Plasticity of the heat shock response and development of thermotolerance during embryonic development of Lake Whitefish (*Coregonus clupeaformis*)

A Thesis
Submitted to the Faculty of Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the degree of

Master of Science
in
Biology
University of Regina

By
Katherine Jean Sessions
Regina, Saskatchewan
September, 2015

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UNIVERSITY OF REGINA

FACULTY OF GRADUATE STUDIES AND RESEARCH

SUPERVISORY AND EXAMINING COMMITTEE

Katherine Jean Sessions, candidate for the degree of Master of Science in Biology, has presented a thesis titled, *Plasticity of the heat shock response and development of thermotolerance during embryonic development of Lake Whitefish (Coregonus clupeaformis)*, in an oral examination held on August 31, 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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Committee Member: Dr. Christopher Somers, Department of Biology

Committee Member: Dr. Harold Weger, Department of Biology

Chair of Defense: Dr. Kyle Hodder, Department of Geography
Abstract
Thermal discharge produced by industrial facilities has the potential to impact development of fish by increasing background temperature of the surrounding area. The overall objectives of this study were to: 1) Examine the interplay between how temperature and frequency of heat shock and post-heat shock recovery time can modulate the heat shock response to a subsequent more severe stressor; 2) determine if this interplay is the same at different embryonic stages; and 3) assess whether transient heat shocks confer protection to the embryo. These objectives were addressed by exposing embryos to repeated transient heat shocks and high-level heat shocks and then quantifying heat shock protein mRNA levels and whole animal responses such as, percent survival, time to hatch and morphometrics. Reverse transcription quantitative real-time PCR revealed that transient heat shocks increased or attenuated the heat shock response to severe heat shock depending on the post-transient recovery period. The most frequent transient heat shocks, every 3 days, had the lowest levels of hsp70 mRNA of all heat shock treatments. Embryonic stages had similar results, however, it seemed older embryos had a larger capacity to respond to heat shock as determined by greater increases in heat shock protein mRNA levels. Transient heat shock regimes were also able to confer protection to embryos exposed to a severe 4 h +18 °C high-level heat shock. In general, transient heat shock regimes seem to have some benefit to the embryo allowing for a quicker response to stress and therefore, greater tolerance.
Acknowledgement

I would like to thank my supervisor Dr. Richard Manzon for his support and guidance throughout my degree program. I would also like to thank my collaborators, Dr. Douglas Boreham, Dr. Christopher Somers and Dr. Joanna Wilson for their support.

I would like to thank Daniel Stefanovic for teaching me certain laboratory techniques and proper fish husbandry care. Also, would like to thank Chelsey Fonger for her assistance in rearing Lake Whitefish and for data collection of morphometrics. Lastly, I would like to thank Dr. Gavin Simpson for his guidance in statistical approaches for my data.

I would like to acknowledge the work of Daniel Stefanovic and Dr. Lori Manzon for their work on real-time PCR primer design and optimization for Lake Whitefish prior to my arrival as a Master student.

This work was funded by contracts with Bruce Power (Douglas Boreham, Christopher Somers, Joanna Wilson, and Richard Manzon) and grants from the Natural Sciences and Engineering Research Council of Canada (Joanna Wilson, Christopher Somers and Richard Manzon). I, Katherine Sessions, was funded, in part, by awards and scholarships from the Faculty of Graduate Studies and Research (FGSR) at the University of Regina. I also received travel grant funding from FGSR and Fisheries Society of the British Isles.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>3c3d</td>
<td>+3 °C heat shock every 3 days</td>
</tr>
<tr>
<td>3c3d-6</td>
<td>+3 °C heat shock every 3 days with a 6 h post-transient recovery period</td>
</tr>
<tr>
<td>3c6d</td>
<td>+3 °C heat shock every 6 days</td>
</tr>
<tr>
<td>3c6d-18</td>
<td>+3 °C heat shock every 3 days with an 18 h post-transient recovery period</td>
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</tr>
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</tr>
<tr>
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<td>+12 °C heat shock every 6 days with a 3 h post-transient recovery period</td>
</tr>
<tr>
<td>12c6d-6</td>
<td>+12 °C heat shock every 6 days with a 6 h post-transient recovery period</td>
</tr>
<tr>
<td>CA</td>
<td>California</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHSE-214</td>
<td>Chinook Salmon cell line</td>
</tr>
<tr>
<td>con</td>
<td>Control</td>
</tr>
<tr>
<td>Cq</td>
<td>cycle number</td>
</tr>
<tr>
<td>Cq1</td>
<td>number of qPCR cycles required to detect a single target molecule</td>
</tr>
<tr>
<td>dpf</td>
<td>days post fertilization</td>
</tr>
<tr>
<td>E</td>
<td>efficiency of amplification</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
</tbody>
</table>
gDNA  genomic deoxyribonucleic acid
Hsc70  70 kDa heat shock cognate
Hsf   heat shock transcription factor
Hsp70  70 kDa heat shock protein
Hsp90α 90 kDa heat shock protein-alpha
Hsp90β 90 kDa heat shock protein-beta
Hsps   Heat shock proteins
MCMC  Markov Chain Monte Carlo
MIQE  minimum information for publication of quantitative real-time PCR experiments
mRNA  messenger ribonucleic acid
MS-222  ethyl3-aminobenzoate methanesulfonate
ON    Ontario
qPCR  quantitative real-time polymerase chain reaction
RNA   ribonucleic acid
RT-qPCR reverse transcription quantitative real-time polymerase chain reaction
Overview and Objectives

Temperature is important throughout fish life history and impact fish behavior (Hutchison and Maness, 1979), distribution (Brander et al., 2003), growth rate (Handeland et al., 2008), swimming performance (Lee et al., 2003), reproductive performance (Webb et al., 1999), developmental rate (Gillooly et al., 2002), and cellular processes (Mark et al., 2005). Changes in any of these factors may increase or decrease overall fitness in fish and this is in part why rising temperature could profoundly impact fish.

Thermal effluents or thermal discharge into water bodies can raise background temperatures in and around outlet areas. A range of effects have been shown on fish migrating through or living in these thermal discharge areas. The nature of these effects varies with species and the habitat and can include impacts on survival, food abundance and reproductive mode (Cooke et al., 2004; Holland et al., 1974; Young and Gibson, 1973). Very little research is being conducted on the potential impact these effluents can have on developing embryos. Griffiths and Hydro, 1979, examined impact of incubation temperature on time to hatch and survival and found a decrease in survival and time to hatch in Lake Whitefish, Coregonus clupeaformis, with increasing incubation temperature. For instance, a 3 °C rise in temperature for 25% of developmental time decreased time to completion of hatch by 13 days (Griffiths and Hydro, 1979).

One area of research which remains poorly investigated is embryo's response or acclimation to temperature changes at the cellular and physiological levels and the longer-term impacts. One area of particular importance is the heat shock response, as it could have the potential to impact tolerance to stress in later life stages of fish. The heat
shock response is characterized by the upregulation of heat shock proteins (Hsps). In general, these proteins act as molecular chaperones to maintain cell homeostasis during times of stress but are also important in the normal functioning of the cell (Feder and Hofmann, 1999). Importantly, the heat shock response has a degree of plasticity which can be altered depending on thermal history. This plasticity has been observed in response to natural seasonal variation (Fangue et al., 2003; Padmini and Usha Rani, 2008), population difference, (Nakano and Iwama, 2002; Narum et al., 2013) and acclimation (Lund et al., 2006). Goby fishes, *Gillichthys mirabilis*, sampled during summer months have increased basal protein levels of heat shock protein 90 (Hsp90) and as a result required a more severe heat shock prior to initiating the heat shock response and upregulating heat shock protein expression (Dietz and Somero, 1992). Also, Killifish, *Fundulus heteroclitus*, from southern populations that experienced monthly mean temperatures 13 °C higher than northern populations tended to show greater tolerance to thermal stress (Fangue et al., 2006). Controlled laboratory experiments which acclimated fish to elevated temperatures and different environmental conditions confirm these findings (Bennett and Beitinger, 1997; DuBeau et al., 1998; Healy and Schulte, 2011; Lund et al., 2006; Stitt et al., 2014).

The long-term objectives of my research were to understand how repeated transient heat shocks affect short-term and long-term embryo responses to stress and to determine if embryos could develop tolerance. Specifically, the objectives of my research were:

1. Determine if repeated low-level transient thermal stress throughout embryogenesis will impact the nature of the heat shock response to high-level stressors.
2. Determine how embryonic stage impacts the heat shock response and the interaction between low-level transient heat shock and high-level heat shock.

3. Determine if repeated mid-level transient thermal stress during embryogenesis alters the heat shock response, survival and development following exposure to an acute severe stressor.

4. Determine what role does the recovery period between transient heat shock (low or mid-level) and high-level shock (i.e. post-transient recovery) play in the observed embryo responses.

The ability of embryos to respond to stress and congruently develop correctly is of great importance for the long-term fitness of an organism. In meeting these objectives I will gain knowledge into the potential impacts of transient stress associated with thermal discharge on embryonic fish and their ability to respond to stress. Moreover, this work begins to address the question, how does thermal discharge impact development and can it have potential protective effects through transient stress.
Chapter 1: Repeated transient heat shocks attenuate the heat shock response in embryonic Lake Whitefish
**Introduction**

Exposure to environmental stressors during the incubation period present a unique challenge to the embryos as they are unable to physically (behaviourally) escape the stressor. Lake Whitefish are a particularly relevant Northern Hemisphere species in that their peak spawning activity occurs in late fall, early winter. Thus, their embryos develop at near zero temperatures during the winter months usually under the cover of ice (Hart, 1931). Several studies have shown that Lake Whitefish embryos and juvenile/adult fish are particularly sensitive to elevated temperatures and thermal stress, even in comparison to other cold-water fishes (Coutant, 1977). Given their sensitivity to temperature, I propose they will serve as a good model to explore the impacts of thermal pollution on developing embryos. Near shore habitats, habitat where Lake Whitefish spawn can be impacted by thermal effluents from once-through cooling systems used by a variety of industrial processes (e.g. power generation). These systems uptake large amounts of cold water from lakes, rivers, reservoirs or oceans and use it for cooling prior to returning the waste water back to the source at higher than ambient temperatures. Due to the variable environment of this thermal plume embryos incubating in these affected areas may be exposed to stress transiently, by experiencing periods of low to high thermal stress intermitted by periods of recovery. These transient thermal stress events may trigger a heat shock response, which is a universal cellular stress response, to protect the cell and thus embryo from the potential detrimental effects of the stressor (Alsop and Vijayan, 2008).

The heat shock response is an evolutionarily conserved cellular stress response found in all taxa, studied to date, from bacteria to humans (Georgopoulos and Welch, 1993). In general, heat shock proteins (Hsps) are upregulated when an accumulation of
denatured proteins occur within the cell and function as a molecular chaperone to maintain homeostasis and function in protein folding, repair and transport (Feder and Hofmann, 1999; Georgopoulos and Welch, 1993; Lindquist, 1986). They also play roles in mediating cell signaling events and inhibiting programmed cell death pathways (Beere, 2005). Numerous heat shock proteins have been identified and grouped into families based on the molecular weight. In general, heat shock proteins can be classed as constitutive or inducible, with constitutive forms being expressed at low levels at all times and the inducible forms only being expressed in response to stress (Feder and Hofmann, 1999). Two key families in fish are the Hsp70 and the Hsp90 families. The Hsp70 family function as molecular chaperones by binding to small peptides, nascent polypeptide chains, proteins that have been targeted to the wrong cellular compartment, mutated proteins and certain oligomeric proteins in the process of assembly or disassembly (Parsell and Lindquist, 1993). This family contains one of the most inducible and extensively studied heat shock protein, heat shock protein 70 (Hsp70). The Hsp70 family also has a constitutively expressed gene named heat shock cognate 70 (Hsc70). Likewise the Hsp90 family contains both inducible and constitutive isoforms, Hsp90α and Hsp90β, respectively. Hsp90 family members also function as molecular chaperones and are additionally important in mediating steroid binding to steroid nuclear receptors located in the cytoplasm (Parsell and Lindquist, 1993). Finally, both families function to inhibit apoptotic pathways (Beere, 2005).

In adult fish, environmental conditioning has been observed to alter the characteristics of the heat shock response (Barua and Heckathorn, 2004). This plasticity in the heat shock response can lead to long-term adaptation. For instance, in fish, heat shock response varies along latitudinal gradients and with thermal history
(Fangue et al., 2006; Nakano and Iwama, 2002; Narum et al., 2013). Southern populations of Killifish that experience monthly mean temperatures 13 °C higher than northern populations have an attenuated heat shock response when challenged with acute heat shock (Fangue et al., 2006). In Lake Whitefish, this attenuation of the response has also been observed through acclimation (temporary responses to recent temperatures) (Zak, 2015). Beyond their role as housekeeping molecular chaperones and stress inducible proteins (Parsell and Lindquist, 1993) Hsps have also been shown to function in the regulation of developmental events (Rupik et al., 2011). Lens development in zebrafish, *Danio rerio*, under non-stressful conditions, requires the regulated expression of *hsp70* during embryogenesis (Blechinger et al., 2002). Yet embryos are still able to induce *hsp70* mRNA at all embryonic stages examined when exposed to a heat shock (Krone and Sass, 1994; Lele et al., 1997). Given their importance for normal development and combating stress during development, it is of interest to know if embryos exhibit a plastic heat shock response under varying environmental conditions.

The impact of acclimation temperature and thermal history on the heat shock response in juvenile and adult fishes has been studied in a variety of species (Barua and Heckathorn, 2004). However, there are little to no data pertaining to the impact of thermal history during the embryonic period on the heat shock response. To this end, in the present study I set out to investigate the effect of repeated low-level transient heat shocks on the heat shock response to a subsequent high-level thermal stressor at different embryonic stages. The primary objectives of the study were to 1) determine if low-level transient heat shocks impact embryos response to high-level heat shock; 2) determine how embryonic stage may impact the interaction between transient heat shock and high-level heat shock; 3) determine if the recovery time between the initial transient
shock and subsequent high-level heat shock will impact the embryos response.

Understanding the effects of transient heat shock, intended to mimic potential exposure from thermal effluents, on embryos, on their heat shock response and any associated plasticity or adaptation may provide insight into the potential impacts of thermal effluents on this and other cold water species.

Materials and Methods

Animal Collection and Husbandry

Eggs and sperm were collected from Lake Whitefish caught by short set gill nets near Fishing Islands of Lake Huron adjacent to South Bruce Peninsula, Ontario, Canada in November, 2012 (Eme et al., 2015). Eggs were fertilized in the field (Eme et al., 2015; Mueller et al., 2015) and transported to the aquatic facility at the University of Regina. Once received, embryos were incubated in mini-McDonald bell jars with continuous flowing, filtered water maintained at 3 °C. All procedures involving animals were approved by the University of Regina President’s Committee on Animal Care and conducted in accordance with the guidelines of Canadian Council on Animal Care.

Experiment

This study consisted of multiple components including a chronic low-level transient stressor, acute high-level stressor and variable recovery period between the two aforementioned stressors. Repeated low-level transient heat shocks (here after transient heat shock) were delivered throughout development and served as the chronic stressor intended to prime the embryo’s heat shock response system. At two points in development (64 and 82 days post fertilization (dpf)) embryos were subsequently exposed to an acute high-level heat shock and sampled. Finally, the recovery period between the last transient heat shock and high-level heat shock (here after post-transient
recovery) varied to determine if timing between stressors affected the embryo’s heat shock response.

**Transient Heat Shock Protocol**

McDonald bell jars containing approximately 10,000 embryos were randomly assigned to one of four transient heat shock treatments as follows: Control (no transient heat shock; control temperature of 3 °C); a heat shock of +3 °C every three days (3c3d); a heat shock of +3 °C every six days (3c6d); or a heat shock of +6 °C every six days (6c6d) (Table 1). The transient heat shocks began at 15dpf and were applied continually every three or six days as appropriate until hatching. These heat shocks would last 1 h in duration and were administered by transferring the mini-McDonald bell jars to a circulating system at the appropriate heat shock temperature after which the bell jars were returned to the main system at 3 °C.

**High-Level Heat Shock Protocol**

To investigate the potential long-term impact of transient thermal stress on Lake Whitefish embryos and the heat shock response, embryos from the four aforementioned transient heat shock treatments were exposed to one of eight different high-level heat shocks at 64 and 82dpf. Embryos 64dpf were considered to be around 56 % developed while embryos 82dpf were around 59 % developed (Sreetharan et al., 2015). The main difference between these stages was size of embryo and amount of vascularisation occurring within the embryo. High-level heat shocks were administered to the embryos 6 or 18 h after receiving transient heat shocks. During the 6 or 18 h post-transient recovery embryos were maintained at the control temperature of 3 °C.
Table 1: Heat shock experimental treatment groups for embryos that received either 6 or 18 h recovery period between low-level transient heat shock and high-level heat shock

<table>
<thead>
<tr>
<th>Transient Heat Shock</th>
<th>Control</th>
<th>+3°C every 3 days</th>
<th>+3°C every 6 days</th>
<th>+6°C every 6 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con/12x1</td>
<td>3c3d//12x1</td>
<td>3c6d//12x1</td>
<td>6c6d//12x1</td>
</tr>
<tr>
<td></td>
<td>Con/15x1</td>
<td>3c3d//15x1</td>
<td>3c6d//15x1</td>
<td>6c6d//15x1</td>
</tr>
<tr>
<td></td>
<td>Con/18x1</td>
<td>3c3d//18x1</td>
<td>3c6d//18x1</td>
<td>6c6d//18x1</td>
</tr>
<tr>
<td></td>
<td>Con/0x4</td>
<td>3c3d//0x4</td>
<td>3c6d//0x4</td>
<td>6c6d//0x4</td>
</tr>
<tr>
<td></td>
<td>Con/12x4</td>
<td>3c3d//12x4</td>
<td>3c6d//12x4</td>
<td>6c6d//12x4</td>
</tr>
<tr>
<td></td>
<td>Con/15x4</td>
<td>3c3d//15x4</td>
<td>3c6d//15x4</td>
<td>6c6d//15x4</td>
</tr>
</tbody>
</table>

// represents recovery period
Within each box: Transient heat shock//High-level heat shock
Embryos were then exposed to one of eight high-level heat shock groups as follows: +0 °C (control temperature of 3 °C); +12 °C; +15 °C or +18 °C with each temperature group receiving a heat shock duration of 1 or 4 h (Table 1). After transient heat shock and the subsequent recovery period (6 or 18 h) high-level heat shocks were administered by placing 75 embryos from each transient heat shock regime into 250 ml polypropylene jars that were floated in a water bath at the appropriate high-level heat shock temperature. Following the appropriate 1 or 4 h heat shock, the polypropylene jars containing the embryos were transferred to a 3 °C water bath for a 2 h recovery period prior to sampling. Embryos from each treatment groups were snap frozen in liquid nitrogen and stored at -80 °C for later analysis of hsp70, hsp90α, hsp90β and hsc70 mRNA levels.

**RNA Isolation and cDNA Synthesis**
Total RNA was extracted by homogenizing two embryos in TRizol reagent according to manufacturer’s instructions (Invitrogen-life technologies, Mississauga, ON). MIQE guidelines were followed by verifying purity and quality of sample and quantifying RNA (Bustin et al., 2009). Purity of RNA was verified and concentration was quantified spectrophotometrically (NanoDrop, Thermo Scientific) (A_{260}/A_{280} and A_{260}/A_{230} ratios>1.8). RNA quality was further assessed through gel electrophoresis and evaluated based on the presence of ribosomal RNA bands. RNA was stored at -80 °C until further processing. For each sample, first strand cDNA was prepared using 1 µg of total RNA and the Qiagen, quantiTect Reverse Transcription kit according to the manufacturer’s instructions (Qiagen, Mississauga, ON). cDNA was stored at -20 °C.

**Reverse Transcription Quantitative Real-Time PCR Analysis**
Primers for reverse transcription quantitative real-time PCR (RT-qPCR) were designed using cDNA sequences available in GenBank as previously described.
(Stefanovic et al., 2015) (hsp90α, GenBank accession no. KP893539; hsp90β, GenBank accession no. KP893540; hsp70, GenBank accession no. KP861983; hsc70, GenBank accession no. HQ287746; β-actin, GenBank accession no. KP893542 and GAPDH, GenBank accession no. KP893543) (Table 2). For each primer pair amplification efficiency was determined by performing standard curves on pooled heat shocked and control samples and was shown to meet MIQE guidelines (efficiency between 90-110%).

RT-qPCR gene expression profiling assays were carried out on a Bio-Rad CFX-Connect real time detection system (Hercules, CA) using SSoAdvance™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA), 250 nM of forward and reverse primers and 1.0 µL of 2x diluted cDNA in a total reaction volume of 20 µL. Each qPCR run consisted of 1 cycle of 95°C for 2 min and 40 cycles of 5 s at 95°C and 30 s at primer specific annealing temperature (Table 2). Melt curve analysis, which confirms the presence of a single amplicon was performed at the completion of each qPCR run. MIQE guidelines were met by using no template controls. The absence of gDNA contamination in cDNA samples was confirmed by the absence of additional amplicons when using primer pairs (β-actin) that span an intron. To correct for variations in reverse transcription efficiency and minor variations in RNA and cDNA template loading, samples were normalized to two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin. These genes were shown to be stable during heat shock treatment (Stefanovic et al., 2015).
Table 2: Primers used for RT-qPCR of heat shock protein genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>GenBank Accession no.</th>
<th>Annealing Temperature (°C)</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP90α</td>
<td>Forward</td>
<td>KP893539</td>
<td>65</td>
<td>AGT CGT GGG GAA AGG ATT GT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>KP893539</td>
<td>65</td>
<td>TGA ATA AGG TTG AAA GCA GCA GA</td>
</tr>
<tr>
<td>HSP90β</td>
<td>Forward</td>
<td>KP893540</td>
<td>60</td>
<td>CCT TTC TAT TTT CCT GCG TC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>KP893540</td>
<td>60</td>
<td>TGT TTC CGT TGA CTG TCT TT</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward</td>
<td>KP893542</td>
<td>65</td>
<td>GTG GCG CTG GAC TTT GAG CA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>KP893542</td>
<td>65</td>
<td>ACC GAG GAA GGA GGG CTG GA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>KP893543</td>
<td>60</td>
<td>CCG TCC GTC TGG AGA AGG C</td>
</tr>
<tr>
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**Statistical Analysis**

Data were analyzed using R Studio version 0.98.977 (Team, 2014). The effects of thermal stress on target gene expression were investigated using the MCMC.qpcr package (Matz, 2013). This package implements a poisson-lognormal generalized mixed model to infer fold changes in target genes in response to fixed factors as well as variation attributable to random differences in technical replicates. The model fitting processes involves a Bayesian MCMC sampling scheme that incorporates references genes as priors. For this approach the MCMC.qpcr package empirically transforms raw Cq values into molecular counts as follows:

\[ \text{Count} = E^{(Cq_1 - Cq)}, \text{rounded to integer} \]

Where:

- \( E \) = efficiency of amplification
- \( Cq_1 \) = number of qPCR cycles required to detect a single target molecule
- \( Cq \) = cycle number for sample

**Approximation of Cq1 Value**

\( Cq_1 \) can be approximated based on the efficiency of amplification using empirical formula \( Cq_1 = 79-21.5E \) (\( E=\)efficiency of amplification) which was developed for Light Cycler 480 (Matz, 2013). The current study used Bio-Rad CFX-Connect where \( Cq_1 \) values were experimentally determined for a sub-sample of genes by doing four-fold template dilutions. Matz, 2013, found that there was no difference in model fit or model approximation of molecular counts when using the same average size \( Cq_1 \) for all genes versus a more accurately approximated \( Cq_1 \) value for each individual gene. This was also found to be true for the sub-sample of genes used in this study’s experimental estimation. When three separate models using empirical formula \( Cq_1 \) values, experimentally
estimated Cq1 values, or averaged Cq1 values were plotted against one another the approximated molecular count of a sample did not differ nor did that variation of that sample. Thus, for this analysis a Cq1 value of 37 was used as suggested by Matz, 2013.

**Modeling of Heat Shock Proteins mRNA Levels**

Each embryonic stage was analyzed separately, and within an embryonic stage duration of high-level heat shock was analyzed separately. The effects of thermal stress were modeled with transient heat shock combined with post-transient recovery period (Transient heat shock regime) and high-level heat shock as fixed factors. Transient heat shocks were grouped as follows: Control (no heat shock), 3c6d-6 (i.e. +3 °C every 6 days with a 6 h post-transient recovery), 3c3d-6 (+3 °C every 3 days, with a 6 h post-transient recovery), 6c6d-6 (+6 °C every 6 days, with a 6 h post-transient recovery), 3c6d-18 (+3 °C every 6 days, with an 18 h post-transient recovery), 3c3d-18 (+3 °C every 3 days, with a 18 h post-transient recovery), 6c6d-18 (+6 °C every 6 days with a 18 h post-transient recovery). High-level heat shocks were grouped as follows: +0 °C (no heat shock), +12 °C, +15 °C, +18 °C. Control treatments for both transient heat shock (Control) and high-level heat shock (+0 °C) were set as reference groups (here after control reference group). For the +0 °C 1 h high-level heat shock experimental group, transient heat shock regime and control embryos were sampled after the 4 h high-level heat shock. Due to missing data, caused by loss of treatment prior to sampling, treatment 3c6d-18 (+3 °C every 6 days with an 18 h post-transient recovery) was completely removed from the 64dpf analysis. Control genes, GAPDH and β-actin, were added as priors to the model and functioned to normalize for variations in cDNA and were allowed 1.2 fold changes between samples. Posterior means were plotted relative to the control reference group and error bars represented 95% credible intervals, which are the Bayesian
analog of confidence intervals (Matz, 2013). Effects were deemed statistically significant when credible intervals did not overlap.

**Results**

**Overview**

Transient heat shock impacted how embryos responded to high-level heat shock. Moreover, post-transient recovery played an important role in the interaction between transient and high-level heat shock. Overall, transient heat shock seemed to attenuate the upregulation of inducible genes in response to high-level heat shock provided the recovery period between the two events was sufficiently long. In general, a decrease in constitutive hsp mRNA was observed. Embryonic stage also had an impact on this interaction between transient and high-level heat shock with later stage embryos showing greater increases in hsp mRNA levels.

**hsp70**

Heat shock protein 70 mRNA levels varied in response to the transient heat shock regimes in the absence of high-level heat shock (Fig 1-IA, 1-IE, 2-IA, 2-IE). Embryos were sampled 12 and 24 h after the last 1 h transient heat shock for those transient regimes that did not receive a high-level heat shock. In embryos sampled 12 h post-transient heat shock (i.e. 6 h post-transient recovery group) hsp70 mRNA levels varied by as much as 2 fold above control reference group while embryos sampled 24 h post-transient heat shock (i.e. 18 h post-transient recovery) varied by as much as 2 fold below control reference group. Most notable was the observation that the 3c3d-18 transient heat shock regime had lower hsp70 mRNA levels than control reference group and most other regimes for both embryonic stages.
An interaction between high-level heat shock and transient heat shock regime was observed. This interaction varied by transient heat shock regime, duration of high-level heat shock, and embryonic stage (Fig. 1, Fig. 2). High-level heat shock increased *hsp70* mRNA levels relative to control reference group in all instances except for the embryos exposed to the 3c3d-18 transient heat shock regime and a 1 h high-level heat shock (Fig. 1, Fig. 2). In several instances transient heat shock regimes resulted in an attenuated increase in *hsp70* mRNA levels relative to the no transient control groups. This effect was greatest in the 64dpf embryos exposed to a 1 h high-level heat shock delivered 18 h after the last transient heat shock (Fig. 1, I.B-D). In the case of transient heat shock regime, 3c3d-18, this reduction in *hsp70* mRNA levels relative to no transient controls was statistically significant and the resultant *hsp70* mRNA levels did not differ from the control reference group. A similar attenuation of the *hsp70* response was observed in embryos 64dpf exposed to the 3c3d-18 transient heat shock regime and 4 h high-level heat shocks of +15 °C and +18 °C (Fig 2, I.B-D). In embryos 82dpf exposed to a 1 or 4 h high-level heat shock *hsp70* mRNA levels exposed to the various transient regimes did not vary from the no transient heat shock control embryos except in the 3c3d-18 transient regime. In this later group a significant reduction in *hsp70* mRNA was observed following a high-level heat shock of +15 and +18 °C (Fig 1-IIG-H, 2-IIG-H).
**Figure 1.** The effect of low-level transient heat shock regimes on expression of normalized *hsp70* mRNA 2 h after a 1 h high-level heat shock was examined at different embryonic stages. Normalized expression was determined using R-package MCMC.qpr (Matz, 2013). Expression values were plotted as fold change relative to the control embryos that received no heat shock in log2 scale. Posterior means and 95 % credible intervals as error bars are presented. All transient regimes in +0 °C group were sampled with the 4 h high-level heat shock group, so sampling occurred 13 h post-transient heat shock for 3c6d-6, 3c3d-6 and 6c6d-6 while the other three transient regimes were sampled 24 h post-transient heat shock. Graph I represents data from embryos 64dpf (~56 %) while graph II represents embryos 82dpf (~59 %). Transient heat shock regimes include a repeated low-level transient heat shock and a 6 or 18 h recovery period received prior to exposure to the 1 h high level heat shock and is labeled as follows: for 6 h recover post-transient heat shock, 3c6d-6 (+3 °C every 6 days), 3c3d-6 (+3 °C every 3 days), 6c6d-6 (+6 °C every 6 days) and for a 18 h recovery post-transient heat shock, 3c6d-18 (+3 °C every 6 days), 3c3d-18 (+3 °C every 3 days), 6c6d-18 (+6 °C every 6 days). Control embryos were never given a transient heat shock and therefore received no recovery period. Each sub-graph represents a 1 h high-level heat shock at a given temperature as follows: +0 °C (no heat shock; sub-graph A, E), +12 °C (sub-graph B, F), +15 °C (sub-graph C, G) and +18 °C (sub-graph D, H) from a control temperature of 3 °C. Embryos were given 2 h of recovery at 3 °C before being sampled.
Figure 2. The effect of low-level transient heat shock regimes on expression of normalized hsp70 mRNA 2 h after a 4 h high-level heat shock was examined at different embryonic stages. Normalized expression was determined using R-package MCMC.qpr (Matz, 2013). Expression values were plotted as fold change relative to the control embryos that received no heat shock in log2 scale. Posterior means and 95 % credible intervals as error bars are presented. Graph I represents data from embryos 64dpf (~56 %) while graph II represents embryos 82dpf (~59 %). Transient heat shock regimes include a repeated low-level transient heat shock and a 6 or 18 h recovery period received prior to exposure to the 1 h high-level heat shock and is labeled as follows: for 6 h recover post-transient heat shock, 3c6d-6 (+3 °C every 6 days), 3c3d-6 (+3 °C every 3 days), 6c6d-6 (+6 °C every 6 days) and for a 18 h recovery post-transient heat shock, 3c6d-18 (+3 °C every 6 days), 3c3d-18 (+3 °C every 3 days), 6c6d-18 (+6 °C every 6 days). Control embryos were never given a transient heat shock and therefore received no recovery period. Due to missing data, treatment 3c6d-18 (+3 °C every 6 days-with an 18 h post-transient recovery) was completely removed from 64dpf analysis. Each sub-graph represents a 4 h high-level heat shock at a given temperature as follows: +0 °C (no heat shock; sub-graph A, E), +12 °C (sub-graph B, F), +15 °C (sub-graph C, G) and +18 °C (sub-graph D, H) from a control temperature of 3 °C. Embryos were given 2 h of recovery at 3 °C before being sampled.
**hsc70**

The response to transient heat shock regimes, with respect to *hsc70* mRNA levels, in younger 64dpf embryos differed starkly from that in the older 82dpf embryos (Fig. 3, 4). In 64dpf embryos, *hsc70* mRNA levels varied considerably with no strong discernible trends (Fig. 3-IA-D, 4-IA-D). In some transient heat shock regimes *hsc70* mRNA levels increased while in others there were no noticeable differences relative to control reference group. In contrast, there was a strong trend of *hsc70* mRNA levels being downregulated in embryos 82dpf for all transient heat shock regimes except for 3c3d-6 (Fig. 3-IIE, 4IIE). This trend of lower *hsc70* mRNA levels was also observed in all 82dpf embryos exposed to a high-level heat shock when compared to the control reference group with 6c6d-6 transient heat shock regime showing a significant decrease compared to no transient controls(Fig. 3-IIIF-H, 4IIF-H). No transient control embryos showed downregulation when exposed to a 1 h high-level heat shock (Fig. 3, IIF-H) while exposure to a 4 h high-level heat shock did not noticeably impact *hsc70* mRNA levels relative to control reference group (Fig. 4-IIIF-H).

**hsp90**

Transient heat shock regimes appeared to have the greatest effect on *hsp90α* and *β* mRNA levels with the effects of high-level heat shock being less consistent (Fig. 5, Fig. 6). Transient heat shock regimes in most instances decreased *hsp90α* and *β* mRNA levels relative to control reference group. This was most pronounced in 64dpf embryos where the decreases were more consistent and of greater magnitude (Fig. 5-IA, 6-IA). In the case of the 82dpf embryos, *hsp90α* and *β* levels remained similar to control reference group or decreased slightly, with the greatest decreases predominating 24 h after the last transient heat shock (i.e. 18 h post-transient recovery period) (Fig. 5-IIIE, 6-IIIE).
**Figure 3.** The effect of transient heat shock regimes on expression of normalized hsc70 mRNA 2 h after a 1 h high-level heat shock was examined at different embryonic stages. Normalized expression was determined using R-package MCMC.qpr (Matz, 2013). Expression values were plotted as fold change relative to the control embryos that received no heat shock in log2 scale. Posterior means and 95% credible intervals as error bars are presented. All transient regimes in +0 °C group were sampled with the 4 h high-level heat shock group, so sampling occurred 13 h post-transient heat shock for 3c6d-6, 3c3d-6 and 6c6d-6 while the other three transient regimes were sampled 24 h post-transient heat shock. The graph I represents data from embryos 64dpf (~56%) while the graph II represents embryos 82dpf (~59%). Transient heat shock regimes include a repeated low-level transient heat shock and a 6 or 18 h recovery period received prior to exposure to the 1 h high-level heat shock and is labeled as follows: for 6 h recovery post-transient heat shock, 3c6d-6 (+3 °C every 6 days), 3c3d-6 (+3 °C every 3 days), 6c6d-6 (+6 °C every 6 days) and for a 18 h recovery post transient heat shock, 3c6d-18 (+3 °C every 6 days), 3c3d-18 (+3 °C every 3 days), 6c6d-18 (+6 °C every 6 days). Control embryos were never given a transient heat shock and therefore received no recovery period. Each sub-graph represents a 1 h high-level heat shock at a given temperature as follows: +0 °C (no heat shock; sub-graph A, E), +12 °C (sub-graph B, F), +15 °C (sub-graph C, G) and +18 °C (sub-graph D, H) from a control temperature of 3 °C. Embryos were given 2 h of recovery at 3 °C before being sampled.
**Figure 4.** The effect of low-level transient heat shock regimes on expression of normalized *hsc70* mRNA 2 h after a 4 h high-level heat shock was examined at different embryonic stages. Normalized expression was determined using R-package MCMC.qpr (Matz, 2013). Expression values were plotted as fold change relative to the control embryos that received no heat shock in log2 scale. Posterior means and 95 % credible intervals as error bars are presented. Graph I represents data from embryos 64dpf (~56 %) while graph II represents embryos 82dpf (~59 %). Transient heat shock regimes include a repeated low-level transient heat shock and a 6 or 18 h recovery period received prior to exposure to the 1 h high-level heat shock and is labeled as follows: for 6 h recover post-transient heat shock, 3c6d-6 (+3 °C every 6 days), 3c3d-6 (+3 °C every 3 days), 6c6d-6 (+6 °C every 6 days) and for a 18 h recovery post transient heat shock, 3c6d-18 (+3 °C every 6 days), 3c3d-18 (+3 °C every 3 days), 6c6d-18 (+6 °C every 6 days). Control embryos were never given a transient heat shock and therefore received no recovery period. Due to missing data, treatment 3c6d-18 (+3 °C every 6 days-with an 18 h post-transient recovery) was completely removed from 64dpf analysis. Each sub-graph represents a 4 h high-level heat shock at a given temperature as follows: +0 °C (no heat shock; sub-graph A, E), +12 °C (sub-graph B, F), +15 °C (sub-graph C,G) and +18 °C (sub-graph D, H) from a control temperature of 3 °C. Embryos were given 2 h of recovery at 3 °C before being sampled.
**Figure 5.** The effect of low-level transient heat shock regimes on expression of normalized hsp90a (■) and hsp90β (■) mRNA 2 h after a 1 h high-level heat shock was examined at different embryonic stages. Normalized expression was determined using R-package MCMC.qpr (Matz, 2013). Expression values were plotted as fold change relative to the control embryos that received no heat shock in log2 scale. Posterior means and 95 % credible intervals as error bars are presented. All transient regimes in +0 °C group were sampled with the 4 h high-level heat shock group, so sampling occurred 13 h post-transient heat shock for 3c6d-6, 3c3d-6 and 6c6d-6 while the other three transient regimes were sampled 24 h post-transient heat shock. Graph I represents data from embryos 64dpf (~56 %) while graph II represent embryos 82dpf (~59 %). Transient heat shock regimes include a repeated low-level transient heat shock and a 6 or 18 h recovery period received prior to exposure to the 1 h high-level heat shock and is labeled as follows: for 6 h recover post-transient heat shock, 3c6d-6 (+3 °C every 6 days), 3c3d-6 (+3 °C every 3 days), 6c6d-6 (+6 °C every 6 days) and for a 18 h recovery post-transient heat shock, 3c6d-18 (+3 °C every 6 days), 3c3d-18 (+3 °C every 3 days), 6c6d-18 (+6 °C every 6 days). Control embryos were never given a transient heat shock and therefore received no recovery period. Each sub-graph represents a 1 h high-level heat shock at a given temperature as follows: +0 °C (no heat shock; sub-graph A, E), +12 °C (sub-graph B, F), +15 °C (sub-graph C, G) and +18 °C (sub-graph D, H) from a control temperature of 3 °C. Embryos were given 2 h of recovery at 3 °C before being sampled.
**Figure 6.** The effect of low-level transient heat shock regimes on expression of normalized $hsp90\alpha$ (■) and $hsp90\beta$ (▲) mRNA 2 h after a 4 h high-level heat shock was examined at different embryonic stages. Normalized expression was determined using R-package MCMC.qpr (Matz, 2013). Expression values were plotted as fold change relative to the control embryos that received no heat shock in log2 scale. Posterior means and 95 % credible intervals as error bars are presented. Graph I represents data from embryos 64dpf (~56 %) while graph II represents embryos 82dpf (~59 %). Transient heat shock regimes include a repeated low-level transient heat shock and a 6 or 18 h recovery period received prior to exposure to the 1 h high-level heat shock and is labeled as follows: for 6 h recover post-transient heat shock, 3c6d-6 (+3 °C every 6 days), 3c3d-6 (+3 °C every 3 days), 6c6d-6 (+6 °C every 6 days) and for a 18 h recovery post-transient heat shock, 3c6d-18 (+3 °C every 6 days), 3c3d-18 (+3 °C every 3 days), 6c6d-18 (+6 °C every 6 days). Control embryos were never given a transient heat shock and therefore received no recovery period. Due to missing data, treatment 3c6d-18 (+3 °C every 6 days with an 18 h post-transient recovery) was completely removed from 64dpf analysis. Each sub-graph represents a 4 h high-level heat shock at a given temperature as follows: +0 °C (no heat shock; sub-graph A, E), +12 °C (sub-graph B, F), +15 °C (sub-graph C, G) and +18 °C (sub-graph D, H) from a control temperature of 3 °C. Embryos were given 2 h of recovery at 3 °C before being sampled.
In most instances, the pattern of \textit{hsp90}α and β mRNA downregulation in 64dpf embryos was also observed in embryos receiving a 1 or 4 h high-level heat shock (Fig. 5-IB-D, 6-IB-D). The most consistent exception to this trend was in the 3c3d-18 transient heat shock regime which showed no change relative to control reference group. In contrast, 82dpf embryos showed a much more variable hsp90α and β mRNA response to high level-heat shock and transient heat shock regimes. A 1 h high-level heat shock did not impact \textit{hsp90}β mRNA levels except for 3c3d-18 transient heat shock regime which showed a significant decrease in \textit{hsp90}β mRNA levels compared to no transient control for that particular high-level heat shock temperature (Fig. 5-IIIF-H). Embryos exposed to a 4 h high-level heat shock had a decrease in \textit{hsp90}β mRNA levels in all transient heat shock regimes except for two cases (3c6d-18 receiving a +12 °C heat shock; 3c6d-6 receiving a +15 °C heat shock and +18 °C heat shock) (Fig. 6-IIIF-H).

In most instances, \textit{hsp90}α mRNA levels in embryos 82dpf did not differ relative to control reference group. However, \textit{hsp90}α was observed to be slightly upregulated in a +15 and +18 °C 1 h high-level heat shock, though this increase was not significant to the no transient controls (Fig. 5-IIIF-H). In another instance, embryos within the 4 h high-level heat shock experimental groups, downregulated \textit{hsp90}α predominantly in the transient heat shock groups that received an 18 h post-transient recovery (Fig. 6-IIIF-H). This was significant in the 6c6d-18 transient heat shock regime at all high-level heat shock temperatures and for all transient heat shock regimes with an 18 h post-transient recovery that received a +15 °C heat shock.

\textbf{Discussion}

In the present study, I show that transient heat shocks throughout embryogenesis affect how embryos responded to subsequent high-level heat shock. This impact was
dependent on the nature of the transient heat shock regime, the duration of the high-level heat shock and the embryonic stage. One key notable finding was the observation that induction of hsp70 mRNA levels was significantly lower in groups that received the most frequent transient heat shock and the 18 h recovery period prior to high-level heat shock. This attenuated heat shock response indicates that Lake Whitefish embryos can respond to chronic low-level stressors (i.e. transient heat shock) with a plastic heat shock response. This plasticity in the heat shock response could point to adaptive potential of early life stages to environmental stressors that could affect later life stages.

In fish, both Hsp70 and Hsp90α are both inducible Hsps and as such mRNA and protein levels will increase in response to heat and other stressors (Basu et al., 2002; Iwama, 1998; Iwama et al., 1998; Krone et al., 1997; Rupik et al., 2011). In the present study, I consistently observed significant increases in hsp70 mRNA levels in response to high-level heat shock. On the other hand, in most instances hsp90α mRNA levels in embryonic Lake Whitefish were not elevated in response to heat stress, a finding that is consistent with previous work (Stefanovic et al., 2015). However, this observation is not a phenomenon that can be generalized to all fish embryos as hsp70 and hsp90α are inducible at the mRNA in response to thermal stress in Zebrafish and Atlantic Cod, *Gadus morhua*, embryos and an immortalized embryonic Chinook Salmon, *Oncorhynchus tshawytscha*, cell line (CHSE-214) (Krone and Sass, 1994; Krone et al., 1997; Palmisano et al., 1999; Skjærven et al., 2011). Importantly, in Lake Whitefish, this observation is stage or environment specific and not a result of a fundamental difference in the Lake Whitefish hsp90α gene and it not being inducible. Support for this claim comes from the fact that Hsp90α can be upregulated in later life stages of Lake Whitefish. For instance, hsp90α mRNA levels were elevated following heat shock in both year of
young fry and 2 year old juvenile Lake Whitefish (Stefanovic et al., 2015; Zak, 2015). In young of the year, this response was delayed compared to the increase in hsp70 and confirms that hsp90α is a heat inducible Hsp in early life history stages of Lake Whitefish (Stefanovic et al., 2015).

Several parameters might contribute to the lack of an upregulation of hsp90α in Lake Whitefish embryos. When comparing levels of hsp90α mRNA levels from non-stressed Lake Whitefish embryos, mRNA levels were very similar to those of hsp70. In contrast, hsc70 and hsp90β mRNA levels was about 6 fold higher than hsp70 or hsp90α, indicating hsp90α expression is maintained low as a typical inducible heat shock protein but is not being upregulated in response to stress. In Atlantic Cod, another cold-water species, hsp90α and hsp70 was found to be inducible to heat stress throughout development (Skjærven et al., 2011). This disparity between these two species may be due to differences in embryonic incubation temperature which may impact cellular metabolism or experimental design differences. For instance, Atlantic Cod were not permitted a post-heat shock recovery prior to sampling for mRNA analysis (Skjærven et al., 2011). In the current study embryos received at 2 h post-heat shock recovery period and although one might suggest mRNA levels decreased to control levels during this period of time, this is not the case. All data to date indicate that in embryonic Lake Whitefish the heat shock response is slow and long lived (Stefanovic et al., 2015). It is plausible that with the very low incubation temperatures the upregulation of hsp90α may require extraordinarily long time. An alternate explanation is that energy resources are diverted to transcription and translation of hsp70 exclusively. Due to the delayed induction of hsp90α in juvenile Lake Whitefish compared to hsp70
(Stefanovic et al., 2015), it is reasonable to think that given a longer post-heat shock recovery period an increase in \textit{hsp90}α mRNA levels might also be detected in embryos.

Heat shock cognate 70 and \textit{hsp90}β are constitutive HSPs and thus are not upregulated in response to thermal or other types of stress (Boone and Vijayan, 2002; Liu et al., 2011). This finding has been consistently observed across taxa and confirmed to be the case in embryos of Zebrafish (Krone and Sass, 1994; Krone et al., 1997; Santacruz et al., 1997), Lake Whitefish (Stefanovic et al., 2015) and Pool frog, \textit{Rana lessonae} (Simoncelli et al., 2010). The findings of my study are consistent with previous work in that I did not observe an increase in \textit{hsc70} or \textit{hsp90}β in Lake Whitefish embryos following acute heat shock. However, in the present study I did show that in Lake Whitefish embryos chronic thermal stress when administered as transient heat shocks results in a decrease in \textit{hsc70} and \textit{hsp90}β mRNA expression levels. This research is the first to show, to the best of my knowledge, the downregulation of constitutive heat shock proteins in embryos exposed to chronic low-level stress (i.e. transient heat shock). This may occur to help prevent Hsp toxicity, from over expression of Hsps, which could directly interfere with ongoing processes in the cell (Feder and Hofmann, 1999; Krebs and Feder, 1997; Lindquist, 1993). The potential long-term impacts, if any, of these shifts in Hsp expression levels on Lake Whitefish embryos or later life history stages remains to be determined. In Zebrafish, disruption of the Hsp90α gene resulted in the failure of proper somatic muscle development and Zebrafish embryos that are immotile (Etard et al., 2007; Lele et al., 1999; Rupik et al., 2011). However, in the short-term, this downregulation of constitutive \textit{hsps} may function to increase survival in the face of continually repeated heat shock assault.
The heat shock response undergoes development and maturation during embryogenesis. This was evident by my observations that the heat shock response in 64dpf and 82dpf embryos differed in response to both transient and high-level heat shock, and that this was the case for multiple Hsps. The upregulation in hsp70 in following high-level heat shock was approximately 6 fold greater in the 82dpf embryos than 64dpf embryos. Likewise, the capacity to downregulate hsc70 in response to transient heat shock was only observed in the older 82dpf embryos. For Lake Whitefish, early embryonic stages may be more sensitive to thermal discharge. At different life stages, sensitivity to stress and the ability to respond to those stressors can vary (McKim, 1977; Verriopoulos and Moraïtou-Apostolopoulou, 1982). When examining differences in embryos (right before hatch) and larvae (‘swim up’ and beginning to transition to exogenous feeding), Fuzzen et al., 2011, found that Rainbow Trout, Oncorhynchus mykiss, larvae had a potentially suppressed response to hypoxia than embryos 28dpf. The authors suggest that this suppression of the heat shock response in larvae but not embryos may be a function of the cortisol stress response suppressing the heat shock response (Basu et al., 2001). Embryonic Rainbow Trout lack the capacity to initiate a cortisol stress response (Alsop and Vijayan, 2008). Knowledge of the development and maturation of the various stress responses and their protective abilities at different life stages is important to better understand the potential impacts of changing environments on an organism.

Stress responses have a degree of plasticity that lends flexibility to an organism in a changing environment. One hypothesis states that Hsp protein levels within the cell remain elevated for a significant duration following a stress event and that these residual Hsps can serve a protective function in response to subsequent stressors (Anestis et al.,
2007; Dietz, 1994; Lund et al., 2006; Mosser and Bols, 1988; Viant et al., 2003). In the present study, I showed that Lake Whitefish embryo have a plastic heat shock response in that repeated low-level transient heat shocks allowed for a more rapid response to these low-level heat shocks and an attenuated response to subsequent high-level heat shock. For embryos in the thermal discharge this may mean higher tolerance to thermal assault. In the present study, repeated low-level transient heat shocks resulted in an observed increase in hsp70 mRNA levels 12 hours after a +3 °C 1 h heat shock. In contrast, Stefanovic et al., 2015 observed no significantly increase in hsp70 mRNA levels until 48 h after a 2 h heat shock of +3 °C. The repeated heat shocks may have a cumulative effect as they may permit the accumulation Hsp70 protein in the cell. This accumulation would be consistent with an expected half-life for Hsp70 of several days and the consequences of synthesizing additional Hsp70 may be long lasting and cumulative (Hofmann, 1999) and thus quicken time to induction and subsequent down regulation of the response.

**Summary and Conclusions**

The heat shock response of embryonic Lake Whitefish was observed to be plastic to repeated transient heat shocks. Embryos receiving an 18 h post-transient recovery were observed to have an attenuated response to high-level heat shock. This attenuation suggests that Lake Whitefish that are transiently heat shocked are able to increase protein levels to a sufficient amount in the cell in a shorter period of time; perhaps via previously accumulated protein or mRNAs. This may have long-term benefits to a species that is thermally quite sensitive. If thermal plumes are increasing the background temperature in areas where Lake Whitefish are developing the plasticity of the heat shock response may allow embryos protection against continual thermal assaults.
Chapter 2: Development of thermotolerance during Lake Whitefish embryogenesis
Introduction

Temperature can have profound effects on the development of fish. Increases in incubation temperature can increase rate of development, decrease survival and the portion of yolk available for formation of new tissue (Gray, 1928; Pepin, 1991). Collectively, these factors can contribute to population level impacts and thus a clear understanding on how embryos respond to fluctuating temperatures is important. Numerous industrial processes (i.e. power generation, pulp and paper mills, fuel refineries) use once-through cooling systems. The usage of cool water for industrial cooling processes has the potential to alter water temperatures and thus affect local fish. Thermal effluents from these processes range from 0.5 to 13 °C warmer than intake temperatures (Bruce Power, 2005). The potential effects of these thermal effluents on fish have focused on juvenile and adult migrating fishes (Bamber, 1995; Gray et al., 1977; Young and Gibson, 1973). However, for embryos that are spawned within the thermal plume, these effluents may present a particularly significant challenge as embryos are immobile and lack the ability to leave less than ideal conditions. Thus, this rise in background temperature may present challenges during development that can have lasting impacts on fitness in later life stages. Numerous studies have shown that increases in embryonic incubation temperature can lead to an increase in mortality, shorter developmental times and may contribute to lower body conditions at hatch (Damme et al., 1992; Guma’a, 1978; Ojianguren et al., 1999). If embryos are within an affected area, thermal effluents can expose embryos to unique, fluctuating periods of thermal stress and recovery to optimal conditions. These fluctuating temperatures may be detrimental or have beneficial properties depending on the degree of stress and/or impacted species.
Lake Whitefish are a cold adapted freshwater species that supports some of the largest and most valuable commercial fisheries in the Laurentian Great Lakes (Kinnunen, 2003). Relative to other freshwater fishes, Lake Whitefish are very sensitive to elevations in water temperatures (Coutant, 1977). Moreover, as a fish that spawns in the near shore gravel shoals in the late fall early winter, Lake Whitefish embryos may be exposed to thermal effluents from the numerous industrial facilities that line the shores of the Great Lakes. Embryonic development in Lake Whitefish usually takes place under the cover of ice at temperatures ranging from 0.5-6 °C with much of this time being spent close to 2 °C. At these low incubation temperatures development is long and slow, lasting between 80 and 180 days (Brooke, 1975; Price, 1940). Laboratory studies show that Lake Whitefish embryos optimal incubation temperature is between 2-6 °C (Brooke, 1975; Price, 1940). Given the very low optimal temperatures, increases in incubation temperature even transiently could impact Lake Whitefish development and how these embryos respond to stress. These features make Lake Whitefish a good model to study the effects of chronic transient stress and the potential for protective adaptation via the heat shock response.

One of the most universal responses to thermal stress is the heat shock response which has been shown to occur throughout fish life history from embryogenesis to adults (Boone and Vijayan, 2002; Graser et al., 1996; Kothary et al., 1984; Pan et al., 2000; Sessions, 2015; Stefanovic et al., 2015; Zafarullah et al., 1992). This evolutionarily conserved cellular stress response is characterized by the upregulation of heat shock proteins. Heat shock proteins function as molecular chaperones involved in routine cellular functions such as protein folding and transport and mediating cell signaling and immune system function. In addition, they function as stress proteins to repair and
prevent aggregation of non-native proteins and suppress cell death pathways (Feder and Hofmann, 1999; Georgopoulos and Welch, 1993; Lindquist, 1986). During embryogenesis, heat shock proteins play an important role in correct formation of embryonic and extra-embryonic structures and cytoprotection for the embryo (Heikkila, 1993a; Heikkila, 1993b; Krone et al., 1997; Rupik et al., 2011). Multiple families of heat shock proteins have been characterized in Lake Whitefish including inducible isoforms such as Hsp70, Hsp90α and Hsp47 as well as constitutive forms such as Hsc70 and Hsp90β (Stefanovic et al., 2015).

The impacts of increased background temperature through acclimation (temporary responses to recent temperatures) on a fish stress response have been extensively studied. The minimum temperature required to induce heat shock protein upregulation (induction temperature) and critical thermal maxima are positively correlated with increase in acclimation temperature (Bennett and Beitinger, 1997; Buckley and Hofmann, 2002; Dietz, 1994; Fangue et al., 2003; Lund et al., 2006). This type of plasticity has been shown to have significant benefits to the organism via the development of thermotolerance. Thermotolerance is the idea that a priming low-level heat event will give an organism increase cellular resistance to a subsequent high-level heat shock or alternate stressor (Ealy and Hansen, 1994). In Rainbow Trout fibroblasts, synthesis of heat shock proteins was shown to be paramount for the development of thermotolerance by coinciding with an increase in percent survival in response to high-level heat shock (Mosser and Bols, 1988). The recovery period between the thermal stressors has been shown to play an important role in the development and the decay of tolerance (Landry et al., 1982). This phenomena, however, has not been studied extensively in embryos. In cultured rat embryos, a priming heat shock allowed for the acquisition of thermotolerance
which protected embryos from a subsequent more severe teratogenic heat treatment (Walsh et al., 1987). Shimada, 1985 found acclimation of embryonic Japanese Medaka, *Oryzias latipes*, to an elevated but non-lethal temperature allowed for increase hatchability if the embryos were exposed to a typically lethal temperature and alluded to heat shock proteins as a possible culprit. In Lake Whitefish, I showed that low-level repeated transient heat shock throughout development modified the heat shock response to a more severe heat shock (Chapter 1). It is of interest to understand if the link between heat shock proteins and development of thermotolerance can be shown during embryonic stages as this link could increase tolerance of the embryos to repeated thermal stress assaults that the thermal discharge may produce.

The overall aim of the current study was to determine if repeated transient heat shocks of Lake Whitefish embryos conferred protection to subsequent severe thermal stress. More specifically, I wanted to determine: 1) how mid-level transient heat shocks impacted the heat shock response to a subsequent stressor; 2) whether transient heat shocks increase survival in embryos exposed to more severe stressors; 3) does the post-transient recovery period between the successive stressors affect the embryos response; and 4) does the transient heat shocks have an impact on time to hatch and morphology at hatch.

**Methods**

*Embryo and Animal Husbandry*

Eggs and sperm were collected from Lake Whitefish caught by short set gill nets at the Fishing Islands of Lake Huron adjacent to South Bruce Peninsula, Ontario, Canada in November, 2013. Eggs were fertilized in the field (Eme et al., 2015; Mueller et al., 2015) and transported to the aquatic facility at the University of Regina. Once received,
Embryos were incubated in mini-McDonald bell jars with continuous flowing, filtered water maintained at 4 °C. All procedures involving animals were approved by the University of Regina President’s Committee on Animal Care and conducted in accordance with the guidelines of Canadian Council on Animal Care.

**Experiment**

This study consisted of multiple components. The first was performing repeated transient heat shocks throughout development that were intended to serve as a chronic stressor that might prime the embryo for subsequent stressors. The second component was to perform a more severe subsequent thermal stress event. This experimental design permitted the examination of how priming heat shocks impact the embryo’s response to severe stress and the potential for protective effects. Finally, the post-transient heat shock recovery period was altered to determine if it played a role in the embryos’ response to the high-level heat shock and how this recovery period might impact an observed potential protective benefit of the transient heat shocks.

**Transient Heat Shock Protocol**

Ten mini-McDonald bell jars containing approximately 5,000 embryos were randomly assigned to one of three different transient heat shock treatments as follows: Control (no transient heat shock; control temperature of 4 °C); a heat shock of +9 °C every six days (9c6d) or a heat shock of +12 °C every six days (12c6d) (Table 3). The transient heat shock treatments began at 19dpf and were applied continually every six days until a high-level heat shock exposure was performed.
Table 3: Heat shock experimental treatment groups for embryos that received either 3 or 6 h recovery period between mid-level transient heat shock and high-level heat shock

<table>
<thead>
<tr>
<th>Transient Heat Shock</th>
<th>Control</th>
<th>+9°C every 6 days</th>
<th>+12°C every 6 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con//0x1</td>
<td>9c6d//0x1</td>
<td>12c6d//0x1</td>
</tr>
<tr>
<td></td>
<td>Con//15x1</td>
<td>9c6d//15x1</td>
<td>12c6d//15x1</td>
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<tr>
<td></td>
<td>Con//18x1</td>
<td>9c6d//18x1</td>
<td>12c6d//18x1</td>
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<tr>
<td></td>
<td>Con//0x4</td>
<td>9c6d//0x4</td>
<td>12c6d//0x4</td>
</tr>
<tr>
<td></td>
<td>Con//15x4</td>
<td>9c6d//15x4</td>
<td>12c6d//15x4</td>
</tr>
</tbody>
</table>

// represents recovery period
Within each box: Transient heat shock//High-level heat shock
Transient heat shocks were 1 h in duration and were administered by transferring the mini-McDonald bell jars to a circulating system at the appropriate heat shock temperature after which the bell jars were returned to the primary circulating system at 4 °C.

**High-Level Heat Shock Protocol**
To investigate the potential long term impact of repeated transient heat shocks, embryos from the three aforementioned transient heat shock treatments were exposed to one of six different high-level heat shocks at 62dpf. High-level heat shocks were administered to the embryos following a 3 or 6 h recovery period at 4 °C after receiving their last transient heat shocks. Embryos were subsequently exposed to a 1 or 4 h high-level heat shock grouped as follows: +0 °C (Control, held at 4 °C); +15 °C; +18 °C and sampled 2 h post high-level heat shock (Table 3). In total 162 embryos from each transient heat shock group were exposed to each of the six different high-level heat shock treatments. High-level heat shocks were administered by distributing these into 4 polypropylene jars maintained, in a water bath, at the appropriate temperature (Table 3). Following the appropriate 1 or 4 h high-level heat shock, the polypropylene jars and embryos were transferred to a 4 °C water bath for a 2 h recovery prior to sampling. A portion of embryos (12) from each treatment group were sampled and preserved by snap freezing in liquid nitrogen for later RNA analysis of multiple heat shock protein genes. The rest were raised in control conditions for whole animal response analysis.

**Whole Animal Response**
A total of 150 embryos from each experimental group were monitored for whole animal responses following the high-level heat shock treatment. These embryos were distributed into four petri dishes (100 mm) and were held at a constant 4 °C until hatch. Ninety percent of the water in all petri dishes was replaced with fresh 4 °C dechlorinated
water daily. The number of dead and hatched embryos was recorded daily with all dead embryos being removed daily. Death was classified as an embryo being opaque and cloudy. Lake Whitefish were collected within 48 h of hatch, euthanized with an overdose of ethyl 3-aminobenzoate methanesulfonate (tricaine methanesulphonate (MS-222)) anesthetic and then preserved in 10% neutral buffered formalin for later morphological analysis. Measurements for morphological analysis were done using an Olympus SZX10 stereomicroscope (Olympus America Inc., Center Valley, PA) with an Infinity 1 USB 2.0 Scientific Digital Camera attachment and Infinity Analyze 5.0 software (Lumenera Corporation, Ottawa, ON). Total body length and eye diameter of each Lake Whitefish hatchling were measured in mm to two significant digits according to the method of Sreetharan et al., 2015.

**Quantitative Real-Time PCR**

Total RNA was extracted by homogenizing two embryos in TRIzol reagent according to manufacturer’s instruction (Invitrogen-life technologies, Mississauga, ON). MIQE guidelines were followed by verifying purity and quality of sample and quantifying RNA (Bustin et al., 2009). Purity of RNA was verified and concentration was quantified spectrophotometrically (NanoDrop, Thermo Scientific) \( \frac{A_{260}}{A_{280}} \) and \( \frac{A_{260}}{A_{230}} \) ratios>1.8). RNA quality was further assessed through gel electrophoresis and evaluated based on the presence of ribosomal RNA bands. RNA was stored at -80 °C until further processing. For each sample, first strand cDNA was prepared using 1 µg of total RNA and the Qiagen quantiTect Reverse Transcription kit according to the manufacturer (Qiagen, Mississauga, ON). cDNA was stored at -20 °C.

Primers for quantitative-real time PCR (RT-qPCR) were designed from cDNA sequences available in GenBank as previously describe (Stefanovic et al., 2015) (hsp90α,
GenBank accession no. KP893539; hsp90β, GenBank accession no. KP893540; hsp70, GenBank accession no. KP861983; hsc70, GenBank accession no. HQ287746; β-actin, GenBank accession no. KP893542 and GAPDH, GenBank accession no. KP893543) (Table 2). For each primer pair amplification efficiency was determined by performing standard curves on pooled heat shocked and control samples and was shown to meet MIQE guidelines (efficiency between 90-110%).

RT-qPCR gene expression profiling assay were carried out on a Bio-Rad CFX-Connect real time detection system (Hercules, CA) using SSoAdvance™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA), 250 nM of forward and reverse primers and 1.0 µL of 2x diluted cDNA for a total volume of 20 µL. Each qPCR run consisted of 1 cycle of 95 °C for 2 min and 40 cycles of 5 s at 95 °C and 30 s at primer specific annealing temperature (Table 2). Melt curve analysis, which confirms the presence of a single amplicon was performed at the completion of each qPCR run. MIQE guidelines were met by using no template controls. The absence of gDNA contamination in the cDNA was confirmed through the use of primer pairs (β-actin) which span an intron. To correct for variations in reverse transcription efficiency and minor variations in RNA and cDNA template loading, samples were normalized to two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin. These genes were shown to be stable during heat shock treatment (Stefanovic et al., 2015).

Statistical Analysis
For Hsp gene expression analysis the mRNA levels of the aforementioned genes were investigated using MCMC.qpcr package (Matz, 2013; Team, 2014). The analyses were performed as detailed in Chapter 1. In brief, target genes consisted of hsp70, hsc70, hsp90α and hsp90β. Hsp levels were used as the dependent variables and transient heat
shock regimes (transient heat shock including appropriate post-transient recovery period) and high-level heat shock were used as independent categorical variables. Transient heat shock regimes were grouped as follows: Control (no heat shock), 9c6d-3 (i.e. +9 °C every 6 days with a 3 h post-transient recovery), 12c6d-3 (+12 °C every 6 days, with a 3 h post-transient recovery), 9c6d-6 (+9 °C every 6 days, with a 6 h post-transient recovery), 12c6d-6 (+12 °C every days with a 6 h post-transient recovery). High-level heat shocks were grouped as follows: +0 °C (no heat shock), +15 °C, +18 °C. The treatment group receiving no transient heat shock (no transient control) and no high-level heat shock (+0 °C) was set as reference group (here after control reference group) for comparison with all other treatments groups. Reference genes, GAPDH and β-actin, were added as priors to the model and functioned to normalize for variations in cDNA and were allowed 1.2 fold changes between samples. Results for Hsp genes are reported as posterior means expressed relative to the control reference group and error bars represented 95 % credible intervals as calculated by Bayesian analysis. Differences between groups were accepted as statistically significant when credible intervals were non-overlapping.

Whole embryo effects were modeled using a gamma distribution generalized linear model with a log link function and time to hatch and morphometric measurements as dependent variable (Team, 2014). The effect of transient heat shock regimes on survival at each high-level heat shock was examined using two direct measurements: percent survival and survival statistics. Percent survival was determined by dividing the number of hatched by the total number of hatched and dead embryos recorded. For specific high-level heat shock treatments, survival statistics were performed for each transient heat shock regime with a Log-rank test for curve comparison (GraphPad Prism 6.03).
Results

Hsp mRNA Levels
Heat shock protein 70 mRNA levels were affected by transient heat shock, post-transient recovery and high-level heat shock. High-level heat shock significantly increased hsp70 mRNA levels with peak levels reaching 14-fold greater than those of the control reference treatment (Fig. 7). Post-transient recovery period seemed to have a larger impact on the degree of the response than did the temperature at which embryos were repeatedly heat shocked. Embryos provided with a 3 h post-transient recovery period more robustly upregulated hsp70 mRNA in response to a 1 h high-level heat shock than the embryos in other transient heat shock regimes (Fig. 7-IB-C). Moreover, embryos in this group maintained hsp70 mRNA in the no high-level heat shock group (+0 °C for 1 h) which was four-fold greater than the control reference group (Fig. 7-IA). Similar results were observed with the 4 h high-level heat shock treatments exposed to transient heat shocks as they increased hsp70 mRNA levels more robustly to a +15 °C heat shock than their respective no transient control embryos (Fig. 7- IIE). However, when the high-level heat shock was further increased to +18 °C for 4 h the response of embryos in the different transient groups did not differ (Fig. 7-IIF).
**I.** +0°C/1hr

![Graph A](image)

**II.** +0°C/4hr

![Graph D](image)

**B.** +15°C/1hr

![Graph B](image)

**E.** +15°C/4hr

![Graph E](image)

**C.** +18°C/1hr

![Graph C](image)

**F.** +18°C/4hr

![Graph F](image)

**Transcript Heat Shock Regimes**

Control 9c6d-3 12c6d-3 9c6d-6 12c6d-6
**Figure 7.** The effect of mid-level transient heat shock regimes on expression of normalized \( hsp70 \) mRNA 2 h after a 1 or 4 h high-level heat shock was examined. Normalized expression was determined using R-package MCMC.qpr (Matz, 2013). Expression values were plotted as fold change relative to the control embryos that received no heat shock in log2 scale. Posterior means and 95% credible intervals as error bars are presented. Graph I represents data from a 1 h high-level heat shock while graph II represents a 4 h high-level heat shock. Transient heat shock regimes include a repeated mid-level transient heat shock and a 3 or 6 h recovery period received prior to exposure to the 1 or 4 h high-level heat shock and is labeled as follows: for 3 h recovery post-transient heat shock, 9c6d-3 (+9 °C every 6 days), 12c6d-3 (+12 °C every 6 days) and for a 6 h recovery post-transient heat shock, 9c6d-6 (+9 °C every 6 days), 12c6d-6 (+12 °C every 6 days). Control embryos were never given a transient heat shock and therefore received no recovery period. Each sub-graph represents high-level heat shock temperature as follows +0 °C (no heat shock, A and D), +15 °C (B and E) and +18 °C (C and F) from a control temperature of 4 °C. Embryos were given 2 h of recovery at 4 °C before being sampled.
Neither high-level heat shock nor the transient heat shock regimes altered \textit{hsp90α}, \textit{hsp90β} or \textit{hsc70} mRNA levels (Fig. 8, 9, respectively). When changes were observed in mRNA levels of these genes they were at most a 2 fold deviation from the control reference group values with relatively large 95 % credible intervals and did not seem to follow any discernible trend. The exception to this was a slight elevation of \textit{hsp90α} mRNA levels in embryos exposed to a 4 h high-level heat shock (Fig 8-1IE-F). This increase in \textit{hsp90α} mRNA levels was only observed in no transient controls and transient regimes that received a 3 h post-transient recovery. Overall, it seems Lake Whitefish embryos induce only \textit{hsp70} in response to thermal stress and transient heat shocks and facilitated a more robust response.

\textbf{Survival}

Percent survival in control and transiently heat shocked embryos that had never received a high-level heat shock ranged from 88 to 97 percent (Table 4). This high survival rate was also observed in embryos that received a 1 h high-level heat shock of +15 or +18 °C. In contrast, those embryos exposed to a 4 h high-level heat shock had significantly lower survival. Survival in embryos receiving a 4 h +15 °C heat shock ranged from 74 to 83 percent but this survival rate was not affected by the different transient heat shock treatments (Survival Analyses, \textit{p} > 0.05) Embryos exposed to a 4 h +18 °C heat shock had the lowest survival rates which ranged from 21 to 42 percent (Table 4). In this instance, survival statistics did detect significant differences between the transient heat shock regimes (Fig 10). Transient regimes with a 6 h post-transient recovery had the highest survival rate over time among the embryos exposed to the +18 °C 4 h high-level heat shock, with 12c6d-6 transient heat shock regime having the highest percent survival.
Figure 8. The effect of mid-level transient heat shock regimes on expression of normalized \textit{hsp90\textalpha} (■) and \textit{hsp90\textbeta} (□) mRNA 2 h after a 1 or 4 h high-level heat shock was examined. Normalized expression was determined using R-package MCMC.qpr (Matz, 2013). Expression values were plotted as fold change relative to the control embryos that received no heat shock in log2 scale. Posterior means and 95 \% credible intervals as error bars are presented. Graph I represents data from a 1 h high-level heat shock while graph II represents a 4 h high-level heat shock. Transient heat shock regimes include a repeated mid-level transient heat shock and a 3 or 6 h recovery period received prior to exposure to the 1 or 4 h high-level heat shock and is labeled as follows: for 3 h recover post-transient heat shock, 9c6d-3 (+9 °C every 6 days), 12c6d-3 (+12 °C every 6 days) and for a 6 h recovery post-transient heat shock, 9c6d-6 (+9 °C every 6 days), 12c6d-6 (+12 °C every 6 days). Control embryos were never given a transient heat shock and therefore received no recovery period. Each sub-graph represents high-level heat shock temperature as follows: +0 °C (no heat shock, A and D), +15 °C (B and E) and +18 °C (C and F) from a control temperature of 4 °C. Embryos were given 2 h of recovery at 4 °C before being sampled.
Figure 9. The effect of mid-level transient heat shock regimes on expression of normalized \textit{hsc70} mRNA 2 hours after a 1 or 4 h high-level heat shock was examined. Normalized expression was determined using R-package MCMC.qpr (Matz, 2013). Expression values were plotted as fold change relative to the control embryos that received no heat shock in log2 scale. Posterior means and 95 % credible intervals as error bars are presented. Graph I represents a 1 h high-level heat shock while graph II represents a 4 h high-level heat shock. Transient heat shock regimes include a repeated mid-level transient heat shock and a 3 or 6 h recovery period received prior to exposure to the 1 or 4 h high-level heat shock and is labeled as follows: for 3 h recover post-transient heat shock, 9c6d-3 (+9 °C every 6 days), 12c6d-3 (+12 °C every 6 days) and for a 6 h recovery post-transient heat shock, 9c6d-6 (+9 °C every 6 days), 12c6d-6 (+12 °C every 6 days). Control embryos were never given a transient heat shock and therefore received no recovery period. Each sub-graph represents a high-level heat shock temperature as follows: +0 °C (no heat shock; A and D), +15 °C (B and E) and +18 °C (C and F) from a control temperature of 4 °C. Embryos were given 2 h of recovery at 4 °C before being sampled.
Table 4: Percent survival of heat shock treatments

<table>
<thead>
<tr>
<th>Percent Survival (%)</th>
<th>High-Level Heat Shock Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+0°C</td>
</tr>
<tr>
<td>Control</td>
<td>95.2</td>
</tr>
<tr>
<td>9c6d-3\text{\textsuperscript{1}}</td>
<td>88.0</td>
</tr>
<tr>
<td>12c6d-3\text{\textsuperscript{2}}</td>
<td>93.5</td>
</tr>
<tr>
<td>9c6d-6\text{\textsuperscript{3}}</td>
<td>95.1</td>
</tr>
<tr>
<td>12c6d-6\text{\textsuperscript{4}}</td>
<td>97.0</td>
</tr>
</tbody>
</table>

\text{\textsuperscript{1}:+9°C heat shock every 6 days with a 3 h post-transient recovery period}
\text{\textsuperscript{2}:+12°C heat shock every 6 days with a 3 h post-transient recovery period}
\text{\textsuperscript{3}:+9°C heat shock every 6 days with a 6 h post-transient recovery period}
\text{\textsuperscript{4}:+12°C heat shock every 6 days with a 6 h post-transient recovery period}
**Figure 10.** Percent survival over the number of days after embryos received a 4 h high-level heat shock of +18 °C is shown. Each curve represents a transient heat shock regime as follows: Control (no heat sock), 9c6d-3, 12c6d-3, 9c6d-6 and 12c6d-6. Black solid line represents control embryos. Black patterned lines represent the transient regime of +9 °C every 6 days (9c6d) while gray patterned lines represent the transient heat shock regime of +12 °C every 6 days (12c6d). The dashed pattern indicates a post-transient recovery time of 3 h (…-3) while the dotted pattern represents a post-transient recovery period of 6 h (…-6). Log-rank test determined the curves are significantly different (df = 4, p-value < 0.0001).
In contrast, the transient heat shock regimes with the 3 h post-transient recovery period did not show improved survival but rather had slightly lower survival rates than the controls.

**Mean age and Morphology at Hatch**

Mean age at hatch was not significantly impacted by thermal stressors as applied in this experiment with the average time to 50 % hatch being 129dpf. However, it was observed that +9 °C transient heat shock triggered hatch in Lake Whitefish embryos as the experiment progressed and embryos reached an age of 91-103dpf. The triggering of hatch for 9c6d treated embryos resulted in the completion of hatch in this group a full 13 days earlier than in the control group that was never exposed to thermal stress. As overall timing was not impacted by thermal stress it was expected that morphology of hatch would also not be impacted. However, the models showed a significant interaction between transient heat shock regime and high-level heat shock for total body length and eye diameter. The exception to this was the group that received a 4 hour high-level heat shock and where total body length was measured. This group did not show a significant interaction but each thermal variable, transient heat shock regime and high-level heat shock, had a significant effect. For embryos that received an either 1 or 4 h high-level heat shock the predicted means for total body length and eye diameter ranged from 12.1 mm to 12.6 mm and 0.92 mm to 0.97 mm respectively (Fig. 11 and 12). Though statistical significance was observed these predicted mean differences were rather small that I did not deem them biologically relevant.
**Figure 11.** The effect of mid-level transient heat shock regimes on total length and eye diameter at hatch while exposed to transient and 1 h high-level heat shock during embryogenesis was examined. Predicted means and 95% confidence intervals were presented. Transient heat shock regimes include a repeated mid-level transient heat shock and a 3 or 6 h recovery period received prior to exposure to the 1 h high-level heat shock and is labeled as follows: for 3 h recover post-transient heat shock, 9c6d-3 (+9 °C every 6 days), 12c6d-3 (+12 °C every 6 days) and for a 6 h recovery post-transient heat shock, 9c6d-6 (+9 °C every 6 days), 12c6d-6 (+12 °C every 6 days). Control embryos were never given a transient heat shock and therefore received no recovery period. Each line represents a high-level heat shock temperature as follows: +0 °C (no heat shock; circle and black), +15 °C (square and dark gray) and +18 °C (triangle and light gray) from a temperature control of 4 °C. After high-level heat shock embryos were allowed to develop in optimal conditions then preserved at hatch for later analysis.
Figure 12. The effect of mid-level transient heat shock regimes on total length and eye diameter at hatch while exposed to transient and 4 h high-level heat shock during embryogenesis was examined. Predicted means and 95% confidence intervals were presented. Transient heat shock regimes include a repeated mid-level transient heat shock and a 3 or 6 h recovery period received prior to exposure to the 4 h high-level heat shock and is labeled as follows: for 3 h recover post-transient heat shock, 9c6d-3 (+9 °C every 6 days), 12c6d-3 (+12 °C every 6 days) and for a 6 h recovery post-transient heat shock, 9c6d-6 (+9 °C every 6 days), 12c6d-6 (+12 °C every 6 days). Control embryos were never given a transient heat shock and therefore received no recovery period. Each line represents a high-level heat shock temperature as follows: +0 °C (no heat shock; circle and black), +15 °C (square and dark gray) and +18 °C (triangle and light gray) from a control temperature of 4 °C. After high-level heat shock embryos were allowed to develop in optimal conditions then preserved at hatch for later analysis.
**Discussion**

This study showed that embryos exposed to repeated transient heat shocks initiate a more robust heat shock response to a subsequent stressor and develop some degree of thermotolerance. For example, transiently heat shocked embryos exposed to a +15 °C 4 h high-level heat shock had larger increases in *hsp70* mRNA levels than no transient control embryos receiving the same high-level heat shock. Moreover, transient heat shock regimes with a sufficiently long post-transient recovery period had increased survival to a subsequent severe heat shock. For embryos in the wild these repeated transient heat shocks may provide a degree of protection from larger environmental assaults such as a more severe thermal stress from the industrial effluents or a chemical stressor or some other natural or anthropogenic stressor.

In fish, aspects of thermal history such as seasonal variation, population difference and acclimation temperature have been shown to influence thermotolerance (Bennett and Beitinger, 1997; Dietz and Somero, 1992; Oksala et al., 2014; Schaefer and Ryan, 2006). Fish acclimated to higher then optimal temperature show an increase in basal protein levels of inducible Hsp70 (Anestis et al., 2007; Dietz, 1994). This accumulation of Hsp70 protein levels may impact the response to a subsequent stressor by increasing the temperature threshold for induction (Dietz, 1994; Lund et al., 2006; Selvakumar and Geraldine, 2005). It has also been shown this elevation in Hsps can increase survival to a subsequent more severe stressor. This has been well documented in multiple cell lines and has been observed in multiple organisms including several fish species (DuBeau et al., 1998; Landry et al., 1982; Mosser and Bols, 1988; Todgham et al., 2005). For example, survival of RTG-2 cells following a severe stressor increased with the amount of time spent at the initial heat shock, with those conditions that produced
maximal Hsp levels having the highest survival. This protective effect was long lasting as percent survival remained high even as Hsp’s protein synthesis gradually declined (Mosser and Bols, 1988). In the present study, I have shown transient heat shocks more robustly increase hsp70 mRNA levels to high-level heat shock in Lake Whitefish embryos. However, robust responses did not correspond to increased survival in embryos. Although increased survival was observed in +18 °C 4 h high-level heat shock in transient heat shock regimes, with a 6 h post-transient recovery, this was not correlated with higher hsp70 mRNA levels. This lack of correlation could be related to the relatively quick turnover of mRNA, and it is possible Hsp proteins levels were elevated and coincided with increased survival. If transient regimes with a sufficiently long recovery period produced sufficient amount of protein in a shorter amount of time this could explain the increase in survival that was observed. The recovery period after an initial stressor was also observed to be important in the development of thermotolerance in this and other fish species (DuBeau et al., 1998; Todgham et al., 2005). Exposure to thermal shock increased survival to a subsequent osmotic stressor where highest survival coincided with the post-heat shock recovery period where induction of Hsp70 occurred (DuBeau et al., 1998; Todgham et al., 2005). Todgham et al., 2005, observed that it took 8 h after the priming heat shock for Hsp70 protein levels to significantly increase which also coincided with increase survival to a severe osmotic shock.

Transient heat shock may provide some benefits to Lake Whitefish embryos; however, the associated thermal stress and increased temperatures may impact time to hatch or have other potentially detrimental effects (Brooke, 1975; Griffiths and Hydro, 1979; Lee et al., 2015; Mueller et al., 2015; Patrick et al., 2013). In this study, time to mean age at hatch was not significantly different between temperature treatments. My
finding is consistent with another study that showed time to hatch in Lake Whitefish was not affected by weekly transient heat shocks of up to +5 °C (Lee et al., 2015). However, in Griffiths and Hydro, 1979, a 5 °C fluctuation above the 2 °C baseline decreased time to 50% hatch by about 26 days compared to hatch time of embryos exposed to a 2 °C fluctuation, while an 8 °C fluctuation in temperature advanced 50% hatch by about 9 weeks. Likewise an advanced hatch in Lake Whitefish was observed in response to temperature fluctuation greater than +8 °C above baseline (Patrick et al., 2013). Finally, incubation of Lake Whitefish embryos in situ in the near-shore shoals of Lake Huron potentially affected by the Bruce Power thermal plume resulted in an early hatch time than those embryos incubated at reference sites (Thome et al., 2015). These disparities between findings of the various studies discussed above are likely related to differences in magnitude, frequency and timing of the thermal stressors.

**Summary and Conclusion**

Heat shock response may be a key player in adaptation to a more variable severe environment for embryonic Lake Whitefish. Transient heat shocks were able to produce a more robust heat shock response and confer protection for embryos to a severe heat shock. Future work should determine if more robust heat shock response can increase tolerance to stress in later life stages. In Zebrafish, it was observed that a higher incubation temperature or a more variable temperature regime during development increased critical thermal maximum of adults (Schaefer and Ryan, 2006). However, thermal stress during development may have its own consequences on later life stages. For example, early hatch may have consequences for young of the year fish. Some studies have shown early hatch to impact size and condition of newly hatched larvae (Brooke, 1975; Laurel et al., 2008; Murray and McPhail, 1988). Also, early hatch may
mean hatching when food availability is low, which has been shown to decrease larvae survival (Freeberg et al., 1990). Examining these areas in early life stages will allow us to continue to provide information on the potential benefits and consequences of thermal discharge and how these trades-offs between increase tolerance and potential advance hatch may lead to adaptation or deleterious consequences for Lake Whitefish developing in a thermal discharge.
General Summary, Conclusions and Future Works

Thermal history can modify the heat shock response by manipulating basal heat shock protein levels within the cell. As acclimation temperature increases, the cell can respond by increasing basal levels of inducible Hsp70 (Buckley et al., 2001; Hofmann and Somero, 1995; Maloyan et al., 1999; Tomanek and Somero, 2002). Due to this, the dynamics or regulatory mechanisms of the response are impacted. One of the main interactors of Hsp70 is heat shock factor 1 (Hsf1) (Nollen and Morimoto, 2002). This is a transcription factor that mediates transcription of heat shock proteins. Elevated acclimation temperature results in an increase in the temperature at which a heat shock response is initiated, a process that may be mediated through regulation of Hsf1 binding activity (Buckley and Hofmann, 2002). Higher basal levels of Hsp could mean an increase in the inactive Hsf1 reservoir in the cell which in turn could result in modulation of the response.

In my study, I show post-transient recovery period can alter hsp70 mRNA levels by producing a more rapid and robust response, similar response or an attenuated response to a subsequent stressor relative to no transient heat shock controls. This suggests that embryos exposed to transient heat shock regimes may have a higher basal level of hsp70 thus allowing embryos to respond in a shorter amount of time than embryos never exposed to thermal stress. Transient heat shock regimes that received no high-level heat shock were sampled on a delayed time-line with respect to their transient heat shock in the sense they were sampled with those receiving high-level heat shocks. Thus, they were sampled 6, 9, 12 or 24 h after the removal of the transient heat shock. Six hours after transient heat shock I observed increased hsp70 mRNA levels but levels had returned to those in the reference control group by 9-12 h post-transient heat shock. It is
conceivable that this decrease in mRNA levels is triggered in part by accumulation of protein and it is this accumulation of protein that subsequently results in the observed downregulation of mRNA levels in transient heat shock regimes sampled 9-12 h post. In my study, I also observed lower hsp70 mRNA levels 24 h after transient heat shock and an attenuated heat shock response to high-level heat shock. It is at this time point I think protein levels had become sufficient within the cell that new mRNA is not being synthesized and existing mRNA is being degraded producing the observed drop in hsp70 mRNA levels. One approach to confirm this hypothesis would be examining protein levels in a time series after repeated transient heat shocks. This modulation of mRNA levels to transient heat shocks and the ability for transient regimes with a 6 h post-transient recovery to increase survival to a high-level heat shock adds confidence that transient regimes may have higher basal levels of hsp70 and are able to produce protein in a shorter period of time than embryos never exposed to heat stress.

Finally, the fact that the timing and frequency of the transient heat shocks seemed to have a more important function in modulating the response than the absolute temperature suggests the amount of temperature fluctuation within a thermal discharge is important to examine and to regulate. The greatest attenuation of the heat shock response was observed following low-level transient heat shocks every 3 days (chapter 1) and thus it may be this frequency that has the greatest impact on embryos. It would be interesting to determine if increasing the frequency of mid-level transient heat shocks would still confer protection if a substantially recovery period (>12 hours) was provided prior to a high-level stressor. Alternatively, is this the tipping point beyond which more deleterious effects are observed. If thermal discharges have a high degree of fluctuation the protective effects observed in this study could be diminished with more deleterious
effects on development such as increase malformations, decrease in survival and condition at hatch. The line between potential protective and negative consequences could be fine and be easily pushed by the degree of temperature fluctuations within the thermal plume.
References


Zak, M. (2015). Intensity of heat shock response is reduced in Lake Whitefish (*Coregonus clupeaformis*) acclimated to temperatures above or below thermal optimum.