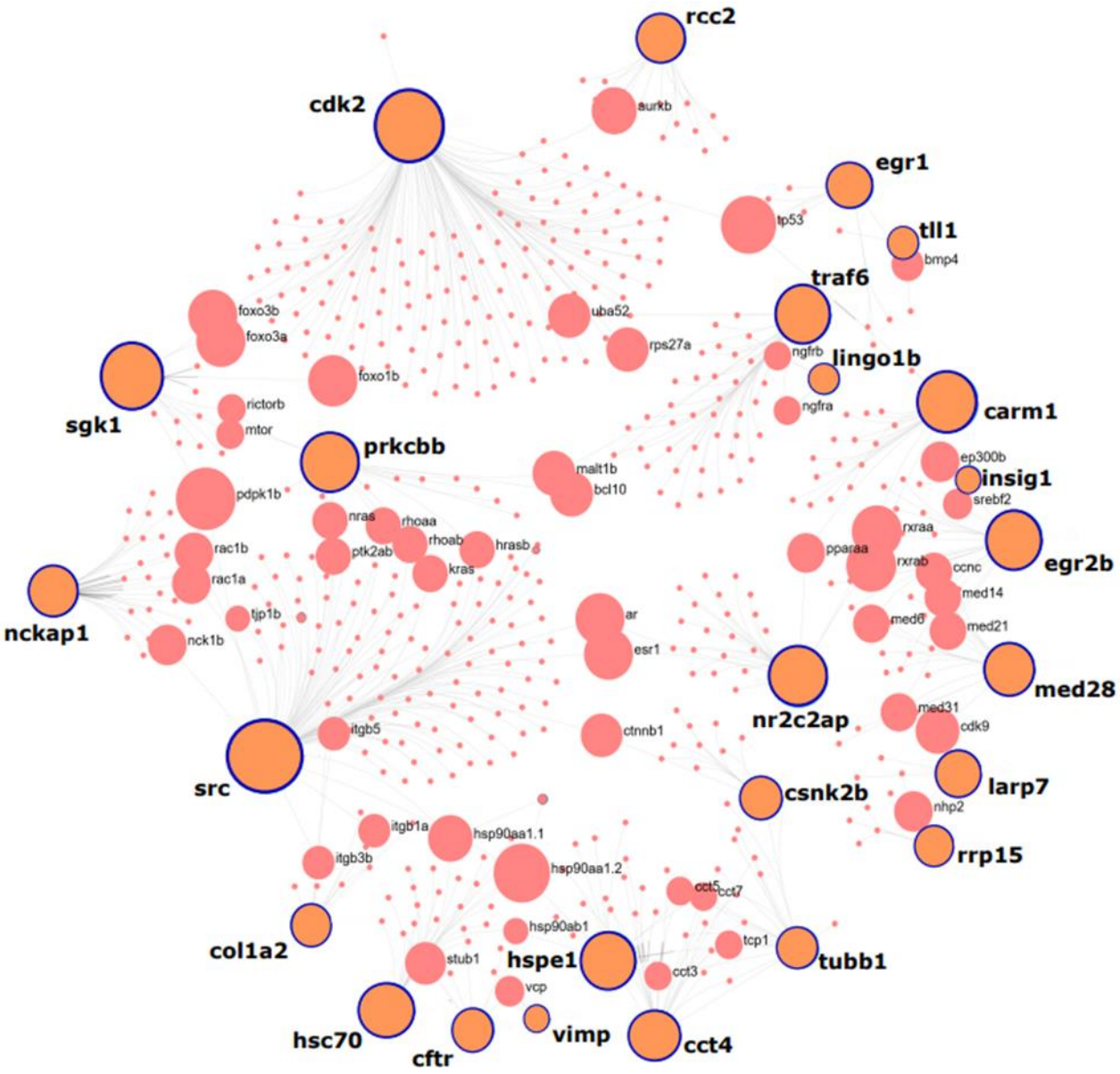

Figure 4.6 Aggregate most differentially expressed transcript network demonstrating predicted protein-protein interactions across the most upregulated and downregulated transcripts of northern and southern lake sturgeon, *Acipenser fulvescens*, populations, within Manitoba, Canada, acclimated to 16˚C, 20˚C, and 24˚C for 30 days in early development, measured using RNAseq. Orange transcript circles outlined in blue with bolded names indicate hub transcripts within the network. Hub size and naming is scaled according to their degree of interaction with surrounding hubs (Zhou and Xia, 2019).

4.5 Discussion

In the current study, we demonstrated wide-ranging conserved and population-specific transcriptional responses as environmental temperatures increased in two geographically and genetically distinct endangered populations of lake sturgeon. These responses spanned wide ranging cellular protective processes induced during acclimation to thermally stressful conditions. Specifically, these processes related to the cellular machinery involved in transcriptional and translational regulation, mitochondrial function, oxidative damage, and immunocompetence. Overarching transcript expression patterns indicated population-specific capacity for gene transcription as environmental temperatures increased, with divergent transcript response trajectories and a higher magnitude of transcriptional change apparent in the southern Winnipeg River population. Conserved and population-specific biological process responses illustrated that this transcriptional plasticity promoted whole-organism acclimation to elevated temperatures in developing lake sturgeon with potential for adaptive responses at the population scale. Ultimately, these processes enabled lake sturgeon to acclimate to environments with increasing temperature and highlight the physiological consequences once acclimatory capacity and sublethal thresholds have been exceeded.

4.5.1 Transcriptomic Responses to Increasing Temperatures

As temperatures increased from 16°C to 20°C, analysis of altered biological processes revealed shared functional mechanisms conserved between the lake sturgeon populations, as well as population-specific processes unique to southern Winnipeg River and northern Burntwood River sturgeon. When compared to other acclimatory and thermal stress experiments, the transcriptional plasticity observed in the Winnipeg River and Burntwood River lake sturgeon populations as temperatures increased from 16 to 20°C (21.6 and 10.5%, respectively) was on the upper end of transcriptional plasticity detected for other fish species (4-20%; Logan and Somero, 2011; Garvin et al., 2015), especially so for southern Winnipeg River population of lake sturgeon. Both populations upregulated biological processes involved in transcriptional and translational activity, which may lead to protein level changes that impact whole organism responses to thermal stress (Buccitelli and Selbach, 2020). Additionally, induced changes in transcript expression associated with biological processes involving cellular damage responses, mitochondrial function,

and pathogenic infection potentially highlight sublethal thresholds and physiological impacts of thermal stress as temperatures increase (Jeffries et al., 2018).

Population-specific responses indicated induction of protective mechanisms in the southern Winnipeg River population and further markers of cellular damage in northern Burntwood River sturgeon. Lake sturgeon from the Winnipeg River upregulated processes involved in protein cycling, maintenance of transcriptional mechanisms, membrane modification, and electron transport chain function, protecting themselves from the potential effects of oxidative species formed under elevated temperatures (Gomez-Gallardo et al., 2016). Uniquely, differential transcript expression showed that Winnipeg River sturgeon may have relocalized telomerases to cajal bodies, highly transcriptionally active sites within the nucleus (Ogg and Lamond, 2002; Cioce and Lamond, 2005). We speculate that telomere relocalization may have protected the cajal bodies from the effects of a changing environment and in the process, preserved transcriptional machinery as well as plasticity. In contrast, Burntwood River lake sturgeon upregulated transcripts related to damage responses at the DNA, mRNA, chromosome, cell, and anatomical structure levels, which may suggest widespread damage across many levels of biological organization, especially so in the context of decreased physiological performance for this northern population (Chapter 3). Transcripts associated with cellular respiration was also upregulated as well as mitochondrial respiratory chain complex III, which if not present in adequate amounts can lead to proton leakage during ATP production, mitochondrial dysregulation, and oxidative damage (Guo et al., 2013; Chandel et al., 2020). Previous work demonstrated a decreased metabolic and antioxidant capacity in Burntwood River sturgeon under thermally stressful conditions, relative to their Winnipeg River counterparts (Bugg et al., 2020). Thus, this combination of transcriptional and whole-organism physiological responses suggests that reduced transcriptional plasticity in northern Burntwood River sturgeon compromises ATP production, metabolic function, and cellular protective mechanisms, leading to increased damage, potentially from the production of reactive oxygen species (Filho, 2007; Portner and Lannig, 2009; Paital et al., 2016; Portner et al., 2017; Birnie-Gauvin et al., 2017). However, the southern Winnipeg River population exhibited enhanced transcriptional responsiveness, induction of protective mechanisms, as well as maintained antioxidant and metabolic capacity as temperatures increase from 16°C to 20°C (Bugg et al., 2020).

Further temperature increases from 20°C to 24°C elicited the suppression of processes promoting mRNA transcription and protein translation across both populations of sturgeon,

indicating strong thermal thresholds for plasticity to environmental change. Many of these processes, including production of cellular transcriptional and translational machinery, as well as the maintenance of mitochondrial function, were induced between 16°C and 20°C, but were ultimately decreased as temperatures increase from 20°C to 24°C. This responsive trajectory in the shape of an inverted U as temperatures increase, may be indicative of sublethal thresholds that are likely predictive of long-term impacts to organismal fitness (Jeffries et al., 2015; Jeffries et al., 2018).

Downregulation of population-specific processes as temperatures increased from 20°C to 24°C indicated that further temperature elevation induced unique effects across northern and southern Manitoba populations of lake sturgeon. Sturgeon from the Winnipeg River demonstrated decreases in cell cycling and damage responses, while sturgeon from the Burntwood River exhibit increased disruption of transcriptional, translational, protein, mitochondrial, and developmental processes. Overall, these downregulated processes demonstrated increased cellular stress and the inhibition of integral components of cellular control for both sturgeon populations, as well as increased disruption and specific aspects of mitochondrial dysregulation in Burntwood River sturgeon exposed to high temperature thermal stress.

4.5.2 Network Analysis

Predictive transcript network analysis of the aggregate most highly differentially expressed transcripts across all contrasts highlighted functional processes possibly significant for thermal acclimation. Key hub transcripts in these regulatory networks involved cell cycling, protein folding, transcriptional regulation and immune responsive mechanisms with functional enrichment as well as overarching REVIGO analysis indicating the recruitment of similar biological processes. Together, these highlighted transcripts and processes indicate the importance of these systems in the transcriptomic response to thermal acclimation across different populations of developing lake sturgeon.

Throughout temperature acclimation, hub transcripts and pathways (pathway: mitotic cell cycle; hub transcripts: c*dk2*, *src*, *rcc2*) critical in the process of cell cycling and proliferation were altered. These transcripts were largely involved with the G1/S phase transition, and associated DNA replication (Taylor and Shalloway 1996; Bacevic, et al., 2017; Guo et al., 2021) indicating processes disrupted by thermal stress. This G1 checkpoint specifically detects DNA damage prior

to entering the S phase of DNA synthesis, preventing proliferation of irreversible DNA damage in future cells (Bertoli et al., 2013). From 16°C to 20°C these processes were upregulated in both populations of sturgeon, however as temperatures increase to 24°C they decrease, suggesting a reduction in the regulation of cell cycling functions and potentially a proliferation of DNA damage as cells replicate in high temperature environments (Bartek et al., 2004).

Increased temperatures also modulated protein folding and cycling processes (pathways: protein folding, response to heat; hub transcripts: *hspe1*, *hsc70*, *vimp*, *cct4*) in the face of thermal stress and reactive oxygen species formation. These chaperones have wide ranging functions throughout the cell involving the maintenance of mitochondrial proteins under stress, cytosolic protein folding and ligation, degradation and recycling of misfolded proteins in the endoplasmic reticulum, and preservation of proteins in the cytoskeletal structure of the cell, respectively (Hartman et al., 1992; Place and Hoffman, 2001; Jackson and Hewitt 2016; Vallin and Grantham, 2019). Modulation of these chaperone proteins, paired with an observed decrease in metabolic rate for lake sturgeon in temperatures of 24°C from previous research (Bugg et al., 2020), suggest that under thermal stress cells transcriptionally modified heat shock protein production and decreased mitochondrial oxygen consumption to suppress formation of reactive oxygen species, adaptively stabilizing the existing cellular proteome to enhance long-term fitness and survival (Storey and Storey, 2011).

In addition to changes in protein folding mechanisms, differential expression of transcripts involved in transcriptional regulation (hub transcripts: *carm1* and *larp7*) further highlight methylation and alternative splicing processes. Histone level methylation (*carm1*) alters the structure and availability of DNA for transcription, promoting phenotypic modification at the DNA level, allowing lake sturgeon to rapidly respond to environmental change (Burggren and Crews, 2014). Additionally, post-transcriptional modifications by pre-mRNA splicing (*larp7*; Frilander and Barboric, 2020) can provide enhanced transcriptional plasticity, with the potential for enhanced adaptation to environmental change (Steward et al., 2022). Together, these two processes enhance the ability of lake sturgeon to modify the production of mRNAs prior to translation, and likely promote phenotypic plasticity towards increasing temperatures.

Finally, elevated temperatures altered immune system processes (pathways: adaptive immune response, inflammatory response, immune system processes; hub transcripts: *traf6, prkcbb, egr-1, egr2b, insig1*), as sublethal thresholds were breached. Both populations of sturgeon

additionally had increased transcriptional markers associated with viral infection, suggesting immune compromise and increased susceptibility to pathogens as temperatures increased, and a sublethal threshold of 20°C for immunocompetence. As the above hub genes are key innate and adaptive immune regulators (Lim et al., 2015; Choi, 2005; Wei et al., 2017; Miao et al., 2017; Zhang et al., 2019; Trizzino et al., 2021), modification of their transcription impacts many immune processes throughout their regulatory network. Endemic viral pathogens likely infect both studied populations of lake sturgeon, other populations throughout the lake sturgeon range, and, research suggests, sturgeon species throughout North America (Drennan et al., 2006; Clouthier et al., 2013; Clouthier et al., 2020; Mugetti et al., 2020; Stilwell et al., 2022). Thus, future increases in temperature will not only have direct effects on the lake sturgeon's thermally responsive mechanisms, but likely indirect effects on pathogen tolerance and survival as combining stressors mount which may extend to other species of sturgeons as well (Dittmar et al., 2014; Alfonso et al., 2020).

4.5.3 Mitochondrial Limits under Thermal Stress

Based on transcriptional evidence, mitochondrial function is likely inhibited across both populations of lake sturgeon as temperatures increased to 24°C. Many mitochondrial processes were upregulated in response to initial thermal stress at 20°C but declined under these higher temperatures. Transcriptional markers of dysfunction and resulting damages were apparent at lower temperatures in northern Burntwood River sturgeon and were more extreme when compared to southern Winnipeg River sturgeon. Aside from the direct effects of decreased ATP production and increased reactive oxygen species damage, a decline in mitochondrial function also has implications for stress signaling, viral immune response, and formation of heme as well as iron cluster assemblies required for oxygen transport and many of the above-mentioned cellular processes (Tovar et al., 2003; Ajioka et al., 2006; Lin et al., 2006; Qin et al., 2010; Karnkowska et al., 2016; Hernansanz-Agustin et al., 2020; Braymer et al., 2021). The impairment of these widespread mitochondrial processes in developing lake sturgeon likely inhibited the function of the mitochondria itself, related cellular systems, and ultimately gill function, with the potential for population-specific thresholds impacting whole-organism fitness to changes in environmental temperatures (Iftikar and Hickey, 2013; Jeffries et al., 2018).

4.5.4 Plasticity as an Adaptive Trait

All thermally responsive molecular pathways first rely on the ability of lake sturgeon to transcriptionally respond to environmental change. Without the necessary translational machinery in place, mRNA abundance changes cannot modify the proteome or phenotype (Vornanen et al., 2005; Smith et al., 2013; Akbarzadeh et al., 2018). Thus, as thermal thresholds are reached and lake sturgeon face a reduction in the ability to transcribe mRNA's, produce ribosomes, and maintain mitochondria, those consequences likely negatively impact the organism's ability to respond to changes in their environment with impaired and diminished mRNA, protein, and energy production.

Increasing temperatures induced post-transcriptional modifications (alternative splicing and gene silencing via miRNA) in both populations of lake sturgeon, potentially enhancing translational plasticity. Conserved responses of alternative splicing and gene silencing via miRNA suggest that these mechanisms are part of the inducible stress response of sturgeons, similar to other studied fishes, and likely play a role in environmental acclimation (Somero, 2018; Healy & Schulte, 2019; Tan et al., 2019; Shiina and Shimizu, 2020; Verta and Jacobs, 2021; Thorstensen et al., 2022; Steward et al., 2022). However, these processes were ultimately downregulated as temperatures increased to 24˚C, indicating thermal thresholds on post-translational as well as transcriptional responsiveness. The conservation of these mechanisms across both populations of lake sturgeon, as temperatures increase and under stressful conditions in various other species of fish, highlight the importance of post-transcriptional plasticity across the evolution of fish lineages.

As temperatures surpass thermal thresholds, and the effectiveness of some transcriptional and translational mechanisms are diminished, overall physiological plasticity is likely reduced. Fishes' susceptibility to the effects of additional stressors are thereby likely increased by compromising their adaptive ability to respond at multiple levels of biological organization (Smith et al., 2013; Brennan et al., 2022). As temperatures increased, transcription modifying processes were more highly preserved in Winnipeg River sturgeon that historically experience higher temperature thermal environments in the wild. As these populations are genetically distinct, experience different thermal environments, and have been geographically separated throughout recent history (McDougall et al., 2017; Bugg et al., 2020), the preservation of these transcriptional mechanisms may themselves be an adaptive mechanism, enhancing the southern Winnipeg River population's ability to respond to environmental change. Enhanced transcriptional plasticity likely

results in the observed increases in growth, metabolism, thermal tolerance, and survival of the southern Winnipeg River population under thermally stressful conditions, when compared to their Burntwood River counterparts (Bugg et al., 2020).

4.5.5 Ecological Relevance

In the current study, temperatures of 20°C and above limited the transcriptional plasticity of developing lake sturgeon, which would ultimately diminish their plasticity to other stressors. As temperatures continue to increase in the wild, the combined effects of thermal, oxidative, and pathogen related stressors will likely limit the survival of lake sturgeon populations within Manitoba, Canada, with increased severity for northern subarctic populations throughout the province (Bugg et al., 2020). In recent years, population designation units have been reclassified, grouping lake sturgeon inhabiting northern regions with those from several other populations in the province to form a larger designation unit (COSEWIC 2006; COSEWIC 2017). However, new evidence suggesting large discrepancies in transcriptomic, physiological, and likely genetic differences between populations, as well as their natural thermal environments and tolerance thresholds (McDougall et al., 2017; Bugg et al., 2020; Bugg et al., 2021b) indicate that they are differentially susceptible to increasing temperatures and require distinct management strategies for conservation.

Currently, temperatures in the Winnipeg River exceed 20°C for approximately 50 days per year, while this temperature threshold is projected to be exceeded during June, July, and August in the Burntwood River in the coming decades (Manitoba Hydro, 2015; Vincent et al., 2015; Zhang et al., 2019; Bugg et al., 2020). These seasonal increases in temperature occur when newly hatched sturgeon are in their first few months of life, which are the same life stages examined in the current study and in the recent thermal tolerance research (Bugg et al., 2020; Bugg et al., 2021b). During these early developmental stages, lake sturgeon from both populations use a variety of pre- and post-transcriptional mechanisms to modify their acclimatory capacity as temperatures increase to 20°C, but these mechanisms are largely downregulated by 24°C when many sturgeon are not able to survive chronically elevated temperatures. Additionally, endemic viral pathogens are persistent in both northern and southern populations of lake sturgeon throughout the province, with higher abundance and probability of detection in younger fish (Clouthier et al., 2013; Clouthier et al., 2020), and as temperatures increase. With the present experimental evidence, we suggest that if

river temperatures continue to increase as projected (Manitoba Hydro, 2015) and there are not sufficient thermal refugia, these physiological limitations will likely impede future lake sturgeon recruitment, especially in the most endangered northern populations. Therefore, increasing temperatures may influence recruitment, movement patterns, and distributions of lake sturgeon within Manitoba, as sturgeon shift to areas of thermal refuge to preserve energy and persist in the face of environmental change (Moore et al., 2020; Robertson et al., 2021). However, even with sufficient refugia, fish do not always travel to habitat with increased suitability due to life history constraints (Sutton et al., 2007; White et al., 2019). If lake sturgeon are unable to redistribute themselves in areas of thermal refuge, evidence suggests that the impacts of increasing environmental temperature may be more severe, impact population-level selection processes and survival based on observed population-specific whole-organism physiological responses as well as transcriptional plasticity to elevated temperatures.

4.6 Data Accessibility

The data is available in supplementary files and the raw sequencing reads are available at the National Center for Biotechnology Information Sequence Read Archive with the accession number PRJNA815828. The Trinity assembly and Trinotate annotation file are available via figshare (https://doi.org/10.6084/m9.figshare.19209753.v1).

4.7 Acknowledgements

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Credit Author Statement

W.B., W.G.A. and K.J. conceived and designed the experiments. W.B., W.G.A. and K.J. collected gametes from wild spawning lake sturgeon, while W.B. reared juveniles and collected samples. W.S.B., M.J.T., and K.E.M., conducted bioinformatic analysis of transcriptional data. W.B., M.J.T., K.E.M., W.G.A. and K.J. wrote, reviewed and edited the manuscript. W.G.A. and K.J. acquired funding and aided in supervision throughout the experimentation and review processes.

Chapter 4 demonstrated transcriptome-wide response in the gill of developing lake sturgeon following acclimation to elevated temperatures, with population-specific and acclimation-specific effects, lower thermal thresholds in the northern BWR population indicated by increased damage responses, relative to southern WR sturgeon at 20ºC, and sub-lethal thresholds on transcriptional plasticity for both populations as temperatures increased to 24ºC. Chapter 5 lays the foundation for investigating these effects as they apply to innate immunity, while Chapter 6 targets innate immune, fatty acid, and stress responses of developing lake sturgeon to an immune stressor following thermal acclimation.

Chapter 5. Survival and gene expression responses in immune challenged larval lake sturgeon (*Acipenser fulvescens*)

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5.1 Abstract

Larval lake sturgeon, *Acipenser fulvescens*, reared in hatcheries for stock enhancement of wild populations may be susceptible to early opportunistic bacterial infection. Thus, we examined survival and whole-body mRNA transcript abundance of both stress- (*StAR, GR1,* and *HSP70*) and immune-related (*MyD88* and *IL-1β*) genes in 30 days post fertilization larval lake sturgeon following immune challenge with lipopolysaccharides (LPS). Larval sturgeon were exposed to 0, 25, 50, 100, 150, and 200 $\mu g.ml^{-1}$ LPS and sampled after 30 min, 4 h, and 48 h, with exposure concentrations and sampling timepoints chosen based on previous experimental exposures in other fish species. Mortality was zero in 0 and 25 $\mu g.ml^{-1}$ LPS; 37.5% in 50 $\mu g.ml^{-1}$ LPS and 100% in the higher concentrations. Expression of *MyD88* and *StAR* mRNA were positively correlated and increased with time in the 50 $\mu g.ml^{-1}$ LPS treatment. There was an influence of both treatment and time on *IL-1β* mRNA, with expression 10-fold higher than controls after 4 h. Expression of *HSP70* mRNA was suppressed within 30 min of 50 $\mu g.ml^{-1}$ LPS exposure and remained so throughout the time course. Correlated mRNA transcript abundance of *GR1* with *MyD88, StAR* and *IL-1β* suggests a potential relationship between the innate immune and stress responses of larval Lake Sturgeon during this early developmental stage. Data presented suggest that larval Lake sturgeon largely responded with predicted changes in gene expression of immune related and stress response genes following an LPS challenge. This study provides a foundation for future research examining the effects of hatchery and naturally occurring stressors on the immune responses of larval lake sturgeon.

5.2 Introduction

Common opportunistic bacteria can infect many diverse fishes in aquaculture facilities (Declercq et al., 2013; Xu et al., 2019). Research describing the impacts of these common aquaculture pathogens has largely focused on commercially valuable food fishes (Wagner et al., 2011; Chistiakov et al., 2007), with less emphasis on species of conservation concern, specifically the conservation aquaculture of sturgeons.

Bacterial pathogens can infect cultured sturgeons resulting in widespread mortality (Li et al., 2008; Karatas et al., 2010; Zhang et al., 2011; Xu et al., 2015; Jiang et al., 2018). In Manitoba, Canada, lake sturgeon *Acipenser fulvescens*, are cultured in hatcheries yearly and released as part of ongoing stock enhancement programs (McDougall et al., 2014). As it has been demonstrated that some sturgeon species are slow to fully develop their immune systems (Gradil et al., 2014a; Gradil et al., 2014b), larval lake sturgeon may rely on innate immune responses during early developmental stages, which could leave them vulnerable to pathogenic infection and may in part explain high mortalities often observed during early rearing in this species. While free-flowing riverine environments typically have low levels of bacterial endotoxins (usually < 1 $\mu g.ml^{-1}$; Rapala et al., 2002; Wichmann et al., 2004), hatchery environments may compound the effects of pathogenic bacteria with high densities of vulnerable eggs and larvae, accumulation of waste by-products, lower flow rates than may be expected in natural water ways, and the accumulation of biofilms on hatchery surfaces or sturgeon eggs themselves, which could collectively lead to increased endotoxin concentrations (Papadopoulou and Wiklund, 2018; Rios-Castillo et al., 2018). Under hatchery conditions, increases in the presence of bacterial pathogens such as *Flavobacterium sp.,* have been associated with higher rates of lake sturgeon embryonic mortality (Fujimoto et al., 2012). Additionally, as larval sturgeon hatch, they may be exposed to *Flavobacterium*, *Aeromonas*, and *Pseudomonas spp.*, that have colonized their egg surfaces and could induce mortality in early development (Fujimoto et al., 2018).

The cell walls of gram-negative bacteria contain endotoxic components that can illicit an innate immune and stress response in hosts (Swain et al., 2008; Xiang et al., 2008; Novoa et al., 2009). Lipopolysaccharides (LPS) are bacterial endotoxins that have been used to study the immune and stress responses of variety of fish species across taxa, including sturgeons (Balm et al., 1995; Dalmo et al., 2000; Xie, et al., 2006; Haukenes et al., 2008; Xiang et al., 2008; Novoa et al., 2009). Cellular responses to gram-negative bacteria and LPS begin with Toll-like receptors

(TLRs), which can help differentiate between and activate specific immune responses against a wide variety of pathogens (Pietretti and Wiegertjes 2013; Pasman and Kasper 2017). Recently, RNA sequencing experiments in three species of sturgeon from Asia; a hybrid species (*Huso dauricus* x *Acipenser schrenckii*); amur sturgeon, *Acipenser schrenckii*, and dabry's sturgeon, *Acipenser dabryanus,* reported transcriptional responses to bacterial pathogens with a variety of TLRs including TLR 4, likely upregulated due to its responsiveness to bacterial LPS (Park and Lee, 2014). These receptors rely largely on intracellular signaling conducted by myeloid differentiation primary response 88 (MyD88) dependent signaling pathways and activation of nuclear factor kappa-light-chain-enhancer of activated B cell (NF-kB) to induce a transcriptional innate immune response (Zhu et al., 2013; Jiang et al., 2018; Li et al., 2018; Lou et al., 2018). The NF-kB and related transcription initiation factors reaching the nucleus of the cell can induce the production of pro-inflammatory cytokines such as interleukin 1 beta (IL-1β) (West and Heagy, 2002; Xiang et al., 2008; Novoa et al., 2009; Zhu et al., 2013). As circulating levels of IL-1β and other cytokines increase, the pituitary is stimulated to release α-MSH (alpha-melanocyte-stimulating hormone) and N-Ac-β-endorphin, leading to physiological and behavioral changes in bacteria infected fish (Dantzer, 2006; Metz et al., 2006).

Increased circulating levels of IL-1β also promote the release of corticotropin-releasing factor from the hypothalamus of vertebrates, stimulating increased release of adrenocorticotropin (ACTH) from the pituitary and ultimately the production of cortisol during infection (Shintani et al., 1995; Turnbull and Rivier, 1999). Thus, the hypothalamic-pituitary-internal (HPI) axis is responsive to infection, which results in increases in cortisol production and changes in the immune response (Maule and Schreck, 1991; Pijanowski et al., 2015). The rate limiting step in the production of cortisol is regulated by steroidogenic acute regulatory protein (StAR), which is responsible for the transportation of cholesterol from the outer to the inner mitochondrial membrane inside the interrennal cells of the head kidney where cortisol is synthesized (Stocco et al., 2005). Once synthesized and released into circulation in fish, cortisol can then bind to glucocorticoid receptors (GR) resulting in further physiological changes involved in development, cellular signaling, and the immune response (Wendelaar Bonga, 1997; Castillo et al., 2008; Pijanowski et al., 2015). However, studies investigating the effects of LPS on the HPI axis, glucocorticoid activation, and cortisol production in pallid sturgeon, *Scaphirhynchus albus* were inconclusive (Huakenes et al., 2008).

Finally, heat shock protein 70 (HSP70) is well known for its role as a chaperone with critical functions in protein folding, transport, and assembly (Lindquist, 1992; Liberek et al., 2008; Roberts et al., 2010) and in the cellular response to thermal stress (Logan and Somero, 2011; Tomanek and Somero, 1999). However, HSP70 also plays important roles in the cellular response to pathogens (Srivastava, 2002; Zhang et al., 2011). Specifically, HSP70 functions acutely to induce the inflammatory responses of the innate immune system, aids in extracellular antigen presentation (Wang et al., 2006; Lou et al., 2018), and is involved in the early stages of the immune response to bacterial infection (Elibol-Flemming et al., 2009).

The goal of this study was to use the mRNA transcript abundance of innate immune and endocrine glucocorticoid responsive genes to investigate components of the molecular responses of 30 days post fertilization (DPF) larval lake sturgeon following exposure to LPS. We used LPS from *Pseudomonas aeruginosa*, an opportunistic gram-negative bacterium implicated as one of the most common bacterial infections among freshwater fish under culture conditions (Kitao et al., 1993; Thomas et al., 2014). We hypothesized that LPS would initiate a bacterial immune and glucocorticoid response in larval lake sturgeon that would be detectable by changes in the mRNA transcript abundance of genes critical in the response to bacterial infection. Thus, assayed genes (*MyD88, IL-1β, StAR, GR1,* and *HSP70*) were chosen based on their roles in the activation of the innate immune and glucocorticoid stress responses. Globally, threatened and endangered sturgeons face an array of stressors which disrupt physiological equilibria and may result in susceptibility to pathogens (Marketon and Glaser, 2008; Tort, 2011). Thus, by studying the cellular responses of immunologically vulnerable larval lake sturgeon to LPS in a laboratory setting, we can develop a framework to better understand the impacts of bacterial pathogens in hatcheries and natural environments, where there are compounding effects determining an individual's survival (Moe et al., 2012).

5.3 Methods

5.3.1 Lake Sturgeon Husbandry

In May 2019, gametes were collected from wild-caught female and male lake sturgeon downstream from the Pointe du Bois generating station on the Winnipeg River, Manitoba, Canada (50º17′52′′N, 95º32′51′′W). Gametes were transferred to the University of Manitoba, Winnipeg, Manitoba, for fertilization. Eggs from a single female were fertilized using 0.1 ml of

diluted sperm. After 1 min of fertilization, embryos were washed three times with dechlorinated water and then immediately allowed to adhere to a mesh egg mat at approximately 500 embryos per egg mat (25.4 cm L x 24.1 cm W) with circulation of fast flowing (~120 L min^{-1}), oxygenated, water at 12^{o}C until hatch following methods from Earhart et al. (2020b).

Post hatch, larvae were transferred to two 9 L flow-through aquaria with aeration and bio-balls as substrate. Temperature was maintained at 12^{o}C until 13 DPF, after which temperature was increased at 1^{o}C day^{-1} until 16^{o}C. Freshly hatched artemia (Artemia International LLC; Texas, USA), were provided as a starting diet at 19 DPF, prior to yolk-sac absorption, after which tank substrate was removed over a 7-day period (Earhart et al., 2020a). Lake sturgeon were fed to satiation three times daily on a diet of artemia until 30 DPF when experimental trials began. All animals in this study were reared and sampled under guidelines established by the Canadian Council for Animal Care and approved by the Animal Care Committee at the University of Manitoba under Protocol #F15-007.

5.3.2 LPS Trials

The LPS trials conducted on larval lake sturgeon were based on previously published protocols (Dalmo et al., 2000; Novoa et al., 2009). Briefly, larvae were exposed to concentrations of 0, 25, 50, 100, 150, or 200 μg.ml^{-1} of LPS from *Pseudomonas aeruginosa* (Sigma-Aldrich, St. Louis, Mo, USA) for 48 h. Groups of 10 larval lake sturgeon, five rearing $aquaria^{-1}$, were transferred to one of two replicate treatment tanks containing 400 ml of gently aerated aquarium water for each treatment. After a 1 h period, to reduce the effects of acute handling stress, each tank was dosed once with the designated concentration of LPS diluted with ultrapure water to a total volume of 8 ml for each of the treatments. Larval lake sturgeon were then monitored for mortality every 15 min for the first 8 h, and then at least every 3 h for the following 40 h. Three larval lake sturgeon were sampled from each tank for a total of n=6 $treatment^{-1}$ sampled at 30 min and 4 h, and all remaining larvae were sampled at the final 48 h timepoint (n = 5-8). Larval lake sturgeon were euthanized by immersion in a 250 mg.L^{-1} solution of MS-222 buffered with an equal volume of sodium bicarbonate. The whole-body was then preserved in RNA*later* (Thermo Fisher Scientific, Waltham, USA), held in 4^{o}C for 24 h and stored at -80^{o}C until RNA extraction and mRNA transcript quantification. Trials used 30 DPF larval lake sturgeon with a mean length of 23.9 ± 1.1 mm, body mass of 416.6 ± 32.5 mg, and

Fulton's condition factor (Fulton, 1911; calculated as K = ((mass/length^3)*100)) of 0.31 ± 0.031 (n = 30 for all measurements, mean +/- SD).

5.3.3 RNA Extraction, cDNA synthesis, and qPCR

Total RNA was extracted from larval lake sturgeon from the 0, 25, and 50 µg.ml^{-1} LPS treatment groups using a PureLink RNA mini Kit (Invitrogen; Ambion Life Technologies) following the manufacturer's instructions. Whole-body larval lake sturgeon were homogenized in 500 µl of lysis buffer, for 10 min at 50 Hz using a TissueLyser II (Qiagen, Germantown, MD, USA). Total RNA purity and concentration for all samples was assessed using a Nanodrop One (Thermo Fischer Scientific) and gel electrophoresis was used to evaluate RNA integrity. After extraction, RNA samples were stored at -80ºC. A qScript cDNA synthesis kit (Quantbio; Beverly, Massachusetts) was then used, following the manufactures instructions, to synthesize cDNA from 1 µg of DNAse treated total RNA. The synthesis was conducted using a SimpliAmp Thermal Cycler (ThermoFisher; Waltham, Massachusetts) with cycling conditions of 1 cycle of 22ºC for 5 min, 1 cycle of 42ºC for 30 min, and 1 cycle of 85ºC for 5 min and a hold at 4ºC. Following synthesis, cDNA samples were stored at -20ºC.

Real-time quantitative polymerase chain reactions (RT-qPCR) for each gene of interest (*MyD88, IL-1β, StAR, GR1, HSP70*; Table 5.1) and the reference gene (*RPS6*) were conducted using 5 µl of Bio-Rad SsoAdvanced Universal SYBR Green Supermix (Bio-Rad; Hercules, California), 0.1 to 0.05 µl of 100 µM primers, 2 µl of cDNA per sample, and nuclease-free water adjusted for each assay to bring the total volume of each well to 10 µl. Primer sequences for *GAPDH*, *RPL7*, *RPS6*, *β-actin* and *EFA1-α* were tested for stability in mRNA transcript abundance across treatments and time points, but only *RPS6* remained stable in its expression and thus was used as the reference gene for mRNA quantification. For all assays, each well contained 0.025 µl forward and 0.025 µl reverse primer, except *RPS6* which used 0.05 µl forward and 0.05 µl reverse primer well^{-1}. The cDNA for all assays, except *StAR*, was diluted 1:10 with nuclease-free water post-synthesis and prior to RT-qPCR assays. For the *StAR* assay, 2 µl of undiluted cDNA was included. Primers for *MyD88* and *IL-1β* were designed from an annotated white sturgeon liver transcriptome (Doering et al., 2016). The sequences were aligned against publicly available transcripts using NCBI BLAST (Johnson et al., 2008) and shared highly conserved regions with the Chinese sturgeon *Acipenser sinensis* (*MyD88*; Sequence ID:

HM776034.1; 98% identity), Japanese sturgeon *Acipenser schrenckii* (*MyD88*; Sequence ID: KU238084.1; 97% identity), and dabry's sturgeon (*IL-1β*; Sequence ID: MF818014.1; 96% identity). Primers for *StAR*, *GR1*, and *RPS6* were from Earhart et al. (2020a). The *HSP70* primers were from Bugg et al. (2020). The expression of all genes of interest were normalized to the expression of the reference gene *RPS6*. Expression was then analyzed after applying the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001). Expression of *MyD88*, *StAR*, *GR1*, and *HSP70* were then normalized to the 30 min 0 $mg.L^{-1}$ LPS control treatment group. Expression of *IL-1β* was normalized to the 4 h, 0 $mg.L^{-1}$ control treatment group to aid visualization of the data, as there was relatively high levels of mRNA transcript abundance of this gene across all treatments at the 30 min time point.

Table 5.1 Primer sequences used to investigate changes in the mRNA transcript abundance of genes in the innate immune and stress response of larval lake sturgeon, *Acipenser fulvescens*, following exposure bacterial lipopolysaccharides. *RPS6* used as a reference gene. Efficiencies are listed as a percentage.

Gene	Forward	Reverse	Efficiency (%)
MyD88	CACATGCGTCACTGTCAAGG	AGCATCACCAGCGAACTCAT	96.0
IL-1β	CACCAGCGAGATCTTTGACTT	GCTCATCTTGCGTTCTCTTCT	90.8
StAR	CCGAGCAAAAAGGCTTCA	TTGGGCCGAAGAACAATACAG	99.7
GR1	TTTGCAGCTCCCACATGTAA	TCTTGTGTGCTCGGATGAAG	104.9
HSP70	CGTTCACTCGGACTTTAACTTTAATTT	AACTGTCCTAAAGAACTGCCTTATCC	99.5
RPS6	CTGGCTGGATTCTGATTTGGATG	ATCTGATTATGCCAAGCTGCT	96.9

5.3.4 Statistical Analysis

The mRNA transcript abundance data were analyzed using two-factor ANOVA's with LPS treatment dosage and time as well as their interactions included in the model as fixed effects. Time to mortality was analyzed using a one-factor ANOVA across LPS treatment dosages. Shapiro-Wilk's and Levene's tests were used to assess normality of data and homogeneity of variance. Normality was also visually inspected using fitted residual plots. If assumptions of either normality or homogeneity were violated, a ranked, log, or square root transformation was applied to the data set. Following the ANOVAs, post-hoc tests were performed with Tukey's honestly significant difference tests from the "multcomp" package (Hothorn et al., 2008). Spearman's correlation was used to investigate the relationship between all genes of interest as expression data did not fit a normal distribution. Only significant correlations are reported. All statistical analyses were performed using R 3.6.1 with a significance level (α) of 0.05.

5.4 Results

5.4.1 Mortality

There was no mortality in the 0 and 25 $\mu g.ml^{-1}$ LPS treatment groups; 37.5% in 50 $\mu g.ml^{-1}$ LPS and 100% in higher concentrations. Lake sturgeon at 100 $\mu g.ml^{-1}$ and 200 $\mu g.ml^{-1}$ LPS all succumbed within 1 h post-exposure, while mortalities in the 50 $\mu g.ml^{-1}$ treatment occurred within 4 h of exposure ($P < 0.05$).

5.4.2 Gene Expression

There was an overall effect of time on the mRNA transcript abundance of *MyD88* (Figure 5.1A; $P < 0.005$; $F = 7.3$) with increased expression in the 50 $\mu g.ml^{-1}$ LPS treatment group throughout the 48 h LPS trial exposure. In the 50 $\mu g.ml^{-1}$ LPS treatment, *MyD88* mRNA transcript abundance was 5.2 and 3.4-fold higher after 48 h than at 30 min and 4 h, respectively (Figure 5.1.1A; $P < 0.05$). The mRNA transcript abundance of *MyD88* and *StAR* as well as *MyD88* and *GR1* were positively correlated ($P < 0.0001$, $\rho = 0.66$, $n = 52$, Figure 2A; $P < 0.005$; $\rho = 0.40$; $n = 52$, Figure 5.1.1B, respectively).

There was an interaction between treatment and time on the mRNA transcript abundance of *IL-1β* (Figure 5.1B; $P < 0.0001$; $F = 5.4$). In the 25 $\mu g.ml^{-1}$ LPS treatment after 4 h, *IL-1β* mRNA was depressed 4.2 fold, but increased 10-fold in the 50 $\mu g.ml^{-1}$ treatment relative to controls at the same time point ($P < 0.005$). The expression of *IL-1β* mRNA also changed over time across LPS treatments. At 4 h, both control and 25 $\mu g.ml^{-1}$ treated sturgeon decreased mRNA transcript abundance of *IL-1β* relative to their 30 min timepoints, 22.7 and 23.7- fold, respectively ($P < 0.001$). In contrast, the 50 $\mu g.ml^{-1}$ treatment showed no decline in *IL-1β* mRNA transcript abundance between 30 min and 4 h. Between 4 h and 48 h, the 25 $\mu g.ml^{-1}$ treatment increased expression 5.2-fold, while the 50 $\mu g.ml^{-1}$ treatment decreased expression 13-fold ($P < 0.005$).

There was an effect of time on the mRNA transcript abundance of *StAR* (Figure 5.1C; $P < 0.05$; $F = 3.4$) with increased expression in the 50 $\mu g.ml^{-1}$ LPS treatment group throughout the 48 h LPS trial exposure. In the 50 $\mu g.ml^{-1}$ treatment after 48 h, *StAR* mRNA transcript abundance was elevated 2.4-fold compared control 0 $\mu g.ml^{-1}$ treatment at the same timepoint, and 4.4-fold relative to the 50 $\mu g.ml^{-1}$ treatment at 30 min ($P < 0.05$). The expression of *GR1* mRNA was not significantly affected by treatment or time (Figure 5.1D). The mRNA transcript abundance of *StAR* and *GR1* were positively correlated ($P < 0.0001$; $\rho = 0.65$; $n = 53$, Figure 2D).

There was an effect of treatment on the mRNA transcript abundance of *HSP70* (Figure 5.1E; $P < 0.0001$; $F = 18.8$), and within 30 min of LPS exposure, larval lake sturgeon in the 50 $\mu g.ml^{-1}$ treatment had 11.4-fold lower expression than sturgeon in the 0 $\mu g.ml^{-1}$ control treatment (Figure 5.1E; $P < 0.05$). After 4 h, sturgeon in the 50 $\mu g.ml^{-1}$ treatment had 6-fold lower expression than controls ($P < 0.05$). In this same treatment, *HSP70* mRNA transcript abundance displayed an increasing trend throughout the time course, with increased expression at 4 h and 48 h timepoints relative to 30 min, however this was not statistically significant ($P = 0.06$). At the 48 h timepoint and 50 $\mu g.ml^{-1}$ treatment, *HSP70* mRNA transcript abundance was 3.3-fold lower than 25 $\mu g.ml^{-1}$ levels ($P < 0.05$), but not significantly different from controls.

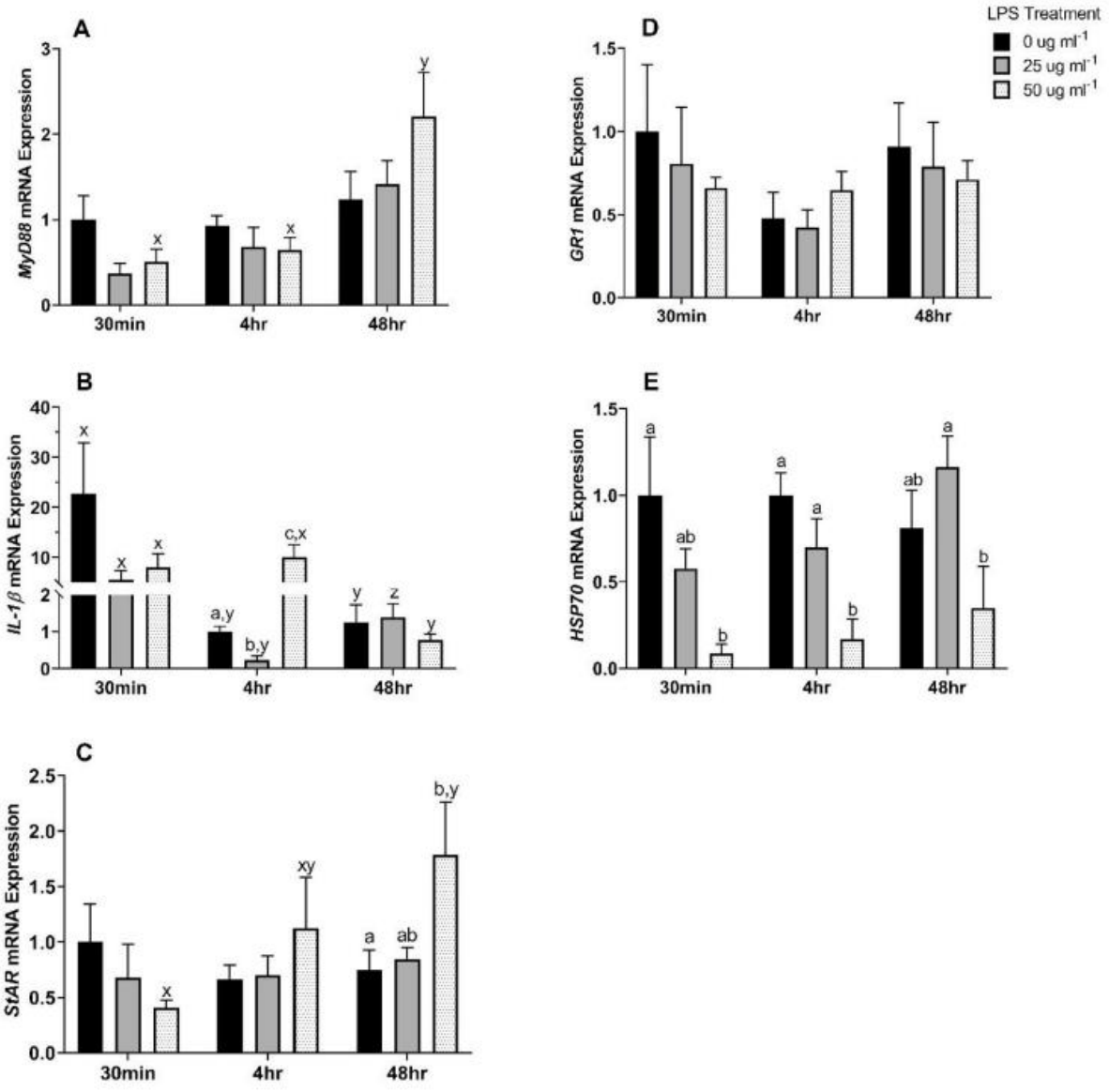

Figure 5.1 Whole-body mRNA transcript abundance of *MyD88*, *IL-1β*, *StAR*, *GR1*, and *HSP70* of larval lake sturgeon, *Acipenser fulvescens*, exposed to 0, 25, and 50 μg.ml^{-1} lipopolysaccharides over a timeseries of 30 min, 4 hr, and 48 hr.

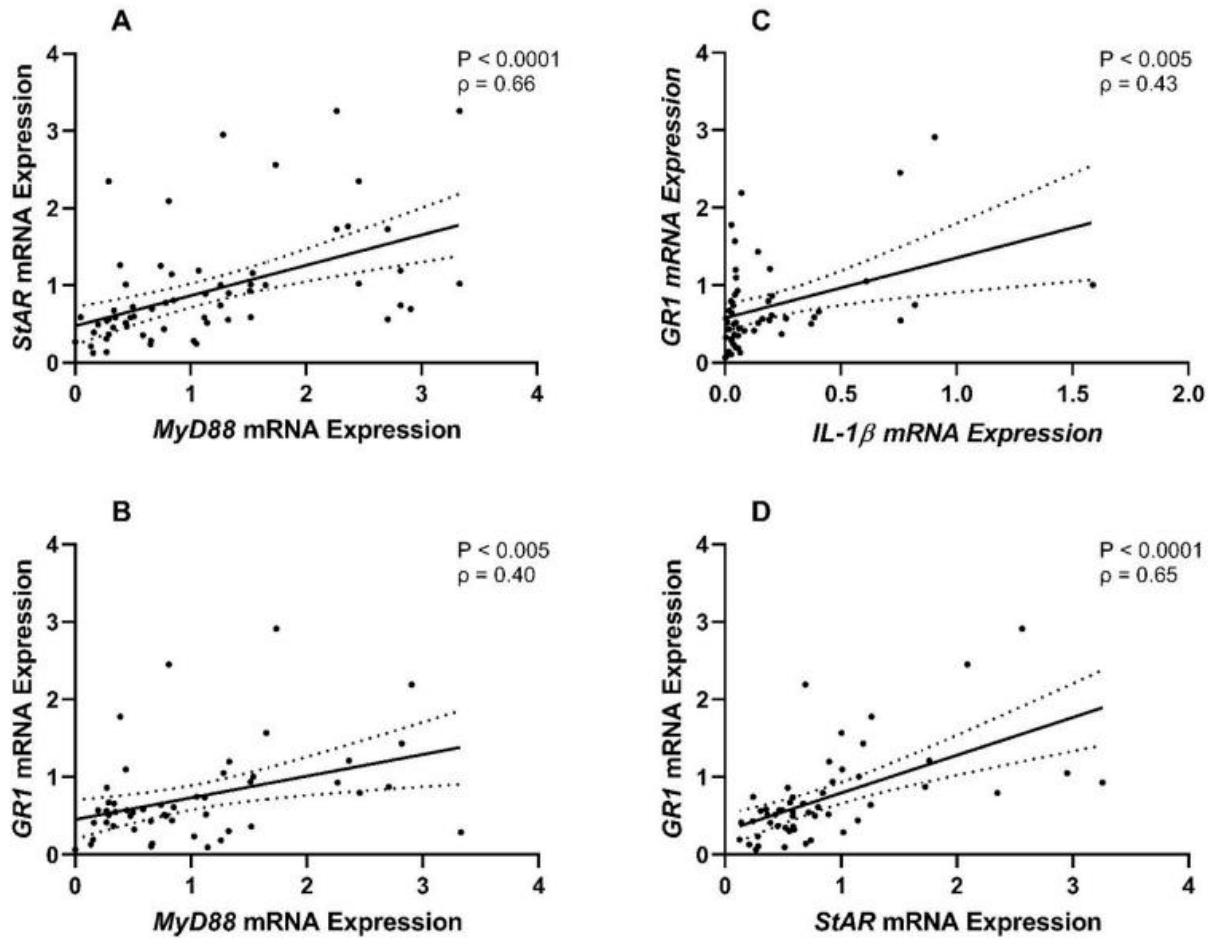

Figure 5.2 Whole-body mRNA transcript abundance relationships of innate immune and stress responsive genes (A) MyD88 and StAR, B) MyD88 and GR1, C) IL-1β and GR1) of larval lake sturgeon, *Acipenser fulvescens*, following exposure to 0, 25, and 50 μg.ml^{-1} lipopolysaccharides over a timeseries of 30 min, 4 h, and 48 h. Statistical significance was determined by Spearman's correlation. The solid line throughout the graph represents the best fit straight line surrounded by dotted lines representing the 95% confidence interval. Dots represent individual larval lake sturgeon (n = 52–53).

5.5 Discussion

In the current study, we investigated the molecular responses of lake sturgeon to an exposure to LPS and demonstrated changes in the mRNA transcript abundance of multiple genes involved in the immune and glucocorticoid stress response. Mortality was observed in LPS treatments higher than 25 $\mu g.ml^{-1}$, consistent with previous experimental findings from environmental exposure trials in larvae of Atlantic halibut, *Hippoglossus hippoglossus L*., and zebrafish, *Danio rerio* (Dalmo et al., 2000; Novoa et al., 2009). In the 50 $\mu g.ml^{-1}$ LPS treatment, mRNA transcript abundance of *MyD88* increased over time, suggesting increased intracellular signaling through TLR related pathways and a physiological innate immune response (Arras and Alper, 2013). In zebrafish, MyD88 was identified as a key transcriptional initiator required for recognition and stimulation of early stage innate immune responses to bacterial pathogens *E. tarda*, *Salmonella typhimurium*, *A. hydrophila* as well as LPS (Van der Vaart et al., 2013; Srivastava et al., 2017). Similarly, in other teleosts, *MyD88* mRNA transcript abundance is induced following exposure to bacterial stimuli in Japanese flounder *Paralichthys olivaceus*, rock bream *Oplegnathus fasciatus*, Indian major carp *Cirrhinus mrigala*, roughskin sculpin *Trachidermus fasciatus,* and silvery pomfret *Pampus argenteus* (Takano et al., 2006; Whang et al., 2011; Basu et al,, 2012; Ciahong et al., 2015; Gao et al., 2017). In dabry's sturgeon, hybrid sturgeon, and amur sturgeon, either mRNA transcript abundance of *MyD88* or the *TLR's* (*2*,*4*,*5*, and *8*) were upregulated in response to bacterial infection (Luo et al., 2018; Wang et al., 2018; Jiang et al., 2018). Thus, it is likely that the MyD88 signaling pathway is a common cellular response to bacterial pathogens in many Actinopterygian clades.

Expression of *IL-1β* mRNA was elevated in the 50 $\mu g.ml^{-1}$ LPS treatment (which had 37.5% mortality) compared to controls at 4 h, but depressed in the 25 $\mu g.ml^{-1}$ treatment. Similarly, experiments in zebrafish demonstrate LPS concentrations which do not induce mortality suppress *IL-1β* mRNA transcript abundance, however, in treatments where there is some mortality, like the 50 $\mu g.ml^{-1}$ LPS treatment for larval lake sturgeon, mRNA transcript abundance of *IL-1β* mRNA is increased (Novoa et al., 2009). In zebrafish, *IL-1β* mRNA transcript abundance is activated directly through MyD88 dependent pathways in response to bacterial pathogens and LPS (Watzke et al., 2007; van der Vaart et al., 2013). However, expression of *IL-1β* can be induced independent of MyD88 through the PI3Kδ-AKT-GSK3 pathway or TLR4 related MyD88 independent signaling

(Carpintero et al., 2013; Agarwal, 2016). In other sturgeons exposed to *Aeromonas hydrophila, IL-1β, MyD88,* and *TLR4* mRNA transcript abundance was induced, further indicating the importance of these genes in the sturgeon response to bacterial pathogens (Jiang et al., 2018; Lou et al., 2018). In the current study, within 30 min of the trial beginning, elevated mRNA levels of *IL-1β* across all treatments potentially indicate a prolonged upregulation of this gene potentially caused by acute handling stress and transfer to experimental units, as observed in common carp, *Cyprinus carpio* (Metz et al., 2006). Thus, future experiments should use a longer pre-trial period post-transfer, to reduce the potentially confounding effects of handling stress.

The activation of the HPI axis in response to LPS has been documented in numerous teleosts including rainbow trout *O. mykiss*, plaice *Pleuronectes platessa* L., Nile tilapia *O. mossambicus*, and yellow perch *Perca flavescens* (Wedemeyer, 1969; White and Fletcher 1985; Balm et al., 1993; Balm et al., 1995; Haukenes and Barton 2004; Pepels et al., 2004; Haukenes et al., 2008). However, when pallid sturgeon were injected with similar concentrations of LPS to teleosts, a significant cortisol response was not induced (Haukenes et al., 2008). In larval lake sturgeon, increases in *StAR* mRNA transcript abundance have been observed to coincide with endogenous cortisol production and activation of the HPI axis (Earhart et al., 2020a). In the current study, *StAR* mRNA transcript abundance increased over time in the 50 $\mu g.ml^{-1}$ LPS treatment and was elevated relative to controls after 48 h. This increased *StAR* expression is likely indicative of either ACTH or α-MSH activation at this early developmental stage (Metz et al., 2006), leading to cortisol production. Although *StAR* expression could be influenced by the production of other steroids (e.g. Huffman et al., 2012), cortisol is the dominant steroid regulating homeostasis at approximately 30 DPF in larval sturgeon (Simontacchi et al., 2009). Thus, positively correlated expression of *StAR* and *MyD88* in addition to the relationship of *StAR*, *MyD88*, and *IL-1β* to *GR1* across timepoints and LPS concentrations demonstrates that larval lake sturgeon responses to LPS exposure at the molecular level link both the innate immune and glucocorticoid stress responses.

Glucocorticoids and their receptors play a major role in the stress and immune response, regulating apoptosis, cellular differentiation, migration of immunocytes as well as inhibition of NF-kB and pro-inflammatory cytokine production such as IL-1β with expression regulated by cortisol synthesis (Maule and Schreck 1991; Basu et al., 2003; Stolte et al., 2009; Pijanowski et al., 2015). However, LPS treatment induced *GR1* mRNA transcript abundance in common carp, with subsequent increases observed in pro-inflammatory cytokine production (Stolte et al., 2008a,

Stolte et al., 2008b). In the current study, no change in *GR1* mRNA transcript abundance was observed in larval lake sturgeon following exposure to LPS, perhaps influenced by the use of full body samples, which may mask tissue-specific alterations in GR expression. Tissue-specific evaluation of *GR1* mRNA transcript abundance and longer overall trial exposures may better demonstrate differential regulation in larval lake sturgeon exposed to LPS.

Heat shock protein 70 aids in antigen presentation, promoting macrophage activation in teleosts by signaling through TLR4 and NF-kB and MyD88 pathways resulting in pro-inflammatory cytokine production (Vega et al., 2008; Stolte et al., 2009; Roberts et al., 2010). Experimentally, increases in HSP70 protein or mRNA have been observed in the livers and head kidneys of teleosts following infection and exposure to *Vibrio anguillarum*, *Edwardsi ictaluri*, and LPS, in rainbow trout *O. mykiss*, channel catfish *Ictalurus punctatus*, and grass carp *Ctenopharyngodon idella*, respectively (Ackerman and Iwama, 2001; Elibol-Flemming et al., 2009; Zhang et al., 2011). In contrast, larval lake sturgeon in the current study exhibited a whole-body downregulation of *HSP70* mRNA following exposure to LPS. As *HSP70* can be responsive to many types of stressors, its expression may have been influenced by handling as observed in the *IL-1β* response. However, in yellow perch and haddock *Melanogrammus aeglefinus,* exposed to handling stress, neither acute nor long term changes in HSP70 mRNA transcript abundance were observed (Afonso et al., 2008; Eissa et al., 2017), suggesting that exposure to LPS was the transcriptionally suppressive influence in larval lake sturgeon. Additionally, recent studies have suggested that *HSP70* mRNA transcript abundance is downregulated in the spleen of hybrid sturgeon and the liver of amur sturgeon following infection from *A. hydrophila* and *Yersinia ruckeri*, respectively (Jiang et al., 2018; Li et al., 2018). However, in the head kidney of dabry's sturgeon, *HSP70* mRNA transcript abundance increased following infection from *Aeromonas hydrophila* (Lou et al., 2018). As the head kidney is the primary site of cortisol synthesis, a bacterial induced glucocorticoid response may influence the expression of *HSP70* specifically in this organ, with its role in the GR signaling complex (Kirschke et al., 2014). In the current study, the mRNA transcript abundance of *HSP70* was not correlated with that of other studied genes involved in the innate immune and glucocorticoid stress responses, suggesting a separate mechanism for its suppression in high concentrations of LPS, possibly related to increased NF-kB activation (Chen et al., 2006), although this was not specifically addressed in this study. These discrepancies between sturgeon studies may be the result of species, tissue, or pathogen specific *HSP70* mRNA

transcript abundance patterns, but indicate different responses when compared to teleosts.

5.6 Conclusion

This study has laid the foundation for future investigation of larval lake sturgeon immunity and demonstrates relationships between the larval lake sturgeon innate immune and HPI axis related responses to LPS. Thus, future studies should examine the combined effects of environmentally relevant stressors and LPS on the mRNA transcript abundance of additional pro-inflammatory genes (e.g., TNF-α, IL-6, IL-8) and physiological responses of lake sturgeon to further our understanding of their ability to respond to bacterial infection.

5.7 Acknowledgements

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Credit Author Statement

W.B., W.G.A. and K.J. conceived and designed the experiments. W.B., W.G.A. and K.J. collected gametes from wild spawning lake sturgeon, while W.B. reared juveniles, conducted lipopolysaccharide exposure trials, and collected samples. W.S.B. conducted gene expression and data analysis. W.B., W.G.A. and K.J. wrote, reviewed and edited the manuscript. W.G.A. and K.J. acquired funding and aided in supervision throughout the experimentation and review processes.

Chapter 5 demonstrated a relationship between innate immune and stress responsive transcriptional processes following immune stimulation. Chapter 6 builds upon these findings to further explore the effects of thermal acclimation on sub-lethal and lethal thresholds in the innate immune, stress, and fatty acid responses of developing lake sturgeon.

Chapter 6. Elevated temperatures dampen innate immune responses of developing lake sturgeon (*Acipenser fulvescens*)

6.1 Abstract

Chronic temperature stress may leave freshwater fishes vulnerable to opportunistic pathogens, particularly during early life stages. Lake sturgeon, *Acipenser fulvescens*, populations within the northern expanse of their range in Manitoba, Canada, may be susceptible to high temperature thermal stress and pathogenic infection. We acclimated developing lake sturgeon for 21 days to two ecologically relevant temperatures (16 and 20˚C). Both acclimation treatments were exposed to 0, 30, and 60 $\mu g.ml^{-1}$ bacterial lipopolysaccharides (endotoxins), as an immune stimulus, for 48 h, with samples taken 4 and 48 h during trial exposures and following a 7-day recovery period. We then measured whole body transcriptional (mRNA) responses involved in the innate immune, general stress, and fatty acid responses following acute exposure to bacterial endotoxins. Data detailing the responses involved in the above physiological processes revealed that while overall expression levels were higher at 20˚C under control conditions, during bacterial stimulus 16˚C reared lake sturgeon produced a more robust response with higher induced expression of the above responses than their 20˚C acclimated counterparts. Additional whole-animal performance metrics (critical thermal maximum and metabolic rate) demonstrated acclimation-specific responses indicating compromised metabolic capacity following the initiation of immune related responses. Our study showed that acclimation to 20˚C during early development impaired the activation of molecular pathways involved in the immune, stress, and fatty acid responses of lake sturgeon, highlighting the effects of chronic thermal stress on transcriptional activation and recruitment of downstream biological processes in this endangered species.

6.2 Introduction

Globally, mounting environmental pressures of temperature change, flow alteration, and extreme weather events lead to decreased productivity and imperil the fish populations that inhabit freshwater systems (Milly et al., 2008; Reid et al., 2019; Dudgeon, 2019). These environmental changes are often accompanied by alterations in pathogen populations and abundance, leaving freshwater species to overcome the effects of compounding stressors, which may leave them more vulnerable to their combined effects (Marcos-Lopez et al., 2010; Paull and Johnson, 2011; Dittmar et al., 2013; Miller et al., 2014). As temperatures increase, some fishes show plasticity to changes in their thermal environment, but this phenotypic flexibility to temperature alteration may make them more susceptible to the effects of pathogens, and ultimately shape evolutionary trajectories, through additional pathogen related selection pressures, for these species and populations as mortality occurs (Dittmar et al., 2013; Schade et al., 2014; Chapter 4). However, until recently little research has focused on the effects of these combined stressors under laboratory conditions, especially in species of conservation concern (Bugg et al., 2021a). Conservation hatcheries rear fish for release to bolster and supplement wild populations, however, to ensure their success, an understanding of abiotic and biotic factors effecting survival outcomes is key. As host-pathogen-environment interactions determine survival outcomes for fish following release in wild environments (Jeffries et al., 2014; Teffer et al., 2022), it is necessary to study the interactions of these combining stressors in fishes of conservation concern, which are released by hatcheries to bolster natural populations worldwide.

Early development is a critical period in the life cycles of most freshwater fishes as it is a transitionary period accompanied by a shifting of biological function, organ development, narrowing energy reserves, and is often accompanied by high levels of mortality (Sifa and Mathias, 1987; Wieser, 1991; Rombough, 1994). During this period, fish may have limited swimming ability and often must persist within their environmental circumstances, making them vulnerable to increases in temperature (Kopf et al., 2014; Brandt et al., 2021). Additionally, many fishes do not have the organ and cellular systems in place to recruit an adaptive immune response at hatch and in early development, leaving them reliant on their innate immune capabilities until later in life (Chantanachookhin et al., 1991; Petrie-Hanson and Ainsworth, 2001; Magnadottir et al., 2006; Reyes-Lopez et al., 2018). Thus, limited mobility, immune capabilities, and exposure to thermal stress may compromise the defenses of developing larval

freshwater species and leave them susceptible to the effects of opportunistic viral, bacterial, and fungal pathogens which are pervasive throughout freshwater ecosystems and can lead to widespread mortality. Ultimately, in this critical period of early development, the ability to functionally sustain molecular responses against multiple stressors likely has a role in determining interindividual survival and may influence population level outcomes (Dittmar et al., 2013).

Innate immunity relies on two specific mechanisms in order to respond to pathogens: the reception of the immune stimulus, resulting in an intracellular immune stimulating cascade, and the following transcriptional initiation, activating the production of immune related compounds, known as the immune response. The ability of the innate immune system to detect pathogens largely relies on pattern recognition receptors, such as toll-like receptor 4 (TLR4) which detects gram negative bacteria by their outer lipopolysaccharide (LPS) structure (Magnadottir, 2006; Amarante-Mendes et al., 2018). Once activated TLR4 can respond through two different activation pathways, either through myeloid differentiation primary response 88 (MyD88)-dependent activation or through the use of toll-like receptor adaptor molecule 1 (TICAM-1) signaling, both of which induce the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) to translocate into the nucleus, bind to DNA, and initiate the transcription of mRNAs coding for pro inflammatory cytokines and other immune compounds (Figure 6.1A; Vaure and Liu, 2014; Srivastava et al., 2017; Amarante-Mendes et al., 2018). Post transcription, these mRNAs are then translated into proteins by ribosomes attached to the endoplasmic reticulum prior to further processing in the golgi apparatus where these proteins are packed into transport vesicles, ultimately to be secreted outside of the cellular membrane (Blank et al., 2014). Following secretion, immune responsive proteins such as cytokines (TNFa, IL-1β and IL-8; Turnbull and Rivier, 1999), antibodies (IgM; Lobo PI, 2016; Yu et al., 2020), complement activators (C3; Holland and Lambris, 2002), and enzymes (Lysozyme-C; Saurabh and Sahoo, 2008) initiate a variety of immunomodulatory activities to suppress and destroy invading bacteria. Additionally, the activation of innate immune responses can further induce changes in long chain fatty acids, either through mitochondrial β oxidation limited by the mitochondrial transporter carnitine palmitoyltransferase I (CPT1; Coccia et al., 2014; Norambuena et al., 2015) or the formation of immune precursors through cytokine-induced, phospholipase A2 mediated, cleavage (PLA2; Okamura et al., 2021; Nguyen et al.,

2022). However, many of these innate immune mechanisms may be ultimately compromised by the effects of chronic thermal stress, associated allostatic load, and the resultant energic costs related to elevation of the stress response (Schreck, 2010; Schreck and Tort, 2016).

Under environmental conditions with limited allostatic load (physiological stress caused by abiotic or biotic environmental factors, Samaras et al., 2018), infection, the triggering of the immune response, and the detection circulating cytokines by the hypothalamus can stimulate an increased release of adrenocorticotropin (ACTH) from the pituitary and ultimately an increase in the production of cortisol responsive to infection (Shintani et al., 1995; Turnbull and Rivier, 1999; Figure 6.1B). This synthesis of cortisol is rate limited by the transport of cholesterol into the mitochondrial membrane of the interrenal cells of the head kidney, a key step regulated by steroidogenic acute regulatory protein (StAR; Stocco et al., 2005). Once cortisol enters circulation and cells, cytosolic glucocorticoid receptors (GR1) can then bind to it with the help of molecular chaperones (HSP70 and HSP90) (Hutchinson et al., 1994; Bamberger et al., 1996; Bekhbat et al., 2017). This complex can then translocate into the nucleus and bind to GRE sites on DNA itself, transcriptionally regulating further cellular signaling mechanisms and physiological responses critical in the maintenance of homeostasis and organismal survival (Marchi and van Eeden, 2021). However, if stressful conditions are present before pathogenic encounter, sub-lethal thermal thresholds may be breached, increasing GR and circulating glucocorticoid levels instead leading to suppression of the nuclear translocation of the immune stimulating transcription factor NF-kB (Bekhbat et al., 2017; Jeffries et al., 2018), resulting in inactivation of the cytokine response, immunocompromise, and subsequent infection by opportunistic pathogens (O’ Connor et al., 2000; Stolte et al., 2008a; Tort et al., 2011; Irwin and Cole, 2011; Rebl et al., 2020; Alfonso et al., 2020; Aversa-Marnai et al., 2021).

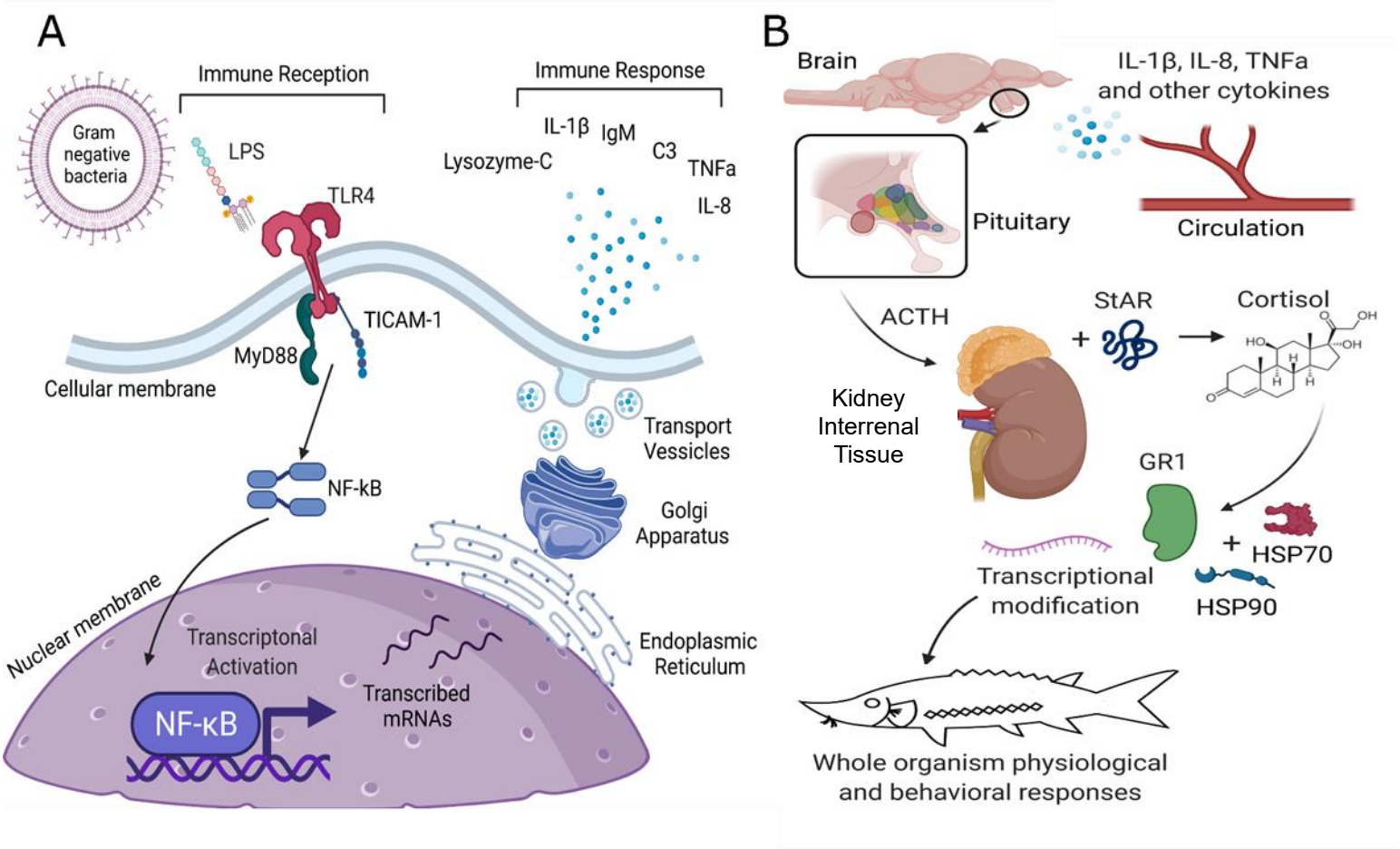

Figure 6.1 Diagrams outlining hypothetical A) immune reception, transcriptional activation, and immune responses to bacterial lipopolysaccharides as well as stimulation of the B) stress response via circulating cytokines in developing lake sturgeon, *Acipenser fulvescens*. Note, StAR and cortisol production take place in the interrenal tissue of the head kidney of fish, pictured here is a kidney for graphical demonstration. The authors would like to thank Miri E. Seo for her willingness to share her artwork and have it included in Figure B.

Chronic thermal stress thus has the potential to elevate the allostatic load, influence the production of glucocorticoids, and disrupt the innate immune responses of organisms, preventing them from adequately protecting themselves from encounters with environmentally pervasive opportunistic pathogens (McEwen and Wingfeld, 2003; Roth et al., 2010; Seppaelae & Jokela, 2011; Dittmar et al., 2013). Specifically, increases in temperatures can alter development, energy utilization, ion balance, metabolism, numerous cellular processes and the transcriptional mechanisms that underly these physiological mechanisms (Dengiz Balta et al., 2017; Jonsson and Jonsson, 2019; Rebl et al., 2020; Chapter 4). This phenomenon has been practically observed in a hatchery context where the additive effects of thermal stress in addition to hatchery related stressors influence the immune capacity of rainbow trout, *Oncorhynchus mykiss* (Rebl et al., 2020), acute thermal stress modulating immune activity in black sea trout, *Salmo labrax* (Dengiz Balta et al., 2017), and chronic elevated temperatures modulating immune processes in lake sturgeon, *Acipenser fulvescens* (Chapter 4). The effects of these compounding environmental influences can interrupt transcriptional processes and result in long lasting impacts at the whole organism level (Heijtz et al., 2011; Galindo-Villegas et al., 2012; Jonsson and Jonsson, 2019).

Sturgeons are some of the most endangered species on the planet and are reared in hatcheries for subsequent release to enhance wild populations. However, recent research has indicated that in both wild and hatchery environments, sturgeons are susceptible to a variety of fungal, viral, and bacterial pathogens (Li et al., 2017; Coleman et al., 2018, Soto et al., 2022, Mugetti et al., 2020, Clouthier et al., 2013, Clouther et al., 2020, Fujimoto, 2012, Fujimoto et al., 2018, Jiang et al., 2018; Luo et al., 2018; Santi et al., 2019 Aversa-marnia et al., 2021; Stilwell et al., 2022; Chapter 4). Additionally, sturgeons are likely more susceptible to these opportunistic pathogenic infections in early development as their adaptive immune development is slower than that of many other fish species (Gradil et al., 2014a; Gradil et al., 2014b). Thus, evaluation of these vulnerabilities at a key point in early development may provide insights into the ability of sturgeons to respond to pathogens using their innate immune capabilities while coping with the effects of thermal stress.

In Manitoba, Canada, river temperatures where endangered populations of lake sturgeon live are projected to increase by 2.1 – 3.4°C by 2050 (Manitoba Hydro, 2015). These anticipated increases in temperature would elevate temperatures above the 20°C, impacting the metabolic,

morphological, and transcriptional physiology of lake sturgeon (Chapters 3 and 4), from May to October for many rivers throughout the province, during a period of the year when newly hatched lake sturgeon are developing (Bugg et al., 2020; Bugg et al., 2021b). While previous research has demonstrated that prolonged periods of temperatures 20˚C and above can be thermally stressful and have negative physiological consequences for developing lake sturgeon (Bugg et al., 2020, Chapter 4), there has been little evaluation of the immune capabilities of this species in early development or the immune capabilities of sturgeons under thermal stress (Bugg et al., 2021a).

The goal of this study was to investigate the ability of thermally stressed developing lake sturgeon to produce an innate immune response when stimulated with bacterial LPS. Using a transcriptional profiling approach (Jeffrey et al., 2020), we targeted genes in the innate immune, stress, and fatty acid pathways responsive to bacterial infection. I then paired this strategy with standard physiological metrics of hepatosomatic index (HSI), critical thermal maximum (CT_{max}), and metabolic rates, to assess the immune responses and physiology of developing lake sturgeon exposed to bacterial LPS following acclimation to environmentally relevant temperatures of 16 and 20˚C. I hypothesized that heightened stress responses associated with acclimation to increased temperatures would elevate glucocorticoid and receptor levels, impairing the transcriptional activation of the innate immune system following exposure to bacterial LPS. Thus, I predicted that the immune responses of 16˚C acclimated sturgeon would be more highly induced than that of their 20˚C counterparts, following exposure to the bacterial stimuli. Additionally, I predicted that due to the metabolic demand required to acclimate to 20˚C and initiate an immune response, we would observe a decrease in HSI in 20˚C acclimated sturgeon, compared to their 16˚C acclimated counterparts, as well as alteration of CT_{max} and metabolic rates in both acclimation treatments following exposure to LPS, when compared to unexposed sturgeon.

6.3 Methods

6.3.1 Lake Sturgeon Husbandry

In May of 2021, gametes from wild-caught female and male lake sturgeon were harvested from individuals at the Pointe du Bois Generating Station on the Winnipeg River, Manitoba,

Canada (50° 17′ 52″ N, 95° 32′ 51″ W). Once collected, eggs and sperm were transported to the University of Manitoba animal holding facility in Winnipeg, Canada for fertilization. Upon arrival, fertilization occurred immediately using the eggs from three females and 0.1 ml of diluted sperm from six males to produce three maternal families. After 1 min of fertilization, embryos were washed three times with dechlorinated water and immediately placed on mesh egg mats. Once adhered to the egg mats, flow of well oxygenated water at 12ºC was applied over the eggs using submersible fans until hatch (Earhart et al., 2020b).

Post hatch, larvae were transferred to three 9 L flow-flow through aquaria with aeration and bio-balls as substrate, with each maternal family initially reared in a separate tank. Starting at 13 days post fertilization (DPF) the rearing temperature was increased 1ºC day^{-1} until 16°C. Once temperatures reached 16°C on 17 DPF, developing sturgeon were transferred into 5 replicate 9 L flow through aquaria. Each aquaria was stocked with 100 sturgeon from each maternal family (n = 300 sturgeon $replicate\ tank^{-1}$ to reduce stress and mortality by decreasing stocking density; Aidos et al., 2020). Beginning at 19 DPF freshly hatched artemia (Artemia International LLC; Texas, USA), were provided as a starting diet before yolk sack absorption had been completed, while tank substrate was removed over a 7 day period (Earhart et al., 2020a). Lake sturgeon were fed freshly hatched artemia to satiation three times daily until LPS challenges began.

Next, at 33 DPF preparations for acclimation began, reducing the stocking density of sturgeon further. For each acclimation temperature (16 and 20°C), 4 replicate 9 L tanks were stocked with approximately 170 sturgeon each, evenly distributed from the 5 replicate initial rearing tanks for a total of 680 sturgeon $acclimation\ temperature^{-1}$. Sturgeon remained in these tanks until they were moved to the LPS trial experimental setup at 52 and 56 DPF for 20°C and 16°C treatments respectively. Throughout initial rearing and acclimation, mortality and rearing temperature was monitored at least twice daily. All animals in this study were reared and sampled under guidelines established by the Canadian Council for Animal Care and approved by the animal Care Committee at the University of Manitoba under protocol #F15-007.

6.3.2 Acclimation

Acclimation for the 20°C treatment began at 34 DPF, increasing the temperature 1°C day^{-1} until 20°C was reached at 37 DPF and this temperature was maintained until LPS trials began 21

days later (59 DPF; Figure 6.2), throughout the 48 h duration of trials (61 DPF), and during the 7 day recovery period (68 DPF). For sturgeon acclimated to 16°C, this temperature was maintained from 16 DPF, until the beginning of the LPS trials (63 DPF), throughout the 48 h trials (65 DPF), and during the 7 day recovery period (72 DPF). Acclimation and experimental trails were staggered by four days in an effort to make accumulated thermal units similar across both treatment groups (Accumulated thermal units at beginning of trials: 16°C = 950, 20°C = 980).

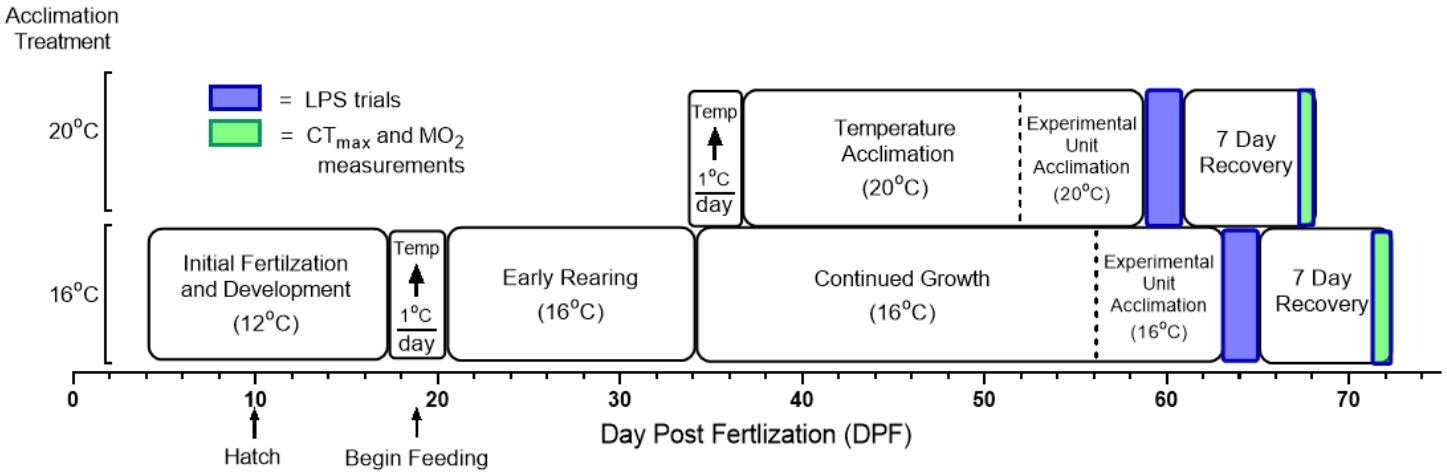

Figure 6.2 Experimental design and timeline for lake sturgeon, *Acipenser fulvescens*, fertilization, early rearing, acclimation, lipopolysaccharide exposures, and measurements of critical thermal maximum (CT_{max}) as well as metabolic rates following a 7 day recovery. All time periods are measured as days post fertilization (DPF).

In addition to temperature acclimation, developing lake sturgeon were also acclimated to the environment of the LPS trial experimental units for one week prior to experimentation (beginning at 56 and 52 DPF for 16 and 20°C treatments respectively), to avoid possible stress related effects of handling and transfer (Bugg et al., 2021a). Each LPS trial experimental unit was 30 cm long × 25 cm wide × 7 cm deep with drainage holes cut into the side at the 3.3 L volume mark to allow for water to flow out of the unit during acclimation. A total of approximately 75 sturgeon (selected evenly from the 4 acclimation tanks) were stocked into each of 6 experimental units for each acclimation temperature (16 and 20°C), totaling 12 experimental units, with duplication for each of the future LPS treatments (e.g. 2 replicate tanks for each concentration of 0, 30, and 60 $\mu g.ml^{-1}$ LPS for each temperature, n = 12 units). Throughout experimental unit acclimation developing sturgeon were provided with flow through water and aeration via an air stone. During the first 6 days of acclimation, sturgeon were fed artemia to satiation however they were fasted 24 h before the initation of LPS trials to reduce variability due to food consumption, and ammonia accumulation during the static bath exposures. Throughout both stages of acclimation and recovery post LPS trials, water temperature was recorded every 15 min by HOBO Water Temperature Pro v2 Data Loggers (Onset Computer Corporation; Bourne, MA, USA).

6.3.3 LPS Trials and Sampling

The LPS trials conducted on developing lake sturgeon were based on previously established protocols (Dalmo et al., 2000; Novoa et al., 2009; Bugg et al., 2021a). Developing lake sturgeon were exposed to concentrations of 0, 30, and 60 $\mu g.ml^{-1}$ of LPS from *Pseudomonas aeruginosa* (Sigma-Aldrich; St. Louis, Mo, USA) for 48 h. Prior to the beginning of the LPS trial 300 ml of water was removed from each experimental unit so that the total volume of the unit was 3 L. Each unit was then dosed once with the designated concentration of LPS diluted with ultrapure water to a total volume of 36 ml. Following introduction of LPS into the experimental units, each unit was monitored every 15 min for the first 8 h and then at least every 2 h for the following 40 h. As flow-through water was removed for the duration of the trials, ammonia was also measured every 6 h to monitor any potential accumulation, but never reached concentrations higher than 1 $mg.L^{-1}$ in any treatment. Throughout the duration of the trials, moribund fish were removed, recording their body mass (weighed to 0.0001 g), total length (measured to nearest 1 mm), and

liver wet mass (weighed to 0.0001 g). These measurements were then used to calculate both the Fulton's condition factor (K; Fulton, 1911) and hepatosomatic index (HSI; wet mass of the liver, W_{liver} divided by the wet mass of the body W_{body}) for each individual mortality as follows:

$$K = \frac{mass\ (g)}{total\ length\ (cm)^3}\ x\ 100 \qquad HSI = \frac{W_{liver}(g)}{W_{body}(g)}\ x\ 100$$

Following the conclusion of the 48 h LPS exposure, flow-through water was then returned to each experimental unit, expelling water containing LPS through drainage. Fish remained in these flow-through units for 7 days to recover from their LPS exposures. Throughout the experiment, developing sturgeon were sampled at 4 h and 48 h during LPS exposure as well as following 7 days of recovery post LPS exposure. At 4 h, 48 h and following 7 days of recovery, 5 developing lake sturgeon from each replicate experimental unit were euthanized by immersion in 250 mg L^{-1} solution of MS-222 buffered with an equal amount of sodium bicarbonate. Weights (to 0.0001g) and lengths (measured to the nearest 1 mm) were quickly taken from each fish. Whole body sturgeon were then placed in CryoELITE cryogenic vials (DWK Life Science; Milville, NJ, USA) and immediately flash frozen in liquid nitrogen, after which they were stored in -80°C until processing.

At the beginning of LPS trials, 10 sturgeon from each acclimation treatment were sampled directly from acclimation tanks, taking measurements of weight, length, and liver size as described above, as well as preservation of whole-body samples. These samples represent sturgeon that went through thermal acclimation but did not go through the experimental unit acclimation and will be henceforth referred to as negative controls. In contrast, sturgeon that went through both thermal and experimental unit acclimation, were exposed to 0 μg.ml^{-1} LPS at the beginning of LPS trials, and were sampled 4 h, 48 h and after a 7 day recover period, will be referred to as handling controls.

6.3.4 Post Sampling Processing

Whole body samples of sturgeon collected both prior to and during LPS trails were then homogenized so that they could be used to measure the gene expression from each individual sturgeon sampled. Each whole fish was individually homogenized using a pestle and mortar in liquid nitrogen. All homogenized samples were then returned to storage at -80°C until further analysis.

6.3.5 Primer Design

Primers were designed to target genes involved in the innate immune, stress, and fatty acid responses of lake sturgeon to the combined effects of elevated temperature and bacterial infection (Table 6.1). Many primers were used based on previously conducted lake sturgeon immune (*MyD88*, *IL-1β*; Bugg et al., 2021a), stress (*StAR*, *GR1*, *HSP70*, *HSP90a*; Bugg et al., 2020; Earhart et al., 2020a), and fatty acid-based research (*PLA2*, *CPT1*; Yoon et al., 2022). However, primers for other targets in immune responsive pathways and potential reference genes (*TLR4, TICAM-1, NF-kB, TNFa, IL-8, C3, Lysozyme-C, IgM, RPL13a, eEF1A1,* and *RPL4*) were designed from lake and white sturgeon, *Acipenser transmontanus,* tissue specific transcriptomes. All results from transcriptome searches were aligned against publicly available transcripts using NCBI BLAST (Johnson et al., 2008) with primers designed over conserved regions between the query and the search result(s). Original transcript sources, results from related species with highly conserved regions, percent identities and accession numbers for each publicly available transcript from NCBI BLAST results are listed below.

A lake sturgeon head kidney transcriptome was used to design primers for *TLR4*, *NF-kB*, *TNFa*, *IL-8*, and *IgM* (Thorstensen et al., *In prep*). Annotated lake sturgeon sequences for *TLR4* demonstrated conserved regions with 98.3 and 94.7% identity to the sterlet sturgeon, *Acipenser ruthenus,* and the American paddlefish *Polydon spathula* (Transcripts XM_034909094.1 and XM_041234367.1, respectively). Similarly, *NF-kB* and *TNFa* transcripts shared 98 and 98.5% identity, respectively, with published transcripts from the sterlet sturgeon (Transcripts XM_034013617.2 and XM_034909934.1, respectively). Lake sturgeon head kidney transcripts annotated as *IL-8* shared 94.8 and 94.5% identity to published transcripts from the Siberian sturgeon, *Acipenser baerii*, and the sterlet sturgeon, respectively (Transcripts MK140599.1 and XM_034035867.2, respectively). Finally, transcripts annotated as *IgM* shared conserved regions of 96.4, 95.2, 95, 95, and 93.4% identity to previously annotated transcripts from the Siberian sturgeon, beluga sturgeon, *Huso huso*, sterlet sturgeon, Russian sturgeon, *Acipenser gueldenstaedtii*, and Japanese sturgeon, *Acipenser schrenckii*, respectively (Transcripts KC734558.1, DQ257633.1, DQ257636.1, DQ257634.1 and DQ257635.1, respectively).

Primers for *C3* and *TICAM-1* were designed from an annotated and published white sturgeon, liver transcriptome (Doering et al., 2016) while primers for *Lysozyme-C* were designed from a lake sturgeon liver transcriptome (Thorstensen et al., *In prep*). Transcripts for both *C3* and

TICAM-1 shared conserved regions with 98% identity to previously annotated transcripts from the sterlet sturgeon (Transcripts XM_034016062.2 and XM_034911846.1, respectively). Transcripts for *Lysozyme-C* shared conserved regions with 98.6, 99.4 and 99.1% identity to previously annotated transcripts from the sterlet sturgeon, Chinese sturgeon, *Acipenser sinensis*, and Dabry's sturgeon, *Acipenser dabryanus*, respectively (Transcripts XM_034058580.2, MF280234.1, MF135537.1, respectively).

A lake sturgeon gill transcriptome was used to design primers for potential reference genes *RPL13a*, eEF*1A1*, and *RPL4* (Thorstensen et al., *In prep*; Bugg et al., *In prep*). Transcripts for *RPL13a* shared conserved regions with both sterlet sturgeon and the American paddlefish, 98.6 and 96% identity, respectively (Transcripts XM_034908381.1 and XM_041240517.1, respectively). Similarly, transcripts for *eEF1A1* shared conserved regions with the sterlet sturgeon, American paddlefish, and additionally Dabry's sturgeon, with identities of 96.4, 95.4, and 98.4%, respectively (Transcripts XM_034915679.1, XM_041240787.1 and MH790258.1, respectively). Finally, transcripts annotated as *RPL4* shared conserved regions with previously annotated transcripts from the Siberian sturgeon, sterlet sturgeon, and American paddlefish, with identities of 99.8, 99.4 and 97.2%, respectively (Transcripts MG722839.1, XM_034049385.2 and XM_041218712.1, respectively).

Table 6.1 Primer sequences for lake sturgeon, *Acipenser fulvescens*. Target genes (*TLR4* to *CPT1*) were selected based on their roles in innate immune, stress, and fatty acid responses to pathogenic infection. Reference genes (*RPL13a* to *RPL4*) were chosen as candidates based on their stability in other pathogen-based experiments, however in the current experiment were largely unstable under the compounding effects of both temperature and bacterial LPS. Efficiencies are listed as a percentage.

Gene	Forward	Reverse	Efficiency (%)
TLR4	AAGCTGACGGTTGTGGATAC	GCTGTGCCAAGTGACTGATA	103.4
MyD88	CACATGCGTCACTGTCAAGG	AGCATCACCAGCGAACTCAT	95.96
TICAM-1	GAAGCTCGCTAGAAGGACATAC	GAGAAGGATGCTCTGAGAAATGA	101.04
NF-kB	CAGAGCTTGCACTACAGCCT	TGGGTTCACTCAATGGCAGG	92.13
TNFa	AGGAGCGGTCTCTACTTCGT	TGTGCGACAGATATACGGGC	94.4
IL-8	CAGGCAGATCCAGAATGTAGAG	CCAGATTTCAAAGTGGCAATGA	100.9
IL-1β	CACCAGCGAGATCTTTGACTT	GCTCATCTTGCGTTCTCTTCT	99.9
C3	AGGGCTCTCTCATCCTTTACT	CAGACCCACTTCAAACTCCTT	91.03
Lysozyme-C	CTGCCAAACTGGGTGTGTCT	TGTTGTGGTTCACTGCCTGA	98.31
IgM	GGTGTTTCTCCTCTCGCCAT	GTCAGGCTAACTTCCCCGTT	97.7
StAR	CCGAGCAAAAAGGCTTCA	TTGGGCCGAAGAACAATACAG	92.1
GR1	TTTGCAGCTCCCACATGTAA	TCTTGTGTGCTCGGATGAAG	96.24
HSP70	CGTTCACTCGGACTTTAACTTTAATTT	AACTGTCCTAAAGAACTGCCTTATCC	98.7
HSP90a	GATCACACGAGCGGATTTGC	ATGTTGTGCTCTGTCCTGCG	92.9
PLA2	GCGGGCACAGTTAATACCCA	CCCTAACCCACAGTAGCAGC	96.9
CPT1	CAGAAGAAAGCTGGACAGAGAG	CATACGCTCATACTGGGAAGTG	92.3
RPL13a	TGAAGTACCTTGCGTTCCTG	TCTCACTGTCCTCCAGAAGAT	96.2
eEF1A1	TCAAGTATGCCTGGGTGTTG	GAGGGAGATGTCAATGGTGATG	97.3
RPL7	TGCTTAGGATTGCTGAGCCG	GATCTTTCCGTGACCCCGTT	100.2
RPS6	CTGGCTGGATTCTGATTTGGATG	ATCTGATTATGCCAAGCTGCT	98.7
β-actin	GAAGTCCAGGGCGACATAGC	TGAAGATCCTGACCGAGCGA	99.8
RPL4	CCGAGGGAGTCCTAAGCGAA	GGAGCCTTGAAGACAGCAGG	92.07

6.3.6 RNA extraction, cDNA synthesis and qPCR

Total RNA was extracted from the whole body homogenates of developing lake sturgeon from all treatment groups using RNeasy Plus Mini Prep Kits (Qiagen; Germantown, MD, USA) following the manufacturer's instructions. Whole body homogenates were additionally homogenized in 500 ul of lysis buffer for 5 min at 50 Hz using a TissueLyser II (Qiagen; Germantown, MD, USA). Total concentration, integrity, and purity of RNA for each sample were assessed using a Nanodrop One (Thermo Fisher Scientific) and gel electrophoresis, respectively. Synthesis of cDNA was conducted using a SuperScript IV First-Strand Synthesis System with ezDNase Enzyme (Quantbio; Beverly, MA, USA) from 500 ng of total RNA following the manufactures instructions. Genomic DNA was first removed using 1 µl of ezDNAse Enzyme prior to cDNA synthesis. Synthesis of cDNA was then performed using 1 µl of 50 ng.µl^{-1} random hexamers to anneal to the template RNA, followed by reverse transcription using 1 µl of SuperScript IV Reverse Transcriptase. Synthesis was conducted using a SimpliAmp Thermal Cycler (Thermo Fisher Scientific) with cycling conditions of one cycle of 23ºC for 10 min, one cycle of 55ºC for 10 min and one cycle of 88ºC for 10 min with a final hold at 4ºC.

Real-time quantitative polymerase chain reactions (RT-qPCR) for each gene of interest (*TLR4, MyD88, TICAM-1, NF-kB, TNFa, IL-8, IL-1β, C3, Lysozyme-C, IgM, StAR, GR1, HSP70, HSP90a, PLA2,* and *CPT1*) and potential reference genes (*RPL13a, eEF1A1, RPL7, RPS6, β-actin,* and *RPL4*) were conducted using 5 µl of PowerUp SYBR Green Master Mix (Applied Biosystems; Bedford, MA, USA), 0.1-0.025 µl of each forward and reverse 100 µM primers, 5-10 µl 1:10 nuclease free water diluted cDNA well^{-1} with additional nuclease free water adjusted for each assay to bring the total volume of each well to 12-16 µl based on the amount of cDNA included. Assays for potential reference genes *RPS6* and *RPL7* included 0.1 µl each of forward and reverse primer well^{-1}, while all other assays included 0.025 µl each of forward and reverse primers well^{-1}. All assays used 5 µl 1:10 diluted cDNA well^{-1} and had a total reaction volume of 12 µl except for the *StAR* assay which used 10 µl of 1:10 diluted cDNA well^{-1} and a total volume of 16 µl.

Potential reference genes (*RPL13a, eEF1A1, RPL7, RPS6, β-actin,* and *RPL4*) were tested for stability in expression across acclimation treatments, LPS concentrations, and timepoints, however only *RPS6* remained stable across these different criteria. Thus, NORMA-Gene, a robust method for qPCR normalization based on the expression of target genes (Heckmann et al., 2011), was used for normalization of mRNA transcript abundance, inputting the expression of all 22

assayed genes to produce the lowest theoretical variance in normalization (ibid). Post normalization, expression was then analyzed after applying the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001). Expression of all target genes was then normalized to the expression of the pre-trial 16°C (negative) control group.

6.3.7 Post-trial Metabolism and Thermal Tolerance

During the 7 day recovery from LPS trials, measurements of both the metabolic rates (resting and forced) and critical thermal maximum (CT_{max}) of sturgeon treatments surviving the trials were taken. Metabolic rates were measured on day 6 post-trial, while the CT_{max} of the same fish were measured the following day, day 7 post-trial.

Whole body metabolic rate ($\dot{M}O_2$) was measured by intermittent flow respirometry (Loligo Systems, Viborg, Denmark) following the previously established protocols with some modifications (Yoon et al., 2021). The respirometry system consisted of 16 borosilicate metabolic chambers with oxygen sensor spots (PreSens, Regensburg, Germany) on which fiber optic cables were situated in order to read oxygen saturation in percent air saturation at 1 Hz by Witrox 4 Oxygen Meter (Loligo Systems, Viborg, Denmark). The volumes of chamber and tubing were 44.60 ± 3.58 mL (mean ± S.D.) and 3.51 ± 0.90 mL, respectively. At each sampling point fish (n = 8 per treatment) were haphazardly taken from rearing tanks and chased for 15 min to induce maximum metabolic rate. Then, fish were immediately placed into chambers, and $\dot{M}O_2$ was measured for the next 6 h.

We used $\dot{M}O_2$ slopes with $r^2 \geq 0.9$ to ensure linearity in the oxygen consumption (Chabot et al. 2021). Before and after each experiment, $\dot{M}O_2$ was measured for 15 min without fish to assess background respiration, and background respiration data were used to linearly interpolate over each $\dot{M}O_2$ recording session (Rodgers et al. 2016). Then, all $\dot{M}O_2$ data were corrected by subtracting all corresponding background respiration data. Maximum metabolic rate was chosen as the highest $\dot{M}O_2$ during the first three measurements whereas routine metabolic rate was estimated by averaging $\dot{M}O_2$ of the last two hours, excluding the first four hours of acclimation. Because we measured routine metabolic rate, we chose to report the difference ($\Delta\dot{M}O_2$) and ratio (MMR/RMR) between routine and maximum metabolic rate as metabolic scope, both of which are analogous to absolute and factorial aerobic scope in the literature (Halsey et al., 2018).

After measurement of metabolic rates, critical thermal maximum (CT_{max}) was measured following previously established protocols with some modification (Bugg et al., 2020). At the end of metabolic rate measurements, the 8 sturgeon were transferred from metabolic chambers directly into the CT_{max} arena placing them into individually labeled experimental units (~200 ml of water volume and 9.5 cm long × 5 cm wide with mesh-screened sides to allow water to flow through each unit; Yusishen et al., 2020) with well oxygenated circulating water held at the respective acclimation temperature. An additional 16 fish which had not had their metabolic rates measured were added to each CT_{max} trial for a total of 24 fish per trial. The position of all fish in the experimental units in the CT_{max} arena was assigned via random number generator. Temperature in the recirculating water bath was heated at approximately 0.3°C min^{-1}, by a Isotemp recirculating heater (Fisher Scientific; Hampton, USA) until fish were unable to right themselves after a physical disturbance. When sturgeon were unable to right themselves, the final CT_{max} temperature was recorded, the fish was euthanized and mass and length measurements were recorded as previously described.

6.3.8 Statistical Analysis

Data taken prior to the beginning of LPS trails for negative control fish including, weight, length, condition factor, HSI were analyzed using a Welch Two Sample t-test comparing 16ºC acclimated lake sturgeon to their 20ºC acclimated counterparts.

Differences in mortality between acclimation treatments and LPS concentrations throughout trials were assessed using Cox proportional hazards models using the 'survival' and 'survminer' R packages (Kassambara et al., 2019; Therneau, 2015) with covariates of both acclimation treatment and LPS concentration included in the model. A pairwise comparison was then conducted to compare mortality across both concentrations and acclimation temperatures, using the 'pairwise_survdiff' function in the 'survminer' package as well as a Bonferroni correction to correct for the effects of multiple comparisons. Assumptions of the hazard model was evaluated using the 'cox.zph' function in the 'survival' package.

Data collected from each mortality (weight, total length, condition factor, HSI) were analyzed to determine if there was a relationship with time to mortality using a spearman's correlation to identify which physiological metrics were most indicative of time to survival in sturgeon exposed to LPS. Mortalities were only apparent in the 20˚C acclimation treatment

exposed to 60 μg.ml^{-1} LPS, thus these data are representative of mortality under these conditions (n = 142).

The mRNA transcript abundance of all target genes was analyzed using three-factor ANOVAs to investigate changes in gene expression across treatments, including acclimation treatment, LPS concentration, and time in the model as fixed effects. A subset of the total data, including negative control samples following acclimation, 48 h exposer to 30 μg.ml^{-1} LPS, and a 7 day recovery was then analyzed using two-factor ANOVAS with acclimation treatment and gene as fixed effects in the model, to focus on the sub-lethal tolerance thresholds of LPS exposure.

Principal component analyses were conducted using the 'factomineR' (Le et al., 2008) and 'factoextra' (Kassambara and Mundt, 2021) packages in R, including the subset data with negative control samples following acclimation, 48 h exposer to 30 μg.ml^{-1} LPS, and a 7 day recovery (n = 48). Contributions and vector directions of the variance in gene expression observed in principal component analysis were illustrated using a contributions plot and variable plot, with only target genes that exceeded the expected average contributed towards variance in the overall PCA included.

Semi-partial Spearman's correlation were used to investigate the relationship between the mRNA transcript abundance of each gene and that of other studied genes, as well as whole-body condition factor, cortisol level, and lysozyme enzyme activity, of sturgeon sampled during LPS trials, using the 'ppcor' package (Seongho, 2015). This analysis was used to control for the effect of both LPS concentration and time during calculation of spearman's correlation coefficients and was conducted individually for each acclimation temperature (16 and 20°C, n =80 and 61, respectively) to highlight the differences in expression relationships between the two acclimation treatments. The difference between the two values between acclimation treatments was then calculated (ρ16°C – ρ20°C) and is presented as delta. All values are reported as estimated Spearman's rho (ρ).

Metabolic rate and CT_{max} of developing lake sturgeon, during recovery from LPS exposure, were analyzed with two-factor ANOVA's including both LPS concentration and acclimation temperature as well as their interactions in the model as fixed effects. For all ANOVA's Shapiro-Wilk's and Levene's tests were used to assess normality of data and homogeneity of variance. Additionally, normality was visually inspected using fitted residual plots. If assumptions of either normality or homogeneity were violated, either a ranked, long, or square root transformation was

applied to the data set. Following evaluation of main and interactive effects, post-hoc tests were performed with Tukey's honestly significant difference tests from the 'multcomp' package (Hothorn et al., 2008). All statistical analyses were performed using R 4.0.0 (R Core Team, 2022), with a significance level (α) of 0.05.

6.4 Results

6.4.1 Pre-trial Physiology and Gene Expression

There was no significant difference in the weight, length, or condition of developing lake sturgeon from different acclimation treatments, however sturgeon acclimated to 20°C had a 47% reduction of their HSI, the relative weight of the liver to the body, when compared to sturgeon acclimated to 16°C ($P < 0.0001$). Figures detailing this physiological data can be found in Supplemental File A.6.1.

6.4.2 In-trial Mortality and Physiological Relationships

Physiological metrics of weight, length, and HSI demonstrated significant correlations with the time to mortality of sturgeon exposed to 60 $\mu g.ml^{-1}$ concentrations of LPS ($P < 0.005$) in which all sturgeon perished. Condition factor (K) demonstrated no significant relationship ($P > 0.1$). The metric with the strongest correlation with time to mortality was HSI ($\rho = 0.36$; $P < 0.0001$) while weight and length demonstrated similar correlative relationships ($\rho = 0.28$ and 0.26, respectively; $P < 0.005$). Figures demonstrating these correlative relationships can be found in Supplementary Figure A.6.2

6.4.3 LPS Induced Molecular Modifications

6.4.3.1 ANOVAs

6.4.3.1.1 Three-factor ANOVAs

There was an effect of acclimation temperature, either individual or interactive, on the mRNA transcript abundance of all studied genes ($P < 0.05$) across immune reception, immune response, fatty acid response, and stress responsive biological processes. There were interactive effects of LPS exposure, temperature, and time on the mRNA transcript abundance of genes

MyD88, *GR1*, *HSP70*, *HSP90a*, *Lysozyme-C*, *IL-1β*, and *PLA2* ($P < 0.05$) and combined effects of temperature:time and LPS:time on *IL-8*, *TICAM-1*, *CPT1*, *StAR*, *C3*, *NF-kB*, and *TLR4* ($P < 0.05$; further ANOVA data can be found in Supplementary Table A.6.1; Supplementary Figures A.6.3-6).

Multiple comparison tests revealed that all immune reception genes (*TLR4*, *MyD88*, *TICAM-1*, and *NF-kB*) for 16ºC acclimated sturgeon demonstrated an upregulation over the time course of LPS exposure in 30 $\mu g.ml^{-1}$ while a similar upregulation was not observed in 20ºC acclimated counterparts ($P < 0.05$) or in 0 $\mu g.ml^{-1}$ exposed sturgeon, but with similar expression patterns in 60 $\mu g.ml^{-1}$ exposed sturgeon. Immune responsive genes demonstrated more nuanced changes in expression with, *C3* and *TNFa* increasing for only 16ºC acclimated sturgeon throughout the time course ($P < 0.05$), while *IL-1β*, *IL-8,* and *Lysozyme-C* demonstrated similar levels of responsiveness in 16 and 20ºC. However, *IgM* was higher in 20ºC sturgeon across sampling points ($P < 0.05$). Fatty acid responsive genes *CPT1* and *PLA2* were more responsive to LPS stimulus in 16ºC acclimated sturgeon than 20ºC acclimated fish ($P < 0.05$), with higher peak mRNA transcript abundance following exposure and persistent increases in expression of *PLA2* ($P < 0.05$). Finally, stress responsive genes *StAR* and *GR1* demonstrated upregulated responses throughout the exposure and recovery to LPS in 16ºC while their expression was not modified by LPS stimulus in 20ºC acclimated sturgeon ($P < 0.05$). Chaperones *HSP70* and *HSP90a* were upregulated across both acclimation temperatures during LPS exposure, with a higher magnitude of induction for *HSP70* in 16ºC acclimated sturgeon ($P < 0.05$). Detailed results for three-factor ANOVA analyses are provided in Supplemental Table A.6.1.

6.4.3.1.2 Two-factor ANOVAs

There was a gene by acclimation temperature interaction ($P < 0.0001$; Figure 5.2.3) for each analyzed time point. Prior to the beginning of LPS trials, acclimation to 20°C induced increased mRNA transcript abundance of genes across biological processes (Figure 6.3A; $P < 0.05$) compared to 16ºC acclimated sturgeon. The mRNA transcript abundance of both *MyD88* and *NF-kB*, involved in immune reception, increased expression approximately 1.5-fold in 20ºC compared to 16ºC acclimated sturgeon ($P < 0.05$). Additionally, *C3* and *Lysozyme-C* expression involved in the immune response were both increased 4.8-fold and 2.4-fold, respectively, when compared to 16°C acclimated sturgeon ($P < 0.05$). Mitochondrial fatty acid transporter *CPT1* also

increased its expression throughout acclimation 3.1-fold so in 20°C acclimated sturgeon versus their 16°C acclimated counterparts ($P < 0.05$). Finally, *GR1 HSP70* and *HSP90a*, all involved in the endocrine stress response, increased expression 2.2, 2.0, and 1.9-fold, respectively, in 20°C compared to 16°C acclimated sturgeon ($P < 0.05$).

However, following 48 h exposure to 30 $\mu g.ml^{-1}$ LPS, 16ºC acclimated sturgeon had higher mRNA transcript abundance across biological processes, when compared to their 20 ºC acclimated counterparts at the same time point and LPS exposure (Figure 6.3B; $P < 0.05$). Intracellular signaling molecules *MyD88* and *TICAM-1* were 78.5% and 48.8% more highly expressed in 16ºC acclimated lake sturgeon, when compared to 20ºC acclimated sturgeon ($P < 0.05$). While all immune responsive genes were qualitatively higher in expression for 16ºC sturgeon (with the exception of *IgM*), only *IL-1β* demonstrated significantly higher expression 2.2-fold higher in 16ºC acclimated sturgeon compared to their 20ºC acclimated counterparts ($P < 0.05$). In contrast to the observed trend of the other immune responsive genes, *IgM* was 58% higher in 20ºC acclimated sturgeon ($P < 0.05$). Fatty acid responsive genes *PLA2* and *CTP1* were much more highly induced in 16ºC acclimated sturgeon 5.6 and 2.5-fold when compared to their 20ºC acclimated counterparts ($P < 0.05$). For stress responsive genes, only *HSP70* was differentially expressed between acclimation treatments, with 2-fold higher expression in 16ºC acclimated sturgeon.

Following a 7 day recovery, these increased mRNA responses in 16ºC acclimated lake sturgeon observed during LPS exposure were persistent, with higher mRNA transcript abundance across immune reception, fatty acid, and stress responsive processes when compared to 20ºC acclimated sturgeon (Figure 6.3C; $P < 0.05$). Immune reception components of the toll like receptor signaling complex *TLR4*, *MyD88*, and *TICAM-1* were all upregulated in 16ºC compared to 20ºC acclimated lake sturgeon 1.9, 1.5 and 2.6-fold respectively ($P < 0.05$). Fatty acid responsive genes *PLA2* and *CPT1* had mRNA transcript abundance 5.5 and 1.5-fold higher in 16ºC acclimated sturgeon when compared to their 20ºC acclimated counterparts ($P < 0.05$). Stress responsive genes *StAR*, *GR1*, and *HSP70* were also all elevated in 16ºC versus 20ºC acclimated lake sturgeon, 2, 1.4 and 2.3-fold respectively ($P < 0.05$).

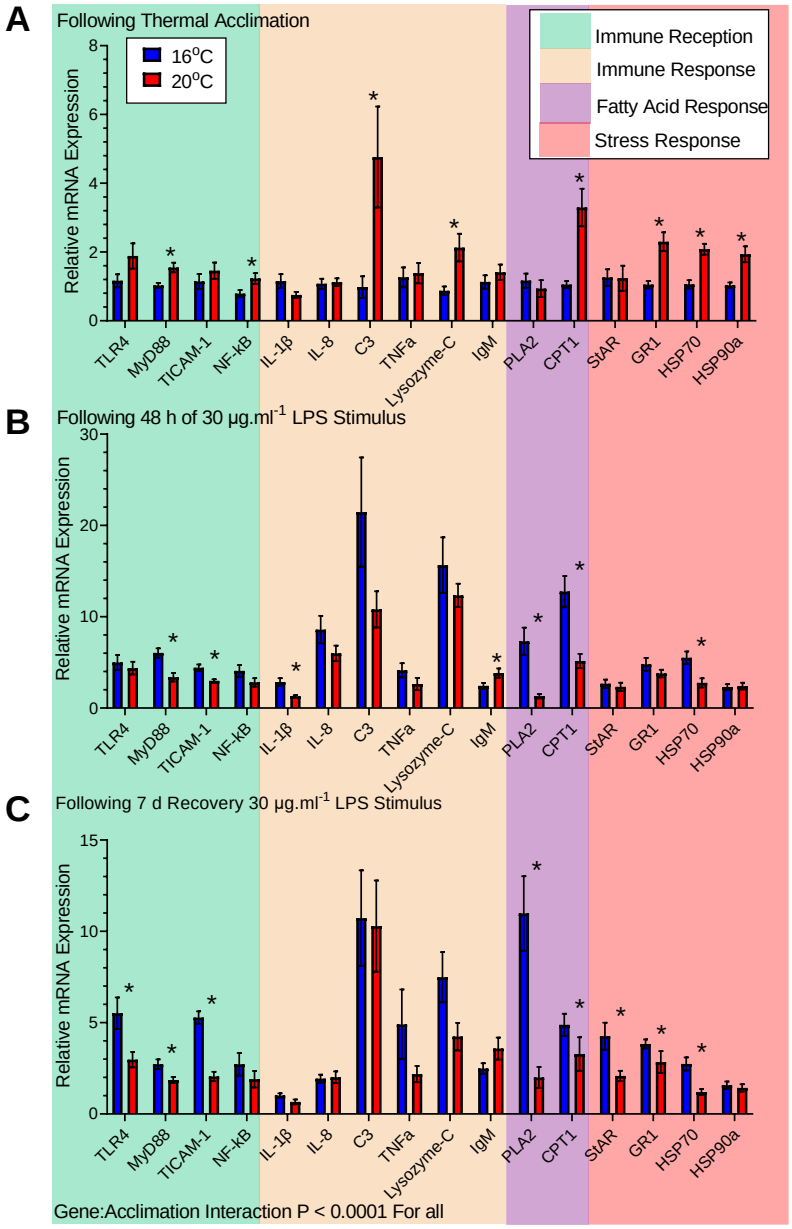

Figure 6.3 Relative mRNA transcript abundance of genes involved in immune reception (*TLR4, Myd88, TICAM-1, NF-kB*), immune response (*IL-1β*, *IL-8, C3, TNFa, Lysozyme-C, IgM*), fatty acid responses (*PLA2, CPT1*), and stress responses (*StAR*, *GR1*, *HSP70*, *HSP90a*) in developing lake sturgeon, *Acipenser fulvescens*, following A) acclimation to 16 and 20°C for 21 days, B) 48 h exposure to 30 µg.ml^{-1} bacterial lipopolysaccharides (LPS), and C) a 7 day recovery from LPS exposure. * indicates significance between 16 and 20°C acclimation treatments for a given gene ($P < 0.05$; Two-factor ANOVA). Data are expressed as mean ± S.E.M (n = 9-10).

6.4.3.2 Principal Component Analysis

Principal component analysis (Figure 6.4A) demonstrated separation between 16 and 20ºC acclimation treatments across their responsive trajectories to acclimation, 48 h LPS exposure, and 7 day recovery. Principal component 1 along the x-axis explained 43.6% of variation, while principal component 2 on the y-axis explained 13.1%. As developing sturgeon responded to LPS exposure both acclimation treatments separated out from the left to the right side of the y axis, however with different responsive trajectories as 16ºC acclimated sturgeon moved to the bottom right-hand quadrant, while those acclimated to 20ºC moved to the upper left. Following recovery these responses also differed with 16ºC acclimated sturgeon moving to the upper right quadrant, and 20ºC acclimated sturgeon regressing to the upper left. Thus, the mRNA transcript abundance of the acclimation treatments had different response trajectories, but also differences in the magnitude of their response, with 16ºC acclimated sturgeon moving further across the axes than their 20ºC acclimated counterparts, in response to LPS exposure.

The contributions of variables to observed variation in the principal component analysis was distributed across biological processes, with at least one gene from each contributing more than the average expected value, to the overall variation observed (Figure 6.4B). Genes passing this average expected contribution threshold were *TLR4*, *MyD88*, and *TICAM-1* involved in immune reception, *Lysozyme-C* and *IL-8* involved in the immune response, *HSP70*, *StAR*, and *GR1* in the stress response, and *CPT1* involved in fatty acid responses. Variable plots further demonstrate the response trajectory which the variation in mRNA transcript abundance of these genes contributed to the principal component analysis, with all genes over the expected contribution threshold moving to the right hand side of the y-axis (Figure 6.4C).

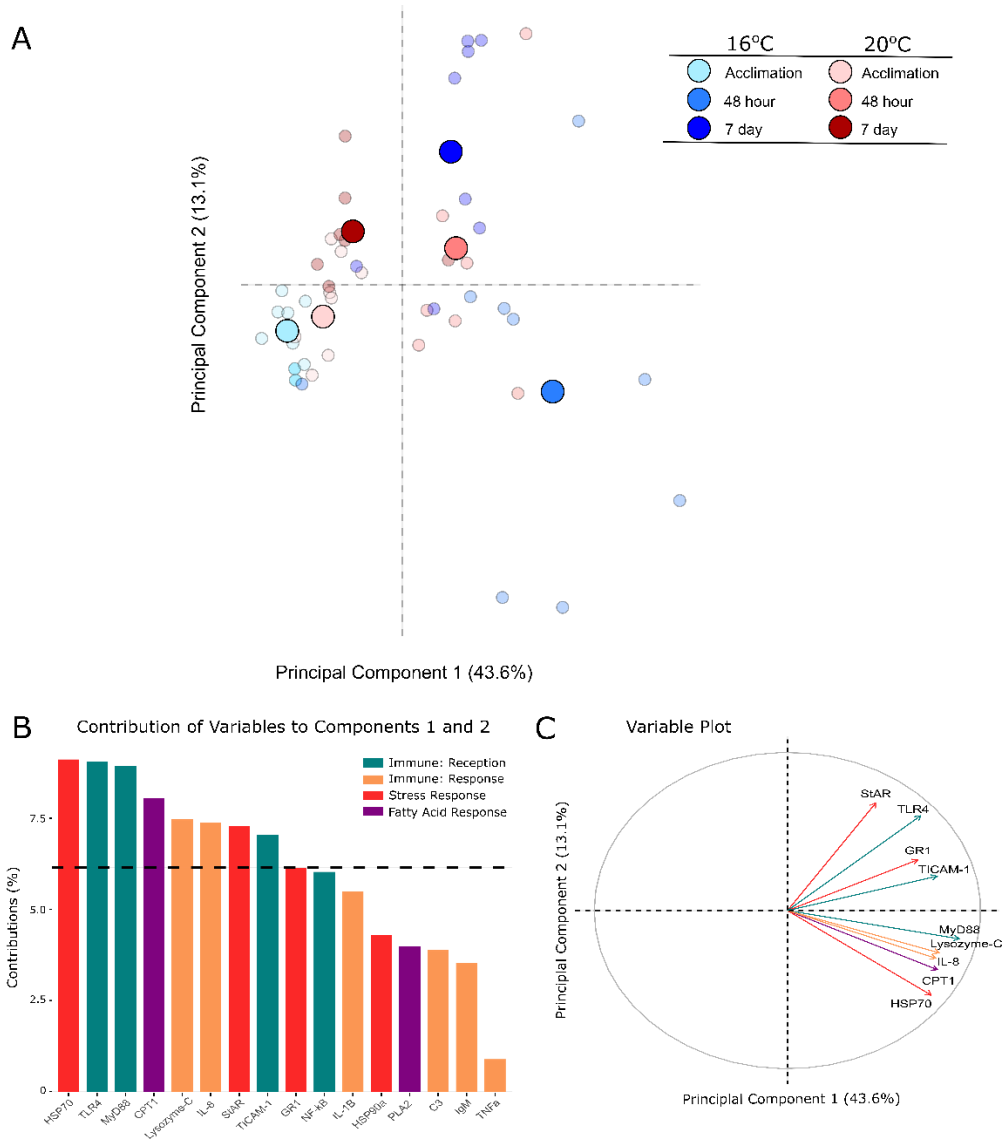

Figure 6.4 A) Principal component analysis, B) contributions of variables to components 1 and 2, and C) variable plots for 16 and 20°C acclimated, developing lake sturgeon (*Acipenser fulvescens*) following thermal acclimation, exposure to 30 µg.ml^{-1} of bacterial lipopolysaccharides (LPS) for 48 h, and a 7 day recovery period (n = 48). For A, each lightly colored point represents an individual, while the larger darker colored points represent the centroid for each treatment group. The dotted line in B represents the default average contribution value expected for the contribution of each gene to the overall observed variation. For simplicity, only genes that exceeded the average expected contribution were included in the variable plot (C).

6.4.3.3 Correlative Relationships

The mRNA transcript abundance relationships of developing lake sturgeon differed between acclimation treatments throughout acclimation, LPS exposure, and recovery, with higher spearman's correlation coefficients across 16ºC acclimated sturgeon when comparing genes across biological processes (Figure 6.5A). Many genes involved in the downstream responses to immune reception of LPS (*IL-8*, *C3*, *Lysozyme-C*, *PLA2, CPT1, GR1, HSP70, and HSP90a*) demonstrated strong correlative relationships (ρ values ≥ 0.6) with intracellular immune signaling molecules *MyD88* or *TICAM-1* for 16ºC acclimated sturgeon. Comparatively, no transcriptional relationships with *MyD88* or *TICAM-1* were as strong in 20ºC acclimated sturgeon, and only one as strong when comparing the relationships of every studied gene (*IL-8* to *IL1-β*). The largest differences in correlative relationships between the treatments can be found in the expression of stress responsive genes *GR1*, *HSP70*, and *HSP90a* (Figure 6.5B) while the relationships of *StAR* with every other gene were relatively consistent across the acclimation treatments. There were also inconsistencies between the acclimation treatments in *C3* and *Lysozyme* in their relationship with fatty acid responsive genes *PLA2* and *CPT1* and *C3* with elements of immune reception.

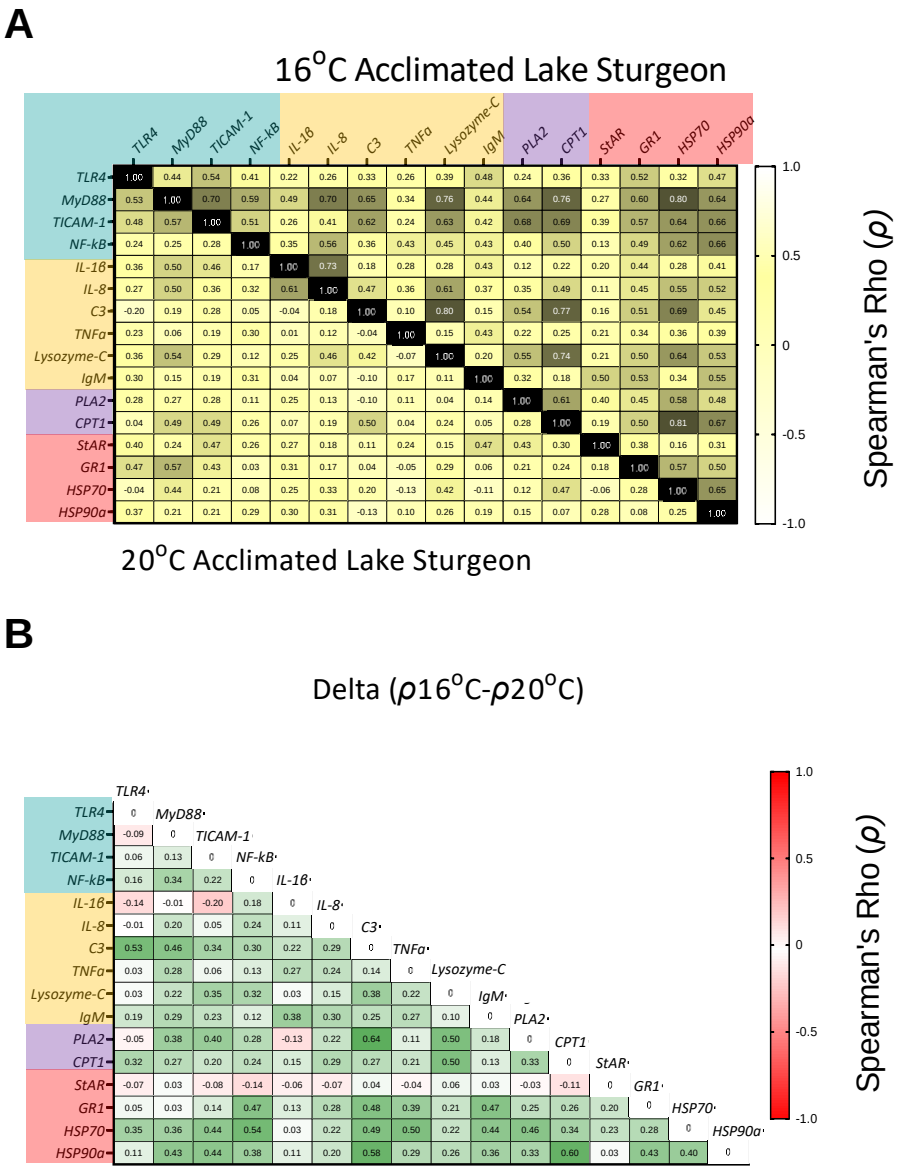

Figure 6.5 Gene expression relationships of developing lake sturgeon, *Acipenser fulvescens*, acclimated to A) 16°C as well as 20°C and B) the delta (difference in Spearman's rho (ρ) between the two acclimation treatments), following trial exposures to 0, 30, and 60 µg.ml^{-1} concentrations of bacterial lipopolysaccharide (LPS) during pretrial conditions, 4 and 48 h following exposure, as well as after 7 days of recovery. Correlative relationships between the mRNA transcript abundance of each gene were quantified while accounting for variance in both LPS exposure concentration and timepoint using semi-partial Spearman's correlations. Color overlays on gene names indicate the biological process to which they belong with green being

immune reception, yellow immune response, purple fatty acid response, and red stress response. Values are reported as Spearman's rho (ρ).

6.4.4 Post-trial Metabolism and Thermal Tolerance

While CT_{max} was not compromised by the effects of LPS exposure in either acclimation treatment (Supplementary Figure A.6.7), several metabolic traits were suppressed following exposure to LPS, but only in 16°C reared sturgeon. Measurement of metabolic rate prior to CT_{max} trials did not impact the resulting CT_{max} for sturgeon who had their metabolic rate measured, from any treatment ($P > 0.05$). There was no effect of LPS exposure or acclimation temperature on the resting metabolic rate of developing lake sturgeon, however, there were effects on forced metabolic rate (FMR), delta, and metabolic scope, all of which demonstrated significant interactions between acclimation treatment and LPS concentration ($P < 0.01$; Figure 5.2.6). Further, multiple comparisons revealed specific differences between acclimation treatments and across LPS concentrations.

In 16°C acclimated sturgeon, FMR was reduced in both 30 and 60 $\mu g.ml^{-1}$ concentrations of LPS compared to control sturgeon, 38.5 and 40.9%, respectively ($P < 0.01$; Figure 5.2.6B), however there was no change between control and 30 $\mu g.ml^{-1}$ treated sturgeon acclimated to 20°C. This resulted in a 30.3% increase in FMR for 20°C acclimated sturgeon exposed to 30 $\mu g.ml^{-1}$ LPS when compared to their 16°C acclimated counterparts exposed to the same LPS concentration ($P < 0.05$). Multiple comparisons for delta indicated a decrease of 73.6 and 88.6% for 16°C acclimated lake sturgeon following exposure to LPS concentrations of 30 and 60 $\mu g.ml^{-1}$, respectively when compared to controls acclimated to the same temperature ($P < 0.001$; Figure 5.2.6C). However, there was no change in delta for 20°C acclimated fish. This again resulted in higher delta, similar to FMR, between the acclimation treatments exposed to 30 $\mu g.ml^{-1}$ LPS ($P < 0.05$) with a 55.5% higher delta in 20°C acclimated sturgeon than their 16°C counterparts exposed to the same LPS concentration. Finally, metabolic scope also decreased between handling control and LPS exposed sturgeon in the 16°C acclimation treatment, 41.6 and 54.7% for 30 and 60 $\mu g.ml^{-1}$ LPS treatments when compared to handling control fish, respectively ($P < 0.005$; Figure 5.2.6D), with no effect of LPS exposure on the metabolic scope of sturgeon acclimated to 20°C.

There was a significant interaction between acclimation treatment and LPS concentration on the CT_{max} of sturgeon following a 7 day recover from LPS trials ($P < 0.05$; $F = 4.24$; Supplementary Figure A.6.7). Sturgeon acclimated to 20°C and exposed to 30 $\mu g.ml^{-1}$ concentrations of LPS had a CT_{max} 0.44°C higher than their control counterparts not exposed to LPS ($P < 0.001$). There were no differences in CT_{max} for 16°C reared sturgeon across LPS exposure

concentrations. Across acclimation temperatures, sturgeon acclimated to 20°C had CT_{maxes} 2.71 and 3.13°C higher than their 16°C reared counterparts, for control and 30 $\mu g.ml^{-1}$ concentrations of LPS, respectively ($P < 0.0001$).

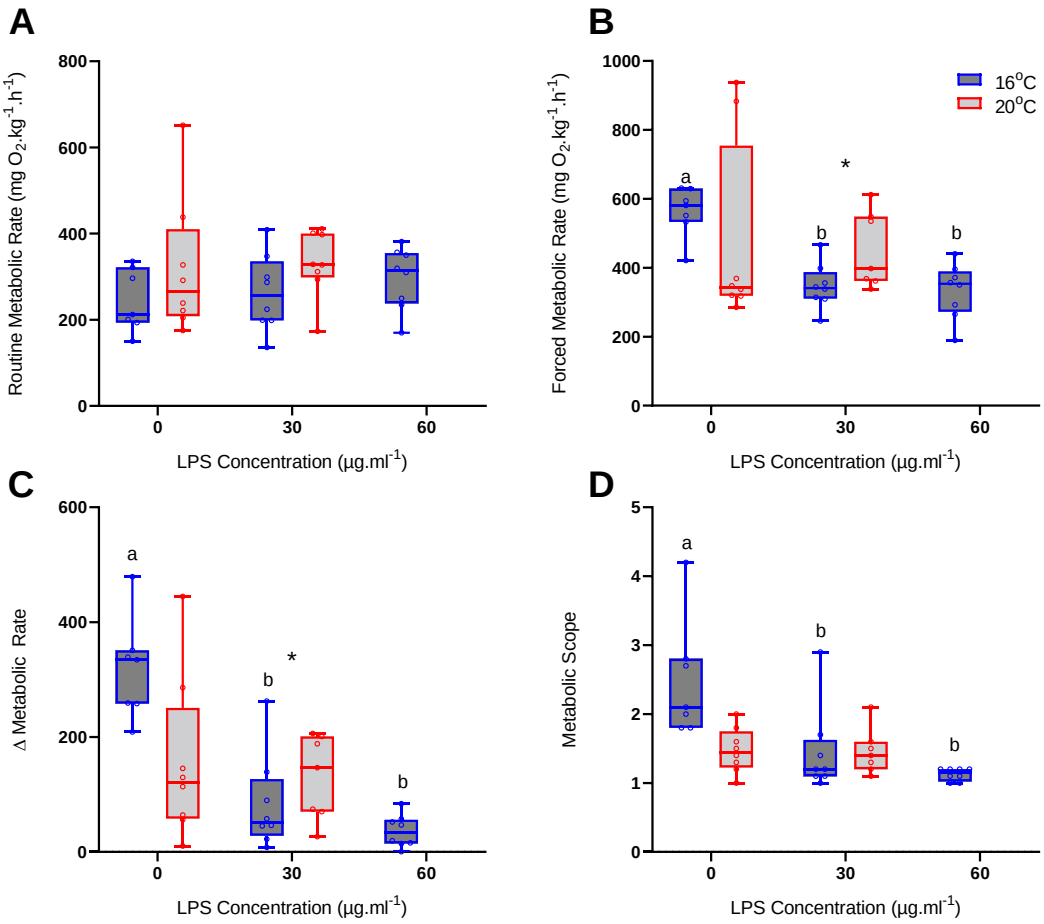

Figure 6.6 A) Resting metabolic rate, B) forced metabolic rate, C) delta metabolic rate, and d) metabolic scope of 16 and 20°C acclimated developing lake sturgeon, *Acipenser fulvescens*, following a 6 day recovery from 48 h exposure trials in lipopolysaccharide concentrations of 0, 30, and 60 µg.ml^{-1}. Differences between treatments were determined by two-factor ANOVA ($P < 0.05$) followed by Tukey's honestly significant different post-hoc test. *'s represent significance between 16 and 20°C acclimation treatments within a lipopolysaccharide exposure concentration. Lowercase letters a and b represent significance across treatment concentrations within a single acclimation treatment (n = 7-8).

6.5 Discussion

In the current study, we demonstrate the effects of ecologically relevant summer temperatures on the innate immune responses of developing lake sturgeon. Increased environmental temperatures influenced the expression of every studied gene, inducing the endocrine stress response and fatty acid oxidation during acclimation, and inhibiting activation of innate immune, fatty acid, and stress-responsive biological processes when lake sturgeon were challenged with bacterial LPS. Acclimation temperatures used in the current study were approximately 2-3°C below maximum sustained summer temperatures for this population of lake sturgeon and persisted for only a small portion of the duration as would likely be experienced in the wild (Bugg et al., 2020; Bugg et al., 2021b). These results indicate the presence of seasonal sub-lethal thresholds on innate immunity for wild populations of lake sturgeon throughout Manitoba, Canada, which may be especially vulnerable to the effects of pathogens during early development (Clouthier et al., 2020). While developing sturgeon may be plastic in the face of changes in their thermal environment (Bugg et al., 2020; Pennman et al., 2022; Earhart et al., *In prep*), this enhanced plasticity, induction of the glucocorticoid stress response, and decrease in energy stores, may elicit a tradeoff and diminish their ability to mount an effective immune response against opportunistic pathogens, especially in early life.

6.5.1 Pre-trial Physiology and In-Trial Mortality

While there was no difference in weight, length, and condition, prior to the beginning of trials there was a decrease in HSI in 20°C acclimated sturgeon when compared to their 16ºC reared counterparts, and HSI was the strongest relationship to time to mortality during LPS exposures. As HSI is an indication of fatty acid and glycogen stores accrued during development (Chellappa et al, 1995; Rossi et al., 2017; Morrison et al., 2020), these findings suggest that the effects of increasing temperature compromise the acquisition of these energy reserves, and that this relative reduction increases the vulnerability of developing sturgeon to pathogenic stressors. Similar to the findings of the current study, research on pallid, *Scaphirhynchus albus,* and white sturgeon, *A. transmontanus*, suggests increased rearing temperatures resulted in high levels of mortality (50-60%) once a pathogen was introduced, while there was limited mortality (< 10%) in lower temperature acclimation treatments (Coleman et al., 2022; Stilwell et al., 2022). These

findings suggest strong thermal thresholds for pathogen induced mortality, which may be associated with the energetic state of developing sturgeon.

6.5.2 Acclimation and LPS Induced Molecular Modifications

At the end of acclimation, prior to LPS trials, lake sturgeon acclimated to 20°C had increased mRNA transcript abundance of genes involved in immune reception, immune responsiveness, fatty acid responses, and stress responses. Increased expression of immune receptive (*MyD88* and *NF-kB*) as well as immune responsive genes (*C3* and *Lysozyme-C*), may indicate a stress responsive role for their expression, as observed in channel catfish, *Ictalurus punctatus* and large yellow croaker, *Larimichthys crocea* (Small and Bilodeau, 2005; Sun et al., 2017). Interestingly, mRNA transcript abundance of a transporter involved in the rate limiting step of long chain fatty acid oxidation (*CPT1;* Coccia et al., 2014) was also induced in 20ºC following acclimation. Similar to observations in thermally stressed Atlantic salmon (Norambuena, et al., 2015), increases in the oxidation of fatty acids and decreases in HSI in 20ºC acclimated sturgeon likely indicate that sturgeon in this treatment were using fatty acid stores for energy, and not accruing them in the liver throughout development. Additionally, facets of the glucocorticoid stress-signaling complex and stress response (*GR1*, *HSP70*, and *HSP90a*) were all more highly induced in 20ºC acclimated lake sturgeon, indicating an activation of the glucocorticoid stress response (Kirschke, et al., 2014). Together these responses highlight the effects of thermal acclimation, inducing stress related gene expression and mobilization of energy reserves in developing lake sturgeon exposed to environments with increased temperatures. Although these mRNA level responses were all induced by thermal acclimation, they did not adequately protect developing lake sturgeon from the combined effects of thermal stress and bacterial endotoxin exposure.

While measured transcriptional responses were elevated in 20ºC sturgeon following acclimation, after exposure to LPS, responses of 16ºC acclimated sturgeon were more highly induced across biological processes. There was increased expression of both *TLR4* related intracellular signalers (*MyD88* and *TICAM-1*) suggesting a stronger activation of the innate immune reception and stimulation of the signaling cascade in 16ºC acclimated sturgeon (Deguine and Barton, 2014; Tanekhy, 2014). Immune responsive transcripts (*IL-1β*, *IL-8*, *C3*, *TNFa*, and *Lysozyme-C*) trended higher in 16ºC acclimated sturgeon, when compared to their

20ºC acclimated counterparts, showing potential differences in the magnitude and timing of innate immune responses induced by LPS exposure (Bennoit and Craig, 2020). Fatty acid responses, both involved in immune precursor production (*PLA2*) and energy production (*CPT1*), were also more highly induced in 16ºC acclimated sturgeon indicating a higher capacity to respond with both innate immune and energetically intensive processes likely related to the abundance of fatty acid precursors (Angosto and Mulero, 2014; Arnemo et al., 2017). There was also increased expression of *HSP70* in 16ºC acclimated sturgeon following LPS exposure, which has multifaceted roles in stress signaling, but also in both antigen presentation and protection from inflammation (DeNagel and Pierce, 1992; Sarlin et al., 1994; Zugel and Kaufmann, 1999). Overall, LPS exposure induced the mRNA transcript abundance of the measured biological processes in 16ºC acclimated sturgeon higher than their 20ºC acclimated counterparts, likely protecting them from mortality under high endotoxin concentrations.

Following a 7 day recovery from LPS exposure, elevated mRNA responses in 16°C acclimated sturgeon were persistent, remaining higher than their 20ºC acclimated counterparts across immune reception, fatty acid, and stress responsive processes. The continued elevated expression of immune reception related processes indicates a prolonged impact of LPS exposure. As the upregulated components of the LPS responsive receptor complex (*TLR4*, *MyD88*, and *TICAM-1*; Palsson-McDermott and O'Neill, 2004; Srivastava et al., 2017) are abundant in myeloid, innate immune responsive cells (Kawamoto and Minato, 2004), their proliferation and elevated abundance likely enhances the detection and signaling responses to pathogens (Zhang et al., 2019). Fatty acid responses, involved in beta oxidation and eicosanoid production, additionally remained elevated increasing energy production and availability of immune precursors (Norambuena et al., 2015; Okamura et al., 2021; Nguyen et al., 2022). Stress responsive gene elevation following recovery, involved facets of the cortisol producing and signaling complexes (*StAR, GR1, HSP70*) likely indicating a prolonged activation of HPI-axis related responses. Together these responses highlight the prolonged effects of bacterial LPS with the potential for persistent effects on immune reception, fatty acid and stress responses of sturgeon following thermal acclimation.

Temperature played a powerful role in modulating the transcriptional responses of developing lake sturgeon, with impacts on biological processes prior to, during, and following exposure to bacterial LPS. Overall, sturgeon acclimated to 20ºC were less transcriptionally

responsive than their 16ºC counterparts, with modification of the magnitude of transcriptional response of various immune related processes (Bennoit and Criag, 2020). A potential mechanistic explanation lies in the effects of accumulating thermal stress on crosstalk between GR and the innate immune transcription factor NF-kB (Bekhbat et al., 2017). Under stressful conditions GR may be upregulated as observed in the 20ºC acclimation treatment, which can increase the affinity of binding proteins, inhibiting the translocation of NF-kB into the nucleus and impeding the activation of the immune response as well as downstream processes (i.b.i.d.). Further experimentation would be required to validate this hypothesis, but collective evidence from this study supports the role of thermal stress in the inhibition of the immune response of developing lake sturgeon.

Although many of the gene expression responses were largely different between acclimation treatments, there was an induction of immune responses involving *IL-8*, *Lysozyme-C* and *C3* across both treatments consistent with other studies of the sturgeon immune response (Li et al., 2017; Lou et al., 2018; Valipour et al., 2018; Hohne et al., 2021). These innate immune mechanisms may play an enhanced role in sturgeon pathogen defense due to their more primitive nature, especially in early development (Magnadottir, 2006; Wang et al., 2009; Huber-Lang, 2018). However, the induction of these responses was not enough to protect 20ºC acclimated sturgeon from mortality, indicating the importance of peripheral immune responsive mechanisms in pathogen defense (e.g. unstudied receptor, complement, cytokine, and antimicrobial peptide [etc.] activated immune response pathways). Ultimately the combined effects of gene expression alteration, different responsive trajectories, and weakening of molecular relationships decreased the survival of 20ºC acclimated sturgeon exposed to high concentrations of bacterial lipopolysaccharides.

6.5.3 Post-trial Metabolism and Thermal Tolerance

Following one week of recovery from the effects of LPS exposure, 16°C acclimated lake sturgeon showed depressed maximum metabolic rates and scope, when compared to non-exposed sturgeon, however, thermal tolerance was not compromised in either treatment. As the immune response is energetically costly to induce (Martin and Krol, 2017), this decrease in metabolic capacity may show a cost-benefit relationship to its activation. Sturgeon in 20ºC did not as strongly activate an immune response, or demonstrate a persistent response, potentially

due to an absence of energy reserves, as indicated by a decreased HSI, and ability to mobilize them to fuel an energetically costly immune response. However, at high concentrations of bacterial endotoxin this inability to respond was costly, as sturgeon in this treatment suffered complete mortality, while the 16°C fish had higher survival. These results are in contrast to a similar study exposing mosquitofish, *Gambusia holbrooki*, to LPS, where one week following exposure, mosquitofish demonstrated increased metabolic scope (Bonneaud et al., 2016). Similarly, in pathogen exposed zebrafish, *Danio rerio*, routine metabolic rate increased in high temperature treatments following immune stimulus (Bennoit and Craig, 2020) and in the sockeye salmon, *Oncorhynchus nerka*, similar results were observed, with a minimal metabolic cost and a highly energetically efficient innate immune response to infection (Polinski, 2021). While there is limited research on metabolic cost of immune activation in fishes, these results may indicate a higher energetic cost for innate immune activation in lake sturgeon when compared to more recently derived fish lineages. This increased energetic cost may have resulted in compromising metabolic scope with prolonged implications for metabolic capacity once the immune system is strongly induced by a pathogen in developing lake sturgeon.

6.6 Acknowledgements

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Chapter 7. Thesis Conclusions

Temperature fundamentally influenced whole organism performance, transcriptional plasticity, and sub-lethal thresholds of developing lake sturgeon in Manitoba, Canada with population-specific thermal tolerances and sub-lethal thresholds. Elevated temperatures in early development altered early physiological condition and influenced the responses of young of year sturgeon to stocking related cold stress later in development (Chapter 2). Lake sturgeon from distinct northern and southern populations exhibited population-specific tolerance thresholds in growth, metabolism, thermal tolerance, and transcriptional responses to thermal acclimation (Chapter 3). Whole-transcriptome responses from these same populations indicated the induction of transcriptionally plastic responses as temperatures increased from 16 to 20^{o}C, but tolerance declined as temperatures increased further to 24^{o}C, suggesting sub-lethal limitations on plasticity, and vulnerability to compounding stressors (Chapter 4). As temperatures increased from 16 to 20^{o}C, the transcriptional responsiveness of the lake sturgeon innate immune system was compromised, potentially resulting in increased vulnerability to pathogens, and elevated mortality (Chapters 4 and 6). As environmental temperatures within the rivers of Manitoba are anticipated to increase in the coming years (Manitoba Hydro, 2015), it will be important to take the potentially necessary precautions (e.g. assessing population level health, increasing stocking of lake sturgeon, thermoregulating rivers, etc.) to protect these geographically distributed populations of lake sturgeon which demonstrate population-specific sub-lethal thresholds for whole organism performance, transcriptional plasticity, and vulnerability to temperature increases.

Currently, northern populations of lake sturgeon are reared in hatcheries yearly and released to sustain and bolster wild populations. There are opportunities to increase hatchery rearing temperatures to promote growth in these developing fishes. However, the results of these studies on whole organism performance (Chapters 2 and 3) demonstrate both short- and long-term impacts of elevated rearing temperatures. Thus, increasing rearing temperatures should be strongly considered, with impacts on growth performance as well as the potential for long term impacts on responses to stressors later in life, such as stocking or future environmental change. The results of stocking experiments indicate that sturgeon reared in elevated temperatures during early development may suffer increased stress with all studied lake sturgeon demonstrating

increased responses to cold as temperatures get further from their acclimation. Thus, hatchery acclimation and stocking procedures should mimic environmental temperatures, to lower stress upon release. These experiments also demonstrate population-specific phenotypes across multiple year classes of developing lake sturgeon indicating differences in their performance, and tolerance thresholds.

These performance differences were then investigated using RNAseq revealing that as developing lake sturgeon approach sub-lethal thermal limits, transcriptional plasticity is limited (Chapter 4). This diminished ability to cope with additional environmental changes likely makes them more susceptible to the effects of compounding stressors (Smith et al., 2013). As stressors like temperature, pathogens, and hypoxia are often co-occurring (McBryan et al., 2013), and may be impactful in the benthic environments of early developing sturgeon (Secor and Niklitschek, 2001), it may be important to examine their cumulative effects, especially in the context of natural environments with increasing temperatures. Currently, lake sturgeon demonstrate sub-lethal effects of reduced transcriptional plasticity to elevated temperatures, but these sub-lethal thresholds may be lower once combined stressors are encountered.

To investigate temperature influences on the transcriptional plasticity of the immune response of developing lake sturgeon, first an understanding of their immune related transcriptional must be established. Exposing sturgeon to varying concentrations of bacterial lipopolysaccharides as an immune stimulant revealed that there was an intertwined induction of both the innate immune and stress responses (Chapter 5). These responses were persistent over a 48 hr time course exposure and demonstrated correlation between innate immune signaling molecules and stress responsive mRNAs. Using the resulting framework, a more thorough study focused on thermal influences on the responses of developing lake sturgeon to an immune stimulus was able to be constructed.

Developing sturgeon acclimated to elevated temperatures demonstrated higher transcriptional expression of immune related compounds, but following pathogenic stimulus with bacterial lipopolysaccharides, had diminished responses compared to their lower temperature acclimated counterparts (Chapter 6). Lake sturgeon are exposed to thermal stress and environmentally pervasive pathogens both in hatchery and in wild environments and may be more susceptible to increasing temperatures, especially during early development. As climate

change effects will likely exacerbate cross-species pathogen transmission and expand range boundaries (Bebber et al., 2015; Carlson et al., 2022) it will be critically important to understand how pathogens impact the development and recruitment of lake sturgeon. This study investigated the effects of current summer temperatures on the innate immune responses of lake sturgeon, demonstrating impacts on the ability of a southern Manitoba population of lake sturgeon to transcriptionally induce processes involved in innate immune reception, immune responses, fatty acid responses, and stress responses during exposure to bacterial endotoxins. Riverine temperatures are projected to continue to increase (Manitoba Hydro, 2015) throughout Manitoba in the coming years. These data suggest that the effects of increasing temperatures will impact the immunocompetency of lake sturgeon during a critical period of early development during which lake sturgeon already experience high mortality (Sifa and Mathias, 1987; Wieser, 1991; Rombough, 1994). The effects of elevated temperatures will likely most strongly influence populations in the northern end of the lake sturgeon range, as these groups demonstrate lower sublethal thresholds to the effects of increasing temperature (Chapter 3 and 4). Further research should investigate the health effects of increasing temperatures on these leading edge, sub-arctic populations, to determine their susceptibility to pathogens as temperatures increase.

In the wild, lake sturgeon throughout Manitoba will continue to face the effects of increasing environmental temperatures (Manitoba Hydro, 2015). These studies demonstrated the effects elevated temperatures for 21-60 days, however currently wild sturgeon from southern populations, and in the future northern populations as well, are likely exposed to these temperatures, and higher, for prolong periods (Chapter 3). As these studies were performed on sturgeon which were lab reared, an analysis of the effects of thermal stress on the health status of wild sturgeon populations across the province would provide further evidence of the pathogens which infect sturgeon, their physiological status, and insight into the changing immune health of populations as environmental temperatures increase. By using non-lethal sampling procedures of peripheral tissues or mucus, managers may can assess the immune and physiological status of wild populations without inducing unnecessary harm, providing better insights on the real-world impacts of increasing temperatures on lake sturgeon immune capacity (Jeffries et al., 2021; Thorstensen et al., 2022). If there is indeed decreased physiological capacity, health and immune capacity in the northern populations of lake sturgeon, as current research may suggest, management actions should be taken to protect these critically endangered populations before

they are lost.

Throughout these experiments, elevated temperatures demonstrated sub-lethal and lethal effects on developing lake sturgeon (Chapters 2, 3, 4, and 6) which may be influenced by environmentally pervasive pathogens. Currently little is known about the pathogens that are endemic to these populations, but the available research suggests that lake sturgeon may be especially susceptible to their effects during early development (Gradil et al., 2014a; Gradil et al., 2014b; Clouthier et al., 2020). As yearly temperatures are often highest in these crucial early developing months (June-August), this period will represent a life history period when lake sturgeon will be particularly vulnerable. Evidence suggests that there are a variety of viral pathogens that infect sturgeon species in the wild (Drennan et al., 2006; Clouthier et al., 2013; Mugetti et al., 2020), and an array of pathogens which may inflict mortality in an aquaculture setting (Groff et al., 1996; Fujimoto, 2012, Coleman et al., 2018; Fujimoto et al., 2018; Jiang et al., 2018; Stilwell et al., 2022; Soto et al., 2022) which could be transferred to wild populations if not managed appropriately (Bouwmeester et al., 2020). As early mortality is an impediment to the success of both conservation hatcheries and natural recruitment, it is necessary to determine *what kind* and *to what extent* these pathogens may influence mortality in developing lake sturgeon. The effects of pathogens can be compounded by increasing temperatures, which in addition to increasing the susceptibility of sturgeon to infection, may enable range expansions for pathogens (Bebber et al., 2015). Thus, determining which pathogens are present and how they affect lake sturgeon populations will be an important and ever-evolving challenge as temperatures increase.

Taken together, these studies demonstrate the effectiveness of using whole organism performance metrics paired with transcriptional studies to understanding the sub-lethal tolerances of lake sturgeon, and more broadly freshwater fishes. While studying the effects of increasing temperatures on lake sturgeon physiology in a laboratory can provide some insights into the impacts of environmental change, the natural environment represents a complex mix of compounding stressors which cannot be precisely simulated in captivity. Based on the findings of these laboratory-based studies, future research may aid in determining the impacts of pathogens on the health of wild lake sturgeon populations, how temperature increases effect their habitat use, and the availability of thermal refuge areas to mitigate the sub-lethal effects of

thermal stress. Although a shift towards field-based studies may require modification of research techniques, the increasing utility of non-lethal sampling (Jeffries et al., 2021; Thorstensen et al., 2022) may provide an enhanced opportunity to physiologically assess wild sturgeon in suggested studies. With the addition of a gill sample or mucus swab taken during tag placement, movement studies to evaluate the thermal refuge use of lake sturgeon can be combined with assessments of their health and transcriptional physiology. These combined techniques can provide insight into the environments lake sturgeon are using in Manitoba, how those environments may be changing, and the physiological consequences to wild lake sturgeon populations. Overall, the examination of physiological plasticity and sub-lethal thresholds has great potential for understanding the environmental challenges faced by organisms in the wild and aid the conservation research of imperiled freshwater species.

Currently, the northern (Nelson River and Burntwood River) and southern (Winnipeg River) populations of lake sturgeon in these studies reside in the same management unit, which encompasses most of the lake sturgeon populations in southern Manitoba (COSEWIC, 2017). However, based on the population-specific differences in genetics (McDougall et al., 2015), transcriptional responses (Chapter 2, 3, and 4), whole organism performance (Chapters 2 and 3) sub-lethal thermal thresholds, and observed population declines (COSEWIC, 2006; COSEWIC, 2017) considering these northern populations separately for conservation purposes may be prudent. Under the most recent status evaluation, most northern populations of lake sturgeon, including those in the Churchill, Nelson, and Hayes Rivers are classified in separate, government managed, designated units. If these northern populations are indeed more vulnerable to the effects of increasing temperatures, as current data suggests, it may be necessary to combine these populations into a new designated or management units to aid in their conservation against the anticipated effects of a warming environment.

Appendix

A.2 Supplementary Material for Chapter 2. The effects of population and thermal acclimation on the growth, condition, and cold responsive mRNA transcript abundance of age-0 lake sturgeon (*Acipenser fulvescens*)

Table A.2.1 ANOVA results for condition factor and mRNA transcript abundance of genes *HSP70*, *HSP90a*, *HSP90b*, *CIRP* and *SCD*. The results for genes represent the effects cold shock following stocking under hatchery representative conditions for northern (Nelson River) southern (Winnipeg River) populations of lake sturgeon, *Acipenser fulvescens*, within Manitoba, Canada. Time represents at the end of a 1 day and 1 week following cold stocking. Acclimation treatment demonstrates either northern lake sturgeon acclimated to 16 or 20^{o}C in early development. Stocking represents different lab simulated temperatures of 8, 6, and 4^{o}C. All factors are included in the model as fixed effects.

Comparison throughout development	Measurement	Sources of Variation	Num df	Den df	F Value	P Value
Population Comparison	Condition Factor	Population	1	148	28.0223	4.25E-07
		Time	5	148	1.474	0.2016
		Population:Time	5	148	2.002	0.0816
Acclimation Treatment Comparison	Condition Factor	Acclimation	1	148	44.344	5.05E-10
		Time	5	148	4.523	0.000724
		Acclimation:Time	5	148	0.791	0.55808
Comparison during cold shock	Measurement	Sources of Variation	Num df	Den df	F Value	P Value
Population Comparisons	*HSP70*	Population	1	130	1.5901	0.209576
		Stocking Temperature	2	130	3.8245	0.024326
		Time	2	130	4.6937	0.01076
		Population:Stocking Temperature	2	130	0.1699	0.843949
		Population: Time	2	130	11.2565	3.10E-05
		Stocking Temperature: Time	4	130	3.575	0.008427
		Population:Stocking Temperature:Time	4	130	0.3747	0.826347
	HSP90a	Population	1	132	3.6286	0.05897
		Stocking Temperature	2	132	17.53	1.77E-07
		Time	2	132	0.4097	0.66469
		Population:Stocking Temperature	2	132	0.6317	0.5333
		Population: Time	2	132	22.9794	2.73E-09
		Stocking Temperature: Time	4	132	8.8457	2.33E-06
		Population:Stocking Temperature:Time	4	132	1.2137	0.30805
	HSP90b	Population	1	133	0.7911	0.3754
		Stocking Temperature	2	133	0.0337	0.9668
		Time	2	133	1.0564	0.3506
		Population:Stocking Temperature	2	133	0.3019	0.7399
		Population: Time	2	133	0.4761	0.6222
		Stocking Temperature: Time	4	133	0.243	0.9135
		Population:Stocking Temperature:Time	4	133	0.4012	0.8075
	CIRP	Population	1	131	0.2578	0.6125
		Stocking Temperature	2	131	0.1346	0.8742
		Time	2	131	2.0138	0.1376
		Population:Stocking Temperature	2	131	0.3777	0.6862
		Population: Time	2	131	0.5632	0.5708
		Stocking Temperature: Time	4	131	0.8429	0.5004
		Population:Stocking Temperature:Time	4	131	0.6015	0.6622
	SCD	Population	1	128	4.6177	0.033527
		Stocking Temperature	2	128	3.2715	0.041144
		Time	2	128	6.8544	0.001486
		Population:Stocking Temperature	2	128	1.1876	0.308282
		Population: Time	2	128	0.8658	0.423175
		Stocking Temperature: Time	4	128	0.7429	0.564447
		Population:Stocking Temperature:Time	4	128	0.4016	0.807238
	Condition Factor	Population	1	161	76.1805	3.12E-15
		Stocking Temperature	2	161	4.9437	0.008247
		Time	2	161	1.2288	0.295366
		Population:Stocking Temperature	2	161	1.4579	0.235779
		Population: Time	2	161	0.2256	0.798284
		Stocking Temperature: Time	4	161	0.7608	0.552242
		Population:Stocking Temperature:Time	4	161	1.4262	0.227623
Acclimation Treatment Comparisons	*HSP70*	Acclimation Treatment	1	131	0.495	0.4829661
		Stocking Temperature	2	131	9.5461	0.0001348
		Time	2	131	2.9652	0.0550215
		Acclimation Treatment:Stocking Temperature	2	131	1.7998	0.1693951
		Acclimation Treatment: Time	2	131	0.0245	0.9757811
		Stocking Temperature: Time	4	131	4.043	0.0040033
		Acclimation Treatment:Stocking Temperature:Time	4	131	0.2678	0.8982129
	HSP90a	Acclimation Treatment	1	132	0.2483	0.61909
		Stocking Temperature	2	132	12.4567	1.11E-05
		Time	2	132	15.1429	1.20E-06
		Acclimation Treatment:Stocking Temperature	2	132	1.1809	0.31021
		Acclimation Treatment: Time	2	132	4.6082	0.01163
		Stocking Temperature: Time	4	132	10.7533	1.42E-07
		Acclimation Treatment:Stocking Temperature:Time	4	132	1.9144	0.11173
	HSP90b	Acclimation Treatment	1	136	0.0694	0.7926
		Stocking Temperature	2	136	2.2558	0.1087
		Time	2	136	0.3003	0.7411
		Acclimation Treatment:Stocking Temperature	2	136	0.9219	0.4002
		Acclimation Treatment: Time	2	136	0.9922	0.3734
		Stocking Temperature: Time	4	136	0.4178	0.7956
		Acclimation Treatment:Stocking Temperature:Time	4	136	0.4934	0.7406
	CIRP	Acclimation Treatment	1	131	0.0288	0.86541
		Stocking Temperature	2	131	1.4389	0.24092
		Time	2	131	3.457	0.03443
		Acclimation Treatment:Stocking Temperature	2	131	0.2012	0.81798
		Acclimation Treatment: Time	2	131	1.0752	0.34423
		Stocking Temperature: Time	4	131	1.4055	0.23569
		Acclimation Treatment:Stocking Temperature:Time	4	131	0.3753	0.82594
	SCD	Acclimation Treatment	1	129	0.0142	0.90549
		Stocking Temperature	2	129	3.8138	0.02459
		Time	2	129	10.0133	9.07E-05
		Acclimation Treatment:Stocking Temperature	2	129	0.6495	0.524
		Acclimation Treatment: Time	2	129	0.8929	0.41198
		Stocking Temperature: Time	4	129	2.5866	0.03995
		Acclimation Treatment:Stocking Temperature:Time	4	129	0.3863	0.81815
	Condition Factor	Acclimation Treatment	1	161	22.6457	4.31E-06
		Stocking Temperature	2	161	1.2449	0.2907
		Time	2	161	1.9827	0.141
		Acclimation Treatment:Stocking Temperature	2	161	0.3604	0.6979
		Acclimation Treatment: Time	2	161	1.5505	0.2153
		Stocking Temperature: Time	4	161	0.9223	0.4525
		Acclimation Treatment:Stocking Temperature:Time	4	161	0.3571	0.8388

A.3 Supplemental Material for Chapter 3. Effects of Acclimation Temperature on the Thermal Physiology in two geographically distinct Populations of Lake Sturgeon (*Acipenser fulvescens*)

Table A.3.1 ANOVA results for mass, length, condition factor, hepatosomatic index (HSI), resting metabolic rate (RMR), maximum metabolic rate (MMR), forced aerobic scope (FAS), and mRNA transcript abundance of genes *HSP70*, *HSP90a*, *HSP90b*, *HIF-1α*, Na^+/K^+ *ATPase-α1* as well as enzymatic activation of glutathione peroxidase (GPx). The results for genes and enzymatic activities represent the effects of acclimation to 16, 20, and 24ºC on northern and southern populations of lake sturgeon, *Acipenser fulvescens*, within Manitoba, Canada. Time represents at the end of a 30 day acclimation, immediately following critical thermal maximum trials, and after a 3 day recovery. All factors were included in the model as fixed effects.

Measurement	Sources of Variation	Num df	Den df	F Value	P Value
Mass	Population	1	60	23.801	8.23E-06
	Acclimation Treatment	2	60	41.98	3.96E-12
	Population:Acclimation Treatment	2	60	3.522	0.0358
Length	Population	1	60	17.863	8.35E-05
	Acclimation Treatment	2	60	31.519	4.88E-10
	Population:Acclimation Treatment	2	60	3.012	0.0568
Condition Factor	Population	1	60	2.016	0.1609
	Acclimation Treatment	2	60	2.545	0.0869
	Population:Acclimation Treatment	2	60	3.678	0.0311
HSI	Population	1	60	14.228	0.000392
	Acclimation Treatment	2	60	39.15	2.31E-11
	Population:Acclimation Treatment	2	60	0.068	0.934735
RMR	Population	1	34	30.77	3.34E-06
	Acclimation Treatment	2	34	19.836	1.95E-06
	Population:Acclimation Treatment	1	34	2.816	0.1025
MMR	Population	1	34	10.3163	0.002881
	Acclimation Treatment	2	34	6.9968	0.002851
	Population:Acclimation Treatment	1	34	1.5511	0.221489
FAS	Population	1	34	2.2413	0.14359
	Acclimation Treatment	2	34	4.3208	0.02128
	Population:Acclimation Treatment	1	34	0.375	0.54439
HSP70	Population	1	112	0.7726	0.381304
	Acclimation Treatment	2	112	2.403	0.095097
	Time	2	112	46.3865	2.11E-15
	Population:Time	2	112	0.8663	0.423286
	Population:Acclimation Treatment	2	112	0.2002	0.818872
	Time:Acclimation Treatment	4	112	2.4737	0.04842
	Population:Acclimation Treatment:Time	4	112	3.1861	0.016087
HSP90a	Population	1	115	20.1266	1.73E-05
	Acclimation Treatment	2	115	1.8135	0.1677208
	Time	2	115	425.0012	< 2.2e-16
	Population:Time	2	115	1.2901	0.2791975
	Population:Acclimation Treatment	2	115	1.6435	0.1978149
	Acclimation Treatment:Time	4	115	5.1326	0.0007678
	Population:Acclimation Treatment:Time	4	115	7.7011	1.56E-05
HSP90b	Population	1	112	2.9871	0.086687
	Acclimation Treatment	2	112	1.9822	0.14257
	Time	2	112	5.667	0.004525
	Population:Time	2	112	0.1936	0.824237
	Population:Acclimation Treatment	2	112	0.8703	0.421645
	Acclimation Treatment:Time	4	112	0.1188	0.975552
	Population:Acclimation Treatment:Time	4	112	0.2939	0.88142
HIF-1α	Population	1	112	3.8984	0.05079
	Acclimation Treatment	2	112	11.7044	2.42E-05
	Time	2	112	2.3847	0.09678
	Population:Time	2	112	1.0739	0.34517
	Population:Acclimation Treatment	2	112	0.7231	0.4875
	Time:Acclimation Treatment	4	112	1.7356	0.14712
	Population:Acclimation Treatment:Time	4	112	0.1404	0.96685
Na^+/K^+ ATPase-α1	Population	1	115	5.3429	0.02259
	Acclimation Treatment	2	115	0.2848	0.75267
	Time	2	115	3.1672	0.04582
	Population:Time	2	115	0.2603	0.77124
	Population:Acclimation Treatment	2	115	0.4461	0.64122
	Acclimation Treatment:Time	4	115	0.2714	0.89587
	Population:Acclimation Treatment:Time	4	115	0.1655	0.95546
GPx	Population	1	111	5.7359	0.0183
	Acclimation Treatment	2	111	2.0871	0.12889
	Time	2	111	1.2561	0.28879
	Population:Time	2	111	0.1034	0.90183
	Population:Acclimation Treatment	2	111	0.6289	0.53509
	Acclimation Treatment:Time	4	111	1.9946	0.10025
	Population:Acclimation Treatment:Time	4	111	3.1255	0.01771

A.4 Supplemental Material for Chapter 4: Transcriptome-wide patterns reveal conserved and population-specific responses to increasing acclimation temperatures in developing lake sturgeon (*Acipenser fulvescens*)

Table A.4.1 Number of total uniquely annotated, upregulated, and downregulated transcripts, as well as upregulated and downregulated enrichment terms returned through gene enrichment with enrichR (Chen et al., 2013; Kuleshov et al., 2016; Xie et al., 2021), using the "GO Biological Processes_2018", "GO Molecular Function 2018", "GO Cellular Component 2018", "KEGG 2016" databases and filtering for an adjusted p value < 0.05.

Specific Contrast: Across Populations	Total Uniquely Annotated Genes	Upregulated Genes	Downregulated Genes	Upregulated Enrichment Terms	Downregulated Enrichment Terms
16	539	284	255	6	2
20	2,158	741	1417	4	67
24	2,279	1,115	1,164	17	86
Within Burntwood River					
16v20	2,345	1,042	1,321	2	117
16v24	1,109	483	626	6	9
Within Winnipeg River					
16v20	2,903	1,111	1,792	3	113
16v24	2,636	1,156	1,480	10	116

Supplemental Files A.4.1-8 are available and attached in a compressed zipped folder entitled "Chapter 4 Supplemental Files wsb_thesis" but were too large to append to the document.

A.6 Supplemental Material for Chapter 6: Investigating the effects of elevated temperatures on the innate immune responses of developing lake sturgeon (*Acipenser fulvescens*)

Table A.6.1 ANOVA results for critical thermal maximum (CT_{max}), resting metabolic rate (RMR), maximum forced metabolic rate (FMR), delta, metabolic scope and the mRNA transcript abundance of genes involved in the innate immune responses, *MyD88*, *GR1*, *IL-8*, *TICAM-1*, *Lysozyme-C*, *CPT1*, *IL-1β*, *StAR*, *C3*, *NF-kB*, *TNFa*, *TLR4*, *IgM*, *HSP90a*, and *PLA2*. These results represent the effects of thermal acclimation to 16 and 20°C for lake sturgeon, *Acipenser fulvescens*, within Manitoba, Canada. Time represents 4 h during exposure to bacterial lipopolysaccharides, 48 h during exposure to bacterial lipopolysaccharides, and following a 7 day recovery. LPS represents the different exposure concentrations of 0, 30, and 60 $\mu g.ml^{-1}$ bacterial lipopolysaccharide. Metabolic rate measurements and CT_{max} were taken on days 6 and 7, respectively during recovery. All factors were included in the model as fixed effects.

Physiological Metric	Measurement	Sources of Variation	Num df	Den df	F Value	P Value
post-trial recovery MO_2 and CT_{max}	CTmax	Treatment	115	1	260.338	<2e-16 ***
		LPS	115	2	2.83	0.0632
		Treatment : LPS	115	1	4.239	0.0418 *
	RMR	Treatment	34	1	2.484	0.124
		LPS	34	2	0.886	0.422
		Treatment : LPS	34	1	0.027	0.87
	FMR	Treatment	33	1	0.183	0.67148
		LPS	33	2	3.518	0.04123 *
		Treatment : LPS	33	1	12.534	0.00121 **
	Delta	Treatment	33	1	0.581	0.45139
		LPS	33	2	14.754	2.65e-05 ***
		Treatment : LPS	33	1	9.875	0.00353 **
	Metabolic Scope	Treatment	33	1	0.366	0.54912
		LPS	33	2	12.724	8.01e-05 ***
		Treatment : LPS	33	1	7.8	0.00863 **

Table continued on next page…

Three-way ANOVA Gene Expression Analysis	*MyD88*	Temperature	144	1	10.5896	0.0014173 **
		LPS	144	2	2.0786	0.1288465
		Time	144	2	113.034	< 2.2e-16 ***
		Temperature:LPS	144	2	1.5683	0.2119424
		Temperature:Time	144	2	33.3917	1.219e-12 ***
		LPS:Time	144	3	31.4905	1.031e-15 ***
		Temperature:LPS:Time	144	2	9.0052	0.0002065 ***
	GR1	Temperature	144	1	19.93	1.610e-05 ***
		LPS	144	2	0.702	0.497291
		Time	144	2	26.4002	1.707e-10 ***
		Temperature:LPS	144	2	1.4208	0.24488
		Temperature:Time	144	2	16.1478	4.707e-07 ***
		LPS:Time	144	3	4.1821	0.007144 **
		Temperature:LPS:Time	144	2	3.9141	0.022117 *
	IL-8	Temperature	144	1	16.2058	9.151e-05 ***
		LPS	144	2	33.0061	1.587e-12 ***
		Time	144	2	103.0855	< 2.2e-16 ***
		Temperature:LPS	144	2	5.4684	0.00514 **
		Temperature:Time	144	2	14.2577	2.241e-06 ***
		LPS:Time	144	3	14.1037	4.132e-08 ***
		Temperature:LPS:Time	144	2	2.4515	0.08975 .
	TICAM-1	Temperature	144	1	0.0877	0.7675
		LPS	144	2	2.0303	0.135
		Time	144	2	12.5937	9.112e-06 ***
		Temperature:LPS	144	2	0.8077	0.4479
		Temperature:Time	144	2	24.147	9.048e-10 ***
		LPS:Time	144	3	18.7428	2.554e-10 ***
		Temperature:LPS:Time	144	2	1.3533	0.2616
	HSP70	Temperature	144	1	13.1875	0.000391 ***
		LPS	144	2	4.9647	0.008221 **
		Time	144	2	84.8483	< 2.2e-16 ***
		Temperature:LPS	144	2	3.054	0.050236 .
		Temperature:Time	144	2	31.3446	5.003e-12 ***
		LPS:Time	144	3	21.499	1.452e-11 ***
		Temperature:LPS:Time	144	2	14.6933	1.560e-06 ***
	Lysozyme-C	Temperature	144	1	3.1637	0.077404 .
		LPS	144	2	5.7101	0.004107 **
		Time	144	2	99.0261	< 2.2e-16 ***
		Temperature:LPS	144	2	1.0916	0.33844
		Temperature:Time	144	2	7.1579	0.001087 **
		LPS:Time	144	3	18.8845	2.198e-10 ***
		Temperature:LPS:Time	144	2	7.1341	0.001111 **
	CPT1	Temperature	141	1	12.2893	0.0006112 ***
		LPS	141	2	3.7265	0.0264795 *
		Time	141	2	28.7576	3.353e-11 ***
		Temperature:LPS	141	2	5.3849	0.0055768 **
		Temperature:Time	141	2	27.9978	5.764e-11 ***
		LPS:Time	141	3	24.496	8.091e-13 ***
		Temperature:LPS:Time	141	2	1.726	0.1817274
	IL1-ß	Temperature	142	1	0.1371	0.71176
		LPS	142	2	137.5081	< 2.2e-16 ***
		Time	142	2	43.7184	1.605e-15 ***
		Temperature:LPS	142	2	1.2378	0.29314
		Temperature:Time	142	2	11.67	2.031e-05 ***
		LPS:Time	142	3	21.1893	2.112e-11 ***
		Temperature:LPS:Time	142	2	4.5556	0.01209 *
	StAR	Temperature	136	1	1.9038	0.169919
		LPS	136	2	0.0146	0.985518
		Time	136	2	1.3468	0.263525
		Temperature:LPS	136	2	1.5035	0.226022
		Temperature:Time	136	2	5.7472	0.004017 **
		LPS:Time	136	3	8.705	2.528e-05 ***
		Temperature:LPS:Time	136	2	0.307	0.736166
	C3	Temperature	144	1	27.0093	6.805e-07 ***
		LPS	144	2	2.0522	0.13219
		Time	144	2	16.6353	3.164e-07 ***
		Temperature:LPS	144	2	0.6371	0.53033
		Temperature:Time	144	2	14.4846	1.855e-06 ***
		LPS:Time	144	3	11.9578	4.889e-07 ***
		Temperature:LPS:Time	144	2	2.7122	0.06978 .
	NF-kB1	Temperature	135	1	11.0453	0.001145 **
		LPS	135	2	1.7208	0.182833
		Time	135	2	33.4792	1.557e-12 ***
		Temperature:LPS	135	2	1.6191	0.201892
		Temperature:Time	135	2	6.3789	0.002254 **
		LPS:Time	135	3	7.7385	8.286e-05 ***
		Temperature:LPS:Time	135	2	0.4272	0.653237
	TNFa	Temperature	136	1	4.9869	0.027174 *
		LPS	136	2	0.524	0.593355
		Time	136	2	12.3301	1.199e-05 ***
		Temperature:LPS	136	2	1.6694	0.192197
		Temperature:Time	136	2	4.6095	0.011563 *
		LPS:Time	136	3	5.0163	0.002495 **
		Temperature:LPS:Time	136	2	0.9981	0.37127
	TLR4	Temperature	142	1	0.5485	0.46015
		LPS	142	2	3.1112	0.04760 *
		Time	142	2	12.6395	8.874e-06 ***
		Temperature:LPS	142	2	1.7599	0.17579
		Temperature:Time	142	2	4.224	0.01652 *
		LPS:Time	142	3	2.9961	0.03287 *
		Temperature:LPS:Time	142	2	0.6392	0.52922
	IgM	Temperature	142	1	38.8382	4.938e-09 ***
		LPS	142	2	1.1831	0.309343
		Time	142	2	16.3713	4.001e-07 ***
		Temperature:LPS	142	2	0.6656	0.515553
		Temperature:Time	142	2	0.5406	0.583586
		LPS:Time	142	3	4.0072	0.008969 **
		Temperature:LPS:Time	142	2	1.3141	0.271976
	HSP90a	Temperature	143	1	68.2292	8.901e-14 ***
		LPS	143	2	15.7094	6.802e-07 ***
		Time	143	2	58.3946	< 2.2e-16 ***
		Temperature:LPS	143	2	0.7631	0.46809
		Temperature:Time	143	2	5.3288	0.00586 **
		LPS:Time	143	3	17.5479	9.363e-10 ***
		Temperature:LPS:Time	143	2	11.3516	2.657e-05 ***
	PLA2	Temperature	139	1	37.4268	9.096e-09 ***
		LPS	139	2	3.415	0.03566 *
		Time	139	2	19.6777	2.986e-08 ***
		Temperature:LPS	139	2	0.0452	0.95582
		Temperature:Time	139	2	9.9269	9.337e-05 ***
		LPS:Time	139	3	10.655	2.382e-06 ***
		Temperature:LPS:Time	139	2	3.8104	0.02449 *

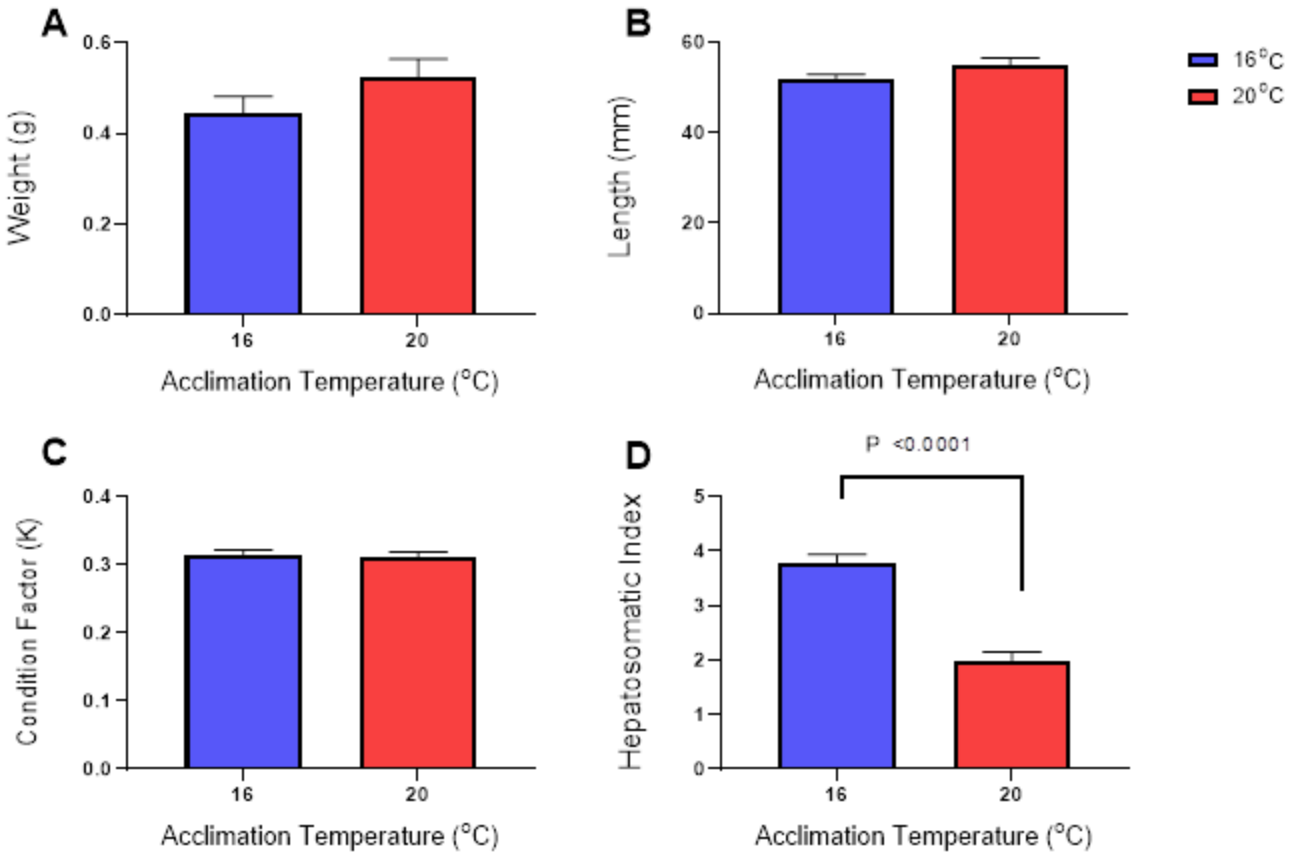

Figure A.6.1 A) Weight (g), B) length (mm), C) condition factor (K), and D) hepatosomatic index (HSI) of developing lake sturgeon, *Acipenser fulvescens*, following 28 days acclimation to 16 and 20°C. Significant differences between treatments were determined by a Welch Two Sample t-test ($P < 0.05$). Data are expressed as mean +/- SEM ($n = 10$).

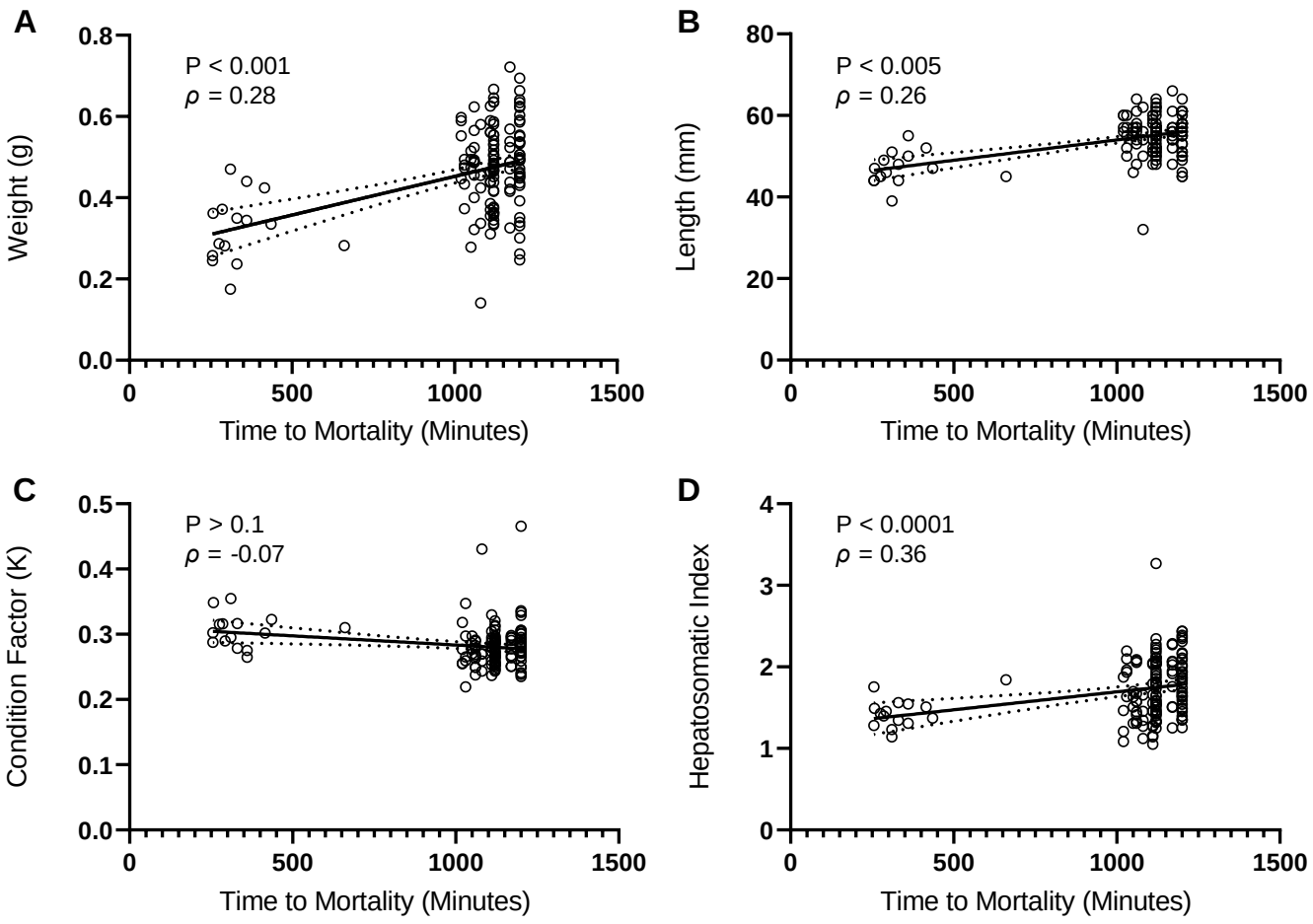

Figure A.6.2 The relationship between A) weight (g), B) length (mm), C) condition factor (K) and D) hepatosomatic index (HSI) and time to mortality of 20°C acclimated developing lake sturgeon, *Acipenser fulvescens*, following exposure to 60 µg.ml^{-1} lipopolysaccharides. Significance was determined by Spearman's correlation. The solid line throughout the graph represents the best fit straight line surrounded by dotted lines representing the 95% confidence interval. Open circles represent individual lake sturgeon (n = 142).

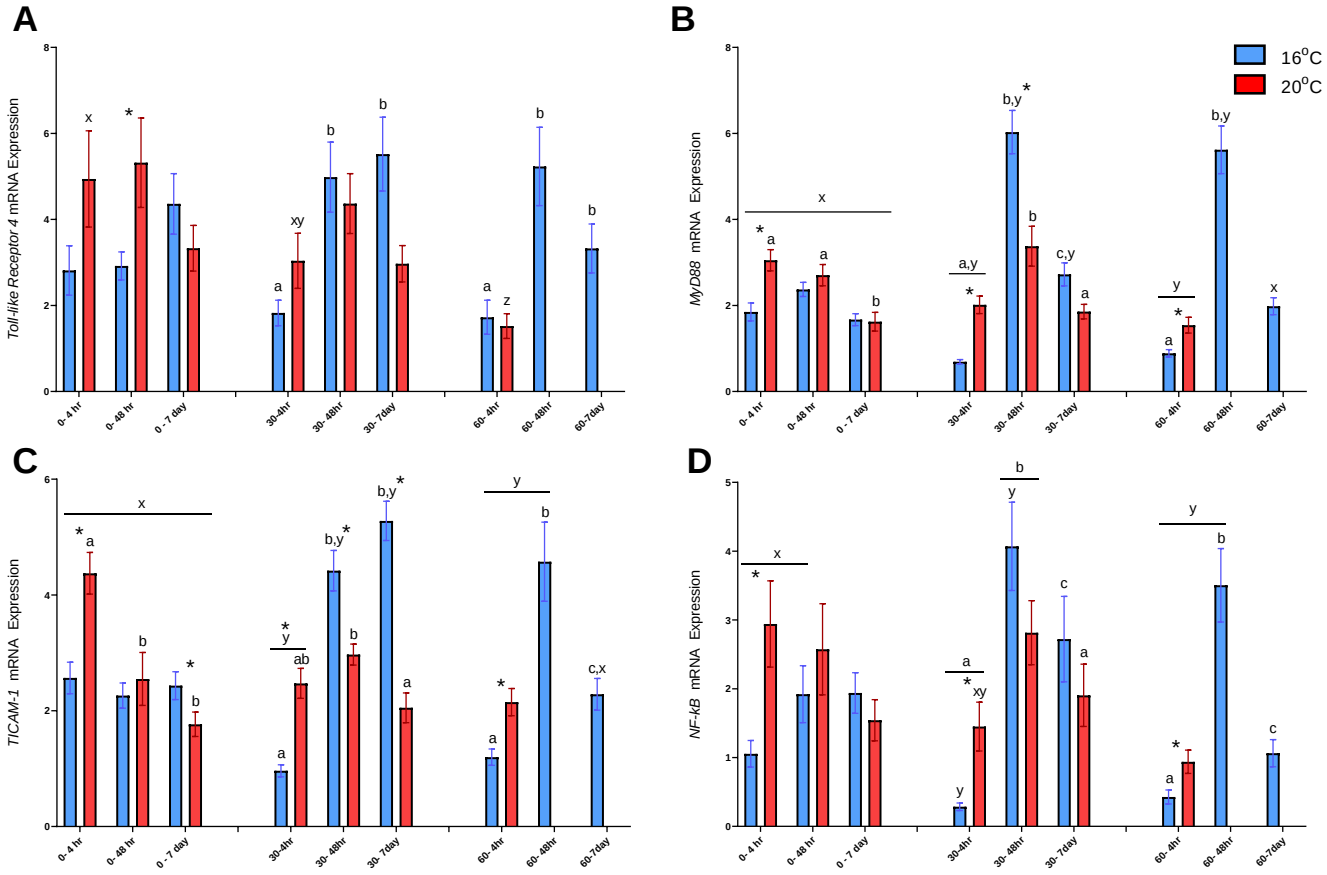

Figure A.6.3 Whole-body mRNA transcript abundance of genes involved in innate immune reception A) *Toll-like Receptor 4*, B) *MyD88*, C) *TICAM-1*, and D) *NF-kB* in developing lake sturgeon, *Acipenser fulvescens*, acclimated to 16 and 20ºC and then exposed to 0, 30, and 60 $\mu g.ml^{-1}$ of bacterial lipopolysaccharides, over a timeseries of 4 h exposure, 48 h exposure, and following a 7 day lipopolysaccharides-free recovery. Asterisks represent significance between acclimation treatments. Lowercase letters a, b, c represent significance between timepoints, within an exposure concentrations and timepoint. Lowercase letters x, y, z represent significance in a given timepoint and acclimation temperature, throughout exposure concentrations ($P < 0.05$; three-factor ANOVA). Data are expressed as +/- SEM (n = 8-10).

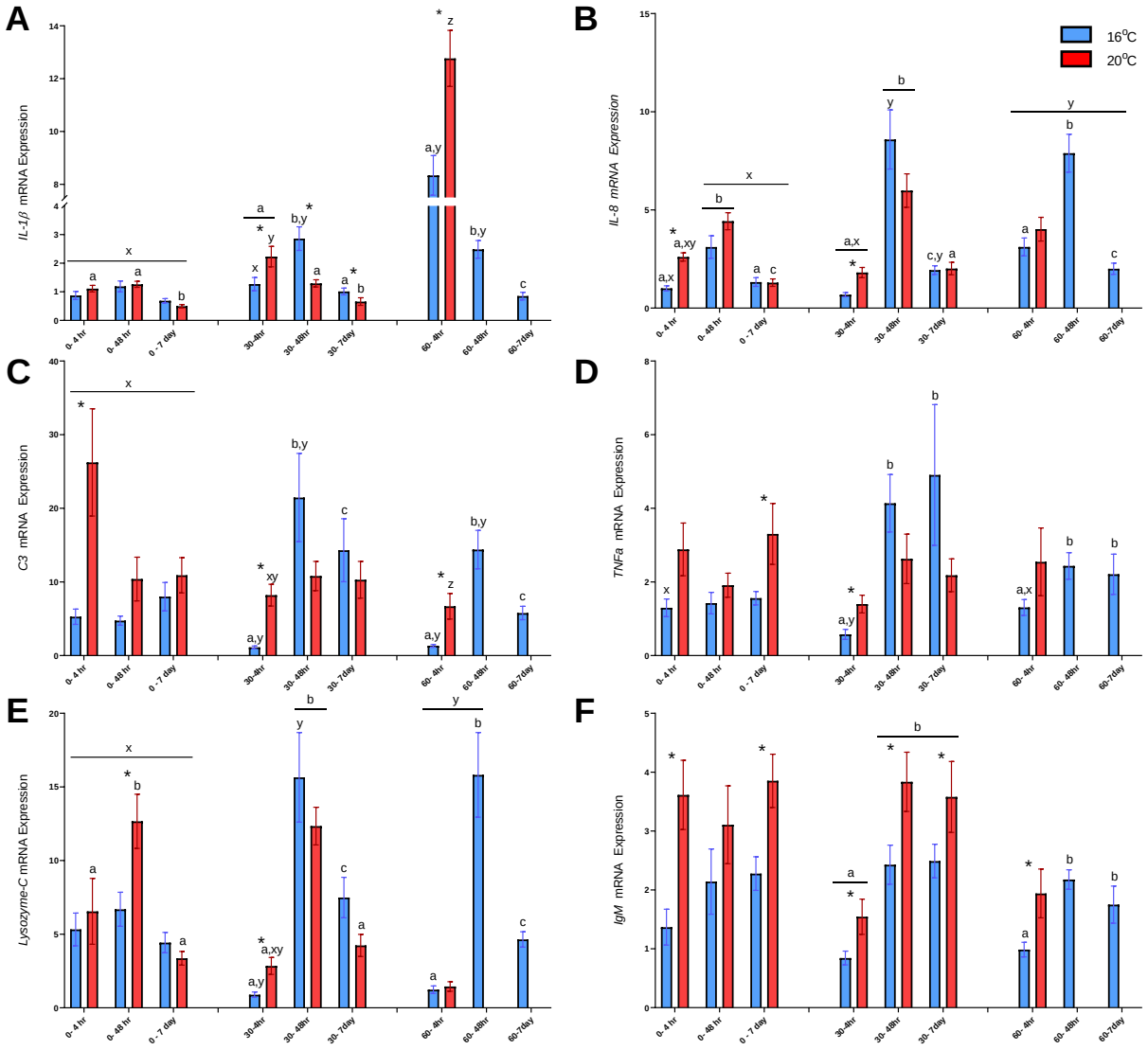

Figure A.6.4 Whole-body mRNA transcript abundance of genes involved in the innate immune response A) *IL-1β*, B) *IL-8*, C) *C3*, D) *TNFa*, E) *Lysozyme-C*, and F) *IgM* in developing lake sturgeon, *Acipenser fulvescens*, acclimated to 16 and 20ºC and then exposed to 0, 30, and 60 µg.ml^{-1} of bacterial lipopolysaccharides, over a timeseries of 4 h exposure, 48 h exposure, and following a 7 day lipopolysaccharides-free recovery. Asterisks represent significance between acclimation treatments. Lowercase letters a, b, c represent significance between timepoints, within an exposure concentrations and timepoint. Lowercase letters x, y, z represent significance in a given timepoint and acclimation temperature, throughout exposure concentrations ($P < 0.05$; three-factor ANOVA). Data are expressed as +/- SEM (n = 9-10).

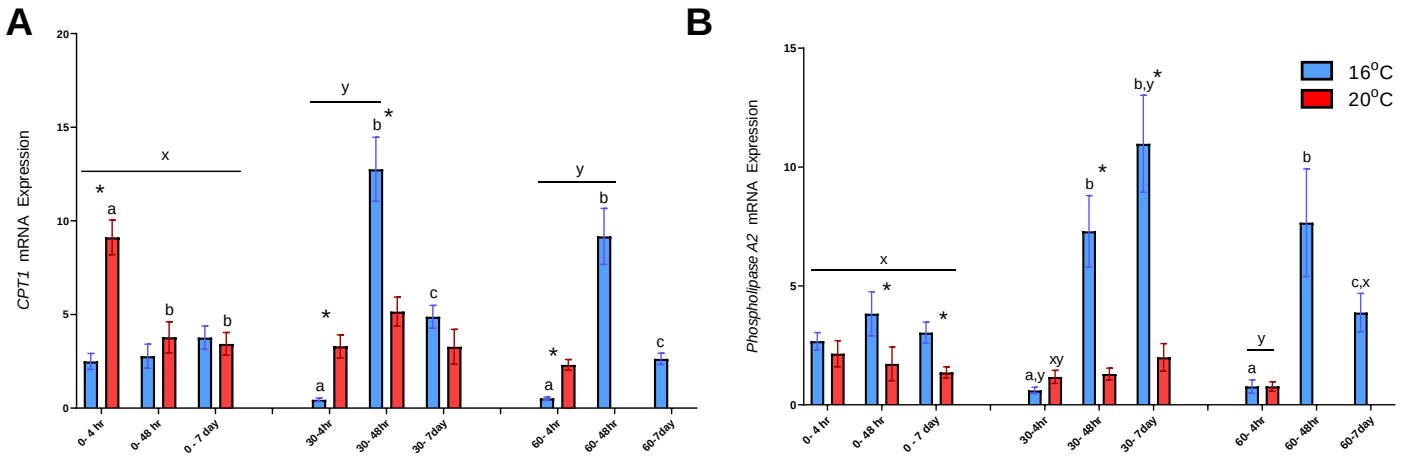

Figure A.6.5 Whole-body mRNA transcript abundance of genes involved in the fatty acid response A) *CPT1* and B) *Phospholipase A2* in developing lake sturgeon, *Acipenser fulvescens*, acclimated to 16 and 20ºC and then exposed to 0, 30, and 60 µg.ml^{-1} of bacterial lipopolysaccharides, over a timeseries of 4 h exposure, 48 h exposure, and following a 7 day lipopolysaccharides-free recovery. Asterisks represent significance between acclimation treatments. Lowercase letters a, b, c represent significance between timepoints, within an exposure concentrations and timepoint. Lowercase letters x, y, z represent significance in a given timepoint and acclimation temperature, throughout exposure concentrations ($P < 0.05$; three-factor ANOVA). Data are expressed as +/- SEM (n = 8-10).

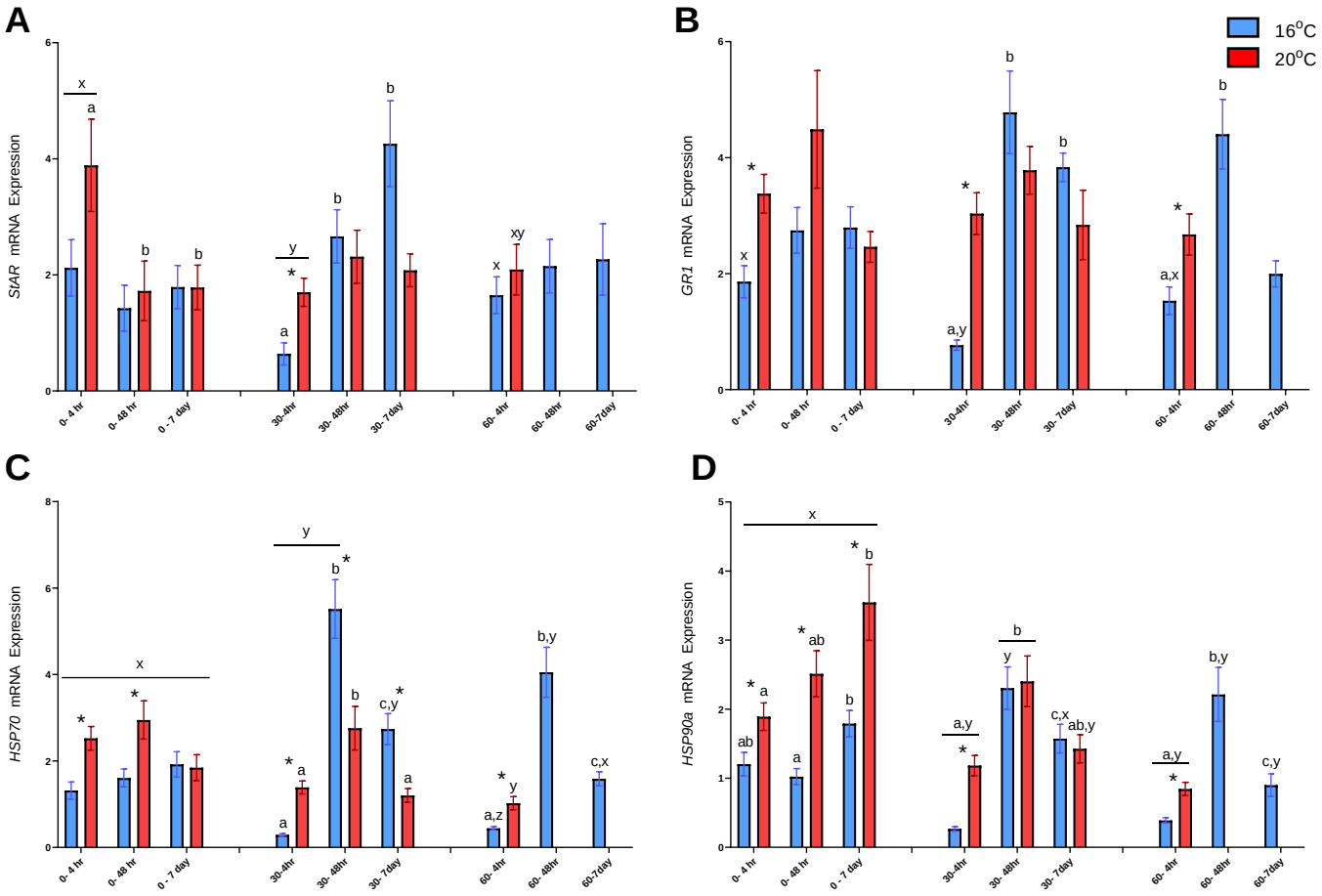

Figure A.6.6 Whole-body mRNA transcript abundance of genes involved in the glucocorticoid stress response A) *StAR*, B) *GR1*, C) *HSP70*, D) *HSP90a* in developing lake sturgeon, *Acipenser fulvescens*, acclimated to 16 and 20°C and then exposed to 0, 30, and 60 µg.ml^{-1} of bacterial lipopolysaccharides, over a timeseries of 4 h exposure, 48 h exposure, and following a 7 day lipopolysaccharides-free recovery. Asterisks represent significance between acclimation treatments. Lowercase letters a, b, c represent significance between timepoints, within an exposure concentrations and timepoint. Lowercase letters x, y, z represent significance in a given timepoint and acclimation temperature, throughout exposure concentrations ($P < 0.05$; three-factor ANOVA). Data are expressed as +/- SEM (n = 7-10).

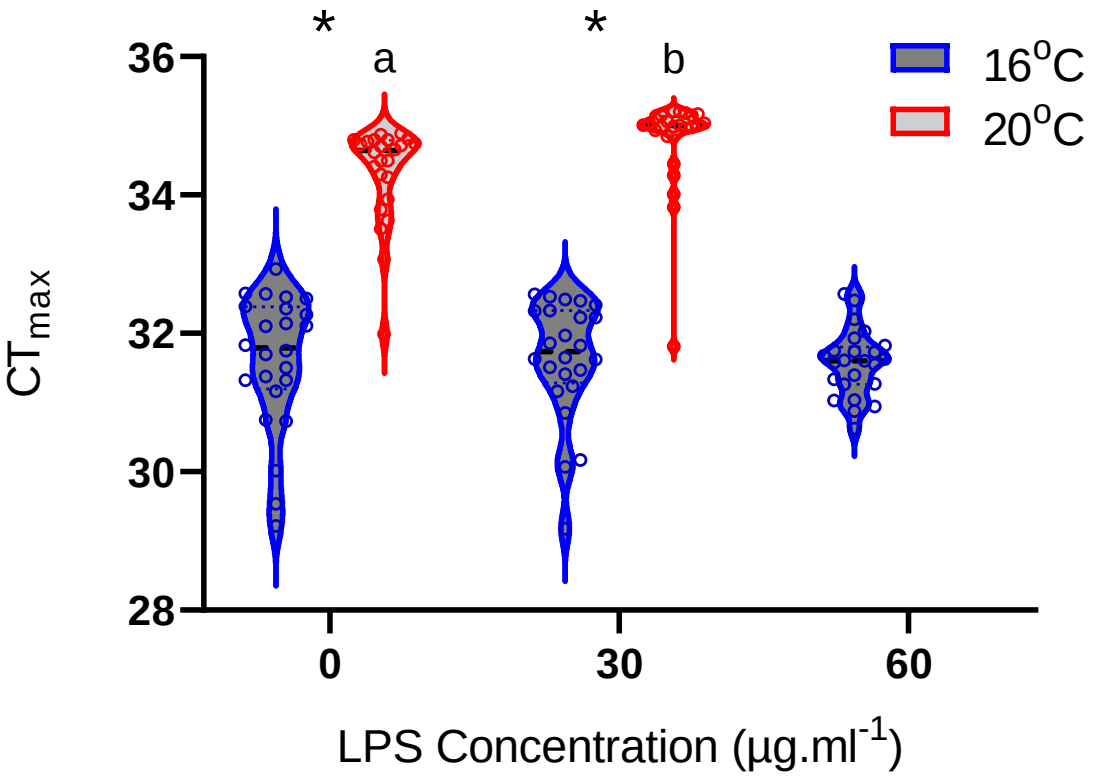

Figure A.6.7 Critical thermal maximum (CT_{max}) of 16 and 20°C acclimated developing lake sturgeon, *Acipenser fulvescens*, following a 7 day recovery from 48 h exposure trials in lipopolysaccharide concentrations of 0, 30, and 60 µg.ml^{-1}. Differences between treatments were determined by two-factor ANOVA ($P < 0.05$) followed by Tukey's honestly significant different post-hoc test. *'s represent significance between 16 and 20°C acclimation treatments within a lipopolysaccharide exposure concentration. Lowercase letters a and b represent significance across treatment concentrations within a single acclimation treatment (n = 24).

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