# ENVIRONMENTAL STRESSOR EFFECTS ON WHITEFISH EMBRYOGENESIS

# THE COMBINED EFFECTS OF THERMAL AND RADIOLOGICAL STRESS ON THE EMBRYONIC DEVELOPMENT OF LAKE WHITEFISH (*COREGONUS CLUPEAFORMIS)*

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TITLE: The combined effects of thermal and radiological stress on the embryonic development of lake whitefish (*Coregonus clupeaformis*)

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## Lay Abstract

Mild heat shocks (HS) have been observed to induce a cellular heat shock response (HSR) that may protect animals from a subsequent lethal radiation exposure. The presence of a HSR and adaptive response during embryonic development was investigated in lake whitefish (LWF; *Coregonus clupeaformis*). HSR was induced in LWF embryos exposed to HS at gastrulation but not at the eyed stage of development. Radiation exposure at the eyed stage resulted in increased mortality at hatch, decreased time to hatch, and decreased hatchling size. Mild HS prior to lethal radiation exposure had no protective effect and no adaptive response was observed.

**Abstract**

Lake whitefish (*Coregonus clupeaformis*; LWF) are a cold-adapted freshwater species that are of both economic and cultural value. These fish spawn in lake areas where their embryos are exposed to thermal power plant effluents that may contain low levels of thermal, radiological and chemical stressors. Many studies on LWF embryonic development have looked at the individual effects of these stressors, but few have looked at the potential for combined effects. The combined effects of thermal and radiological stress were of interest due to growing evidence that mild thermal stress can produce an adaptive response, through the induction of the heat shock response (HSR), when followed with subsequent ionizing radiation stress. This thesis examined the combined impacts of thermal and radiological stress during LWF embryogenesis. LWF embryos were exposed to mild heat shocks (HS; Δ3 or 9°C) prior to a high dose of acute $^{137}$Cs gamma rays at 2, 6 and 24 hours post heat shock during the gastrulation or eyed stage. Heat shocked embryos were collected at each developmental stage and assessed for induction of heat shock protein (Hsp) genes. Following exposure, embryos were raised until hatch where mortality, morphometry, and embryo weight were measured. Mild HS induced *Hsp70* mRNA expression at gastrulation, but not at the eyed stage. Embryos at hatch were not impacted by thermal or radiological exposure at the gastrulation stage. During the eyed stage, acute radiation treatment increased mortality and decreased body size at hatch. Mild HS prior to radiation did not provide protective effects and no adaptive response was observed. This thesis better defines the combined effects of thermal and radiological stress on the embryonic development of LWF. It also suggests that the

ontogeny aspects of heat shock responses and radiosensitivity are important to consider for future adaptive response studies.

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**List of Abbreviations**

CANDU: Canadian deuterium uranium

CLM: Chronic lethal maximum

CTM: Critical thermal maximum

DNA: Deoxyribonucleic acid

dpf: Days post fertilization

EPIC: Environmental Protection from Ionizing Contaminants

HS: Heat shock

Hsps: Heat shock proteins

HSR: Heat shock response

ILT: Incipent lethal temperature

$LD_{50/30}$: median lethal dose killing 50% of the population within 30 days

$LD_{50(7Days)}$: median lethal dose killing 50% of the population within 7 days

LWF: Lake whitefish

mRNA: Messenger ribonucleic acid

# Chapter 1

# Introduction

### 1.1 Thermal power generation

Thermal power generation accounts for approximately 35% of national energy production in Canada (Canadian Electricity Association, 2014). These power plants differ from one another in terms of which fuel source they use, which may include coal, oil, gas or nuclear fission. About 16% of Canada's electricity comes from nuclear power, with 19 reactors providing 13.5 GWe of power capacity. In Ontario, there are 18 nuclear reactors that account for ~66% of the power produced by the province (Canadian Electricity Association, 2014). Approximately half of this production (~31.3% of Ontario's total production) is provided by Bruce Power's eight reactors which generate a maximum of 6300 MW at full capacity (Bruce Power, 2005).

Thermal power plants use thermal energy to convert water into steam, which moves a turbine, and then is converted back to liquid. To accomplish this, various cooling methods are used, with once-through cooling being a common process (Macknick, *et al.*, 2012). Once-through cooling relies on large amounts of water resulting in many thermal power plants to be located near oceans, large rivers or lakes. This cooling process uses an open loop system where water is pulled continuously from the water source, converted to turbine steam, condensed back to liquid, and then discharged back into the environment. Depending on the size of the power plant, water consumption can be upwards to 100 000 L $MW^{-1}$ $h^{-1}$ (Macknick, *et al.*, 2012). Large lakes, including those in the Great Lakes basin, are chosen for the location of once-through cooling thermal power plants due to the volume of water available and the large populations surrounding them. These plants can account for the withdrawal of over 400 billion litres of water per day from all the

Great Lakes (Great Lakes Commission, 2011). However, as large as once-through cooling volume may seem, the majority of the water is not actually consumed, but rather returned into the lakes.

A number of nuclear power plants utilize the combination of once through cooling and nuclear fission in the production of electricity (Steed, 2007). The majority of nuclear reactor designs involve a neutron moderator and nuclear fuel located in the reactor core. This neutron moderator is a medium that reduces the speed of the fast neutrons, thereby turning them into thermal neutrons capable of sustaining a nuclear chain reaction and producing energy. Neutrons are released from the core, through the moderator, and then through a heat exchanger. The energy from this transfer converts water into steam to rotate the turbines. In Canada, all nuclear power reactors are Canadian Deuterium Uranium (CANDU) reactors (Steed, 2007). These reactors utilize heavy water, containing deuterium (a hydrogen isotope), as a neutron moderator. Overall these processes create enormous amounts of energy, but have the potential to release pollutants into the environment.

### 1.2. Environmental stressors

Nuclear power plants that utilize once-through cooling have the potential to release thermal, chemical (morpholine, sodium hypochlorite, hydrazine), and radiological stressors into the environment (Bruce Power, 2005, 2010). This discharge may affect development in aquatic species that spawn in nearshore waters. The water that is heated and used to power the turbines is condensed and returned to the water source at warmer

temperatures (compared to ambient intake temperature), resulting in thermal plumes. As well, there is potential for the discharge from nuclear power plants to contain trace amounts of ionizing radiation and several compounds important for pH and corrosion control, and preventing settlement of biological organisms in the system. Whether separate, or in combination, these stressors may have an impact on the development and growth of aquatic biota that reside in the areas near the discharge site. Fish species are known to spawn in shallow parts of the lake near these discharge sites (Bruce Power, 2005), potentially exposing embryos to these stressors throughout development. My thesis focuses on the potential interaction between thermal and radiological stressors on lake whitefish development.

### 1.2.1. Thermal stress

Temperature has long been regarded as an important abiotic factor for fish development and survival (reviewed Blaxter and Hallers-Tjabbes, 1992; López-Olmeda and Sánchez-Vázquez, 2011; Rombough, 1997). Different species have different optimal temperature ranges, where within these ranges, there is optimal growth and performance (reviewed in Brett, 1969, 1971; López-Olmeda and Sánchez-Vázquez, 2011). However, once temperatures start approaching the limits of this range, increased mortality, changes in behaviour and developmental abnormalities may occur (reviewed in Brett, 1969, 1971; López-Olmeda and Sánchez-Vázquez, 2011). Thermal effluents have been shown to affect different physiological aspects in fish including behavior (Kelso, 1976), size (Bennett, 1972), gametogenesis (Luksiene, *et al.*, 2000), and altering overall thermal tolerance (Holland, *et al.*, 1974). A more recent concern has been the effects of thermal

effluents on embryonic development. Many fish spawn in shallow waters near shore where regions are potentially impacted by industrial discharges. Discharge water can reach up to 37°C and a temperature difference of upwards of 10°C compared to intake water (Madden, *et al.*, 2013). These increases in water temperature can have an impact fish, particularly on cold water-resident fish that are less tolerant to increases in water temperature (reviewed in Fangue, 2006).

Embryogenesis represents a particularly sensitive period for fish exposed to environmental stressors (reviewed in Jezierska, *et al.*, 2009; López-Olmeda and Sánchez-Vázquez, 2011; Rombough, 1997; Von Westernhagen, 1988), where developing embryos lack mobility to avoid such stressors, and may develop long-term or permanent modifications. The effects of different incubation temperatures on embryonic development has been studied in a number of fish species (Brooke, 1975; Marsh, 1985; Murray & McPhail, 1988; reviewed in Pepin, 1991; Price, 1934, 1940). Within their thermotolerance range, warmer temperatures increase the rate of development, while at more extreme temperatures, there is increased mortality and developmental abnormalities (reviewed in Pepin, 1991). During zebrafish (*Danio rerio*) embryo development, increased temperature has been shown to increase developmental rates, decrease embryo length, increase oxygen consumption and mutation rates (reviewed in López-Olmeda and Sánchez-Vázquez, 2011). Studies on lake whitefish (*Coregonus clupeaformis*) have quantified differences in development rate, growth, heart rate, $O_2$ consumption, and mortality for a wide range of incubation temperatures (0.5ºC-12 ºC; Brooke, 1975; Eme et al., 2015; Lim *et al.*, in press; Mueller et al., 2015; J. W. Price, 1940). Studies

consistently observed increased mortality (>30%; Brooke, 1975; Lim, *et al.*, in press; Mueller *et al*., 2015; Price, 1940) and decreased time to 50% hatch (>50%; Brooke, 1975; Lim, *et al.*, in press; Mueller et al., 2015) at 8°C compared to 2°C incubation temperature. These studies also observed a decrease in body length (>5%; Brooke, 1975; Lim, *et al.*, in press; Mueller et al., 2015) and decreased body mass (>40%; Lim, *et al*., in press; Muller *et al*., 2015) at hatch. Increased incubation temperatures led to increased $O_2$ consumption and heart rates (Eme *et al.*, 2015).

Impacts of fluctuating thermal regimes such as those that occur naturally in the environment have also been studied in fish embryo development (Bestgen and Williams, 1994; Kupren, *et al.*, 2011; Lim, *et al.*, in press; Targońska *et al.*, 2014; Thome *et al*., 2016). Recent *in-situ* work has shown that lake whitefish embryos which developed at sites near a nuclear power plant thermal discharge experienced advanced growth, potentially impacting post-hatch larval survival (Thome *et al*., 2016). A study by Lim *et al.*, (in press) on lake whitefish exhibited no significant effect on mortality when exposed to different combinations of seasonal temperature changes over the span of weeks and days (+/-Δ1°C, +/-Δ3°C). Lee *et al*. (2016) had similar results where weekly 1 hour Δ3°C heat shocks had no significant effect on mortality or morphology.

### 1.2.2. Ionizing radiation

Industrial effluent from nuclear power plants contain very low levels of ionizing radiation (Bruce Power, 2014). In CANDU plants, low levels of tritium may be released through once through cooling due to the use of heavy water as a neutron moderator.

These levels equate to around $10^{14}$ Bq released annually, which on average results in concentrations of 20 Bq/L (Bruce Power, 2014). The provincial limit is 7000 Bq/L, which is magnitudes higher that what has been found near discharge sites (Ontario Power Generation, 2014).

Ionizing radiation can either directly or indirectly ionize (reviewed in Hall and Giaccia, 2006). The source used in this thesis is $^{137}$Cs which produces gamma rays of energy 662 keV, causing indirect ionization. Indirect ionization causes excitation of intermediate molecules (e.g. water), which produces free radicals. Free radicals can cause damage to DNA, leading to base damage, single strand breaks, and/or double strand breaks (reviewed in Hall and Giaccia, 2006). This damage can lead to increased repair mechanism to compensate for damage, increased mutation rates, increased cell death, and result in death (reviewed in Hall and Giaccia, 2006). The level of damage depends on both the overall cumulative dose as well as the rate at which the dose is given (reviewed in Hall and Giaccia, 2006). Low dose rates generally result in less damage, allowing the cells to increase the rate of cell damage repair that is equal to or greater than the rate of damage (reviewed in Hall and Giaccia, 2006).

Aquatic organisms such as fish are well known to tolerate much higher dose rates than humans, with a toxic effect dose limit set >180 times higher than human dose limits (0.5 and 0.003 mGy/day respectively; Bréchignac and Barescut, 2003). Studies have a calculated a range of acute irradiation $LD_{50/30}$s (median lethal dose which is killing 50% of the population within 30 days) ranging from 3.75 to 100 Gy, depending on the fish species (reviewed in Donaldson and Foster, 1957; Harrison and Anderson, 1996). This

difference in radioresistance between species is often the result of evolutionary adaption to different environmental extremes and differences in epigenetics (reviewed in Harrison and Anderson, 1996). These $LD_{50/30}$ values decrease when comparing adults to earlier parts of embryonic development (reviewed in Donaldson and Foster, 1957; Harrison and Anderson, 1996).

Fish embryonic development has been shown to be particularly sensitive to ionizing radiation (reviewed in Laale and Lerner, 1981). During embryogenesis, particularly close to fertilization, there is a greater amount of dividing cells which increases the risk of cell death and embryonic mortality (reviewed in Harrison and Anderson, 1996). As development progresses towards organogenesis, there is a greater chance for malformation and abnormalities when organs are developing (reviewed in Harrison and Anderson, 1996). Studies on rainbow trout (*Salmo gairdnerii*) exposed to acute irradiation during development show a range in $LD_{50/30}$ from 1 Gy at the gamete stage, to 4.1-9.0 Gy at the eyed stage, and 15 Gy at the adult stage (Welander, 1954; Welander and Donaldson, 1948). A study on lake whitefish embryos had similar increases in radioresistance with an acute irradiation $LD_{50/hatch}$ of 5 Gy at 1dpf, upwards to 15 Gy at 30dpf (organogenesis, Thome, *et al*., 2017). At acute irradiation doses, fish embryos have been shown to have increased mortality, increased abnormalities, earlier hatch and smaller size at hatch (*Fundulus heteroclitus*, Bell and Hoar, 1950; *Fundulus heteroclitus* , Hinrichs, 1925; *Carassius auratus*, Konno, *et al.*, 1955; *Danio rerio*, Miyachi *et al*., 2003; *Coregonus clupeaformis*, Thome, *et al*., 2017; *Salmo gairdnerii*, Welander, 1954; *Salmo gairdnerii*, Welander and Donaldson, 1948).

In nature, doses may be quite low and these fish may be exposed for long periods of time, especially during development when embryos are unable to move away from stressors. Few studies have looked at the impacts of chronic exposure on embryonic development in fish. Many of these studies have been compiled in the EPIC (Environmental Protection from Ionizing Contaminants) database (Sazykina and Kryshev, 2003) or reviewed by Real *et al* (2004). Embryonic mortality has been shown following exposure down to 0.5 mGy/day (*Gambusia affinis*, Trabalka and Allen, 1977). In contrast, chronic radiation can also have stimulatory effects, such as an increase in fecundity (*Gambusia affinis*, Blaylock, 1969; *Oncorhynchus tshawytscha* Donaldson and Bonham, 1970), an increase in development rate (*Prosopium cylindraceum*, Lim, 2016; *Danio rerio*, Simon, *et al.*, 2011; *Coregonus clupeaformis*, Thome, *et al*., 2017) and stimulated growth during development (*Prosopium cylindraceum*, Lim, 2016; *Coregonus clupeaformis*, Thome, *et al*., 2017). Radiation stimulated growth was also seen in lake whitefish (*Coregonus clupeaformis*) when exposed to fractionated doses throughout development (Mitz, 2016).

### 1.2.3. Combined stressors

Compared to laboratory settings, organisms in the environment are rarely exposed to only one stressor, but potentially experience multiple stressors in various combinations. When exposed to multiple stressors, biological outcomes may occur that differ from what is predicted to happen based on the results from individual exposures alone (Holmstrup *et al*., 2010). Generally, there are three different scenarios that may occur. Firstly, an additive response may be seen in which the effect of combining two

stressors is equal to the sum of the effect of each stressor individually. Secondly, a synergistic response may be seen in which the outcome is greater than additive. Thirdly, outcomes can also be adaptive or protective (also referred to as antagonistic), in which the response is less than additive. Radiological and other stressors have been shown to result in what is known as an adaptive response, where a low dose stress can protect cells or whole-organisms from future high dose exposure (Tang and Loke, 2015). Low doses of radiation (Cassidy, *et al.*, 2007; Choi *et al*., 2010) and thermal stressors (Boreham and Mitchel, 1994; Shen *et al.*, 1991) have been shown to induce an adaptive response to reduce damage from a subsequent high dose ionizing radiation challenge dose. Variables such as dose, duration and magnitude of exposure, and timing between exposures can impact the type of combined stress response.

An extensive review has looked at the different approaches to laboratory multiple stressor studies in aquatic animals with ionizing radiation being the common stressor (reviewed in Vanhoudt, *et al.*, 2012). The other stressors have included salinity, water content, temperature, metals, food levels, and oxygen content (reviewed in Vanhoudt *et a*l., 2012). Of these studies, many have examined the combination of radiation and temperature in medaka (*Oryzias lattipes*) adults (Egami and Etoh, 1966; Etoh and Egami, 1967) and embryos (Shimada and Egami, 1984; Shimada, 1985; Shimada, *et al.*, 1985a, 1985b). Heat treatment (+Δ13°C for 30min) before radiation exposure (10 Gy) in medaka embryos resulted in radioresistance of germ cells in hatchlings (Shimada and Egami, 1984; Shimada, 1985; Shimada, *et al.*, 1985a, 1985b). An adaptive response was seen in lake whitefish embryos using a heat treatment prior to radiation (Thome, *et al.*, 2017). A

2 hour, Δ3°C or Δ9°C heat shock, 6 hours prior to an acute 10 Gy irradiation increased radioresistance and reduced mortality 30%. However, this adaptive response was not seen at irradiations 24 hours post heat shock (Thome, *et al*., 2017). Many questions still remain about the exact time course of this response, the potential mechanism and how it changes over the course of embryonic development.

### 1.3. Heat shock proteins and the heat shock response

The heat shock response (HSR) is found universally throughout all models and is characterized by the transcription and translation of a family of proteins known as heat shock proteins (Hsps, Lindquist and Craig, 1988). Hsps are a family of cellular proteins that are highly conserved and present in all organisms that have been examined (Feder and Hofmann, 1999; Morimoto, *et al.*, 1990; Welch, 1993), including fish (Iwama, *et al.*, 1998). Extensive research on model species has revealed multiple heat shock families, including Hsp90, low molecular weight heat shock proteins, and Hsp70. In unstressed cells, some of these proteins have constitutive functions and some have inducible functions that are necessary for several aspects of protein metabolism (Hightower, 1991; Morimoto *et al*., 1990; Welch, 1993). During general stress, Hsps, such as Hsp70, act as molecular chaperones that interact with proteins that have been damaged by stress to prevent alteration or denaturation (Hightower, 1991; Morimoto *et al*., 1990). While the term ‘heat shock response’ originated from early observations of *Drosophilia* exposed to severe heat shock (Tissieres, *et al.*, 1974), heat shock proteins can also be upregulated by various other forms of stress such as UV, γ-rays, chemicals and general oxidative stress

(Matsumoto *et al*., 1994; Santoro, 2000). This oxidative stress may cause DNA damage which induce Hsps, particularly Hsp70 (Calini, *et al.*, 2003).

In fish, the induction of heat shock proteins are well documented in cell lines and various tissues from whole animals (reviewed in Iwama *et al*., 1998). Generally, these studies suggest a correlation between increased levels of Hsps and exposure to ecologically relevant range of stressors. These observations suggest that the stress response is most likely playing a role in enhancing the survival and health of stressed fish. Fish make an excellent model for studying HSR because of the variety of stressors found in their natural environment, all of which may increase Hsp expression, and that they are easily exposed to numerous experimental conditions in the laboratory setting.

Many species, in particular fish (reviewed in Basu *et al.*, 2002; Iwama *et al*., 1998), exhibit characteristic and distinctive patterns of *Hsp* expression during the various stages of development, including embryogenesis (reviewed in Feder and Hofmann, 1999). A study on medaka embryos showed that *Hsp70* and *Hsp60* were not inducible prior to beginning of gastrulation, where they were considerably less tolerant to heat stress compared to developmental stage 19 or later (Werner, *et al.*, 2001). A similar trend was shown in zebrafish embryos where *Hsp70* mRNA was expressed after the gastrula period under heat-shock conditions (reviewed in Yamashita, *et al.*, 2010). Krone and Sass (1994) observed low levels of constitutive *hsp90α* in developing zebrafish, but this gene was strongly induced following a heat stress in the gastrula and later stage embryos. These expression patterns correlate with thermotolerance patterns seen in a study where zebrafish embryos were most susceptible to heat shock at early cleavage stages and

acquired increased resistance as they progressed through the blastula and gastrula stages (Majima and Ingalls, 1966). Therefore, these studies suggest that gastrulation is the first stage in fish embryo development where heat shock first induces *Hsps* expression and the HSR.

An interesting aspect of zebrafish development was observed in a study that noticed that constitutive *Hsp70* was maternally derived in zebrafish (Santacruz, *et al.*, 1997). This brings up questions on and whether this phenomenon is observed in other fish, if other heat shock proteins are transferred, whether the health of the mother affects transfer amount, and whether this transfer provides protection for the embryo (Basu *et al*., 2002). Though limited in other fish species, studies on other model organisms have shown maternal transfer of Hsps and *Hsp* mRNAs (e.g. *Hsp90, Hsp70 and Hsc70,* Bensaude, *et al.*, 1983; reviewed in Heikkila, *et al.*, 1997). A study on sea urchin (*Strongylocentrotus purpuratus*) embryos showed that *Hsp*90 protein synthesis increased abruptly at the morula stage due to the selective activation of translation of stored maternal mRNA (Bédard and Brandhorst, 1986). However, heat shocked eggs or zygotes did not result in translational activation of *Hsp90* mRNA. Thus, is likely that the heat-shock response in embryos is dependent on the production of new, non-maternal, mRNA (Bédard and Brandhorst, 1986).

An important aspect to note is that both the magnitude and duration of heat shocks have significant effects on the expression of Hsps in fish (reviewed in Iwama *et al*., 1998). A study on adult cutthroat trout (*Oncorhynchus clarki*) showed that heat shocks ranging from Δ10-16.2°C for 2 hours elevated Hsp70 protein levels as early as 1 hour

post recovery, and were maintained in some instances upwards to 3 weeks later (Mazur, 1996). In lake whitefish (*Coregonus clupeaformis*) juveniles and embryos, heat shocks as low as Δ3°C for 2 hours could significantly increase *Hsp70* mRNA expression levels (Stefanovic, *et al.*, 2016). Embryos at the fin flutter stage exposed to Δ3, Δ6, and Δ9°C heat shocks for 2 hours were able to maintain *Hsp70* mRNA expression levels for upwards to 48 hours, but showed a slow and long induction rate (~1.07x fold increase 2 hours post HS, and ~1.28x fold increase 48 hours post HS; Stefanovic, *et al*., 2016). In YOY juveniles, induction of *Hsp70* mRNA expression levels increased by ~1.8x fold at 2 hours post HS, but returned to baseline by 24 hours (Stefanovic, *et al*., 2016). This study on lake whitefish showed that embryos had a slower and longer induction rate, while YOY juveniles had a larger yet shorter induction rate (Stefanovic, *et al.*, 2016).

Transient exposure to elevated temperatures has been shown to have a cross-protective effect against exposure to other forms of normally lethal stress (Santoro, 2000), with this cytoprotective effect of Hsps being attributed to one of the major Hsps, Hsp70 (Lee *et al*., 2001). Cytoprotective effects have been demonstrated in different models with a combination of heat shock and radiation exposure (Boreham *et al.*, 1997; Mitchel and Morrison, 1982; Shen *et al*., 1991) including fish (Egami and Etoh, 1966; Etoh and Egami, 1967; Shimada and Egami, 1984; Shimada, 1985; Shimada *et al.*, 1985a, 1985b; Thome, *et al.*, 2017). The proposed molecular mechanism behind this adaptive response includes the initiation of DNA repair, bystander signaling, stress response induction and free radical scavenging (Jolly and Meyer, 2009). Specifically, it is believed that heat shocks create low levels of DNA damage, signaling for an increase in

Hsps, and thus providing proteins for protection and repair against higher levels of DNA damage in the future. *Hsp70* overexpression has been shown to act as a radioprotective mechanism towards the first event of DNA damage and increases long term viability (Calini *et al.*, 2003).

### 1.4. Lake whitefish

Lake whitefish (*Coregonus clupeaformis*) are coregonid fish that are native to North America and are found in many areas, such as rivers, brackish water, and the Great Lakes (Macpherson, *et al.*, 2010). These fish are part of a multimillion dollar commercial fishing industry, important recreational fish species, and are both culturally and economically valuable to the Aboriginal and Indigenous Peoples of North America (Madenjian *et al.*, 2002; Nalepa, *et al.*, 2005). Lake whitefish generally occupy cool, deep waters during the summer months and have an optimal temperature range of 12-16°C. During spawning, which occurs for approximately two weeks in the late fall and early winter, these fish migrate to shallower waters near shore (Ebener, *et al.*, 2010). Approximate temperatures during spawn are generally between 6-8°C and the embryos incubate in water temperatures ranging from 0.5- 6°C (Brooke, 1975; Hart, 1930; Price, 1934, 1940). Given the small and low optimal range for this cold-water adapted species, it is not surprising that temperature shifts have significant effects (Cingi, *et al.*, 2010; Yocom and Edsall, 1974), specifically during embryogenesis and larval stages (Brooke, 1975; Eme et al., 2015; Lee *et al.*, 2016; Lim *et al.*, in press; Mueller et al., 2015; Price, 1940; Rombough, 1997, Thome *et al.*, 2016)). Most embryonic mortality occurs early in

development before the beginning of organogenesis (Brooke, 1975). At warmer temperatures, a second mortality event can occur close to the hatching stage (Price, 1940). Thermal shifts that occur between gastrulation to organogenesis have been shown to increase cost of development and mortality (Mueller *et al.*, 2015), while also altering heart rate and oxygen consumption (Eme *et al*., 2015).

Studies on lake whitefish have examined other factors besides temperature, including the effects of chemical and radiological stressors on development. Lake whitefish had increased mortality and decreased body size when exposed to morpholine and hypochlorite (Thome, *et al*., 2017), important industrial cooling water additives, and stimulated body growth when exposed to chronic low dose ionizing radiation (0.06mGy/day - 1mGy/day, Thome, *et al*., 2017). The combined effects of thermal and radiological stressors had a potential adaptive response in lake whitefish (Thome, *et al.*, 2017) but much remains unknown about the mechanism and dynamics of this interaction.

Lake whitefish have many aspects that make them an ideal model organism for research. One reason being the detailed developmental staging series previously been described (Price, 1934; Sreetharan *et al*., 2015). As well, their time-to-hatch, depending on incubation temperature, can range from ~80-200 days (Brooke, 1975; Lim *et al.*, in press; Mueller *et al*., 2015; Price, 1934, 1940), allowing for longer and more precise exposures. Lake whitefish have a translucent chorion, allowing for accurate interpretation of developmental staging and mortality (Sreetharan *et al*., 2015). As mentioned previously in this thesis, there have been many studies using lake whitefish, giving a solid understanding of the species, but there is still much unknown. Therefore, the combination

of the value of lake whitefish in North America and their potential exposure and vulnerability to various anthropogenic stressors in nature make this species an excellent model of study.

### 1.5. Thesis objectives

This thesis will investigate the effect of multiple stressors in lake whitefish embryos with a particular focus on better understanding the underlying interaction between temperature and radiation treatment. While there have been many studies looking at the interaction of these two stressors in other models, information is lacking in fish models and the kinetics of this interaction. Utilizing radiation as a diagnostic tool, since doses used in this study are much higher than what would ever be found in the environment, one can better understand the mechanism behind the heat shock response, and the combination of both thermal and radiological stressors. The objective of this study was to determine the time course of the protective effect of an acute heat shock (HS) against lethal acute radiation in lake whitefish and how this effect changes throughout development.

Since *Hsp* mRNA expression is slower to induce earlier in development, I predict that lake whitefish will show a lower adaptive response at earlier developmental stages when exposed to acute lethal radiation. Later in embryogenesis, the *Hsp* mRNA expression will increase and the adaptive response will be greater, resulting in lower mortality to radiation after heat shock, compared to mortality with radiation alone. *Hsp* mRNA levels will correlate with protective effect seen in all stages of embryonic

development. This work aims to provide a better understanding of one of many combinations of stressors that fish populations may face when exposed to industrial effluent. Only by beginning to understand the mechanism that certain combinations of stressor regimes induce, can there be better protection of important freshwater species and potential transfer of this knowledge to other significant models.

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# Chapter 2

# The combined effects of thermal and radiological stress on the embryonic development of lake whitefish (*Coregonus clupeaformis*)

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## 2.1. Abstract

There is growing evidence that mild hyperthermia stress can produce an adaptive response through a heat shock response (HSR), when followed with subsequent ionizing radiation stressor. The purpose of this study was to examine the combined effects of both thermal and radiological stressors on embryonic development in the lake whitefish (LWF; *Coregonus clupeaformis*). LWF embryos were administered 3 and 9°C heat shocks (HS) for 2 hours, and subsequently exposed to high dose acute $^{137}$Cs gamma rays at 2, 6 and 24 hours post heat shock at either the gastrulation or eyed stage. HS embryos were collected at each developmental point and assessed for induction of heat shock protein (Hsp) genes. Following exposure, embryos were raised until hatch where mortality, hatch dynamics, morphometry, and embryo weight were measured. Both 3°C and 9°C HS induced *Hsp70* mRNA expression at gastrulation, but HS at the eyed stage did not alter *Hsp70* expression. Radiation treatment alone and in combination with mild heat shocks during gastrulation stage had no significant effects on embryos at hatch. During the eyed stage, acute radiation treatment increased mortality, decreased time to 50% hatch, decreased body size and decreased yolk consumption at hatch. However, mild HS prior to radiation was not different in any of these endpoints compared to radiation alone. Although *Hsp70* mRNA expression was induced, no adaptive response was seen. However, the possibility of an adaptive response, as seen in other species, cannot be ruled out in lake whitefish.

## 2.2. Introduction

Fish embryonic development is known to be susceptible to many stressors (reviewed in Jezierska, *et al.*, 2009; Rombough, 1997; Von Westernhagen, 1988). Specifically, ionizing radiation caused negative impacts on fish embryogenesis (reviewed in Laale and Lerner, 1981). During embryogenesis, predominantly after fertilization, there is a greater number of dividing cells which if exposed to radiation, can increase the risk of cell death and embryonic mortality (reviewed in Harrison and Anderson, 1996). As the embryo progresses through development, vital organs begin to form and there is a greater chance for malformation and abnormalities (reviewed in Harrison and Anderson, 1996). Susceptibility to ionizing radiation decreases as the fish embryos become more developed and embryos have been reported to increase their radioresistance up to 10-fold from fertilization to hatch (reviewed in Harrison and Anderson, 1996; Welander, 1954). Exposure to acute radiation doses in fish embryos increased mortality and abnormalities, while also resulting in earlier hatch and smaller hatchlings (Bell and Hoar, 1950; Hinrichs, 1925; Konno, *et al.*, 1955; Miyachi *et al*., 2003; Thome, *et al*., 2017; Welander, 1954; Welander and Donaldson, 1948). Chronic low dose radiation in developing fish embryos resulted in both growth retardation (reviewed in Blaylock and Trabalka, 1978) and growth stimulation (Lim, 2016; Thome *et al*., 2017). Growth stimulation has also been shown with fish embryos exposed to fractionated radiation dosing (Mitz, 2016). The difference in effects between studies may be due to the differences in species, dose and dose rate (reviewed in Hall and Giaccia, 2006).

Other stressors in combination with radiological stress result in what is known as an adaptive response, where a low dose stress can protect organisms from future high dose exposure (Tang and Loke, 2015). Specifically, thermal stressors induce an adaptive response in yeast (Boreham and Mitchel, 1994), mice (Shen, 1991), and adult fish (Egami and Etoh 1966; Etoh and Egami, 1967) to reduce damage from a subsequent high dose ionizing radiation exposure. Mild hyperthermia stress in fish embryos, has shown an adaptive response when followed by an acute lethal radiation dose (Shimada and Egami, 1984; Shimada, 1985; Shimada, *et al.*, 1985a, 1985b; Thome *et al.*, 2017). Some multiple stressor work on lake whitefish (*Coregonus clupeaformis*) embryos has shown a protective effect with 3°C and 9°C heat shock prior to acute radiation dose of 10 Gy (Thome *et al*., 2017), however questions still remain about the exact kinetics of this response and how it changes throughout development.

The proposed molecular mechanism during the adaptive response is thought to include the initiation of DNA repair and free radical scavenging (reviewed in Jolly and Meyer, 2009). It is believed that this protective effect is a result of heat shock induced DNA damage, which signals for an increase of heat shock proteins (Hsps) and initiates the heat shock response (HSR). Hsps are a family of highly conserved proteins and response protects cells and organisms against oxidative stress, caused by ionizing radiation, and prevents cell death (Park *et al*., 2000). The overexpression of *Hsp70* acts as a radioprotective mechanism towards the first event of DNA damage and increases survivability (Calini *et al*., 2003). Hsp expression has been studied extensively in fish (reviewed in Iwama *et al*., 1998) and stage-dependent Hsp expression is seen throughout

embryonic development (Krone *et al.*, 2003; Krone *et al.*, 1997; Krone and Sass, 1994; Lele *et al.*, 1997). This stage-dependent expression may potentially result in different magnitudes of adaptive response depending on the point in development.

Lake whitefish (*Coregonus clupeaformis*) present an excellent model for analyzing the effects of radiological and thermal stress. This is primarily due to long embryonic developmental periods of up to 200 days when reared at colder temperatures (0.5-2°C; Brooke, 1975, Lim *et al*, in press), combined with a clear chorion (egg shell), which allows for precise acute exposures at specific developmental stages. Lake whitefish are quite sensitive to increases in water temperature in the embryonic and early life-history stages (Brooke, 1975; Lim *et al.*, in press; Mueller *et al*., 2015; Price, 1934, 1940). Studies have examined the effects of radiological stress exposures in lake whitefish embryos after acute, chronic low dose, and fractionated dose irradiations (Mitz, 2016; Thome *et al*., 2017). Heat shock proteins (*hsp70, hsc70, hsp90α, hsp90β, hsp47*) are expressed in lake whitefish embryos and they mount an HSR, as measured by induction of *hsp70*, in response to heat shock (HS) of at least 3°C for 2 hours (Stefanovic *et al*., 2016). Hsp expression varied prior to and post hatch in lake whitefish with differences in the induction of heat shock response, the types and numbers of Hsps involved in the response, and the overall duration of the response (Stefanovic et al., 2016). During the fin flutter stage of development, only *hsp70* was inducible with HS, where a HS as low as 3°C increased mRNA levels by ~1.3x fold for 48 hours post HS (Stefanovic *et al*., 2016). A 9°C HS for at least 1 hour induced *hsp70* immediately post HS (Stefanovic, *et al*., 2016). Whether Hsp induction is possible at earlier developmental

stages is not yet clear, nor is whether an HSR would result in an adaptive response to subsequent radiological stress.

The purpose of this study was to examine the combined effects of both thermal and radiological stressors on embryonic development in lake whitefish. Embryos were exposed to mild heat shocks prior to irradiations at two critical points of development, gastrulation and eyed stage. Timing between stressor exposures varied during a 24 h time span to assess at which times post heat shock an adaptive response was present. After exposure to both stressors, embryos were reared until hatch and mortality, hatch duration, and time to hatch were determined. Morphometrics and dry weights were measured at hatch to provide insight into the combined effects on embryonic growth and metabolic efficiency. Embryos were collected post heat shock, but prior to irradiation, to determine *Hsp70* mRNA expression at the time of irradiation exposure. *Hsp70* mRNA expression is typically lower in early embryonic development in other species (reviewed in Heikkila et al., 1986), therefore we hypothesized that lake whitefish would have lower *Hsp70* mRNA expression and a smaller HSR at the gastrulation stage compared to the eyed stage. When exposed to heat shock prior to acute lethal radiation, we predicted an adaptive response and lower mortality; *Hsp70* mRNA expression levels were predicted to correlate with any adaptive response.

## 2.3. Methods

### 2.3.1. Lake whitefish embryo collection and rearing

Lake whitefish adults were gill-netted on November 10, 2016 from Lake Huron (Stokes Bay, 44.994609, -81.385711). Eggs and milt were stripped from these fish (males= 54, females= 11) and gametes combined in a common pool. Following collection, 2000 mL of egg and milt mixture was divided and placed into each of four 1 L Nalgene containers and mixed with 500 mL of lake water and 1 mL Ovadine (0.5% iodine, for disinfection). After 30 minutes, eggs were strained of all fluid and rinsed three times with lake water, placed back into Nalgene containers, filled with lake water and placed into coolers on ice. Cleaned embryos were transported in clean lake water on ice to McMaster University. Embryos were then placed evenly into McDonald Bell hatching jars at 5ºC.

At 5 days post fertilization (dpf), ~28000 embryos were transferred from the bell hatching jars at 5 °C into 280 sterile petri dishes (100mm x 20mm) at a density of ~50 embryos/dish with ~75ml of dechlorinated city water. Embryos were raised in custom fridges as described in Mitz *et al*. (2014) set at a constant 2ºC. Water temperature was monitored using HOBO® data loggers (accurate to ±0.2 ºC; TidbiT v2 Temperature Data Logger UTBI001; Onset Computer Corporation, Bourne, MA) measuring every 10 minutes in ~100 mL of dechlorinated water in a glass beaker adjacent to petri dishes in each fridge. Temperature loggers gave a mean water temperature of 2.1±0.2ºC (±standard deviation). Petri dishes underwent 100% water changes daily for the first 30

days, then 2-3 times weekly until all embryos hatched. The developmental stages used for heat shock and radiation experiments were determined by visual inspection of embryos under light microscopy and staged according to Sreetheran *et al*. (2015) and occurred at 7 days (gastrulation) and 60 days (eyed) post fertilization.

### 2.3.2 Heat shocks

After 48 h at 2 °C, the embryos reached the gastrulation stage of development (7 dpf) and 140 petri dishes were exposed to transient heat shocks at Δ0°C (n=60 dishes), Δ3°C (n=40 dishes), or Δ9ºC (n=40 dishes) for 2 hours. Heat shocks were administered by removing water from dishes and replacing with pre-heated water (75ml). After heat shock, dishes were transferred immediately back to the 2ºC fridge and water was allowed to return to 2 °C. Heat shocked non-irradiated embryos were snap frozen at 2 (20 embryos/dish, n=5), 6 (20 embryos/dish, n=5), and 24 (20 embryos/dish, n=5) hours post heat shock for each temperature treatment. Samples were stored at -80 C for RNA extraction. This same process was repeated at 60 dpf for the remaining 140 petri dishes.

### 2.3.2. Acute irradiation post heat shock

To assess the protective effect of hyperthermia, embryos were exposed to acute radiation at 2, 6, and 24 hours post heat shock. Irradiation consisted of acute exposures of 662 keV gamma rays were using a $^{137}$Cs source (Thome, *et al.*,2017). Embryos (n=50, 5 replicates) were transferred from experimental petri dishes to 12.5 $cm^2$ vented cap cell culture flasks and placed on ice immediately prior to acute irradiation. Embryos were irradiated on an ice slurry with either 10 Gy (at 7 dpf) or 20 Gy (at 60 dpf). No heat

shock, radiation only controls (Δ0°C) were irradiated but without prior heat shock and sham controls (Sham) were brought to the source, but not irradiated. Post irradiation, embryos were transferred back in the petri dishes until hatch.

### 2.3.3. Embryo endpoints

Dishes from all treatment groups were checked multiple days a week and dead or hatched embryos were removed and recorded. Daily mortality in each dish was used to calculate the cumulative percent mortality at hatch. Hatch duration was measured by taking the difference in days between first and last hatch and time to 50% hatch was taken as the median value between first and last hatch. Hatched fish were fixed in 10% neutral buffered formalin for 1 week and then transferred to 50% ethanol. All hatchlings from the eyed stage exposure were imaged and measured using a Zeiss AX Zoom V16 microscope (Carl Zeiss AG) equipped with a CANON SL6 digital camera. A dorsal image was used to measure total body length and a lateral image was used to measure yolk area and eye diameter. Yolk area was measured assuming the yolk sac was an ellipse shape.

Following imaging, the hatchling body was dissected from the yolk and specimens were dried separately in a 70°C oven for 24 hours. Dry weights of yolk-free body and yolk were measured using a ±0.01 mg fine scale balance (Mettler-Toledo XA105DU). A subset of 75 control embryos was fixed at 1 dpf and weighed. A yolk conversion efficiency (YCE) was calculated according to the equation:

$$YCE(\%) = \frac{yolk\ free\ body\ dry\ mass}{(1\ dpf\ yolk\ dry\ mass - yolk\ dry\ mass)} \times 100$$

### 2.3.4. *Hsp70* gene expression

Total RNA was isolated from a pool of 10 embryos from a single treatment using TRIzol® Reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Burlington, ON, Canada). RNA quality and purity were confirmed using agarose gel electrophoresis and spectrophotometry ($A_{260}/A_{280}$ ratio > 1.8), respectively. First strand cDNA was synthesized from 2 µg total RNA by first treating with a DNAse I kit (Sigma Aldrich, Oakville, ON, Canada) and then using Promega M-MLV Reverse Transcriptase (Fischer Scientific, Burlington, ON, Canada) following manufacturer's instructions. mRNA levels were determined using quantitative real-time PCR (qPCR) with a CFX Connect Real-Time Detection System (Bio-Rad, Mississauga, ON, Canada). Previously validated primers for *β-actin* and *Hsp70* were used (Stefanovic et al., 2016). qPCR reactions were performed using 40ng cDNA, 500 nmol of each primer and LuminoCt® SYBR® Green qPCR ReadyMix™ (Sigma Aldrich, Oakville, ON, Canada) to a total volume of 25 µl under the following conditions: 1 cycle of 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 65 °C for 30 s. Melt curve analysis, no template controls and no reverse transcription controls were used to confirm the presence of a single amplicon and the absence of genomic DNA contamination. A pool of cDNA from control and HS samples was generated to serve as an inter-run calibrator (IRC); duplicates of the IRC were included in each qPCR run to monitor for any run-to-run variation which could be adjusted for during statistical modeling.

### 2.3.5. Statistical analysis

Statistical analysis was conducted using GraphPad Prism 6 and R Studio version 0.98.977. Using GraphPad Prism 6, the percent mortality, time to 50% hatch, and the hatch duration between replicate dishes were compared between HS treatments within a given irradiation time post heat shock using ordinary one-way ANOVA followed by Tukey's HSD test. Due to non-normal distribution, hatchling weights and morphometrics were compared using a Kruskal-Wallis one-way ANOVA followed by Dunn's pairwise multiple comparison test.

For mRNA expression levels, R Studio version 0.98.977 and a previously validated Bayesian, Markov Chain Monte Carlo (MCMC) sampling scheme were used (Matz, *et al.*, 2013; Stefanovic *et al*., 2016). *Hsp70* mRNA levels were modelled in the following way. Each embryonic stage (gastrulation and eyed) was analyzed separately. The effects of thermal stress were modelled with HS temperature group (control, 3 and 9°C) and post-HS recovery as fixed factors. The reference gene *β-actin* was added as a prior to the model and function to account for variations in cDNA. Posterior means from the control for *Hsp70* and the 2 HS temperatures were plotted as log abundance with the 95% credible intervals as the error bars. The credible intervals are the Bayesian analog of confidence intervals. Effects were deemed statistically significant when credible intervals did not overlap. Statistically significant differences between HS temperature groups within a given post-HS recovery time are noted on the plots.

## 2.4. Results

### 2.4.1. Mortality and hatch dynamics

Background mortality measured from gastrulation stage to hatch was approximately 80% (Fig. 1A). Application of mild or moderate heat shock during the gastrulation stage did not alter the mortality of fish at hatch compared to the sham group (Fig.1A). At 2 hours post heat shock, mortality was not different between sham and radiation alone (Δ0°C) or the combination of 3°C HS and radiation. However, percent mortality was significantly increased with the combination of 9°C HS and radiation compared to embryos that received no heat shock or irradiation (Sham; Fig. 1A). At 6 hours post heat shock, the radiation alone and combination with both 3 and 9°C HS significantly increased percent mortality. At 24 hours post heat shock, radiation alone or HS in combination with radiation did not result in any significant change in percent mortality (Fig.1A). Due to high mortality at hatch from gastrulation stage exposures, no fixed specimens from this group were utilized for morphometric endpoints.

During the eyed stage exposure, the 3°C HS alone, but not 9°C HS alone, resulted in a significant increase in percent mortality at hatch compared to sham (Fig.1B). At 2 hours post heat shock, radiation alone and HS and radiation combinations had significantly higher mortality compared to sham. As well, the combination of 9°C HS and radiation resulted in a significantly higher mortality than the combination of 3°C HS and radiation. At 6 and 24 hours post heat shock, radiation alone significantly increased

mortality compared to sham, however the combination of heat shock and radiation was not significantly different from radiation treatment alone (Fig. 1B).

Time to 50% hatch was not altered by HS alone, radiation alone or HS in combination with radiation at 6 and 24 hours post heat shock during gastrulation stage exposures (Fig. 2A). However, the combination of 9°C HS and radiation at 2 hours post heat shock resulted in fewer days to 50% hatch when compared to sham. During the eyed stage exposure, HS alone had no significant effect on time to 50% hatch when compared to sham (Fig. 2B). Radiation alone and the combination of HS and radiation treatments at 2, 6, and 24 hours post heat shock resulted in significantly shorter times to 50% hatch (Fig.2B).

There was no significant effect of any of the treatments during gastrulation exposure on hatch duration (Fig. 2C). During the eyed stage exposure, the 9°C HS alone resulted in significantly longer hatch duration, compared to sham (Fig. 2D). Radiation alone or in combination with HS at any time post HS did not significantly effect hatch duration compared to shams.

### 2.4.2. Body growth and yolk consumption at hatch

Body length, eye diameter and yolk area at hatch were not significantly affected by a mild HS at the eyed stage, compared to sham (Fig. 3A,B,C). Radiation treatment alone resulted in significantly smaller body lengths, smaller eye diameters, and larger yolk sac areas, regardless of time post HS. However, the treatments that combined heat shock and radiation were not different from radiation treatment alone (Fig. 3A,B).

Dry body mass and dry yolk mass at hatch were not significantly affected by HS alone at the eyed stage compared to sham (Fig.4A,B). Radiation treatment alone resulted in significantly smaller body mass and larger yolk mass at hatch. However, the combination of heat shock and radiation were not different from radiation treatment alone (Fig.4A,B). Yolk conversion efficiency (YCE) in HS only treatment was not different from sham (Fig. 4C). Radiation treatment alone at the eyed stage resulted in significantly lower YCE at hatch. At 6 hours post HS, the combination of 9°C HS and radiation resulted in a significantly lower YCE (27.5%) compared to both sham (45.9%) and radiation alone treatment (33.0%). YCE was not different across the HS and radiation combination treatments, when radiation was given at 2 and 24 hours post HS, compared to radiation alone treatment (Fig. 4C).

### 2.4.3. *Hsp70* mRNA expression

HS temperature and recovery time significantly affected the induction of *Hsp70* mRNA expression at gastrulation but not at the eyed stage. During gastrulation, *Hsp70* mRNA expression levels were not affected immediately post HS. A 3°C HS significantly upregulated gene expression at 2 and 6 hours post heat shock, but returned to baseline at 24 hours post heat shock (Fig.5A). A 9°C HS upregulated *Hsp70* mRNA expression at 2 hours post heat shock but not at 6 or 24 hours post heat shock (Fig. 5A). During the eyed stage, neither the 3 or 9°C HS resulted in significantly different *Hsp70* mRNA levels at any time post HS compared to control (Fig. 5B).

## 2.5. Discussion

Lake whitefish were used to examine the effects of combined stressors (priming mild heat shock, followed with an acute lethal radiation dose) on embryonic development. Our study found that acute radiation treatment alone and in combination with heat shock generally had no significant effect on mortality or hatch dynamics. However, at the eyed stage, an acute exposure to a high dose of radiation had significant effects on mortality at hatch, hatch dynamics, morphometrics and hatchling weights. Heat shocks prior to irradiation generally did not provide an adaptive response. Heat shocks during gastrulation significantly increased *Hsp70* mRNA expression levels quickly after exposure, but these levels were not maintained for 24 hours. No change was seen in *Hsp70* mRNA expression levels when embryos were heat shocked during eyed stage.

### 2.5.1 Radiation increases mortality rates and decreases time to hatch

In lake whitefish embryos, a 10 Gy dose of radiation at the gastrulation stage generally did not alter mortality at hatch, compared to shams, except when given 6 hours post heat shock (Fig. 1A). This was unexpected since 10 Gy was the $LD50_{hatch}$ for lake whitefish irradiated at this stage of development in a prior study (Thome *et al*., 2017). At the eyed stage, a 20 Gy dose ($LD50_{hatch}$; Thome *et al*., 2017) significantly increased mortality at hatch, regardless of when it was given with respect to the heat shock (Fig.1B). Radiosensitivity decreases throughout embryo development in fish, with lake whitefish having an $LD50_{hatch}$ of 5 Gy at 1 dpf, and as high as 15 Gy at organogenesis (30 dpf; Thome *et al.*, 2017). Welander (1954) found an $LD50_{hatch}$ of 0.7 Gy immediately

post fertilization, in rainbow trout (*Salmo gairdneri*), using x-rays, which increased to 6 Gy at the germ ring stage. During gastrulation in general, there is still the initial dividing of cells which increases the risk of cell death and embryonic mortality when exposed to radiation (reviewed in Harrison and Anderson, 1996). The eyed stage is a more developed form of the lake whitefish in which most organs are fully formed, blood circulation begins and the eyes are fully pigmented (Sreetharan *et al*., 2015), therefore less susceptible to radiation damage (reviewed in Harrison and Anderson, 1996).

Generally, radiation treatment during the gastrulation stage had no significant effect on hatch dynamics (Fig.2A,C), however, radiation exposure during the eyed stage significantly decreased time to 50% hatch (Fig.2B,D). On average, time to 50% hatch decreased by ~25 days in the irradiated embryos compared to sham. With low dose rates of x-rays or gamma rays during early development (1-1000mGy/d), time to hatch decreased in zebrafish (Gagnaire *et al*., 2015; Miyachi, *et al.*, 2003; Simon, *et al.*, 2011), similar to what was seen in this study. It was suggested that low dose radiation had a stimulatory effect, increasing metabolic functions and earlier stage onset (Simon *et al*., 2011). However, in contrast to these findings, some studies have shown that high acute irradiation (1-10 Gy) doses on the embryos of zebrafish (McAleer *et al*., 2005; Praveen Kumar *et al*., 2017) and hermaphroditic fish (*Kryptolebias marmoratus*; Rhee, *et al.*, 2012) had delayed time to hatch. Overall, these studies suggest that time to hatch is a stress sensitive marker.

### 2.5.2. Radiation decreases embryonic growth and yolk consumption at hatch

Radiation treatment during the eyed stage significantly decreased body length, eye diameter, body weight, and yolk size and conversion efficiency at hatch (Fig. 3, 4). A similar decrease in body length at hatch was seen in lake whitefish embryos acutely exposed to 7.75 Gy at the gastrulation stage and 15.51 Gy at organogenesis (Thome *et al*., 2017). Atlantic cod (*Gadus morhua*) embryos exposed to UV radiation had smaller standard lengths at hatch (Lesser, *et al*., 2001). Previous work in amphibian species (*Rana blairi*) showed that tadpoles exposed as embryos to UV radiation, grew and developed slower than controls (Smith, *et al.*, 2008; Worrest and Kimeldorf, 1975). Chronic low dose radiation (0.1-0.3 mGy/day) in lake whitefish (Thome *et al*., 2017) and in round whitefish (*Prosopium cylindraceum*; Lim, 2016) embryos resulted in increased body size and decreased yolk size at hatch. This hormetic growth stimulation in lake whitefish embryos was also seen with fractionated dosing (Mitz, 2016). However, this is generally not the case, with majority of chronic low dose radiation studies on aquatic species showing a retardation in growth (reviewed in Blaylock and Trabalka, 1978). The reductions in both growth and growth efficiency when exposed to radiological stress is commonly seen in fish exposed to other kinds of stress (eg. temperature, hypoxia, chemical, metal), particularly when at an early development stage (reviewed in Rice, 1990; Bonga, 1997). Fish respond to stressors by changing physiological function to reallocate energy for the purposes of coping to stress (reviewed in Schreck et al., 2001). One of these functions may be yolk conversion efficiency, which is directly related to fish size and growth rate (reviewed in Blaxter, 1969).

### 2.5.3. Mild heat shock in combination with radiation does not affect embryo development

Mortality from acute radiation exposures was generally not modified by the prior thermal stress. Priming heat shocks in combination with radiation did not result in any significant differences in mortality at hatch when compared to radiation alone at the gastrulation stage. This was unexpected, since it has previously been shown in lake whitefish, that a mild heat shock of 3 or 9°C at the gastrulation stage decreased mortality and resulted in an adaptive response at 6 hours post heat shock, but was gone at 24 hours post heat shock (Thome *et al*., 2017). As well, heat shock induced adaptive responses have protected against radiation induced mortality in mice (Shen et al., 1991), cell culture (Boreham *et al.*, 1997; Shimada, 1985) and yeast (Boreham and Mitchel, 1994; Mitchel and Morrison, 1982). The variability of the adaptive response may be due to difference in genetic variation and quality in lake whitefish embryos collected from season to season. Background mortality between Thome *et al.,* (2017) and this study varied greatly, suggesting lower quality embryos, which may have affected the heat shock and adaptive response. Large variations in background mortality are difficult to control for in studies that utilize wild collected fish. This is particularly true for lake whitefish, which spawn in a short time window, once per year, in difficult field conditions because we cannot easily control for sampling location, number of females and males available for in vitro fertilization, spawning condition, and where in the spawn we can collect fish.

### 2.5.4. *Hsp70* mRNA expression in relation to the adaptive response

Several types of stress, including heat, UV, gamma-rays and chemicals, that cause DNA damage induce a heat shock response (HSR) and in particular *hsp70* (Calini *et al*., 2003). Heat shock proteins (Hsps) have been proposed to play a significant role in DNA repair after UV or gamma-ray irradiation. Hsps and the HSR have been examined in a variety of embryonic animal systems including fish (reviewed in Heikkila, *et al.*, 1986). The HSR and Hsps during normal zebrafish embryonic development has been studied extensively (Krone et al., 1997, 2003; Krone and Sass, 1994; Lele et al., 1997, 1999; Sass, *et al.*, 1996, 1999). Lake whitefish expressed Hsps, including Hsp70, in development and can mount an HSR in response to thermal stress (Stefanovic, *et al.*, 2016).

During gastrulation, *Hsp70* mRNA expression levels were significantly increased following heat shocks, maintained until 6 hours, and returned to baseline by 24 hours (Fig.5A). During the eyed stage, *Hsp70* mRNA expression levels did not change following heat shocks (Fig.5B). This suggests that a HSR was illicited at the gastrulation stage, which was similar to findings that reported embryonic whitefish and juveniles were capable of eliciting an HSR (Stefanovic *et al*., 2016). However, similar conditions (3 and 9°C HS for 2 hours) in this experiment resulted in constantly elevated *Hsp70* mRNA levels upwards to 48 hours post heat shock in embryonic lake whitefish (Stefanovic *et al*., 2016). This difference in HSR may be due to differences in developmental stages at which the experiments took place; gastrulation and eyed stage in this study and fin flutter/vitelline circulation stage in the other study (Stefanovic *et al*., 2016). Hsp

expression during embryonic development is a stage-dependent phenomena (reviewed in Heikkila et al., 1986). Developmental stage-dependent responses to HS were reported in Medaka (*Oryzias latipes*), where a 15°C HS resulted in an increase in *Hsp70* proteins in late, but not early embryos (pre-gastrulation; Werner *et al*., 1986). In zebrafish (*Danio rerio*), a 1 hour 9°C HS induced *Hsp70* mRNA expression during and after gastrulation, but not before gastrulation (Yamashita, *et al*., 2010). These studies suggest that some fish embryos are able to induce *Hsp70* mRNA expression as early as gastrulation and continue this HSR through development.

## 2.6. Conclusion

This study demonstrated that acute high dose irradiation had significant effects on lake whitefish exposed at the eyed stage of development where it was shown to increase mortality, decrease time to hatch, growth and yolk consumption at hatch. High background mortality at hatch due to poor embryo quality and the point of development at which exposures occurred may have affected the results from gastrulation stage. The combination of mild heat shock and acute irradiation at the gastrulation and eyed stage showed no significant adaptive or synergistic response when looking at mortality, hatch dynamics, growth and yolk consumption at hatch. *Hsp70* mRNA levels and the HSR were inducible at the gastrulation stage but not at the eyed stage of development. Overall, even though the HSR was induced, no adaptive response was seen. However, it can not be ruled out that the adaptive response is not possible in lake whitefish.

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## 2.9. Figures

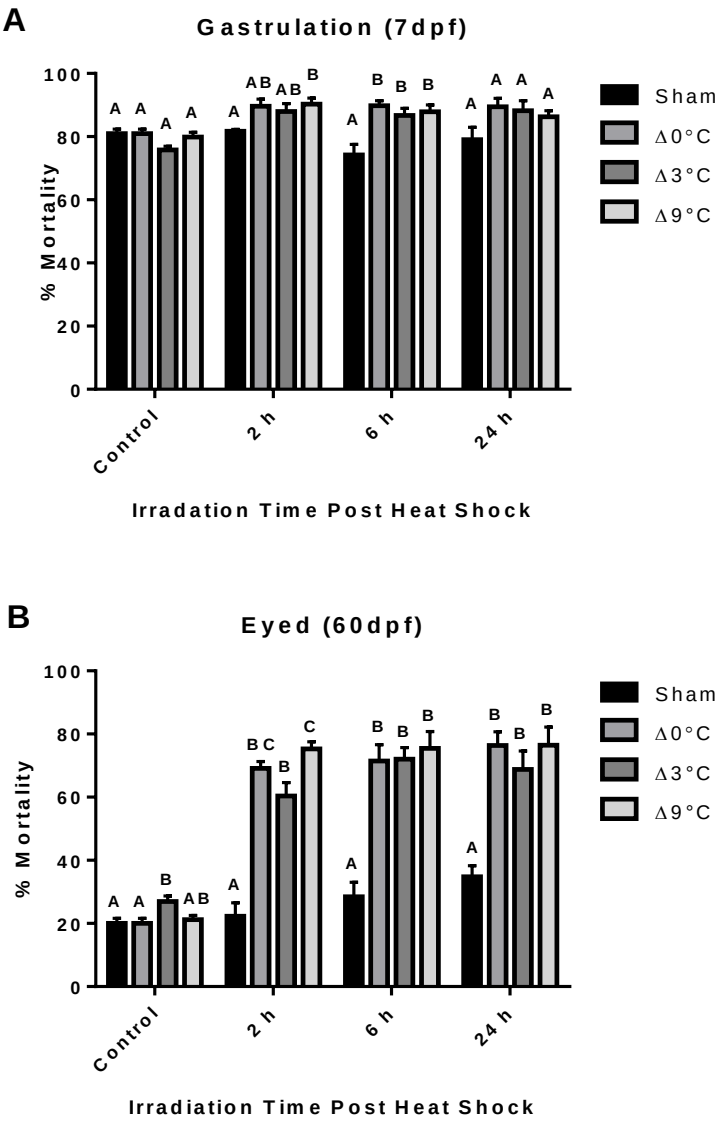


**Figure 1.** Mean mortality (from gastrulation to hatch or eyed stage to hatch) of lake whitefish embryos exposed to various combinations of heat shocks and radiation. Embryos were given a 2 hour heat shock (HS) at (A) gastrulation (Day 7; n=5), or (B) eyed stage (Day 60; n=5 for all, except for Control - Sham; Control - Δ0°C; Control - Δ3°C; 2h - Δ3°C; 6h – Δ0°C, where n=4), followed by a 10 Gy (gastrulation) or 20 Gy (eyed stage) acute radiation exposure at 2, 6 or 24 hours post heat shock. The total percent mortality was calculated at hatch. Percent mortality was compared between HS treatments within a given irradiation time post heat shock using one-way ANOVA followed with Tukey's HSD test. Letters denote statistical differences between heat shock treatments. Bars represent means ± SEM.

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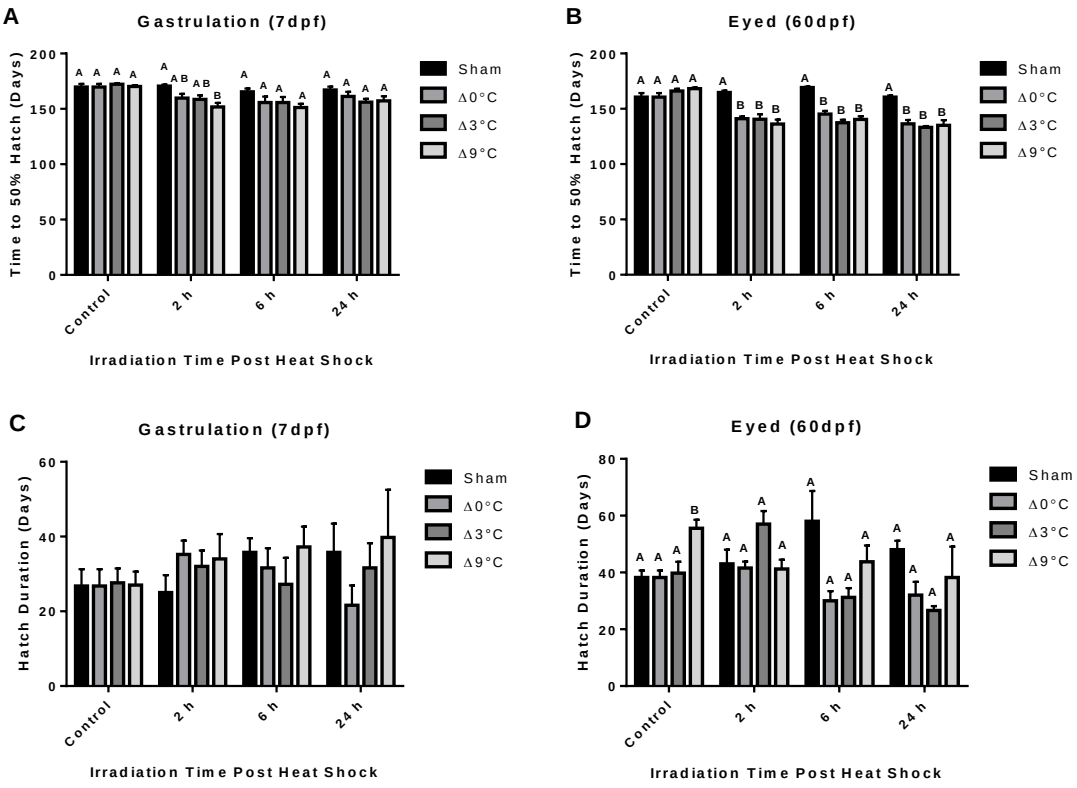


**Figure 2**. Hatch dynamics of lake whitefish embryos exposed to various combinations of heat shocks and radiation. Embryos were given a 2 hour heat shock (HS) at (A,C) gastrulation (Day 7; n=5), or (B,D) eyed stage (Day 60; n=5 for all, except for Control - Sham; Control - Δ0°C; Control - Δ3°C; 2h - Δ3°C; 6h – Δ0°C, where n=4), followed by a 10 Gy (gastrulation) or 20 Gy (eyed stage) acute radiation exposure at 2, 6 or 24 hours post heat shock. The time to median hatch (A,B) and the hatch duration, measured as the time between the first and last hatch (C,D), were calculated for each replicate dish and median hatch and hatch duration were compared between HS treatments within a given irradiation time post heat shock using a one-way ANOVA with Tukey's HSD test. Letters denote statistical differences between heat shock treatments. Bars represent means ± SEM.

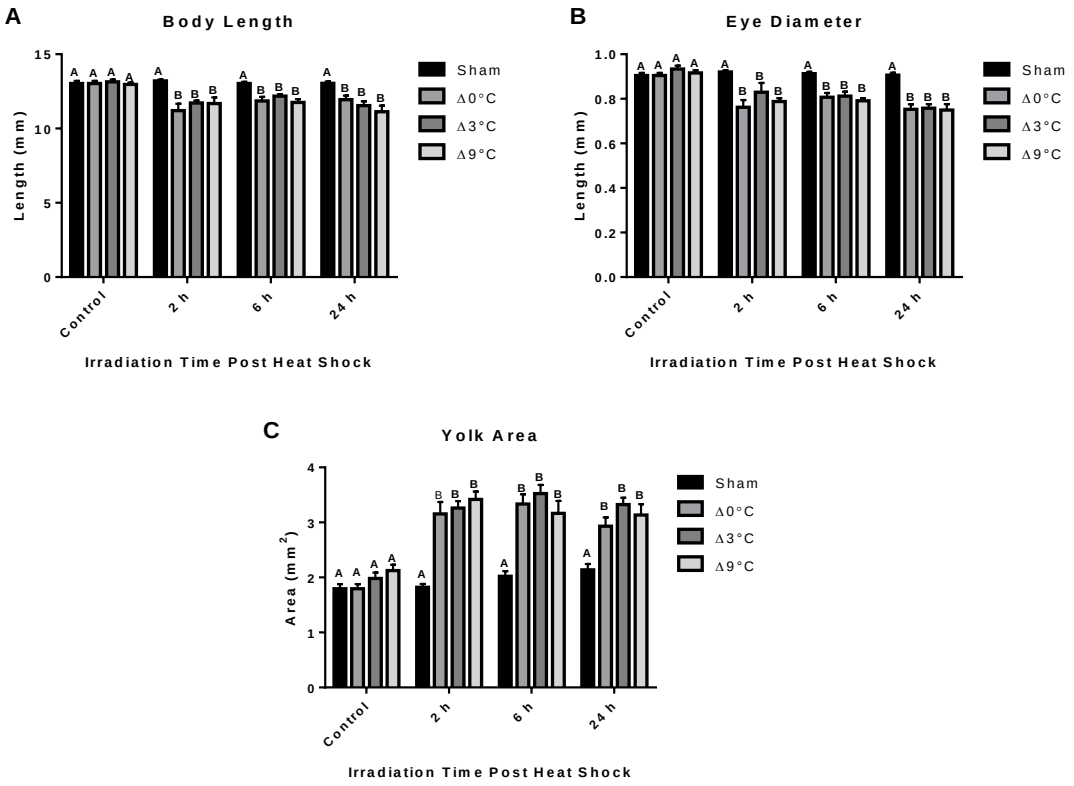


**Figure 3**. Morphometric measurements on preserved hatchlings exposed to heat shock (HS) and radiation treatment at the eyed stage. Body lengths (A) and eye diameters (B) were measured. Yolk area (C) was calculated assuming ellipse shape of yolk (Area=πab) using measured yolk width (a) and height (b). Morphometrics were compared between HS treatments within a given irradiation time post heat shock using a Kruskal-Wallis one-way ANOVA because of non-normal distribution, followed with Dunn's pairwise multiple comparison test. Letters denote statistical differences between heat shock treatments. Bars represent means ± SEM. (Control – Sham, n=57; Control - Δ0°C, n=57; Control – Δ3°C, n=80; Control – Δ9°C, n= 94; 2h – Sham, n=100; 2h - Δ0°C, n=33; 2h – Δ3°C, n=31; 2h – Δ9°C, n= 27; 6h – Sham, n=81; 6h - Δ0°C, n=37; 6h – Δ3°C, n=28; 6h – Δ9°C, n= 22; 24h – Sham, n=53; 24h - Δ0°C, n=39; 24h – Δ3°C, n=48; 2h – Δ9°C, n= 31)

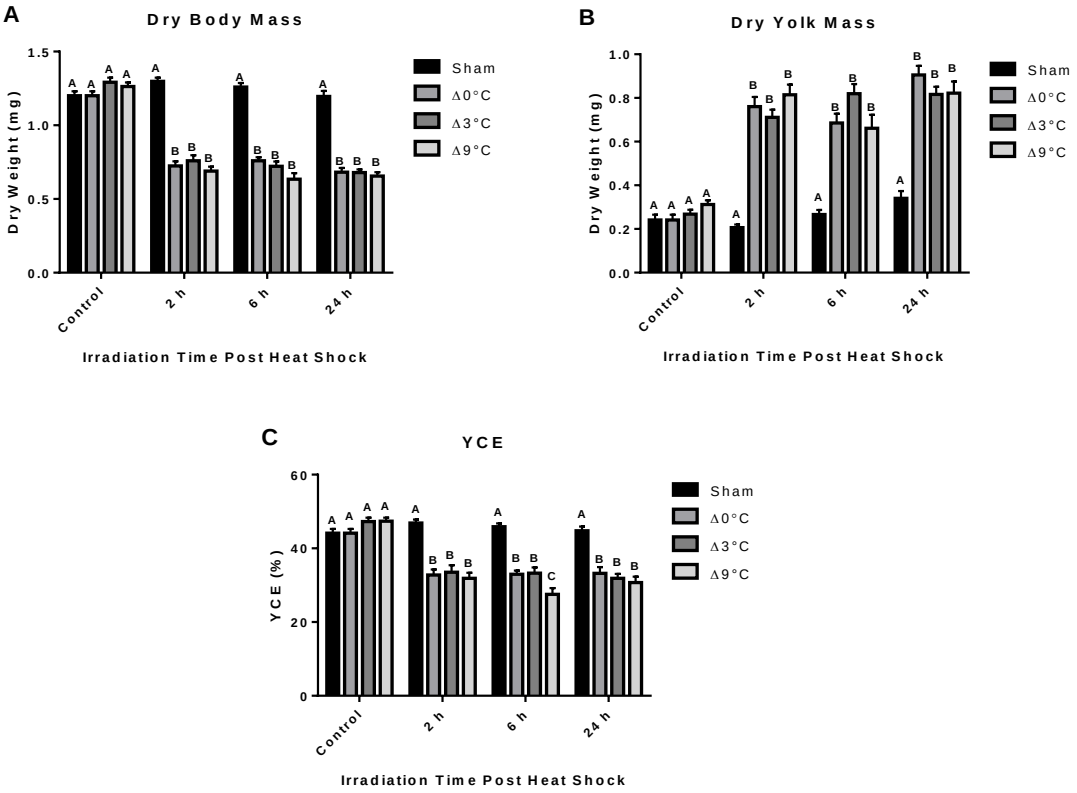


**Figure 4**. Dry weight measurements on preserved hatchlings exposed to heat shock (HS) and radiation treatment at the eyed stage. Yolk-free body (A) and yolk weights (B) were measured. A yolk conversion efficiency (YCE; C) was calculated based on body and yolk weights. Dry weights were compared between HS treatments within a given irradiation time post heat shock using a Kruskal-Wallis one-way ANOVA because of non-normal distribution, followed with Dunn's pairwise multiple comparison test. YCE was compared between HS treatments within a given irradiation time post heat shock using a one-way ANOVA with Tukey's HSD test. Letters denote statistical differences between HS treatments. Bars represent means ± SEM. (Control – Sham, n=57; Control - Δ0°C, n=57; Control – Δ3°C, n=80; Control – Δ9°C, n= 94; 2h – Sham, n=100; 2h - Δ0°C, n=33; 2h – Δ3°C, n=31; 2h – Δ9°C, n= 27; 6h – Sham, n=81; 6h - Δ0°C, n=37; 6h – Δ3°C, n=28; 6h – Δ9°C, n= 22; 24h – Sham, n=53; 24h - Δ0°C, n=39; 24h – Δ3°C, n=48; 2h – Δ9°C, n= 31)

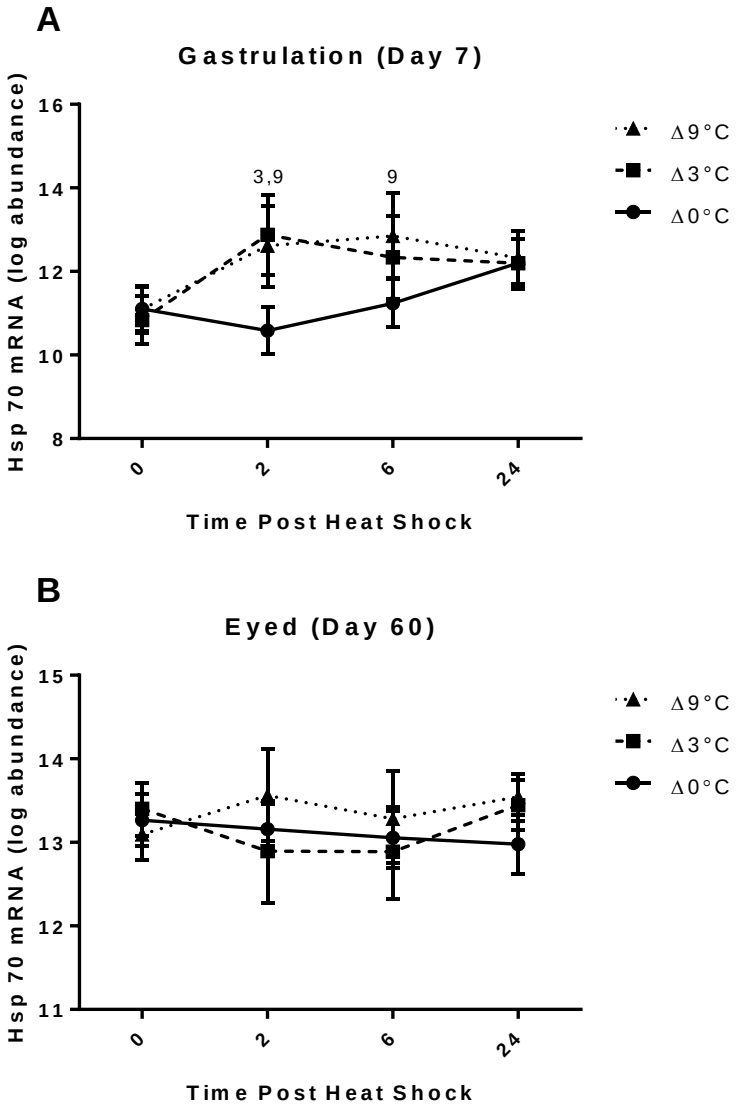


**Figure 5**. *Hsp70* mRNA levels (log abundance arbitrary units) for lake whitefish embryos at (A) gastrulation (n=5 for all, except, 2h – Δ9°C, n=4) or (B) eyed stage (n=5 for all except, 24h - Δ0°C, n=4; 2h – Δ3°C, n=3) following a heat shock (HS) of 3, or 9 °C above the acclimation temperature of 2 °C for 2 h. Δ0°C did not receive a heat shock. Following HS, fish were returned to control temperatures and allowed to recover for three different durations (2, 6, or 24 h) prior to sampling. Data were modeled using a Poisson-lognormal generalized mixed model fitted with a Bayesian Markov Chain Monte Carlo model and β-actin as a prior. Data represent the posterior means ± 95% credible intervals. Differences between treatment groups were accepted as statistically significant if the 95% credible intervals were non-overlapping. Data points labeled with 3, or 9, indicate that the 3, or 9 °C HS temperature group within that recovery time is significantly different from the control group**.**

# Chapter 3

# Discussion

The goal of this thesis was to investigate the effects of combined thermal and radiological stressors during the embryonic development of lake whitefish. The effects of single stressor exposures during lake whitefish embryonic development has been investigated for thermal (Brooke, 1975; Eme *et al*., 2015; Lee *et al*., 2016; Lim *et al.*, in press; Mueller *et al*., 2015; Price, 1940; Stefanovic *et al*., 2016; Thome, *et al*., 2016), radiological (Mitz, 2016; Thome, *et al*., 2017a), and chemical (Lim, 2016; Thome, *et al*., 2017b) stressors. These classes of stressors have been of interest due to their potential presence in once-through cooling discharge waters from thermal power plants. In the environment, aquatic species may be exposed to a combination of these stressors, which may result in additive, synergistic or antagonistic interactions. While a recent study has looked at the combination of thermal and radiological stressors using lake whitefish embryos (Thome, *et al*., 2017a), the mechanism behind the interaction of these stressors was not determined. Like the study by Thome et al (2017a), this thesis used radiation doses many orders of magnitude (>1000-fold) greater than what is considered to be environmentally relevant to produce an appropriate level of response (ie. embryo mortality) that would allow for visualization of an adaptive response (Bréchignac and Barescut, 2003). We observed that acute radiation exposure during the eyed stage of development in lake whitefish embryos resulted in decreased time to 50% hatch, decreased body size, decreased yolk consumption, and increased mortality at hatch. When embryos were treated with a HS prior to an acute radiation exposure, an adaptive response was not observed. However, a heat shock response (HSR) based on *Hsp70* expression was not induced at the eyed stage. A HSR was induced at gastrulation

suggesting that an adaptive response may be possible in LWF embryos but that it will be dependent on the developmental stage, the magnitude and timing of the HS, and the radiation dose.

### 3.1. Stress and effects on growth and hatch timing

Chapter 2 examined the effects of ionizing radiation exposure on lake whitefish embryos. Lake whitefish embryos that were exposed to acute radiation stress during the gastrulation (7.75 Gy; Thome *et al*., 2017a), organogenesis (15.5 Gy; Thome, *et al*., 2017a), and eyed stage (20 Gy; Chapter 2) of development had an earlier time to 50% hatch and decreased body size at hatch. These studies suggest a positive relationship between time to hatch and hatchling size. For whitefish embryos exposed to acute radiation stress, both time to hatch and hatchling size was decreased, at least for early to mid development stages. This response was not limited to acute radiation stress, but appears to be a generalized stress response in fish embryos (reviewed in Bonga, 1997).

Reductions in growth and earlier time to hatch are commonly seen in fish exposed to different kinds of stress, particularly when the stressor is experienced during an early development stage (reviewed in Rice, 1990; Bonga, 1997). Reduction in growth and earlier hatch have been reported in numerous single stressor experiments on lake whitefish embryos, including experiments with temperature and chemical exposures. Higher incubation temperatures have been reported to decrease time to hatch and decrease body size at hatch in lake whitefish (Brooke, 1975; Griffiths, 1980; Mueller *et al*., 2015). Thermal shifts during development have decreased time to hatch and

decreased body size (Muller *et al*., 2015). Chemical stressors decreased time to 50% hatch by ~20 days with exposure from fertilization to hatch to constant morpholine (anti-corrosive; >500mg/L) in lake whitefish; body size at hatch decreased concomitantly (Thome, *et al*., 2017b). Round whitefish (*Prosopium cylindraceum*) exposed to morpholine at concentrations >500 mg/L for the entirety of embryonic development showed decreased time to hatch and decreased body size at hatch (Lim, 2016). Early hatching and reduction in body size at hatch have been shown in other fish species exposed to increased incubation temperatures (reviewed in Blaxter, 1991), acute radiation (reviewed in Blaylock and Trabalka, 1978), and heavy metals (reviewed in Jezierka *et al*., 2009) during embryogenesis. The effects of morpholine on hatchling size and time to hatch has not been studied in any other fish species.

A reduced hatchling size and earlier hatch with irradiation could have been the result of stress affecting the growth efficiency of the embryo. Decreased growth efficiency can be reflected in a decreased yolk conversion efficiency (%; YCE), where embryos are overall less efficient at converting their yolk into body mass (Section 2.4.2). YCE is not a commonly studied endpoint, with many studies focusing just on yolk size (reviewed in Schreck *et al*., 2001). Fish size and growth rate during the yolk absorption period from fertilization to post-hatch, are functions of the amount of yolk present, the rate of yolk absorption and the efficiency of converting yolk into somatic tissue (reviewed in Schreck *et al*., 2001). Lake whitefish embryos exposed to stresses of various types not only had decreased time to hatch and reduced body size at hatch, but also showed stress increased yolk size (Section 2.4.2; Lee *et al.*, 2016; Lim *et al.*, in press;

Mueller *et al*., 2015; Thome, *et al*., 2017b). Fish have been shown to cope and respond to stress by changing physiological functions to reallocate energy towards a stress response through compensatory energy partitioning (reviewed in Rombough 1994; Schreck *et al*., 2001). YCE may be an indicator of this energy reallocation, which is directly related to fish size and growth rate (reviewed in Blaxter, 1969). YCE, body size and time to hatch decreased in lake whitefish embryos as they were exposed to increasing incubation temperatures (Mueller *et al*., 2015; Lim *et al*., in press), acute radiation (Section 2.4.2) and morpholine (Thome *et al*., 2017b). Overall, these studies suggest that a positive correlation between hatch time and hatchling size, which may be affected by YCE. Since YCE can be impacted by stress and has the potential to affect growth and time to hatch, future studies may wish to examine the specific mechanisms that alter YCE and growth efficiency such as metabolic enzymes or metabolite profiles.

### 3.2. Yearly variation in embryo radiosensitivity

In this thesis, a 10 Gy nominal dose during gastrulation in lake whitefish embryos increased mortality at hatch by ~10-15% (embryos collected in 2016; Chapter 2) and 35-40% (embryos collected in 2015; Appendix A). Previously, a 10 Gy nominal dose at gastrulation resulted in ~65-80% mortality at hatch (embryos collected in 2012-2014; Thome *et al*., 2017a). The discrepancies between the radiation induced mortality in this thesis and in the study by Thome *et al*. (2017a) may have been due to yearly differences in radiosensitivity. Parameters that reflect inherent radiosensitivity of an organism can include the nuclear material content, cell repopulation, tissue and organ regeneration, and biological repair (reviewed in Harrison and Anderson, 1996). Functions such as tissue

and organ regeneration and biological repair involve metabolic processes, which can be modified by environmental factors (reviewed in Harrison and Anderson, 1996). In the case of fish embryos, these metabolic and repair processes could be affected by yearly variations in embryo quality due to environmental factors (see section 3.3). Stressed embryos may reallocate energy resources towards maintaining repair mechanisms, changing overall radiosensitivity.

The challenge for examining the adaptive response is that experiments must be designed to include potential antagonistic interactions between stressors. A reasonable positive response, well above background, is needed to detect if that response is decreased. To do this, it is best to be in the middle of the dose response curve. Low amounts of radiation-induced mortality in each year of this study (Chapter 2; Appendix A) made it difficult to distinguish or measure any protective effect of HS. Due the low radiation-induced mortality, this study was unable to adequately test the hypothesis on adaptive response. To increase radiation-induced mortality and compensate for yearly differences in radiosensitivity, future studies could utilize a range of acute radiation doses at each developmental point. Using previously established $LD_{50/hatch}$ radiation doses, multiple doses above and below the $LD_{50/hatch}$ dose could be selected to give a range of mortality at hatch. Including a number of irradiation doses would more likely provide sufficient mortality needed to observe an adaptive response, despite possible changes in radiosensitivity between years.

### 3.3. Yearly variation in background mortality and embryo quality

An indicator of fish health and the quality of the embryos between years is background mortality, the naturally occurring mortality from fertilization to hatch in embryos reared under control conditions (reviewed in Shulʹman and Love, 1999). Background mortality was 80% (embryos collected in 2016; Chapter 2) and 60% (embryos collected in 2015; Appendix A) for this study, and ~15% in previously published research (embryos collected in 2012-2014; Thome, *et al*., 2017a). Experimental rearing conditions between this thesis and Thome *et al*. (2017a) were nearly identical, suggesting that a difference in overall embryo quality may be the cause of such a large difference in background mortalities between the studies. Quality of embryos may change year to year and across the spawning season. The quality of milt and egg can be influenced by many factors such as feeding regime; environmental factors; quality of the feed; variations between individuals; age, weight, length of the fish; stress; pollutant exposures; fungi, and bacteria; uptake of nutritive and genetic materials; and physiochemical properties of water (pH, salinity and temperature and dissolve oxygen; reviewed in Bobe and Labbé, 2010; Ochokwu, *et al.*, 2015; Schreck, *et al.*, 2001).

Many factors during the adult lake whitefish collection may have influenced resulting embryo quality each year, including the condition of the fish at the time of collection and the timing of collection within the spawning period. Spawning fish were collected using gill netting. Gill netting is a highly stressful method of fish collection where fish may be held for upwards to 24 hours before being removed. This constant stress to the fish may decrease gamete viability in fish (reviewed in Bonga, 1997). In

2015-2016, some of the fish were already dead when pulled into the boat, suggesting stressful conditions overnight in the gill net. In 2016-2017, approximately one third of the fish were dead at collection. Utilizing less stressful fishing methods such as fish traps may improve survival, but due to the severe conditions during spawning season and the number of fish required for experiments, this was not possible.

The timing of gillnetting varies each year and is very weather dependent. The spawning period of lake whitefish is fairly short, only 10-14 days, which further decreases sampling opportunities. The timing of fishing within the spawning period is an important factor that affects fertilization capacity (number of eggs fertilized) in fish (reviewed in Kjorsvik, *et al.*, 1990). Fish eggs may be viable anywhere from 1 hour to 2 weeks post ovulation depending on the species, with over-ripening and decreased hatching rate occurring after this period (reviewed in Kjorsvik, *et al.*, 1990). Immature or unripened eggs have a much lower hatching rate compared to eggs taken during the viable ovulation window (reviewed in Kjorsvik, *et al.*, 1990). The timing of fishing may be an important consideration for minimizing variations between experiments. During the 2015-2016 fishing season (Appendix A), lake whitefish were collected near the end of the spawn, as was indicated with majority of the females being spent (already deposited their eggs). Only a small amount of eggs were retrieved, with a portion of them being pale and unhealthy in colour (reviewed in Kjorsvik, *et al.*, 1990). During the 2016-2017 fishing season (Chapter 2), half of the females were ripe and the other half were unripe (were not spent) suggesting this collection took place near the beginning of the spawn. Of the eggs collected, some were pale yellow and mixed with blood, suggesting unripened and poor-

quality eggs (reviewed in Kjorsvik, *et al.*, 1990). Ideally, fish should be collected mid-spawn to maximize the number of ripe individuals collected and the quality of the gametes.

To improve fish embryo quality, some modifications to the sample collection process may be advantageous. Any fish that are dead or presumed dead should not be used. Viability and quality may differ between individuals, so separating eggs by females, rather than pooling eggs from different females at fertilization, would decrease the chance of mixing viable and unviable eggs together. Monitoring each set of fertilized eggs for a few days after fertilization could determine which females had viable eggs (ie. larger number fertilized and greater % surviving), and those with less viable eggs could be disposed to save resources and time. After a set period of time, the embryos from individual females with high embryo survival could be pooled together to reduce maternal effects on experiments. Timing in spawn could be monitored by observing the number of spent versus unspent females. If some or the majority of fish are unspent, efforts should be made to go back out in the field within a few days to catch females closer to the middle of spawn.

### 3.4. Heat shock response in cold-water species

The ontogeny of the heat shock response (HSR) has been studied extensively in model fish species, including zebrafish (*Danio rerio*; Krone *et al.*, 2003) and medaka (Werner, *et al*., 1986). In both model species, heat shocks (HS) induced *Hsp70* mRNA expression and an HSR as early as gastrulation and the HSR was maintained through the

rest of development (*Oryzias latipes*; Werner, *et al*., 1986; *Danio rerio,* Krone *et al.*, 2003; Yamashita, *et al*., 2010). Contrary to this well documented response pattern, data in this thesis suggest that lake whitefish embryos may not be capable of mounting an HSR after exposure to a HS at the eyed stage, even though a HSR was possible at gastrulation. A 9°C HS for 2 hours at gastrulation induced *Hsp70*; gene induction remained elevated at 2 and 6 hours post heat shock but returned to baseline at 24 hours post heat shock (Figure 5A, Section 2.4.3). Yet, no HSR was seen at the eyed stage when exposed to the same HS (Figure 5B, Section 2.4.3). However, HSR induction with similar HS conditions (3 and 9°C HS for 2 hours) has been previously documented at the fin flutter stage (Stefanovic *et al*., 2016). At fin flutter, lake whitefish embryos had slowly elevated *Hsp70* mRNA levels for upwards of 48 hours post heat shock (Stefanovic *et al*., 2016). Lake whitefish are not the only species to show a stage specific HSR during development. In Atlantic salmon (*Salmo salar*), every stage of development from gastrulation to hatch showed an increase in *Hsp70* mRNA expression in response to HS, except for the end of the segmentation stage (~prior to the eyed stage; Takle, *et al*., 2005). The cause of the loss of HSR at the eyed stage is unknown. One major distinction across HSR studies in embryos is the natural incubation temperature of the species. HSR was induced as early as gastrulation and then inducible at all subsequent developmental stages (Werner, *et al*., 1986; Yamashita, *et al*., 2010) in tropical, warm-water species while HSR was not inducible prior to gastrulation and at the eyed stage (Chapter 2; Takle, *et al*., 2005), in temperate, cold-water species. This may suggest that HSR ontogeny in warm-water model species may not be universally applied to cold-water fish species. The ontogeny of

HSR in cold-water fish is not well studied and future studies on coregonids, salmonoids and other cold-water species may provide better insight on species-specific HSR.

This thesis used a cold-water species incubated at 2°C to study Hsps and HSR. Studies on the HSR during embryogenesis in cold water species is limited, however there are studies that have looked at Hsp expression in adult cold-water fish. Some cold-water species had lower levels of constitutive Hsps under normal incubation temperatures compared to warm-water species. Specifically, a study showed that three cold water Antarctic *Trematomus* species had significantly lower levels of 70 kDa Hsp isoforms than their temperate confamilial from New Zealand waters (Carpenter and Hofmann, 2000). One species of Antarctic notothenioid fish, *Trematomus bernacchii*, has lost the ability to induce Hsps in response to exposure to acute thermal stress due to constant subzero temperatures (Buckley, 2004). Seasonal temperatures have affected constitutive Hsp expression in fish (*Pimephales promelas, Salmo trutta, Ictalurus natalis and Ambloplites rupestris*; Fader, *et al.*, 1994). During winter months, Hsp70 protein levels were lowest, the highest Hsp 70 protein levels were in spring (Fader, *et al.*, 1994). It could be inferred that for lake whitefish embryos that incubate during winter months, constitutive levels of Hsps may be lower compared to embryos from species who spawn in warmer months.

Cold incubation temperatures may reduce the rate of induction of the HSR in lake whitefish embryos. The rates of biochemical processes are determined by the kinetic energy of the reactants and rates of protein-based activity (e.g. enzymatic catalysis) and are altered by changes in temperature (reviewed in Somero, 1995). At 2°C, cellular processes in the lake whitefish embryos slow down, including transcription and

translation. At such low temperatures, induction of *Hsps* may take hours to days, versus minutes to hours as seen in warm-acclimated species (reviewed in Lindquist, 1986). Overall, differences between cold water and warm water species, such as spawning season and incubation temperatures, may have significant impacts on HSR. These differences must be taken into account when generalizing results from warm-water to cold-water species.

### 3.5. Conclusions and future directions

This thesis has provided new insight into the ontogeny of both heat shock response (HSR) and radiosensitivity in lake whitefish. The information gathered from this thesis and from other studies (Stefanovic, *et al.*, 2016; Thome, *et al*., 2017a) on lake whitefish embryos have provided the means for improving and directing a future adaptive response study. One important point to consider for future adaptive response studies is the ontogeny aspect of radiosensitivity. Radiosensitivity has been shown to change throughout lake whitefish embryonic development (Section 2.4.1; Appendix A; Thome, *et al.*, 2017a). To observe an adaptive response in lake whitefish embryos, the appropriate radiation dose must be applied at each chosen developmental stage to induce a sufficient level of mortality. This thesis strongly suggested yearly variations in embryo radiosensitivity, implying that using previously established $LD_{50/hatch}$ radiation dose responses may be difficult to apply in future studies. A solution to this could be adding more doses during the experiment to create a range of mortality. These doses would be both higher and lower than previously established $LD_{50/hatch}$ to account for yearly variations in radiosensitivity.

The ontogeny of HSR is another aspect to consider for future studies on adaptive response. HSR was inducible at gastrulation and fin flutter, but not at the eyed stage (Section 2.4.3; Stefanovic *et al*., 2016). Whether HSR can be induced during many important developmental stages is still unknown. To better understand HSR at each of these stages, various combinations of HS durations and magnitudes would need to be tested. Future studies may want to use larger HS magnitudes (Δ12-15°C) to potentially illicit a faster and longer HSR. The tradeoff for increasing the HS magnitude is potentially using HS that are too high and that may kill the embryos. This information, coupled with previous studies (Stefanovic, *et al*., 2016), would help provide the rationale in choosing developmental points for future adaptive response studies.

Once sufficient information has been obtained on the ontogeny of the HSR and radiosensitivity in lake whitefish, a successful adaptive response study can be designed. Using information on HSR during development, appropriate developmental stages can be selected where a HSR is known to occur. Once developmental points are identified, radiosensitivity at those stages can be analyzed, and appropriate radiation doses can be selected. Finally, lake whitefish embryos can be subjected to thermal and radiological stress to determine if an adaptive response is possible. Realistically, this process would have to occur over more than one season due to the large amount of work and embryos required. Overall, radiosensitivity, HSR, and choice of developmental points are key factors that must be considered for any future adaptive response study to be successful.

HSR is induced by multiple forms of stress other than heat, including UV, γ-rays, chemicals and general oxidative stress (Matsumoto *et al*., 1994; Santoro, 2000). While

this thesis focused on mild HS followed by radiation as a possible multiple stressor design, other multiple stressor experiments are possible. A future avenue for research could include looking at the ability of other stressors to induce HSR in lake whitefish embryos. Specifically, rather than applying a mild thermal shock, a chronic low dose irradiation could potentially induce a similar HSR and potentially infer an adaptive response. Chronic low dose radiation exposure to mice increased Hsp70 levels after 4 weeks of exposure, inducing a HSR (Nogami *et al*., 1993). A HSR has been induced by thermal stress in lake whitefish (Section 2.4.3; Stefanovic *et al*., 2016), but the effects of chronic low dose radiation on heat shock protein expression in this species is still unknown. As with heat shocks, kinetics of the HSR could vary depending on magnitude and duration of the chronic low dose irradiation treatment, as well as the stage of development at the time of exposure. Manipulation of these variables would provide a better understanding of HSR in developing fish, while also providing an overall better understanding of the underlying mechanism of the HSR.

If chronic low dose radiation could induce a HSR in lake whitefish embryos, future work could include studying the potential of chronic low dose radiation providing an adaptive response. Specifically, by reversing the order of stressors from this thesis, future studies could apply chronic low dose radiation, followed by an acute thermal challenge HS in lake whitefish embryos. A large acute heat shock would rapidly create more damage than a HSR could account for, killing the organism (reviewed in Lindquist, 1986). However, if a HSR has been mounted prior to acute thermal stress, this may provide a protective effect. Many studies have looked at a priming mild heat shock,

followed by a subsequent challenge radiation treatment (Boreham and Mitchel, 1994; Shen, *et al.*, 1991; Thome, *et al*., 2017a), but there have been no studies on the effect of low dose radiation prior to an acute thermal heat shock. Previous work on lake whitefish and chronic low dose radiation have shown stimulated growth in embryos at doses as low as 0.06 mGy/day (Thome, *et al*., 2017a), and it would be interesting to see if this would translate to a protective effect when exposed to an acute HS.

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# Appendix A

# Effects of thermal and radiological stressors on mortality in lake whitefish (*Coregonus clupeaformis*) embryos from the 2015-2016 season

## A.1. Introduction

In addition to the experiments described in Chapter 2, lake whitefish embryos were collected in 2015 and utilized in experiments to study the interaction between temperature and radiation stress. The main differences between the experiments in Chapter 2 and this appendix are the developmental time points chosen and the timing between heat shock and irradiation. In 2015, the adaptive response was examined at gastrulation and organogenesis (versus gastrulation and eyed stage in Chapter 2). Further, embryos were irradiated at 2, 4, 8, 16 and 24 hours post heat shock (versus 2,6 and 24 hours post heat shock in Chapter 2). In both cases, embryos were give no heat shock or a 3 or 9°C heat shock for 2 hours, followed by an acute irradiation with a $^{137}$Cs at 10 or 15 Gy.

## A.2. Methods

### 2.3.1. Lake whitefish embryo collection and rearing

Lake whitefish adults were gill-netted on November 1, 2015 from Lake Huron. Eggs and milt were stripped from these fish (males= 14, females= 3) and gametes combined in a common pool, following methods described in Chapter 2.

At 5 days post fertilization (dpf), ~4500 embryos were transferred from the bell hatching jars at 5°C into 90 sterile petri dishes (100mm x 20mm) at a density and temperature as described in Chapter 2. Temperature loggers gave a mean water temperature of 2.2±0.3ºC (±standard deviation). The developmental stages occurred at 7 days (gastrulation) and 30 days (organogenesis) post fertilization (dpf).

### 2.3.2 Heat shocks

After 48 h at 2 °C, the embryos reached the gastrulation stage of development (7 dpf) and 54 petri dishes were exposed to transient heat shocks at Δ0°C (n=18 dishes), Δ3°C (n=18 dishes), or Δ9ºC (n=18 dishes) for 2 hours as described in Chapter 2. This same process was repeated at 30 dpf for the remaining 36 petri dishes.

### A.2.3. Acute irradiation post heat shock

Embryos were exposed to acute radiation at 2, 4, 8, 16, and 24 hours post heat shock at gastrulation (Day 7) or 4, 16 and 24 hours post heat shock at organogenesis (Day 30) using the same methods from Chapter 2. Embryos were irradiated on an ice slurry with either 10 Gy (at 7 dpf) or 15 Gy (at 30 dpf). No heat shock, radiation only controls (Δ0°C) were irradiated.

### A.2.3. Statistical analysis

Statistical analysis was run using GraphPad Prism 6. The percent mortality replicate dishes were compared using a two-way ANOVA followed by Tukey's HSD test.

### A.3. Results

There were no differences in mortality between embryos given no heat shock, 3°C heat shock or 9°C heat shock at either gastrulation (Fig. A1-A) or organogenesis (Fig. A1-B). Radiation treatment alone induced 95-100% mortality for both developmental points. Mortality was not different between embryos that were irradiated and those that

received irradiation at 2, 4, 8, 16 and 24 hours after heat shock, regardless of whether the irradiations were at gastrulation (Fig. A1-A) or organogenesis (Fig. A1-B).

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## A.5. Figures

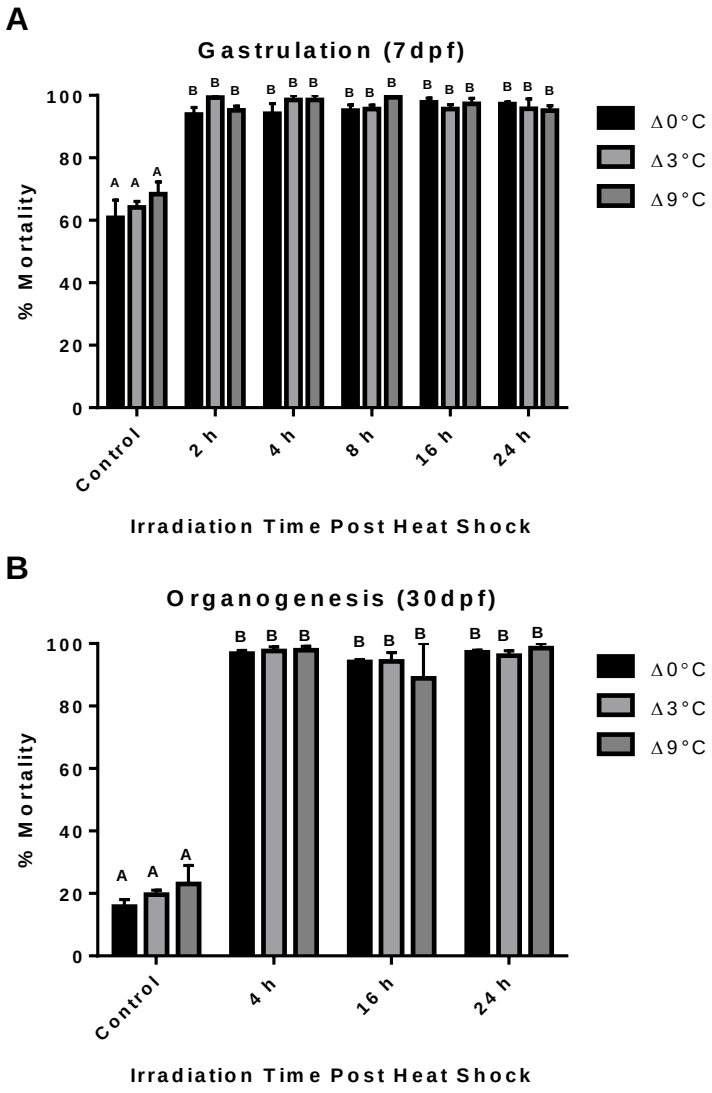


**Figure A1.** Mean mortality (from gastrulation to hatch or organogenesis to hatch) of lake whitefish embryos exposed to various combinations of heat shocks and radiation. Embryos were given a 2 hour heat shock at (A) gastrulation (Day 7; n=3), or (B) organogenesis (Day 30; n=3), followed by a 10 Gy (gastrulation) or 15 Gy (organogenesis) acute radiation exposure at 2, 4, 8, 16, or 24 hours post heat shock (gastrulation) or 4, 6, or 24 hours post heat shock (organogenesis). The total percent mortality was calculated at hatch. Percent mortality was compared between treatments using two-way ANOVA followed with Tukey's HSD test. Letters denote statistical differences between treatments. Bars represent means ± SEM.

# Appendix B

## Acute thermal $LD_{50(7days)}$ heat shocks in lake whitefish (*Coregonus clupeaformis*) embryos at gastrulation

## B.1. Introduction

Temperature is an increasingly relevant environmental factor that affects fish biology and distribution (reviewed in Somero, 2010). Increases in water temperature are due to many factors including natural causes, climate change, and anthropogenic sources (reviewed in Feder & Hofmann, 1999; Somero, 2010). Thermal effluents affect fish physiology including behaviour (Kelso, 1976) and size (Bennett, 1972). The effects of thermal waste have become an increasing concern when present during embryonic development. Many fish spawn in shallow parts of the lakes, rivers and streams and are potentially affected by industrial pollution. Additionally, embryonic development denotes a particularly sensitive period for exposure to environmental stressors (reviewed in Iguchi, *et al.*, 2001; Jezierska, *et al.*, 2009; McKim, 1977; Rombough, 1997). Fish have an optimal thermal range, where falling outside of this range can result in increased levels of mortality and developmental mutations (Pepin, 1991).

Thermotolerance in fish has been investigated using several experimental approaches, such as the incipent lethal temperature (ILT), critical thermal maximum (CTM) and chronic lethal maximum (CLM) methodologies (reviewed in Beitinger, *et al.*, 2000). ILT involves plunging the fish directly into various lethal temperatures, CTM involves raising the temperature constantly, linearly until a sublethal endpoint, while CLM is a variation of CTM, but uses much lower temperatures and rates of temperature change. All three approaches generate valuable, albeit different, information concerning the temperature tolerance of a species. CTM and CLM have become more prevalent in

recent studies (reviewed in Becker and Genoway, 1979; López-Olmeda and Sánchez-Vázquez, 2011; Lutterschmidt and Hutchison, 1997) due to the environmental relevance of the delta temperature (T) experienced by fish species, and the decreased handling stress. ILT is a useful technique in that the length of the experiment is shortened, however, the duration of the HS are not taken into account. Research on the limits and lethal dose in terms of duration and magnitude of heat shocks is limited, especially during development. This information may be pertinent to better understanding the thermotolerance of fish populations, while also being a useful tool in future multiple stressor studies. Thermotolerance through development has been looked at in fish (reviewed in Pörtner & Peck, 2010; reviewed in Rombough, 1997), but by analyzing lethal doses of temperature throughout development in greater depth, the change in thermotolerance over development can be better defined. This study investigated the thermotolerance at gastrulation in lake whitefish (*Coregonus clupeaformis*) embryos. This was accomplished by exposing lake whitefish embryos during the gastrulation stage to various magnitudes and durations of acute thermal stress, and measuring mortality to create an $LD_{50}$ curve for mortality after 7 days, denoted $LD_{50(7days)}$.

## B.2. Methods

### B.2.1. Lake whitefish embryo collection and rearing

Lake whitefish were collected at the same time as experiments in Appendix A and in-vitro fertilization was conducted as described in Chapter 2. One day post fertilization (dpf), ~8000 embryos were distributed into 160 sterile petri dishes (100mm x 20mm) at

the same density and temperature described in Appendix A. The developmental stage occurred at 8 days (gastrulation) post fertilization (dpf).

### B.2.2. Determining $LD_{50(7days)}$ with keyboarding

Keyboarding (Fig. B1) is a method that was designed to gauge a bracket of heat shock durations to determine $LD_{50(7\ Days)}$ acute exposure times with minimal embryo use (Mitz *et al.*, unpublished). Pools of embryos were transferred from 2°C into a beaker at one heat shock temperature. At 1 minute intervals (2 minute intervals for 25 and 30°C), 1 embryo was removed and placed into an individual well of a 48 well plate with 2°C water. Mortality was measured after 7 days and keyboards (Fig. B2) and approximate $LD_{50(7\ Days)}$ acute exposure times (min) were calculated (Table B1), based on the midpoint between the first embryo mortality and the last embryo surviving.

### B.2.3 $LD_{50}$ heat shocks

To estimate $LD_{50(7\ Days)}$ acute exposure times and determine reasonable times for the experiment at gastrulation (8 dpf), keyboarding was first completed 1 week prior (1dpf) to gastrulation. 42 embryos were transferred from 2°C into a beaker at one heat shock temperature (Δ10°C, Δ15°C, Δ20°C, Δ25°C, or Δ30°C). One minute intervals were used for Δ10°C, Δ15°C, and Δ20°C heat shocks and 2 minute intervals were used for Δ25°C, and Δ30°C heat shocks. Mortality was determined at 7 days and $LD_{50(7\ days)}$ acute exposure times for 1dpf were calculated. To determine the $LD_{50(7\ Days)}$ acute exposure times at gastrulation (8 dpf), heat shocks (HS) were administered to embryos at 8 dpf at 5 different heat shock temperatures (Δ10°C, Δ15°C, Δ20°C, Δ25°C or Δ30°C). Using

$LD_{50(7Days)}$ acute exposure times for Δ25°C and Δ30°C determined from keyboarding at 1 dpf (Fig. B2) and $LD_{50(7Days)}$ acute exposure time estimations for gastrulation for Δ10°C, Δ15°C, and Δ20°C based from Mitz *et al.* (unpublished); two heat shock durations lower and two heat shock durations greater than the approximated $LD_{50(7Days)}$ acute exposure time were used to create a bracket that would most likely encompass the actual $LD_{50(7Days)}$ acute exposure time for gastrulation (Table B1). These values were chosen to be approximately semi-logarithmic. Embryos (n=50, 3 replicates) were transferred from petri dishes into beakers at each temperature, and after each duration of time, embryos were transferred back into dishes with 2°C water. No heat shock control was also added for each HS temperature. Embryos were observed and mortality was noted at 7 days post heat shock. Cumulative survival curves were constructed using probit analysis and $LD_{50(7Days)}$ acute exposure durations were calculated for each temperature.

**B.3. Results**

Using a limited number of embryos, keyboarding gave approximate $LD_{50(7Days)}$ acute exposure times at 1 dpf of 40 minutes for Δ25°C and 6 minutes for Δ30°C, but was inconclusive for Δ10°C, Δ15°C and Δ20°C (Fig. B2). These approximations combined with results from a previous study (Mitz *et al.,* unpublished) were used to build the experimental design where more embryos and petri dish replicates were used to determine approximate $LD_{50(7Days)}$ acute exposure times at gastrulation (8 dpf) for each HS (Table B1). Cumulative mortality curves were constructed for Δ10°C, Δ20°C, Δ25°C, and Δ30°C HS of embryos at gastrulation for five durations; mortality was dependent on

the magnitude and duration of the HS (Fig. B3). Heat shock (HS) $LD_{50(7Days)}$ acute exposure durations at gastrulation increased from 3 minutes at a HS of Δ30°C, to 104 minutes at HS of Δ20°C, to 1500 minutes at HS of Δ10°C (Fig. B2). As temperature increased, $LD_{50(7Days)}$ acute exposure durations decreased following an inverse logarithmic relationship as described by the equation, $y= 44400e^{-0.33x}$ (Fig. 4B). Due to fungal growth, all Δ15°C HS durations had 100% and no $LD_{50(7Days)}$ was calculated.

**B.4. Discussion and conclusion**

Lake whitefish embryos at gastrulation showed a logarithmic relationship when comparing the magnitude of heat shocks (HS) against the duration. Generally, as HS increased from Δ10°C to Δ30°C, acute exposure times to reach $LD_{50(7\ Days)}$ decreased exponentially from over 1000 minutes to just 3. Neitzel and Becker (1985) showed that acute HS of Δ12°C for 1-8 hours decreased survival of chinook salmon (*Oncorhynchus tshawytscha*) cleavage eggs. Chinook salmon embryos showed a similar mortality trend as this study, where embryos could survive 8 hour exposures at Δ13°C, but could only survive Δ14.5°C for 2 hours (Neitzel and Becker, 1985). A study by Lamadrid-Rose and Boehlert (1988) using acute thermal exposure times (8- 24 minutes) looked at acute cold shock in tropical fish species (larval mahimahi, *Coryphaena hippurus*, manini, *Acanthurus triostegus*, and juvenile striped mullet, *Mugil cephalus)* where mortality increased with increasing absolute delta T compared to controls. As well, earlier stage eggs were more sensitive to heat shock (100% mortality for 0h post-fertilization, hpf, compared to 80% mortality for 12 hpf and 5% mortality for 24 hpf at Δ15°C), further

demonstrating increased thermotolerance through development (Lamadrid-Rose and Boehlert, 1988). Therefore, depending on whether fish species experience a large enough positive (heat shock, reviewed in Fry, 1971) or negative (cold shock, reviewed in Donaldson, *et al.*, 2008) delta T will result in increased mortality as the delta T diverges farther from the normal environmental temperature.

The acute lethal HS used in this study may be of future use when looking at multiple stressor experiments. Utilizing acute lethal HS and analyzing endpoints such as the expected mortality and malformation rate in fish species, other stressors can be used to determine synergistic or adaptive responses. As an example, radiological stress had adaptive responses with mild heat shock prior to a lethal radiation dose in lake whitefish (Thome *et al*., 2017), mice (Shen *et al*., 1991), and cell culture (Boreham *et al*., 1997). As well studies have examined low dose chronic radiation prior to challenge with acute lethal HS in cell culture (Takahashi, 2001). Having an extensive knowledge on HS $LD_{50(7days)}$ exposure durations will be a useful diagnostic tool in future studies that attempt to better understand mechanisms of thermotolerance in fish species, which has potential to be generalized to other organisms. Further research into developmental thermal tolerance in lake whitefish is required to determine whether thermotolerance continues to increase through development, or decrease at critical periods.

## B.5. References

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## B.6. Tables

**Table B1.** Durations of heat shocks (HS) used on lake whitefish embryos at gastrulation stage (8 dpf). Approximate $LD_{50(7days)}$ acute exposure durations for Δ25°C and Δ30°C used for 8 dpf were calculated from keyboarding HS exposures at 1 dpf. Approximate $LD_{50(7days)}$ acute exposure durations for Δ10°C, Δ15°C, and Δ20°C were estimated from preliminary work by Mitz *et al*. (unpublished). Values for Time 1, 2, 3, 4 for each HS were chosen to encompass the approximate $LD_{50(7days)}$ acute exposure durations and to be approximately semi-logarithmic. These durations were chosen to create cumulative mortality curves to find actual $LD_{50(7days)}$ acute exposure durations at 8 dpf.

| Duration of HS | Δ10°C (hrs) | Δ15°C (hrs) | Δ20°C (min) | Δ25°C (min) | Δ30°C (min) |
|---|---|---|---|---|---|
| **Control** | 0 | 0 | 0 | 0 | 0 |
| **Time 1** | 6 | 6 | 50 | 7 | 1 |
| **Time 2** | 12 | 12 | 150 | 20 | 3 |
| **~$LD_{50(7days)}$** | 24 | 24 | 300 | 40 | 6 |
| **Time 3** | 84 | 84 | 500 | 65 | 10 |
| **Time 4** | 168 | 168 | 800 | 90 | 15 |

## B.7. Figures

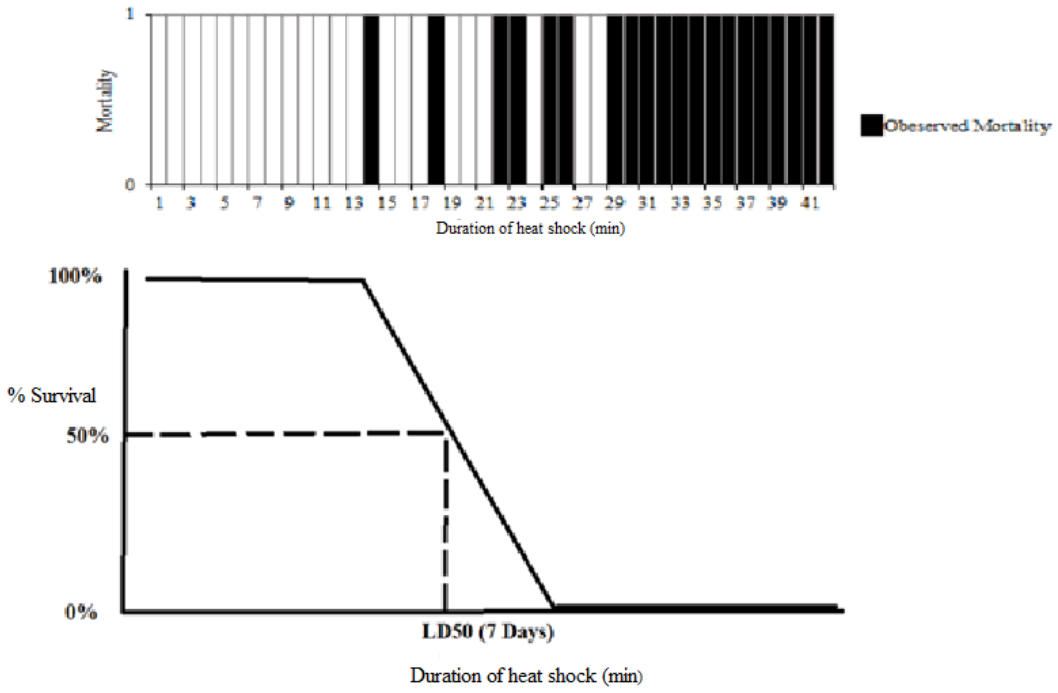


**Figure B1**. An example of keyboarding from which an approximate survival curve and $LD50_{7Days}$ was found. Approximate HS $LD_{50(7days)}$ duration was determined to be the duration of heat shock at the midpoint between the first embryo mortality (black bar) and last embryo surviving (white bar).

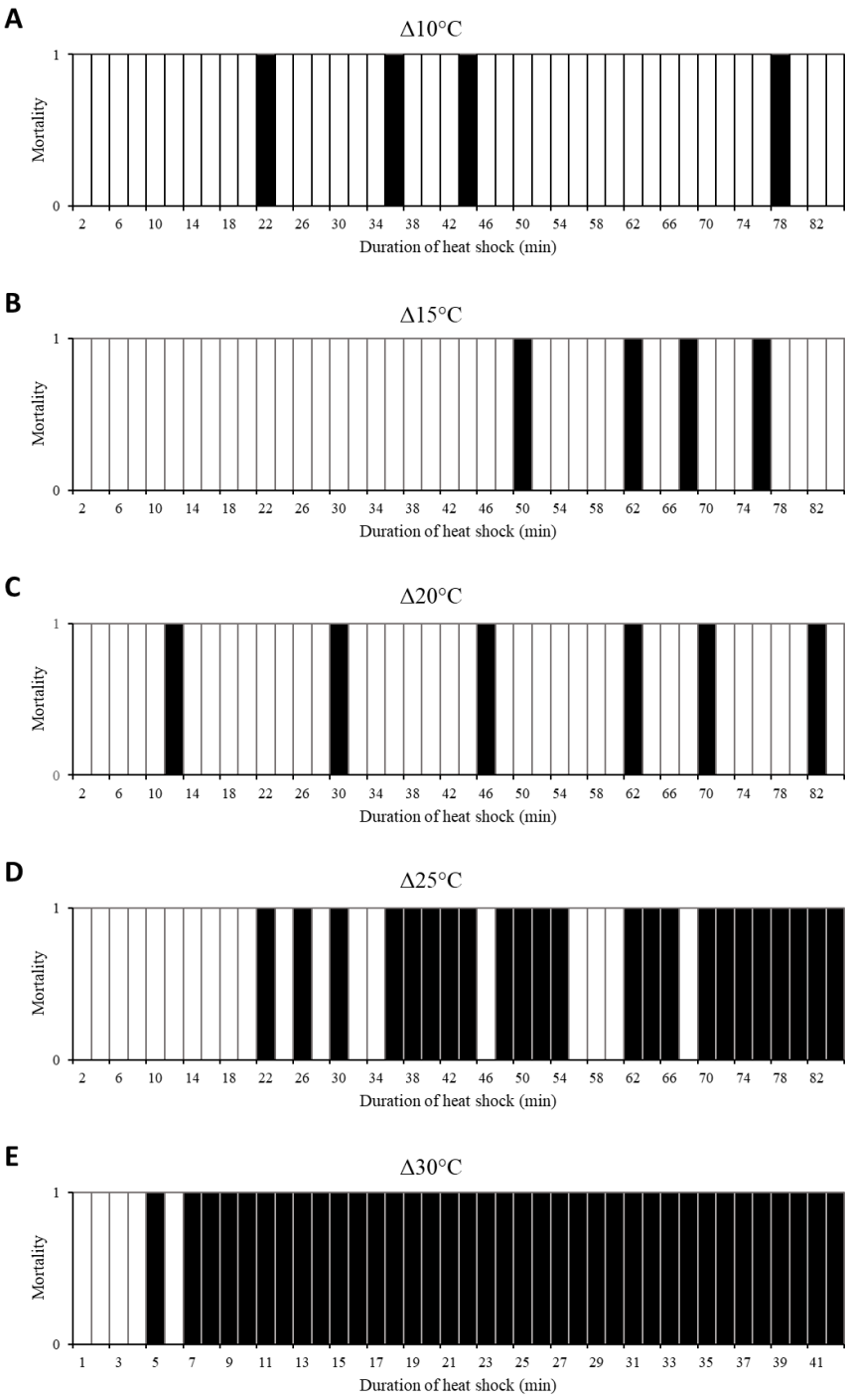


**Figure B2.** Keyboarding of mortality of lake whitefish embryos at 1 dpf. Embryos were exposed to Δ10°C (A), Δ15°C (B), Δ20°C (C), Δ25°C (D), and Δ30°C (E) heat shocks (HS) at varying durations (min) and placed into 48 well plates. Each black box represents 1 embryo in each well that was an observed mortality at that specific duration of HS. (n=42 total embryos per HS temperature, n=1 embryo per well)

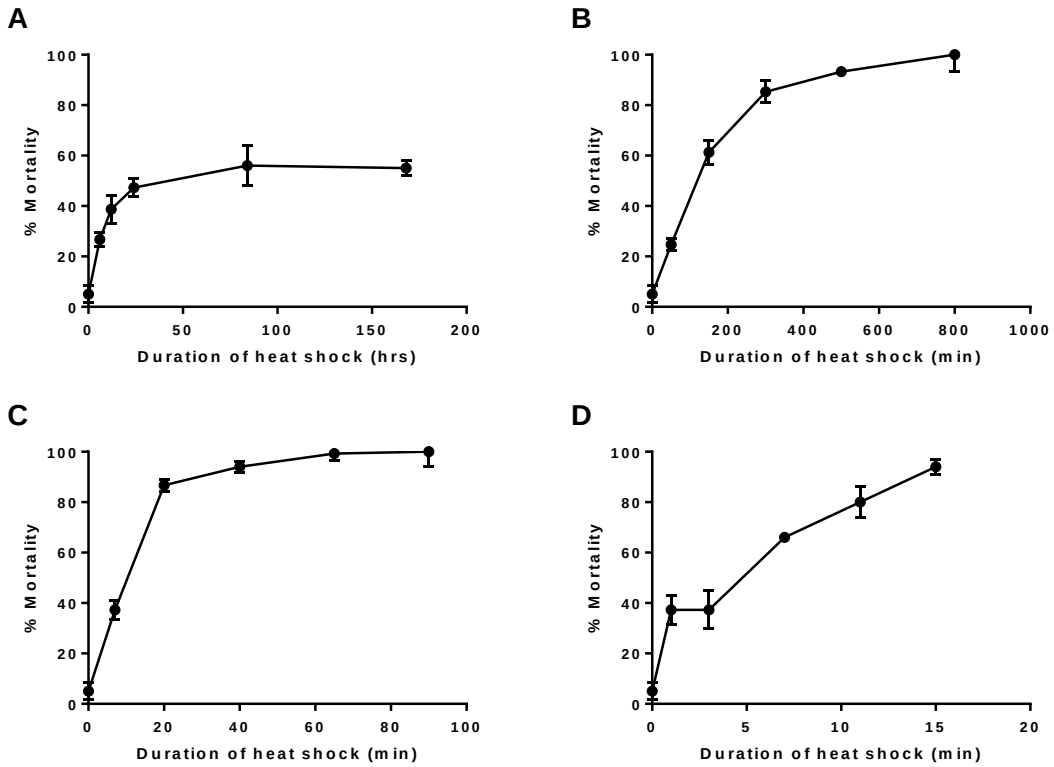


**Figure B3.** Lake whitefish embryonic mortality from acute heat shock exposure at various durations of heat shock during gastrulation (Day 8). Cumulative percent mortality 7 days after heat shocks at A) Δ10°C, B) Δ20°C, C) Δ25°C, and D) Δ30°C. (n=3 at each duration of heat shock)

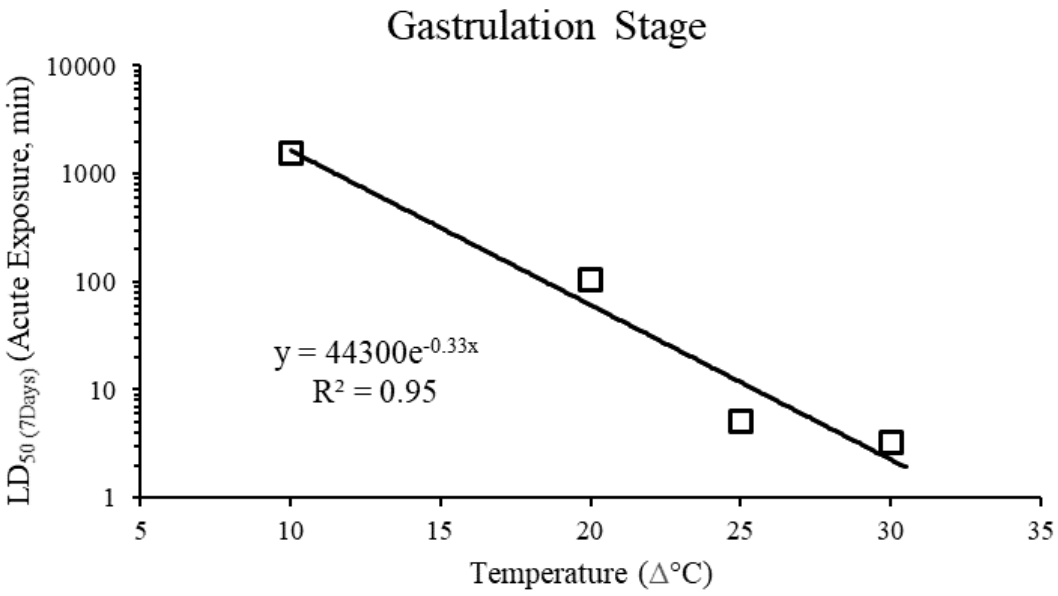


**Figure B4.** $LD_{50(7\ Days)}$ acute exposure times for various heat shock (HS) temperatures on lake whitefish embryos at gastrulation. $LD_{50(7\ Days)}$ acute exposure times (min) were calculated for Δ10°C, Δ20°C, Δ25°C, Δ30°C using probit analysis and plotted on a semi-logarithmic scale. Line of best was plotted and calculated to be $y= 44400e^{-0.33x}$, with an $R^2=0.95$.