THERMAL STRESS AND THE HEAT SHOCK RESPONSE IN EMBRYONIC
AND YOUNG OF THE YEAR JUVENILE LAKE WHITEFISH (COREGONUS
CLUPEAFORMIS)

A Thesis
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By
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Regina, Saskatchewan
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Daniel Ivan Stefanovic, candidate for the degree of Master of Science in Biology, has presented a thesis titled, *Thermal Stress and the Heat Shock Response in Embryonic and Young of the Year Juvenile Lake Whitefish (Coregonus Clupeaformis)*, in an oral examination held on April 20, 2015. The following committee members have found the thesis acceptable in form and content, and that the candidate demonstrated satisfactory knowledge of the subject material.

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ABSTRACT

I investigated the effects of thermal stress associated with changing environments and industrial thermal pollution by characterizing the kinetics of the heat shock response (HSR) throughout Lake Whitefish (*Coregonus clupeaformis*) development. Lake Whitefish are a cold water species that spawn in the late fall to early winter. Their embryos develop at 0.5 – 6 °C, usually under the cover of ice. The HSR is a universal response to thermal and other types of stressors that offer protection at the cellular level. This response is characterized by the synthesis of a group of highly conserved proteins called the heat shock proteins (Hsps). Here I isolated five different *hsp* cDNAs from Lake Whitefish and quantified changes in mRNA transcript levels in response to varying heat stress conditions in embryos and young of the year (YOY) juveniles. Lake Whitefish were subjected to three different heat shock temperatures (3 °C, 6 °C or 9 °C above control) and six different heat shock durations (0.25, 0.50, 1, 2, 3, and 4 hr) followed by a 2 hr recovery period prior to sampling. In addition, the duration of the HSR was examined by varying the post-heat shock recovery time period prior to sampling. In this recovery experiment, Lake Whitefish were permitted to recover for 1, 2, 4, 8, 12, 16, 24, 36, and 48 hr following a 2 hr heat shock. My data suggest that Lake Whitefish embryos may be resilient to short bouts of heat shock. In embryos, *hsp70* mRNA levels were elevated following a 2 hr, 9 °C heat shock. However, levels of the *hsp70* did not increase in response to any of the 3 °C or 6 °C heat shocks, irrespective of heat shock duration. It is also significant that embryos did not upregulate mRNA levels of the normally inducible *hsp90a* or *hsp47*, in response to any of the heat shock exposures. These embryo data are different from those in 60 days post-hatch YOY
juveniles. In YOY juveniles, all three inducible hsp\textsubscript{s} were upregulated in response to both a 6 °C and 9 °C heat shock, suggesting that YOY juveniles will more readily initiate a HSR and that this response is more robust in that it involves multiple hsp\textsubscript{s}. In embryos, once triggered, the HSR was relatively long lasting with hsp70 mRNA levels remaining elevated 48 hr post-heat shock. In contrast, in YOY juveniles the HSR was shorter lived with hsp70 levels beginning to decrease as early as 4 - 8 hr post-heat shock. In summary, my data indicates that Lake Whitefish can initiate a HSR during embryogenesis. In comparison to YOY juveniles, embryos are more resistant to heat stress as they only initiated a HSR at relatively high heat shock temperatures and this response was limited to increases in hsp70. However, once initiated the HSR was relatively long lasting. Collectively, these data will help us better understand the potential impact of thermal stress associated with changing environments and industrial thermal pollution on development of this and other coldwater fish species.
ACKNOWLEDGEMENTS

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<table>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>α</td>
<td>Alpha</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>ATPase</td>
<td>Adenosine triphosphatase</td>
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<td>B</td>
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<td>kDa</td>
<td>Kilodaltons</td>
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<td>Polymerase chain reaction</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>qRT-PCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
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1. GENERAL INTRODUCTION

1.1 Changing environments

Water temperature is one of the many important environmental factors affecting fish. Increases in water temperature can occur due to natural causes, climate change or anthropogenic sources. Fish inhabiting rivers or streams can encounter daily or circadian fluctuations in water temperature that can range from 1 °C to more than 15 °C (Contantz et al., 1994, Johnson, 2004). Increases in water temperature from natural causes, climate change and anthropogenic pollution are of growing concerns for our environment.

Climate change has become top priority in the past 2 – 3 decades and its impact is expanding. The latest models indicate a 0.2 °C increase in mean global temperatures per decade for the next two decades, and a 1.8 to 4.0 °C increase by the year 2100 (IPCC, 2007). Increases in temperature have direct implications on our freshwater ecosystems, including cold- and cool-water fisheries which are declining as warmer/drier conditions reduce their habitat (IPCC, 2007). In addition to climate change, one of the most common anthropogenic sources of thermal water pollution comes from industrial plants (i.e., power, paper and pulp mills). These facilities use once-through cooling systems that extract cool water and return it to the water source at a higher than ambient temperature.

In the United States alone, there are approximately 425 power plants that use once-through cooling systems, making up about a third of their total electricity generation (UCS, 2012). In Canada, most fossil electric power facilities and all nuclear power facilities use or feature once-through cooling systems and thus are located near major bodies of water (NRTEE, 2010). Industrial thermal pollution, coupled with climate
change, can increase the water temperature of surrounding lakes, rivers and streams by as much as 8 to 15 °C (Island et al., 1993; Langford, 2001). These increases in water temperature can have a large impact on cold water-resident fish that are less tolerant to increases in water temperature (Fangue et al., 2006)

1.2 Lake Whitefish

Lake Whitefish (*Coregonus clupeaformis*) are a cold water species of ecological, economic and cultural importance to the Laurentian Great Lakes and inland lakes of Canada and the United States. In addition to supporting a multimillion dollar commercial fishery, Lake Whitefish are also an important recreational fish species and one of cultural and economic value to the Aboriginal and Indigenous Peoples of North America (Madenjian et al., 2002; Mohr and Nalepa, 2005). Lake Whitefish are cold water benthivores with optimal temperatures of 12 -16 °C. These fish occupy cool, deep waters in the warm summer months and spawn in shallower waters in the cold months; in this way they function to link near-shore and offshore habitats (Rennie et al., 2009). Spawning occurs in the late fall to early winter months at water temperatures between 6 – 8 °C and the embryos incubate in water temperatures ranging from 0.5 – 6 °C (Hart, 1930; Price, 1940; Faber, 1970; Brooke, 1975). Given that they have low optimal temperatures as adults and very low incubation temperatures as embryos, it is not surprising that Lake Whitefish are sensitive to increases in water temperature (Cingi et al., 2010; Yocom and Edsell, 1974) particularly in the embryonic and early life-history stages (Price, 1930; Brook, 1975; Rombough, 1988; Finn, 2007). Recently our research group has shown that embryos are sensitive to thermal shifts and identified organogenesis through gastrulation as critical windows during embryonic development (Eme et al.,
2015; Mueller et al., 2015). In these studies it was shown that changes in temperatures during the critical windows can alter heart rate and oxygen consumption (Eme et al., 2015), and result in increased cost of development and mortality (Mueller et al., 2015). Given their value as a fish species in North America and their vulnerability at elevated temperatures, Lake Whitefish are an excellent model or sentinel species to study the effects of thermal stress associated with changing environments and industrial thermal pollution.

1.3 The heat shock response

The heat shock response (HSR) is one of the most universal responses to stress and is characterized by the transcription and translation of a family of proteins known as the heat shock proteins (Hsps) (Lindquist and Craig, 1988). Heat shock proteins are molecular chaperones that interact with proteins damaged by thermal, oxidative, or chemical stress to prevent their aggregation and denaturation (Hightower, 1991; Morimoto, 1998; Wickner, 1999). Beyond their role as stress proteins, Hsps also play an important role during routine intracellular processes (Blechinger et al., 2002). As such, Hsps may exhibit patterns of both constitutive and inducible expression (Heikkila, 1993a; Heikkila, 1993b). The constitutively expressed Hsps (i.e. Heat shock cognate 70; Hsc70) are active during non-stressful conditions and are usually thought of as housekeeping proteins; crucial for intracellular localization, regulation, secretion, and protein degradation (Feder and Hofmann, 1999; Fink, 1999). The inducible Hsps (e.g. Hsp70) are generally present at lower levels under normal conditions, but their concentrations increase in response to stress.
The Hsps have been grouped into six families according to their molecular mass. The major families are: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and the small heat shock proteins (sHsps ≤30kDa). Each of these families contain closely related genes and proteins that demonstrate different levels of inducibility, intracellular location, and functional properties (Gething, 1997). The Hsp genes are highly conserved and have been found in all organisms from bacteria to plants and mammals. For instance, one of the most studied and conserved Hsps, inducible Hsp70, has an amino acid similarity of 42% to more than 57% between organisms as diverse as *E. coli* and humans, respectively (Bardwell and Craig, 1984; Gupta and Golding, 1993; Schlesinger, 1990). By comparison other highly conserved proteins such as ATPase, glutamine synthetase, and RNA polymerase are all in the range of 13 - 58%, 7 - 41%, and 22 - 30%, respectively (Gupta and Golding, 1993). This high similarity and their universal presence across species, suggests that Hsps have an evolutionary importance and a role in protecting protein homeostasis (Feder and Hofmann, 1999; Lindquist, 1986). Hsps are also known as molecular chaperones. Chaperones are defined as any protein that interacts with, stabilizes, or aids another protein in obtaining its functionally active conformation, without being present in its final structure (Hartl and Hayer-Hartl, 2009; Hartl et al., 2011). These chaperones recognize hydrophobic amino acid side-chains exposed by non-native protein conformations and promote their folding through ATP-dependent cycles of binding and release (Hartl and Hayer-Hartl, 2009). Therefore, Hsps play an important role in optimizing the efficiency of protein folding and releasing them into the functioning protein pool (Hartl et al., 2011; Wickner, 1999). Protein biogenesis is among the most energetically expensive processes of an organism, and as a result a large portion
of their energy is dedicated to maintaining the existing cellular protein pools (Houlihan, 1991). Hence, having a cellular response dedicated to limiting the energetic costs of environmentally induced protein damage and the ability to maintain protein homeostasis is important to an organism exposed to stressors.

The HSR is a highly regulated cellular response and is controlled by a number of transcriptional and post-transcriptional mechanisms that allow for the rapid synthesis of Hsps (Mosser et al., 2000; Wu et al., 1990). The HSR is transcriptionally regulated by the activation of heat shock transcription factors (HSFs). Several HSFs have been identified, with the best studied being HSF1 and HSF2. HSFs bind to key promoter regions, called heat shock elements (HSEs), of Hsp genes, upregulating Hsp gene transcription (Morimoto et al., 1992). The activation of HSF1 is linked to an increase of denatured or misfolded proteins in response to a variety of different stress stimuli (Morimoto, 1998; Voellmy, 2004). Under non-stress conditions HSF1 exists as a monomer complexed with Hsp90 and Hsp70. With stress the increase of denatured or misfolded proteins titrate away the Hsps from the HSF1/Hsp complex freeing it to undergo activation (Akerfelt et al., 2010). As free HSF1 accumulates the monomeric HSF1 will form active HSF1 trimers, which are transported into the nucleus and hyperphosphorylated. The active nuclear HSF1 trimer binds to HSE and upregulates transcription of the downstream Hsp (Prahlad and Morimoto, 2009). Once activated Hsps themselves are known to attenuate or repress their own synthesis by mediating the activity of HSF1 (Morimoto, 1998; see Fig. 1). There are several mechanisms in which the effects of thermal stress promote the preferential translation of Hsps. A few of these mechanisms are: a block in pre-mRNA processing (Yost and Lindquist, 1986), the
promotion of Hsp mRNA stability (Petersen and Lindquist, 1988), and genes that promote the translation efficiency (McGarry and Lindquist, 1985). The absence of introns, in the case of inducible Hsp70 gene is also thought to be important in its rapid transcriptional upregulation and processing (Ingolia et al., 1980). Likewise the return to homeostasis following the removal of the stressor involves repression of Hsp gene expression, which also occurs via a number of tightly regulated mechanisms (Petersen and Lindquist, 1989).

1.4 The heat shock response in fish

When behavioral responses are not practical, fish will rely on their physiological and cellular responses to reduce the effects of temperature changes or thermal stress. For instance, Lake Whitefish begin to show behavioral (i.e. avoidance) changes at temperatures well below their lethal maximum (Yocom and Edsall, 1974). These changes in behavior may be helpful in determining stressful temperatures, but changes at a cellular level, may be the early warning signs of sub-lethal heat stress, which will later be apparent at an organismal or population level. Fish make excellent models to study their HSR since they encounter a variety of stressors in their natural environment, and can be easily exposed to a number of experimental conditions in a laboratory setting. The HSR in fish cells is similar to other vertebrates; Hsps are upregulated in response to different types of stressors. The HSR in fish has been well studied (Iwama et al., 1998; Basu et al., 2002) and many of these studies have focused on Hsp induction in response to aquatic contaminants (Ryan and Hightower 1996; Vijayan et al., 1998; Williams et al., 1996). Sub-lethal concentrations of pulp mill effluent and sodium dodecyl sulfate resulted in an increase in Hsp70 protein levels in liver tissues of rainbow trout and
Figure 1. Regulation of the heat shock response and the HSF attenuation cycle.

Activation of the HSF1 coincides with the appearance of misfolded proteins (due to stress) and the recruitment of Hsps to prevent the appearance of denatured proteins. HSF1 is a monomer in the control state in the cytoplasm and/or nucleus through interactions with Hsps such as Hsp70 and Hsp90. Activation of HSF1 results in trimerization of the HSF1 where it then binds to DNA or more specifically the HSE. Transcription then leads to the upregulation of Hsps, which then leads to the ability of the cell to maintain cellular homeostasis by refolding of misfolded proteins, returning them to their native state.
Chinook salmon (Vijayan et al., 1998). Increased levels of various Hsps have also been
detected in various tissues of fish exposed to polycyclic aromatic hydrocarbons, heavy
metals, pesticides, and arsenite (Vijayan et al., 1998; Duffy et al., 1999; Williams et al.,
food deprivation, hypoxic conditions, and heat shock had an effect on Hsp70 and Hsp90
protein levels in young of the year gilthead sea bream. Hence, the induction of different
types of Hsps varies depending on tissue and nature of the stressor in addition to varying
between developmental stages in fish (Basu et al., 2001; Nakano and Iwama 2002;
Martin et al., 2001; Santacruz et al., 1997). In fish, certain Hsps also play an important
role during development in addition to their role as stress adapting proteins. For
example, constitutive Hsps in fish, e.g. heat shock cognate 70 (Hsc70) and Hsp90β have
been demonstrated to have more specific regulatory roles within developing and
differentiating cells (Csermely et al., 1998; Mosser et al., 2000; Nagai et al., 2000). The
inducible Hsps in fish, such as Hsp70, Hsp90α and Hsp47 are generally present at lower
levels under normal conditions, but increase their concentration in response to stress.
Studies have shown that fish can possess high levels of these constitutive Hsps (Currie
and Tufts, 1997; Boone and Vijayan, 2002) that are regulated in a spatial and temporal
manner during zebrafish embryogenesis (Krone et al., 1997; Lele et al., 1999; Sass et al.,
1999). For example, Krone and Sass (1994) demonstrated that constitutive levels of
hsp90β were high relative to hsp90α and only weakly upregulated following heat shock.
They also showed hsp90α was present at lower levels in developing embryos but was
strongly upregulated following heat shock in gastrula and late stage zebrafish embryos.
This has also been shown with hsp70 and hsp47 gene expression, where they were
present at lower levels but highly upregulated in response to heat shock (Pearson et al., 1996; Krone et al., 1997; Lele et al., 1997). This suggests that specific Hsps, such as the Hsp90 isoform, may be differentially regulated and have different roles during development.

1.5 Hypotheses and objectives

Given potential for environmental change in response to both climate change and anthropogenic pollution, I set out to investigate the impacts of these types of thermal shifts on embryonic and YOY juvenile Lake Whitefish, a thermally sensitive coldwater species. As one of the first cellular indicators of temperature stress, the HSR represents ideal metrics for this study. To this end, I used laboratory studies to characterize the HSR and quantify changes in gene expression levels. These data will help us better understand the nature and degree of thermal stress coldwater fishes may be experiencing in the wild. So I hypothesize that both embryos and YOY juveniles will upregulate Hsp expression in response to thermal stress. However, I expect the nature of the HSR, and the specific kinetic profiles will differ with developmental stage. I further hypothesize that YOY juvenile Lake Whitefish will be more sensitive to thermal stress and posses a more rapid induction and a shorter lived recovery of the HSR than embryonic Lake Whitefish. The objectives of my thesis were as follows:

1. Develop the necessary reverse transcription, quantitative real-time PCR (qRT-PCR) assays to quantify inducible (hsp47, hsp70, hsp90α) and constitutive (hsc70, hsp90β) Hsp mRNA.

2. Characterize the kinetics of induction of the HSR in embryonic and YOY juvenile Lake Whitefish.
3. Characterize the kinetics of recovery of the HSR in embryonic and YOY juvenile Lake Whitefish.
2. MATERIALS AND METHODS

2.1 Experimental animals

Adult Lake Whitefish (*Coregonus clupeaformis*) were collected using bottom-set gillnets from spawning shoals at Blackstrap Lake in Dundurn, SK (51° 47’20”N) and Lake Huron, ON (44° 48’0”N) in November 2011. Male and Female fish in full breeding condition were separated, placed in holding tanks, then stripped of milt and eggs. All female eggs were pooled, wet fertilized from a pool of milt, water hardened, and transported in 1 L plastic jars back to the University of Regina’s aquatics facility. Embryos were then incubated at 2-4 °C in 2 L mini-egg hatching bell jars (Aquatic Eco-Systems Inc., Apopka, FL, USA) with a constant flow of aerated dechlorinated water, to ensure embryos were at a gentle roll. Embryos were incubated using a biologically filtered recirculated water with 25% fresh water changes performed every week, and dead embryos removed daily. Post-hatch YOY Lake Whitefish were reared in 77 L glass aquaria or 700 L fiberglass tanks. Newly hatched fry and YOY juveniles were fed a marine fish larval and weaning feed (Otohime-Marubeni Nisshin Feed Company, Chuo-ku, Tokyo, Japan). All animal care, handling and experimentation were approved by the University of Regina’s President’s Committee on Animal Care and performed in accordance with the Canadian Council on Animal Care (CCAC) guidelines.

2.2 Heat shock experiments

2.2.1 Induction of HS in embryos and YOY juveniles

Lake whitefish embryos at 102 days post fertilization (dpf) or at the fin flutter, vitelline circulation stage (Shreetharan et al., in press) were exposed to a HS of 3, 6, or 9
°C above their 2 °C control incubation temperature for 0.25, 0.50, 1, 2, 3 or 4 hr, then returned to water at the control temperature for a 2 hr recovery period prior to sampling. HS was administered by transferring embryos to 400 ml beakers containing 2, 5, 8, or 11 °C water and placing the beakers in a water bath at 2, 5, 8 or 11 °C. Three replicates were made from each of the HS treatments, durations and controls.

Two month post hatch YOY juvenile lake whitefish were exposed to a HS of 3, 6, or 9 °C above their 14 °C control temperature for 0.25, 0.50, 1, 2, 3 or 4 hr and returned to 14 °C for a 2 hr recovery period prior to sampling. HS was administered by transferring YOY juveniles to 38 L aquaria containing 14, 17, 20, or 23 °C water. Three YOY juvenile fish were sampled from each of the HS treatments, durations and controls, euthanized using 0.005% tricaine methanesulfonate (MS-222; 0.05 g MS-222/L of water) buffered with 0.01% sodium bicarbonate, immediately frozen in liquid nitrogen and stored at -80 °C until further analysis.

2.2.2 HS recovery of embryos and YOY juveniles

Lake whitefish embryos at 105 dpf or at the fin flutter, vitelline circulation stage (Shreetharan et al., in press) were exposed to a HS of 3, 6, or 9 °C above their 2 °C control incubation temperature for 2 hr, then returned to water at the control temperature for 1, 2, 4, 8, 12, 16, 24, 36 or 48 hr prior to sampling. HS was administered as reported for the induction experiment above. Three replicates were made from each of the HS treatments, recovery durations and controls.

Two month post hatch YOY juvenile lake whitefish were exposed to a HS of 3, 6, or 9 °C above the 14 °C control temperature for 2 hr and allowed to recover for 1, 2, 4, 8, 12, 16, 24, 36 or 48 hr prior to sampling. HS was administered as previously described
for the induction experiment. Three replicates were sampled from each of the HS treatments, recovery durations and controls.

2.3 Quantitative real-time PCR analysis of hsp gene expression.

Total RNA was isolated from a pool of 5 embryos or a whole YOY juvenile Lake Whitefish using TRIzol® Reagent (Invitrogen Life Technologies, Burlington, ON, Canada) according to the manufactures protocol. RNA quality and purity were confirmed using agarose gel electrophoresis and spectrophotometry ($A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios > 1.8, respectively). First strand cDNA was synthesized from 1 μg total RNA using the QuantiTect® Reverse Transcription Kit (Qiagen, Mississauga, ON, Canada) following manufacturer’s instructions. *Hsp* mRNA levels were obtained using quantitative real-time PCR (qRT-PCR) with a CFX Connect Real-Time Detection System (Bio-Rad, Mississauga, ON, Canada) as outlined below. Partial cDNA fragments for *hsp90α, hsp90β, hsp70, hsc70, hsp47, β-Actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* were amplified, subcloned and sequenced with a variety of PCR based techniques and have been submitted to GenBank (*hsp90α* KP893539; *hsp90β* KP893540; *hsp70* KP861983; *hsc70* HQ287746; *hsp47* KP893541; *β-actin* KP893542 and *GAPDH* KP893543). Gene specific real-time quantitative PCR primers were designed from the aforementioned sequences and an existing *hsc70* partial sequence (Accession no. HQ287746) using NetPrimer software (Premier Biosoft, Palo Alto, CA, USA; Table 1). All primers were validated to meet MIQE guidelines (Bustin et al., 2009) as detailed below. To determine qRT-PCR conditions, including annealing temperature, reaction efficiency and template concentration; triplicate standard curves using serially diluted cDNA (2.0 – 0.0000002 ul of cDNA) were performed with each primer pair set to
calculate amplification efficiency which ranged from 97 – 110% for all primers. Details on primer pairs, annealing temperature and amplification efficiency can be found in Table 1. qRT-PCR reactions were performed in triplicate using 0.5 μl cDNA, 500 nmol of each primer and $2 \times$ SsoAdvanced™ SYBR® Green Supermix (Bio-Rad, Mississauga, ON, Canada) to a total volume of 20 μl under the following conditions: 1 cycle of 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60-65 °C for 30 s. Melt curve analysis, no template controls and no reverse transcription controls all confirmed the presence of a single amplicon and the absence of DNA contamination.

A pool of heat shocked and control samples were used to produce a large pool of cDNA in which a standard curve was performed for all primer pairs (see above) relating threshold cycle to cDNA amount. This pool was included in triplicate in all assays to serve as an inter-run calibrator (IRC) to account for any run to run variance. The CFX Connect Real-Time Detection System and software were used to quantify mRNA transcript levels for the 5 different hsp5. Gene specific transcript levels were normalized to transcript levels of the reference genes β-Actin and GAPDH (run in triplicate) and when required any inter-run variation in fluorescence levels using a triplicate IRC was loaded in every run.

2.4 Statistical analysis

All analyses were conducted using Graphpad Prism 6 (Graphpad Software, La Jolla, CA, USA) and data are presented as means ± standard error of the mean (s.e.m.). All data were log transformed to ensure homogeneity of variance. Statistical comparisons were carried out using a two-way analysis of variance (ANOVA) to determine if there were any interaction effects between HS duration and temperature,
differences between HS temperatures within a given duration, or between durations at a particular HS temperature. If the overall ANOVA model detected significant differences ($P<0.05$) a Tukey’s multiple comparisons post hoc test was performed to test for differences in mRNA levels within a heat shock temperature and within heat shock durations (or recovery durations). Alpha was set at 0.05.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ – 3’)</th>
<th>Annealing Temperature</th>
<th>Amplification Efficiency</th>
</tr>
</thead>
</table>
| hsp90α | F: AGT CGT GGG GAA AGG ATT GT  
               R: TGA ATA AGG TTG AAA GCA GCA GA | 65 °C | 104.0% |
| hsp90β | F: CCT TTC TAT TTT CCT GCG TC  
               R: TTG TTC CGT TGA CT TTT CTT | 60 °C | 98.2% |
| hsp70  | F: TCA TTA CAG TCC CCG CCT AC  
               R: TCA CCT CAA AGA TCC  
               CAT CC | 65 °C | 107.8% |
| hsc70  | F: CCT GTC CTC CAG CAC CCA AG  
               R: AGC CTC CGA CCA GAA CGA TG | 60 °C | 107.3% |
| hsp47  | F: ATG GGC AAG ATG GAG GAG AG  
               R: TCA GAC CAA GTT CAC  
               CAA GAG | 60 °C | 104.8% |
| GAPDH  | F: CCG TCC GTC TGG AGA AGG C  
               R: GAA GTG GTC GTT CAG  
               AGC AAT G | 60 °C | 100.1% |
| β Actin| F: GTG GCG CTG GAC TTT GAG CA  
               R: ACC GAG GAA GGA GGG CTG GA | 65 °C | 97.7% |
3. RESULTS

3.1 hsp70 and hsc70 mRNA levels in embryonic and YOY juvenile Lake Whitefish

A significant interaction effect for hsp70 mRNA induction was observed between heat shock (HS) duration and temperature and the overall two-way ANOVA model detected significant differences in hsp70 mRNA levels both between HS temperatures within a given duration, and between durations at a particular HS temperature (Table 2; Figs. 2A and B). Furthermore, pairwise differences in hsp70 mRNA levels were only observed following a 9 °C HS in both embryonic and YOY juvenile LWF (Table 2; Figs. 2A and B). Differences in the HSR of embryos and YOY juveniles were observed with respect to both the duration of HS required to elicit a significant increase in hsp70 mRNA and the duration at which peak expression levels were reached. The levels of hsp70 mRNA began to increase significantly in embryos following a 2 hr, 9 °C HS and reached peak levels after 4 hrs; these levels corresponded to 2.3 fold and 4.0 fold increases over controls, respectively (Fig. 2A). hsp70 mRNA was significantly increased in YOY juveniles after a 1 hr, 9 °C HS and expression peaked at 3 hr (Fig. 2B). In embryos, but not YOY juveniles, a significant interaction effect between HS temperature and duration on hsc70 mRNA was detected (Table 2). The duration of HS had a significant effect on hsc70 expression in both embryos and YOY juveniles, but the magnitude of HS did not, with the exception of the 9 °C HS, 0.25 hr, embryonic treatment group (Table 2; Figs. 2C and D). In embryos, a decrease in hsc70 from 0.25 to 1 hr was observed, followed by an increase to peak levels in the 2 hr HS duration group (Fig. 2C), whereas levels in YOY juveniles showed an initial decrease from 0.25 to 0.5 hr then increased slightly in the 1 hr
HS group (Fig. 2D). However, HS temperature treatment groups followed the same fluctuating pattern over the different HS durations and no pairwise differences were detected in YOY juveniles (Fig. 2D).

3.2 *hsp90α, hsp90β, and hsp47* mRNA levels in embryonic and YOY juvenile LWF

The mRNA levels of typically inducible *hsp90α* and *hsp47* as well as the constitutive *hsp90β* in embryonic and YOY juvenile Lake Whitefish were quantified in response to various HS temperatures and durations. In embryos, the expression of *hsp90β* (Fig. 3A), *hsp90α* (Fig. 3C) and *hsp47* (Fig. 3E) varied significantly in response to both HS temperature treatments and duration and a significant interaction between HS duration and temperature treatments was detected (Table 2). With the exception of the 9 °C HS treatment group, pairwise differences between temperature treatments were not detected within a HS duration (Figs. 3A and C). *hsp90β* mRNA levels differed between the various durations within a HS temperature treatment in a similar manner for all temperatures examined; a decrease in mRNA levels between 0.25 and 1 hr, followed by an increase to peak levels at 4 hr (Fig. 3A). Surprisingly, both *hsp90α* and *hsp47* are typically inducible and show an increase in transcription with HS, but behaved like the constitutive *hsp90β* in embryos (Figs. 3C and E). The mRNA levels of *hsp90β* in YOY juveniles did not differ significantly in response to the various HS temperature treatments, however significant differences were observed in response to HS duration and an interaction between HS duration and HS temperature treatments was detected (Table 2; Fig. 3B). No pairwise differences in *hsp90β* expression were detected. In contrast to our observation with embryos, *hsp90α* and *hsp47* mRNA levels in YOY juveniles were upregulated in response to HS (Figs. 3D and F). We observed significant differences in
the expression of *hsp90α* and *hsp47* mRNA levels between HS temperature treatments within a duration (Table 2). Significant differences in gene expression were not observed between the controls, 3 °C and 6 °C HS treatment groups, but *hsp90α* expression in the 9 °C HS group differed significantly from both the other three temperature groups and with HS duration (Fig. 3D). In the 9 °C HS group, *hsp90α* mRNA began to increase after a 1 hr HS, by 3 hr it was significantly higher than all other groups and continued to increase up to the final time point of 4 hr (Fig. 3D). Similarly, a 9 °C HS resulted in a significant upregulation of *hsp47* gene expression relative to all other groups after 3 hr and 4 hr at the elevated temperature, but *hsp47* also increased significantly in response to a 6 °C HS for 3 hrs (Table 2; Fig. 3F).

### 3.3 Recovery of *hsp* mRNA levels in embryonic and YOY juvenile Lake Whitefish

Overall, our HS recovery experiment indicated that the HSR is slower to respond, but longer in duration in embryos as compared to YOY juveniles (Fig. 4). We also observed that smaller HS will also trigger an increase in *hsp70* mRNA levels in embryos, but that this increase is only observed 8 - 16 hr following the 2 hr HS (Fig. 4A). In embryos, *hsp70* mRNA levels varied significantly between HS temperature treatments within a recovery duration, between recovery durations within a HS temperature treatment, and an interaction effect was observed (Table 3; Fig. 4A). The elevation of *hsp70* mRNA in the 6 °C and 9 °C HS groups at 16 hr post HS was the first significant change from control levels and by 24 hr post HS all three HS temperature groups were significantly higher than controls (Fig. 4A). *hsp70* mRNA remained elevated in the 6 °C and 9 °C groups until 36 and 48 hr post HS, respectively (Fig. 4A). Interestingly, we observed a significant delay in the upregulation of *hsp70* mRNA levels in 105 dpf
embryo (fin flutter, vitelline circulation stage) from the kinetics of recovery experiments (Fig. 3A) in comparison to the 102 dpf (fin flutter, vitelline circulation stage) embryos from kinetics of induction experiment (Fig. 1A). Whereas *hsp70* mRNA increased following a 2 hr HS of 9 °C in the kinetics of induction experiment, we did not observe a similar increase *hsp70*
Figure 2. Kinetics induction of *hsp70* and *hsc70* mRNA levels of embryonic and YOY juvenile Lake Whitefish.  *Hsp70* (A, embryos; B, YOY juveniles) and *hsc70* (C, embryos; D, YOY juveniles) mRNA levels for Lake Whitefish following a heat shock (HS) of 3, 6, or 9 °C above the acclimation (control) temperature of 2 °C (embryos) or 14 °C (YOY juveniles) for one of six durations (0.25, 0.50, 1, 2, 3 or 4 hr). Following HS fish were returned to control temperatures for a 2 hr recovery period prior to sampling. Data represent the mean ± SEM for three biological replicates normalized to β-actin and GAPDH. Statistical significance was determined by two-way ANOVA and Tukey’s multiple comparisons test. The overall two-way ANOVA model indicated that both temperature and duration significantly affected *hsp70* and *hsc70* levels and that there was an interaction effect between the two factors (P <0.0001). Pairwise significant differences (P < 0.05) are indicated as follows: within a HS temperature and between HS durations the data points that differ significantly are labelled with different letters; data points labelled with an asterisk differ significantly from control within a given HS duration. AEU = arbitrary expression units.
Figure 3. Kinetics induction of hsp90β, hsp90α and hsp47 mRNA levels of embryonic and YOY juvenile Lake Whitefish. Hsp90α (C, embryos; D, YOY juveniles), hsp90β (A, embryos; B, YOY juveniles), and hsp47 (E, embryos; F, YOY juveniles) mRNA levels for Lake Whitefish following a heat shock (HS) of 3, 6, or 9 °C above the acclimation (control) temperature of 2 °C (embryos) or 14 °C (YOY juveniles) for one of six durations (0.25, 0.50, 1, 2, 3 or 4 hr). Following HS fish were returned to control temperatures for a 2 hr recovery period prior to sampling. Data represent the mean ± SEM for three biological replicates normalized to β-actin and GAPDH. Statistical significance was determined by two-way ANOVA and Tukey's multiple comparisons test. The overall two-way ANOVA model indicated that both temperature and duration significantly affected hsp90β, hsp90α and hsc47 levels and that there was an interaction effect between the two factors (P <0.0001). Pairwise significant differences (P < 0.05) are indicated as follows: within a HS temperature and between HS durations the data points that differ significantly are labelled with different letters; data points labelled with an asterisk differ significantly from control within a given HS duration. AEU = arbitrary expression units.
Table 2: Two-way ANOVA statistics for the kinetics of HSR induction in embryonic and YOY juvenile Lake Whitefish.

<table>
<thead>
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<th></th>
<th>HS Temperature Factor</th>
<th>HS Duration Factor</th>
<th>Interaction</th>
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<tr>
<td></td>
<td>F (DF_n, DF_d)</td>
<td>P</td>
<td>F (DF_n, DF_d)</td>
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<tr>
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<td>131.7 (3, 48)</td>
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<tr>
<td>hsc70</td>
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<td>41.85 (5, 48)</td>
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<td>89.93 (5, 48)</td>
</tr>
<tr>
<td>hsp47</td>
<td>19.09 (3, 48)</td>
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</tr>
<tr>
<td>hsp90β</td>
<td>8.142 (3, 48)</td>
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<td>29.29 (5, 48)</td>
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<td><strong>Juveniles</strong></td>
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<tr>
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mRNA until 16 hr after at 2 hr HS of 9 °C, however, levels in these embryos remained elevated 48 hrs post HS (Fig 3A). In contrast, in YOY juveniles hsp70 mRNA levels increased rapidly, within 1 hr, following a 2 hr, 9 °C HS but returned to control levels somewhere between 4 and 8 hr post HS (Fig 3B).

The overall two-way ANOVA for YOY juveniles reported that the levels of hsp70 mRNA varied significantly in response to different HS temperatures and recovery periods and there was a significant interaction effect between these two factors (Table 3). However, upon analysis with Tukey’s post-hoc test the only pairwise statistically significant differences observed were increased hsp70 mRNA levels (1 - 4 hr post HS) in the 9 °C HS treatment (Fig. 4B). A 1 hr recovery from a 2 hr, 9 °C HS was sufficient to elicit a significant increase in hsp70 mRNA relative to all other HS temperature groups (i.e., control, 3 °C and 6 °C; Fig. 4B). Levels continued to increase until 2 hr post HS then gradually declined so that by 8 hr post HS they were comparable to those of the other temperature groups (Fig. 4B).

When the expression of other hsps was examined during HS recovery experiment, hsc70 mRNA levels in embryos, but not YOY juveniles, varied significantly in response to HS temperature and recovery period (Table 3). However we did not observe an interaction between HS temperature and recovery period (Table 3) nor were any pairwise differences detected (Figs. 4C and D). Preliminary analysis of embryonic hsp90α, hsp90β, and hsp47 mRNA levels (data not shown) were consistent with our findings for these genes in the HSR induction experiment (Fig. 3) and the hsc70 findings in the recovery experiment (Figs. 4C and D). Similar trends between the induction and recovery experiments were also observed in YOY juveniles whereby hsp90α and hsp47,
but not the constitutive *hsp90β*, were significantly upregulated in response to HS (Table 3; Fig. 5). Although pairwise differences were not observed, *hsp90α* was elevated to a greater degree between the 2 to 12 hr recovery period following a 9 °C HS than in response to the other temperatures used (Table 3; Fig. 5A). *hsp47* expression following a 9 °C HS was significantly higher than controls after a 1 or 2 hr recovery, higher than all other groups at 4 hr post HS, then declined to levels comparable to the other treatment groups (Table 3; Fig. 5C).
Embryos

A

YOY Juveniles

B

C

D

Hsp70 mRNA levels (AEU)

Hsc70 mRNA levels (AEU)

HS Recovery (hr)

HS Recovery (hr)
Figure 4. Kinetics recovery of \textit{hsp70} and \textit{hsc70} mRNA levels of embryonic and YOY juvenile Lake Whitefish. \textit{hsp70} (A, embryos; B, YOY juveniles) and \textit{hsc70} (C, embryos; D, YOY juveniles) mRNA levels for Lake Whitefish following a heat shock (HS) of 3, 6, or 9 °C above the acclimation (control) temperature of 2 °C (embryos) or 14 °C (YOY juveniles) for 2 hr. Following HS fish were returned to control temperatures and allowed to recover for nine different periods (1, 2, 4, 8, 12, 16, 24, 36 or 48 hr) prior to sampling. Data represent the mean ± SEM for three biological replicates normalized to β-actin and GAPDH. Statistical significance was determined by two-way ANOVA and Tukey’s multiple comparisons test. The overall two-way ANOVA model indicated that both temperature and duration significantly affected \textit{hsp70} and \textit{hsc70} levels and that there was an interaction effect between the two factors (P <0.0001). Pairwise significant differences (P < 0.05) are indicated as follows: data points labelled with an asterisk differ significantly from control within a given HS recovery period. AEU = arbitrary expression units.
Figure 5. Kinetics recovery of hsp90α, hsp90β and hsp47 mRNA levels of YOY juvenile Lake Whitefish. hsp90α (A), hsp90β (B), and hsp47 (C) mRNA levels for Lake Whitefish following a heat shock (HS) of 3, 6, or 9 °C above the acclimation (control) temperature (14 °C) for 2 hr. Following HS fish were returned to control temperature and allowed to recover for nine different periods (1, 2, 4, 8, 12, 16, 24, 36 or 48 hr) prior to sampling. Data represent the mean ± SEM for three biological replicates normalized to β-actin and GAPDH. Statistical significance was determined by two-way ANOVA and Tukey’s multiple comparisons test. The overall two-way ANOVA model indicated that both temperature and duration significantly affected hsp90α, hsp90β and hsp47 levels and that there was an interaction effect between the two factors (P <0.0001). Pairwise significant differences (P < 0.05) are indicated as follows: data points labelled with an asterisk differ significantly from control within a given HS recovery period. AEU = arbitrary expression units.
Table 3: Two-way ANOVA statistics for the kinetics of HS recovery experiment in embryonic and YOY juvenile Lake Whitefish.

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<th>HS Temperature Factor</th>
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<tr>
<td>hsp70</td>
<td>57.69 (3, 72)</td>
<td>&lt; 0.0001</td>
<td>62.36 (8, 72)</td>
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<td>12.39 (24, 72)</td>
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<td>15.84 (24, 72)</td>
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<td>0.2244 (24, 72)</td>
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<td>12.23 (8, 72)</td>
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<td>3.864 (24, 72)</td>
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<tr>
<td>hsp90β</td>
<td>0.2629 (3, 72)</td>
<td>0.8519</td>
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<td>&lt; 0.0001</td>
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<td>0.9994</td>
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4. GENERAL DISCUSSION

Temperature is one of the most pervasive variables affecting fish biology and as one of the most universal cellular stress responses, the HSR in fish has been well studied (Iwama et al., 1998; Basu et al., 2002). However, little is known about the nature of this response during embryonic development and the early life history stages of fishes. Here we show that Lake Whitefish embryos are capable of eliciting a HSR in a manner that differs from that of YOY juveniles (2 months post-hatch). Compared to YOY juveniles, embryos required a higher, sustained HS prior to upregulating hsp70 gene expression, but hsp70 levels remained elevated for longer post HS. Moreover, only hsp70 was upregulated in embryos, unlike YOY juveniles where all three of the characteristically inducible hsps (hsp90α, hsp70, and hsp47) were upregulated in response to HS. Hsps and the HSR have been examined in a variety of embryonic animal systems (see Heikkila et.al, 1986) including fish. In particular, function of the HSR and Hsps during normal embryonic development in the zebrafish model system has been studied extensively (Krone and Sass, 1994; Sass et al., 1996; Krone et al., 1997; Lele et al., 1997; Lele et al., 1999; Sass et al., 1999; Martin et al., 2001; Krone et al., 2003; Martin et al., 2002).

The first indication that embryonic Lake Whitefish were capable of mounting a HSR was observed following a 2 hr, 9 °C HS and peak hsp70 levels were reached following a 4 hr, 9 °C HS (Fig. 2). By comparison, zebrafish embryos responded as early as the gastrula stage with an increase in hsp70 mRNA following a 1 - 1.5 hr, 5.5 °C HS, peak hsp70 expression occurred after a 8.5 °C HS, and an 11.5 °C HS resulted in a small increase in hsp70 mRNA and coincided with rapid mortality of all embryos (Krone et al.,
Lake Whitefish embryos may be slower to respond to HS due to their lower metabolic rate as a result of their lower ambient water temperatures (2 – 4 °C versus 27.5 °C for zebrafish). However, in Atlantic salmon (Salmo salar) embryos housed at 8 °C, hsp70 mRNA expression was elevated following a 1 hr, 8 °C HS and no recovery period (Takle et al., 2005). In addition, the magnitude of the HSR of Atlantic salmon was dependent on stage of development; some stages (1st and 25th somite) showed no increase in hsp70 mRNA expression in response to HS (Takle et al., 2005).

Correspondingly, Werner et al. (2001) reported developmental stage-dependent responses to HS in the warm water Medaka (Oryzias latipes), such that a 30 min, 15 °C HS resulted in an increase in Hsp70 and Hsp60 proteins in late embryos, but not early embryos. Likewise, Lake Whitefish embryos may show stage-dependent responses to HS. In the current study we were only able to examine the HSR at the fin flutter, vitelline circulation stage; thus embryos earlier or later in development may prove to be more or less sensitive to HS.

The HSR of Lake Whitefish embryos differed from their YOY juvenile counterparts and from embryos of other species with respect to two other typically inducible hsps (hsp90α and hsp47). In the present study, we did not observe any significant increases in either hsp90α or hsp47 mRNA levels even after a 4 hr, 9 °C HS followed by a 2 hr recovery (Fig. 3). hsp90α is expressed at relatively low levels under non-stress conditions in late stage zebrafish embryos, but is strongly upregulated following a 1 hr HS of 6 or 9 °C (Krone and Sass, 1994; Sass et al., 1996). Likewise, Lele et al. (1997) demonstrated that zebrafish embryos upregulate hsp47 mRNA after a 1 - 2 hr, 9 °C HS. Similar findings were reported with embryos of the African clawed frog.
(Xenopus laevis) where \textit{hsp90\(\alpha\)} was upregulated following a 1 hr, 15 °C HS (Taherian et al., 2010). The expression of constitutive \textit{hsp90\(\beta\)} and \textit{hsc70} was not upregulated by HS in Lake Whitefish embryos and this result is consistent with the zebrafish model (Lele et al., 1997; Lele et al., 1999). Collectively our data suggest that Lake Whitefish embryos have only one heat inducible Hsp (Hsp70). However, further research is required to confirm these findings as our observations may be related to the embryonic stages examined and the nature of the Lake Whitefish HSR. Much of Lake Whitefish embryonic development typically occurs at 0.5 – 6 °C, therefore, the rates of protein denaturation may be significantly reduced even with a HS of 9 °C. Given that protein denaturation and aggregation are thought to be the primary signals to upregulate \textit{hsp} gene expression (Morimoto et al., 1993; Parsell and Lindquist, 1993), it is conceivable that multiple inducible \textit{hsps} are simply not necessary for the survival of Lake Whitefish embryos.

The HSR of Lake Whitefish YOY juveniles was similar to other fish species with \textit{hsp90\(\alpha\)}, \textit{hsp70} and \textit{hsp47} mRNA levels increasing in response to HS. A 1 hr, 9 °C HS with a 2 hr recovery period was sufficient to result in a significant increase in \textit{hsp70} mRNA levels, but a 3 hr, 9 °C HS was required to produce significant increases in \textit{hsp90\(\alpha\)} and \textit{hsp47} mRNA. No increases in any of the inducible Hsps were observed following a 0.25 - 4 hr HS of 3 or 6 °C. The threshold temperature of \textit{hsp} induction generally falls between 5 – 10 °C above the ambient temperature (Lindquist, 1986, Parsell and Lindquist 1993; and Feder and Hofmann, 1999). In YOY gilthead seabream, but not rainbow trout, Hsp70 and Hsp90 protein levels were significantly higher than control values following a 1 hr, 5 °C HS and returned to normal within 24 hr (Cara et al.,
A subsequent experiment on rainbow trout found that a 10 °C HS resulted in 100% mortality, but a 5 °C HS combined with 7 days of food deprivation increased both Hsp70 and Hsp90 protein levels (Cara et al., 2005). Interestingly, this food deprivation and the associated increase in Hsps appeared to provide a protective benefit as 100% of those YOY rainbow trout deprived of food survived a 10 °C HS. In our study we observed 100% survival in YOY lake whitefish exposed to a 9 °C HS for 4 hr in the absence of any other protective stressor. These findings suggest that either Lake Whitefish juveniles are particularly resilient to changes in temperature, or the observations may be related to maturational differences between Lake Whitefish and rainbow trout. Basal non-stress levels of Hsp70 and 90 mRNA and protein were shown to increase in the first 46 dph in the silver sea bream (Sparus sarba; Deane and Woo, 2003). Similarly, Takle et al. (2005) reported hsp70 mRNA levels were dependent on the stage of development in Atlantic salmon embryos. Future research should aim to more closely investigate these subtle, but potentially important differences in YOY fishes.

In the present study we also examined the kinetics of recovery from HS in both embryos and juveniles. Lake Whitefish embryos had a sustained HSR, evidenced by hsp70 mRNA levels that remained elevated for 36 - 48 hr post HS; this was much longer than the 8 - 12 hr observed in YOY juveniles. However, this sustained HSR may not be extraordinary given the low rearing and recovery temperature (2 – 4 °C) of Lake Whitefish embryos. Takle et al., (2005) observed elevated hsp70 mRNA in early stage (gastrula to 45th somite) Atlantic salmon embryos for 24 hr following a 1 hr HS from an 8 °C control temperature. The embryonic period (especially early embryogenesis) is very susceptible to the teratogenic effects of thermal stress (Krone et al., 2003; Werner et al.,
2003; Hattori et al., 2004) and the protective role of Hsps in protecting against thermally induced teratogenesis has been well-documented (Mirkes, 1997; Edwards et al., 1997; Luft and Dix, 1999). Thus, given the reduction in metabolism associated with lower temperatures (Johnston and Dunn, 1987; Podrabsky and Somero, 2004), our results are consistent with observations of other vertebrates.

In summary, we have shown that embryonic Lake Whitefish are capable of eliciting a HSR that differs from those of both YOY juvenile Lake Whitefish and other fish species. Lake Whitefish embryos required longer, higher heat shocks to induce the HSR and this response was limited to the upregulation of hsp70 mRNA. This result is in contrast to the HSR of YOY juveniles where all three of the inducible hsps (hsp90α, hsp70, and hsp47) were upregulated. Furthermore, Lake Whitefish embryos had a HSR that lasted nearly six times longer than that of YOY juveniles. Collectively, these data suggest that embryonic lake whitefish are more resistant to the effects of HS, with respect to the induction of a HSR response, but once initiated the HSR can be long-lived. Future work will provide information on cool and cold water fish and if their embryos are more resilient to thermal stress then once thought. Also my baseline data sets the foundation for a better understanding of cold water fish to long term adaptation to environments which may be experiencing thermal stress. This information is also very valuable to various stakeholders (i.e. power companies, First Nations Peoples, Governments, etc) which will allow them to manage the release of thermal effluents or thermally stressful events.
5. REFERENCES


