# ENVIRONMENTAL STRESSOR EFFECTS ON WHITEFISH EMBRYOGENESIS

# THERMAL, MORPHOLINE, AND RADIATION STRESSOR EFFECTS ON THE EMBRYONIC DEVELOPMENT OF LAKE (*COREGONUS CLUPEAFORMIS)* AND ROUND WHITEFISH (*PROSOPIUM CYLINDRACEUM)*

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TITLE: Thermal, morpholine, and radiation stressor effects on the embryonic development of lake whitefish (*Coregonus clupeaformis*) and round whitefish (*Prosopium cylindraceum*)

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## Lay Abstract

Lake and round whitefish are cold-adapted freshwater species. Both species play important ecological roles, with lake whitefish generally perceived as more economically and culturally important. Many studies have detailed lake whitefish embryonic development under constant stressors (e.g. temperature) but there are relatively few studies on round whitefish embryonic development. Both species experience seasonal temperature fluctuations in nature and may experience additional anthropogenic temperature, chemical, and radiation stress due to discharge from once-through cooling processes at thermal power plants, which may contain low levels of morpholine and radiation. Our study suggests that round whitefish embryos are more sensitive to elevated temperature and morpholine levels, but less impacted by chronic low-dose irradiation relative to lake whitefish embryos. The growth and development of both species are significantly affected by seasonal temperature changes.

**Abstract**

Lake and round whitefish are cold-adapted freshwater species with similar life histories and spawning behaviours. There have been several studies on the embryonic development of both species (particularly for lake whitefish), most utilizing constant temperatures. However, temperatures fluctuate in the field due to natural (e.g. seasonal changes) and anthropogenic (e.g. water discharged from once-through cooling processes) effects. Releases from once-through cooling processes may contain low levels of chemicals (e.g. morpholine) and radiation (e.g. tritium). This thesis examined and compared the impacts of thermal, morpholine, and radiation stressors on lake and round whitefish embryogenesis.

To examine the effects of fluctuating incubation temperatures, lake and round whitefish were reared at constant temperatures, with seasonal temperature declines/inclines, transient temperature spikes, or seasonal temperature changes combined with temperature spikes. Round whitefish embryos had significantly higher mortality when reared at 8°C compared to lake whitefish, and seasonal temperature changes impacted development rate, growth, and hatch dynamics for both species. Temperature spikes had relatively little effect on development.

The effects on embryonic development of chronic morpholine and low-dose radiation exposures were examined in round whitefish to compare with existing data in lake whitefish. Round whitefish embryos were more impacted by morpholine than lake whitefish (larger effects on growth and mortality at relatively lower concentrations) and

less impacted by low-dose radiation (little effect on growth or hatch dynamics). Post-hatch, round whitefish embryos reared at 8°C, with rapid seasonal inclines, or with 500 mg $L^{-1}$ morpholine had elevated mortality. All irradiated embryos had decreased mortality post-hatch compared to non-irradiated embryos. Thus, embryonic exposure to all stressors examined appears to alter post-hatch survival.

This thesis better defines the effects of fluctuating incubation temperatures, chronic morpholine, and chronic radiation exposures on the embryonic development of lake and round whitefish. It also suggests that embryonic incubation conditions are important beyond hatching.

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## Contributions

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**List of Abbreviations**

CANDU: Canadian deuterium uranium

DNA: Deoxyribonucleic acid

dpf: Days post fertilization

dph: Days post-hatch

ANOVA: Analysis of variance

SEM: Standard error of the mean

mRNA: Messenger ribonucleic acid

bpm: Beats per minute

$O_2$: Oxygen

$S_A$: Seasonal (+1ºC/week warming period)

$S_B$: Seasonal (+2ºC/week warming period)

$SP_A$: Temperature spikes (between ~43-99dpf)

$SP_B$: Temperature spikes (from 1dpf until hatch)

M#: M refers to morpholine; # refers to concentration in # mg $L^{-1}$

TLD: Thermoluminescent dosimeter

UV-B: Ultraviolet - B

# Chapter 1

# Introduction

Incubation temperature has long been regarded as an important abiotic factor for fish embryo development (see Blaxter, 1992; Rombough, 1997). Different species have varying optimal temperature ranges, in which embryos develop and hatch normally. As incubation temperature approaches the limits of this range, there can be strong effects on embryo development including changes to development rate, body growth rate, yolk consumption rate, increased abnormality rate, and decreased survival to hatch; especially in species that possess narrow ranges and/or long development periods (see Blaxter, 1992; Pepin, 1991). While survival decreases towards either end of the optimal range, at colder sub-lethal temperatures embryos develop more slowly with decreased growth and yolk consumption rates, and at warmer sub-lethal temperatures embryos develop and grow more rapidly with less efficient yolk-to-body conversion (see Blaxter, 1992). Due to increased global concern regarding elevated water temperatures, there has been a resurgence in studies regarding the impacts of temperature on embryo development, especially in areas impacted by human activities. The studies described in this thesis focus on impacts of temperature on the cold-adapted freshwater lake and round whitefish, for which some developmental impacts of temperature have already been described (e.g. Eme et al., 2015; Mueller et al., 2015; Patrick et al., 2013).

## 1.1 Lake whitefish and seasonal temperature changes

Lake whitefish are native to North America and can be found in many areas including the Great Lakes, many northern lakes/rivers (such as Great Slave Lake), and even brackish waters (Macpherson et al., 2010; Rawson, 1951). During the spawning

season, typically late autumn, lake whitefish will lay their eggs in shallow water as water temperatures decline (~6-7°C). These eggs are fertilized, incubate over winter in cold water (~0.5-4°C), and hatch in the spring as water temperatures increase (Price, 1940). As a result of this long incubation period, embryos are exposed to large seasonal temperature changes, spanning a range of over 6°C. The long incubation period provides the opportunity to observe and measure cumulative low level impacts of stressors at key points of embryonic development that would otherwise be difficult with more rapid developing warm-adapted species. Several studies have identified and described the different stages of development in lake whitefish including a 31-stage guide outlined by Price (1940) and a more recent 18-stage guide developed by Sreetharan et al. (2015). These stage guides highlight key development characteristics including first cell divisions, eye colour onset, and behavioural differences such as fin flutter. Using these guides, studies have quantified differences in development rate, growth, heart rate, $O_2$ consumption, and mortality for a wide range of constant incubation temperatures (e.g. 0.5ºC-12 ºC; Brooke, 1975; Eme et al., 2015; Mueller et al., 2015; Price, 1940). Studies like those conducted by Brooke (1975) found an increased development rate at warmer temperatures, in which embryos reared at 2ºC took nearly 150% longer to reach the eye pigmentation stage than those reared at 7.8ºC. Studies also consistently observed >35% increased mortality (Brooke, 1975; Mueller et al., 2015; Price, 1940), a >50% reduction to 50% hatch (Brooke, 1975; Mueller et al., 2015), and ~50% shorter hatch windows (defined as the length of time between the first and last embryo hatching; Brooke, 1975; Mueller et al., 2015) at 8ºC compared to cooler 2ºC incubation temperatures. Embryos

raised at warmer temperatures (e.g. 8ºC) had shorter incubation periods, decreased body size, increased yolk size, and overall less efficient growth (Mueller et al., 2015).

Despite these advances in understanding lake whitefish embryonic development, there has been conflict regarding an 'optimal' incubation temperature range. The optimal incubation temperature range includes temperatures where there is increased survival, decreased abnormality incidence, and more efficient growth. Price (1940) stated that 0.5ºC, (the lowest temperature he tested) was the optimal rearing temperature for lake whitefish, while Brooke (1975) suggested this range should be higher (3.2-8.1ºC). A later study by Mueller et al. (2015) found that whitefish embryos developed more efficiently and with notably reduced mortality at 2ºC, below the range suggested by Brooke (1975). In addition, initial lower incubation temperatures (e.g. 2ºC) followed by higher incubation temperatures (e.g. 5ºC) at a later developmental stage could significantly reduce mortality compared to being solely raised at warmer temperatures (Mueller et al., 2015).

At constant temperatures, heart and oxygen consumption rates increased as incubation temperatures increased (see Benfey and Bennett, 2009; Dabrowski et al., 1984; Eme et al., 2015). It was also found that exposing embryos to different temperatures at important developmental stages (such as moving 5ºC embryos to 8ºC at the organogenesis stage), could significantly alter heart rate and oxygen consumption rate (Eme et al., 2015). As a result, arguably more importantly than a specific incubation temperature range, the work by Mueller et al. (2015) and Eme et al. (2015) demonstrated that lake whitefish embryos possess moments in development in which changing

temperature significantly affects embryonic development. These moments are referred to as critical windows.

Critical windows have been described as moments in development where an organism is more susceptible to sub-lethal stressors, in that it will adjust its phenotype due to intrinsic or extrinsic factors (such as temperature). For instance, if an 8ºC embryo were to be shifted to a 2ºC incubation treatment at gastrulation, it would have an increased survival rate, later 50% hatch, and grow more efficiently than a constant 8ºC embryo, suggesting that the gastrulation stage is a critical window for temperature in lake whitefish embryos (Mueller et al., 2015). This can be seen on the level of a specific tissue, organ, or organ system (see Burggren & Reyna, 2011). After a stressor and/or the initial response have ended, the organism may be able to return to a state similar to if the stressor had not occurred. In other cases, the response to the stressor permanently alters development, with effects that can be observed at the adult stage (Burggren & Reyna, 2011). By combining the possible critical window period identified by Mueller et al. (2015) with studies that monitor temperature changes in the field (e.g. Patrick et al., 2013), a critical window is predicted to coincide with the seasonal transition from autumn to winter. As water temperatures begin to rapidly decrease during this time, lake whitefish embryonic development and survival may be significantly affected. Unfortunately, most previous studies on lake whitefish embryonic development have used constant incubation temperatures or regimes that lake whitefish would unlikely experience in the field. Even for studies that recreated field temperature conditions (e.g. Patrick et al, 2013), there was no distinguishing between natural and anthropological effects, and no information on

differences in development or growth rate, if any. A more recent study by Thome et al. (2016) reared embryos in the field, some of which were reared in areas impacted by anthropological temperature stressors. This study revealed significantly increased hatch size when exposed to anthropological impacts compared to seasonal changes alone, but lacks information on embryonic development before hatch, and these embryos were not exposed to the water temperature incubation decrease associated with the transition from late to early fall (Thome et al., 2016).

Without a complete understanding of embryo development under field conditions, it is unclear if the effect of natural and anthropological temperature stressors is being accurately estimated. This is a cause of concern considering both the cultural significance of lake whitefish to several first nations groups (Bruce Power, 2005) and the economic impact lake whitefish have for Canada, bringing in over $3.6 million dollars in 2014 for Ontario alone (DFO, 2014).

## 1.2 Round whitefish

While there have been a relatively large number of laboratory studies on lake whitefish embryo development over the last eight decades, there is surprisingly little information on round whitefish, another cold-adapted Great Lakes species. Most round whitefish studies focus on adult populations, such as where they inhabit and their diets (both of which are similar to lake whitefish populations; e.g. Macpherson et al., 2010). While many small lakes generally have one of the two whitefish species, larger lakes often have both species present in the same area. In this scenario, each species has

different preferred prey species, in which round whitefish exhibit mainly generalist eating behaviour and there is low competition (Eberts et al., 2015; Macpherson et al., 2010). The lack of studies on round whitefish embryo development is generally attributed to relatively lower perceived commercial and cultural importance, in addition to overall lower species abundance. As a result, there is currently a deficit in the understanding of round whitefish embryo development which needs to be addressed. One of the few studies to look at round whitefish was by Normandeau (1969). Compared to the lake whitefish embryos observed by Sreetharan et al. (2015) with an average diameter of ~3.19 mm at fertilization, round whitefish embryos were notably larger (~3.9mm average diameter) and had a distinct yellow-orange colouration (Normandeau, 1969). These eggs were laid and fertilized in shallow water with a gravel and rubble substrate, as seen with lake whitefish. Round whitefish also spawn in late autumn (the last two weeks of November) and their embryos begin to hatch in spring (mid-April; Normandeau, 1969). When raised in the lab, round whitefish were found to possess similar development trends to lake whitefish. These trends included higher mortality and shorter development time at higher temperatures (Griffiths, 1980). Interestingly, the majority of mortality for round whitefish embryos occurred late in development, and rose steeply once above 7ºC, notably lower than what lake whitefish embryos tolerate (Griffiths, 1980; Price 1940). As such, round whitefish embryos have been suggested to be more temperature sensitive and/or have a lower thermal limit than lake whitefish. Griffiths (1980) also predicted there to be little effect of chronic ≤2ºC water temperature elevations or acute ≤5.5ºC water temperature elevations on round whitefish embryo survival. Unfortunately, there is

little information on other developmental characteristics such as hatch size and development rate differences. In regards to the effects of field conditions on round whitefish embryo development, there is very little information other than mortality and hatch timing data, and the individual impacts of natural and anthropological effects are unknown (see Patrick et al., 2013). This is particularly troubling when taking into consideration the lower species abundance and theoretically lower thermal limit of round whitefish. For instance, even if average water temperature levels are within the optimal temperature range for lake whitefish, there may be significant, long-term effects on development of round whitefish embryos. If this is true, environmental guidelines for anthropological activities that influence water temperature may need to be lowered to reduce the possibility of adverse effects.

### 1.3 Warm water effluent as an environmental stressor

Embryos developing in the wild not only face natural seasonal temperature fluctuations, but also temperature changes due to anthropological activities such as thermal power plants. Thermal power plants are important for Canada's energy production, providing ~35% of the national demand and are found across the country (CEA, 2014). These power plants can be found near possible whitefish spawning habitats, like the Great Lakes. When these power plants generate energy, water is drawn from nearby water sources (e.g. the Great Lakes) to transfer energy in the form of heat. This water is eventually re-circulated back to the water source, significantly warmer, as thermal effluent (Zeng et al., 2002). This process is known as 'once-through cooling'.

The amount of water used for this process can vary depending on several factors such as the amount of energy generation needed. For facilities on the Great Lakes basin facilities, ~470 billion litres of water are utilized for the once-through cooling process every day (GLC, 2011). The amount of heat transferred to the water source is dependent on energy generation needs and the temperature of the source water. As such, once-through cooling processes raise water temperature several degrees Celsius above ambient (ranging from ~0.5-10 ºC, with an average of ~3ºC in winter 2014) near discharge sites. Transient temperature spikes (durations ranging from <30minutes to >2hours, most~1 hour) could further elevate temperatures near discharge sites, which may impact the development of nearby embryos (Thome et al., 2016; unpublished raw data). While these temperature recordings were not taken directly from discharge sites, they identify two different timescales for temperature elevations. Small, acute temperature changes can occur anywhere between once every 1-3 days, with larger and more persistent temperature elevations occurring once every 2-3 weeks (Thome et al., 2016).

### 1.4 Chronic morpholine exposure as an environmental stressor

In addition to thermal effluent, discharges from power plants may contain low levels of chemicals used to prevent corrosion and damage to pipes involved in cooling water systems. One such chemical is morpholine. Morpholine increases pH, neutralizing the effects of compounds such as carbon dioxide (Nordmann and Fiquet, 1996). Morpholine is thought to have a relatively short biological half-life (below 24h), and is not metabolized easily by biological systems (Tanaka et al., 1978). In Ontario,

morpholine has a provincial water quality objective set at 0.004 mg $L^{-1}$ (OMOEE, 1994), but regulatory limits for levels released by thermal power plants can far exceed this (e.g. 25 mg $L^{-1}$; Bruce Power, 2005).

These regulatory limits were likely set based on previous morpholine studies. The majority of these studies used terrestrial species (e.g. Tanaka et al., 1978) with relatively few studies on the effects on aquatic organisms. Of those studies, the majority involved acute exposures and studied adult fish (e.g. Brandão et al., 1992). The only known study that chronically exposed lake whitefish embryos observed significant increases in body size at hatch at as low as 1 mg $L^{-1}$, significantly decreased growth at 500 mg $L^{-1}$, and increased mortality at 1000 mg $L^{-1}$ (Thome, 2015). As morpholine induces elevated pH levels of water ranging from a pH of ~8.1 (10 mg $L^{-1}$ morpholine) to a pH of ~9.8 (1000 mg $L^{-1}$ morpholine), it is important to study the effects of elevated pH alone, such as via the administration of NaOH to water. Increased pH was not found to have a significant effect on mortality, but increased growth at pH 8 and 8.5, and decreased growth at pH 10 for lake whitefish embryos (Thome, 2015). This suggests that sub-lethal effects such as increases in growth may be attributed more to pH changes than morpholine itself. There is currently no known information of the effects of morpholine or pH on round whitefish embryos, which is concerning as they share similar habitats to lake whitefish and are thus also likely to experience exposure to thermal power plant effluent.

1.5 Chronic low-dose irradiation as an environmental stressor

In 2013, nuclear power plants generated energy for over 60% of Ontario via nuclear fission. There are several methods to harness energy from nuclear fission processes, such as via a pressurized water reactor design. Similar to other thermal power generation, several cooling loops are utilized to transfer the energy as heat, eventually utilizing steam to rotate turbines and generate electricity (see Rosen, 2001; Zeng et al., 2002). All nuclear power plants in Canada utilize Canadian Deuterium Uranium (CANDU) operation procedures, which aim to protect the public and the environment from radiation. CANDU reactors use heavy water, which contains deuterium, to moderate neutrons and dissipate heat. When deuterium captures a neutron from the fission process, it becomes tritium. Annual releases of tritium by nuclear power plants, such as Bruce Power, as effluent into surrounding lakes and streams are well under 40 Bq/L, which is under the provincial limit in Ontario of 7000 Bq/L (Bruce Power, 2014). When tritium is released in effluent from nuclear plants, there may be low levels of ionizing radiation (Ontario Power Generation, 2015). Ionizing radiation can either directly or indirectly ionize (Hall and Giaccia, 2006). The source used in this thesis is $^{137}$Cs which produces gamma rays, 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 (Hall and Giaccia, 2006). This damage can in turn lead to increased repair mechanisms in an effort to compensate, increased mutation incidence (which may be beneficial, detrimental, or silent), increased cell death, and/or cause the death of the

organism.

The level of damage that occurs depends on both the cumulative dose and dose rate. Lower dose rates generally result in less damaging effects as cell repair mechanisms are more likely to be able to keep up or surpass the rate of damage (Hall and Giaccia, 2006). Aquatic organisms such as fish are well known to tolerate much higher dose rates than humans, with a deleterious effect dose limit set >180 times higher than human dose limits (0.5 and 0.0027 mGy/day respectively; see Bréchignac & Barescut, 1992). Acute radiation exposure studies on fish embryos have found $LD_{50}$ levels nearly 10 times higher at hatch (10Gy) compared to fertilization (1Gy; Ward et al., 1971), with significantly larger body sizes at hatch after being irradiated with 0.025 Gy (Miyachi et al., 2003). Unfortunately, there are very few studies on chronic fish embryo exposures. Of those studies, there have been a variety of effects such as increased mortality at 0.5 mGy/day (Trabalka and Allen, 1977), increased development rate at 1 mGy/day (Simon et al., 2011), and growth stimulation at as low as 0.06 mGy/day (Thome, 2015). While the work by Thome (2015) addresses the effects of acute and chronic radiation exposures on lake whitefish embryos, there is no information on the impacts of radiation on round whitefish embryos. Similar to possible morpholine exposures, round whitefish may experience chronic, low-dose rate exposure to radiation during their embryonic development. This can have arguably beneficial effects (e.g. increased growth efficiency) or detrimental effects (e.g. increased mortality) depending on the sensitivity of round whitefish to radiation stressors. Without this information, current low-dose radiation in areas around

nuclear power plants may inadvertently be affecting the survival of round whitefish throughout embryo development and beyond.

## 1.6 Thesis objectives

This thesis addresses the lack of information of relevant field conditions on the embryonic development of lake and round whitefish. One such condition is fluctuating incubation temperatures. While there have been many studies on lake whitefish embryo development, these have mainly taken place at constant temperatures, ignoring the impacts of seasonal water temperature changes (autumn to winter, winter to spring) and anthropological effects (e.g. water temperature spikes caused by thermal effluent from once-through cooling processes). By teasing apart both the individual and combined effects of seasonal changes and temperature spikes, researchers can better predict and model the effects of rearing in the field that were overlooked by earlier, relatively simpler, constant incubation temperature setups. To address this lack of information on fluctuating incubation temperatures, Chapter 2 describes the impact of several fluctuating regimes on lake whitefish embryos, including rearing embryos with seasonal temperature changes, temperature spikes, and both combined. Embryos raised under these regimes were compared to lake whitefish embryos raised constantly at 2, 5, or 8ºC. Development effects were assessed in terms of survival to hatch, morphology, developmental stage onset, hatch dynamics, and heart rate.

On the other hand, round whitefish embryos have had few development studies even at constant temperatures, with little information on their growth and development

rates throughout incubation. To get a basic understanding of round whitefish development, Chapter 3 includes studies on round whitefish embryo development across two years when raised at the constant temperatures of 2, 5, or 8ºC. Round whitefish embryos were also incubated under fluctuating temperature regimes. The regimes used include all those studied in Chapter 2, while adding two regimes with an extended number of temperature spikes from a base temperature of either 2ºC or a transient increase along a seasonal temperature regime (e.g. 8ºC->11ºC, 7ºC->10ºC, … as seasonal temperatures declined). Embryos raised under these regimes were compared to those raised constantly at 2, 5, or 8ºC.

To better understand the sensitivity of round whitefish embryos to environmental stressors, Chapter 3 of this thesis explores the impacts of chronic morpholine and chronic irradiation exposure. To study the impacts of morpholine on round whitefish, embryos were chronically exposed to a range of morpholine levels at 5ºC; appropriately matched pH controls were included to distinguish effects from the chemical and its impact on water pH level. For instance, significant differences between exposure to 1000 mg $L^{-1}$ morpholine and pH 10 may indicate significant effects from the chemical properties of morpholine itself. Finally, to evaluate the effects of chronic irradiation, round whitefish embryos were exposed to a range of low-dose irradiation rates at 2.5ºC. Development effects were assessed in terms of survival to hatch, survival 30 days post-hatch (dph), morphology, developmental stage onset, hatch dynamics, and oxygen consumption rate.

A better understanding of temperature, morpholine, and irradiation environmental stressors on lake and round whitefish embryos will prove invaluable for evaluating and

setting appropriate water quality and effluent guidelines, as well as help develop better lab rearing protocols to more accurately capture the effects that may be seen in the field. This work aims to provide a foundation for future multiple-stressor studies, which combine multiple environmental stressors. Only through the understanding of a wide variety of different stressor regimes can there be better protection of important freshwater species such as lake and round whitefish.

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# Chapter 2

# The effects of fluctuating temperature regimes on the embryonic development of lake whitefish (*Coregonus clupeaformis*)

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## 2.1 Abstract

Most laboratory studies on fish embryogenesis occur at constant temperature, even for species that experience wide seasonal temperature changes in nature. Natural incubation temperatures can be altered by human activities such as thermal effluents, which may increase average and/or transiently raise temperature around discharge sites. These fluctuating incubation temperatures may have significant effects on embryogenesis. In this study, lake whitefish (*Coregonus clupeaformis*) were reared under constant (2, 5, or 8°C) and fluctuating temperature regimes. Fluctuating temperature treatments had a base temperature of 2°C with: 1) seasonal temperature changes modeled by gradual declines/inclines, 2) repeating Δ 3°C, 1hr temperature spikes, or 3) both seasonal temperature declines/inclines and Δ 3°C temperature spikes. We compared survival to hatch, morphometrics, and heart rate measurements at three development stages; fin flutter, protruding mouth, and pre-hatch. Embryos raised at a constant 8°C displayed >3 times elevated mortality, but fluctuating temperature regimes had no increase in mortality compared to embryos reared at constant 2°C. Embryos exposed to both seasonal changes and temperature spikes had elevated heart rate measurements across all developmental stages compared to embryos reared at constant 2°C. Embryos reared under seasonal temperatures had the largest changes in body growth and yolk consumption between the protruding mouth and pre-hatch stages. Our study suggests that seasonal temperatures may have significant effects on development, growth, and heart rate of lake whitefish embryos.

## 2.2 Introduction

Temperature has long been regarded as an important abiotic factor for fish embryo development (see Blaxter, 1992; Rombough, 1997). Different species have varying optimal temperature ranges, in which embryos develop and hatch normally. As incubation temperature approaches the limits of this range, there can be strong effects on embryos including changes to development rate, body growth rate, yolk consumption rate, incidence of abnormality, and survival to hatch, especially in species that possess narrow ranges and/or long development periods (see Blaxter, 1992; Pepin, 1991). Due to increased global concern regarding elevated water temperatures, there has been a resurgence in studies regarding the impacts of temperature on embryo development, including areas impacted by human activities. Our study focuses on the impacts of temperature on a cold-adapted freshwater species, lake whitefish, building on previous work to describe some developmental impacts of fluctuating incubation temperatures (e.g. Eme et al., 2015; Mueller et al., 2015; Patrick et al., 2013).

Lake whitefish are native to North America and can be found in many areas including the Great Lakes area, many northern lakes/rivers, and even brackish waters (Macpherson et al., 2010; Rawson, 1951). They are highly valued both culturally, to several first nations groups (Bruce Power, 2005), and economically, bringing in over $3.6 million dollars in 2014 for Ontario alone (DFO, 2014). During late autumn, lake whitefish spawn in shallow water as water temperatures decline (~6-7°C). Their eggs are fertilized, incubate over winter (~0.5-4°C), and hatch in the spring as water temperatures increase (~6°C; Price, 1940).

Previous studies have quantified differences in embryo development, for a wide range of constant incubation temperatures (e.g. 0.5 ºC-12 ºC) and development stages (e.g. Brooke, 1975; Eme et al., 2015; Mueller et al., 2015; Price, 1940). Studies like those conducted by Brooke (1975) found an increased development rate at warmer temperatures, in which embryos reared at 2ºC took nearly 150% longer to reach the eye pigmentation stage than embryos reared at 7.8ºC. Studies have consistently observed >35% increased mortality, a >50% reduction to median hatch, and ~50% shorter hatch windows at 8ºC compared to cooler 2ºC incubation temperatures (defined as the length of time between the first and last embryo hatching; Brooke, 1975; Mueller et al., 2015; Price, 1940). Embryos raised at warmer temperatures had shorter incubation periods, decreased body size, increased yolk size, and overall less efficient growth (Mueller et al., 2015). Studies by Eme et al. (2015) and Mueller et al. (2015) utilized fluctuating thermal regimes which involved shifting embryos to either warmer or colder conditions at different stages of development. Their research found significant changes in embryo development, such as reduced mortality, when being transferred from a warmer (e.g. 8ºC) to a colder (e.g. 2ºC) incubation temperature at gastrulation compared to being solely raised at the warmer temperature (Mueller et al., 2015).

By combining the timing of temperature changes monitored in the field (e.g. Patrick et al., 2013; Thome et al., 2016) and the work completed by Mueller et al. (2015), there is likely a “critical window” to temperature that coincides with the seasonal transition from autumn to winter. Critical windows have been described as moments in development where an organism is more susceptible to sub-lethal stressors (such as

temperature), in that it will adjust its phenotype due to intrinsic or extrinsic factors (Burggren & Reyna, 2011). As water temperatures rapidly decrease during this sensitive developmental period, lake whitefish embryonic development may be significantly affected.

Embryos developing in natural bodies of water face both seasonal temperature fluctuations and temperature changes due to anthropogenic activities. Thermal power plants are one such source of thermal effluent that can be found near possible whitefish spawning habitats in the Great Lakes (Fietsch, 2011). When thermal power plants generate energy, water is often used from lakes or rivers to remove waste heat. This water is eventually re-circulated back to the source, significantly warmer, as thermal effluent (Zeng et al., 2002). This process is known as 'once-through cooling'. The amount of warming caused by this process can vary depending on several factors including the amount of energy generation needed and the temperature of the source water. Warm water effluent from this process can raise substrate water temperature several degrees Celsius above ambient (ranging from ~ Δ 0.5-10ºC, with an average of ~ Δ 3ºC) near discharge sites (Thome et al., 2016). Temperature spikes (durations range from <30minutes to >2hours, average ~1 hour) can transiently elevate temperatures near discharge sites, which may impact the development of nearby embryos (Thome et al., 2016, unpublished raw data). A field study suggested that these temperature changes occur once every 1-3 days, with larger and more persistent changes occurring once every 2-3 weeks (Thome et al., 2016). It is important to note that the aforementioned field study recorded substrate water temperature away from the discharge site, providing time and

volume for the heat to dissipate. As such, warm water effluent may raise water temperature even further directly at the discharge site itself. While lake whitefish embryos may be exposed to natural and/or anthropogenic temperature fluctuations, very few laboratory studies incorporate fluctuating incubation temperature regimes. Of those studies, the individual effects of seasonal changes and transient warm effluent spikes across development were unclear (e.g. Patrick et al., 2013).

In this study, we raised lake whitefish embryos under constant incubation temperatures (2, 5, or 8ºC) and under three fluctuating temperature regimes reflecting natural seasonal changes, temperature spikes, or seasonal changes with temperature spikes. We assessed if there were significant differences across different incubation treatments by evaluating changes in development rate, growth via morphological measurements, heart rate, and survival until hatch. We hypothesized that exposure to seasonal temperatures would result in significantly larger and more efficient growth and the temperature spikes were expected to decrease body size at hatch and increase yolk consumption compared to constant temperature regimes. This study recreated simplified field incubation temperature conditions in the lab and compared a variety of morphometrics at different developmental stages under these fluctuating temperature conditions.

## 2.3 Methods

### 2.3.1 Lake whitefish embryos

Lake whitefish adults were gill-netted on November 29, 2014 from Lake Huron (44° 42' 37.74" N and 81° 18' 38.94" W). Eggs and milt were stripped from 6 male and 3 female fish, mixed randomly, and fertilized *in vitro*. Fertilized eggs were placed in lake water, disinfected with Ovadine (0.5% iodine) for 30 minutes, and rinsed several times with additional lake water. Cleaned embryos were transported on ice to McMaster University in containers of approximately 50% embryos, 50% clean lake water. Embryos were placed into McDonald Bell hatching jars (see Mitz et al., 2014) at 5ºC at a density of ~5000 per litre of dechlorinated city water. 1 day post fertilization (dpf), ~5400 embryos were distributed into 108 sterile petri dishes (100mm x 20mm) at a density of 50±1 (± standard deviation) embryos/dish. Each dish contained ~75mL of dechlorinated city water. 54 dishes were placed at 8 ºC, 18 at 5ºC, and 36 at 2ºC. At 1dpf, chorion diameter was 3.07±0.19 mm (± standard deviation) with a yolk area of 4.54±0.26 $mm^2$ (± standard deviation).

### 2.3.2 Embryo rearing & fluctuating temperature regimes

Embryos were raised in custom fridges as described in Mitz et al. (2014) set at 8, 5, and 2ºC. These fridges averaged temperatures of 8.0±0.2, 5.3±0.4, and 2.0±0.4ºC (±standard deviation) respectively. 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 5 minutes in ~100 mL of

dechlorinated water in a glass beaker adjacent to petri dishes in each fridge. Petri dishes underwent 100% water changes 2-3 times per week, regardless of incubation treatment, until all embryos hatched. Dechlorinated water used for water changes was chilled within 0.1ºC of the incubation treatment group temperature (e.g. 4.9-5.1ºC for the 5ºC group). Water changes were carried outside of rearing fridges on ice.

To generate seasonal (Seasonal) temperature incubations, 36 dishes of 8ºC lake whitefish embryos were gradually exposed to decreasing temperatures at a rate of -1ºC/week until reaching 2ºC, starting 1dpf. The embryos were kept at 2ºC for 8 weeks, and then were gradually exposed to warming temperatures at a rate of +1ºC/week until complete hatch at 6°C. The decline from 8ºC to 2ºC took 6 weeks, and the ascent from 2ºC to 6ºC took 4 weeks. The average temperature for this regime was 3.7±1.3 ºC (±standard deviation). For temperature spike (Spike) treatments, 18 dishes of 2ºC lake whitefish embryos were held at 2ºC for the first 1.5 months of development. Embryos were then exposed to Δ3ºC temperature spikes every 2-3 days, coinciding with water change days. Temperature spikes lasted one hour, and then the dishes were then returned to 2ºC (taking ~1h for temperature to return to a stable 2ºC; monitored via HOBO® data Logger). These dishes would then remain at 2ºC until the next temperature spike. Following an 8 month period of temperature spikes, embryos were held at 2ºC until they hatched. The average temperature for this regime was 2.1±0.5 ºC (±standard deviation). A combined seasonal and spikes (Seasonal & Spike) incubation regime included the gradual cooling (-1ºC/week until reaching 2ºC) and warming periods (+1ºC/week until they all hatched) for the first and last 6 weeks of incubation respectively, coupled with the

temperature spikes for the intermediate 8 weeks. The average temperature for this regime was 3.8±1.4 ºC (±standard deviation).

2.3.3 Development stage onset, mortality, and 50% hatch

Throughout the incubation period, two dishes per incubation group were randomly selected and observed daily under an AXIO Zoom V16 microscope (Carl Zeiss AG) to determine when a treatment group reached each development stage. Mortality was monitored daily and dead embryos were removed. Upon initiation of the hatch window (defined as the length of time between the first and last embryo hatching), the number of hatched embryos was monitored daily for each incubation group until all embryos hatched. This allowed for the calculation of 50% hatch for each incubation group.

2.3.4 Measurements at fin flutter, protruding mouth, and pre-hatch

Three major development stages (see Sreetharan et al., 2015) were chosen during the latter half of development to measure heart rate and morphometrics of the embryos. Fin flutter was indicated by onset of sustained pectoral fin movement, protruding mouth was indicated by a visibly protruding and open mouth, and pre-hatch was defined as the first day there were at least 3 hatches within a treatment group. One embryo per dish was randomly selected to serve as a sample of each incubation treatment group (i.e. 18 embryos per incubation treatment). Heart rate was measured via direct observation of embryos under an AXIO Zoom V16 microscope (Carl Zeiss AG). Embryos were placed in a custom built water-jacket observation chamber (described in Eme et al., 2015). The

observation chamber was filled with dechlorinated tap water at the same incubation temperature (e.g. 5ºC embryos were observed at 5ºC). All fluctuating temperature regime groups had a base incubation temperature of 2ºC during the fin flutter and protruding mouth stages, and thus were observed at 2ºC. The Seasonal and Seasonal & Spike groups were incubated at 3ºC at the pre-hatch stage. This was due to entering the warming period of seasonal regimes. As such, they were incubated at 2ºC for an hour prior to being placed in the observation chamber. Embryos were given ~10 min to rest individually in the observation chamber prior to measurements. Average heart rate (bpm) was estimated by visual counting for 1 min (in triplicate) with ~45 seconds between each measurement. Following measurement, embryos were placed in 10% formalin for 7 days, and then transferred to 50% ethanol for an additional 42-44 days. Embryos were then dissected and imaged on their ventral and lateral sides using an AXIO Zoom V16 microscope (Carl Zeiss AG).

Morphometrics were carried out on the images of the fixed embryos using the AxioVision Release 4.8 length measurement tool (see Sreetharan et al., 2015), including body length, body width, and yolk area. Body length was measured with the embryo on its ventral side and defined as the distance between the most anterior location of the head to the distal edge of the caudal fin. Body width was measured halfway down the embryo laid on its lateral side and defined as the distance between the dorsal and ventral sides of the body perpendicular to the body length measurement. Yolk areas were calculated by measuring using two perpendicular yolk diameters, assuming an ellipse shape. The embryos' body and yolk were dissected apart and then dried for 23-25h in a 70 °C oven

(model 1500EM, VWR/Sheldon, Cornelius, OR, USA) and weighed to ±0.01 mg (Mettler-Toledo XA105DU) to determine dry body mass and dry yolk mass.

### 2.3.5 Statistical analyses

Individual hatchlings measurements (i.e. body weight, yolk weight, body length) and mortality were averaged across replicate dishes for each treatment to generate an overall mean. Heart rates were measured for individual hatchlings in triplicate, and this mean was averaged across each replicate dish to generate an overall mean. Statistical analyses were run using each dish in a treatment as a replicate. Mortality was compared across treatment groups using a one-way Analysis of Variance (ANOVA), with a post-hoc Tukey Test when the ANOVA was significant (Sigmaplot 11). To compare across temperature regimes and development stages, all other variables were compared utilizing two-way ANOVAs with a post-hoc Holm-Sidak test when the ANOVA was significant (Sigmaplot 11). Data throughout this study is presented as the mean ± SEM unless otherwise stated, with $\alpha=0.05$ for all ANOVA and post-hoc testing.

## 2.4 Results

### 2.4.1 Development stage onset, hatch dynamics, and mortality

As increased incubation temperatures generally result in earlier hatch and a shorter hatch window, embryonic development was scaled to median hatch (100% developed) and presented as a percentage. This allowed for a better comparison of development rate compared to utilizing the pre-hatch stage, which was heavily influenced

by the onset of the hatch window. From this, higher incubation temperatures were observed to result in later onset of the protruding mouth and pre-hatch development stages and smaller hatching windows (Table 2.1). Of the constant temperature groups, only the 2ºC control group had a notably early pre-hatch stage onset. The Spike group produced similar development stage onset as the 2ºC control group, but a relatively delayed pre-hatch stage. The Seasonal and Seasonal & Spike groups had advanced fin flutter and protruding mouth stage onset (Table 2.1). Mortality was significantly elevated in the constant 8°C group only (Fig. 2.1).

2.4.2 Body growth, yolk consumption, and heart rate

Body mass and length significantly increased with development stage for all treatments (Fig. 2.2A, 2.3A). The constant 8°C group had the smallest proportional body mass gain near the end of development (comparing between the protruding mouth and pre-hatch stages), while the Seasonal group had the largest proportional body mass gain (Fig. 2.2A). Incubation at colder constant temperatures resulted in larger body lengths relative to warmer incubation temperature groups (e.g. embryos raised at 2°C were significantly larger than embryos reared at 8°C). The Spike group had the largest body length at the pre-hatch stage. Unlike body mass and length, body width was not significantly different between treatments at the fin flutter and protruding mouth stages. At the pre-hatch stage, the colder incubation treatments (2°C and Spike) had the largest body widths. Of the warmer incubation treatments, only the 5°C, Seasonal, and Seasonal

& Spike groups had significant width growth between the fin flutter and pre-hatch stages (Fig. 2.3B).

Unlike body mass and length, dry yolk mass and yolk area significantly decreased with development stage for all treatments (Fig. 2.2B, 2.4). The constant 8°C group had the smallest yolk sac consumption, while the Seasonal group had the largest proportional yolk sac consumption (Fig. 2.2B). Incubation at colder constant temperatures resulted in smaller yolk areas relative to warmer incubation temperature groups. In addition, yolk area decreased significantly with development stage for the 2°C and Spike groups between fin flutter and pre-hatch and again between protruding mouth and pre-hatch (Fig. 2.4).

Only the 8°C, 5°C, and Seasonal & Spike groups consistently had higher heart rates than the 2°C treatment at all development stages examined. Heart rate increased significantly with development stage for all treatments excluding Seasonal & Spike, which did not increase significantly between the fin flutter and protruding mouth stages (Fig. 2.5).

## 2.5 Discussion

Our study observed that compared to the constant 2 and 5°C temperature regimes, fluctuating incubation temperature regimes did not have a significant effect on mortality, but did result in significantly altered development stage lengths, increased growth, and higher heart rates. As has been observed in previous studies, there was decreased growth, lower yolk consumption, and higher mortality at warmer incubation temperatures

(Mueller et al., 2015; Price, 1940), especially near the end of the development period at 8°C.

2.5.1 Mortality rates at constant incubation temperature

While there have been several studies on lake whitefish mortality at different temperatures, there have been some conflicting results on the impact of temperature on the mortality curve. Mortality has been seen to increase in a stepwise manner (Mueller et al., 2015) or exhibit a U-shaped curve (Brooke, 1975) as incubation temperature increased. However, mortality in our study followed neither of these trends, and was only significantly elevated at 8°C. A similar finding was reported when incubation temperatures between 0.5°C and 8°C were compared by Price (1940), in which mortality nearly doubled for embryos raised at 8°C compared to embryos raised between 2 and 6°C. As Brooke (1975) and Price (1940) utilized circulating water incubation setups, we focused our comparisons on other studies that reared embryos in static water. From this, it was found that the mortality in our study was ~20% lower at 2°C, ~40% lower at 5°C , and ~10% lower at 8°C compared to studies that also incubated embryos in petri dishes (Mueller et al., 2015; Sreetharan et al., 2015). These differences in observed mortality may have been due to differences in embryo rearing between studies such as less frequent water changes (Mueller et al., 2015; Sreetharan et al., 2015) and/or parental gamete quality between studies. One measure of maternal health status and egg quality is the amount of yolk deposited within each egg. If a mother is unable to sustain a healthy diet, their eggs are often smaller and contain less yolk (see Brooks et al., 1997). Stressed

mothers may also deposit varying levels of mRNA and hormones (e.g. thyroid hormones, cortisol, and sex steroids) into eggs which could impact the expression of genes that may be crucial to survival shortly after fertilization (see Brooks et al., 1997). As the embryos in our study had roughly 9 times the yolk area at 1dpf compared to Sreetharan et al. (2015), there may have also been differences in egg deposits.

### 2.5.2 Temperature spike regimes alter development stage onset, body size, and heart rate

Fluctuating incubation temperature regimes had no significant effect on mortality, but impacted development stage onset. As hypothesized, the Spike treatment had the least effect of the fluctuating temperature regimes, with the pre-hatch stage beginning slightly delayed compared to the 2°C treatment group (Table 2.1). This limited effect is likely a result of being exposed to the smallest average temperature increase (~0.1°C warmer than the constant 2°C incubation) and that the temperature increases only occurred after organogenesis and therefore after the critical window for temperature induced survival (Mueller et al., 2015).

There was another critical window following the fin flutter stage which influenced dry body mass at hatch and $O_2$ consumption (Mueller et al., 2015). While our study did not observe a larger body mass at hatch for the Spike regime, the return to the lower/less fluctuating constant 2°C incubation temperature may have caused the significantly increased body length at hatch (Fig. 2.3A). There was an increased heart rate at the protruding mouth stage, which occurred after the onset of the temperature spike period of development (Fig. 2.5). Heart rate can be used as a proxy for $O_2$ consumption, suggesting

that these temperature spikes transiently increased $O_2$ consumption (Eme et al., 2015). Temperature spikes of +Δ3°C are known to result in elevated heat shock protein responses in lake whitefish embryos (Stefanovic et al., 2016). Stress responses often involve increases in metabolic rates, and may be the cause of the elevated heart rate (see Bonga, 1992). Despite this period of elevated heart rate, there was no significant effect on the embryos' yolk consumption rates (Fig. 2.3B, 2.5). This would suggest that exposure to acute, infrequent temperature spikes caused by warm water effluent may result in more efficient growth. However, it is important to note that the magnitude, duration, and number of temperature spikes may fluctuate more in the field and would likely occur throughout the development period, instead of only for 8 weeks (see Patrick et al., 2013; Thome et al., 2016). These larger and more frequently changing temperatures may cause reductions in both growth and growth efficiency as is commonly seen in fish exposed to stress, particularly when at an early development stage (see Bonga, 1997; Rice, 1990).

### 2.5.3 Seasonal temperature changes alter development stage onset, hatch dynamics, and body and yolk sizes

The Seasonal treatment had no significant effect on mortality. However, it had a large effect on development stage onset. Being exposed to warmer temperatures early in development resulted in development stage onset similar to the 8°C treatment (e.g. having eye colour onset around the same period). However, once the incubation temperature had sufficiently decreased, development rate slowed and was more similar to constant 2°C group (data not shown), and then increased to the rate of the constant 5°C group during

the seasonal warming period at the end of development. The change in development stage onset from an 8°C rate to a 2°C rate has also been documented in the fluctuating temperature regime utilized by Mueller et al. (2015). Shifting lake whitefish embryos from a rearing temperature of 8°C to 2°C between gastrulation and organogenesis resulted in a significantly increased development stage window compared to embryos reared at constant 8°C, and with similar development stage window as embryos reared at constant 2°C (Mueller et al., 2015).

Seasonal regime embryos had the largest body size growth and the largest yolk consumption of all treatments near the end of development, suggesting that Seasonal regime embryos grew more rapidly than constant temperature embryos. In addition, the seasonal temperature incline at the end of development (modeling the entrance into spring) resulted in Seasonal regime embryos having the shortest hatch window. The accelerated hatch of Seasonal regime embryos may have developed to allow for matching hatch to prey availability in spring, trading internal energy stores for potential external sources as suggested by several studies (e.g. Brander, 1994; Chambers & Trippel, 1997). However, it is unclear if that is the only reason considering conflicting opinions on the matching of hatch to food availability where some studies support a relationship (see Freeberg et al., 1990), report a minimal relation (see Claramunt et al., 2010), or even suggest an opposite causal relationship (see James et al., 2003). It is possible that lake whitefish hatch dynamics may be more associated with temperatures that suggest the avoidance of ice cover, rather than the presence of food, as seen in other coregonid species which hatch as early as February due to low ice build-up (Eckmann, 1987). While

there may not necessarily be an abundance of food at this time, it may allow for the avoidance of predators and/or other food competitors (Eckmann, 1987), as well as provide the opportunity to better develop swim muscles and techniques compared to embryos still inside the chorion. Although the chorion is crucial for early embryo protection, the chorion limits the amount of room to grow, room to move, as well as limits oxygen uptake (Eme et al., 2015).

2.5.4 Seasonal temperature changes combined with temperature spikes alter development stage onset, hatch dynamics, heart rate, and body and yolk sizes

Combining the Seasonal and Spike incubation regimes had no significant effect on survival to hatch, and had similar development and hatch timing to the Seasonal regime embryos. When comparing hatch dynamics of our study with the study by Patrick et al. (2013), the Seasonal & Spike treatment group had notably delayed 50% hatch (10 days later), and a longer hatch window (15 days longer, data not shown). This may seem surprising as their study had a lower average temperature (~2.6 vs. ~3.8°C), and it has been well documented that warmer incubation temperatures result in earlier hatch (see Brooke, 1975; Eme et al., 2015; Mueller et al., 2015; Patrick et al., 2013; Price 1940). The difference in hatch timing between these studies is most likely due to differences in incubation regimes. The largest of these differences occurred during the seasonal temperature incline near the end of development, in which embryos of study experienced a relatively gradual (+1°C/week) increase, and the embryos in the Patrick et al. (2013) study were exposed to a more rapid +3°C/week. Temperatures during the end of

development likely have the greatest influence on hatch dynamics, as the majority of our hatches occurred at ~6°C, similar to what was seen by Patrick et al. (2013) and previous findings in the field (Price, 1940). Despite Patrick et al. (2013) not incorporating separate seasonal and temperature spike regimes, our study suggests that any effect on development or hatch dynamics caused by the addition of +Δ3°C temperature spikes is negligible compared to effects of seasonal changes.

Lake whitefish embryos have not only been raised in the laboratory, but have also been reared in the field both outside and within range of thermal power plant discharge. A field study documented significantly increased average temperatures and more fluctuating temperature coinciding with increased body size and yolk consumption in lake whitefish embryos exposed to thermal effluent compared to seasonal changes alone (Thome et al., 2016). Interestingly, our study did not have significant growth differences between Seasonal and Seasonal & Spike groups, with a trend for larger yolk sacs for the Seasonal & Spike group (Fig. 2.3B, 2.5). This is surprising, as the Spike regime embryos generally had larger bodies than the constant 2°C treatment embryos used in our study, suggesting that the Seasonal & Spike group should also have grown larger than the Seasonal regime embryos, as would be predicted by Thome et al. (2016). The difference in growth between these two studies was likely the result of our Seasonal and Seasonal & Spike regimes being more similar and warmer on average (~3.7 and ~3.8°C vs. ~1.5 and ~2.5°C respectively in Thome et al., 2016). The presence of long, low magnitude temperature increases in the field have a greater effect than temperature spikes on average temperature. This in turn resulted in a larger variation in growth rate and time to grow.

While embryo development for Seasonal and Seasonal & Spike regime embryos was found to be relatively similar (i.e. growth, development, hatch dynamics, mortality), only the Seasonal & Spike regime embryos had significantly elevated heart rate across the latter half of development. As heart rate can be used as a proxy for oxygen consumption, this suggests that the Seasonal & Spike group had a higher average metabolic rate than the embryos reared with constant 2°C, Spike, and Seasonal regimes. As the growth for Seasonal & Spike regime embryos was not significantly larger than Seasonal regime embryos, it is possible that the Seasonal & Spike regime embryos were allocating more of their energy stores to other processes. This matches previous work, where exposure of adult fish to stressors, such as temperature changes, has resulted in reduced growth/growth efficiency and increased metabolic rates (see Blaxter, 1997; Bonga, 1992; Rice, 1990). The higher metabolic rates of these embryos may be due to allocating extra energy to stress response, such as the aforementioned heat shock response (Stefanovic et al., 2016). Although we had originally hypothesized that all the fluctuating temperature regime groups would result in less efficient growth and have higher metabolic rates than the 2°C constant treatment group, it appears that either an incubation with continuous temperature fluctuations or the combination of several different temperature fluctuations (i.e. natural and anthropological effects) is necessary to cause significantly increased stress, and subsequently observe effects on growth and energy use.

### 2.5.5 Conclusions

The fluctuating temperature regimes used in this study were simplified versions of both natural variation and anthropogenic sources, and had significant effects on development, growth, and heart rate measurements of lake whitefish embryos but no significant effect on mortality. Seasonal temperature changes during and shortly after the transition from fall-to-winter and winter-to-spring resulted in earlier development onset. These significant effects support the previous findings of critical windows for lake whitefish embryonic development (Eme et al., 2015; Mueller et al., 2015) by identifying significant changes in development and growth rates as incubation temperature changed during the development stages of gastrulation-organogenesis and fin flutter- hatch. Our study also suggests that changing from fluctuating temperature regimes (e.g. Spike) to constant temperatures can reduce the effect of those changes (e.g. lower metabolic rate, higher growth rate) compared to consistent fluctuating temperature regimes. As such, when evaluating policies regarding the release of thermal stressors (i.e. warm water effluent) into the environment, it is important to put place emphasis on avoiding increased exposures during the seasonal temperature transition into and out of winter water temperatures in order to avoid impacting embryonic development. Future studies that investigate fluctuating temperature regimes should focus on recreating more fluctuating temperature spikes (i.e. occurrence, magnitude), extending these temperature spikes to encompass the entire development period, and combining these spikes with seasonal temperature changes for a better re-creation and understanding of field conditions.

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Tables & Figures

**Table 2.1**. Time to onset of each development stage for lake whitefish embryos incubated with different temperature regimes. Average temp refers to the mean temperature during incubation (± standard deviation). Time is reported as percent of development time with the days post fertilization (dpf) in parentheses. 50% Hatch values indicate time to median hatch.

| **Treatment Group** | | **Average Temp (°C)** | **Fin Flutter** | **Protruding Mouth** | **Pre-hatch** | **50% Hatch** |
|---|---|---|---|---|---|---|
| **Constant** | 2ºC | 2.0 (±0.4) | 49% (86) | 62% (109) | 78% (137) | 100% (176) |
| | 5ºC | 5.3 (±0.4) | 50% (51) | 64% (65) | 85% (87) | 100% (102) |
| | 8ºC | 8.0 (±0.2) | 51% (34) | 72% (48) | 88% (59) | 100% (67) |
| **Fluctuating** | Spikes | 2.1 (±0.5) | 49% (83) | 60% (102) | 84% (144) | 100% (171) |
| | Seasonal | 3.7 (±1.3) | 44% (53) | 57% (69) | 89% (108) | 100% (121) |
| | Seasonal & Spikes | 3.8 (±1.4) | 43% (53) | 57% (69) | 88% (107) | 100% (122) |

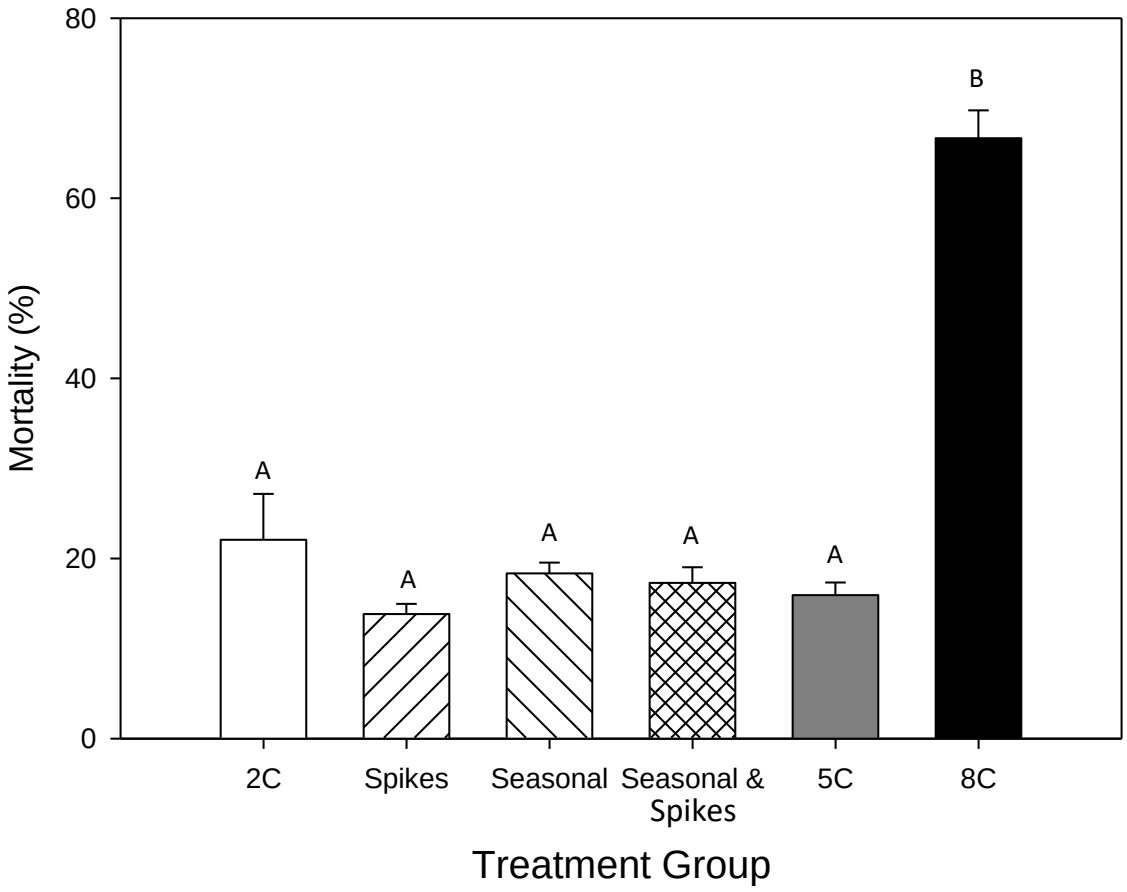
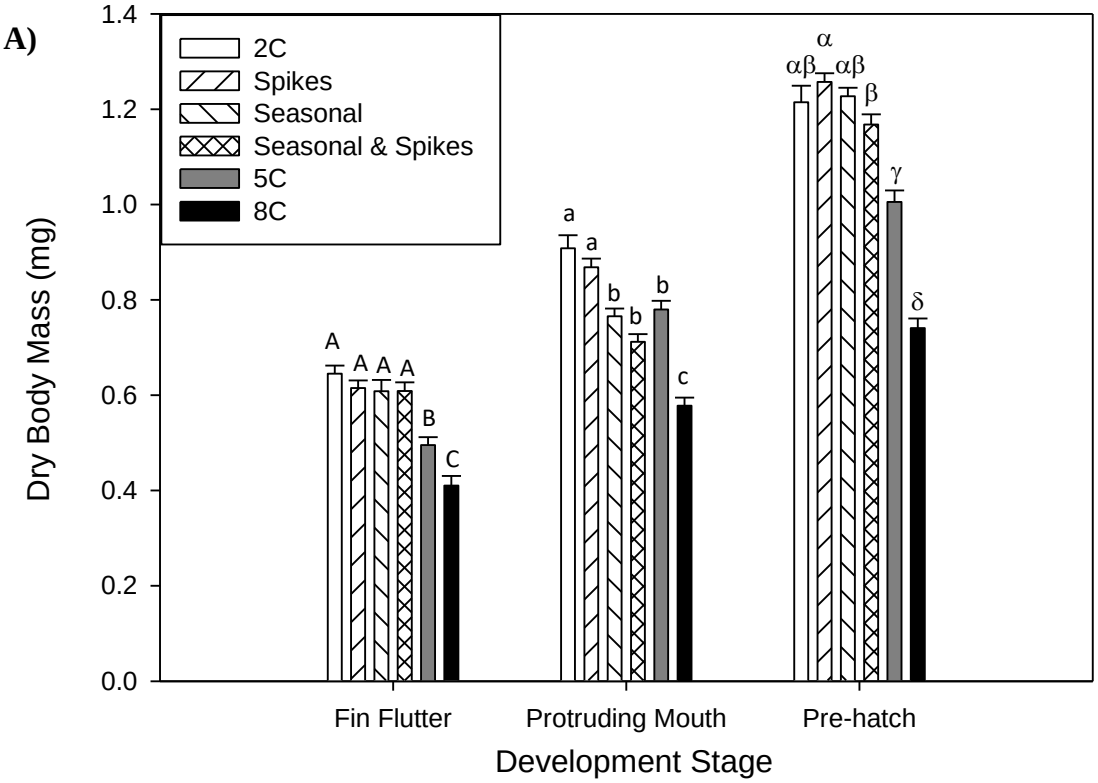



**Fig. 2.1**. Mean mortality (from fertilization until hatch) of lake whitefish embryos under different incubation treatments. Means without the same letter are significantly different from each other (Tukey post hoc comparison across all treatment groups, 1-way ANOVA, $p<0.05$). Error bars indicate SEM (N=18 for each treatment).

**A)**


2C
Spikes
Seasonal
Seasonal & Spikes
5C
8C
Dry Body Mass (mg)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A
A
A
A
B
C
a
a
b
b
b
c
α
αβ
αβ
β
γ
δ
Fin Flutter
Protruding Mouth
Pre-hatch
Development Stage

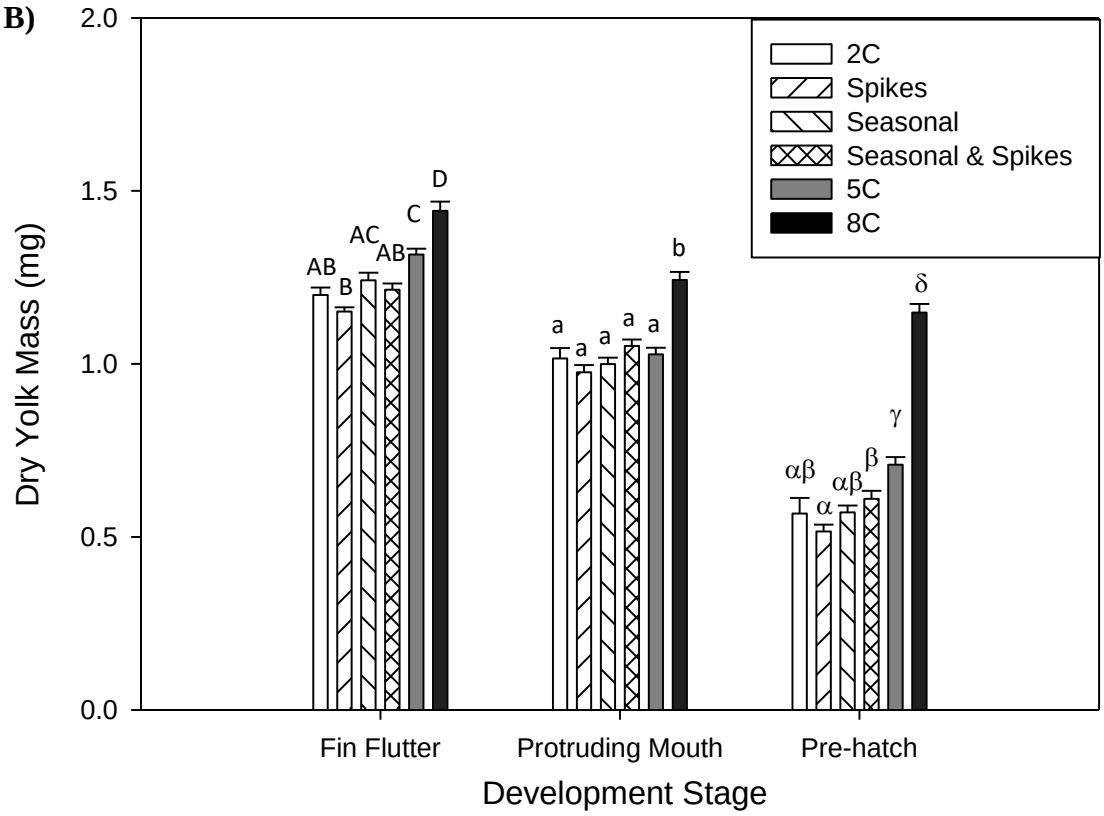
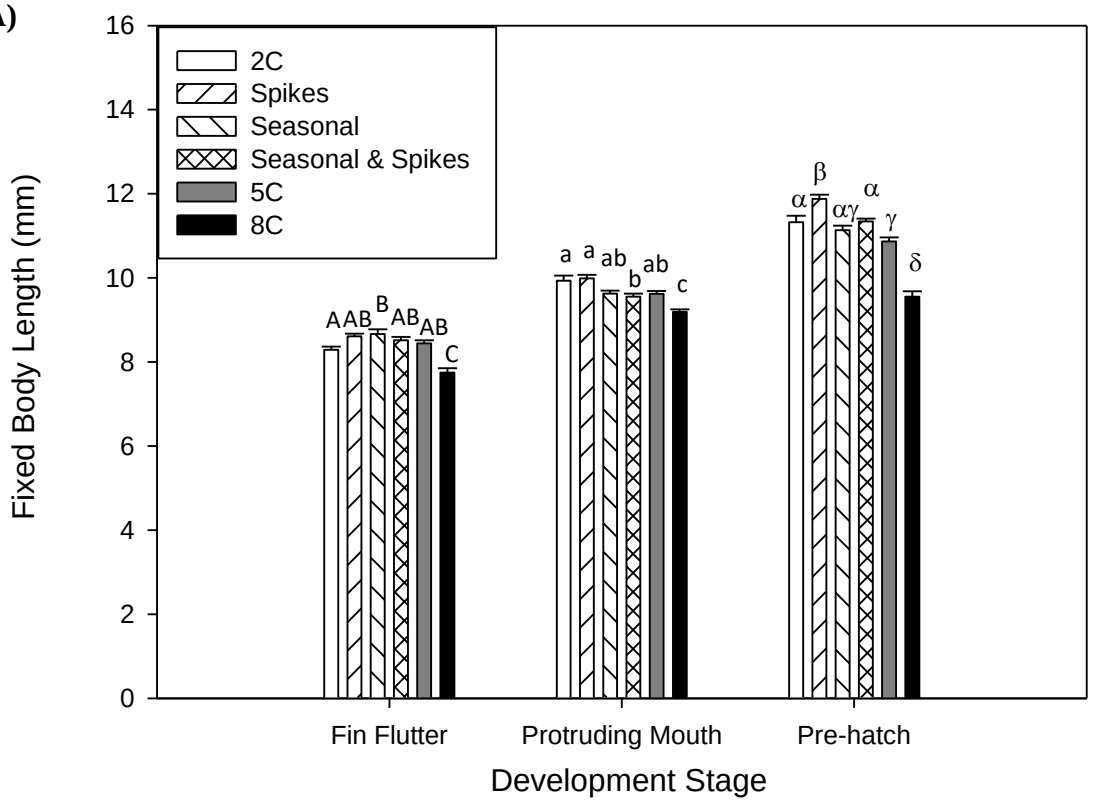



**Fig. 2.2**. Mean dry body (A) and yolk (B) mass of lake whitefish embryos under different incubation treatments. Means within a development stage without the same letter/symbol are significantly different from each other (Holm-Sidak post hoc comparison across all treatment groups, 2-way ANOVA, $p<0.05$). Mass significantly increased (A) and decreased (B) for all treatment groups across development stages. Error bars indicate SEM (N=18 for each treatment).

**A)**


2C
Spikes
Seasonal
Seasonal & Spikes
5C
8C
Fixed Body Length (mm)
0
2
4
6
8
10
12
14
16
A AB B AB AB C
a a ab b ab c
α β αγ α γ δ
Fin Flutter
Protruding Mouth
Pre-hatch
Development Stage

**B)**

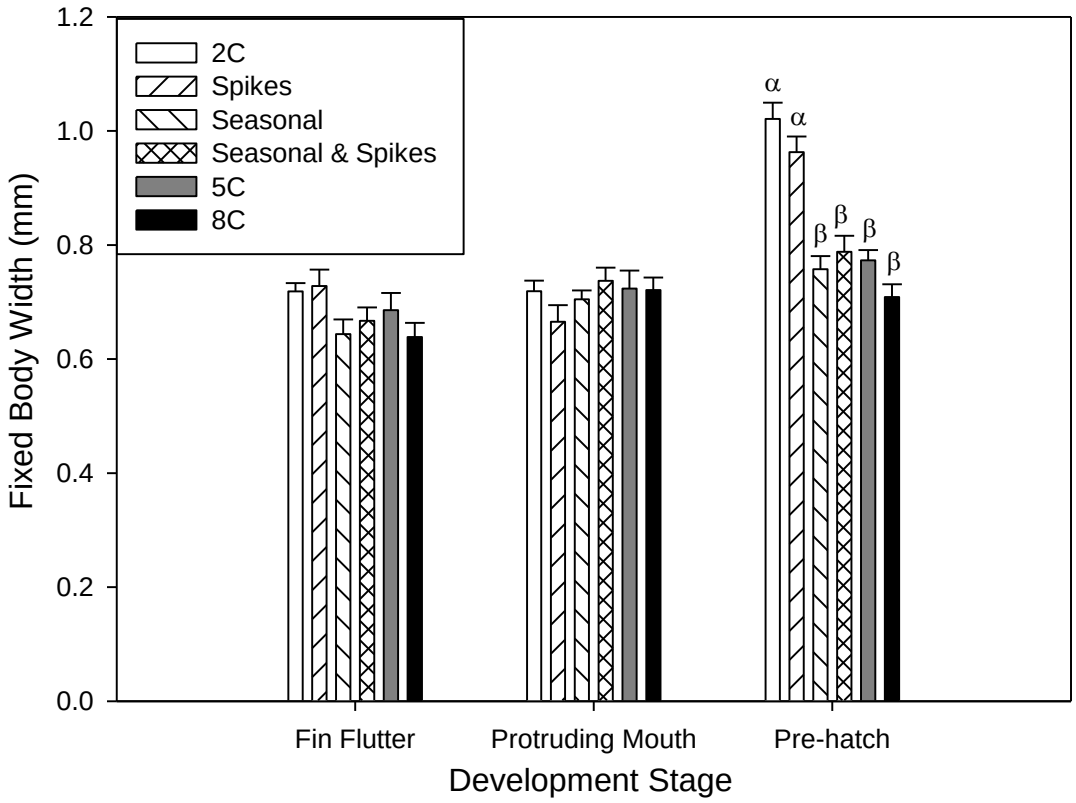



**Fig. 2.3**. Mean fixed (7 days 10% formalin, ~43 days 50% ethanol) body length (A) and width (B) of lake whitefish embryos under different incubation treatments. Means within a development stage without the same letter/symbol are significantly different from each other (Holm-Sidak post hoc comparison across all treatment groups, 2-way ANOVA, $p<0.05$). Body length significantly increased for all treatment groups across development stages. There were no significant body width increases between the fin flutter and protruding mouth stages. Error bars indicate SEM (N=18 for each treatment excluding 8°C at the fin flutter stage with N=9).

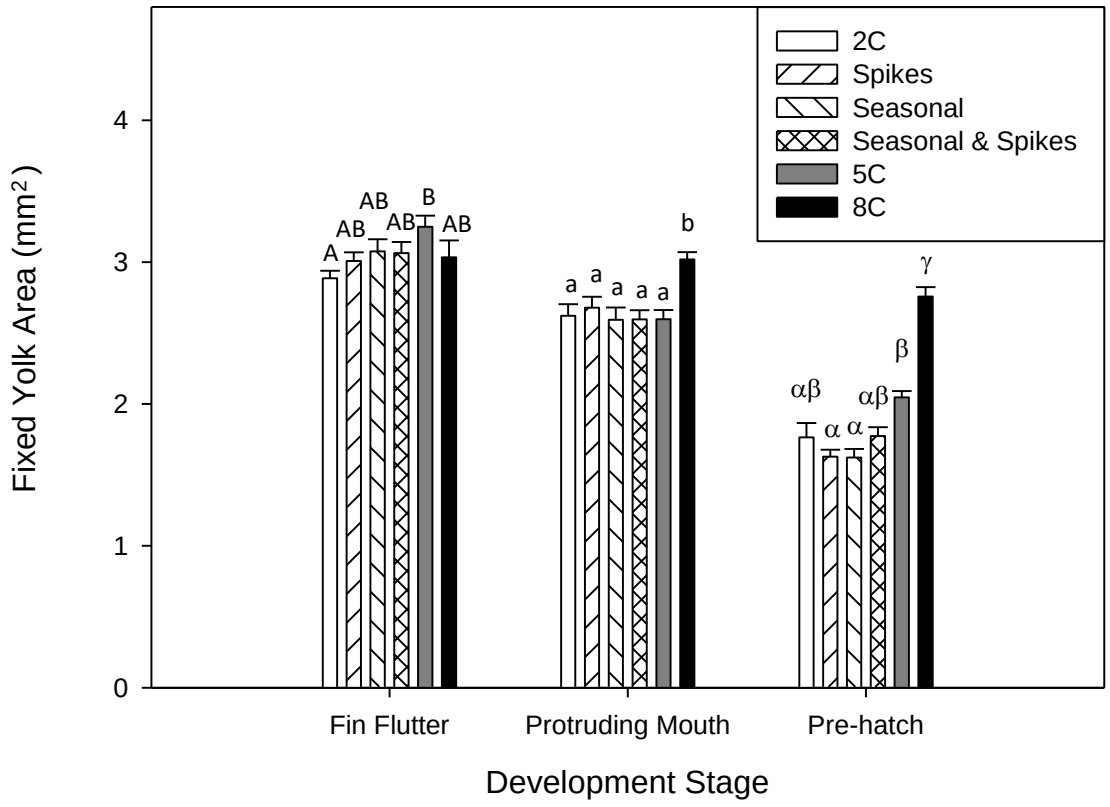



**Fig. 2.4**. Mean fixed (7 days 10% formalin, ~43 days 50% ethanol) yolk areas of lake whitefish embryos under different incubation treatments. Means within a development stage without the same letter/symbol are significantly different from each other. Mass significantly increased for all treatment groups across development stages (Holm-Sidak post hoc comparison across all treatment groups, 2-way ANOVA, $p<0.05$). Error bars indicate SEM (N=18 for each treatment excluding 5°C and Seasonal & Spike groups at the fin flutter stage with N=17).

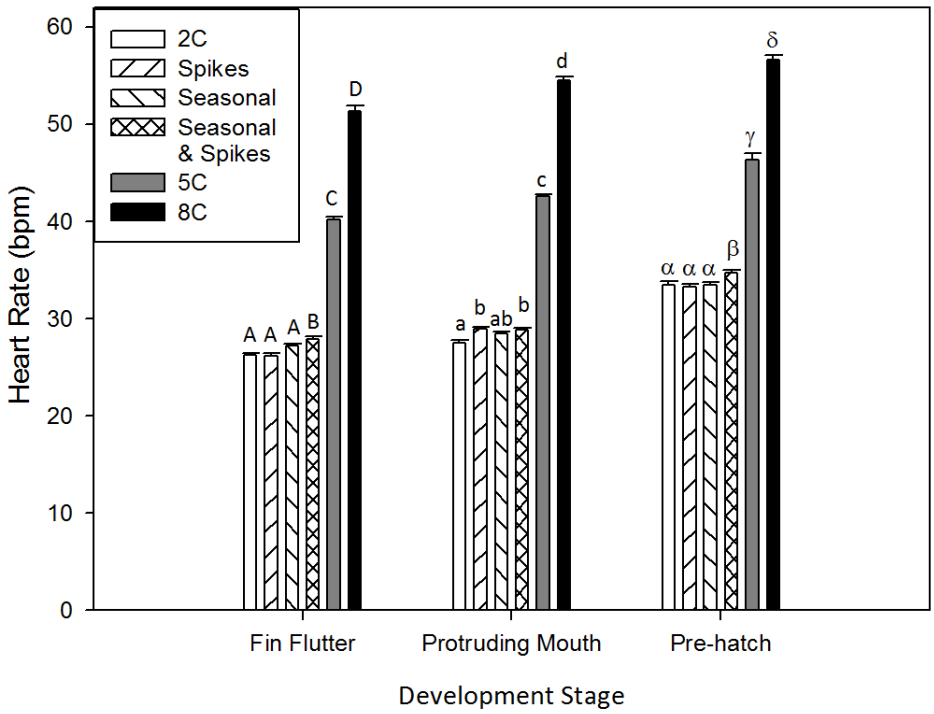



**Fig. 2.5**. Mean heart rates of lake whitefish embryos under different incubation treatments. Means within a development stage without the same letter/symbol are significantly different from each other (Holm-Sidak post hoc comparison across all treatment groups, 2-way ANOVA, $p<0.05$). Heart rate significantly increased for all treatment groups across development stages. Error bars indicate SEM (N=18 for each treatment).

# Chapter 3

# Impacts of temperature, morpholine, and chronic radiation on the embryonic development of round whitefish (*Prosopium cylindraceum*)

Michael Y.-T. Lim, Richard G. Manzon, Christopher M. Somers, Douglas R. Boreham, and Joanna Y. Wilson

## 3.1 Abstract

Round whitefish (*Prosopium cylindraceum*) embryos were reared from fertilization until hatch with constant (2, 2.5, 5, or 8°C) and fluctuating temperatures regimes, chronic morpholine (10-1000 mg $L^{-1}$), or low-dose chronic irradiation 90.10-0.30mGy/day of $^{137}Cs$) to determine whether different environmental stressors can significantly impact development and survival of embryos and hatchlings. We determined mortality to hatch and 30 days post-hatch (dph) and morphometrics, heart rate, and oxygen consumption rate at four development stages; fully eyed, fin flutter, protruding mouth, and pre-hatch. Incubation at 8°C or exposure to ≥500mg $L^{-1}$ morpholine significantly elevated mortality before hatch. Embryos reared at 8°C had elevated mortality post-hatch compared to other constant temperature regimes. Embryos exposed to 500 mg $L^{-1}$, but not 0-100 mg $L^{-1}$, morpholine had delayed development stage onset, shortened hatch windows, and approached 100% mortality 30 dph. Seasonal temperature regimes with + 2°C/week warming periods at the end of the incubation period had >20% elevated mortality post-hatch. Irradiated embryos had >20% reduced mortality post-hatch compared to non-irradiated. Our study suggests that seasonal temperatures significantly increase development rate and shorten hatch timing, and have significant effects on survival after hatch. Round whitefish embryos were found to be more susceptible to morpholine, and are possibly less impacted by chronic low-dose irradiation than lake whitefish embryos.

## 3.2 Introduction

Changes to environmental conditions can have significant effects on fish embryonic development. The rate and duration of these changes can vary widely and may be natural and/or anthropogenic in origin. Some of these conditions include temperature fluctuations (e.g. Eme et al., 2015; Lim et al., Chapter 2; Mueller et al., 2015; Patrick et al., 2013; Thome et al., 2016), chemical exposures (e.g. Brandão et al., 1992; Thome, 2015), and radiation exposures (e.g. Simon et al., 2011; Thome, 2015). Tolerance ranges can differ widely between species for different stressors. Within these ranges, exposures to environmental stressors may have negligible effects on development and survival. However, as these stressors increase in magnitude, rate, duration, and/or complexity, they may cause significant impacts on embryo development. Some of these impacts include altered development rates, body growth rates, yolk consumption rates, abnormality incidence, and survival to hatch (e.g. Blaxter, 1992; Lim et al., Chapter 2; Thome, 2015, Thome et al., 2016). Anthropogenic activities can have strong impacts on environmental conditions and have been under scrutiny for several decades. One aspect under study is the impact on the development and survival of species near thermal power plants due to possible exposure to thermal and chemical stressors from once-through cooling processes. At nuclear power plants, radiation stressors may also be present.

Within these sites, species that have ecological, cultural, and/or economic importance are of greatest concern. One such species is the lake whitefish; a cold-adapted freshwater species native to North America that can be found in the Great Lakes area and many northern lakes/rivers (Macpherson et al., 2010; Rawson, 1951). Lake whitefish

embryo development has been well studied in the context of temperature stress (e.g. Brooke, 1975; Eme et al., 2015; Lim et al., Chapter 2; Mueller et al., 2015; Patrick et al., 2013; Price, 1940; Thome et al., 2016), with a few additional studies on chemical and radiation stressors (e.g. Thome, 2015). Our study focuses on a lesser-known cold-adapted freshwater species, the round whitefish. As both species have similar life histories, spawning behaviour, and embryo incubation conditions, our study will build on the previous work on lake whitefish (e.g. Lim et al., Chapter 2; Patrick et al., 2013; Thome, 2015; Thome et al., 2016) and describe and compare developmental impacts of temperature, morpholine, and radiation stressors to other cold-adapted species.

### 3.2.1 Temperature as an environmental stressor

Lake and round whitefish have similar spawning behaviour, laying and fertilizing eggs in shallow water with a gravel and cobble bottom. Both species spawn in late autumn (water temperatures decline to ~6-7°C), incubate over winter (~0.5-4°C), and hatch in the spring as water temperatures increase (~6°C; Normandeau, 1969; Price, 1940). Compared to lake whitefish embryos, which were transparent and light yellow with an average diameter of ~3.19 mm at fertilization (Sreetharan et al., 2015), round whitefish embryos were >20% wider, and were more opaque with a yellow-orange colouration (Normandeau, 1969). When reared in the lab, both whitefish species were found to possess higher mortality and shorter development times when incubated at higher temperatures (Griffiths, 1980; Price, 1940) although the majority of mortality for round whitefish was found to occur late in development. Once incubation temperatures

were above 7°C, round whitefish mortality approached 100% (Griffiths, 1980), lower than the temperature limits of lake whitefish (100% mortality at ~10ºC; Price 1940). While this suggests that round whitefish embryos are more temperature sensitive that lake whitefish embryos, it is not known whether round whitefish embryos are also more sensitive to fluctuating temperatures or other environmental stressors.

The timing of temperature changes monitored in the field (e.g. Patrick et al., 2013; Thome et al., 2016) combined with studies by Mueller et al. (2015) and seasonal temperature changes recreated in the lab (Lim et al., Chapter 2), strongly suggest that lake whitefish embryos have a "critical window" to temperature coinciding with the seasonal transition from autumn to winter. Critical windows have been described as moments in development where an organism is more susceptible to sub-lethal stressors (such as temperature), in that it will adjust its phenotype due to intrinsic or extrinsic factors (Burggren & Reyna, 2011). As round whitefish have similar spawning habitats and timing, they will experience a similar decline in temperature between autumn and winter. However, it is unknown if they also have a thermal critical window during this developmental period.

Water temperature can be impacted by anthropological activities such as the operation of thermal power plants. These power plants can be found near whitefish spawning habitats in the Great Lakes (Fietsch, 2011). When thermal power plants generate energy, water is often utilized from lakes or rivers to remove waste heat through a process known as 'once-through cooling'. This water is re-circulated back to the source, significantly warmer, as thermal effluent (Zeng et al., 2002). The magnitude of water

temperature elevation caused by this process can vary (ranging from ~ Δ 0.5-10ºC, with an average of ~ Δ 3ºC) depending on several factors including the amount of energy generation needed, the ambient temperature of the source water, and the distance from the discharge site (Thome et al., 2016). Temperature spikes (durations range from <30minutes to >2hours, average ~1 hour) can transiently elevate temperatures near discharge sites, which may impact the development of nearby embryos (Thome et al., 2016, unpublished raw data). These temperature changes can occur once every 1-3 days, with larger and more persistent changes occurring once every 2-3 weeks (Thome et al., 2016). However, this study may underestimate the magnitude and duration of temperature spikes because they recorded substrate water temperature near but not necessarily directly at a discharge site. With this in mind, a study on lake whitefish that incorporated repeating, simplified 1 hour, +Δ3°C temperature spike found significant increases in heart rate and growth compared to regimes without temperature spikes (Lim et al., Chapter 2).

While round whitefish embryos may also be exposed to natural and/or anthropogenic temperature fluctuations, very few laboratory studies incorporate fluctuating incubation temperatures or detail their impacts on round whitefish throughout development (e.g. Patrick et al., 2013). Griffiths (1980) has predicted there to be little effect of chronic ≤2ºC water temperature elevations or acute ≤5.5ºC water temperature elevations on round whitefish embryo survival. Although elevations below 2ºC may have no effect on survival, when fluctuating incubation temperatures result in elevated average incubation temperatures, an ~1ºC elevation resulted in lake whitefish embryos that were significantly larger at hatch and had smaller yolks (Thome et al., 2016). As round

whitefish are more sensitive to higher constant temperatures, it is possible that they may be more susceptible and display larger sub-lethal effects (e.g. growth, metabolic rate changes) to fluctuating temperatures than lake whitefish.

### 3.2.2 Chronic morpholine as an environmental stressor

In addition to thermal effluent, discharges from power plants may contain low levels of chemicals used to prevent corrosion and damage to pipes involved in cooling water systems. One such chemical is morpholine. Morpholine increases pH, neutralizing the effects of compounds such as carbon dioxide (Nordmann and Fiquet, 1996). Morpholine is thought to have a relatively short biological half-life (below 24h), and is not metabolized easily by biological systems (Tanaka et al., 1978). In Ontario, the guideline is set at 0.004 mg $L^{-1}$ for morpholine discharge, but regulatory limits for levels released by thermal power plants are much higher (e.g. 25 mg $L^{-1}$ for the Bruce Power nuclear generating station; Bruce Power, 2005).

There have not been many previous studies on the effect of morpholine on aquatic organisms. Of those studies, the majority studied acute exposures and adult fish (e.g. Brandão et al., 1992). One of the few that have used chronic exposures on fish embryos found significantly increased body size at hatch at as low as 1 mg $L^{-1}$, significantly decreased growth at 500 mg $L^{-1}$, and increased mortality at 1000 mg $L^{-1}$ in lake whitefish (Thome, 2015). Morpholine induces elevated pH levels of water, altering growth as an indirect effect of morpholine exposure. However increased mortality was a direct effect of morpholine exposure at higher concentrations (Thome, 2015).

3.2.3 Low-dose chronic radiation as an environmental stressor

Outflow water from nuclear power plants may contain very low levels of ionizing radiation. This radiation mainly comes from the release of tritium, which is used to both dissipate heat and moderate neutrons (Ontario Power Generation, 2015). Ionizing radiation can either directly or indirectly ionize (Hall and Giaccia, 2006). The source used by Thome (2015) and in our study utilizes $^{137}Cs$, which produces gamma rays. Gamma rays cause indirect ionization, leading to the 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 (Hall and Giaccia, 2006). This damage can in turn lead to increased repair mechanisms to compensate, cause mutations (which may be beneficial, detrimental, or silent), increase cell death, and/or cause the death of the organism (although the latter would only occur at high doses).

The level of damage that occurs and is observed depends on both the cumulative absorbed dose and dose rate. Cumulative absorbed doses are the total doses received (e.g. 70 mGy). Dose rates are the cumulative dose amounts received over a specific length of exposure time (e.g. 10 m Gy/day = 70 mGy/week). Lower dose rates generally result in less damaging effects as cell repair mechanisms are more likely to be able to keep up or surpass the rate of damage (Hall and Giaccia, 2006). The length of exposure (acute or chronic) can also have a large role on development, due to changing periods of sensitivity as the embryo develops (Ward et al., 1971). While there have been several acute radiation studies, there are very few studies on chronic irradiation, and even fewer on developing organisms. Of those studies, there have been a few that exposed fish embryos, observing

a variety of effects such as increased mortality at 0.5 mGy/day (Trabalka and Allen, 1977), increased development rate at 1 mGy/day (Simon et al., 2011), and growth stimulation at as low as 0.06 mGy/day (Thome, 2015).

To determine the effects of temperature, morpholine, and irradiation on round whitefish embryonic development, we reared round whitefish embryos under constant incubation temperatures (2, 2.5, 5, or 8ºC) or under eight fluctuating temperature regimes combining seasonal changes under different spring warming conditions, temperature spikes occurring for different spans of embryonic development, or a combination of seasonal changes and temperature spikes. Embryos were reared with five different morpholine concentrations (0, 10, 100, 500, or 1000 mg $L^{-1}$) or three different pH levels (8, 9, 10) to identify indirect effects of morpholine (incubation temperature 5ºC). Embryos were exposed to $^{137}Cs$ at dose rates of 0, 0.10, 0.16, or 0.30 mGy/day (incubation temperature 2.5ºC). We assessed if there were significant differences across different incubation treatments by evaluating changes in development rate, growth via morphological measurements, oxygen consumption, and survival until hatch. We monitored embryos up to 30 dph to examine effects on survival after the embryonic development period had ended. We hypothesized that exposure to seasonal temperatures would result in significantly larger embryos with increased oxygen consumption throughout development compared to constant temperature regimes. We hypothesized round whitefish would have significantly reduced growth and survival when exposed to morpholine, and significantly increased growth and growth efficiency with chronic, low-dose rate $^{137}Cs$ exposure.

## 3.3 Methods

### 3.3.1 Round whitefish embryos

Round whitefish embryos were acquired on December 2, 2014 and December 13, 2015 from Lake Ontario (43° 51'51.1"N and 78° 44'32.9"W). Milt and eggs were stripped from 3 male and 7 female fish in 2014 and from 10 males and 6 females in 2015, mixed, and fertilized *in vitro*. Fertilized eggs were placed in lake water, disinfected with Ovadine (0.5% iodine) for 30 minutes, and rinsed several times with additional lake water. Cleaned embryos were transported on ice to McMaster University in containers of approximately 50% embryos, 50% clean lake water. Upon arrival at McMaster University in 2014, ~1400 embryos were immediately distributed into 30 sterile petri dishes (100mm x 20mm) at a density of 45±6 (± standard deviation) embryos/dish. In 2015, embryos were first placed into McDonald Bell hatching jars at 5ºC at a density of ~5000 per litre of dechlorinated city water (see Mitz et al., 2014). 1 day post fertilization (dpf), ~8500 embryos were placed into 168 sterile petri dishes (100mm x 20mm) at a density of 51±4 (± standard deviation) embryos/dish. All dishes contained ~75mL of dechlorinated city water. In 2014, 10 dishes were each placed at 8 ºC, 5ºC, and 2ºC. At 1dpf, chorion diameter was 3.49±0.15 mm (± standard deviation) with a yolk area of 6.12±0.48 $mm^2$ (± standard deviation). In 2015, 40, 68, 20, and 40 dishes were placed at 8 ºC, 5ºC, 2.5ºC, and 2ºC respectively. At 1dpf, chorion diameter was 3.71±0.09 mm (± standard deviation) with a yolk area of 8.15±0.83 $mm^2$ (± standard deviation).

3.3.2 Embryo rearing & maintenance

Embryos were raised in custom fridges as described in Mitz et al. (2014) set at 8, 5, 2.5 (2015 only), and 2ºC. These fridges averaged temperatures of 8.0±0.2, 5.3±0.4, and 2.0±0.4ºC (±standard deviation) respectively in 2014, and 7.9±0.1, 5.0±0.1, 2.4±0.3, and 2.1±0.2ºC (±standard deviation) respectively in 2015. 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 5 minutes in ~100mL of dechlorinated water in a glass beaker adjacent to petri dishes in each fridge. Petri dishes underwent 100% water changes 3 times per week, regardless of year or incubation treatment, until all embryos hatched. Due to high mortality during the pre-hatch stage for 2014 embryos, embryos from an 8ºC petri dish were transferred to 24 well plates in an attempt to reduce the rate of water quality deterioration. Despite this, mortality was not significantly different for this plate compared to the other 8ºC petri dishes (data not shown). Dechlorinated water used for water changes was chilled within 0.1ºC of the incubation treatment group temperature (e.g. 4.9-5.1ºC for the 5ºC group). Water changes were carried out on ice.

Round whitefish embryos maintained at constant temperature (2, 5, and 8ºC) were reared alongside lake whitefish embryos (2014 embryo collections; see Lim et al., Chapter 2) to compare development differences and evaluate the rearing of round whitefish embryos in static water. Body length, body width, yolk area, body mass, yolk mass, and heart rate were measured at the fin flutter (indicated by onset of sustained pectoral fin movement), protruding mouth (indicated by a visibly protruding and open

mouth), and pre-hatch (indicated by being unhatched after hatch onset) development stages. Morphometrics on the pre-hatch stage took place after at least one third of plates per treatment group contained at least one hatch on the same day, and/or a minimum of 5 hatches occurred within a single plate. 10 embryos per treatment were selected to serve as a sample of each group, with 1 embryo randomly selected per dish.

In 2015, round whitefish embryos were reared at constant temperatures (2, 5, and 8ºC), under fluctuating temperature regimes, and with chronic exposures to morpholine or gamma irradiation. All embryos (2015 collections) were monitored for body length, body width, head width, eye diameter, yolk area, body mass, yolk mass, chorion mass, and oxygen consumption rate at the fully eyed (indicated by complete pigmentation of the eyes), fin flutter, protruding mouth, and pre-hatch development stages. 5 embryos per treatment were randomly selected to serve as a sample of each group, with a minimum of 1 embryo per dish before sampling another embryo from the same dish.

### 3.3.3 Fluctuating temperature regimes

To generate seasonal (S) temperature changes, 30 dishes of round whitefish embryos initially reared at 8ºC were gradually exposed to decreasing temperatures at a rate of -1ºC/week until reaching 2ºC, starting 1dpf. The embryos were kept at 2ºC for 8 weeks, and then were gradually exposed to warming temperatures at a rate of either +1ºC/week ($S_A$; 7 dishes) or +1ºC every 3-4 days (net 2ºC/week; $S_B$; 3 dishes) until they all hatched (See Fig. 3.1A). The average temperature for the $S_A$ and $S_B$ groups were 3.7±1.9 and 3.7±2.0ºC (±standard deviation) respectively.

For temperature spike ($SP_A$) treatments, 10 dishes of round whitefish embryos were initially held at 2ºC for the first 1.5 months of development. For two months after this constant 2ºC period, these embryos were exposed to +Δ3ºC temperature spikes every 2-3 days (See Fig. 3.1B). Temperature spikes occurred immediately by replacing 2ºC water with 5ºC water and lasted one hour. The dishes were then returned to 2ºC (taking ~1h for temperature to return to a stable 2ºC). These dishes would then remain at 2ºC until the next temperature spike (See Fig. 3.1C). Following this two month period of fluctuating temperature, embryos were held at 2ºC until they hatched. For the extended temperature spike ($SP_B$) treatment, 10 dishes of round whitefish embryos were reared at 2ºC and immediately exposed to Δ3ºC temperature spikes every 2-3 days throughout development (See Fig. 3.1D). The average temperatures for these regimes were 2.2±0.3 for SP and 2.2±0.5ºC for $SP_B$ (±standard deviation).

A combined seasonal ($S_A$ or $S_B$) and spikes ($SP_A$) incubation regime included the gradual cooling (-1ºC/week until reaching 2ºC) and warming periods (+1ºC/week [$S_A$+$SP_A$; 7 dishes] or +2ºC/week [$S_B$+$SP_A$; 3 dishes] until complete hatch) for the first and last 1.5 months of incubation respectively, coupled with the temperature spikes for the intermediate two months (See Fig. 3.1E). The average temperature for the $S_A$+$SP_A$ and $S_B$+$SP_A$ groups were 3.7±1.9 and 3.7±2.0ºC (±standard deviation) respectively. In addition, a combined seasonal ($S_A$ or $S_B$) and extended spikes ($SP_B$) regime consisted of round whitefish embryos which were immediately exposed to Δ3ºC temperature spikes every 2-3 days. These embryos experienced warming periods of either +1ºC/week [$S_A$+$SP_B$; 7 dishes] or +2ºC/week [$S_B$+$SP_B$; 3 dishes] until complete hatch (See Fig. 3.1F).

The average temperature for the $S_A$+$SP_B$ and $S_B$+$SP_B$ groups were 3.7±1.9 and 3.8±2.0ºC (±standard deviation) respectively.

For oxygen consumption measurements, all temperature regimes that incorporated temperature spikes (e.g. spikes or seasonal with spikes) were measured at their base temperatures (e.g. if reared at 2ºC with a Δ3ºC spike to 5ºC, oxygen consumption measurements took place at 2ºC). Exceptions to this were embryos in the seasonal with spikes treatments, which were incubated at 3-4ºC at the pre-hatch stage due to entering the warming period of seasonal regimes. To remain comparable to embryos raised at constant 2ºC, these embryos were incubated at 2ºC for an hour prior to being placed in the chilled recirculating bath. This procedure was also followed for all other temperature regimes that incorporated seasonal temperature changes at the pre-hatch stage. In addition, as the $S_B$, $S_B$+$SP_A$, and $S_B$+$SP_B$ regimes only differed from $S_A$, $S_A$+$SP_A$, and $S_A$+$SP_B$ respectively for during the pre-hatch stage, there were no separate oxygen consumption or morphometric measurements taken for the preceding development stages.

At 1.5 months post fertilization (coinciding with the constant 2ºC period seen with the $S_A$ and $S_B$ regimes), 10 dishes of round whitefish embryos initially raised at 2ºC were transferred onto ice within the 2ºC fridge. These embryos were reared until hatch. This simulated exposure to near freezing temperatures, and is denoted as 0ºC (See Fig. 3.1G). Ice was changed alongside water changeouts (3 times per week). The average temperature for this regime was 1.0±0.6ºC (±standard deviation).

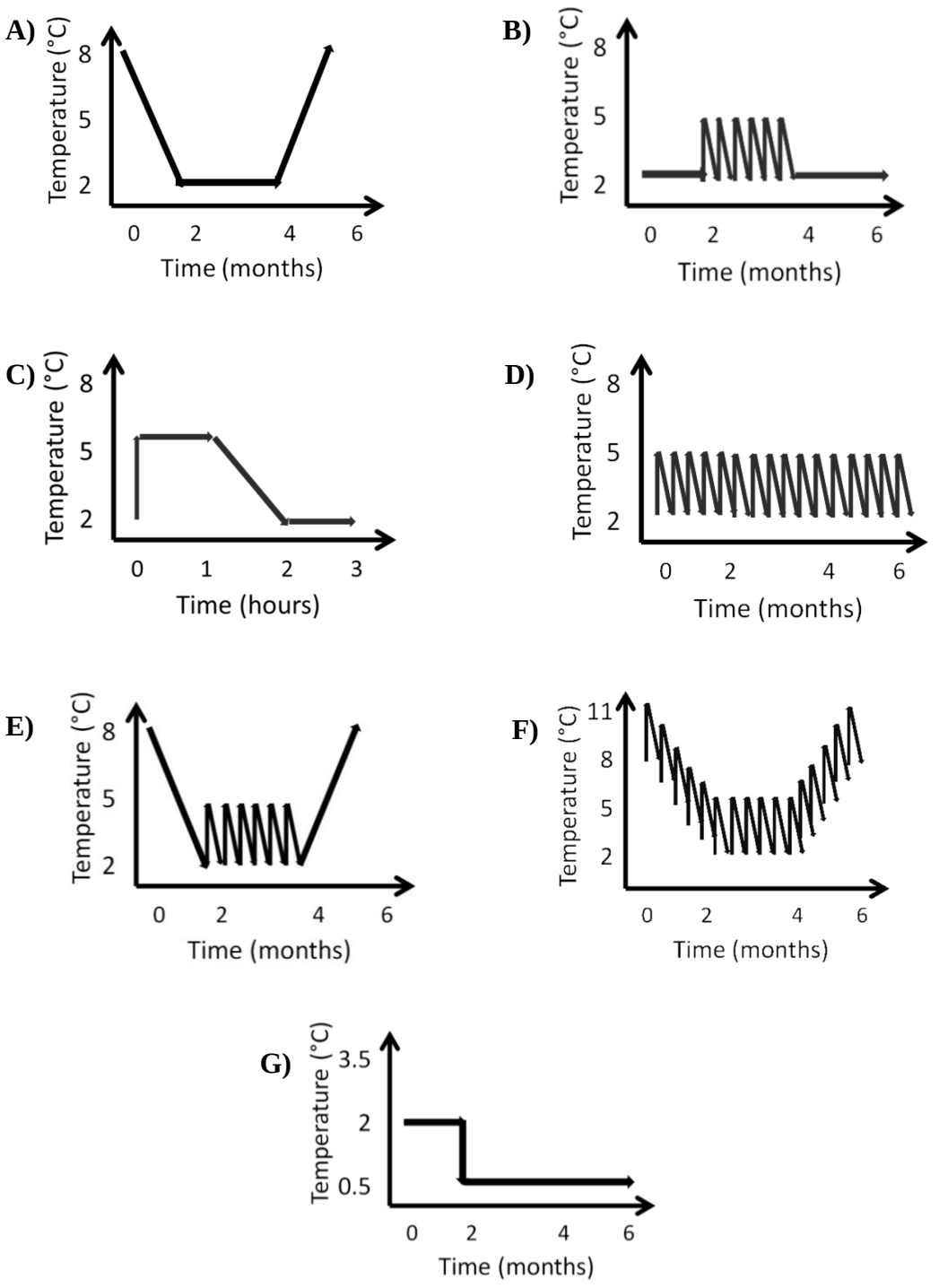



**Fig. 3.1.** Fluctuating temperature regimes: (A) seasonal ($S_A$), (B) spikes ($SP_A$), (D) extended spikes ($SP_B$), (E) seasonal and spikes ($S_A$+$SP_A$), (F) extended seasonal and spikes ($S_A$+$SP_B$), and (G) 0ºC. The process of a temperature spike is also shown in detail (C).

3.3.4 Chronic morpholine & pH exposures

To evaluate the effects of morpholine, 50 dishes (5 doses with 10 dishes/dose) of round whitefish embryos were reared at 5ºC and chronically exposed to 0, 10, 100, 500, or 1000 mg $L^{-1}$ of morpholine (made from laboratory grade morpholine [Sigma-Aldrich]). These exposures are denoted as M0, M10, M100, M500, or M1000 respectively. Concentrations were verified in water with liquid chromatography mass spectrometry (measured by the McMaster Regional Centre for Mass Spectrometry; data not shown).

An additional 18 dishes (3 doses with 6 dishes/dose) of round whitefish embryos reared at 5ºC were chronically exposed to solutions of pH 8, 9, or 10 (made from a 0.1M NaOH stock solution [EMD]). pH values of these samples (and of morpholine samples) were verified with a benchtop pH meter (Mettler Toledo EL20). pH values were recorded daily over 3 days, the longest time between water changes.

These exposures continued until all embryos either died or hatched. Once hatched, round whitefish were placed into new petri dishes where they were exposed to: the same concentration of morpholine/pH they were incubated at before hatch (e.g. M500 treatment embryos were placed into a solution of 500 mg/ $L^{-1}$ morpholine), to control dechlorinated water (e.g. M500 treatment embryos were placed into a solution of 0 mg/ $L^{-1}$ morpholine), or gradually exposed to decreasing morpholine/pH concentrations at a rate of ~25% per water change (e.g. M500 treatment embryos were placed into a solution of ~375 mg $L^{-1}$ morpholine at the first water change).

3.3.5 Chronic irradiation exposures

To evaluate the effects of chronic low-dose irradiation from a $^{137}$Cs source, 20 (4 dishes with 5 dishes/dose) dishes of the round whitefish embryos were raised at 2.5ºC and chronically exposed to 0, 0.10, 0.16, or 0.30 mGy/day. This occurred within a large custom reinforced fridge containing a lead 5-shelf containment unit (see Thome, 2016). Dose was verified with Genesis Ultra TLDs (thermoluminescent dosimeters; Mirion Technologies). Four dosimeters were placed on each shelf to record spatial variation in dose rates. Non-irradiated embryos were reared in the same reinforced fridge outside the containment unit and were handled in the same manner as irradiated embryos. Temperature inside the containment unit was not significantly different from within the reinforced fridge, with an average temperature of 2.7±0.1ºC (±standard deviation).

3.3.6 Development stage onset, mortality, and 50% hatch

Throughout the incubation period of both rearing years, two dishes per incubation group were randomly selected and observed daily under an AXIO Zoom V16 microscope (Carl Zeiss AG) to determine when a treatment group reached each development stage. Mortality was monitored daily and dead embryos were removed. Upon initiation of the hatch window (defined as the length of time between the first and last embryo hatching), the number of hatched embryos was monitored daily for each incubation group until all embryos hatched. This allowed for the calculation of 50% hatch for each incubation group.

### 3.3.7 Heart rate measurements

At the onset of the fin flutter, protruding mouth, and pre-hatch stages, one embryo per dish was randomly selected to serve as a sample of each incubation treatment group at each time point (i.e. 10 embryos per incubation treatment). Heart rate was measured via direct observation of embryos under an AXIO Zoom V16 microscope (Carl Zeiss AG). Embryos were placed in a custom built water-jacket observation chamber (described in Eme et al., 2015). The observation chamber was filled with dechlorinated tap water at the same incubation temperature (e.g. 5ºC embryos were observed at 5ºC). Embryos were given ~10 min to rest individually in the observation chamber prior to measurements. Average heart rate (bpm) was estimated by visual counting for 1 min (in triplicate) with ~45 seconds between each measurement. Average heart rate was later divided by the dry body mass of each tested embryo to calculate the mass-adjusted heart rate. Following heart rate measurements, embryos were placed in 10% formalin for 7 days, and then transferred to 50% ethanol for an additional 42-44 days. Embryos were then dissected and imaged on their ventral and lateral sides using an AXIO Zoom V16 microscope (Carl Zeiss AG).

### 3.3.8 Morphometrics

Morphometrics were carried out on the images of fixed embryos using the AxioVision Release 4.8 length measurement tool (see Sreetharan et al., 2015) following dissecting the body and yolk apart. Body length and yolk areas were measured similarly to Sreetharan et al. (2015). Body width of fixed embryos was also measured, and defined

as the distance between the dorsal and ventral sides of the body perpendicular to the body length measurement halfway down the lateral side of the embryo. Head width measurements were taken with the embryo laid on its ventral side and measured between the sides of the head at the most posterior edge of the eyes. For eye diameter, the embryo was laid on its lateral side and length measured between the two furthest points from the edge of the eye. The embryos' bodies and yolk were then dried for 23-25h in a 70 °C oven (model 1500EM, VWR/Sheldon, Cornelius, OR, USA) and separately weighed to ±0.01 mg (Mettler-Toledo XA105DU) to determine dry body mass and dry yolk mass.

3.3.9 Oxygen consumption

At the fully eyed, fin flutter, protruding mouth, and pre-hatch stages, oxygen consumption rate was measured via observation of embryos via custom built respiratory glass vials with $O_2$ sensor spots in a chilled recirculating bath (see Mueller et al., 2015). These sensor sports were read with a fibre-optic cable (PreSens Precision Sensing GmbH, Regensburg, Germany) connected to an Oxy-4 mini oxygen meter (PreSens). Water temperature was verified every 10 min with both a mercury thermometer and a Thermocouple Thermometer Handheld (VWR International). Embryos were given ~15 min to rest in the bath prior to measurements. Each chamber was recorded for ~30 sec every 10 min. Average oxygen consumption (mg $O_2$ /hr/mg dry body mass) was calculated by averaging across each chamber's change across a 90 min measurement period. An additional blank chamber was run alongside each treatment group to account for any changes caused by microbiota. Following measurement, embryos were placed in

10% formalin for 7 days, and then transferred to 50% ethanol for an additional 23-24 days.

### 3.3.10 Feeding and mortality monitoring post-hatch

Beginning 5-10 dph, hatchlings were fed crushed fish pellets (Otohime Larval Fish Diet; Marubeni Nisshin Feed Co., Ltd.) every 2-3 days (due to low interest in eating shortly after hatch/at low temperatures), followed 1 hour later by 100% water change outs. Temperatures in the fridges hatchlings were held in were increased by 1°C every 2 days to a maximum of 15°C. Once at 10°C, feeds and water changes occurred daily due to more frequent feeding behaviour. Mortality post-hatch was monitored for up to 30 days after the end of the hatch window to evaluate differences in early hatchling mortality across treatment groups.

### 3.3.11 Statistical analyses

Individual hatchlings measurements (i.e. body weight, yolk weight, body length) and mortality were averaged across replicate dishes for each treatment to generate an overall mean. Heart rates were measured for individual hatchlings in triplicate, and this mean was averaged across each replicate dish to generate an overall mean. Statistical analyses were run using each dish in a treatment as a replicate. Mortality was compared across treatment groups using a one-way Analysis of Variance (ANOVA), with a post-hoc Tukey Test when the ANOVA was significant (Sigmaplot 11). To compare across treatments and development stages, all other variables were compared utilizing two-way

ANOVAs with a post-hoc Holm-Sidak test when the ANOVA was significant (Sigmaplot 11). Data throughout this study is presented as the mean ± SEM unless otherwise stated, with $\alpha$=0.05 for all ANOVA and post-hoc testing.

## 3.4 Results

### 3.4.1 Embryos reared under constant temperatures

#### 3.4.1.1 Development stage onset, hatch dynamics, and mortality

As increased incubation temperatures resulted in earlier hatch, embryonic development was normalized to median hatch and expressed as a percentage of development (Table 3.1). Embryos raised at constant incubation temperature showed a general trend for earlier onset of each development stage relative to time to median hatch, and longer hatch windows as incubation temperature decreased. This was particularly emphasized in 2015, where the embryos reared at 8°C were relatively slower than embryos reared at 2 and 5°C (72 vs. 59/57% respectively) to reach the fin flutter stage. However, embryos reared at 8°C developed more quickly, reaching median hatch relatively faster (>13% of development time) compared to embryos raised at 2 and 5°C (Table 3.1). In both years, mortality was significantly elevated with incubation at 8°C compared to embryos reared at 2 and 5°C (Fig. 3.2). While mortality at 8°C was comparable between the years, 2015 embryos reared at 2 or 5°C had significantly elevated mortality (>50%) compared to those reared in 2014 (Fig. 3.2).

3.4.1.2 Growth, yolk consumption, mass-adjusted heart rate, and oxygen consumption rate

Regardless of incubation temperature, embryos collected in 2015 generally had larger (>10%) body mass and smaller (>10%) yolk mass throughout the latter half of development compared to those collected in 2014 (Fig. 3.3A, B). The most growth/yolk consumption occurred at lower incubation temperatures, and was most prominent at the pre-hatch stage (e.g. a stepwise increase in body mass, and as stepwise decrease in yolk mass, as incubation temperature decreased; Fig. 3.3A, B). Before the pre-hatch stage, body and yolk mass was often not statistically different between incubation temperatures, especially for 8 and 5°C embryos (Fig. 3.3A, B).

Colder incubation temperatures had significantly slowed heart rates (>70% lower maximum between embryos reared at 8 and 2°C) at each development stage. Across development stages, heart rate decreased significantly for all incubation temperatures. The embryos reared at 5°C had the greatest reduction (57%) while the embryos reared at 8°C had the least reduction (39%) in heart rate between the fin flutter and pre-hatch stages (Table 3.2). Oxygen consumption rates also generally decreased both as temperature decreased and as development progressed (>60% lower maximum between embryos reared at 8 and 2°C). However, there were no significant trends at the fin flutter and protruding mouth stages. Oxygen consumption for embryo reared at 8°C was found significantly increased at the pre-hatch stage compared to the fin flutter and protruding mouth stages (Table 3.3).

3.4.2 Embryos reared under fluctuating temperatures

3.4.2.1 Development stage onset, hatch dynamics, and mortality

Similar to embryos reared under constant temperature, there was a general trend for later development stage onset and shorter hatch windows as average incubation temperatures increased. Fluctuating temperature groups that included seasonal temperature changes had notably earlier development stage onsets (10-20%) except for the pre-hatch stage, even compared to embryos raised at colder constant incubation temperatures (Table 3.4). The treatments including seasonal temperatures with +2°C/week warming periods ($S_B$, $S_B$+$SP_A$, and $S_B$+$SP_B$) had earlier median hatches (5-7 days shorter hatch windows) compared to seasonal regimes with +1°C/week ($S_A$, $S_A$+$SP_A$, and $S_A$+$SP_B$) warming periods. Compared to the seasonal regimes, the spikes regimes had little influence on development stage onset compared to embryos reared at 2°C other than the slightly later protruding mouth stage onset for the extended spikes ($SP_B$) group (Table 3.4).

Of the different constant and fluctuating temperature regimes, only the constant 8°C regime had significantly elevated mortality (>15%), with slightly elevated mortality for the $S_B$ regime (Fig. 3.4). Following hatch, the 8°C regime and all seasonal regimes with the +2°C/week warming period had significantly elevated mortality (>20% higher than other constant temperature and seasonal +1°C/week warming period embryos respectively; data not shown).

3.4.2.2 Growth, yolk consumption, chorion mass, and oxygen consumption rate

Body length, eye diameter, and head width all generally increased in size across development for constant and fluctuating temperature embryos, except for embryos reared at 8°C, which had no statistically significant differences between the protruding mouth and pre-hatch stages (data not shown). Within a single development stage, lower average temperature generally resulted in embryos of larger size, which was most prominent at the protruding mouth stage (i.e. >70% larger body mass for embryos reared at 0°C compared to those reared under the $S_A$+$SP_B$ regime; Fig. 3.5A). A similar but opposite trend was seen for yolk size, wherein higher average incubation temperatures resulted in larger yolks within development stages. This was most prominently seen at the pre-hatch stage, where the 0°C treatment had >55% smaller yolk mass compared to the $S_A$+$SP_B$ treatment (Fig. 3.5B). Other than the 0°C treatment, the fluctuating temperature treatments had comparable body sizes and yolk consumption to each other, and to constant 2 and 2.5°C treatments. Dry chorion mass was not significantly different across any treatment or development stage other than for 0°C treatment at the pre-hatch stage. The chorion mass at this stage was significantly smaller (>60%) than all other fluctuating and constant temperature regime treatments (data not shown).

Other than the embryos reared under the $S_A$+$SP_B$ regime, which had oxygen consumption rates below (~80%) the consumption rate of embryos reared under the $S_A$ regime (data not shown), there were no significant changes in oxygen consumption across the fluctuating temperature regimes or constant incubation temperatures of 2, 2.5, and 5°C.

### 3.4.3 Embryos exposed to chronic morpholine

#### 3.4.3.1 Development stage onset, hatch dynamics, and mortality

Exposure to 10, 100, and1000 mg $L^{-1}$ morpholine had comparable pH levels as the pH8, 9, and 10 treatments respectively (Table 3.5). 500 mg $L^{-1}$ solutions had an average pH of ~9.5 (see Table 3.5), and embryos exposed to 500 mg $L^{-1}$ exhibited advanced median hatch (15 days) compared to unexposed embryos. However, there were no median hatch advancements for the pH 9 and 10 treatments (Table 3.6).

Before hatch, mortality was significantly elevated for the M500 and M1000 treatments compared to the M0 treatment, reaching 100% for the M1000 treatment (Fig. 3.6). Mortality until 30 dph was >85% elevated for embryos exposed to 500 mg $L^{-1}$ compared to unexposed embryos (i.e. ~93% vs. ~7%). Mortality was not significantly different post-hatch within morpholine treatments regardless of the concentration they were maintained in post-hatch (i.e. embryonic incubation concentrations, ~25% reduction in incubation concentration, or 0 mg $L^{-1}$ morpholine). There were no mortality elevations for pH 8, 9, or 10 treatments.

#### 3.4.3.2 Growth, yolk consumption, chorion mass, and oxygen consumption rate

At the protruding mouth stage, only the embryos in the M10 treatment had significantly larger body mass (>20%) despite having similar body lengths, head widths, and eye diameters as unexposed embryos (see Fig. 3.7A for mass; other body measurement data not shown). At pre-hatch, embryos in the M100 treatment had significantly smaller (>20%) body masses with similar body lengths, head widths, and

eye diameters of unexposed embryos (see Fig. 3.7A for mass; other body measurement data not shown). At pre-hatch, body mass of M500 embryos was ~20% smaller than M100 embryos, and had significantly larger yolk sac mass (Fig. 3.7B) and area (data not shown) than unexposed embryos. The only significant differences in yolk mass were at the protruding mouth and pre-hatch stages for embryos reared in 500 mg $L^{-1}$ morpholine (>15% larger than unexposed embryos; Fig. 3.7B). There were no significant differences in dry chorion mass or oxygen consumption across morpholine treatments (data not shown).

### 3.4.4 Embryos exposed to chronic radiation

#### 3.4.4.1 Development stage onset, hatch dynamics, and mortality

There were no significant development rate differences between irradiated and non-irradiated embryos. 0.10 and 0.16 mGy/day dose rates were significantly lower from the 0.30 mGy/day dose rate, but not significantly different from each other (Table 3.7). Embryos reared under 0.10 and 0.16 mGy/day dose rates had an earlier median hatch compared to controls, resulting in relatively delayed protruding mouth and pre-hatch stage onset (Table 3.8).

While there were no significant differences in mortality between irradiated and non-irradiated embryos before hatch, all irradiated embryos had >20% reduced mortality up to 30 dph compared to control embryos (data not shown).

3.4.4.2 Growth, yolk consumption, chorion mass, and oxygen consumption rate

Within the fully eyed and fin flutter development stages, there was a trend for increased body mass as dose rate increased (Fig. 3.8). However, the embryos irradiated with 0.16 mGy/day had significantly smaller head widths and body lengths compared to non-irradiated embryos at the fully eyed and fin flutter stages respectively (data not shown). There were no other significant differences in growth for the other doses, or at the protruding mouth and the pre-hatch stages. There were no significant effects of dose rate on yolk mass or area (data not shown). Dry chorion mass was significantly larger for the embryos irradiated with 0.16 mGy/day compared to non-irradiated embryos at the fully eyed stage, but was not significantly different across any dose rate at the later development stages (data not shown). There were no significant differences in oxygen consumption across dose rate (data not shown).

## 3.5 Discussion

Lake whitefish have been the focus of more studies than round whitefish due to their higher economic and cultural importance. However, round whitefish are also ecologically important and have some commercial value. Round whitefish populations have also been gaining more attention as a potentially sensitive species, with a significant decline in round whitefish populations (e.g. Lake Ontario) over the last three decades (see Ecometrix Incorporated, 2014). Few studies have been conducted on the embryonic development of round whitefish. Of the few available studies, the only one that examined development rate and mortality utilized constant incubation temperatures, observing

altered embryonic development (e.g. significantly elevated mortality) at 8°C compared to embryos reared at 2 and 5°C (Griffiths, 1980). These developmental changes were greater than seen with lake whitefish (Griffiths, 1980; Lim et al., Chapter 2; Price, 1940), suggesting that round whitefish are more sensitive to temperature stressors and/or have a lower thermal maximum. However, the effects of environmental stressors such as fluctuating temperatures are unknown. Our study examined the potential effects of increased and fluctuating temperatures, morpholine, and radiation stressors on round whitefish embryo development, and contrasted these effects to previous studies on lake whitefish. Our data suggests that compared to lake whitefish embryos, round whitefish embryos are more sensitive to elevated temperatures (Lim et al., Chapter 2), and more susceptible to morpholine but less susceptible to low-dose radiation stressors (Thome, 2015).

### 3.5.1 Incubation at constant elevated temperatures increases mortality and decreases growth

Constant elevated temperature during embryogenesis has been well studied in fish, including the cold-adapted lake whitefish. Elevated incubation temperature in round whitefish resulted in similar effects across years of sampling and compared to previous studies in fish. Specifically, increased mortality, decreased body size, and decreased yolk consumption at hatch were observed at increased incubation temperatures for round whitefish embryos (including this present study; Griffiths, 1980), and the many studies on lake whitefish embryos (e.g. Brooke, 1975; Lim et al., Chapter 2; Mueller et al., 2015;

Price, 1940). The mortality rates of round whitefish embryos in Griffiths (1980) and this study were comparable, surpassing 90% as average temperatures surpassed 7°C. However, we did see significant differences in mortality across sampling years at lower incubation temperatures; mortality was increased in 2015 compared to 2014 at 2 and 5°C. As we used similar rearing conditions, the difference in mortality is likely due to the timing of collection during the spawn (e.g. 2015 had warmer autumn water temperatures) or gamete quality (e.g. greater yolk mass consumption for 2015 embryos between fertilization and fin flutter may suggest relatively low energy density of initial yolk deposits).

### 3.5.2 Seasonal temperatures advance development rate, and hatch dynamics are strongly affected by the warming period at the end of development

As predicted, seasonal changes had the largest influences on the development rate of round whitefish, wherein all stages before pre-hatch occurred significantly earlier relative to total development time. This was very similar to the effects seen on lake whitefish (Lim et al., Chapter 2). Increased warming rates near the end of development advanced median hatch and shortened hatch windows of round whitefish, also similar to what has been seen with lake whitefish (Lim et al., Chapter 2; Patrick et al., 2013; Thome et al., 2016). In agreement with Patrick et al. (2013), round whitefish embryos reared with +2°C/week warming periods had a relatively advanced median hatch, with hatch windows of ~10 days compared to 15-20 days seen with this study's +1°C/week embryos. Interestingly, increasing warming rate not only influenced hatch timing, but also

consistently increased mortality post-hatch ($S_B$, $S_B$+$SP_A$, and $S_B$+$SP_B$ regimes), suggesting that years with increased spring warming will have lower hatchling survival and subsequently smaller recruitment.

As had been observed for lake whitefish (Lim et al., Chapter 2) temperature spikes had little impact on embryonic development, suggesting that variability in incubation temperature may not be sufficient to impact development without elevated average incubation temperature (e.g. the $SP_B$ regime was only ~0.1°C warmer on average compared to the constant 2°C regime). While chronic temperature elevations below 2°C may not affect round whitefish survival (Griffiths, 1980), assuming round whitefish are at least as thermally sensitive as lake whitefish, chronic temperature elevations ~1°C (see Thome et al., 2016) may significantly impact growth and yolk consumption of round whitefish. To have the same magnitude of temperature elevation, round whitefish embryos would require very frequent (e.g. Δ3°C spikes 6 times/day) and/or very large (e.g. Δ15°C spikes 1 time/day) temperature spikes throughout the entirety of development for fluctuating temperatures to impact growth. While our embryos exposed to seasonal temperatures had average incubation temperatures of ~3.7°C, the main causes of this temperature increase were the seasonal declines and inclines near the beginning and end of development. As such there appears to be thermal critical windows for round whitefish embryo development rate at these times, which is very similar to what has been reported for lake whitefish embryos exposed to seasonal temperature regimes (Lim et al., Chapter 2).

3.5.3 Chronic morpholine exposure decreases body growth, advances median hatch, and increases mortality

At 100 mg $L^{-1}$ morpholine, round whitefish embryo growth efficiency was reduced based on significantly reduced body size pre-hatch with no significant reduction in yolk area, which was not seen in lake whitefish at this concentration (Thome, 2015). This suggests that round whitefish embryos may have a lower threshold dose for growth alteration with morpholine exposure. Similar to lake whitefish embryos (Thome, 2015), round whitefish embryos exposed to 500 mg $L^{-1}$ also had significant reductions in body growth and yolk consumption at the pre-hatch stage. However, these differences may also be due to an advanced median hatch and shortened hatch window for the M500 treatment, wherein embryos had less time to grow/use internal energy stores. However, unlike lake whitefish, round whitefish embryos exposed to 500 mg $L^{-1}$ morpholine had significantly elevated mortality compared to unexposed embryos. When morpholine exposure levels were at 1000 mg $L^{-1}$, round whitefish embryos had complete mortality before the protruding mouth stage. While this was also found in lake whitefish embryos (Thome, 2015), the onset of mortality occurred earlier (~10 days) for round whitefish. Collectively, this suggests that round whitefish may be more susceptible to morpholine than lake whitefish.

3.5.4 Chronic low-dose irradiation advances median hatch, promotes more efficient body growth early in development, and reduces mortality post-hatch

Round whitefish embryos exposed to a dose rate of 0.10 or 0.16 mGy/day (but not 0.30 mGy/day) had an earlier median hatch compared to non-irradiated embryos. While this was seen with chronically irradiated lake whitefish embryos (0.11 and 0.19 mGy/day respectively; Thome, 2015), lake whitefish had significantly advanced median hatch for all studied dose rates (0.06-4.40 mGy/day). The lack of median hatch advancement at 0.30 mGy/day for round whitefish embryos coupled with the relatively small median hatch advancement at the lower dose rates for round whitefish (7 days for round whitefish vs. ≥14 days for lake whitefish; Thome, 2015) suggest that hatch dynamics for round whitefish embryos are not as strongly affected by low-dose radiation as lake whitefish embryos. In regards to growth, body size increased for irradiated round whitefish embryos at the fin flutter stage, with no significant decrease in yolk area compared to non-irradiated embryos, suggesting that exposure to increasing dose rates results in increased growth efficiency for round whitefish embryos early in development. However, these impacts on growth were not seen at later development stages. Interestingly, irradiated lake whitefish embryos were significantly smaller at hatch (Thome, 2015). Similarly, yolk size was not significantly different across dose rates at pre-hatch for round whitefish embryos, but yolk sacs were significantly larger for irradiated lake whitefish embryos (Thome, 2015). The reduced/lack of significant differences in median hatch advancement, growth, and yolk consumption suggest that round whitefish embryonic development is less impacted by low-dose radiation compared to lake whitefish.

For both lake and round whitefish, there were no significant changes in mortality between control and irradiated embryos (Thome, 2015). However, embryos that were irradiated had notably reduced mortality up to 30 dph, suggesting that low-dose irradiation provides some survival benefit in early hatchling development. It is possible that cumulative exposure to chronic low-dose irradiation triggered an increase in DNA repair pathways or stress response mechanisms (e.g. increasing constitutive heat shock proteins and/or inducible heat shock protein production) which may help prepare the embryos for future stressors (see Jolly and Meyer, 2009). As such, chronic low-dose irradiation may not impact round whitefish as much as lake whitefish during embryonic development, but it could be beneficial for post-hatch survival.

### 3.5.5 Conclusions

Our study demonstrated that similar to lake whitefish, seasonal temperature changes (i.e. temperature decline from fall-to-winter and incline from winter-to-spring) have strong influences on development rate and timing while small, regular temperature spikes have limited developmental impacts. Round whitefish raised at constant temperature shared many similar trends with lake whitefish (e.g. development and growth rates), but had significantly higher mortality at 8°C, suggesting they are relatively more sensitive to thermal stress. Exposure to high concentrations of morpholine caused significantly higher and earlier mortality in round whitefish compared to lake whitefish embryos, suggesting that round whitefish are additionally more susceptible to morpholine. On the other hand, few effects were documented for the development and

growth of round whitefish exposed to low-dose radiation, suggesting that they may be less impacted by gamma radiation. Chronic exposure to low-dose radiation appeared to have survival benefits post-hatch compared to non-irradiated embryos. Contrary to radiation, higher constant incubation temperature, faster spring warming events, and chronic morpholine exposure (500mg $L^{-1}$) all appear to significantly increase mortality post-hatch. Collectively, this suggests that embryonic incubation conditions can impact the animal post-hatch, and studies that end at hatch likely underestimate the total effects of the stressor. The differences in survival for our different temperature, morpholine, and radiation regimes 30 dph gives emphasis to the importance of designing future studies to quantify survival and growth rate differences beyond hatch.

3.6 Acknowledgements

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Tables & Figures

**Table 3.1**. Time to onset of each development stage for round whitefish embryos incubated at a constant temperature for the duration of development. Time is reported as percent of development time with the days post fertilization (dpf) in parentheses. Average temp refers to the mean temperature during incubation (± standard deviation). 50% Hatch values indicate time to median hatch.

| Treatment Group | | Average Temp (°C) | Fully Eyed | Fin Flutter | Protruding Mouth | Pre-hatch | 50% Hatch |
|---|---|---|---|---|---|---|---|
| **2014** | 2ºC | 2.0 (±0.4) | 38% (60%) | 52% (83) | 66% (106) | 91% (146) | 100% (160) |
| | 5ºC | 5.3 (±0.4) | 35% (34) | 52% (50) | 67% (65) | 93% (90) | 100% (97%) |
| | 8ºC | 8.0 (±0.2) | 39% (26) | 52% (35) | 72% (48) | 88% (59) | 100% (67) |
| **2015** | 2ºC | 2.1 (±0.2) | 43% (60) | 59% (82) | 72% (101) | 94% (132) | 100% (140) |
| | 5ºC | 5.01 (±0.1) | 42% (37) | 57% (50) | 76% (67) | 94% (83) | 100% (88) |
| | 8ºC | 7.9 (±0.1) | 42% (25) | 72% (43) | 82% (49) | 88% (53) | 100% (60) |

**Table 3.2**. Mass adjusted average heart rate (bpm/mg of dry body mass) of round whitefish embryos across development with increasing constant incubation temperatures. The percent of maximum heart rate is in parentheses. Means within a development stage without the same letter/symbol are significantly different from each other (Holm-Sidak post hoc comparison across all treatment groups, 2-way ANOVA, $p<0.05$; N=10 for each group). Heart rates for all groups decreased significantly across development. Average temp refers to the mean temperature during incubation (± standard deviation). Heart rate was measured in 2014 embryos only.

| Treatment Group | Average Temp (°C) | Fin Flutter | Protruding Mouth | Pre-hatch |
|---|---|---|---|---|
| 2ºC | 2.0 (±0.4) | $45.97^{a}$ (100%) | $29.10^{A}$ (63%) | $23.45^{\alpha}$ (51%) |
| 5ºC | 5.3 (±0.4) | $103.85^{b}$ (100%) | $60.60^{B}$ (58%) | $44.32^{\beta}$ (43%) |
| 8ºC | 8.0 (±0.2) | $168.03^{c}$ (100%) | $131.81^{C}$ (78%) | $102.25^{\gamma}$ (61%) |

**Table 3.3**. Average oxygen consumption rate (mg $O_2$/hr/mg of dry body mass) of round whitefish embryos across development with increasing constant incubation temperatures. The percent of maximum oxygen consumption is in parentheses. Means within a development stage without the same letter/symbol are significantly different from each other (Holm-Sidak post hoc comparison across all treatment groups, 2-way ANOVA, $p<0.05$; N=5 for each group). Average temp refers to the mean temperature during incubation (± standard deviation). Oxygen consumption was measured in 2015 embryos only.

| Treatment Group | Average Temp (°C) | Fully Eyed | Fin Flutter | Protruding Mouth | Pre-hatch |
|---|---|---|---|---|---|
| 2ºC | 2.1 (±0.2) | 22.68[a] (100%) | 16.64 (73.37%) | 1.63 (7.19%) | 6.64[A] (29.29%) |
| 5ºC | 5.0 (±0.1) | 49.03[ab] (100%) | 32.70 (66.69%) | 38.35 (78.22%) | 21.80[AB] (44.47%) |
| 8ºC | 7.9 (±0.1) | 63.42[b] (100%) | 27.02 (42.61%) | 22.52 (35.51%) | 57.69[B] (90.98%) |

**Table 3.4**. Time to onset of each development stage for round whitefish embryos incubated with constant and fluctuating temperature regimes for the duration of development. Time is reported as the percent of development time with the days post fertilization (dpf) in parentheses. Average temp refers to the mean temperature during incubation (± standard deviation). 50% Hatch values indicate time to median hatch. See Figure 1 and the methods section for a graphical overview or full description, respectively, of the fluctuating temperature regimes. Data for 2014 embryos are not included.

| Treatment Group | Average Temp (°C) | Fully Eyed | Fin Flutter | Protruding Mouth | Pre-Hatch | 50% Hatch |
|---|---|---|---|---|---|---|
| 0°C | 1.0 (±0.6) | 39% (70) | 59% (107) | 72% (130) | 96% (172) | 100% (180) |
| 2°C | 2.1 (±0.2) | 43% (60) | 59% (82) | 72% (101) | 94% (132) | 100% (140) |
| 2.5°C | 2.4 (±0.3) | 44% (57) | 61% (79) | 78% (100) | 94% (122) | 100% (129) |
| 5°C | 5.0 (±0.1) | 42% (37) | 57% (50) | 76% (67) | 94% (83) | 100% (88) |
| 8°C | 7.9 (±0.1) | 42% (25) | 72% (43) | 82% (49) | 88% (53) | 100% (60) |
| Spikes ($SP_A$) | 2.2 (±0.3) | 43% (59) | 60% (82) | 72% (99) | 95% (130) | 100% (137) |
| Extended Spikes ($SP_B$) | 2.2 (±0.5) | 45% (59) | 62% (81) | 76% (99) | 95% (123) | 100% (130) |
| Seasonal +1°C/week ($S_A$) | 3.7 (±1.9) | 23% (27) | 47% (54) | 63% (72) | 97% (112) | 100% (115) |
| Seasonal +2°C/week ($S_B$) | 3.7 (±2.0) | 25% (27) | 49% (54) | 65% (72) | 100% (110) | 100% (110) |
| Seasonal & Spikes +1°C/week ($S_A$+$SP_A$) | 3.7 (±1.9) | 24% (27) | 47% (54) | 62% (71) | 96% (110) | 100% (114) |
| Seasonal & Spikes +2°C/week ($S_B$+$SP_A$) | 3.7 (±2.0) | 25% (27) | 50% (54) | 66% (71) | 97% (105) | 100% (108) |
| Extended Seasonal & Spikes 1°C/week ($S_A$+$SP_B$) | 3.7 (±1.9) | 23% (26) | 46% (53) | 62% (71) | 94% (107) | 100% (114) |
| Extended Seasonal & Spikes 2°C/week ($S_B$+$SP_B$) | 3.8 (±2.0) | 24% (26) | 50% (53) | 66% (71) | 100% (107) | 100% (107) |

**Table 3.5**. pH measurements of morpholine and NaOH treated water. Treatment groups are given as morpholine concentration (M= morpholine, # refers to concentration in # mg $L^{-1}$) or pH level. pH was tested up to 3 days post-mix for all concentrations. This corresponded to the longest period between water changes. Values represent the mean of 5 replicates (Day 0 and 1) or 3 replicates (Day 2 and 3). Error values indicate SEM.

| Treatment Group | Day 0 | Day 1 | Day 2 | Day 3 |
|---|---|---|---|---|
| M0 | 7.47±0.24 | 7.53±0.11 | 7.58±0.19 | 7.27±0.41 |
| M10 | 8.20±0.19 | 7.76±0.20 | 7.70±0.37 | 7.74±0.27 |
| M100 | 8.88±0.23 | 8.33±0.16 | 7.93±0.41 | 8.00±0.29 |
| M500 | 9.46±0.18 | 8.62±0.22 | 8.32±0.37 | 8.31±0.25 |
| M1000 | 9.78±0.13 | 8.74±0.24 | 8.42±0.38 | 8.43±0.32 |
| pH8 | 8.05±0.04 | 7.65±0.16 | 7.52±0.29 | 7.48±0.34 |
| pH9 | 8.90±0.03 | 7.90±0.14 | 7.73±0.24 | 7.69±0.30 |
| pH10 | 9.91±0.01 | 8.39±0.25 | 7.93±0.23 | 8.04±0.27 |

**Table 3.6**. Time to onset of each development stage for round whitefish embryos chronically exposed to morpholine. Treatment groups are given as morpholine concentration (M= morpholine, # refers to concentration in # mg $L^{-1}$) or pH level, and were chronically exposed from one day post fertilization to hatch. As morpholine alters pH, pH controls were generated by adding NaOH to the M0 treatment group. M10, M100, and M1000 treatments had a pH that was equivalent to pH 8, 9, and 10 respectively; M500 was between pH9 and10 (See Table 3.5). Time is reported as the percent of development time with the days post fertilization (dpf) in parentheses. 50% Hatch value indicates time to median hatch. Protruding mouth, pre-hatch, and 50% hatch values were omitted (-) for the M1000 group due to all embryos dying before the protruding mouth stage. All treatment groups were maintained at 5.0±0.1°C.

| Treatment Group | Fully Eyed | Fin Flutter | Protruding Mouth | Pre-Hatch | 50% Hatch |
|---|---|---|---|---|---|
| M0 | 42% (37) | 57% (50) | 76% (67) | 94% (83) | 100% (88) |
| M10 | 44% (38) | 58% (50) | 80% (69) | 98% (84) | 100% (86) |
| M100 | 44% (38) | 58% (51) | 79% (69) | 95% (83) | 100% (87) |
| M500 | 52% (38) | 70% (51) | 96% (70) | 96% (70) | 100% (73) |
| M1000 | (38) | (51) | - | - | - |
| pH8 | 43% (37) | 59% (51) | 77% (67) | 97% (84) | 100% (87) |
| pH9 | 43% (37) | 60% (52) | 78% (68) | 93% (81) | 100% (87) |
| pH10 | 45% (37) | 63% (52) | 82% (68) | 96% (80) | 100% (83) |

**Table 3.7**. Chronic irradiator dose rates. Dose rates were measured on each shelf using 4 thermoluminescent dosimeters (TLDs) per shelf. Cumulative exposure was calculated to day 122 (pre-hatch stage). Different letters denote statistical differences between dose rates (Tukey post hoc comparison across dose rates, 1-way ANOVA, $p<0.05$). Error values indicate SEM.

| Distance from Source (cm) | Dose Rate (mGy/day) | Day 122: Cumulative Exposure (mGy) |
|---|---|---|
| 67.15 | $0.10\pm0.01^{a}$ | 12.01±1.11 |
| 52.86 | $0.16\pm0.02^{a}$ | 19.52±2.15 |
| 38.58 | $0.30\pm0.03^{b}$ | 36.77±3.75 |

**Table 3.8**. Time to onset of each development stage for round whitefish embryos chronically exposed to $^{137}$Cs gamma radiation. Treatment groups are given as dose rates (mGy/day) and were exposed from one day post fertilization to hatch. Time is reported as the percent of development time with the days post fertilization (dpf) in parentheses. Average temp refers to the mean temperature during incubation (± standard deviation). 50% hatch value indicates time to median hatch.

| Treatment Group | Average Temp (°C) | Fully Eyed | Fin Flutter | Protruding Mouth | Pre-Hatch | 50% Hatch |
|---|---|---|---|---|---|---|
| 0 | 2.4 (±0.3) | 44% (57) | 61% (79) | 78% (100) | 96% (122) | 100% (129) |
| 0.10 | 2.7 (±0.1) | 47% (57) | 65% (79) | 82% (100) | 99% (121) | 100% (122) |
| 0.16 | 2.7 (±0.1) | 48% (58) | 65 (79) | 82% (100) | 99% (121) | 100% (122) |
| 0.30 | 2.7 (±0.1) | 46% (58) | 62% (79) | 79% (100) | 95% (121) | 100% (127) |

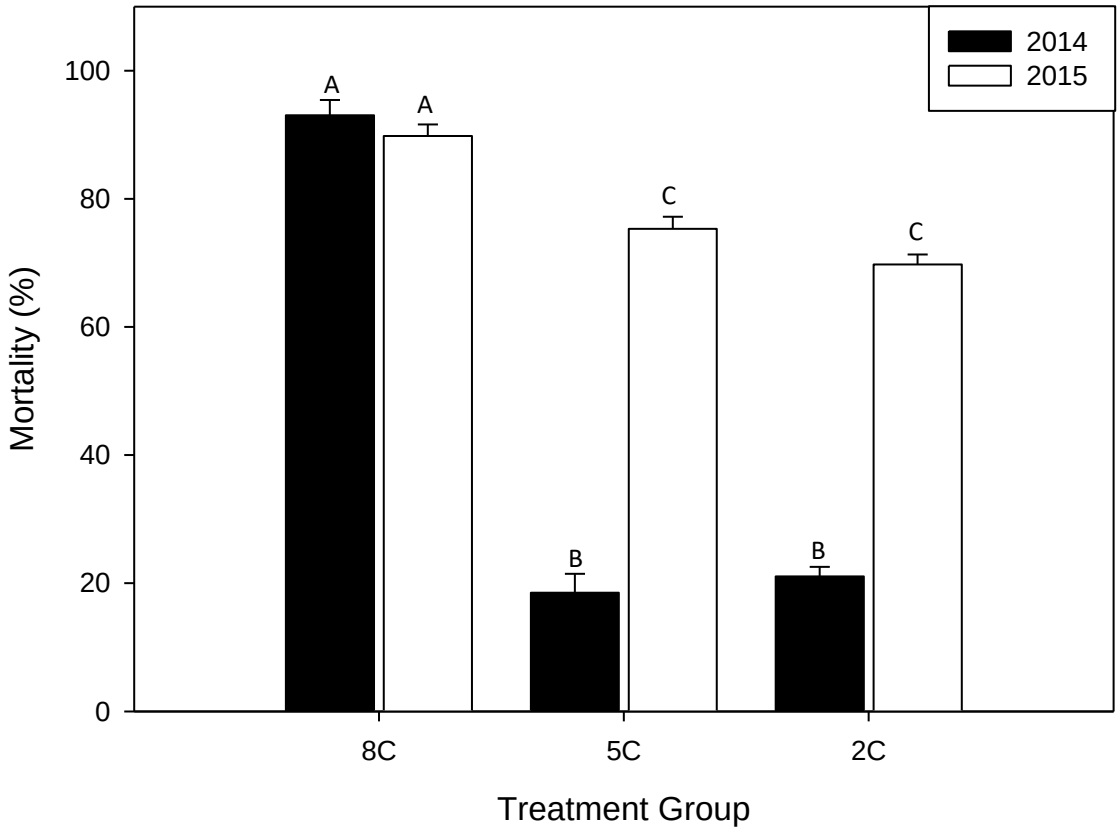
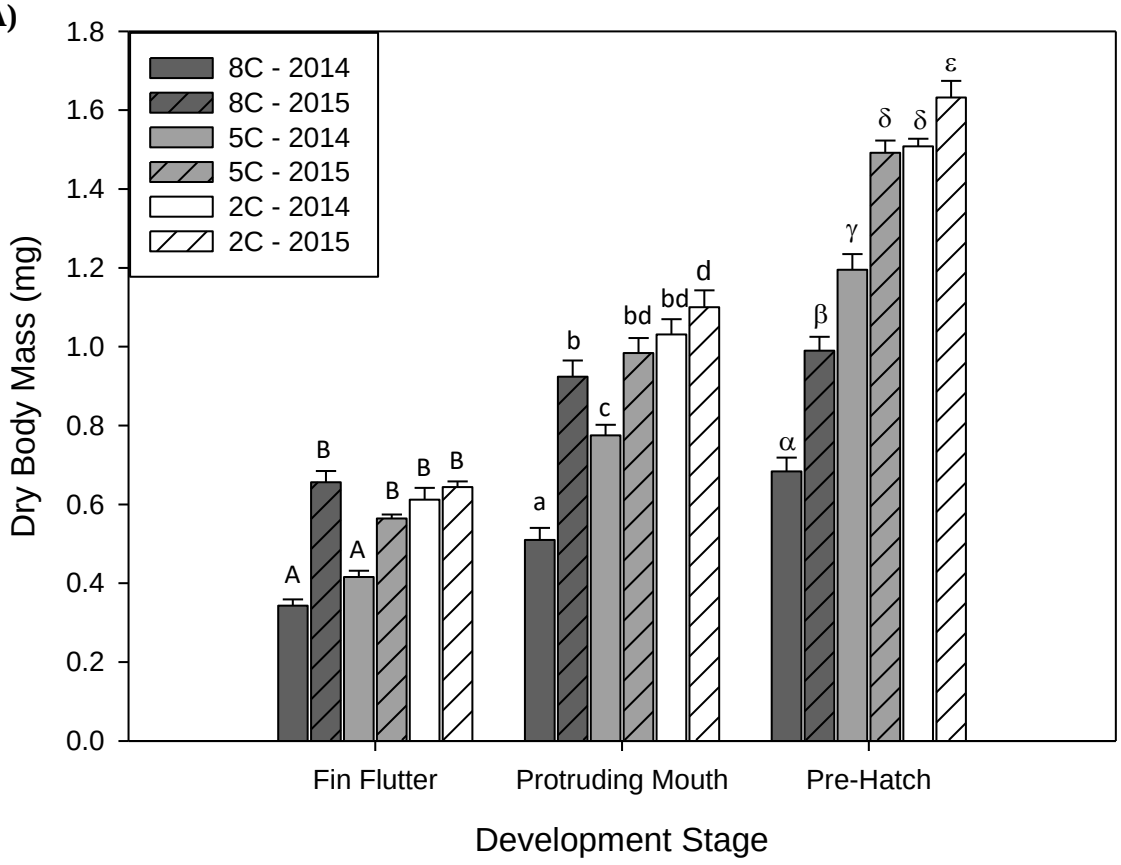



**Fig. 3.2**. Mean mortality (from fertilization until hatch) of round whitefish embryos under constant incubation temperatures. Means without the same letter are significantly different from each other (Tukey post hoc comparison across all treatment groups, 1-way ANOVA, $p<0.05$). Error bars indicate SEM (N=10 for each group).

**A)**


8C - 2014
8C - 2015
5C - 2014
5C - 2015
2C - 2014
2C - 2015
Dry Body Mass (mg)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
A
B
A
B
B
B
a
b
c
bd
bd
d
α
β
γ
δ
δ
ε
Fin Flutter
Protruding Mouth
Pre-Hatch
Development Stage

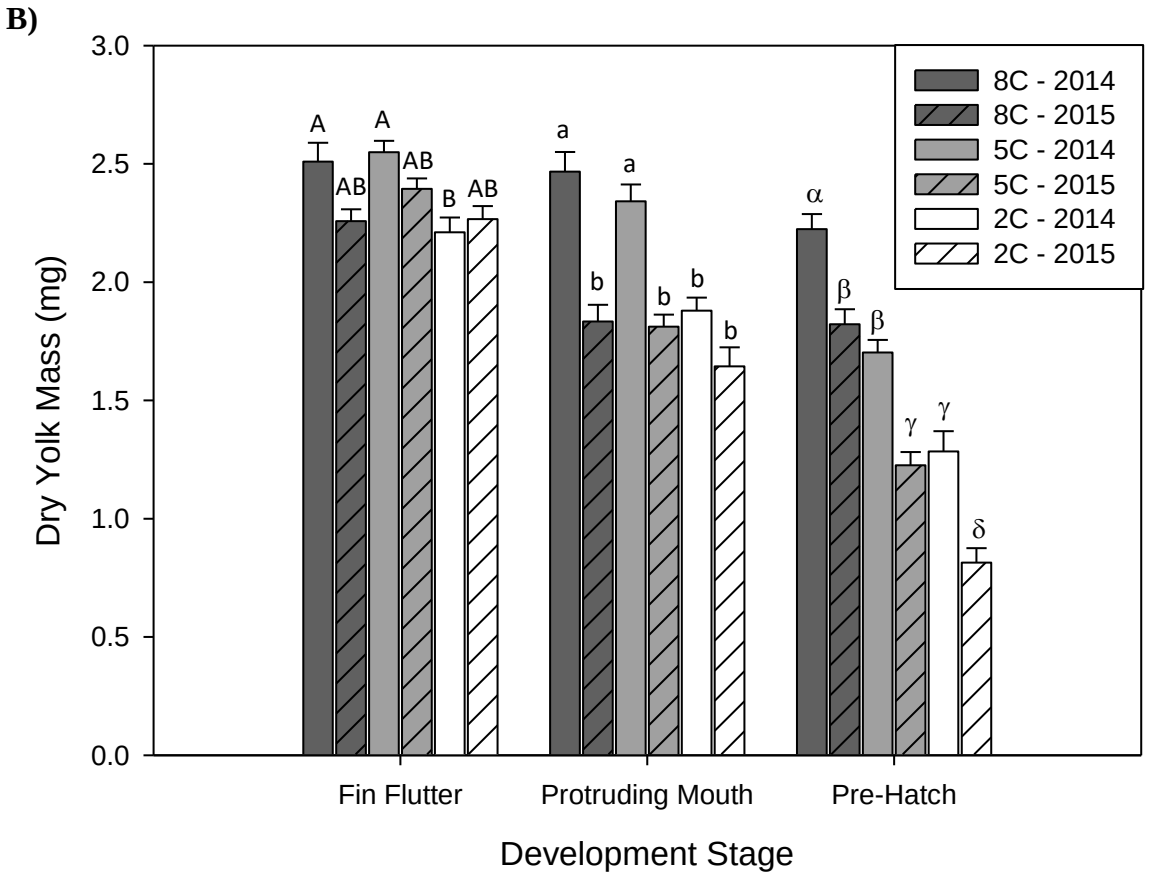



**Fig. 3.3**. Mean dry body (A) and yolk (B) mass of round whitefish embryos under constant incubation temperatures. Means within a development stage without the same letter/symbol are significantly different from each other (Holm-Sidak post hoc comparison across all treatment groups, 2-way ANOVA, $p<0.05$). Mass significantly increased (A) and decreased (B) for all groups across development stages excluding both 8C (2015) masses between the protruding mouth and pre-hatch stages. The 8C (2014) yolk mass did not decrease significantly between the fin flutter and protruding mouth stages. Error bars indicate SEM (N=10 for each 2014 group, N=5 for each 2015 group).

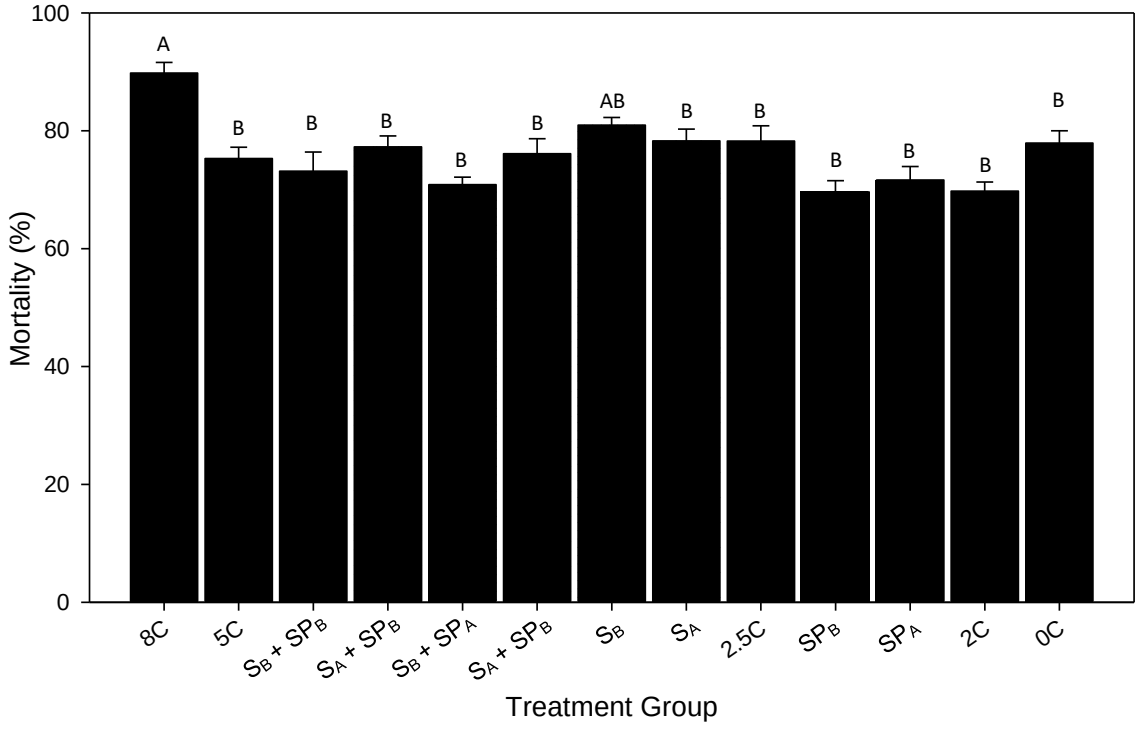
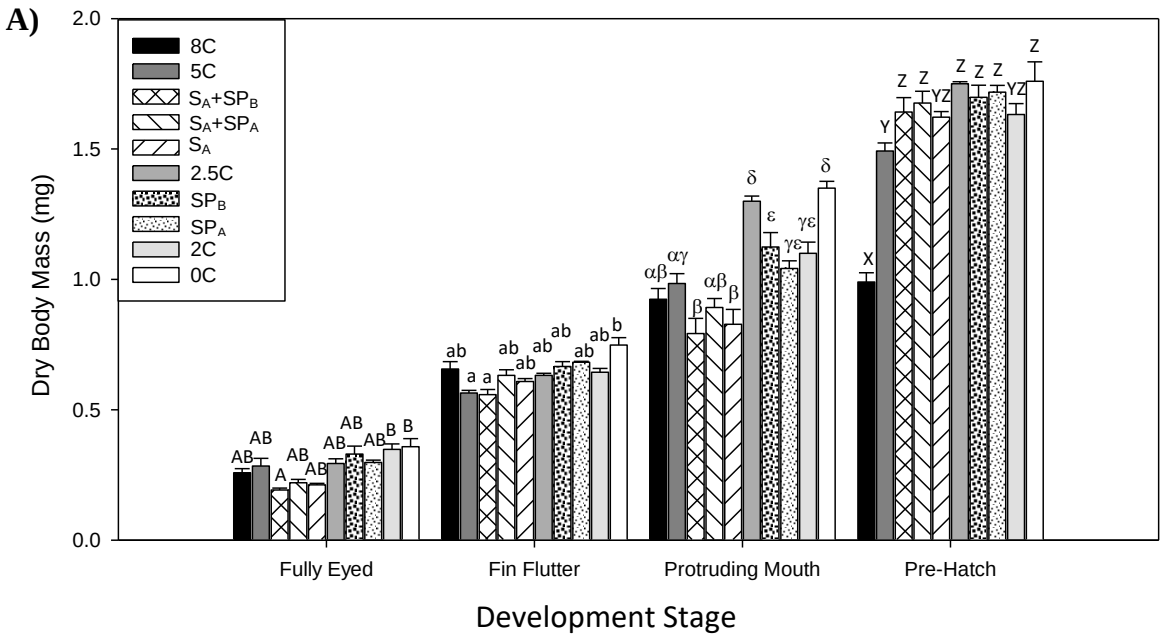



**Fig. 3.4**. Mean mortality (from fertilization until hatch) of round whitefish embryos under different constant and fluctuating temperature regimes (see Table 3.4). Treatments are in order of highest average incubation temperature (left to right). Means without the same letter are significantly different from each other (Tukey post hoc comparison across all treatment groups, 1-way ANOVA, $p<0.05$). Error bars indicate SEM (N=10 for each group excluding regimes with $S_A$ [N=7] and $S_B$ [N=3]).


A)
Dry Body Mass (mg)
2.0
1.5
1.0
0.5
0.0
8C
5C
SA+SPB
SA+SPA
SA
2.5C
SPB
SPA
2C
0C
Fully Eyed
Fin Flutter
Protruding Mouth
Pre-Hatch
Development Stage

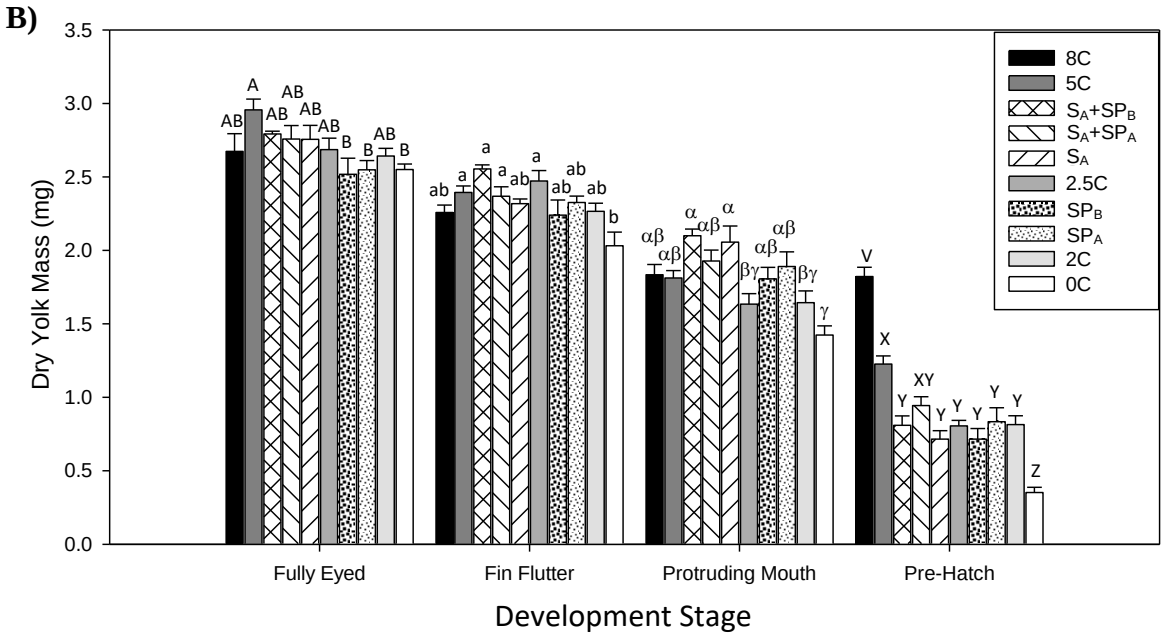

B)
Dry Yolk Mass (mg)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
8C
5C
SA+SPB
SA+SPA
SA
2.5C
SPB
SPA
2C
0C
Fully Eyed
Fin Flutter
Protruding Mouth
Pre-Hatch
Development Stage

**Fig. 3.5**. Mean dry body (A) and yolk (B) mass of round whitefish embryos under constant and fluctuating incubation temperatures. Within a development stage, treatments are in order of highest average incubation temperature (left to right). Bars for the $S_B$, $S_B$+$SP_A$, and $S_B$+$SP_B$ regimes were omitted due to lack of statistical differences with the $S_A$, $S_A$+$SP_A$, and $S_A$+$SP_B$ regimes. Means within a development stage without the same letter/symbol are significantly different from each other (Holm-Sidak post hoc comparison across all treatment groups, 2-way ANOVA, $p<0.05$). Mass significantly increased (A) and decreased (B) for all groups across development stages excluding both 8C masses between the protruding mouth and pre-hatch stages. Error bars indicate SEM (N=5 for each group).

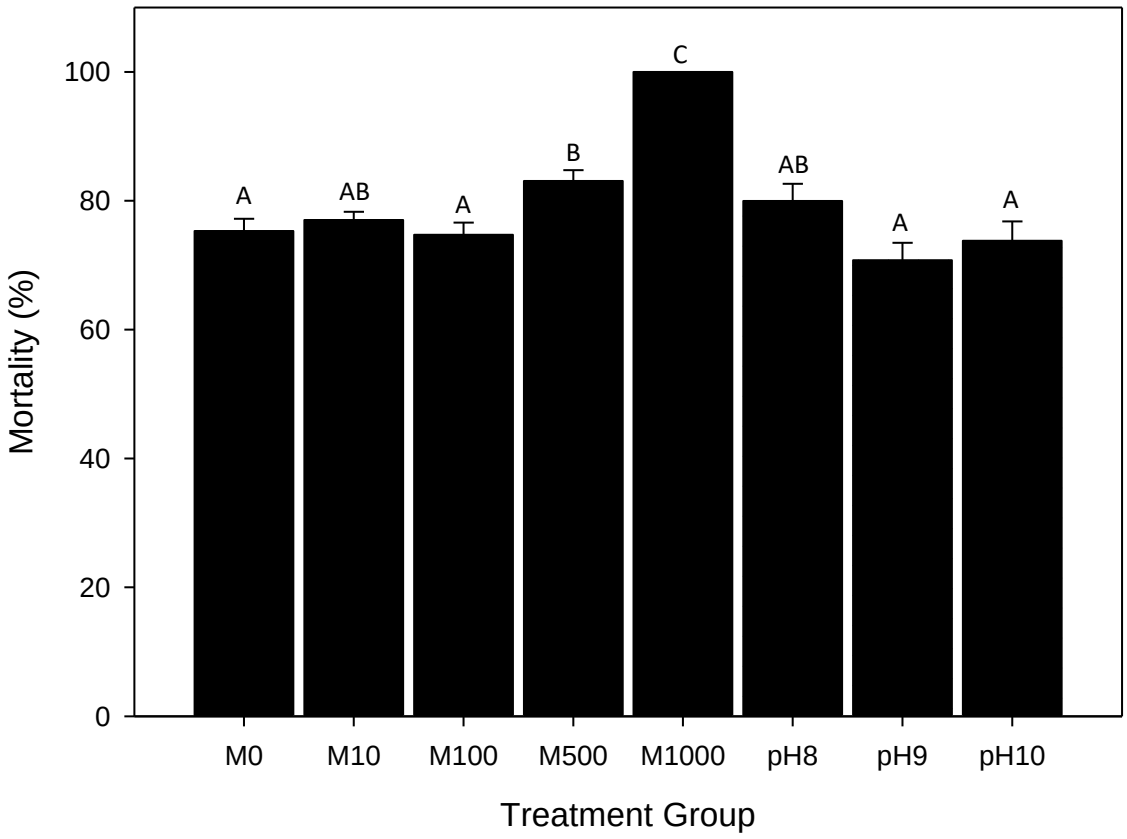
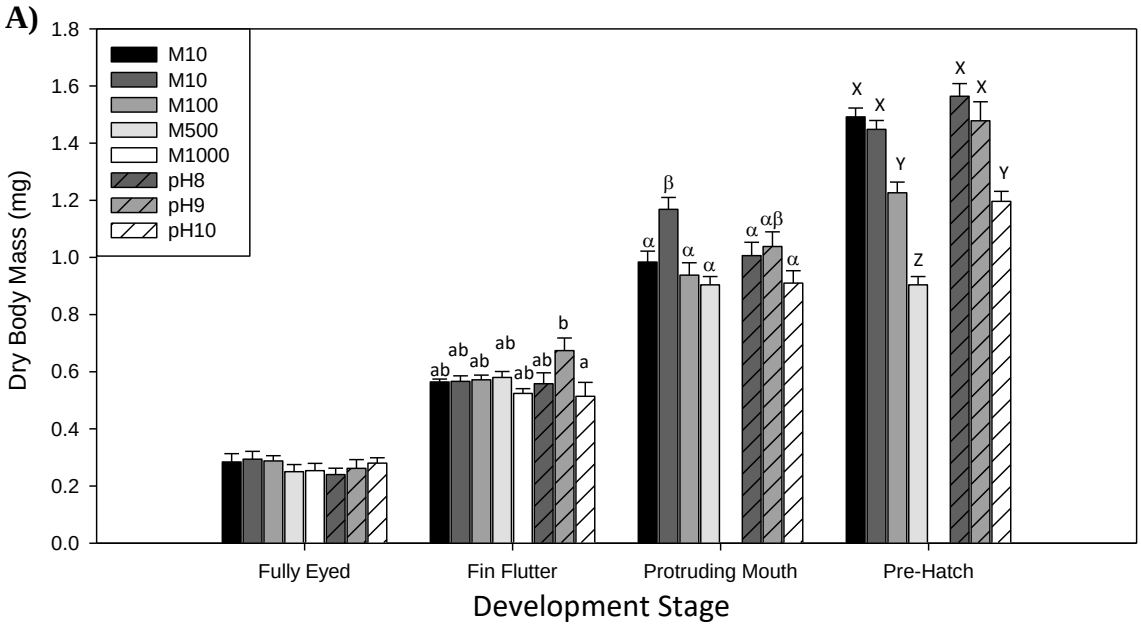



**Fig. 3.6**. Mean mortality (from fertilization until hatch) of round whitefish embryos under different chronic morpholine (mg $L^{-1}$) exposures. All embryos were maintained at 5.0±0.1°C (± standard deviation) and were exposed from one day post fertilization to hatch. As morpholine alters pH, pH controls were generated by adding NaOH to the M0 treatment group. The pH from the M10, M100, and M1000 treatments are equivalent to pH 8, 9, and 10 respectively; the pH from the M500 treatment was between pH 9 and 10 (See Table 3.5). Means without the same letter are significantly different from each other (Tukey post hoc comparison across all treatment groups, 1-way ANOVA, $p<0.05$). Error bars indicate SEM (N=10 for each morpholine group, N=6 for each pH group).


A)
M10
M10
M100
M500
M1000
pH8
pH9
pH10
Dry Body Mass (mg)
Fully Eyed
Fin Flutter
Protruding Mouth
Pre-Hatch
Development Stage

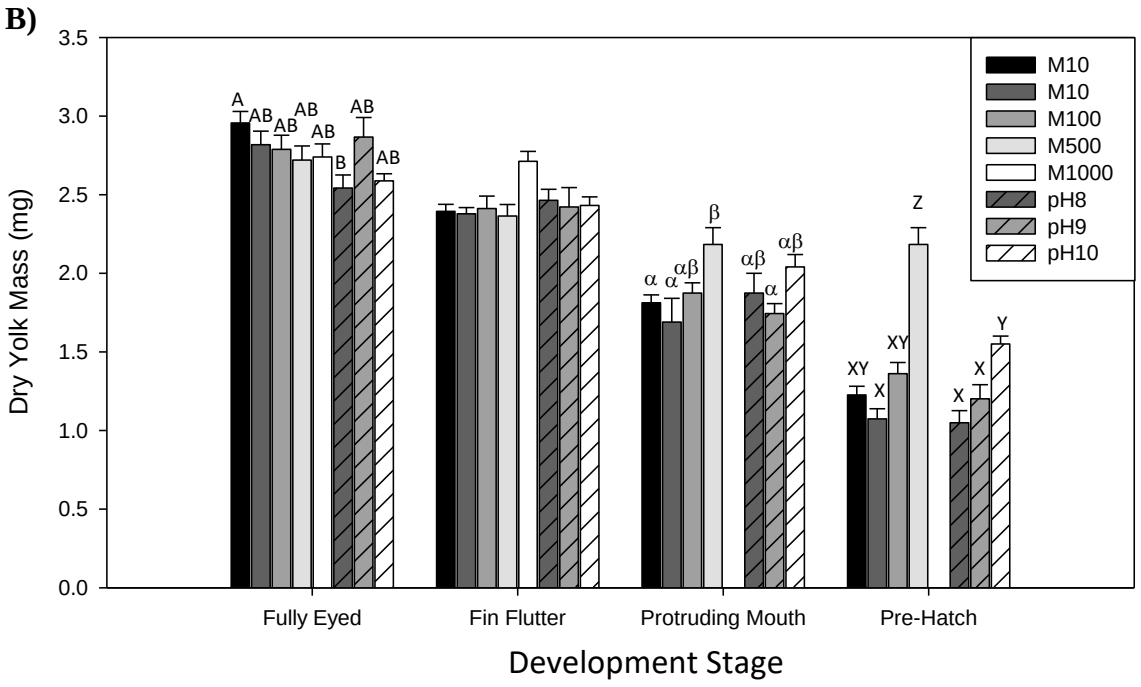

B)
M10
M10
M100
M500
M1000
pH8
pH9
pH10
Dry Yolk Mass (mg)
Fully Eyed
Fin Flutter
Protruding Mouth
Pre-Hatch
Development Stage

**Fig. 3.7**. Mean dry body (A) and yolk (B) mass of round whitefish embryos under different chronic morpholine (mg $L^{-1}$) exposures. All embryos were maintained at 5.0±0.1°C (± standard deviation) and were exposed from one day post fertilization to hatch. As morpholine alters pH, pH controls were generated by adding NaOH to the M0 treatment group. The pH from the M10, M100, and M1000 treatments are equivalent to pH 8, 9, and 10 respectively; the pH from the M500 treatment was between pH 9 and 10 (See Table 3.5). Protruding mouth and pre-hatch stage bars were omitted for the M1000 group due to all embryos dying before the protruding mouth stage. The pre-hatch stage bar for the M500 group is the same as the protruding mouth stage value due to overlap of development stage onset. Means within a development stage without the same letter/symbol are significantly different from each other (Holm-Sidak post hoc comparison across all treatment groups, 2-way ANOVA, $p<0.05$). Error bars indicate SEM (N=5 for each group).

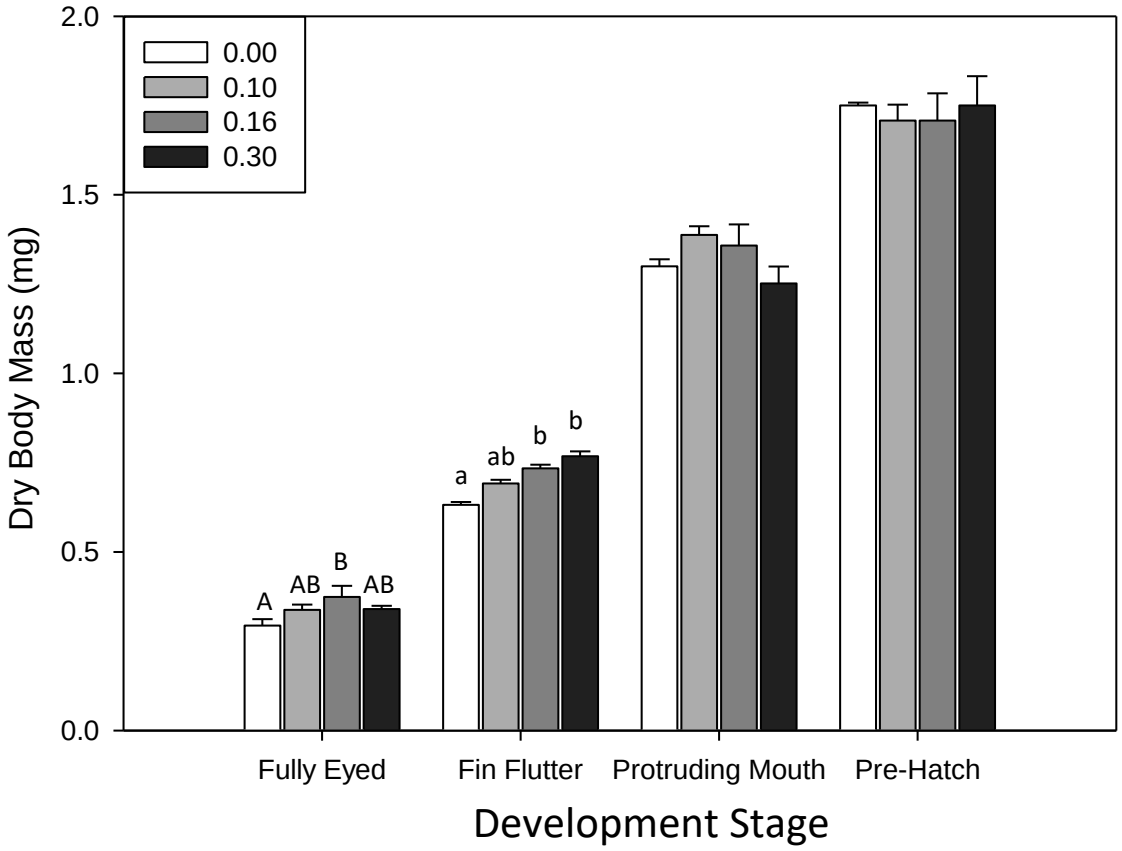



**Fig. 3.8**. Mean dry body mass of round whitefish embryos chronically exposed to radiation. Treatment groups are given as dose rates (mGy/day), and were exposed from one day post fertilization to hatch. Embryos exposed to 0.00 mGy/day were reared at 2.4±0.3°C, while embryos exposed to ≥0.10mGy/day were reared at 2.7±0.1°C (± standard deviation). Means within a development stage without the same letter/symbol are significantly different from each other (Holm-Sidak post hoc comparison across all treatment groups, 2-way ANOVA, $p<0.05$). Mass significantly increased for all groups across development. Error bars indicate SEM (N=5 for each group).

# Chapter 4

# Discussion

The goal of this thesis was to understand and compare the effects of fluctuating temperature regimes, morpholine exposure, and chronic low-dose irradiation on the embryonic development of lake and round whitefish. While both species have been the focus of embryonic development studies, the majority of these studies have taken place at constant temperatures for the entirety of development. However, water temperatures are known to change widely due to natural seasonal temperature changes (i.e. decline in water temperature from autumn to winter, and incline from winter to spring) and transient temperature elevations caused by once-through cooling discharge from thermal power plants. Discharges from power plants may also release low levels of chemicals (e.g. morpholine; used to reduce pipe corrosion) and very low levels of ionizing radiation (from nuclear power plant processes). While morpholine and radiation exposure studies have recently taken place using lake whitefish embryos (Thome, 2015), the effects of these stressors on round whitefish embryo development were unclear. The research on these stressors was completed across two years and is separated by species across two data chapters.

## 4.1 Chronic thermal stressor effects on lake and round whitefish embryos

Lake and round whitefish are cold-adapted freshwater fish, with similar life histories and spawning behaviours (Macpherson et al., 2010; Normandeau, 1969). Water temperature plays an important role both in the initiation of spawning behaviour (start of spawning season correlated to decreased temperatures; Normandeau 1969; Price, 1940) and affects the length of development (decreased development duration for warmer

temperatures). During incubation, water temperatures also alter mortality, development, growth, yolk consumption, oxygen consumption, and heart rates of both species (e.g. Brooke, 1975; Eme et al., 2015; Mueller et al., 2015; Price, 1940). It is unclear if there is an optimal incubation temperature for either species, although the majority of previous studies agree lower incubation temperatures (≤2°C) are generally better for survival and growth efficiency (e.g. Griffiths, 1980; Price, 1940; Mueller et al., 2015). In Chapter 3, round whitefish embryos were raised at several different constant temperature (2-8°C) and fluctuating temperature regimes. Round whitefish embryos were found to be especially sensitive to high incubation temperatures, with significantly increased mortality when reared at 8°C. This increased mortality was even greater for lake whitefish embryos reared at 8°C, suggesting a lower thermal maximum. While average temperatures in the field are unlikely to approach 8°C under current conditions (at discharge sites reaching a transient maximum of 3-5°C from a base of ~1°C; Thome et al., 2016), climate change projections suggest that average water temperatures in the Great Lakes may rise an additional ~5°C (Mortsch and Quinn, 1996). Although lake whitefish embryo survival may not be greatly affected, under climate change projections even water temperatures at reference sites will likely result in increased mortality for the more thermally sensitive round whitefish. Although it may take years to decades to reach this maximal increase, it is important to monitor average water temperatures at reference sites as water temperatures near discharge sites are often ~1°C warmer (not to mention the effects of temperature spikes).

On the other end of the spectrum, the Chapter 3 regime with the coldest incubation temperature involved raising round whitefish embryos on ice, resulting in exposure to near 0°C temperatures. These embryos took the longest to complete development, had the largest average body size at each measured development stage, and were significantly larger at the protruding mouth stage than embryos reared at constant 2°C. Interestingly, round whitefish embryos reared at ~2.5°C also had a significantly larger body size compared to embryos reared at constant 2°C, and had comparable body sizes to the embryos reared on ice at the protruding mouth stage. While this suggests that round whitefish may optimally grow at 2.5°C and 0°C, it is possible that light exposure was a confounding factor; embryos reared at 2.5°C were reared in nearly constant darkness (see experimental setup by Thome, 2015). While lake and round whitefish generally lay their eggs in shallow waters, eggs often fall between gaps in the cobble, and thus may have little exposure to ambient light. Previous research has found that exposure to UV-B radiation alone does not appear to have any effect on survival or growth of coregonid embryos (Häkkinen et al., 2002), but exposure to continuous light or continuous darkness is known to delay hatch and decrease body size in haddock embryos compared to diurnal light cycles (Downing and Litvak, 2002). As haddock have a relatively short development time (~12 days), it is unclear if the same effect would be seen for a species with a longer development duration like lake and round whitefish, or if a near constant darkness regime has the opposite effect and promotes growth. It is important to note that these growth differences may be due to relatively low sampling

sizes, and thus may be an overestimation or underestimation of size for embryos reared at 2.5°C and 0°C respectively.

In regards to oxygen consumption, heart rates appear to be able to serve as a proxy for lake whitefish embryos (Eme et al., 2015). While increases in temperature result in significantly increased heart rates in a stepwise pattern for round whitefish (Table 3.2), this trend was not maintained between the fin flutter and protruding mouth stages for oxygen consumption rates (Table 3.3). While this may suggest that oxygen consumption and heart rates were not as strongly correlated as for lake whitefish, the lack of significance for oxygen consumption rates may reflect differences across sampling years (heart rates in 2014, oxygen consumption rates in 2015), and /or the relatively small sample sizes for each oxygen consumption measurement. In addition to this, oxygen levels are known to decrease as water temperatures increase (Benson and Krause, 1980), and may limit the amount of oxygen available to embryos. As such, future studies should measure both oxygen consumption and heart rates with the same embryos, and correct for different oxygen levels by bubbling in $O_2$ or other gases (e.g. $N_2$) to increase or decrease oxygen levels respectively when setting up the measurement chambers.

## 4.2 Fluctuating temperatures on lake and round whitefish embryos

Chapters 2 and 3 examined the effects of a variety of fluctuating temperature regimes including +Δ3°C temperature spikes, seasonal temperature changes, or some combination of the two. Temperature spikes in this study were based on water temperatures near discharge sites (Thome et al., 2016; unpublished raw data). Based on

this data, and considering the feasibility of replicating these temperature spikes over a long development period, +Δ3°C temperature spikes for 1 hour, 3 times per week was used. Regardless of species, temperature spikes had little effect on embryonic development. This was surprising as temperature spikes of +Δ3°C are known to elicit a stress response in lake whitefish embryos (Stefanovic et al., 2016), and should theoretically require a diversion of energy stores away from growth and therefore reduce growth efficiency. The incorporation of these temperature spikes had little effect on average temperature compared to a base of constant 2°C or seasonal temperature changes (≤0.1°C). This lack of an increase in average temperature likely contributed to the limited effects seen on embryonic development for both species exposed to the fluctuating incubation regimes with temperature spikes. The incorporation of seasonal temperature changes increased average temperature by >1.5°C, and embryos exposed to these seasonal changes had proportionally earlier development stage onset compared to embryos raised at constant temperature. For temperature spikes to reach the same magnitude of average temperature increase as the seasonal regimes, they would either have to be more frequent (e.g. >8 times per day), be larger in magnitude (e.g. ~Δ25°C spikes 1 time per day), and/or last for a longer period of time (e.g. ~12 hours per Δ3°C spike per day) relative to the temperature spikes used in this thesis. It is important to note that in the field there are also gradual, transient, and low temperature increases that were not incorporated in this thesis due to logistical concerns. These temperature increases occur and dissipate on a much larger time scale, and may take weeks to complete (Thome et al., 2016, unpublished raw data). A combination of temperature spikes and longer, low

temperature increases may be sufficient to raise average temperature ~1°C, as seen near power plant discharge sites (Thome et al., 2016), and affect the development and growth of both whitefish species.

In addition to average incubation temperature, fluctuating temperature incubation can have a strong effect on development rate for both species. More specifically, as previously suggested for lake whitefish by Mueller et al. (2015), there appear to be thermal critical windows for development rate for both species between gastrulation to organogenesis and fluctuating temperature incubation during this period has effects that are not just related to mean incubation temperature alone. For instance, there is a stepwise reduction in development rate for both species as seasonal temperatures declined from 8°C to 2°C (i.e. seasonal regime embryos initially developed rapidly, but then much more slowly).

Embryos of both species reared with seasonal temperature changes had the most proportional body growth of all constant/fluctuating incubation regimes between the fully eyed and pre-hatch stages, which may initially seem surprising considering seasonal temperature treatments had an average incubation temperature of ~3.7°C. The majority of studies that examined embryo growth reported an inverse relationship between body size and temperature (e.g. Brooke, 1975; Price, 1940), suggesting the constant 2°C treatment should have had relatively larger body sizes and smaller yolk sacs. However, these previous studies were completed at constant temperatures, and thus neglect to consider the relationship between incubation temperature and growth periods. As observed in previous studies, the majority of growth in lake whitefish occurs during the last 30% of

development (between the protruding mouth and pre-hatch development stages; Sreetharan et al., 2015; Thome, 2015) and was found to be similar for round whitefish embryos in this thesis. During the latter ~50% of development, embryos raised with seasonal temperature regimes were mostly reared at 2°C, where they (and embryos reared at constant 2°C) spent approximately ~50 days at 2°C developing and growing. This allowed the seasonal treatment embryos to approach a similar body size as the constant 2°C treatment embryos by the pre-hatch stage. As similarly seen with lake whitefish embryos (Thome et al., 2016), unless average temperature elevations approach 1°C between January and March (approximately coinciding with the large growth period), there is likely to be little effect on growth for round whitefish embryos.

Exposure to seasonal temperature regimes altered hatch dynamics, where increases in temperature (due to the seasonal temperature incline at the end of development) advanced median hatch and shortened the hatch window for both species. These effects on hatch dynamics were further amplified for round whitefish as the rate of temperature incline increased (i.e. +2°C/week vs. +1°C/week). Although this thesis did not investigate +2°C/week periods for lake whitefish, other studies with more rapid warming periods suggest a similar effect (e.g. Patrick et al., 2013). From studying these different fluctuating temperature regimes, lake and round whitefish embryos appear to take advantage of different periods of natural temperature changes to first develop quickly, grow slowly to a large size, and then hatch rapidly. As such, while overall average incubation temperature data is valuable for a quick estimation on growth and development rates, average temperatures during the latter half of development and

temperature variation during the beginning and end of development give better approximations on growth and development rates respectively.

### 4.3 Chronic morpholine exposure effects on round whitefish embryos

Morpholine, a basic corrosion inhibitor in steam boiler systems, may be released alongside discharge from once-through cooling processes of thermal power plants. Lake and round whitefish embryo experience increased mortality, and altered development and growth rates with chronic exposure to morpholine. When round whitefish embryos were exposed to morpholine (Chapter 3), there were several direct effects including decreased body size (when exposed to ≥100 mg $L^{-1}$ morpholine), advanced median hatch (when exposed to 500 mg $L^{-1}$ morpholine) and increased mortality (when exposed to ≥500 mg $L^{-1}$ morpholine). As lake whitefish embryos exposed to morpholine did not have decreased body sizes at 100 mg $L^{-1}$ morpholine, and only had significantly elevated mortality at 1000 mg $L^{-1}$ morpholine (Thome, 2015), it is likely that round whitefish embryos are more susceptible to morpholine. This increased susceptibility may in part be due to round whitefish embryos having larger chorions, and therefore larger surface areas, compared to lake whitefish. This may allow for larger amounts of morpholine to pass through the chorion, causing effects at relatively lower concentrations and at earlier development time-points compared to lake whitefish.

Interestingly, round whitefish embryos exposed to high concentrations of morpholine (i.e. ≥500 mg $L^{-1}$) had a rough or uneven appearance of the chorion by 50

days of exposure. The roughness appeared to be greater for embryos exposed to 1000 mg $L^{-1}$ morpholine (Fig. 4.1).

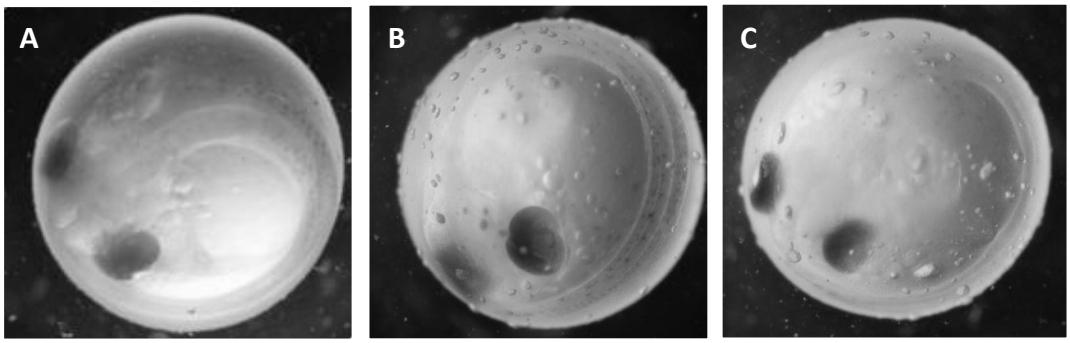



**Fig. 4.1**. Uneven or rough chorion surface in round whitefish embryos reared at 5°C with morpholine at 0 (A), 500 (B) and 1000 mg $L^{-1}$ (C). Embryos are at the fin flutter stage.

As such, morpholine appears to deposit on surfaces over time. It is possible that morpholine may pass through the chorion and settle onto the developing embryo itself. As morpholine deposits increase in number/size, the embryo may be placed under increasing stress (e.g. pH imbalance), resulting in reduced growth and eventually death. This suggests that the biologically effective morpholine concentrations are initially much lower than their corresponding nominal concentrations due to the chorion acting as a barrier. As the length of exposure increases, so does the morpholine concentration within the chorion. It is important to note that the morpholine concentration that results in increased mortality for round whitefish embryos (between 100 and 500 mg $L^{-1}$) is still higher (>25,000 times) than Ontario guidelines, and >4 times higher than regulatory limits for power plants such as Bruce Power (Bruce Power, 2005). As such, we expect

there to be little effect of morpholine on lake and round whitefish embryo survival at discharge sites. However, it is unclear if morpholine concentrations affect growth within regulatory limits (i.e. 10< and ≤25 mg $L^{-1}$).

4.4 Chronic low-dose irradiation effects on round whitefish embryos

Exposure to low-dose and low-dose rate ionizing radiation may occur due to release of tritium from nuclear power plants. Chronic exposure to low-dose irradiation appears to have relatively little effect on the embryonic development of round whitefish. This contrasts with a study on lake whitefish, where exposure to low-dose irradiation advanced hatch and affected growth throughout development at doses as low as 0.06 mGy/day (Thome, 2015). While there is also a slight increase in growth for irradiated round whitefish embryos at early development stages (e.g. fin flutter), this difference is lost at later development stages. As such, round whitefish appear to be less impacted by low-dose irradiation than lake whitefish. The mechanisms for increased growth for irradiated embryos compared to non-irradiated embryos is unclear. It is possible that it may be related to mitochondrial oxidative metabolism, which is noted to be reduced in cells exposed to low-dose irradiation (Krasieva et al., 2011). Decreased mitochondrial metabolism is thought to allow for increased generation/presence of protein and lipid precursors which may in turn allow for increased cell proliferation. As round whitefish embryos do not display differences in growth late in development, it is possible that they become more radioresistant as they develop and/or growth is affected by other rearing conditions such as photoperiod. Compared to regulatory limits for tritium discharge are

on the scale of ~1 nGy/day (see Adam-Guillermin et al., 2012; Bruce Power, 2014), our lowest dose rate with significant growth effects was >160 times greater. As a result, we do not expect significant development rate or growth effects for round whitefish exposed to environmental levels of tritium releases.

4.5 Effects found post-hatch on round whitefish after incubation with an environmental stressor

Following hatch, mortality was seen to be elevated in round whitefish that were incubated at high temperatures (i.e. 8°C) or experienced rapid seasonal temperature inclines (i.e. +2°C/week). As such, embryos that develop under projected climate change conditions or warm/early springs are likely to have reduced hatchling survival. Round whitefish embryos exposed to 500 mg $L^{-1}$ morpholine also had elevated mortality post-hatch, while irradiated round whitefish embryos had decreased mortality post-hatch compared to control embryos. As lake whitefish embryos were not examined post-hatch it is unclear if similar alterations to post-hatch mortality would be seen, or if the concentration/dose rate needed to elicit this effect is below regulatory limits/guidelines. As round whitefish appear to be more sensitive to thermal and morpholine stressors, the magnitude of increased mortality for lake whitefish embryos is likely to be relatively smaller. However, chronic radiation effects on post-hatch mortality effects are likely to be larger for lake whitefish embryos based on relatively greater impacts pre-hatch.

Exposure to different environmental stressors is also known to effect body and yolk sac size at hatch. Suboptimal conditions (e.g. high temperatures, exposure to high

morpholine concentrations) and/or development length decrease growth and/or growth efficiency, leading to smaller bodies and larger yolk sacs. On the other hand, optimal conditions and/or longer development periods often result in the opposite effect. It is important to note that in the event there are growth or yolk consumption differences at a hatch, it is unclear if they will have beneficial or detrimental effects on survival post-hatch due to a multitude of factors such as the presence of predators/prey and swimming performance of the hatchlings. For example, larger body sizes at hatch likely allow for better swimming and foraging ability, but result in increased ease of being spotted by predators and lower internal energy stores. Environmental stressors may cause additional sub-lethal effects such as behavioural changes which can affect survival and recruitment.

## 4.6 Conclusions/Future Directions

This thesis has helped better under the impacts of a variety of fluctuating temperature regimes on lake and round whitefish development, as well as allowed for the comparison of the effects of rearing under different constant temperatures, morpholine concentrations, and ionizing radiation dose rates between the two species. However, the thresholds for some embryonic development effects are unknown. For instance, morpholine concentration between 10 and 500 mg $L^{-1}$ should be examined with smaller increments between concentrations to uncover which concentrations are sufficient to cause altered growth or increased mortality. Radiation dose rates below 0.10 mGy/day (to determine dose-response trend for decreased mortality post-hatch), above 0.30 mGy/day (to determine dose-response trend for altered growth and hatch timing), and large acute

dose rates should also be studied on lake and round whitefish embryos to allow for a more complete comparison between the two whitefish species, and other cold-adapted species.

Another rearing condition factor that is not clearly understood is the effect of photoperiod on the embryonic development of both whitefish species. To study this, initial tests on the effects of light cycles should include constant light, constant dark, and 12 hour light/dark cycle regimes at minimum. Lights of different intensity could be used due to their altered effects on growth and hatch timing of other fish species (Downing and Litvak, 2002). If possible, gradual changes in daylight hours should also be used alongside seasonal declines/inclines (e.g. shorter days as temperatures decrease from fall to winter) to look for interactive effects of natural environmental changes.

Although recent studies and this thesis have given a better understand of several different environmental stressors on the embryonic development of lake and round whitefish, there is very little known about development and survival for these species post-hatch. Based on mortality data in Chapter 3, there appear to be strong effects of incubation regimes (i.e. exposure to high temperatures, high morpholine concentrations, or low-dose irradiation) on survival post-hatch for round whitefish embryos. There may also be additional effects from these incubation regimes such as altered growth or behaviour, which play a role in post-hatch survival. As such, future studies should focus on uncovering and understanding these differences, and their roles on recruitment to the next generation.

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