EFFECT OF ELEVATED EMBRYONIC INCUBATION TEMPERATURE ON WHITEFISH TEMPERATURE PREFERENCE

Effect of elevated embryonic incubation temperature on the temperature preference of juvenile lake (*Coregonus clupeaformis*) and round whitefish (*Prosopium cylindraceum*)

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Lay Abstract

Lake and round whitefish are cold-adapted freshwater species that spawn in shallow coastal water in the Laurentian great lakes. Anthropogenic warming from various sources (e.g. climate change, thermal effluent) has been shown to reduce survival of whitefish embryos, but few studies have investigated the effects of elevated embryonic incubation temperatures on surviving juveniles. Fish typically prefer a small range of temperatures (temperature preference) that they will occupy if given a choice, which can be affected by a variety of factors including early life thermal exposure. This thesis shows round whitefish incubated at elevated temperatures (2°C, 6°C) display decreased temperature preference up to 12 months post-hatch, while lake whitefish had similar temperature preference regardless of incubation temperature. Therefore, this thesis provides more evidence that round whitefish are a more thermally sensitive species.

Abstract

Lake (*Coregonus clupeaformis*) and round (*Prosopium cylindraceum*) whitefish are two species of cold-adapted freshwater fish that spawn in shallow (<10m) cobble beds in the Laurentian great lakes. Developing whitefish embryos are potentially exposed to various anthropogenic sources of warming, including climate change and thermal effluents discharged in the nearshore environment. Several studies have investigated the effects of elevated incubation temperatures on whitefish embryos (particularly lake whitefish) but little work has been done to examine post-hatch effects. Thermal preferenda (temperature preference) describe the range of temperatures an organism will occupy when given a choice and are traditionally thought to be species-specific. Temperature preference can be modulated by a variety of abiotic and biotic factors including environmental temperature and is typically correlated with optimal growth temperature for a species. Assays for thermal preferenda require at least 24 hours, which includes a long tank-acclimation period that limits throughput and thus impacts replication in the study. A shuttle box thermal preference assay was optimized from 24-hours to 4-hours; length of acclimation time and trial length had no significant impact on thermal preference. Whitefish were incubated at natural and elevated temperatures until hatching; all groups were moved to common garden conditions (15°C) during the post-hatching stage. Temperature preference was determined at 12 months of age; lake whitefish were also tested at 8 months. Round whitefish displayed a significant decrease in temperature preference when incubated at 2°C and 6°C compared to 0.5°C. Lake whitefish had similar temperature preferences regardless of age and incubation temperature. This suggests that there is a difference in thermal tolerance between these species, as round whitefish were more sensitive to elevated incubation treatments. This thesis identified a persistent effect of elevated incubation treatments on the thermal preference of juvenile round whitefish, lasting up to 12 months post-hatch, which highlights the importance of examining sub-lethal thermal effects and thermal plasticity of cold-adapted species.

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Contributions

Experimental work in this thesis was designed and conducted by Adam Harman, with Bhuvan Aggarwhal assisting with data collection (Chapter 3), and Hannah Mahoney helping with data collection and analysis (Chapter 3). Guidance provided by Dr. Joanna Wilson, Dr. Meghan Fuzzen, Dr. Douglas Boreham, Dr. Richard Manzon and Dr. Chris Somers. Embryo collection was completed by Adam Harman, Dr. Meghan Fuzzen, and Lisa Stoa, and provided by Tim Drew, MNRF, Sharbot Lake White Fish culture station. Whitefish husbandry was carried out by Adam Harman, Dr. Meghan Fuzzen, Lisa Stoa, and undergraduate volunteers Urvi Pajankar, and Akanksha Arora. All chapters in this thesis were prepared by Adam Harman, and edited by Dr. Joanna Wilson and Dr. Meghan Fuzzen (Chapter 2 only). The manuscripts in preparation (Chapters 2) were also edited by Dr. Meghan Fuzzen, Dr. Douglas Boreham, Dr. Richard Manzon and Dr. Chris Sommers.

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List of Abbreviations

LWF – Lake Whitefish (*Coregonus clupeaformis*)

RWF – Round Whitefish (*Prosopium cylindraceum*)

T_{pref} – Temperature Preference (°C)

DNA – Deoxyribonucleic Acid

ANOVA – Analysis of Variance

ΔT – Change in Temperature

IPCC – Intergovernmental Panel on Climate Change

INCR – Increasing Side of Shuttle Box

DECR – Decreasing Side of Shuttle Box

CANDU – Canadian deuterium uranium

Chapter 1

Introduction

1.1 Ecology of Lake and Round Whitefish

Lake whitefish (*Coregonus clupeaformis*; LWF) are a cold-water adapted species that occupies an extensive range in North America, spanning from Northern Canada to parts of the northern United States (Ebener et al, 2008). LWF are found in the limnetic zone in all the Laurentian Great Lakes. Round whitefish (*Prosopium cylindraceum*; RWF) have a similar distribution and tend to be more littoral, occupying shallower coastal waters. LWF grow larger, mature later and have a longer lifespan than RWF (Morin et al., 1982). LWF have been found to move freely between management zones but show a high degree of natal homing during spawning (Li et al., 2017). While large-scale movements within Lake Huron have not been identified for RWF, and little is known about their site fidelity, large ecological niche (determined with stable isotopes) and microsattelite data suggest mixing within Lake Huron for both LWF and RWF (Graham et al., 2016). LWF and RWF are able to co-exist within the same lake due to differential resource and habitat use.

Since adult LWF occupy deeper waters (18-90m) in the limnetic zone they must migrate to shallow, coastal water to spawn. Whitefish spawn in early to mid-November when water temperatures are cooling towards winter temperatures and the eggs incubate for 5-6 months before hatching in the spring as lake ice melts (Scott & Crossman, 1979). LWF typically spawn when surface water temperature drops below 8°C, whereas RWF begin spawning below 4.5°C (Patrick et al, 2013). Depending on winter conditions, this can lead to several weeks of delay between LWF and RWF spawning. Whitefish typically broadcast eggs on hard stone or cobble substrates at less than 10m of depth and embryos develop within lodgment sites in the substrate (Ebener et al, 2008). These lodgment sites are microclimates in the cobble, creating spatially variable environments for embryonic development. Whitefish eggs will remain in cobble substrate until surface ice begins to melt in the spring (April – May). This is an unusual reproduction strategy among great lakes fishes but is facilitated by stable over-winter temperature with minimal natural fluctuations (Scott & Crossman, 1979). After hatching, larval whitefish remain in the littoral zone where they feed on plankton and grow (Roseman & O'Brian, 2013). Field sampling found high densities of larval whitefish close to shore, with an inverse relationship between density and distance from shore (Roseman & O'Brian, 2013). Therefore, aggregations of whitefish larvae can be found from mixed spawning locations. Larvae reared in different incubation sites will spatially cluster as larvae, experiencing "common garden" (i.e. identical environmental) conditions post-hatch.

LWF are a commercially and culturally important species, supporting the largest commercial fishery in the great lakes and local indigenous communities through subsistence fishing (Ebener et al, 2008). In 2000, the LWF fishery was worth 18 M USD, which accounted for one third of the total revenue of the Great Lakes fisheries (Kinnunen, 2003). Most commercial interest in round whitefish is in Lake Michigan, however due to its small size and fluctuating abundance it cannot support a large fishery (Bailey, 1962).

1.2 Elevated Embryonic Incubation Alters Whitefish Development

LWF and RWF live sympatrically in the great lakes but occupy different zones of the lake. RWF tend to be more littoral, associating with warmer shallow coastal water (Rawson, 1951). Counterintuitively, RWF appear more sensitive to increases in ambient water temperature, including during development (Lim et al., 2017, 2018; Griffiths, 1980). Whitefish embryos incubated at natural temperatures (0.5-2°C) will typically experience low background mortality; increasing incubation temperature will increase mortality (Brooke, 1975; Price 1940; Lim et al., 2017, 2018; Mitz et al., 2019; Eme et al., 2018; Mueller et al., 2017; Lee et al., 2016). While elevated embryonic temperature leads to increased mortality in both species, RWF experience 30-40% higher mortality than LWF when incubated at a constant 8°C (Lim et al., 2017, 2018).

Elevated temperature treatments during whitefish development have typically used either constant (Brooke, 1975; Price 1940; Lim et al., 2017, 2018; Mitz et al., 2019; Eme et al., 2015, 2018; Mueller et al., 2015, 2017; Lee et al., 2016) or seasonal (Patrick et al., 2013; Lim et al., 2017, 2018; Thome, In Press) profiles. A seasonal profile includes a winter decline and a spring incline in temperature, typically at 1 or 2°C per week, and a stable incubation period in between. Alternatively, systems where natural lake water temperatures dictate tank temperatures (Patrick et al., 2013; Thome, In Press) can provide a seasonal temperature profile. The seasonal temperature profile is meant to closely follow or mimic natural lake conditions (Patrick et al., 2013). Increasing incubation temperatures under either seasonal or constant temperature profiles have similar effects on the development of whitefish. Whitefish exposed to constant (Price, 1940; Brooke, 1975; Lim et al., 2017, 2018; Mitz et al., 2019; Eme et al., 2015, 2018; Mueller et al., 2015, 2017; Lee et al., 2016) or seasonal (Patrick et al., 2013; Lim et al., 2017, 2018; Thome, In Press) elevated incubation temperatures of at least +2°C develop more rapidly than those at control/natural temperatures. For example, LWF and RWF raised at 2°C take 176 and 140 days to reach 50% hatch; LWF and RWF reached 50% hatch in 67 and 60 days at 8°C (Lim et al., 2017, 2018). Change in hatch timing is ecologically important as larval emergence is timed with food availability and spring plankton blooms (Pothoven & Nelepa, 2006). A disruption of this timing may lead to whitefish hatching without appropriate or available prey, which could lead to significant larvae mortality.

Whitefish hatching significantly earlier than embryos at lower temperatures appear underdeveloped (e.g. in jaws and fins; Mitz et al., 2019) with altered morphology (Lim et al., 2017, 2018; Price, 1940; Brooke, 1975; Mueller et al., 2015; Eme et al., 2015, 2018; Lee et al., 2016). Early hatched fish had a shorter length, reduced weight (Brooke, 1975; Price, 1940; Mitz et al., 2019; Mueller et al., 2015; Eme et al., 2018; Lee et al., 2016), larger yolk sac, and smaller eyes (Mitz et al., 2019; Lee et al., 2016). The altered morphology and under development at hatching could be problematic for larval whitefish because LWF are primarily visual hunters (Pothoven & Nelepa, 2006) and generally start feeding soon after hatch. Larger yolk sacs combined with less developed fins will likely decrease swimming efficiency in whitefish hatchlings, smaller jaws limits size of prey that can be consumed, and smaller eyes may impact vision. It is unknown whether this phenotype would significantly decrease survival of larval whitefish, as they could potentially survive off the larger yolk sac while continuing to develop and grow. Yet, developing LWF embryos exposed to elevated water temperatures had increased embryonic metabolic and heart rates (Eme et al., 2015). Dechorionated embryos consumed an average of 10 times more oxygen, suggests that the chorion is a barrier to oxygen transport near hatching (Eme et al., 2015). These data imply that embryos exposed to elevated temperatures during development may use energy stores more rapidly due to early hatching.

1.3 Incubation Temperature Induces Phenotypic Plasticity

According to the compensation/allocation model, basal metabolic demand competes with other physiological processes for acquired energy (Killen, 2014). Basal metabolic demand accounts for the energy required for basic function and increases with ambient temperature. Fish in warmer water will have an elevated basal metabolic rate, and therefore less available energy for other activities (aerobic scope). Aerobic scope is the difference between maximal and basal metabolic rate and is generally positively correlated with the fitness of an individual (Killen, 2014). Individuals with higher basal metabolic rates will have increased foraging demands at equivalent ambient temperatures, and therefore may select colder water temperatures in order to compensate. This is a behavioral adaptation that allows fish to maximize aerobic scope in unfavorable conditions. An increase in basal metabolic rate caused by fasting lead to common minnows (*Phoxinus phoxinus*) selecting colder temperatures, highlighting an inverse relationship between basal metabolism and thermal preference (Killen, 2014). Fish embryos display many plastic traits that can be influenced by conditions during development (Jonsson & Jonsson, 2019). In order to maximize survival, fish use the embryonic period to predict thermal conditions in adulthood.

Phenotypic plasticity is the ability of individual genotypes to produce altered phenotypes when exposed to different conditions (Jonsson & Jonsson, 2019). Traits expressed by juveniles and adults can be modified by environmental conditions experienced during embryonic development without changing the DNA sequence. This is primarily accomplished through epigenetic mechanisms, as adaptations can occur within an individual's lifespan. Salinas & Munch (2012) were able to show that fathead minnows (*Pimephales promelas*) performed best at their mother's holding temperature, suggesting they were adaptively programmed to perform best at the expected temperature. Early development plays an important role in the development of behavior, social skills and stress responsiveness in mammals; recent research suggests it might have a similar role in fish (Jonsson & Jonsson, 2019).

Recent studies (Braña & Ji 2000, Ospina-Álvarez & Piferrer 2008, Potter et al. 2011) have shown that embryonic thermal conditions can influence juvenile growth, energy store and sex determination in fish. These changes seem to be an anticipatory response to a predicted adult environment, based on embryonic conditions. Female Atlantic salmon spawning in warmer streams produce larger eggs with increased fitness (Jonsson & Jonsson 2018). However, these adaptations will likely only be beneficial if embryonic conditions persist into adulthood. Acute exposure to increased incubation temperature could cause adaptations to temporary conditions, possibly leading to maladaptation. Whitefish are ectothermic poikilotherms, meaning their body temperature fluctuates considerably, and they do not produce internal heat metabolically (Stevens & Fry, 1970). In order to thermoregulate, whitefish must migrate to more favorable water conditions and avoid unfavorable conditions, as the ambient water temperature is directly correlated with the internal body temperature (Stevens & Fry, 1970). Thus, larval and adult fish, but not embryos, have direct control over the temperature conditions they experience and can modulate their internal body temperature behaviorally.

1.4 Thermal Preferenda

Most motile species exhibit a “thermal preferenda” or a range of preferred temperatures that, when given the opportunity, individuals will tend to aggregate (Reynolds & Casterlin, 1979). This temperature should theoretically correlate with the optimum growth temperature, but there are several other important factors that can influence thermal preferenda, including: photoperiod, salinity, chemicals/pharmaceuticals, age of fish, size of fish, bacterial infection, nutritional state/food availability and biotic factors (as reviewed in Reynolds & Casterlin, 1979). Therefore, it is only possible to assess a species’ optimal growth temperature via measuring their thermal preferenda when these other influential factors are controlled.

Thermal preferenda can be separated into acute and final; typically determined after different lengths of time in a tank system with a gradient or range of temperatures. Acute preferenda are heavily influenced by thermal acclimation (Reynolds & Casterlin, 1979) and are typically observed over short periods ≤2 hours. Acute preferenda have the advantage that they can be more rapidly determined, however you must determine acute preferenda for a range of acclimation temperatures and use these data in a graphical analysis to estimate the final preferenda (Reynolds & Casterlin, 1979). Final preferenda are theoretically only observed over longer periods of time; assays are typically >24 hours. Once acclimated to a novel tank, fish will begin to gravitate towards their final preferenda. The importance of a long period for tank acclimation was generally accepted, however acclimation time did not have a significant effect on the thermal preference of juvenile cutthroat trout (*Oncorhynchus clarkii lewisi;* Macnaughton et al. 2018).

The definition of final preferenda assumes a common temperature preference that all members of the same species will ultimately display (Jobling, 1981). This may be accurate for small warm-water fish, like goldfish (*Carassius auratus*) and bluegill sunfish (*Lepomis macrochirus*), that were used for much of the early preferenda work (Reynolds and Casterlin, 1979) because they experience warm, stable temperatures across their distribution. The same cannot be said for larger temperate species that have consistently dealt with extreme temperature changes over their evolutionary history. Atlantic cod (*Gadus morhua*) display significantly different preferenda across their distribution due to a polymorphic haemoglobin molecule (Petersen and Stefensen, 2003), while juvenile coho salmon (*Oncorhynchus kisutch*) have distinct thermal preferences that align with the thermal profile of home streams (Konecki, 1995). Arctic charr (*Salvelinus alpinus*), which are exposed to repeated freezing and thawing of lakes/streams, experience seasonal changes in preferenda (Mortensen et al., 2007). The idea that a species should display a single temperature preference regardless of previous thermal experience was proposed by Fry in 1947. This perspective of thermal preference oversimplifies the influence of previous thermal experience, especially the importance of early life thermal history. There are functional links between embryonic conditions and adult/juvenile fitness (Weber et al. 2012) and this is likely true for thermal preference.

1.5 Assays of Thermal Preference

Thermal preference is usually determined using a behavioral assay that exposes fish to a range of temperatures and movement is recorded. Temperature preference is calculated as the average (median or mean) occupied temperature during the trial. Therefore, to determine temperature preference, data on water temperature and fish location must be accurately collected over the entire trial.

Traditionally, thermal preferenda experiments were designed using a gradient or acute method (Jobling, 1981). The gradient method involves creating a vertical or horizontal thermal gradient within a tank and tracking the position of fish in the system to determine preferred temperature. Researchers using the gradient method would typically use a total assay time of at least 24 hours, with a long tank-acclimation prior to starting the trial (Reynolds & Casterlin, 1979). The preferenda is determined as the median occupied temperature. The acute method involves acclimating fish to various temperatures around their optimal temperature, then determining the acute thermal preference at each acclimation temperature. The final thermal preference is then graphically calculated from this relationship. As described above, the acute method maintains fish at acclimation temperatures for 2-3 weeks in laboratory prior to testing.

While similar tanks (with a thermal gradient) are typically used for the acute method, the major difference is that the preferenda is determined over a short time frame (2h) compared to the gradient method (>24h; Reynolds & Casterlin, 1979).

Vertical gradients may be problematic by design, as many bottom-dwelling fish species will display an affinity to the bottom regardless of temperature (Edsall, 1999). The same can be said for species that have surface feeding behavior or species with diurnal movements. Vertical migrations are common in marine species (Neilson & Perry, 1990) and could potentially create preferences in vertical depth that are independent of thermal preference when using vertical gradient systems. Being exposed to a large temperature gradient also poses a potential risk to fish that move up or down the water column too rapidly. This can lead to increased physiological stress or even death (Reynolds & Casterlin, 1979). There is no commercially available gradient system, as such they vary considerably in their design and function. Some systems have circulating currents (Edsall, 1999; Konecki, 1995), while others are flow-through (Venables et al., 1973). Many of these systems rely on multiple, simultaneous temperature probes to maintain gradients. These probes must still be constantly re-calibrated to maintain accuracy and pose challenges for maintaining accurate gradients over long periods of time. In this assay, fish are assumed to be making a static choice; in other words, they must remain stationary within the water column. However, fish are usually in a dynamic state of movement (i.e. circling the tank at an approximate depth).

Gradient tanks leave room for human error, as thermal preference is determined by the fish's place in water column. Gradient systems typically use a ruler inside the tank and mark the location of fish in the water column visually at repetitive intervals. There is a physical limit to how rapidly a single researcher can visually obtain data, and as such this limits the temporal density of data. Automating this data collection process would yield significantly more data that is both more accurate and precise. Therefore, a standardized assay that accounts for the dynamic movement of fish might more accurately determine thermal preference.

The shuttle box system is a commercially available system that requires fish to make a dynamic behavioral choice to determine thermal preference; i.e. the fish must continuously swim in the system to maintain a constant temperature. The device consists of two cylindrical tanks connected by a small rectangular section to “shuttle” between the tanks. The water temperature is dynamic and different across the tanks, such that as the fish move between the tanks, the water temperature in each side will change. As the fish experiences water temperature that is cooler or warmer than they prefer, they can shuttle to the other side to a more optimal temperature.

The shuttle box warms and cools at a controlled rate and the sides have a set temperature differential (ΔT) so that the maximum acute thermal stress the fish experience is the ΔT, yet still potentially exposes fish to all possible experimental temperatures. A minimum and maximum system temperature can prevent extremes where fish may suffer or die. The system uses an overhead camera to track fish under infrared light and the software determines the live location of the tracked object. Each compartment is determined to be either the increasing (INCR) or decreasing side (DECR). If on the INCR side, the entire system will increase temperature at a specific rate, and vice versa. This allows the fish to directly control the direction of temperature change within the shuttle box system, which causes the fish to gravitate towards its preferred temperature as it avoids unfavorable conditions. To regulate temperature, system water is pumped through heat-exchange coils in hot and cold-water baths, preventing outside contamination. Trials can be started remotely and do not require human intervention for data collection. Data on fish location and water temperature are collected every second automatically, making observations more accurate and precise. The shuttle box assay requires an active choice by the fish; in other words, they must shuttle between tanks to maintain a constant temperature in the system. Remaining stationary will cause the system to increase/decrease to the point where it becomes uncomfortable for the fish. This potentially limits this assay to species of fish that are bold and mobile.

1.6 Temperature Stress in Whitefish Spawning Habitat

Whitefish developing in natural conditions will experience both natural fluctuations in temperature as well as anthropogenically derived fluctuations. Many industrial power plants that line the shores of the Great Lakes utilize the process of "once-through cooling" to cycle waste heat back into the water source, discharged as a warm effluent. These power plants are typically located on the shore and discharge effluent into shallow coastal waters. Nuclear power plants use once-through cooling to condense steam in electrical generators. Bruce Power has a nuclear power generating station with 8 CANDU reactors (4 on each of two adjacent sites) on the coast of Lake Huron; using data from thermal sensors, the thermal plumes have been modeled. While the dynamics of the thermal plume are subject to change, depending on the recent weather/hydrology patterns, the thermal plume from each site intersects potential whitefish spawning ground, suggesting implications for developing whitefish embryos (Fietsch, 2011). The Bruce Power thermal plume can increase ambient water temperature by ~3°C (Thome et al., 2006). Typical substrate temperature at 10m depth during the stable winter months can range from 0.5°C to 2°C (Schwab, 1999; Patrick et al., 2013; Thome et al., 2016) for reference sites but with the addition of thermal effluent could be as high as 5°C.

Anthropogenic impacts on water temperature are not just through industrial discharge. Increased carbon emissions have led to measurable increases in average temperature globally (IPCC 2013). The greenhouse effect caused by carbon dioxide gas in the atmosphere can lead to an increase in average temperature, as well as an increase in the frequency of extreme weather events (Schneider, 1989). A global increase in average air temperature of up to 4°C, and likely up to 6°C in the Great Lakes region of North America, is expected due to the greenhouse effect by 2100 (IPCC 2013). Data has shown that declining winter ice cover is causing average water temperatures to warm at a rate of 0.1°C per year, which is far in excess of the regional atmospheric warming (Austin & Colman, 2007).

Previous studies (Price, 1940; Brooke, 1975; Griffths, 1980; Lim et al., 2017, 2018; Eme et al., 2015, 2018; Mitz et al., 2019; Mueller et al., 2015, 2017; Lee et al., 2016; Patrick et al., 2013) examining the effect of elevated incubation temperature on whitefish have typically used a temperature range of 0 - 12°C. These studies show that as little as a 2°C increase in incubation temperature can lead to changes in hatch timing and mortality in whitefish; an increase of 3°C has been linked to changes in development and morphology of the hatchlings. The temperatures tested within this thesis (2°C, 5°C, 8°C) coincide with potential anthropogenic increases in ambient water temperature caused by both industrial processes and climate change. In the near future it will be imperative to understand how vulnerable species will respond to rapid changes in temperature, especially those that are subject to substantial exploitation or are the most thermally sensitive. Thus, this thesis research includes both commercially important (LWF) and thermally sensitive (RWF) species that are relevant to the Great Lakes. Understanding the sub-lethal effects of elevated temperature incubation will help identify challenges with the discharge of thermal effluent. Regulation of thermal effluent is typically focused on reducing/limiting mortality of affected fish, and largely ignores potential secondary effects. This research may contribute to a body of evidence that will be used to better manage and regulate discharge of thermal effluent in the Canadian great lakes and plan mitigation strategies needed to deal with climate change.

1.7 Objective

The objective of this thesis is to elucidate the relationship between embryonic thermal exposure and juvenile performance. Previous work has determined the lethal and sub-lethal effects of elevated temperature on whitefish embryos (Price, 1940; Patrick et al., 2013; Lim et al., 2017; Lim et al., 2018; Eme et al., 2015), but little work has looked into post-hatch fitness and performance. A consistent hatchling phenotype has been identified where embryos reared at higher temperatures hatch early with shorter length, reduced weight (Brooke, 1975; Price, 1940; Mitz et al., 2019; Mueller et al., 2015; Eme et al., 2018; Lee et al., 2016), larger yolk sac, and smaller eyes (Mitz et al., 2019; Lee et al., 2016). Whether this early hatch phenotype affects survival and growth in juvenile and adult stages is unknown.

Temperature preference is a non-invasive behavioral endpoint that may be impacted by embryonic temperature. Previous work (Eme et al., 2015) has identified a positive relationship between embryonic incubation temperature and oxygen consumption in LWF. Combined with metabolic studies in other fish species (e.g. Killen, 2014), this suggests a potential change in temperature preference. I hypothesized that whitefish embryos exposed to elevated incubation temperatures would display lower temperature preference compared to control. In Chapter 3, I used a shuttle box behavioral assay to analyze the effect of elevated embryonic incubation on the temperature preference of juvenile lake whitefish incubated at 2°C, 5°C, 8°C and round whitefish incubated at 0.5°C, 2°C, 6°C. Colder temperatures were used for RWF because their embryos experience close to 100% mortality, while LWF experience 60-70% mortality, when incubated at 8°C. Fish were assessed for thermal preference at 8 (LWF only) and 12 (LWF and RWF) months of age, with common garden post-hatch rearing conditions. These experiments provided data to examine species differences (RWF versus LWF) and persistence (8 versus 12 months of age, LWF only) of the impacts of elevated incubation temperatures on post-hatching thermal preferences. I predicted that due to their lower thermal tolerance, RWF would have lower temperature preference than LWF and experience a larger treatment effect. Based on previous studies with respect to body size and temperature preference in lake whitefish (Edsall, 1976; 1999), I predicted LWF at 8 months of age would display warmer T_{pref} than LWF at 12 months of age. I also predicted that whitefish at 8 months of age would show a larger treatment effect than whitefish at 12 months of age.

The traditional assay design for thermal preference typically requires ≥ 24 hours to provide an extended tank acclimation prior to testing (Reynolds & Casterlin, 1979). An assay that takes >24 hours severely limits throughput, which was problematic for collecting data during a fast-growing life stage. Previous studies often limited sample size (Mortensen, 2007; Barker et al., 2018; Larsson 2005; Petersen and Stefensen, 2003; Siikavuopio, 2014), or tested multiple fish at once (Edsall, 1999; Sauter et al., 2001) to minimize total study length. However, increasing sample size by reducing experimental time may better capture the natural variability in thermal preference in fish. In order to use temperature preference for an endpoint with juvenile whitefish, the traditional behavioral assay was first optimized. In Chapter 2, the shuttle box behavioral assay was optimized to increase throughput while maintaining data quality. Several different study designs were tested, and power analyses determined appropriate study designs.

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Chapter 2

Evaluating tank acclimation and trial length for shuttle box temperature preference assays

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2.1 Contributions

Experimental work in this chapter was designed and conducted by Adam Harman. Guidance provided by Dr. Joanna Wilson, Dr. Meghan Fuzzen, Dr. Douglas Boreham, Dr. Richard Manzon and Dr. Chris Somers. Embryo collection was completed by Adam Harman, Dr. Meghan Fuzzen, and Lisa Stoa, and provided by Tim Drew, MNRF, Sharbot Lake White Fish culture station. Whitefish husbandry was carried out by Adam Harman, Dr. Meghan Fuzzen, Lisa Stoa, and undergraduate volunteer Akanksha Arora. This chapter was written by Adam Harman and edited by Dr. Joanna Wilson and Dr. Meghan Fuzzen. The manuscript in submission was also edited by Dr. Meghan Fuzzen, Dr. Douglas Boreham, Dr. Richard Manzon and Dr. Chris Somers.

Evaluating tank acclimation and trial length for shuttle box temperature preference assays

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Running Title: Shuttle box tank acclimation and trial length

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2.2 Abstract

Thermal preferenda are largely defined by optimal growth temperature for a species and describe the range of temperatures an organism will occupy when given a choice. Assays for thermal preferenda require at least 24 hours, which includes a long acclimation to the tank, limits throughput and thus impacts replication in the study. Three different behavioral assay experimental designs were tested to determine the effect of tank acclimation and trial length (12:12, 0:12, 2:2; hours of tank acclimation: behavioral trial) on the temperature preference of juvenile lake whitefish, using a shuttle box system. Average temperature preferences for the 12:12, 0:12, and 2:2 experimental designs were 16.10 ± 1.07 °C, 16.02 ± 1.56 °C, 16.12 ± 1.59°C respectively, with no significant differences between the experimental designs ($p = 0.9337$). Ultimately, length of acclimation time and trial length had no significant impact, suggesting that all designs were equally useful for studies of temperature preference.

2.3 Introduction

Most motile species are thought to exhibit a thermal preferenda or a range of preferred temperatures that individuals will tend to aggregate at when given the opportunity (Reynolds and Casterlin, 1979). This temperature should theoretically correlate with the optimum growth temperature, but there are several other important factors contributing to a thermal preferenda, including photoperiod, salinity, chemical exposure, age and/or size of fish, bacterial infection, nutritional state/food availability, and other biotic factors (Reynolds and Casterlin, 1979).

The definition of final preferenda assumes a common temperature preference that all members of the same species will ultimately display (Jobling, 1981). This may be accurate for small warm-water fish, like goldfish (*Carassius auratus*) and bluegill sunfish (*Lepomis macrochirus*), that were used for much of the early preferenda work (Reynolds and Casterlin, 1979) because they experience warm, stable temperatures across their distribution. The same cannot be said for larger temperate species that have consistently dealt with extreme temperature changes over their evolutionary history. Atlantic cod (*Gadus morhua*) display significantly different preferenda across their distribution due to a polymorphic haemoglobin molecule (Petersen and Stefensen, 2002), while juvenile coho salmon (*Oncorhynchus kisutch*) have distinct thermal preferences that align with the thermal profile of home streams (Konecki, 1995). Arctic charr (*Salvelinus alpinus*) that are exposed to repeated freezing and thawing of lakes/streams, experience seasonal changes in preferenda (Mortensen et al., 2007).

Temperature preference (T_{pref}) in juvenile lake whitefish (*Coregonus clupeaformis*) is inversely related to the size and age of the fish (Edsall, 1999), suggesting that conspecifics of different age classes may show different temperature preferences within the same body of water. Further, the basal metabolic rate of a fish has been correlated to their aerobic scope and their temperature preference (Killen, 2014). Fish with higher basal metabolic rate have both a lower aerobic scope and temperature preference. To compensate for increased metabolic demands, fish with higher basal metabolic rate tend to select colder temperatures when food availability is low (Killen, 2014). Therefore, individual life history traits can account for differences in T_{pref}.

Thermal preferenda assays are conducted in tanks with either a temperature gradient or a choice between different temperatures. These assays require an initial tank acclimation period where fish acclimate to the test arena, followed by a behavioral trial. Traditionally, the total assay (acclimation and trial) have a minimum length of 24 hours (Mortensen et al., 2007; Siikavoupio et al., 2014; Konecki et al., 1995; Petersen and Stefensen, 2002), based on the theory that fish are only displaying their acute temperature preference, rather than their final preferenda, when <24 hours in a new system (Reynolds and Casterlin, 1979). Allowing the fish to remain in the new system for at least 24 hours would theoretically reveal their final preferenda. However, Macnaughton et al. (2018) determined that tank acclimation time had little effect on the final preferenda of juvenile cutthroat trout (*Oncorhynchus clarkia lewisi*), a cold-adapted fresh-water species. Further, a minimum 24-hour assay length per fish has significant disadvantages for sample size and throughput in any study. The ability to assess preferenda would be extremely challenging in experiments that focus on biotic and abotic influences and fast growing life stages because of issues (e.g. length of time for experimental treatment, time out of treatment during the assay, different body sizes) inherent with the total time needed if throughput is ≤ 1 fish per day.

Fish in the juvenile life-stages, including lake whitefish, are in a period of rapid development and growth (Rennie, 2009), and Edsall (1999) reported a relationship between size and temperature preference. Long assay lengths may correspondingly introduce growth as a confounding factor. The influence on preference from seasons, migration, or physiological transitions with small temporal windows (e.g. smoltification), are difficult to determine because of limited throughput. Consequently, many studies (Mortensen, 2007; Barker et al., 2018; Larsson 2005; Petersen and Stefensen, 2002; Siikavuopio, 2014) use low sample sizes and have low statistical power. Alternatively, some studies test multiple fish at one time (Edsall, 1999; Sauter et al., 2001) but the social context likely influences results and individual fish are not truly independent measures. Increasing throughput would have significant advantages for all of these scenarios.

A shuttle box, first described by Neill (1972), is an instrument that determines the temperature preference of aquatic animals by allowing them to choose between two tanks held at different temperatures. Once acclimated to the system, fish will ‘shuttle’ between the two compartments to regulate body temperature, allowing analysis of preferred temperature and avoidance temperatures. This study examined the effect of tank acclimation and trial length on the quality and quantity of data produced to determine thermal preference (T_{pref}) during behavioral assays. We used three distinct experimental designs, starting with a 24-hour total assay length (12 hours tank acclimation:12 hours trial length) as a baseline. It was hypothesized that experimental designs of different lengths (24 hours, 12 hours, 4 hours) would have a limited effect on the determined thermal preference of lake whitefish (*Coregonus clupeaformis*) and that shorter assay designs could increase throughput.

2.4 Methods

Fertilized lake whitefish (LWF) embryos were acquired from Sharbot Lake White Fish Culture Station (Sharbot Lake, ON) on November 30th, 2017. Embryos were incubated under simulated seasonal temperatures until hatch. Embryos were initially held at 8°C and cooled (1°C/week) to 2°C. After 100 days of incubation, embryos were warmed (1°C/week) until hatching. Median hatch occurred at 158 days post fertilization. Hatchlings were placed in petri dishes at 8°C until successful exogenous feeding. Larvae were transferred to tanks and warmed (1°C/week) to 15°C, where they remained until testing (5-6 months). LWF were initially fed *Artemia* nauplii and slowly transitioned to pellet feed (Otohime B1 (200-360 µm) – C2 (920-1,410 µm) larval feed).

The shuttle box system (Loligo®) consists of two cylindrical tanks connected by a small rectangular 'shuttle' to allow movement of animals between the tanks. Each tank is assigned as the increasing (INCR) or decreasing (DECR) side, indicating the direction of temperature change when fish occupy that tank. To accurately regulate temperature, system water was pumped through heat-exchange coils in hot (28°C) and cold (4°C) water baths (60L aquaria) with mixing in separate buffer tanks for each side. A Recirculator 1/4 HP Chiller, Magnetic Drive Centrifugal Pump (300W/600W/950W @ 0°C/10°C/20°C; VWR) and a 400W aquarium heater were used to maintain the temperatures in the cold and warm bath, respectively. Ice was added to the cold bath every 2 hours during shuttle box operation to increase cooling capacity. Polystyrene insulation (1/2"), foam insulation tape (1/4”), and loose fiberglass insulation were used to maintain stable temperatures in the cold-water bath. System water flows (240 mL/min) via gravity through temperature probes and into the shuttle box where counter-directional currents minimize mixing between the two sides. A USB 2.0 uEye Camera tracked larval fish under infrared light (Loligo® Infrared Light Tray), and the Shuttlesoft® software determined the ‘live’ location of the tracked object. Shuttlesoft® uses contrast to identify and track objects and required even, symmetrical overhead lighting; black opaque plastic was used to dim fluorescent lights directly overhead and prevent glare.

In our experiments, we defined distinct static or dynamic modes for the shuttle box; the total assay length was the sum of time for each mode. Static mode (tank acclimation) was used to acclimate the fish to the shuttle box system but was not used to determine temperature preference. In this mode, the shuttle box maintained stable temperatures of 14°C and 16°C with a hysteresis of 0.25°C. Dynamic mode (behavioral trial) was used to determine temperature preference; fish were actively tracked and the entire system would warm or cool (hysteresis = 0.1°C) at a rate of 4°C/hour, depending on whether the fish was in the INCR or DECR tank. In both static and dynamic modes, the difference in temperature across the tanks was Δ 2°C. Hysteresis values were determined experimentally for each operating mode independently to achieve the most stable water temperatures over time. A maximum temperature of 23°C and a minimum temperature of 7°C prevented exposure to extreme temperatures, which could cause stress or mortality (Edsall and Rottiers, 1976).

The orientation of the INCR and DECR tanks and the side to which the fish would be introduced were randomized for each individual, using an online tool (random.org), to limit any potential bias introduced by visual cues or side preference. LWF were randomly selected from their home tank (15°C) and transported to the shuttle box system in 1L glass beakers. LWF were introduced to one side of the shuttle box, with a plastic divider separating the two halves. The assay started immediately after the barrier was removed, initiating acclimation, and continued until the end of the behavioral trial. While data were collected throughout, only data collected during the behavioral trial (dynamic mode) were used for temperature preference analysis. Shuttlesoft® calculates temperature preference (T_{pref}) over time as the median occupied temperature; velocity (cm/s), distance (cm), time spent in INCR/DECR, number of passages and avoidance temperatures were collected in 1 second intervals. The fish remained in the shuttle box throughout the entire assay, without interference or handling. After completion of the assay, fish were removed and measured for total length (±1 mm) and mass (±0.01 g) before returning fish to a separate home tank (15°C).

Three experiments were conducted to test the effect of tank acclimation and trial length on the quality of data, namely 12:12, 0:12, or 2:2 designs representing the number of hours in static mode (tank acclimation) and dynamic mode (behavioral trial), respectively (Figure 1a). Summary statistics were generated for each experimental design to compare the effect of the design on data accuracy and variability. Mean T_{pref} + standard deviation was used to compare the variation between fish, which is the major limit of statistical power. An experimental design was considered equally useful if it produced T_{pref} data that were not statistically different. Power analyses were completed for each experimental design to compare optimal sample sizes at the lowest acceptable power ($1-\beta = 0.60$).

Results and Discussion 2.5

In the first experimental design (12:12), juvenile LWF (n=10) had 12 hours of over-night tank acclimation (9 pm – 9 am) in static mode, followed by 12 hours of behavioral trials (9 am – 9 pm) in dynamic mode. The maximum throughput was 1 fish per day (Figure 2.2e). This design included the longest tank acclimation period, the lowest throughput and was predicted to decrease between-fish variability. The average T_{pref} was 16.10 ± 1.07 °C (Figure 2.1a), which was the lowest standard deviation in average T_{pref} across the experimental designs, as expected.

Available literature suggests that a long tank acclimation period prior to the behavioral trial is required to observe the true temperature preference of a species (Reynolds and Casterlin, 1979). The second design (0:12) explicitly tested the effect of tank acclimation by completely removing it; juvenile LWF (n=9) had a 12-hour behavioral trial (9 am – 9 pm) under dynamic mode with no prior acclimation. One fish was excluded because the system shut down prematurely. Removal of the static period was predicted to increase the variation in T_{pref} between individuals. As predicted, the standard deviation of T_{pref} increased, but not drastically

(Figure 2.1a). Throughput (1 fish/day) remained the same because only the overnight tank acclimation was removed; while 2 fish/day were possible if we ran assays in both day and night, results were more comparable with dynamic mode in the same part of the diurnal cycle (day light). The average T_{pref} was 16.03 ± 1.56 °C (Figure 2.1a), which was not statistically different ($p=0.912$) from the outcome using the baseline design. The data from this experiment were analyzed in 2-hour sub-sets (i.e. 2 hours, 4 hours, 6 hours) to simulate shorter behavioral trial durations (Figure 2.1b). Average T_{pref} was not statistically different ($p=0.1923$) between a 12-hour and a 2-hour behavioral trial length (Figure 2.1b), suggesting that not only was long tank acclimation not required but shorter trials were possible. The advantage of no or limited tank acclimation coupled with a shorter behavioral trial was that throughput could be increased to multiple fish per day, offering the opportunity to increase total sample size or decrease the time needed to assess T_{pref} in different treatment groups.

A third experimental design (2:2) was implemented with 2 hours of tank acclimation and 2 hours of behavioral trial, to increase throughput. Three time periods were used (11 am – 1 pm, 3 pm – 5 pm, 7 pm – 9 pm) instead of one (9 am – 9 pm), which would triple throughput; there was no effect of time of day. This design has not been reported in the literature and this is the first attempt to calculate T_{pref} from such a short assay, to our knowledge. The average T_{pref} was 16.12 ± 1.59°C (Figure 2.1a) and was not significantly different from either alternative experimental design ($p=0.9337$). Further, the standard deviation did not drastically increase (Figure 2.1a), although it was the largest of the tested designs.

Shuttlesoft® automatically calculates the cumulative median of T_{pref} every second, and that data can be compared between individuals and groups. Figure 3 compares individual T_{pref} data to the average, showing the spread of the data as well as the stability over time. A unique aspect of the shuttle box behavioral assay is that a fish must be shuttling between the two sides to maintain a constant temperature within the system; switching sides is an active behavioral choice. Traditional methods require the fish to remain stationary to select a temperature in a gradient. All experimental designs followed a similar pattern of an initial period of high variability, followed by a prolonged period of relative stability (Figure 2.3), suggesting an active choice was made. Therefore, the different designs appear largely equivalent, suggesting that long tank acclimation and long behavioral trials are not necessary to determine T_{pref}, at least for juvenile LWF. This offers the opportunity to increase the throughput on a temperature preference study where confounding variables (e.g. rapid body growth, exposure to abiotic or biotic factors) could significantly impact the data if the traditional design (>24 hours per fish) was used.

Tank acclimation and behavioral trial intervals were chosen based on both scientific evidence and logistics. In all cases, we note the throughput (i.e. how many fish can be tested per week) to highlight the relevant trade off that would impact experimental design choice. While previous literature (Mortensen et al., 2007; Siikavoupio et al., 2014; Konecki et al., 1995; Petersen and Stefensen, 2002) would suggest acclimating fish to the tank for a period of >24 hours, we used a total assay length of 24 hours (12-hour static tank acclimation, 12-hour dynamic behavioral trial) as the baseline. This was chosen because a total assay length of >24 hours would lead to a throughput of only 3 fish/week, which would not have been feasible for a large-scale experiment, particularly with fast growing juvenile fish. Considering the juvenile fish used here (5 months of age), it would be important to account for changes in individual growth during temperature preference studies. A negative correlation between growth and temperature preference has been observed in lake whitefish (Edsall, 1999), which suggests study length could be an influential factor in experiments with fast growing life stages. Increasing throughput could allow testing a wider range of individuals (Figure 2.2e) and may better capture a population's natural variability.

Using the 2:2 design would yield an experiment that is 34 days in length to provide the minimum sample size needed for three treatment groups (Figure 2.2e). Even within 34 days, individual juvenile LWF tested near the beginning of the study would be ~20% younger and 11% smaller (LWF are 9.11 g (± 2.8) versus 10.23 g (± 2.0) at 5 and 6 months, respectively; unpublished data). It would be important to minimize length of time to collect temperature preferenda data and consider the trade-offs between variance and sample size on the statistical power to assess differences across treatment groups. The same can be said when determining T_{pref} within small temporal windows (e.g. smoltification, seasonality, developmental windows) where small sample sizes would limit statistical power. The functional trade-offs between statistical power (1-ß), variance (δ^2), sample size (n), and throughput were investigated using power analysis (Figure 2.2) for the various experimental designs. While experimental design 3 (2:2) led to increased variation in mean T_{pref}, the increased throughput allowed for an increased sample size while still minimizing the total time needed for the experiment. If the number of fish were limited or growth and developmental concerns were not as relevant (e.g. adult fish), then minimizing variation may be more important.

This study used a maximum rate of change of 4 °C/hour, similar to what has been previously reported (Macnaughton et al., 2018; Konecki, 1995; Petersen and Stefensen, 2002). This could have limited the range of temperatures experienced by the juvenile LWF. If a fish occupied the INCR zone for the entire duration of the behavioral trial, the system would have cooled by 8°C, only just hitting the upper temperature limit of the shuttle box. Thus, to reach extreme temperature preferences a fish must exhibit low (<10) passage numbers, a problem when preference is determined by active swimming. This problem could potentially be avoided by increasing the rate of temperature change (Barker et al., 2018), at the expense of possible physical stress. For our experiments, data were excluded only when fish made no passages in the dynamic mode. In all cases, fish made regular passages in at least one mode, indicating they were active and able to explore the entire arena. Hyperactive fish would likewise pose a problem for the system; there was no animal that exhibited so many crosses that the system could not respond and change temperature.

Thermal preferenda can be an important behavioral endpoint but traditionally require long periods of time (>24 hours) to determine. The results of this study show that decreasing the total assay length (24 hours to 4 hours) did not significantly affect the T_{pref} of juvenile lake whitefish. The shuttle box is a powerful behavioral tool and a less restrictive definition of T_{pref} and more flexibility in the assay design would allow T_{pref} as a viable behavioral endpoint for a variety of species and life stages with more experimental power.

(a)

Experimental Design	Sample Size (n)	Average T_{pref} (°C)	Standard Deviation	P-Value
12:12	10	16.10	1.07	-
0:12	9	16.03	1.56	0.912
2:2	9	16.12	1.59	0.971

(b)

Data Sub-set	Average T_{pref} (°C)	Standard Deviation	P-Value
12 hours	16.03	1.56	-
6 hours	16.36	1.14	0.513
4 hours	16.92	1.37	0.241
2 hours	17.06	1.66	0.1923

(c)

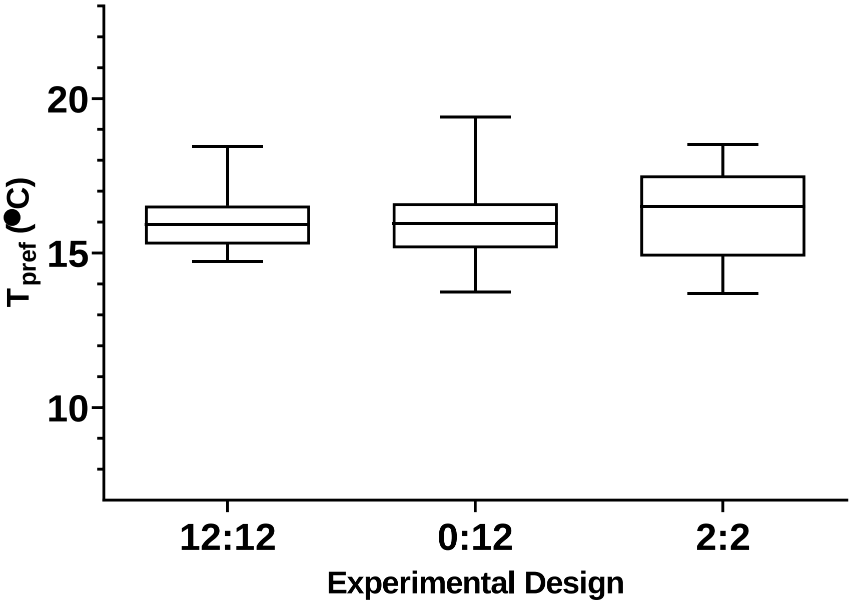
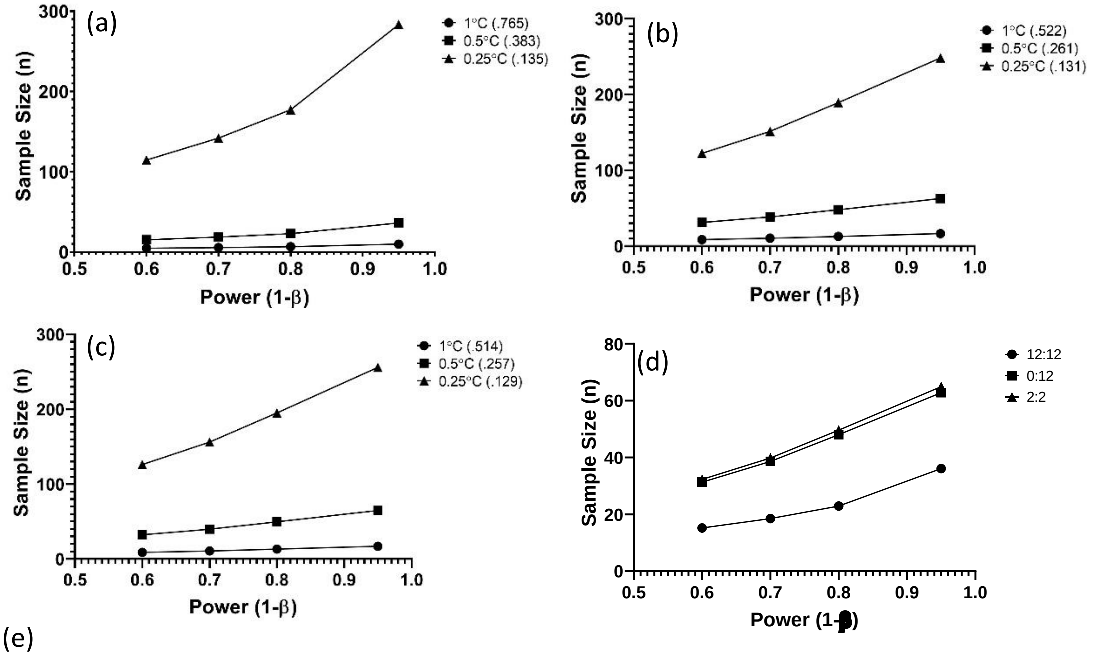


Figure 1: (a) Summary of average temperature preference (T_{pref}) data from three different experimental designs. T_{pref} is calculated as the cumulative median of occupied temperature. 12:12, 0:12, or 2:2 designs representing the number of hours in static mode (tank acclimation) and dynamic mode (behavioral trial), respectively. P-values were determined using one way ANOVA with post-hoc comparisons. (b) Sub-set analysis conducted using the 0:12 experimental design, behavioral trials were sub-set into 2, 4, and 6-hour windows. P-values were determined using ANOVA. (c) Box plot comparing T_{pref} between 12:12, 0:12 and 2:2 experimental designs. The height of the box corresponds to Q1 – Q3, and the bars correspond to the minimum and maximum values. Y-axis represents the thermal range of the shuttle box system.



(e)

Experiment Design	Minimum Sample Size	# of Treatments	Total # of Fish	Throughput (Fish/Day)	Study Length
12:12	15	3	45	1	45 days
0:12	31	3	93	1	93 days
2:2	32	3	96	3	32 days

(f)

$$f = \sqrt{\frac{\sum_{i=1}^{k} p_i * (\mu_i - \mu)^2}{\sigma^2}}$$

where $p_i = n_i / N$,
n_i = number of observations in group i
N = total number of observations
μ_i = mean of group i
μ = grand mean
σ^2 = error variance within groups

Figure 2: (a, b, c) Relationship between sample size (n) and power (1-β) for experimental (Expt) designs 12:12 (a), 0:12 (b), and 2:2 (c), representing the number of hours in static mode (tank acclimation) and dynamic mode (behavioral trial), respectively. Curves were generated using iterative power analysis (pwr package – R). Effect sizes were calculated using panel (f) by predicting expected differences between means. (d) Power analysis using 0.5°C effect sizes, each data series corresponds to an experimental design. (e) Summary of power analysis results. Minimum sample size corresponds to n calculated with 0.5°C effect size and 1-β = 0.6. # of treatments can vary with experimental design, three was chosen as a reasonable example. Total number of fish is minimum sample size times the number of treatments. Study length was calculated by dividing the total number of fish by the throughput of the experimental design, 12:12 = 1/day, 0:12 = 1/day, 2:2 = 3/day. (f) Equation used to calculate effect size (ƒ) for ANOVA.

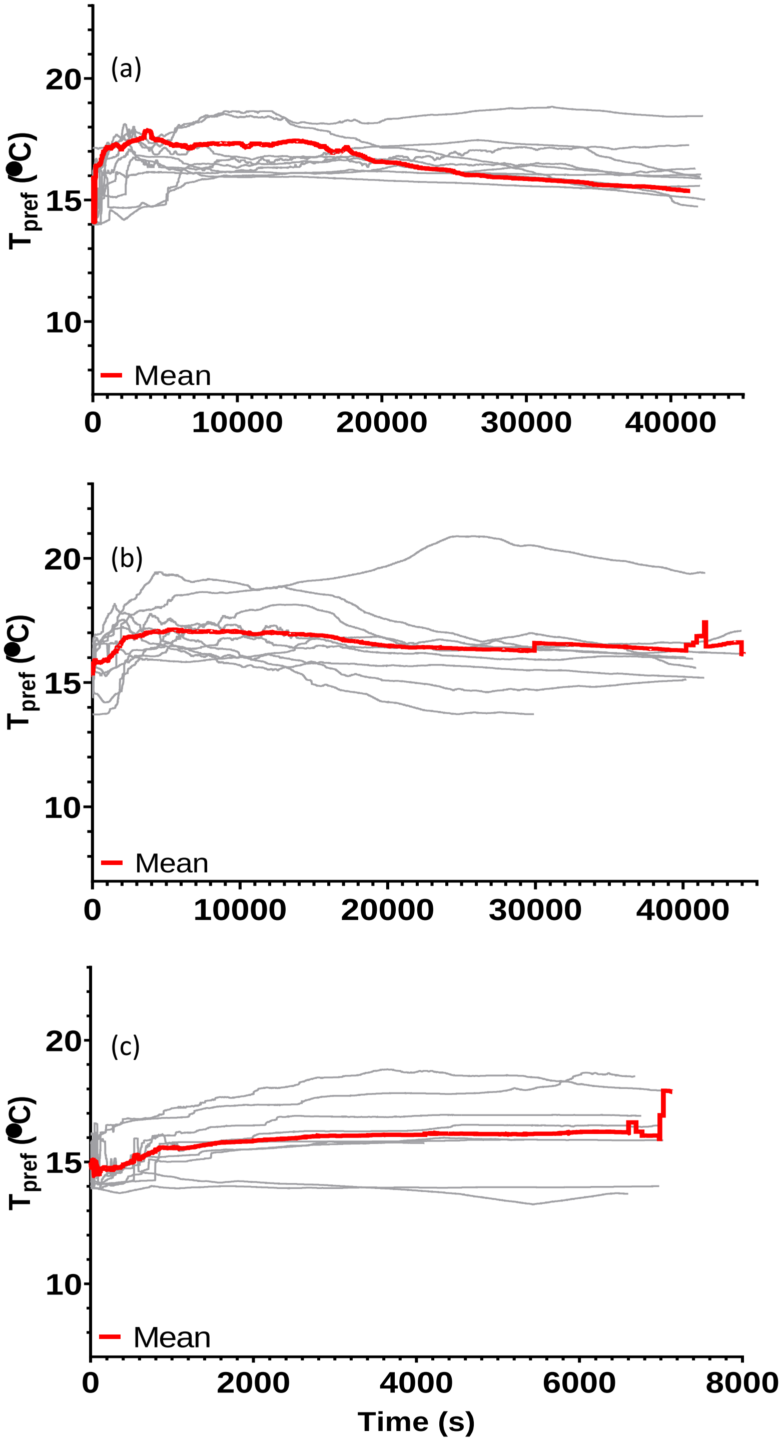


Figure 3: Cumulative median temperature preference (T_{pref}) calculated every 1 second for experimental designs 12:12 (a), 0:12 (b) and 2:2 (c), representing the number of hours in static mode (tank acclimation) and dynamic mode (behavioral trial), respectively. Grey lines represent the T_{pref} of individual fish over time. Red line represents the mean T_{pref} for all fish. Y-axis represents the thermal range of the shuttle box system.

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Chapter 3

Effect of elevated embryonic incubation temperature on the temperature preference of juvenile lake (*Coregonus clupeaformis*) and round whitefish (*Coregonus clupeaformis*)

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3.1 Contributions

Experimental work in this chapter was designed and conducted by Adam Harman and Hannah Mahoney (undergraduate thesis student). Additional assistance in data collection provided by experiential learning student Bhuvan Aggarwhal. Guidance provided by Dr. Joanna Wilson, Dr. Meghan Fuzzen, Dr. Douglas Boreham, Dr. Richard Manzon and Dr. Chris Somers. Embryo collection was completed by Adam Harman, Dr. Meghan Fuzzen, and Lisa Stoa, and provided by Tim Drew, MNRF, Sharbot Lake White Fish culture station. Whitefish husbandry was carried out by Adam Harman, Dr. Meghan Fuzzen, Lisa Stoa, and undergraduate volunteer Urvi Pajankar. This chapter was written by Adam Harman and edited by Dr. Joanna Wilson.

Effect of elevated embryonic incubation temperature on the temperature preference of juvenile lake (*Coregonus clupeaformis*) and round whitefish (*Coregonus clupeaformis*)

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3.1 Abstract

Thermal preference of aquatic organisms can be modulated by abiotic and biotic factors including environmental temperature. Freshwater fish can be exposed to anthropogenic warming from sources such as climate change and thermal effluent and exposure during embryogenesis could result in altered temperature preference and fitness in later life stages. Lake (*Coregonus clupeaformis*) and round (*Prosopium cylindraceum*) whitefish were incubated at natural and elevated temperatures until hatching; all groups were moved to common garden conditions (15°C) during the post-hatching stage. Temperature preference was determined at 12 months of age; lake whitefish were also tested at 8 months. Round whitefish displayed a significant decrease ($p = 0.006$) in temperature preference when incubated at 2°C and 6°C compared to 0.5°C. Lake whitefish had similar temperature preferences regardless of age and incubation temperature. This suggests that there is a difference in thermal tolerance between these species, as round whitefish were more sensitive to elevated incubation treatments. This study identified a persistent effect of elevated temperature on the temperature preference of juvenile round whitefish up to 12 months after the incubation treatment, and highlights how small differences in embryonic thermal experience can produce different outcomes for cold-adapted freshwater fish.

3.2 Introduction

Temperature is a key variable in determining optimal growth conditions and fitness-related activities of fish (Fry, 1947; Magnuson et al., 1979; Jobling 1981). Species-specific thermal tolerance and preference can provide physiological constraints that define the thermal niche of a species and can be used to predict potential consequences of increasing water temperature (Jobling, 1981; Coutant, 1987; Caissie, 2006; Pörtner and Peck, 2010). As global temperatures continue to rise (IPCC 2013), research on the effect of elevated water temperatures has become increasingly relevant. Other anthropogenic sources of warming water, such as thermal effluent, could further exacerbate thermal stress experienced by freshwater fish, especially in periods of limited mobility (e.g. embryonic development). One such source are thermal power plants, which utilize the process of "once-through cooling" to cycle waste heat back into the water source, discharged as a warm effluent into shallow coastal waters. Freshwater fish are highly vulnerable to anthropogenic changes in temperature, especially those requiring cold (<22°C) water (Moyle et al., 2013).

Lake (*Coregonus clupeaformis*; LWF) and round (*Prosopium cylindraceum*; RWF) whitefish are cold-water adapted species that have an extensive range across Canada, with their distribution extending south beyond the Laurentian great lakes (Ebener, 2010). LWF and RWF often occur sympatrically within Canadian freshwater lakes and co-exist due to differential habitat and resource use within the lake (Eberts et al., 2015). Adult LWF tend to occupy deeper (18-90m) limnetic waters, while RWF typically occupy shallow (6-36m) littoral waters (Reigard, 1910;

Rawson, 1951; Bailey, 1962; Cucin & Regier, 1966). LWF are a commercially and culturally important species, supporting the largest commercial fishery in the great lakes and local indigenous communities through subsistence fishing (Ebener et al, 2010). The LWF fishery was worth 18 M USD in 2000, which accounted for one third of the total revenue of the great lakes fisheries (Kinnunen, 2000). Most commercial interest in round whitefish is centered in Lake Michigan, however due to its small size and fluctuating abundance it cannot support a large fishery (Bailey, 1962). Both species of whitefish broadcast spawn in shallow (<10m) cobble beds in late November, where the embryos will remain until hatching at the spring ice melt (April-May; Scott & Crossman, 1979). This is an unusually long incubation period among great lakes fishes, as embryos develop for 4-6 months prior to hatching. After hatching, whitefish larvae are transported by currents to coastal embayments (nursery grounds) where they begin to feed exogenously on zooplankton (Loftus, 1982; Freeberg et al., 1990; Claramunt et al., 2010). Plankton net sampling showed that high densities of larval whitefish can be found in nursery grounds from late April to early June (Loftus, 1982; Ryan et al., 2014).

Whitefish embryos incubated at temperatures reflective of normal winter water (0.5 – 2°C) typically experience low mortality; embryonic mortality increases with incubation temperature ≥ 5-8°C (Price, 1940; Brooke, 1975; Lim et al., 2017, 2018; Mitz et al., 2019; Eme et al., 2015, 2018; Mueller et al., 2015, 2017; Lee et al., 2016; Patrick et al., 2013; Thome, in press). Whitefish exposed to elevated incubation temperatures of at least +2°C above natural incubations develop more rapidly (Price, 1940; Brooke, 1975; Lim et al., 2017, 2018; Mitz et al., 2019; Eme et al., 2015, 2018; Mueller et al., 2015, 2017; Lee et al., 2016; Patrick et al., 2013;

Thome, in press). Whitefish raised at elevated temperature hatch significantly earlier with altered morphology (Lim et al., 2017, 2018; Price, 1940; Brooke, 1975; Mueller et al., 2015; Eme et al., 2015, 2018; Lee et al., 2016) and appear underdeveloped at hatch (e.g. in jaws and fins; Mitz et al., 2019), compared to embryos reared at lower temperatures. Early hatched fish had a shorter length, reduced weight (Brooke, 1975; Price, 1940; Mitz et al., 2019; Mueller et al., 2015; Eme et al., 2018; Lee et al., 2016), larger yolk sac, and smaller eyes (Mitz et al., 2019; Lee et al., 2016). While elevated embryonic temperature affects both species of whitefish similarly, RWF are generally more sensitive. RWF experience 30-40% higher mortality than LWF when incubated at a constant 8°C (Lim et al., 2017, 2018).

Whitefish developing in the great lakes may be exposed to multiple sources of anthropogenic warming, including atmospheric climate change and discharge of thermal effluent. Thermal effluent from the Bruce Power Nuclear Generating Station (Kincardine, ON) is discharged into the nearshore environment, increasing ambient water temperature by ~1-3°C (Thome et al., 2006). Likewise, the expected rise in great lakes water temperature is between 4-6°C by 2100 (IPCC 2013), at a rate of about 0.1°C/year (Austin & Colman, 2007). With typical substrate temperature at 10m depth being 0.5-2°C for the reference sites (Schwab, 1999; Patrick et al., 2013; Thome et al., 2016), this means developing whitefish embryos could be exposed to temperatures as high as 5°C now and up to 8°C within ~30 years at the current rate of warming (Austin & Colman, 2007).

A positive relationship between embryonic incubation temperature and post-hatch metabolic rate has been established in several fish species, including razorback sucker (*Xyrauchen* *texanus*; Bozek et al., 1990), zebrafish (*Danio rerio*; Barrionuevo & Burggren, 1999), Arctic charr (Salvenlinus alpinus; Huuskonen et al., 2003) and Japanese medaka (*Oryzias latipes*; Marty et al., 2010). Fish embryos display many plastic traits that can be influenced by their developmental environment (Jonsson & Jonsson, 2019). To maximize survival and fitness, fish use the embryonic period to predict thermal conditions in adulthood. A recent study on zebrafish (*Danio rerio*) found an inverse relationship between embryonic temperature and relative activity of mitochondrial and glycolytic enzymes that persists to adulthood (Schnurr et al., 2014). Embryonic temperature was positively correlated with the thermal optimum of important metabolic enzymes, suggesting fish incubated at higher temperatures will have a modified response to elevated temperatures as adults (Schnurr et al., 2014).

Fish are ectothermic poikilotherms, meaning their body temperature fluctuates considerably, and they do not produce internal heat metabolically (Stevens & Fry, 1970). Fish are unable to physiologically thermoregulate, so they must migrate to more favorable water conditions and avoid unfavorable conditions to maintain body temperature (Stevens & Fry, 1970). Most motile species exhibit a “thermal preferenda” or a range of preferred temperatures that, when given the opportunity, individuals will tend to aggregate (Reynolds & Casterlin, 1979). This temperature correlates well with the optimum growth temperature of a species (Jobling, 1981), but is influenced by a number of biotic and abiotic factors, including: photoperiod, salinity, chemicals, age and/or size of fish, bacterial infection, and nutritional state/food availability (Reynolds & Casterlin, 1979). Temperature preference (T_{pref}) has been shown to vary across life-stage (Edsall, 1999), season (Mortensen et al., 2007), time of day (Macnaugton et al., 2018) and metabolic state (Killen, 2014). Early life thermal history is proposed to have an influence on the functional response to changes in temperature later in life (Schnurr et al., 2014), suggesting it may be more influential on T_{pref} than previously thought. There are functional links between embryonic conditions and adult/juvenile fitness (Weber et al. 2012). Despite this knowledge, the effect of thermal exposure during development is often overlooked in studies of thermal preference.

The objective of this study is to elucidate the relationship between embryonic thermal exposure and juvenile thermal preference of LWF and RWF. Previous work has determined the lethal and sub-lethal effects of elevated temperature on whitefish embryos (Price, 1940; Brooke, 1975; Lim et al., 2017, 2018; Mitz et al., 2019; Eme et al., 2015, 2018; Mueller et al., 2015, 2017; Lee et al., 2016; Patrick et al., 2013; Thome, in press), but little work has looked into post-hatch fitness and performance. We predicted that juvenile LWF and RWF incubated at elevated temperatures would prefer colder temperatures than those incubated at optimum temperatures for development. Due to their increased thermal sensitivity, we also predicted that RWF would display a colder T_{pref} than LWF incubated at similar temperatures.

3.3 Methods

3.3.1 Study Species

Fertilized LWF embryos were acquired from Sharbot Lake White Fish Culture Station (Sharbot Lake, ON) on November 30, 2017 (12-month-old) or November 27, 2018 (8-month-old).

Spawning RWF were obtained from Lake Ontario (Port Darlington, GPS 43°51′50′′N 78°44′35′′W) on December 10 and 11, 2018. RWF were stripped of eggs and milt and returned to the water. Artificial, in vitro fertilization occurred immediately after stripping. Embryos were disinfected with Ovadine® solution and transported in lake water back to McMaster University. Embryos (160-310; ~200) were plated into 200mm x 20mm sterile petri dishes containing 200mL of dechlorinated water and placed into specialized incubators that maintain stable temperature over time (Mitz et al., 2014). Incubators were initially kept at 8°C and cooled (1°C/week) until they reached a base temperature of 2°C, 5°C or 8°C for LWF or 2°C or 6°C for RWF. Additional RWF embryos were transferred to and maintained on an ice slurry within the 2°C incubator to generate the 0.5°C treatment group. This basal temperature was maintained throughout the "stable" winter period (December – March). After 100 days of incubation, embryos were warmed (1°C/week) until reaching 6°C (RWF) or 8°C (LWF) or 100% hatch. TidbiT® temperature loggers placed in 200mm x 200mm petri dishes with 200 mL of dechlorinated water. Calculated average temperatures including cooling/warming periods were 4.2 ± 1.9°C, 5.2 ± 0.9°C, 7.9 ± 0.3°C for LWF, and 1.7 ± 1.6°C, 3.0 ± 0.7°C, 6.1 ± 0.2°C for RWF. Median hatch for LWF occurred at 50 days post fertilization (8°C), 108 days post fertilization (5°C), and 158 days post fertilization (2°C). Median hatch for RWF occurred at 88 days post fertilization (6°C), 114 days post fertilization (2°C), and 118 days post fertilization (0.5°C). Hatchlings (~10) were placed in 100mm x 20mm petri dishes with 100 mL of water at 6°C (RWF) or 8°C (LWF) until successful exogenous feeding. Water in petri dishes was changed three times a week for embryos and daily for larvae. Larvae were transferred to 1-10L recirculating tanks and warmed (1°C/week) to 15°C, where they remained until testing (8 or 12 months). All treatment groups were maintained in common garden conditions once they were warmed to 15°C. Larval fish were initially fed *Artemia* nauplii and slowly transitioned to pellet feed (Otohime B1 (200-360 µm) – C2 (920-1,410 µm) larval feed).

3.3.2 Behavioral Assay – Shuttle Box

The shuttle box system (Loligo®), first described by Neill et al. (1972), consists of two cylindrical tanks connected by a small rectangular 'shuttle' to allow movement of animals between the tanks. Each tank is designated as the increasing (INCR) or decreasing (DECR) side, indicating the direction of temperature change when fish occupy that tank. To regulate temperature, system water was pumped through heat-exchange coils in hot (28°C) and cold (4°C) water baths (60L chest coolers) with mixing in separate buffer tanks for each side. A Recirculator 1/4 HP Chiller, Magnetic Drive Centrifugal Pump (300W/600W/950W @ 0°C/10°C/20°C; VWR) and 2x400W aquarium heaters were used to maintain the temperatures in the cold and warm bath, respectively. Polystyrene insulation (1/2") and foam insulation tape (1/4") were used to prevent heat loss and maintain stable temperatures in the cold-water bath. System water flowed at 240 mL/min via gravitational pull through temperature probes and into the shuttle box where mixing between the two sides is minimized by counter-directional currents. A USB 2.0 uEye Camera tracked larval fish under infrared light (Loligo® Infrared Light Tray), and the Shuttlesoft® software determined the 'live' location of the tracked object every second. Shuttlesoft® uses contrast to identify and track objects and required even, symmetrical overhead lighting; black translucent plastic was used to dim fluorescent lights directly overhead and prevent glare.

In our experiments, we defined distinct static or dynamic modes for the shuttle box; the total assay length was the sum of time for each mode. Static mode (tank acclimation) was used to acclimate the fish to the shuttle box system but was not used to determine temperature preference. In this mode, the shuttle box maintained stable temperatures of 14°C and 16°C with a hysteresis of 0.25°C. Dynamic mode (behavioral trial) was used to determine temperature preference; fish were actively tracked and the entire system would warm or cool (hysteresis = 0.1°C) at a rate of 4°C/hour, depending on whether the fish was in the INCR or DECR tank. In both static and dynamic modes, the difference in temperature across the tanks was maintained at 2°C. Hysteresis values were determined experimentally for each operating mode independently to achieve the most stable water temperatures over time. A maximum temperature of 23°C and a minimum temperature of 7°C prevented exposure to extreme temperatures, which could cause stress or mortality (Edsall and Rottiers, 1976). The orientation of the INCR and DECR tanks and the side to which the fish would be introduced were randomized for each individual, using an online tool (random.org), to limit any potential bias introduced by visual cues or side preference. Prior to experimentation, whitefish were fasted for 12-20 hours to prevent fouling of the water and to standardize metabolic state. To account for any potential growth over the study duration, the order of sampling among treatment groups was randomized using an online tool (random.org). Whitefish of the appropriate treatment group were randomly selected from their home tank (15°C) and transported to the shuttle box system in blacked-out 1L glass beakers to prevent undue stress. Whitefish were introduced to a randomized side of the shuttle box, with a plastic divider separating the two halves. The assay started immediately after the barrier was removed, initiating acclimation, and continued until the end of the behavioral trial. All shuttle box assays followed the same format of a 2-hour tank acclimation phase in static mode, followed by a 2-hour behavioral trial in dynamic mode; a design based on assay optimization with larval whitefish (Chapter 2). The fish remained in the shuttle box throughout the entire assay, without interference or handling. While data were collected throughout, only data collected during the behavioral trial (dynamic mode) were used for temperature preference analysis. Shuttlesoft® calculates temperature preference (T_{pref}) over time as the median occupied temperature; velocity (cm/s), distance (cm), time spent in INCR/DECR, number of passages and avoidance temperatures were collected in 1 second intervals. After completion of the assay, fish were removed and measured for total length (± 1mm) and mass (± 0.01g) before returning fish to a separate home tank (15°C).

3.3.3 Statistical Analysis

Temperature preference (T_{pref}) for each individual was calculated as the cumulative median of occupied temperature over the course of the behavioral trial. The average T_{pref} was determined by calculating the mean T_{pref} among individuals within each treatment group. Avoidance temperatures were calculated as the mean temperature at which a passage between tanks occurs and was averaged within treatment groups. All data is presented as mean±SD unless otherwise stated. T_{pref} and other measured variables were compared across treatment groups using One-way ANOVA, and Tukey's HSD post-hoc comparison. Linear models including total length and body weight as fixed effects were created to determine the relative effect of total length and body weight on T_{pref}, in addition to the main treatment effect of elevated incubation temperature.

Outliers were identified using the robust regression and outlier removal (ROUT) method, which fits a curve that is not influenced by outliers. The residuals of this fit are then analyzed for any potential outliers and subjected to ordinary least-squares regression once outliers are removed. A relationship between time (s) and temperature change (°C) in the shuttle box was developed to determine the upper functional threshold of the system. This allowed for technical identification of individuals that were too active for the shuttle box system to properly determine T_{pref} due to the limitations of the heating/cooling rates. All statistical analyses were completed in R (version 4.0.0 "Arbor Day"), except for outlier identification which was completed in Graphpad Prism (version 8.4.3). All R scripts used for analysis were uploaded to GitHub (https://github.com/WilsonToxLab).

3.4 Results

In total, 103 (12-month-old) and 87 (8-month-old) LWF, and 83 (12-month-old) RWF were tested for T_{pref} using the Loligo® shuttle box system. A total of 4 outliers were identified and removed using the ROUT method (2 x 8-month-old LWF, 2 x 12-month-old RWF). Differences in treatment group sizes were due to differential mortality in holding tanks during rearing and were not due to experimentation.

3.4.1 Lake Whitefish

LWF at 8-months of age had similar T_{pref} between the 2°C and 8°C treatment groups (Figure 3.1), but both differed from the 5°C treatment. LWF juveniles incubated at 5°C as embryos showed the lowest average T_{pref} (15.12 ± 1.98°C), compared those incubated at 2°C (16.04 ± 1.14°C) and 8°C (16.01 ± 1.74°C; Table 3.1). One-way ANOVA indicated there was a significant difference in average T_{pref} among treatment groups (f = 3.505 , p = 0.346). Tukey's post-hoc comparison identified a significant difference between 5°C and 8°C groups (Adjusted p = 0.040). Upper and lower avoidance temperatures were similar between treatment groups, indicating 8-month-old LWF were on average avoiding temperatures below 15.3°C and above 17.5°C. Eight month old LWF in the 5°C treatment group displayed the lowest activity, travelling an average distance of 173 m, compared to just over 190m at 2°C and 8°C. The 5°C treatment group also shuttled between tanks on average 37 times less than the 2°C group, and 24 times less than the 8°C group (Table 3.1). Total body length was similar between all treatment groups, varying less than 1mm on average (Table 3.1). Likewise, body weight was similar across treatment groups, with the largest difference (11%) between 2°C (1.13 ± 0.32g) and 5°C (1.25 ± 0.39g). Linear models were fit, including body weight and total length as fixed effects, to determine if there was a relationship between size and T_{pref}. Model results (p = .068, p = 0.061) indicated there was no significant interaction between T_{pref} with total length or body weight.

At 12 months of age, LWF from all treatment groups (2°C, 5°C, 8°C) displayed similar T_{pref} (Figure 3.1) and avoidance temperatures (Table 3.1). LWF juveniles incubated at 2°C showed the lowest average T_{pref} (15.27 ± 2.67°C) while those incubated at 5°C displayed the highest T_{pref} (16.02 ± 2.39°C), a difference of only 5%. There were no significant differences in T_{pref} in the 12-months-old LWF, by treatment group (One-way ANOVA, f = 0.765, p = 0.468). Upper and lower avoidance temperatures were comparable between all treatment groups, suggesting 12-month-old LWF were avoiding temperatures below 14.3°C and above 17.4°C on average (Table 3.1). Average total length and body weight were similar across all treatment groups, varying less than 1mm or 0.5g, respectively. As expected, 12-month-old LWF were on average heavier (by 9.3g), and longer (by 58.7mm) than 8-month-old LWF, due to growth over the 4 additional months (Table 3.1). Linear models were fit, including body weight and total length as fixed effects, to determine if there was a relationship between size and T_{pref}. Model results indicated that body weight (p = 0.0678) and total length (p = 0.0607) did not significantly affect T_{pref} at 12-months-old. Body weight data from 8-month-old and 12-month-old LWF were pooled to investigate the effect of size on T_{pref} (Figure 3.1). A linear model was fit, and no consistent relationship between T_{pref} and weight was found for the size range tested (35 – 147mm, 0.51 – 20.9g). To investigate the effect of age on T_{pref} we compared 8-month-old and 12-month-old LWF incubated at the standard temperature of 2°C. Average T_{pref} for 8-month-old LWF was

16.04 ± 1.14°C compared to 15.27 ± 2.67°C for 12-month-old LWF (Table 3.1), which were not statistically different (T-test, $p = 0.147$).

While number of passages and distance travelled were not different between treatment groups at 12-months-old (One-way ANOVA, $f = 0.83$, $p = 0.439$; $f = 2.759$, $p = 0.068$), there was a significant effect of age on activity. 12-month-old LWF performed significantly less passages (T-test, $p < 0.0001$) within the 2-hour dynamic phase compared to 8-month-old LWF. Older LWF crossed an average of 44.4 times per trial, while younger LWF crossed an average of 166.4 times, a difference of 375%. 12-month-old LWF travelled on average 32 m farther than 8-month-old LWF (difference of 17%; Table 3.1).

3.4.2 Round Whitefish

RWF juveniles incubated at 2°C and 6°C as embryos displayed significantly lower T_{pref} (Figure 3.1; One-way ANOVA, $f = 5.509$, $p = 0.00578$) compared to those incubated at the standard temperature 0.5°C. Post-hoc comparisons show that both 2°C and 6°C groups were significantly different than the 0.5°C group (Adjusted $p = 0.0216$, Adjusted $p = 0.0100$), but not different from each other (Adjusted $p = 0.8764$). Juvenile RWF incubated at elevated temperatures displayed a temperature preference that was 1.02°C colder on average (8%). While they followed the same decreasing trend, upper and lower avoidance temperatures were only significantly different between 0.5°C and 6°C treatment groups (Tukey's HSD, adjusted $p = 0.030$; adjusted $p = 0.028$). 12-month-old RWF juveniles typically avoid temperatures outside the range of 13.32 – 15.40°C (Table 3.1). Total distance travelled (One-way ANOVA, $f = 1.885$, $p = 0.159$) and number of passages (One-way ANOVA, $f = 0.522$, $p = 0.596$) were statistically similar between all treatment groups. Total length was not consistent between treatment groups (One-way ANOVA, $f = 15.097$, $p < .00001$) as juveniles in the 2°C group were significantly smaller than those in the 0.5°C and 6°C treatment groups. However, total length was not significantly different between 0.5°C and 6°C treatments (Tukey's HSD, adjusted $p = 0.999$). Body weight followed the same trend as total length, with the 2°C group significantly smaller in body weight on average than the 0.5°C and 6°C groups (One-way ANOVA, $f = 11.374$, $p = .000045$, Table 3.1).

3.4.3 Species Comparison

RWF preferred significantly colder temperatures (13.40 ± 1.35°C) compared to LWF (15.27 ± 2.67°C) incubated at the same temperature (2°C) and age (12-months-old; One-way ANOVA, $f = 2.156$, $p = .1472$). In addition, 12-month-old RWF had the least variability in T_{pref} ($SD_{pooled} = 1.043$) compared to 8-month-old ($SD_{pooled} = 1.422$) and 12-month-old LWF ($SD_{pooled} = 2.528$). 12-month-old RWF had significantly more passages (T-test, $p < 0.0001$) compared to 12-month-old LWF, suggesting a potential species difference in relative activity levels. However, total distance travelled did not follow the same trend, as 12-month-old LWF travelled on average 16 m further (8%) than 12-month-old RWF. RWF at 12-months-old (59 ± 7mm) were more similar in total length to 8-month-old LWF (55 ± 6mm) than 12-month-old LWF (114 ± 10mm), highlighting a difference in juvenile growth rate when raised under similar conditions (Table 1). Body weight followed a similar trend, with 12-month-old LWF being on average ~10x heavier than 8-month-old LWF and 12-month-old RWF.

3.5 Discussion

We predicted that whitefish embryos exposed to elevated temperatures during embryonic development would display decreased T_{pref} compared to those reared in colder incubation conditions that are more reflective of optimal embryo incubation conditions. This prediction was based on the positive relationship between incubation temperature and metabolic rate (Bosek et al., 1990; Barrionuevo & Burggren, 1999; Huuskonen et al., 2003; Marty et al., 2010; Eme et al., 2015) and negative relationship between basal metabolic rate and T_{pref} (Killen, 2014). As predicted, 12-month-old RWF incubated at 2 and 6 °C displayed decreased T_{pref} (13.40 - 2°C, 13.39 - 6°C) compared to those reared at 0.5°C (14.41). This data shows that embryonic incubation conditions can influence juvenile thermal preference and this effect can persist for at least 12 months post-hatch. Yet, there was no clear effect of incubation temperature on T_{pref} for either 8 or 12-month-old LWF incubated at 2, 5, or 8°C, suggesting a species difference in thermal sensitivity. Previous literature (Edsall 1999) identified a relationship between age-class and T_{pref} for juvenile LWF, prompting an analysis for the effect of body weight and total length on T_{pref} in this study, using the LWF which encompassed a larger size range. There was no consistent relationship between size and T_{pref} in the size range tested

(35-147mm), suggesting age class and life history are more important factors than weight or length. Whitefish exposed to elevated temperatures during development may be at a competitive disadvantage *in situ*. Whitefish are poikilothermic ectotherms, and as such many of their life history traits are heavily influenced by temperature (Stevens & Fry, 1970). A change in preferred temperature may have indirect consequences to fitness later in life, as fish may not occupy the most optimal temperature range or habitat for a given life stage.

3.5.1 Effect of Elevated Incubation on Thermal Preference

Most teleost fish are poikilothermic ectotherms, meaning they do not produce internal heat metabolically, and body temperature is heavily influenced by ambient conditions (Stevens & Fry, 1970). To thermoregulate, fish must migrate to more favorable water conditions and avoid unfavorable conditions, as the ambient water temperature is directly correlated with the internal body temperature (Stevens & Fry, 1970). There is a well-established positive relationship between basal metabolic rate and ambient water temperature, in which basal and active metabolic rates decreases with a Q_{10} of 1.6-2.0 as water temperature decreases (Fry & Hochachka, 1970). Basal metabolic rate increases with water temperature up to a threshold point, where oxygen supply to tissue becomes restricted. For example, the basal metabolic rate of largemouth bass (*Micropterus salmoides*) increases with water temperature from 15-30°C but decreases from 30-35°C (Beamish, 1970).

LWF embryos incubated at constant elevated temperatures display increased oxygen consumption and heart rates (Eme et al., 2015); it is unknown whether this difference in metabolic rate persists in the juvenile life stage in LWF. However, a relationship between embryonic incubation temperature and juvenile metabolic rate is reported in other species, including razorback sucker (*Xyrauchen texanus*; Bosek et al., 1990), zebrafish (*Danio rerio*; Barrionuevo & Burggren, 1999), Arctic charr (Salvenlinus alpinus; Huuskonen et al., 2003) and Japanese medaka (*Oryzias latipes*; Marty et al., 2010), suggesting this is likely a more general relationship. A functional link between basal metabolic rate and thermal preference has been established in common minnow (*Phoxinus phoxinus*), which suggests that fish embryos with increased oxygen consumption may prefer colder temperatures as juveniles (Killen, 2014). If this is a general relationship for most fish, it could explain why elevated embryonic incubation treatment resulted in lower T_{pref} in RWF. Whitefish embryos have elevated metabolic rates when exposed to elevated temperatures, which if the treatment effect persists past hatching, could cause whitefish embryos to prefer colder temperatures as juveniles.

Phenotypic plasticity is the ability of individual genotypes to produce modified phenotypes when exposed to different environmental conditions (Jonsson & Jonsson, 2019). Early life thermal history can modulate plastic traits responsible for behavior, social skills, stress responsiveness and personality in mammals (Jonsson & Jonsson, 2019) and may exert similar influences in fish. Increases in ambient water temperature during development causes Atlantic salmon (*Salmo salar*) to have increased growth rates resulting in larger body sizes, which is typically favorable in warmer conditions (Jonsson & Jonsson, 2014). Phenotypically plastic traits help individuals adapt to environmental changes much faster (<1 generation) than would be possible through natural selection (1000s of generations). Most plastic changes are confined to an individual's lifetime, but the extent and degree of plasticity may vary between individuals (Jonsson & Jonsson, 2019). Data from this study suggest plastic changes in T_{pref} can last at least to 12 months in RWF. While on average RWF displayed a T_{pref} of 13.4°C when incubated at 2°C and 6°C, individuals displayed a wide range of T_{pref} (10.13 – 15.75°C; 11.4 – 16.14°C respectively), showing individual differences in plasticity.

While the majority of available literature on thermal effects in whitefish pertains to LWF, there is sufficient data to suggest that RWF are more thermally sensitive. Embryonic mortality increases with increasing incubation temperature for both LWF (Price, 1940; Brooke, 1975; Lim et al., 2017; Mitz et al., 2019; Eme et al., 2015, 2018; Mueller et al., 2015, 2017; Lee et al., 2016; Patrick et al., 2013; Thome, in press) and RWF (Lim et al., 2018; Griffths, 1980). However, RWF experienced 20-30% increased mortality at 2°C and 30-40% increased mortality at 8°C compared to LWF reared under the same conditions (Lim et al., 2017, 2018). This is the primary reason why the two species were not reared in identical temperatures; at 8°C RWF experience near 100% mortality (Lim et al., 2018). Interestingly, we have shown elevated incubation temperatures have a persistent effect on the thermal preference of 12-month-old RWF, but do not in LWF. This supports the notion that RWF are more thermally sensitive because not only was there an effect of embryonic incubation temperature on juvenile T_{pref} in RWF (but not in LWF) but this occurred at lower incubation temperatures (0.5-6° for RWF versus 2-8°C for LWF).

Beyond thermal sensitivity, RWF are more sensitive to chronic exposure of morpholine, a chemical used to prevent corrosion and damage in water pipes, compared to LWF (Lim et al., 2018; Thome et al., 2017). RWF experienced reduced body size (>100 mg/L), and increased mortality at hatch (500 mg/L) at lower concentrations of morpholine compared to LWF (Lim et al., 2018; Thome et al., 2017). RWF have historically had a smaller distribution than LWF in North America, with LWF distribution extending farther south beyond the great lakes. In recent years, population declines have led RWF to be labelled as vulnerable in New York State (Bouton & Stegemann, 1993). Competition with LWF and yellow perch, as well as lake acidification are thought to contribute to this decline, as RWF are not commercially exploited (Bouton & Stegemann, 1993). LWF on the other hand have sustained a large commercial fishery since the 1800s (Ebener et al., 2010), with annual catches between 0.7-11 million kg/yr. Where both species are present LWF are typically more abundant than LWF, outcompeting RWF for shared resources (Eberts et al., 2016). Collectively, these data may suggest that RWF are potentially more generally sensitive to environmental stressors compared to LWF. This needs to be explicitly tested in the future.

3.5.2 Effect of Size on Thermal Preference

Since there was no effect of elevated embryonic incubation on T_{pref} for 12-month-old LWF, an earlier life stage was tested to determine if a treatment effect was present at 8-months-old. While we detected a significant difference between T_{pref} for the 5°C and 8°C treatments in the 8 month old LWF, this was an unexpected result, as we predicted the largest difference in T_{pref} between the lowest (2°C) and highest (8°C) incubation temperatures. Power analyses prior to experimentation determined the statistical power to be 60% so there may be a weak relationship between T_{pref} and incubation temperature for 8-month-old LWF. These results suggest that a treatment effect may be observed if juvenile LWF were tested at a younger age. Ideally, we would test fish immediately post-hatch, and at multiple time points within the first year, but there are biological and logistical challenges that prevent data collection earlier than 8 months.

Younger whitefish are more active and there is a functional limit to the response time of the shuttle box system. 8-month-old LWF were much closer to this threshold (5 passages/min) than 12-month-old LWF. Elevated activity levels have an impact on the thermal capacity of the shuttle box system, with higher activity leading to shorter windows of response time. If the system cannot properly respond, then the temperature of the system water will remain relatively stable. This could potentially lead to occupied temperatures remaining close to the starting temperature, and failure to accurately measure T_{pref}. Shuttlesoft® is the tracking software associated with the Loligo® shuttle box system, and tracks fish using contrast between the fish and the shuttle box. Smaller, fast moving fish are more difficult to track and can be 'lost' by the tracking software during the assay. Traces of fish movement show that it may be possible for juvenile whitefish <50mm to complete undetected rapid passages between the sides of the shuttle box. However, if undetected passages occurred, they are so rapid that the shuttle box system could not adjust the temperature in response to the movement. The shuttle box system water was gravity fed from buffer tanks at a rate of 240 mL/min, which was close to the functional limit of the flow rate in this experimental set-up. LWF that measured <40mm in total length had difficulty maintaining position in the shuttle box due to the current, which may have affected their ability to shuttle between tanks. This suggests that testing LWF younger than 8-months-old (35-71mm) in a shuttle box system would be impractical.

T_{pref} has been determined in juvenile LWF in two previous studies and our data are generally comparable (Table 3.2). T_{pref} varied 15.9 – 18°C for age-0 and 10 - 15.6°C for age-1 LWF, depending on the study (Table 3.2). Differences across the studies may be due to three major factors. First, incubation temperatures may not be similar across studies. Our 2°C incubation group appears to be similar to Edsall (1999), because they performed incubation of embryos by "mimicking nearshore overwinter water temperatures" and the testing dates provided for different aged fish suggest a similar hatching time for fish reared near 2°C. However, the incubation temperature was not provided for either previous study. Second, water temperature in the post-hatching stage are clearly different. Edsall (1999) used much cooler temperatures for all age fishes (Table 3.2). Third, there are differences in experimental design. Both prior studies (Edsall, 1999; Opuszynski, 1974) used vertical thermal gradient systems to determine T_{pref} and both acknowledged the implications of LWF displaying bottom affinity in the vertical test systems. In vertical gradients systems, the coldest temperatures are found at the bottom, so fish associating with the base of the tank may have T_{pref} underestimated. Regardless, T_{pref} for the 2°C incubation group was similar to that reported by Edsall (1999) for both age-0 and age-1 LWF (Table 3.2), and both studies found older fish (age-1) preferred colder temperatures.

Previous studies of LWF thermal preference suggest that thermal preference is different across age-classes, although this could be age and/or size of fish. We also used fish that were age-0 (8 months) and age-1 (12 month) age-classes. In general, younger LWF appear to prefer warmer temperatures when compared to older conspecifics (Table 3.2). A warmer temperature preference at younger ages would be appropriate for the life history of the species. LWF spawn in waters <10m in depth (Ebener et al., 2010), move into nearshore nursery areas immediately post-hatching (age-0), before transitioning into more adult habitat suggesting that LWF go through a natural transition of shallow, warm water to deeper, cooler water as they mature (Ryan et al., 2014). Plankton net sampling in whitefish nursery grounds reveals the highest densities of larval whitefish between April – June (Loftus, 1982; Ryan et al., 2014), suggesting juveniles might begin to leave nursery grounds at approximately 3-4 months of age. Individuals classified as age-0 may occupy warmer coastal waters but age-1 individuals would have naturally migrated to deeper, colder waters.

There were no differences in T_{pref} between 8-month-old and 12-month-old LWF when tested at the same temperatures (2°C; T-test, $p = 0.147$). These results are in contrast with previous studies (Edsall, 1999; Opuszynski, 1974) although in both studies the age-0 cohort included animals younger than ours (4-6 versus 8 months). Both prior studies identified a significant relationship between size and T_{pref} in LWF, yet the age and body weight or length are correlated. The growth rates of the fish in this study were considerably higher than those previously published; LWF had an average weight of 10.54g by 12-months-old, compared to 3.35g for the same age fish in Edsall (1999). The colder holding temperature (8-11°C for Edsall, 1999) likely accounts for this difference in growth rate. Furthermore, Edsall (1999) was able to detect a significant relationship between size and T_{pref} using age-classes that differed in average weight by 0.9 – 2g, while in this study there was no relationship between age-classes that differed by 9.4g (Table 3.2). Regardless, size cannot explain the lack of difference across age classes in our study; length and weight were not significant factors in T_{pref}. Thus, we suggest that age, specifically with respect to life history, is a more important factor than size in determining the decrease in T_{pref} across different aged animals in previous studies but not ours. If whitefish begin migrating from warmer coastal waters to cooler, deeper waters around 4 months of age, as the plankton tow data suggests (Loftus, 1982; Ryan et al., 2014), we would expect age-0 animals <4-6 months of age to have warmer T_{pref}, as was found in both Edsall (1999) and Opuszynki (1974), reflecting a life stage largely located in warmer nursery areas. We used 8-month-old fish for our age-0 class and found no different in T_{pref} across age classes. The age-0 and age-1 classes in our study likely represent the same life stage of fish, expected to have already moved from the nursery areas to colder waters. Thus, we expect T_{pref} would change with life history.

3.6 Conclusion

This study describes a persistent effect of increased embryonic incubation temperature on the thermal preference of juvenile RWF, which has important implications for the great lakes. While RWF are rarely abundant (Mraz, 1964), they have a wide distribution across Canadian freshwater systems and therefore are potentially exposed to many anthropogenic changes in temperature. A benthic water temperature of 2°C is within the "normal" range of winter temperatures in the great lakes but represents winter with low ice cover and was sufficient to alter the preferred temperature of juvenile RWF compared to a colder temperature that represents a winter will extensive ice coverage. That we found altered T_{pref} at 12 months post-hatch demonstrates a developmental plasticity to incubation temperature in this species. Altered T_{pref} could be problematic for developing RWF, as this could cause juveniles to seek out sub-optimal habitats. Coastal embayments provide a thermal refuge during the spring warming (Ryan et al., 2014), and ice-free conditions facilitate a spring bloom of primary productivity which is important for survival of larval whitefish (Faber, 1970). Whitefish seeking cooler water temperatures may avoid prime nursery grounds, which would put them at a disadvantage compared to other conspecifics.

Average water temperatures in the great lakes are increasing at a rapid rate (0.1°C/year; Austin & Colman, 2007) and thermal effluents influence coastal water temperature. Therefore, understanding which species are most thermally sensitive and how incubation temperature influences fish post-hatching will be important for conservation efforts. This is particularly true in RWF which are already considered threatened in at least some of their southern range.

Notably, LWF, an important commercial species, did not have altered T_{pref} with increased incubation temperature, providing another line of evidence that RWF are more thermally sensitive. This study examined two closely related species with comparable reproductive strategies and ranges. Cold-adapted freshwater fish are among the taxa most vulnerable to climate change, but receive a fraction of the research and conservation efforts of terrestrial species (Pacifici et al., 2015). This study highlights the importance of examining sub-lethal thermal effects and thermal plasticity of cold-adapted species. We do not yet suggest a mechanism, which indicates the need for future research. Determining basal metabolic rate in the post-hatching period would be most important to understand the potential role of metabolism in thermal preference and developmental plasticity of round whitefish with respect to different incubation temperatures.

Table 3.1 Preference and avoidance temperatures of lake and round whitefish reared at different incubation temperatures. Fish were reared at different temperatures during embryogenesis and then held at common garden conditions post-hatch. During incubation, water was cooled to a base temperature, provided as the Incubation Treatment below, and held for up to 10 weeks before warming to simulate natural incubations. Temperature preference (T_{pref}) is calculated as the cumulative median of occupied temperature. Avoidance temperatures are calculated as the average temperature at which a passage occurs. Distance is the total distanced travelled, passages represents the number of times the fish shuttled sides in the system; these were based on tracking fish in Shuttlesoft®. All values are mean±SD.

8-month-old LWF

Incubation Treatment	n	T_{pref} (°C)	Upper Avoidance (°C)	Lower Avoidance (°C)	Distance (m)	Number of Passages	Length (mm)	Weight (g)
2°C	31	16.04 (± 1.14)	17.54 (± 0.73)	15.41 (± 0.81)	193 (± 47)	183 (± 113)	55 (± 5)	1.13 (± 0.32)
5°C	29	15.12 (± 1.98)	17.23 (± 1.09)	14.93 (± 1.38)	173 (± 67)	146 (±121)	56 (± 6)	1.25 (± 0.39)
8°C	25	16.01 (± 1.74)	17.74 (± 0.52)	15.63 (± 0.57)	191 (± 60)	170 (± 124)	55 (± 9)	1.18 (± 0.51)

12-month-old LWF

Incubation Treatment	n	T_{pref} (°C)	Upper Avoidance (°C)	Lower Avoidance (°C)	Distance (m)	Number of Passages	Length (mm)	Weight (g)
2°C	31	15.27 (± 2.67)	17.14 (± 2.45)	14.01 (± 2.62)	247 (± 112)	51 (± 95)	114 (± 12)	10.59 (± 3.04)
5°C	40	16.02 (± 2.39)	17.72 (± 2.26)	14.68 (± 1.94)	196 (± 71)	51 (± 65)	114 (± 11)	10.76 (± 3.59)
8°C	32	15.62 (± 2.56)	17.41 (± 1.85)	14.24 (± 2.16)	209 (± 97)	31 (± 55)	114 (± 8)	10.28 (± 2.43)

12-month-old RWF

Incubation Treatment	n	T_{pref} (°C)	Upper Avoidance (°C)	Lower Avoidance (°C)	Distance (m)	Number of Passages	Length (mm)	Weight (g)
0.5°C	27	14.41 (± 1.17)	15.80 (± 0.75)	13.73 (± 0.75)	217 (± 44)	212 (± 81)	62 (± 5)	1.60 (± 0.45)
2°C	31	*13.40 (± 1.35)	15.29 (± 0.93)	13.18 (± 1.01)	189 (± 65)	188 (± 105)	*55 (± 6)	*1.19 (± 0.35)
6°C	23	*13.39 (± 1.00)	15.11 (± 0.90)	13.04 (± 0.79)	198 (± 41)	204 (±79)	62 (± 5)	1.71 (± 0.52)

Table 3.2 Temperature preference (T_{pref}) of juvenile lake whitefish of different ages. Age is provided in years (yrs) and months. Age in months was estimated for previously published studies by assuming median hatch occurs within March – April, as suggested by testing dates for 1-year old fish. T_{pref} from the present study is reported from 2°C treatment groups only. Edsall (1999) used simulated lake water temperature during embryonic incubation. All other T_{pref} data provided by Edsall (1999). Holding temperature refers to water temperature in home tanks from hatch until testing.

Age (yrs)	**Age (months)**	**Size (g)**	**Tpref (°C)**	**Holding Temperature (°C)**	**Source**
0	4-5[a]	2.8	15.9	8-11	Edsall (1999)
0	5-6	1.1-1.7	17-18[b]	-[c]	Opuszynski (1974)
0	5-6	1.9	16.8	8-11	Edsall (1999)
0	8	1.13	16.04	15	Present Study
1	12	10.59	15.27	15	Present Study
1	12-13[a]	3.9	15.6	8-11	Edsall (1999)
1	12-13	5.7	10	-[c]	Opuszynski (1974)

[a] Repeated measure on same cohort of fish.

[b] Temperature preference estimated via inspection by Opuszynski (1974).

[c] Information not available.

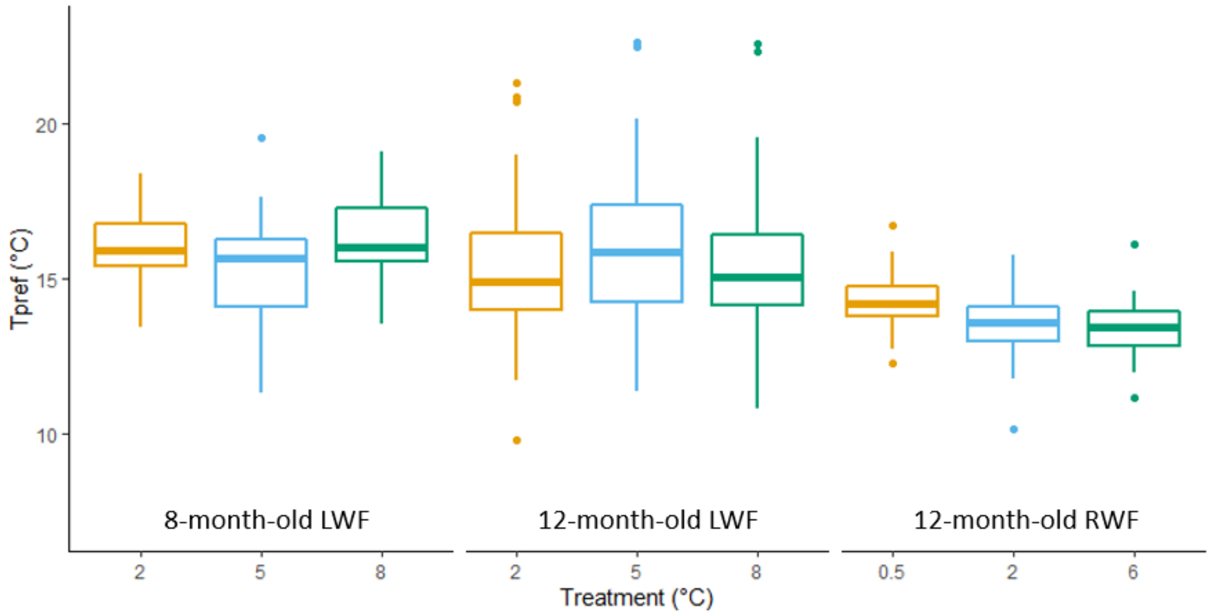

Figure 3.1 Boxplot comparing temperature preference (T_{pref}) between all treatment groups of 8-month-old LWF, 12-month-old LWF and 12-month-old RWF. Solid line indicates median Tpref, edges of the box represent Q1 and Q3, vertical lines represent minimum and maximum values, dots are outliers defined as 1.5 x IQR.

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Chapter 4

General Discussion

4.1 General Discussion

Temperature is an important variable for freshwater fish that influences optimal growth conditions and fitness-related activities (Fry, 1947; Magnuson et al., 1979; Jobling 1981). The sub-lethal effects of thermal exposure during embryonic development are important determinants of juvenile fitness but are often overlooked in studies on thermal tolerance. Traditional thermal preference studies are lengthy due to long periods of acclimation (12-24 hours) prior to testing (Reynolds & Casterlin, 1979) which can limit throughput and power in experiments that examine thermal preference. Further, size had been shown to be a factor in thermal preference in whitefish previously (Edsall, 1999; Opuszynski, 1974) and juvenile fish display rapid rates of growth and development compared to adult life stages (Bacon et al., 2005). Therefore, optimizing the thermal preference behavioral assay to a shorter assay was essential to use thermal preference (T_{pref}) as an endpoint in this developmental study. The primary goal of the optimization was to allow me to determine thermal preference on multiple fish per day and increase throughput in the assay but maintain reasonable statistical power to resolve differences between treatment groups. Increased throughput was essential to limit the extent of growth and age range of fish during the testing periods. The assay development in Chapter 2 was necessary to determine the optimal assay design for lake (*Coregonus clupeaformis*; LWF) and round (*Prosopium cylindraceum*; RWF) whitefish in Chapter 3. Data collected in Chapter 2 showed that a total assay duration of 4 hrs produced statistically similar data to a total assay length of 24 hrs, allowing us to increase throughput while maintaining data quality. Using this optimized design, experiments were designed to test 96 fish within a 32-day window. This confirmed the use of T_{pref} as a viable behavioral endpoint for juvenile fish and offers information on trade-offs between throughput, statistical power, and replication for experimental design. In Chapter 3, I showed that RWF incubated at 2°C and 6°C displayed decreased T_{pref} at 12-months of age compared to those incubated at 0.5°C. In contrast, LWF exhibited similar T_{pref} regardless of incubation temperature (2°C, 5°C, 8°C). These are important findings, as they show lasting implications of early life thermal history, as well as how species differ in response to elevated temperatures.

4.2 Biological Implications of Altered Thermal Preference

This thesis investigated the effect of elevated incubation temperatures on the thermal preference of two cold-adapted fish species and found a persistent effect in RWF at 12-months-old. This is the first study, to our knowledge, to relate elevated incubation temperatures to altered thermal preference post-hatch, which has potentially wide-reaching implications for North American freshwater fish. While whitefish species may be especially vulnerable to changes in embryonic temperature due to their long incubation period (Ebener et al., 2010), freshwater fish requiring cold water (<22°C) are among the taxa considered most susceptible to climate change (Moyle et al., 2013). Climate change encompasses changes in temperature (Webb et al., 2008), atmospheric CO_2 (IPCC 2013), and increased frequency and intensity of weather events (Barnett et al., 2005; Milly et al., 2006). A global increase in average air temperature of up to 4°C, and likely up to 6°C in the Great Lakes region of North America, is expected by 2100 due to the greenhouse effect (IPCC 2013). Declining winter ice cover is causing average water temperatures to warm at a rate of 0.1°C per year, which is far in excess of the regional atmospheric warming (Austin & Colman, 2007). However, with warming at 0.1°C per year it would take around 30 years to reach winter substrate temperatures of $\geq$ 5°C.

Many industrial power plants utilize lake water for their cooling systems, removing excess heat and discharging the water back to the lake as thermal effluent in a process known as once-through cooling. Fish spawning within the thermal plume of the effluent may subject embryos to elevated temperatures during development, while those spawning on the edges of the thermal plume may be exposed to fluctuating temperatures (Thome, 2016). These are point sources of thermal pollution, but collectively affect a large proportion of coastal habitat. There are an estimated 89 thermal power plants in the great lakes using once-through cooling that account for 48% and 46% of total water consumption in Canada and the USA, respectively (Kelso & Milburn, 1979). Temperature data from sites around the Bruce Power Nuclear Generating Station A and B discharge sites on Lake Huron suggest the thermal plumes change ambient water temperature by 1-3°C (Thome et al., 2016). Nuclear power generation is often proposed as a ‘green’ alternative to fossil fuels, as CO_2 emissions are negligible in comparison (Sovacool, 2008). In 2005, 435 nuclear plants supplied 16% of the world's power (International Energy Agency, 2007), which will rise if more countries invest in nuclear energy. It is important that the ramifications of switching to nuclear power are considered, as other alternative sources of electric generation (e.g. solar, wind) do not require massive volumes of water to cool steam generators (Lenzen, 2008). While climate change may be a future threat to freshwater fish, discharge of thermal effluent affects nearshore environments on a constant basis in the present. Fish embryos may be at more vulnerable to thermal effluents as they are non-mobile and cannot escape elevated temperatures.

Because of the depth and proximity to land, the limnetic zone of the lake is most vulnerable to climate change (Jansen & Hesslein, 2004) and the primary zone where thermal effluent is discharged. Whitefish and other great lakes species such as lake sturgeon (*Acipenser fulvescens*), alewife (*Alosa pseudoharengus*) and lake trout (*Salvelinus namaycush*) spawn on shallow, coastal cobble (64–256 mm) beds (Lane et al., 1996) in the limnetic zone. These species are at increased risk from anthropogenic warming as they are cold-water adapted, requiring temperatures below 22°C for optimal growth (Moyle et al., 2013). Thermally sensitive species that live or use (e.g. for spawning and nursery grounds) areas vulnerable to anthropogenic warming will likely be among the first to experience the deleterious effects of elevated water temperatures. The framework from this study could be used to determine the vulnerability of other freshwater species to elevated embryonic temperature. Understanding life stage specific or species differences to elevated temperatures is essential to effectively manage and conserve threatened species like RWF.

Altered T_{pref} in poikilothermic ectotherms could mean that individuals are preferring temperatures outside of their optimal range for growth/development (LWF; 15.5–19.5°C; Edsall, 1999). RWF incubated at 2°C and 6°C displayed a temperature preference that was ~1°C colder than fish incubated at 0.5°C. An altered T_{pref} post-hatch could have consequences on juvenile survival. Whitefish use coastal nursery grounds for growth and development (Scott &

Crossman, 1979), which are typically the warmest part of the lake (Jansen & Hesslein, 2004) due to their depth and proximity to land. At hatch, water temperatures in the great lakes are typically cold (2-4°C) and areas of warmer water (4-8°C) are found in nearshore embayments (Brown and Taylor, 1992). Although larvae are aided in transport by water currents (Ryan et al., 2014), altered T_{pref} could interfere with migration to nursery grounds. Nursery grounds are important for juvenile survival and provide the most optimal habitat for early life (Ryan et al., 2014). Individuals with altered T_{pref} may seek out habitats that conform with their cooler thermal optima, leading to individuals to choose sub-optimal habitats. Therefore, changes in T_{pref} could result in juvenile fish occupying sub-optimal habitat during important life stages, leading to reduced survival rates.

Previous literature (Bozek et al., 1990; Barrionuevo & Burggren, 1999; Huuskonen et al., 2003; Marty et al., 2010) has suggested a correlation between embryonic incubation temperature and basal metabolic rate in juvenile fish. While it is unclear whether standard metabolic rate is correlated with growth in freshwater fish (Álvarez & Nicieza, 2005), juvenile Atlantic salmon (*Salmo salar*) incubated in heated (7.2 ± 0.6°C) river water displayed increased growth rates and attained larger adult body sizes than those incubated in natural (2.6 ± 0.4°C) river water (Jonsson & Jonsson, 2014). Results from Chapter 3 show no effect of elevated incubation temperatures on total length and body weight for 8 or 12-month-old LWF ($p = 0.978$, $p = 0.753$). RWF incubated at 2°C were significantly smaller than those incubated at 0.5°C or 6°C ($p < .00001$), but there were no differences between 0.5 and 6 treatment groups ($p = 0.999$). Total length was only measured at 8 and 12-months in Chapter 3, so growth rates are difficult to estimate. Future studies specifically measuring basal metabolic rate and growth over time from 0 – 12-months could better determine the effect of elevated incubation temperatures on standard metabolic rate, and its effect on growth rates.

4.3 Power Analyses Facilitates Better Experimental Design

Since Platt (1964) and Popper (1968), most aquatic researchers have formatted research questions around formal tests of null hypothesis (H_o). Questions generated in this format will either be answered by rejecting H_o or failing to reject H_o. If a non-significant p-value is obtained, it is interpreted as failing to reject H_o, but does not necessarily indicate that the H_o is true. It is possible that a real effect exists, but researchers failed to detect it due to large sampling variation or small sample size (Peterman, 1990). There is no consensus on what constitutes “sufficiently high” power required to detect an effect, but in general researchers would consider $1-\beta > 0.8$ a lower threshold (Peterman, 1990). Statistical power ($1-\beta$) represents the probability of detecting an effect (if one is present) within a system and is related to the probability of type II error (β). While most studies follow a standard definition of type I error ($\alpha = 0.05$), many aquatic studies overlook the importance of setting limits on power (Peterman, 1990). Failing to reject a false null hypothesis (Type II error) can often be more influential than rejecting a true null hypothesis (Type I error), as important relationships can be overlooked.

Thermal preference studies have often required long experimental timeframes, as individual assays can be longer than 24 hours (Reynolds & Casterlin, 1979); this typically limits sample sizes in experiments that determine thermal preference. There can be considerable variation in T_{pref} within a species (Holland et al., 1974), which can lead to large sampling variation. A correlation between age and thermal preference has been described in rainbow trout (*Salmo gairdneri*), with younger fish preferring warmer water than older conspecifics (McCauly & Pond, 1971; Kwain & McCauly, 1978). Rainbow trout fry display a steady decline of thermal preference until 12-months-old, after which thermal preference begins to plateau (Kwain & McCauly, 1978). Juvenile fish in their first year may have more variable T_{pref} compared to older fish, as they are in a period of rapid growth and development (Bacon et al., 2005). These challenges make it difficult to obtain high power ($1-\beta > 0.80$) when determining the thermal preference of juvenile fish. Power analysis is an effective tool that allows researchers to determine whether sample size and variation were adequate to draw conclusions, but generally is underused within the research community. The power analysis conducted in Chapter 2 could be used as a model for optimizing future studies of thermal preference and to qualify conclusions about non-significant p-values.

To properly conduct a power analysis, preliminary information on sampling variation must be acquired, which can come from closely designed studies in the literature or thoughtfully constructed pilot studies. The studies performed in Chapter 2 were essential to conclude whether power was sufficient to detect effects in whitefish and helped to optimize the behavioral assay. Based on the results of Chapter 2, I determined that the maximum power I would be able to obtain was $1-\beta = 0.6$, which indicates that my design had a 60% chance of detecting an effect if present. While my studies in Chapter 2 only contained LWF incubated at

2°C, the information collected helped determine required sample sizes for experiments involving multiple treatment groups for LWF and RWF. In Chapter 3, I found the variation in T_{pref} was larger for LWF than RWF, meaning predicted experimental power was underestimated for RWF.

An estimated experimental power of 0.6 means there is also a 40% chance that this study design will not detect an existing effect. While 0.6 is less than the proposed threshold (0.8), it was the highest attainable power given the logistical concerns discussed in Chapter 2. With an estimated power of 0.6 a significant treatment effect was detected in RWF, but not in LWF. While designing this experiment, I explicitly limited the total study duration to minimize the impact of growth on T_{pref}, based on a relationship between size and T_{pref} found by previous studies of whitefish thermal preference (Edsall, 1999; Opuszunski 1974). However, analyses in Chapter 3 showed that total length and body weight were not significant factors in determining T_{pref} for juvenile whitefish. Future experiments may be less concerned about the impacts of length and weight as confounding effects on T_{pref} and increase treatment group size to increase statistical power and to increase reliability of results. Careful consideration of age and the age ranges of the juvenile whitefish tested would still be needed.

4.4 Considerations on the Identification of Outliers and Removal of Data

Freshwater fish are motile organisms and the frequency of movement and distance travelled can vary substantially between (Roberts & Angermeir, 2007; Albanese et al., 2011) and within

(Bell, 1976; Behnke, 2002) species. In Chapter 3, a wide range of passage numbers were observed and passage number was strongly influenced by species. On average 12-month-old RWF (201 ± 88) crossed 157 more times compared to 12-month-old LWF (44 ± 72) in the 2-hour behavioral trial (Figure 4.1). However, passage number was not predictive of total distance travelled, which was similar between species, suggesting they are not equivalent measures of relative activity. In this thesis, passages within the static acclimation phase were interpreted as exploratory movement, while passages during the dynamic phase are responses to changing water temperature (i.e. behavioral thermoregulation). There was no significant correlation between passage number and distance travelled for LWF or RWF in Chapter 3, suggesting distance travelled in the dynamic phase was not necessarily the result of thermoregulatory behavior. Distance travelled during the static phase may best represent ‘relative activity’ as fish movements are not influenced by changes in temperature. There were no significant differences in distance travelled during the static phase (One-way ANOVA, f = 0.800, p = 0.450) or dynamic phase (One-way ANOVA, f = 1.693, p = 0.194) in Chapter 3. However, movement was highly variable among individuals, evidenced by the large variation in total distance travelled within treatment groups (12-month-old LWF, SD_{pooled} = 92.9; 8-month-old LWF, SD_{pooled} = 58.8; 12-month-old RWF, SD_{pooled} = 51.5). Since the shuttle box system relies on the positional tracking of the fish in Shuttlesoft®, it is important to consider the potential effects such a large variation in movement could have on the function of the shuttle box.

The shuttle box system detects the position of the fish in real time, but there is a time lag between detecting the location of the fish and activating/deactivating pumps that cause the temperature change. A linear relationship between time and temperature change in the shuttle box system suggests that a passage frequency greater than 5/min is too rapid for the system to respond and results in minimal temperature change within the system. High activity individuals may not be exposed to a range of temperatures required to properly determine T_{pref}. If younger fish are more active, this may add uncertainty to T_{pref} measurements for some age groups, and they may even shuttle too frequently for the system to handle. Thus, it is important to determine a threshold for passages with the experimental set up of the shuttle box and compare this to the mean passage number for a given species and life stage to ensure the set up is rationale to determine T_{pref}. Likewise, it is important to examine passage number for individual fish to identify high passage number individuals where T_{pref} may be inaccurate. In Chapter 3, no fish exceeded the threshold of passages (600) for the entire dynamic phase, but 7 fish were between 400-600 passages (Figure 4.2). Passages were not uniformly distributed across the 2-hour dynamic phase, suggesting that fish with 400-600 passages likely exceeded the 5 passages/min threshold for short durations.

Inactive fish are likewise problematic for the shuttle box system, which relies on fish movement to determine T_{pref}. Fish making <5 passages during a 2-hour behavioral trial may not be actively participating in the behavioral trial. Fish with low passage numbers (<5) had similar distance travelled to fish with high passage numbers, indicating they were still actively moving within the system, but not behaviorally thermoregulating via shuttling. There is little discussion in the literature on whether these individuals are making an active behavioral choice by remaining on one side, or if they are not properly participating in the behavioral trial. Since very few fish were less active or inactive during the dynamic phase, it could suggest that these fish are not actively participating in the trial and their data should be removed. All of these fish passaged in the static phase, indicating they had explored the entire arena. There is considerable individual variation in T_{pref} (Holland et al., 1974), so it is possible these individuals were actively choosing much colder/warmer temperatures than their conspecifics. Due to the length of most behavioral trials, many studies on T_{pref} of fish utilize low sample numbers (Siikavuopio et al., 2014; Barker et al., 2018; Konecki et al., 1995; Larsson, 2005; Petersen & Stefensen, 2003; Macnaughton et al., 2018), which may contribute to the lack of discussion on removing data. This topic deserves more attention and a consensus should be reached on whether these individuals should be excluded as outliers or retained in the dataset. Since it was unclear where the cut off for passage number should be and all fish passaged at least once, I was conservative with removing data solely based on passage number and instead used the ROUT outlier test to identify extreme T_{pref} values.

There is a considerable variety of shuttle box study designs in literature (McCauley et al., 1977; Schurmann et al., 1991; Petersen, 2003; Bear et al., 2007), with no consensus on the appropriate rate of temperature change for system water. In this thesis, a maximum rate of 4°C/hr was used, which was the most common rate among study designs (Macnaughton et al., 2018; Konecki, 1995; Petersen and Stefensen, 2002). Some studies use faster rates (Barker et al., 2015) of up to 15°C/hr, while others use rates as low as 1°C/hr (Siikavuopio et al., 2014). For traditional study designs (>24 hours), the rate of temperature change doesn't impact the range of temperatures an individual is exposed to. However, when using shorter trial durations (e.g. 2

hours in the dynamic phase) a rate of 4°C/hr barely allows fish to reach threshold temperatures (lower = 7°C, upper = 23°C) within the system. A 4°C/hr rate of change creates a confounding relationship between passage number and T_{pref} (Figure 4.2), so that only inactive fish with few passages will have an extreme T_{pref}. When optimizing the assay design, I did not account for whitefish that would prefer temperatures outside their fundamental thermal niche (15.5–19.5°C; Edsall, 1999). Fish that are actively participating in the trial were expected to thermoregulate around this temperature range, and stationary fish selecting cold extremes were not anticipated. In Chapter 3, fish that reached extreme temperatures did so by remaining stationary and did not attempt to thermoregulate via shuttling during the experiment. Because of this, it is difficult to interpret extreme T_{pref} values with this experimental design.

Increasing the rate of temperature change will potentially remove the confounding relationship between passage number and T_{pref} but at the cost of increased thermal stress, which is difficult to quantify in small-bodied fish. Previous studies have suggested an effect of elevated cortisol on T_{pref} (Rey et al., 2015; Cull et al., 2015), so this should be considered when increasing the rate of temperature change. Considering the relationship between passages and T_{pref}, future studies may consider choosing a rate of temperature change that best suits the activity and thermal tolerance of the study species. For example, a more sedentary species may require a slower rate of change to allow time for behavioral thermoregulation, as remaining stationary is not considered an active behavioral choice. On the other hand, a species with a wide range of thermal tolerance may require faster rates to properly expose fish to the entire range of temperatures during a trial and to allow thermoregulatory shuttling at extreme temperatures.

4.5 Conclusion

This thesis has investigated the effects of elevated incubation temperature on thermal preference and highlighted important experimental concerns when using a shuttle box system. The shuttle box is a relatively new scientific instrument and has the potential to be widely used in thermal performance studies of aquatic organisms. Understanding how T_{pref} is affected by certain biological and technical aspects of data collection is vital for accurate interpretation of T_{pref}. In Chapter 2, I investigated the effect of acclimation time and behavioral trial duration and found that they did not significantly affect the measurement of T_{pref} in juvenile whitefish. Based on these results, the experimental design was optimized to reduce acclimation time and experimental duration to provide higher throughput and increased power in subsequent experiments. This facilitated the use T_{pref} as an endpoint for investigating the sub-lethal effects of embryonic thermal exposure. Data from Chapter 3 show that RWF incubated at 2°C and 6°C display altered T_{pref} up to 12 months post-hatch compared to those incubated at 0.5°C, while the T_{pref} of LWF incubated at elevated temperatures was not different. This data supports the idea that RWF are more thermally sensitive (Lim et al., 2017, 2018), and shows differences in plastic response to elevated incubation temperatures of two cold-adapted species. Elevated temperatures during embryonic development caused plastic changes in the T_{pref} of RWF lasting up to 12 months post-hatch, potentially affecting survival and fitness within their first year. Future studies testing older RWF juveniles to investigate the duration of this plastic response could determine if decreased T_{pref} will persist through to sexual maturity. Functional links between embryonic incubation temperature and post-hatch basal metabolic rate have been established (Bozek et al., 1990; Barrionuevo & Burggren, 1999; Huuskonen et al., 2003; Marty et al., 2010), as well as links between basal metabolic rate and T_{pref} (Killen, 2014). This provides a potential framework for the mechanism of altered T_{pref}, but metabolic data on juvenile whitefish is lacking. Future studies collecting post-hatch metabolic data on whitefish incubated at elevated temperatures could help elucidate if elevated metabolic rates are correlated with decreased T_{pref} in juvenile whitefish.

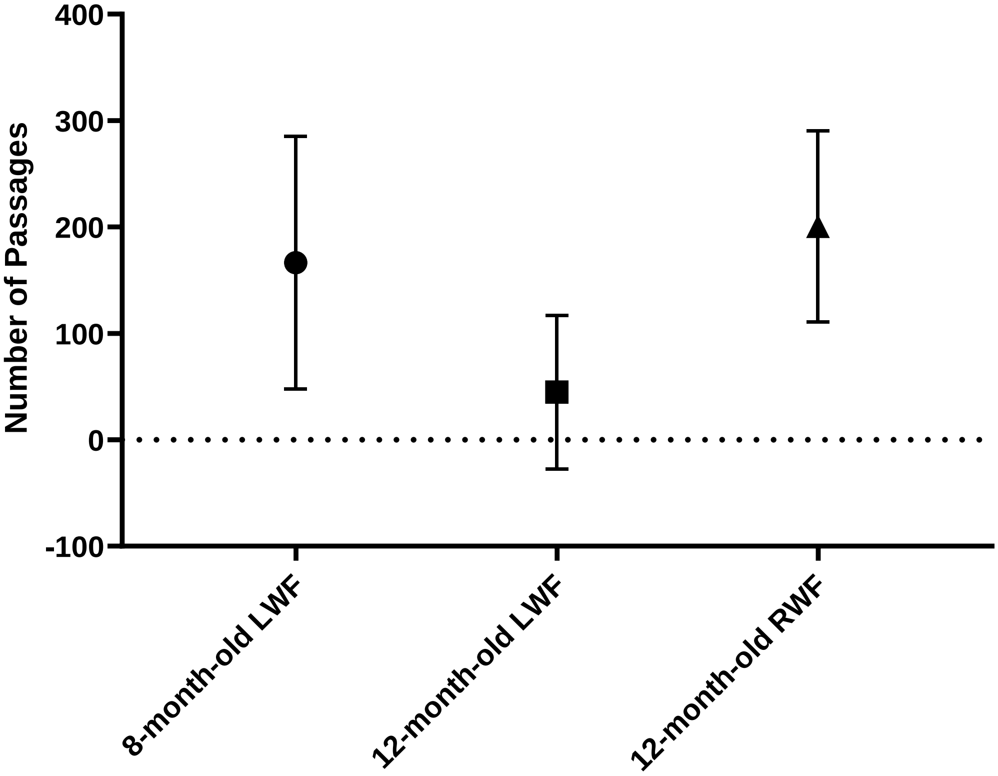

Figure 4.1 Average number of passages for 8-month-old (circle), and 12-month-old (square) lake whitefish (*Coregonus clupeaformis*; LWF) and 12-month-old (triangle) round whitefish (*Prosopium cylindraceum*; RWF). Passages represent a fish shuttling between sides of the shuttle box, which switches the direction of temperature change within the system. Data is presented as mean±SD.

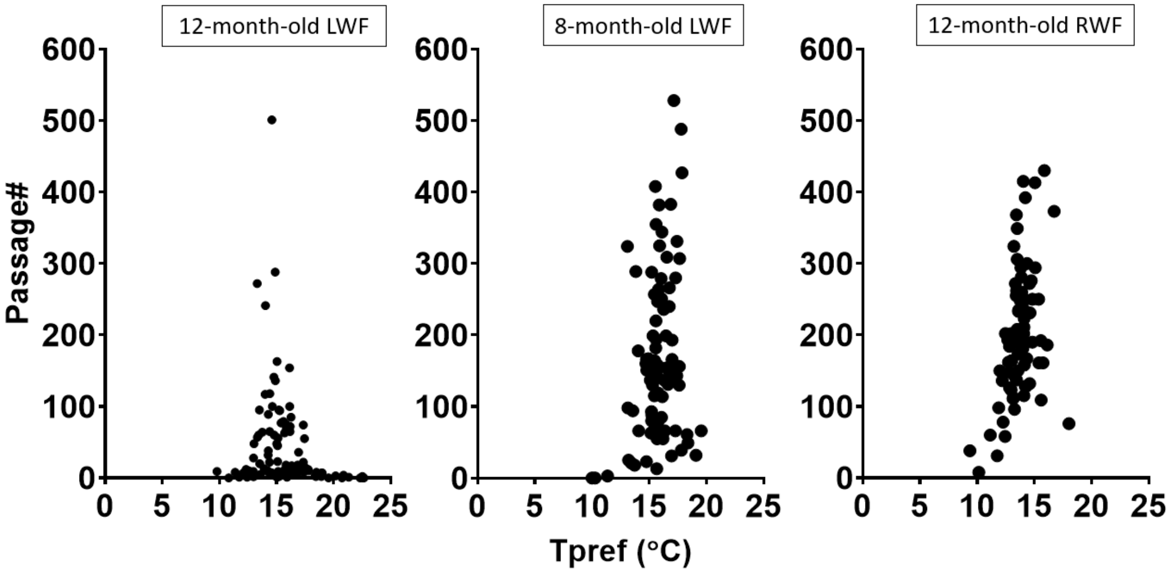

Figure 4.2 Frequency of passage during 2-hour behavioral trial for 8-month old and 12-month old lake whitefish (*Coregonus clupeaformis*; LWF) and 12-month-old round whitefish (*Prosopium cylindraceum*; RWF) plotted against temperature preference (T_{pref}). T_{pref} is calculated as the median occupied temperature during a 2-hour trial. Passages represent a fish shuttling between sides of the shuttle box, which switches the direction of temperature change within the system. Age is determined as the number of months post-hatch. Each data point represents an individual fish.

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