ENVIRONMENTAL STRESSORS AND LAKE WHITEFISH EMBRYOGENESIS

THE EFFECTS OF THERMAL, CHEMICAL AND RADIOLOGICAL STRESSORS ON EMBRYONIC DEVELOPMENT IN LAKE WHITEFISH (*COREGONUS CLUPEAFORMIS*)

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ABSTRACT

Power generation relying on once-through cooling has the potential to release thermal, chemical and radiological stressors into the environment. These discharges may impact development in aquatic species that spawn near cooling water releases. This thesis explores the impacts that stressors associated with industrial cooling water discharges can have on lake whitefish (*Coregonus clupeaformis*) embryonic development.

In-situ incubation in custom designed chambers was used to examine development in thermally impacted areas near a nuclear power plant in Lake Huron. Temperatures at sites near cooling water discharges were significantly warmer and more variable compared to off-site reference locations but were below lethal levels. However, an accelerated development and potential earlier hatch was predicted at these sites based on temperature modelling and morphometric measurements on retrieved embryos.

Chemical (morpholine and sodium hypochlorite) and radiological effects were examined in laboratory reared embryos following both chronic and acute exposures. Embryos became more resistant to radiation as development progressed, while mortality from morpholine exposure was greatest close to hatch. Embryos were minimally impacted by incubation in sodium hypochlorite. Both a synergistic and protective effect were observed in percent mortality when stressors were examined in combination. Low dose sublethal exposure to morpholine and ionizing radiation resulted in changes in hatch timing and stimulation in growth.

Overall, this work helps to advance the understanding of how thermal, chemical and radiation exposure impacts embryonic survival and development, and evaluates potential effects of exposure at environmental levels on lake whitefish.

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CONTRIBUTIONS

The planning and experimental work presented in Chapters 2, 3 and 4 was performed by Christopher Thome under the supervision of Drs. Joanna Wilson and Douglas Boreham. Assistance with experimental work was provided by Charles Mitz on each chapter and by Charlotte Mitz and Shayenthiran Sreetharan on Chapter 3. The manuscripts were prepared by Christopher Thome and edited by Charles Mitz, Charlotte Mitz, Shayenthiran Sreetharan and Drs. Christopher Somers, Richard Manzon, Joanna Wilson and Douglas Boreham.

The experimental design presented in Appendix A was completed by Charles Mitz. Experimental work was conducted by Charles Mitz, Christopher Thome, Mary Ellen Cybulski and Lisa Stoa (Laframboise) under the supervision of Drs. Joanna Wilson and Douglas Boreham. The manuscript was prepared by Charles Mitz and Christopher Thome, and edited by Drs. Christopher Somers, Richard Manzon, Joanna Wilson and Douglas Boreham.

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CANDU: Canadian deuterium uranium

CCW: Condenser cooling water

DDREF: Dose and dose rate effectiveness factor

DSB: Double strand break

EPIC: Environmental protection from ionizing contaminants

HSP: Heat shock protein

LET: Linear energy transfer

LNT: Linear no-threshold

TRC: Total residual chlorine

YCE: Yolk conversion efficiency

Chapter 1

Introduction

1.1 Thermal power generation

Much of our growing electricity demand is supplied through thermal power production. In Canada, thermal power generation in 2013 totalled 133 TWh accounting for approximately 35% of national energy production (CEA 2014). Thermal power plants rely on the same principle of electricity generation. Thermal energy is used to heat water into steam which subsequently rotates a turbine. Mechanical energy of the turbine is then converted into electrical energy. The types of power plants differ in how they generate heat; either through combustion of coal, gas and oil or through nuclear fission. In order for steam systems to operate in a closed loop, steam must be condensed back into water after passing through the turbines. Multiple methods exist to achieve this, including evaporative cooling towers, air-cooled condensing, cooling ponds or once-through cooling (Macknick et al. 2012).

Power plants located on large lakes, rivers or oceans often rely on once-through cooling. Once-through cooling is an open loop system where water is taken directly from a natural source to condense turbine steam and is then discharged back into the environment. This cooling loop is often referred to as the condenser cooling water (CCW). The amount of water required for once-through cooling depends on the type of energy generation and size of the plant, but can be upwards of 100,000 L/MW·h (Macknick et al. 2012). The Great Lakes Basin is home to numerous power plants relying on once-through cooling. These facilities take in 476 billion litres of water per day accounting for 76% of total water withdrawals in the region (Great Lakes Commission 2011). Although withdrawals

are high in once-through cooling, consumption of water is low and most is released back into the source body of water.

Nuclear power generates electricity from energy released during nuclear fission. In order to maintain a chain reaction and sustained energy production, neutrons released during fission must be slowed down to an ideal speed to induce subsequent fission reactions. This is achieved through a neutron moderator which surrounds the nuclear fuel in the reactor core. Several different reactor designs exist. In pressurized water reactors, the neutron moderator is under high pressure to maintain a liquid state. After passing through the reactor, the primary loop neutron moderator passes through a heat exchanger, converting water in the secondary loop into steam to rotate the turbines.

In Canada, all nuclear power reactors are Canadian Deuterium Uranium (CANDU) reactors. These reactors utilize heavy water, containing deuterium, as a neutron moderator. By using heavy water CANDU reactors can run on natural uranium as opposed to light water reactors which require uranium enrichment (Robertson 1978). Nuclear power is an important energy source in Canada, principally in Ontario, where it constituted over 60% of the provincial energy production (CEA 2014).

1.1.1 Bruce Power

Bruce Power is a nuclear generating station located on the eastern shore of Lake Huron, between Kincardine and Port Elgin, Ontario (Figure 1.1). It is the largest operating nuclear power plant in the world generating up to 6,300 MW at full capacity. Bruce Power consists of 8 CANDU pressurized heavy water reactors across two stations. Bruce

A is located on the Northeast of the site and Bruce B to the South, each containing 4 reactors. Both stations rely on Lake Huron water for once through cooling. CCW is taken in through an intake tunnel on the lake bottom 800 m offshore (Bruce Power 2005). After condensing turbine steam, the water is discharged back into the lake through an on shore surface discharge channel. CCW is cycled through the plant at a rate of up to 175 m^3/s for Bruce A and 193 m^3/s for Bruce B (Bruce Power 2005). The aquatic habitat surrounding Bruce Power was the focus of the field study discussed in Chapter 2.

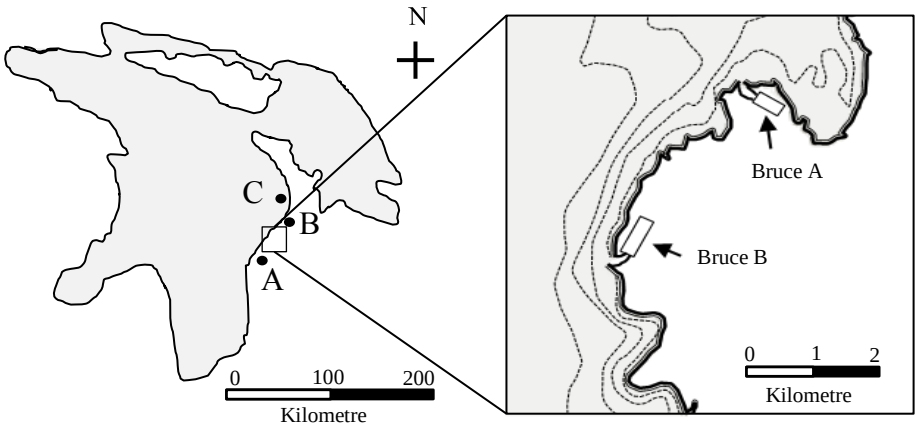

Figure 1.1. Location of Bruce Power on Lake Huron. A) Kincardine, Ontario. B) Port Elgin, Ontario. C) Fishing Islands. Inset: Bruce Power site showing Bruce A and Bruce B units.

1.2 Environmental stressors

Once through cooling can potentially impact aquatic biota through the intake and discharge of large volumes of water. The uptake of water may result in large fish being impinged on intake screens and smaller fish or eggs becoming entrained within the cooling water system (Kelso and Milburn 1979). The focus of this thesis however is on

the effects of stressors associated with cooling water discharges. After condensing turbine steam, warmer water is discharged with the potential to create large thermal plumes. Various chemicals are also added to the steam and cooling water systems to prevent corrosion and biofouling. In addition, discharges from nuclear power plants may contain very low levels of ionizing radiation. These stressors can be of particular concern to aquatic organisms which reside in regions near discharge sites. Numerous fish species are known to spawn in shallow water and developing embryos can therefore be exposed to thermal, chemical or radiological stress throughout embryogenesis.

1.2.1 Thermal releases

Once-through cooling water can be released back into the environment well above ambient temperature. Discharge water can reach 37°C and a temperature difference of upwards of 10°C compared to intake water (Madden et al. 2013). Temperature limits for Bruce Power are 11.1°C above intake during the summer and 13°C in winter, with a maximum absolute discharge temperature of 32°C (Bruce Power 2005). The large volumes of warmer water released create thermal plumes, the extent of which varies depending on meteorological conditions, geographic features and plant operations. Ambient lake temperature will also influence the plume. Normally, warmer discharge water is more buoyant than ambient water and will remain close to the surface as it cools. During winter months however, a sinking thermal plume can occur if ambient water is below 4°C. As discharge water cools to 4°C, where water density is highest, the plume

can sink below the more buoyant lake water resulting in warmer temperatures on lake bottom (Raithby et al. 1988).

Temperature effects on embryonic development in fish have been well documented. Embryos generally have an optimal temperature range within which normal development will occur. Short duration increases can be tolerated, however continued incubation outside of optimal ranges can result in increased mortality and developmental abnormalities (Griffiths 1979). The length of embryonic development is largely dependent on temperature, with colder conditions resulting in slower development rates (Price 1940, Brooke 1975). Incubation temperature can also impact growth, where embryos are larger at hatch and are more efficient at converting yolk energy into body mass when developing at colder temperatures (Mueller et al. 2015). Physiological processes including heart rate and oxygen consumption are also temperature dependent (Eme et al. 2015).

1.2.2 Morpholine

Morpholine ($O(CH_2CH_2)_2NH$) is a secondary amine that is added to steam systems to increase pH, thereby reducing corrosion. Low pH levels, caused mainly by carbon dioxide in the condensate, are corrosive to steel and are mitigated by neutralizing amines such as morpholine (Nordmann and Fiquet 1996). Periodically to prevent the buildup of chemicals, water from the steam system loop is discharged into the CCW channel and released into the environment (Bruce Power 2005). Regulatory limits have been placed on morpholine concentrations in steam and cooling water. The United States Food and Drug

Administration limit morpholine concentrations in the condensate to 10 mg/L (US FDA 2014). In Ontario, provincial water quality objectives have been set at 0.004 mg/L (Ontario MOEE 1994). Regulatory limits for morpholine in the CCW at Bruce Power are above this at 25 mg/L (Bruce Power 2005).

Very little is known about the biological effects of morpholine. Most reported data is from exposure in rats where morpholine acts as an irritant to the respiratory tract when ingested or inhaled (Conaway et al. 1984a). It has a short biological half-life of 24 hours and is not readily metabolized (Tanaka et al. 1978). Morpholine itself is not a strong carcinogen. Exposure in cell culture studies has not produced genotoxic damage (Conaway et al. 1984b). Morpholine fed rats showed a slight increase in hepatocellular carcinoma and angiosarcoma but this was likely due to morpholine reacting with nitrates to form N-nitrosomorpholine (Shank and Newberne 1976).

Few studies have looked at the impacts of morpholine on aquatic biota. Changes in cell growth in algae can occur at concentrations as low as 10 mg/L (Adams et al. 1985). In fish, morpholine effects have only been examined on survival in adult fish with no published data on embryonic development. Acute 96 hour LC_{50} values are on the order of several hundred mg/L depending on the species (Dawson et al. 1975-76, Brandão et al. 1992). Potential impacts of sublethal exposures also remain unknown.

1.2.3 Sodium hypochlorite

Sodium hypochlorite (NaOCl) is utilized in industrial water systems to prevent biofouling. In the Great Lakes Region it is mainly used to control against invasive zebra

mussels (*Dreissena polymorpha*, Rajagopal et al. 2002). Sodium hypochlorite reacts with water to form hypochlorous acid (HOCl), which is responsible for most of its biocidal properties. Hypochlorous acid is a strong oxidizer which acts by disrupting cell membrane structure and enzyme function, and damaging DNA (Fukuzaki 2006). Hypochlorous acid can dissociate to form hypochlorite ions (OCl^-). Both HOCl and OCl^- are referred to as free chlorine. The ratio of HOCl to OCl^- is pH dependent (Fukuzaki 2006). Free chlorine can combine with ammonia to form chloramines and is then referred to as combined chlorine. Chloramines are weaker biocides but are longer lived compared to free chlorine. The sum of free and combined chlorine is known as total residual chlorine (TRC). Limits are in place for chlorine levels in the environment, normally measured as a concentration of TRC. Ontario provincial water quality objectives are 0.002 mg/L (Ontario MOEE 1994) while Bruce Power CCW limits are 0.01 mg/L (Bruce Power 2005).

Chlorine toxicity in adult fish results mainly from hypoxia, which is caused by damage to gill structure leading to reduced gas exchange (Bass et al. 1977) and by increased levels of methemoglobin causing a decrease in oxygen carrying capacity (Grothe and Eaton 1975). There is a large range in reported toxicity data, with LC_{50} values as low as 0.02 mg/L up to greater than 1 mg/L of total residual chlorine (Cooke and Schreer 2010). Similar ranges have been found for embryonic exposure, with reported acute LC_{50} values between 0.04 – 0.4 mg/L (Middaugh et al. 1977, Morgan and Prince 1977). Limited data exist on chronic exposures in embryos or sublethal effects from chlorination.

1.2.4 Ionizing radiation

Under normal operating conditions, once through cooling discharges from nuclear power plants may contain low levels of ionizing radiation. In CANDU plants the major isotope released is tritium due to the use of heavy water as a neutron moderator. Annual releases of tritium can be on the order of 10^{14} Bq, which equates to a concentration of less than 20 Bq/L, well below the provincial limit of 7000 Bq/L (Bruce Power 2014a, Ontario Power Generation 2015). Releases can also include ^{14}C and various other beta/gamma or alpha emitting fission products, at levels several orders of magnitude below tritium. Aquatic species sampled in the vicinity of generating stations may contain slightly elevated levels of tritium and ^{14}C compared to provincial averages. In white sucker (*Catostomus commersonii*) and lake whitefish (*Coregonus clupeaformis*), tritium levels in fish caught near Bruce Power averaged 10 Bq/L and ^{14}C levels averaged 250 Bq/Kg·C, compared to provincial averages of < 4 Bq/L and 225 Bq/Kg·C respectively (Bruce Power 2014a).

Ionizing radiation can be described as directly or indirectly ionizing (Hall and Giaccia, 2006). In directly ionizing, the incident radiation, such as alpha and beta particles, interacts with atoms as it passes through matter and ionizes them. X-rays and gamma rays are described as indirectly ionizing. Incident photons interact with matter through photoelectric effect, Compton scattering or pair production, depending on the energy (Knoll 2000). The secondary electrons produced by these processes are what further interact with atoms to produce multiple ionization events. All of the radiation experiments in this thesis used a ^{137}Cs source producing gamma rays of energy 662 keV. At that

energy range most of the photon interactions are through Compton scattering (Knoll 2000). Most low linear energy transfer (LET) radiation, such as photons, are also described as indirectly acting. Secondary electrons ionize intermediate molecules, mainly water, producing free radicals. These short lived radicals are what cause the majority of biological damage (Hall and Giaccia 2006).

The most critical subcellular target for ionizing radiation is DNA (Hall and Giaccia 2006). Radiation exposure can result in base damage, single strand breaks and double strand breaks (DSB). DSBs are generally considered to be the most biologically significant lesion (Hall and Giaccia 2006). Cells have the capacity to effectively repair moderate amounts of damage. High doses however can result in cell death, most of which occurs through mitotic catastrophe (Vakifahmetoglu et al. 2008). Sublethal damage which is unrepaired or incorrectly repaired can lead to mutations and genomic instability.

A major factor influencing the severity of biological damage from radiation is the dose rate. Generally lower dose rates are less severe because cells have time to repair damage before lethal amounts are accumulated (Hall and Giaccia 2006). Dose rate effects make it difficult to compare between chronic and an acute exposures. Most epidemiological effects in humans have been documented at high doses and dose-rates. To equate these risks to low dose and low dose-rates, a correction factor has been proposed by which to reduce the relative risk. The International Commission on Radiological Protection recommends a dose and dose-rate effectiveness factor (DDREF) of 2 for radiological

protection, although some animal studies have produced values as high as 10 (ICRP 2005).

Epidemiological data on the risk from radiation exposure exists down to approximately 100 mGy (Sanders 2010). Below that several models exist for describing radiation effects. For radiation protection purposes, a linear-no-threshold (LNT) model has been used for extrapolating effects (NRC 2006). This model assumes that any increase in dose above background levels result in an increase in risk. Alternate models exist for describing effects in the low dose region. Supralinearity predicts that risk is higher than predicted based on LNT (Gofman 1992). Considerable experimental data have supported models suggesting that risk is less than linear, and follow either the sub-linear model (Little and Muirhead 1998), threshold model (Hoel et al. 1998) or hormesis, where low doses can reduce risk compared to background levels (Sanders 2010).

It is generally accepted that aquatic biota are more resistant to radiation exposure compared to humans. As such, dose recommendations for aquatic organisms have been set at 400 μGy/day (3.5 Gy/yr), much higher than human dose limits of 50 mGy/year (IAEA 1992). The biological effects on embryogenesis in fish varies between species and can be impacted by factors such as development rate, incubation temperature and embryo size. Generally, sensitivity to radiation decreases throughout development. Most acute studies have looked at the impacts of photon radiation using external x-ray or gamma ray sources. Acute LD_{50} values during embryogenesis range from less than 1 Gy close to fertilization to in excess of 10 Gy closer to hatch (Ward et al. 1971, Wadley and

Welander 1971). Acute exposures have also been shown to impact the length of embryonic development and size, with embryos hatching earlier and growing larger following irradiation (Miyachi et al. 2003).

Fewer studies have looked at the impacts of chronic exposure on embryonic development in fish. Many of these studies have been compiled in the EPIC (Environmental Protection from Ionizing Contaminants) database (Sazykina and Kryshev 2003). Chronic exposures are mostly generated through incubating embryos in water containing differing radioisotope concentrations. However, radioisotope studies make accurate dosimetry difficult due to differences in isotope accumulation and permeability through the chorion. Results can also be confounded due to heavy metal effects depending on the isotopes used. Additionally, different radiation qualities make it difficult to compare to most acute studies. Embryonic mortality has been shown following exposure down to 0.5 mGy/day (Trabalka and Allen 1977). In contrast, chronic radiation can also have stimulatory effects, such as an increase in fecundity (Donaldson and Bonham 1970, Baylock 1969), an increase in development rate and resistance to starvation in newly hatched larvae (Simon et al. 2011).

1.2.5 Combined stressors

In nature, organisms are rarely exposed to single individual stressors but instead are subjected to multiple combined stressors. Exposure to multiple stressors may result in biological responses that differ from what would be predicted based on outcomes from individual exposures (Holmstrup et al. 2010). A synergistic response can occur where the

outcome is greater than additive. Synergistic effects have been shown between thermal and chemical stress (Scheil and Köhler 2009, Hallare et al. 2005). Outcomes can also be antagonistic or protective, where the response is less than additive. Protective effects have been shown between heat shock and radiation exposure (Boreham et al. 1997, Mitchel and Morrison 1982). Variable such as dose/concentration, length of exposure and timing between exposures can impact what type of combined stressor response occurs. Some combinations can be synergistic at one concentration or time points and then protective at another (Boreham et al. 1997).

1.3 Lake whitefish

The species investigated in this thesis is lake whitefish (*Coregonus clupeaformis*), a coregonid fish native to most of Canada and parts of the Northern United States (Scott and Crossman 1973). Lake whitefish are an important commercial fish, particularly within the Great Lakes where they have historically been one of the top catches, a large portion of which has come from Lake Huron (Ebner et al. 2008). In Ontario, commercial landings in 2013 were over 1,000 tonnes at a value of $4.2 million (DFO 2013). Lake whitefish are also a species of cultural importance to numerous first nations groups (Bruce Power 2005).

Lake whitefish grow to an average size of 38 cm and can live in excess of 20 years (Scott and Crossman 1973). Larval fish feed mainly on zooplankton and macroinvertebrates (Johnson et al. 2009). Historically, adult lake whitefish relied on *Diporeia* as prey,

however declines in *Diporeia* caused by invasive zebra mussel (*Dreissena polymorpha*) and quagga mussel (*Dreissena bugensis*) resulted in a shift to alternate food sources, consisting of other zooplankton, Ostracoda, Oligochaeta, Gastropoda and zebra and quagga mussels (Pothoven et al. 2001, Nalepa et al. 2009). Predators include lake trout (*Salvelinus namaycush*), northern pike (*Esox lucius*), burbot (*Lota lota*), walleye (*Sander vitreus*) and sea lamprey (*Petromyzon marinus*, Scott and Crossman 1973). Lake whitefish can reach sexual maturity as early as age 5 (Hart 1931). Adult fish spend most of their life in cold deeper water likely outside the influence of any human interactions on shore. However, mature fish come into shallow water in the fall to spawn in regions that could be impacted by industrial discharges.

Spawning occurs when lake temperatures are between 4.5 - 10°C (Hart 1931). Ripe females broadcast eggs over a depth of generally less than 10 m and a hard rock cobble substrate (Scott and Crossman 1973). Eggs are approximately 3 mm in diameter (Sreetharan et al. 2015). Fertilized embryos incubate throughout the winter and hatch the following spring. The impacts of different incubation temperatures, both constant and fluctuating, have been examined on lake whitefish. However, the effects of morpholine, sodium hypochlorite and ionizing radiation on embryonic development have previously not been studied.

1.3.1 Embryonic development

Detailed developmental staging series for lake whitefish have been documented by Price (1934a, 1934b, 1935) and Sreetharan et al. (2015). The majority of organ development

occurs during the first half of the total time-to-hatch, with the latter half devoted mainly to embryo growth. The optimal incubation temperature for embryonic development is between 0.5 - 6°C, with increased mortality and developmental abnormalities occurring outside this range (Price 1940, Brooke 1975, Griffiths 1979). The average larval size at hatch depends on temperature, ranging from approximately 14 mm at 0.5°C to 8 mm at warmer temperatures (Price 1940, Brooke 1975, Mueller et al. 2015). Most embryonic mortality occurs early in development before the beginning of organogenesis (Brooke 1975). At warmer temperatures a second mortality event can occur close to the hatching stage (Price 1940).

Constant temperature laboratory studies have found an exponential decrease in time to hatch with increasing temperature (Figure 1.2). Reported differences in hatch timing between studies are in part due to differences between the populations of lake whitefish used; Price (1940) from Lake Erie, Brooke (1975) from Lake Michigan, Griffiths (1979) from Lake Simcoe and Mueller et al. (2015) from Lake Huron. Genetic differences have been identified between lake whitefish populations from various Ontario lakes (Ihssen et al. 1981). Additionally, differences in rearing apparatus would also contribute to variations in time-to-hatch (Mitz et al. 2014).

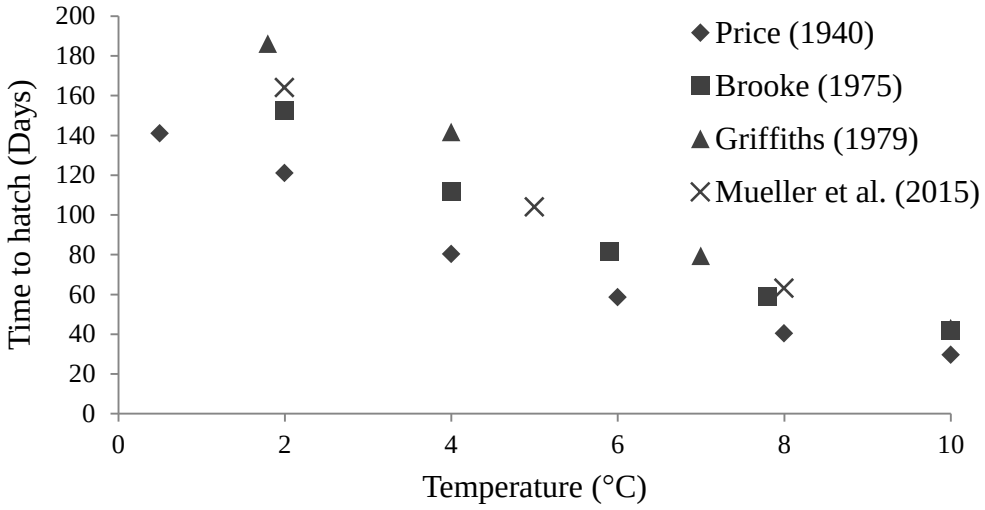

Figure 1.2. Hatch timing for laboratory reared lake whitefish under various constant incubation temperatures. Griffiths (1979) reported time to mean hatch. Price (1940), Brooke (1975) and Mueller et al. (2015) reported time to median hatch.

The impacts of variable thermal regimes have been examined in laboratory. Griffiths (1979) incubated embryos at a baseline temperature for 18 hours/day with transient cycling to a second temperature for 6 hours/day. Embryos were able to survive short duration temperature increases up to 10°C, with smaller temperature cycles reducing the time-to-hatch. To more closely examine actual in-lake temperature fluctuations, Griffiths (1979) and Patrick et al. (2013) raised embryos in water taken directly from Lake Ontario. Embryos were exposed either continually or periodically to water 1,2,3 or 5°C above ambient lake temperature, which resulted in a hatch advance of up to 5 weeks. Although these studies used ambient lake water, embryos were still raised within laboratory rearing apparatus and therefore may not accurately represent temperature effects during in-lake development. Temperature effects on development in lake whitefish have not previously been examined in-situ, with embryos developing in their natural spawning shoals.

1.3.2 Hatching

Hatching in lake whitefish and other teleosts results from a combination of enzymatic chorion digestion and mechanical movement of embryo. Choriolosis begins when hatching enzymes are secreted from hatching gland cells on the embryo body and yolk (Yamagami 1981). The hatching enzyme is stored within hatching glands in its active form in advance of the natural hatching window (Iuchi et al. 1982). This allows the embryo to prematurely trigger hatching in response to a potentially harmful environmental stressor. Hatch can be triggered in aquatic species by numerous factors, including heat (Brooke 1975), mechanical agitation (Griem and Martin 2000), hypoxia (Petranka et al. 1982), or predation cues (Ireland et al. 2007).

1.3.3 Bruce Power habitat

The area surrounding Bruce Power has been identified as a potential spawning ground for lake whitefish. Substrate mapping was conducted in 2009 in regions potentially impacted by the Bruce Power discharge, identifying regions with cobble or boulder as possible spawning grounds (Bruce Power 2010). Approximately 9.22 km^2 or 69% of the region between 2-8 m in depth was identified as a suitable spawning habitat. Gillnetting was run during fall spawning seasons between 2009 and 2011 (Bruce Power 2014b). Ripe male and female fish were caught in all years, with catch per unit efforts ranging from 2-3 ripe females per km and 1-25 ripe males per km depending on the year and location. Despite the netting of spawning adult fish, developing lake whitefish eggs have not been directly observed in the region surrounding Bruce Power. Airlift sampling was conducted over the

substrate during the fall of 2009 and failed to locate any eggs (Bruce Power 2010). However, airlift sampling was only conducted over a small region of the total identified potential spawning grounds.

The lake whitefish embryos used in this thesis were all collected through in-vitro fertilization of eggs from spawning adult fish. In the first experimental season (2011) lake whitefish were gillnetted in front of Bruce Power. In the subsequent three years (2012-2014) embryos were collected from the Fishing Islands, a more productive spawning ground near the base of the Bruce Peninsula, approximately 45 km northeast of Bruce Power (Figure 1.1).

1.3.4 Laboratory rearing

Whitefish embryos have been laboratory reared using a variety of systems including constant flow through (Brooke 1975), air bubbled (Price 1940) and small scale petri dish (Wedekind et al. 2001). Being a cold water developing species, rearing apparatus need to maintain stable temperatures below 8°C in addition to adequate oxygenation and water quality. We designed a custom system for raising lake whitefish embryos incorporating both upwelling hatching jars and standing water petri dishes (Mitz et al. 2014, Appendix A). The system was designed to fit within a 2 door chromatography refrigerator to maintain stable temperatures between 2 – 8°C. Each unit had a bank of 8 upwelling hatching jars holding up to 10,000 embryos per jar, which were continuously fed with recirculated filtered water. Standing water petri dish incubators were housed on the shelves of the unit allowing for small scale rearing of up to 50 embryos per dish. Each

unit could accommodate several hundred dishes, allowing adequate numbers for numerous experiments with multiple doses and replicates. All laboratory lake whitefish rearing described in this thesis, with the exception of chronically irradiated embryos in Chapter 4, was carried out within one of these units.

1.4 Work presented in this thesis

The work presented in this thesis explores the impacts that stressors associated with industrial cooling water discharges can have on lake whitefish embryonic development.

Chapter 2 describes the results of a field based study looking at in-lake development within different thermal environments. Lake whitefish embryos were incubated in-situ near the Bruce Power discharges as well as at offsite reference locations. A custom incubation chamber system was constructed to successfully raise embryos in Lake Huron throughout the winter and allow retrieval of live embryos the following spring. Development at each site was modelled based on recorded temperature data, as well as through morphometric measurements on recovered embryos.

A detailed analysis of exposure to the chemicals morpholine and sodium hypochlorite is examined in Chapter 3. Embryos were exposed both acutely and chronically at concentrations at or above environmental levels. Developmental effects were assessed in terms of survival, hatch dynamics, and morphology.

Radiological effects on development are discussed in Chapter 4. Embryos were exposed to both acute and chronic ^{137}Cs gamma rays. Included in Chapter 4 is a description of the irradiator that was designed and built for chronic exposures. Also examined are the effects of combined stressors, looking specifically at how a mild heat shock or morpholine exposure can alter the response to ionizing radiation.

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Chapter 2

Incubation of lake whitefish (*Coregonus clupeaformis*) embryos in cooling water discharge and the impact of fluctuating thermal regimes on development

Christopher Thome, Charles Mitz, Christopher M. Somers, Richard G. Manzon, Douglas R. Boreham and Joanna Y. Wilson

2.1 Abstract

Thermal discharges released from industrial cooling water systems represent a potential environmental risk to fish species that spawn in nearshore waters. We investigated the impacts of in-situ incubation of lake whitefish (*Coregonus clupeaformis*) embryos in the vicinity of a nuclear generating station. Over three consecutive years, fertilized embryos were deployed in Lake Huron using custom built incubation chambers coupled with data loggers to monitor water temperature. Temperatures at sites in the vicinity of the thermal discharge were significantly elevated compared to reference locations, with average winter temperatures up to 3°C warmer. Temperatures were also more variable at these sites with a significantly greater daily temperature range and rate of change. Embryos developing at discharge sites were significantly larger with smaller yolks than those from the reference stations, suggesting an advanced growth. This increased growth was supported by thermal data, with discharge sites predicted to be more than 10% advanced based on growth rate modelling. These temperature changes and increase in development rate are below thresholds for embryonic mortality but could potentially impact post-hatch larval survival.

2.2 Introduction

Many industrial processes, in particular thermal power generation, rely on natural bodies of water as part of their cooling systems. In once through cooling, water is cycled through the plant removing waste heat or condensing turbine steam, following which warmer effluent water is discharged back into the lake, river or ocean. These discharges can have direct environmental impacts on fish or other aquatic species. Thermal effluents have been shown to influence size (Bennett 1972), behavior (Kelso 1976), gametogenesis (Luksiene et al. 2000), and population structure (Teixeira et al. 2009) in fish. Of particular concern are the effects on embryonic development. Many fish species spawn in shallow nearshore water in regions potentially impacted by industrial discharges. In addition, embryogenesis represents one of the most sensitive periods for exposure to environmental stressors and developing embryos are immobile and unable to avoid suboptimal temperature conditions.

The effects of different constant incubation temperatures on embryonic development have been studied for a variety of fish species. Within tolerance ranges, warmer temperatures will increase the rate of embryonic development, while extreme temperatures outside of this range can induce mortality and developmental abnormalities (Pepin 1991). In comparison to constant temperature regimes, the impacts of fluctuating thermal regimes, such as those occurring naturally in unique ecosystems or resulting from manmade cooling water discharges, are poorly understood. Most studies examining varying incubation temperature have been laboratory based with embryos raised under controlled

conditions (Griffiths 1979, Patrick et al. 2013) and may not accurately describe what occurs during natural in-lake development. Few studies have examined the impacts of thermal discharges in-situ and those that have were limited to non-peer reviewed literature (Griffiths 1987, 1990), or focused on warm water developing species (Sandstrom et al. 1997) that may not be as severely impacted by temperature fluctuations compared to those developing in cold water.

Lake whitefish (*Coregonus clupeaformis*) are a cold water species native to Canada and the Northern United States, including the Great Lakes. Spawning occurs in late fall once lake temperatures have dropped below 10°C. Fertilized eggs are broadcast over coarse substrate at a depth of generally less than 10 m (Hart 1931). Embryos develop within lodgment sites in the substrate throughout the winter and hatch in the spring following ice melt. The optimal range for normal embryonic development is between 0.5 to 6°C (Price 1940, Brooke 1975). Changes in constant incubation temperature will alter the length of the embryonic development period, ranging from 180 days at 0.5°C to 60 days at 8°C (Brooke 1975, Griffiths 1979, Mueller et al. 2015). Periodic increases in temperature have also been shown to reduce the length of embryonic development (Griffiths 1979).

The objective of this study was to examine the impacts of fluctuating thermal regimes associated with industrial cooling water discharges on lake whitefish development in-situ. Fertilized embryos were deployed at control and thermally-affected sites in Lake Huron inside of custom built incubation chambers throughout three consecutive winters. Each site had continuous substrate temperature data for the incubation period, enabling a

comparison between altered thermal environments and control locations under ambient environmental conditions.

2.3 Materials and methods

2.3.1 Study area

Bruce Power is the largest operating nuclear power generating station in the world, located on the eastern shore of Lake Huron between the communities of Kincardine and Port Elgin, Ontario, Canada. It produces up to 6,300 MW of electricity. The Bruce Power site consists of 8 CANDU pressurized heavy water reactors in two stations (4 units in Bruce A and 4 units in Bruce B). Each station relies on once through cooling using Lake Huron water for steam condensation. Condenser cooling water is cycled through at a rate of up to 190 m^3/s per station and returned to the lake via a surface discharge channel. During the winter months, outflow water is released into the lake at temperatures up to 13°C above ambient under normal conditions (Bruce Power 2005).

2.3.2 System design

In-situ incubation chambers were custom built based on a modified design from Manny et al. (1989). Each was designed to hold 100 embryos (Figure 2.1A). Chambers were constructed from three pieces of plastic measuring 27 cm by 15 cm. A center piece, 0.9 cm thick, was cut with 100 circular holes 1.1 cm in diameter, each designed to house a single embryo. Two outer pieces, 0.2 cm thick, were cut with matching 1.1 cm holes.

Outer pieces sandwiched 0.3 cm² nylon mesh to hold embryos in place while allowing free water flow through the chamber. The three pieces were held together using 6 nylon screws.

Between five and seven chambers were deployed at each site attached to a chain and anchor system (Figure 2.1B). The chain system consisted of a 16 m long centre chain (3/16" galvanized chain links) anchored at both ends with a 5 kg claw anchor. Five to seven side chains, 3 m in length, were spaced evenly along the centre chain in alternating directions with an incubation chamber attached to the end. Incubation chambers, side chains, and centre chains were all connected using 3/16" quick links. Data loggers (Onset HOBO UA-002-64) were attached to each system to record substrate temperature at 5 minute intervals. Ultrasonic pingers (Sonotronics EMT-01-1) were deployed to aid in retrieval through signal triangulation using a hydrophone.

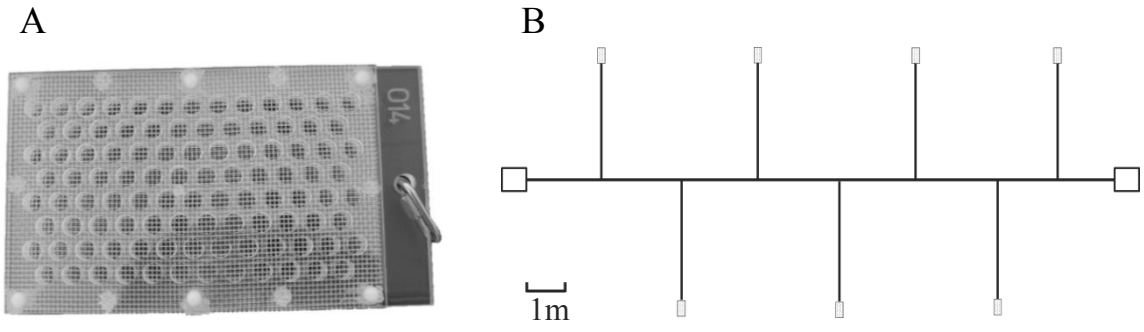

Figure 2.1. Design of incubation chamber system. A) Single chamber with housing for 100 embryos in circular holes, covered on each side with 3 mm² nylon mesh. B) Scale drawing of chain system. Seven side chains and incubation chambers (small rectangles) branched off in alternating directions from a 16 m centre chain, which was secured on both ends with an anchor (large square). Data loggers and ultrasonic pingers were attached to one of the anchors (not shown).

2.3.3 Embryo collection

Lake whitefish embryos were obtained through in-vitro fertilization. Embryos were fertilized in consecutive years on November 22, 2011, November 15, 2012 and November 21, 2013. In 2011, gillnets were set overnight in front of Bruce Power (N 44.3567, W 81.5729). In 2012 and 2013, gillnets were set approximately 45 km northeast of Bruce Power (N 44.7094, W 81.3125). Gametes were stripped from multiple ripe females and pooled with milt from multiple males. Eggs were dry fertilized for 5 minutes, followed by a 5 minute wet fertilization, then disinfected with Ovadine® (5 ml/L, Syndel Laboratories Limited) for 30 min. Embryos were initially reared in the laboratory for approximately 30 days in upwelling hatching jar incubators with a recirculating filtered water supply at 5°C (Mitz et al. 2014). Data loggers recorded laboratory water temperature every 5 minutes (Schlumberger Mini-Diver). All animal procedures were completed with approval from McMaster University's Animal Ethics Review Board. Spawning adult Lake Whitefish were collected under scientific fishing permits from the Ontario Ministry of Natural Resources to J.Y.W.

2.3.4 Deployment and retrieval

Deployment of the incubation chamber systems occurred once embryos in the laboratory had reached the eyed stage. Chambers were loaded with embryos the evening prior to deployment. Chambers were transported and housed overnight in 26 L containers filled with dechlorinated water. These containers were placed in a larger 68 L container filled with ice to maintain an overnight temperature below 5°C. Container water temperature

was monitored with a data logger from the time of chamber loading until deployment. Chambers and chain systems were deployed the following morning with the help of commercial divers (Pollutech Enviroquatics Limited, General Diving Contractors Incorporated). Sites for deployment were selected based on a water depth of 5 – 10 m and a loose rock cobble substrate. Four regions were chosen; a southern reference (R1), a northern reference (R2) and two potentially impacted sites offshore of the Bruce A and B discharges (Sites D2 and D1 respectively, Table 2.1, Figure 2.2). In year 2, two systems were deployed in each discharge zone (labelled a and b). Chambers, chains and anchors were lowered down to the divers fully assembled and were laid out along the lake bottom. The equipment was then partially buried with cobble to prevent movement throughout the winter. Equipment was retrieved the following spring. To determine if transportation and housing overnight impacted embryonic survival, an extra 2 chambers were loaded and brought to the sites but not deployed in each of years 2 and 3. These chambers were then brought back to the laboratory where the embryos were removed and incubated in petri dishes until hatch at a constant 5°C, using a custom designed incubator (Mitz et al. 2014).

2.3.5 Temperature data

Chambers were deployed and retrieved on different days each year, so average site temperature was taken over the same time interval (December 21 – March 16) to allow for comparison between years. The daily average and the daily maximum temperature were calculated at each site. To measure temperature fluctuations, the daily range and the rate of temperature change between consecutive data points were calculated.

Table 2.1. Incubation chamber deployment and retrieval information.

Site	Latitude/longitude	Depth (m)	Deployment date	Retrieval date	Chambers deployed	Chambers retrieved	Embryos deployed	Embryos retrieved
Year 1: 2011-2012								
R1	44.2618, -81.6161	9.14	December 21	April 13	5	5	500	0
D2	44.3483, -81.5888	8.23	December 21	April 13	5	5	500	0
Year 2: 2012-2013								
R1	44.2556, -81.6187	8.63	December 15	March 16	5	3	500	202
D1a	44.3119, -81.6121	7.71	December 15	March 16	5	1	500	26
D1b	44.3212, -81.6107	8.11	December 11	March 16	5	3	500	180
D2a	44.3379, -81.5968	8.11	December 11	March 16	5	2	500	82
D2b	44.3506, -81.5821	8.72	December 11	June 16	5	3	500	0
R2	44.3887, -81.5428	9.33	December 11	June 16	5	4	500	0
Year 3: 2013-2014								
R1	44.2569, -81.6169	5.48	December 15	May 19	7	7	700	0
D1	44.3154, -81.6138	8.53	December 15	May 19	7	7	700	0
D2	44.3539, -81.5794	7.32	December 15	April 19	7	7	700	217
R2	44.3891, -81.5423	7.62	December 15	N/A	7	0	700	0

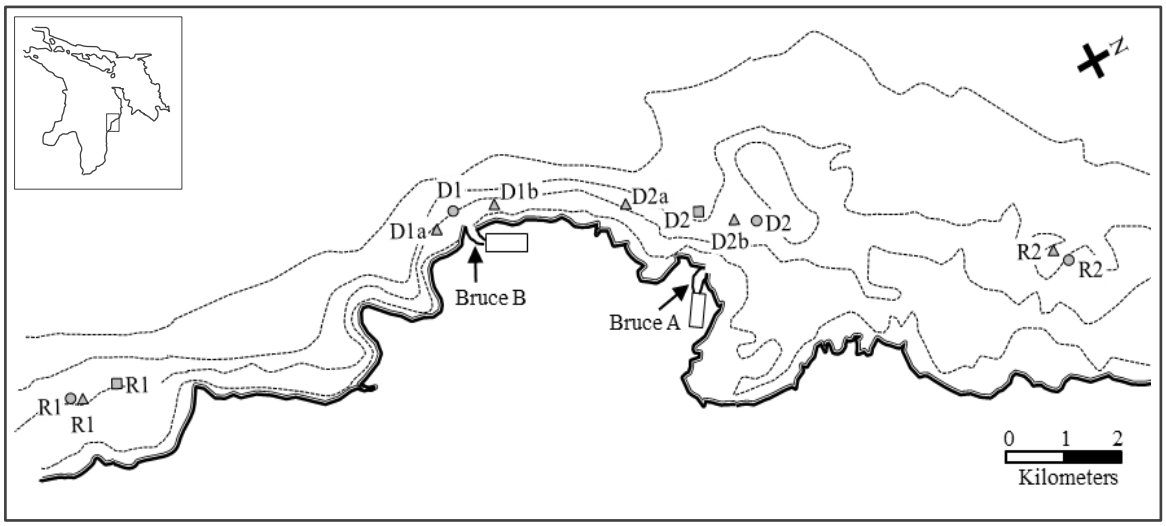

Figure 2.2. Deployment locations of incubation chamber systems in front of Bruce Power, in Eastern Lake Huron (inset). Squares indicate sites in year 1 (2011-2012), triangles in year 2 (2012-2013) and circles in year 3 (2013-2014). Each system was deployed at a depth of 5 – 10 m. Arrows indicate the location of both cooling water surface discharge channels.

2.3.6 Hatch timing and development rate

Brooke (1975), Griffiths (1979) and Mueller et al. (2015) raised different populations of lake whitefish (Lake Michigan, Lake Simcoe and Lake Huron, respectively) at a variety of constant incubation temperatures and found a similar relationship between development rate and temperature. Hatch timing data from the three studies was combined to generate a more generalized model for lake whitefish, with the time to median hatch, T_H (days), decreasing exponentially as a function of temperature, T (°C), according to the equation ($p < 0.01$, $R^2 = 0.98$):

$$T_H = 219e^{-0.159T} \qquad [1]$$

Taking the inverse, the percent of time-to-hatch completed per day, R_T, increases with temperature according to the relationship

$$R_T = 0.457e^{0.159T} \qquad [2]$$

The percent of total time-to-hatch completed at retrieval, D, was calculated using the incremental growth at temperature model

$$D = \sum_{T_o}^{T_i} 0.457e^{0.159T}\Delta t \qquad [3]$$

Increments of development were summed over discrete time intervals Δt. A five minute time interval was used, which was the set recording interval for data loggers. In-lab data logger temperature was used until deployment, after which in lake data logger temperature was used. Incremental development was calculated for each 5 minute time

interval and then summed from fertilization until chamber retrieval. For sites that were retrieved after the hatching window, a time to median hatch was calculated based on the time for incremental growth to summate to 100%.

2.3.7 Morphometric measurements

All live embryos and larval fish retrieved from incubation chambers were fixed in 10% neutral buffered formalin for 1 week then transferred to ethanol for long term storage. Up to 100 fish were imaged and measured at each site. Prior to imaging, embryos were removed from the chorion. Two images, one dorsal and one lateral, were taken for each fish. Dorsal images were used to measure total body length from the anterior tip of the head to caudal fin. Lateral images were used to measure eye diameter along the anteroposterior axis and two perpendicular yolk diameters, from which a yolk area was calculated. All imaging and measurements were completed with the researcher blind to the study site.

Lake whitefish embryos were previously raised in the lab at a constant 5°C, with morphometric measurements taken throughout the entire development period (Sreetharan et al. 2015). Embryos were sampled every 2-3 days and total body length measurements were taken from preserved specimens as described above. Embryo body length, L (mm), increased as a function of % time-to-hatch, D, according to the second order polynomial equation ($p < 0.001$, $R^2 = 0.96$)

$$L = -0.00065D^2 + 0.19D \qquad [4]$$

This regression equation was used to stage embryos to calculate an approximate percent of total time-to-hatch completed at retrieval.

2.3.8 Statistical analysis

Daily average site temperature, daily temperature range and temperature rate of change were compared using a Kruskal-Wallis one-way ANOVA on ranks followed by Tukey's HSD test, because temperature data were not normally distributed. Embryo morphometric measurements were compared using a one-way ANOVA followed by Tukey's HSD test. Statistical analysis was completed using SigmaPlot V11.0.

2.4 Results

2.4.1 Thermal data

Intact data loggers were retrieved from all sites, with the exception of R2 in year 3. The weekly average temperature was compared between sites. In years 1 and 2, lake temperatures in December at the time of deployment were between 4 - 6°C (Figure 2.3A,B). Temperatures decreased until the middle of January, following which they remained stable until increasing again in late February. During January and February of both years, R1 maintained the coldest temperature between 0.5 - 1°C. The remainder of the sites were consistently 0.5 – 1.5°C warmer.

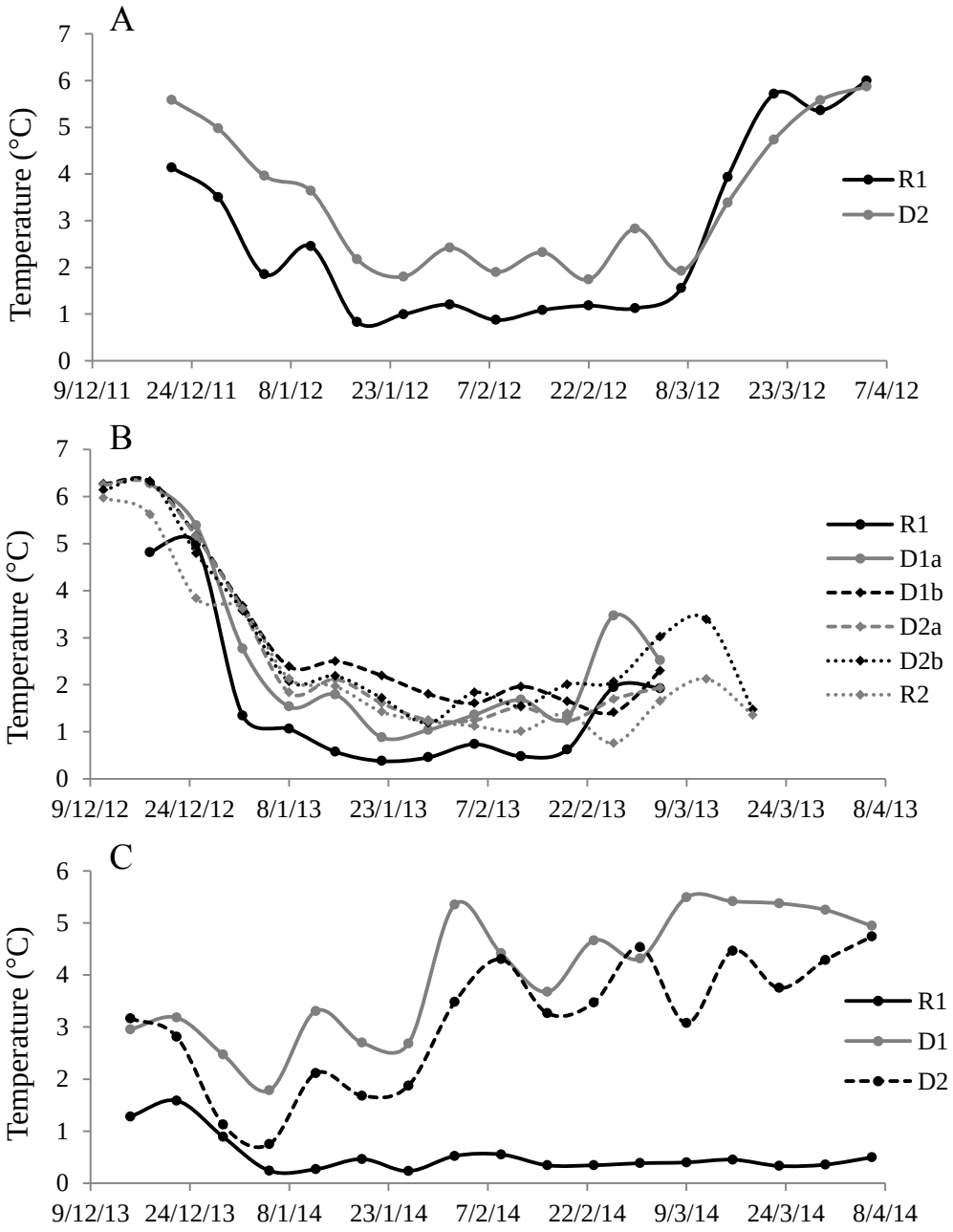

Figure 2.3. Mean weekly temperature at each monitoring site in year 1, 2011-2012 (A), year 2, 2012-2013 (B) and year 3, 2013-2014 (C). Weekly means were calculated from the date of deployment until retrieval in each year.

Temperature patterns differed in year 3, with lake temperatures at 3°C or below at the time of deployment (Figure 2.3C). Temperature at R1 decreased until the beginning of January, before remaining steady below 0.5°C for the remainder of the recording period. Sites D1 and D2 however fluctuated largely, reaching a weekly average as high as 5.4°C at the beginning of February.

In each of the three years, the average temperature across the whole winter was coldest at site R1 (Figure 2.4). The daily average temperature was compared within each year, and the discharge sites were all significantly warmer than R1 (Year 1: $H = 15.485$, $df = 1$, $p < 0.001$, Year 2: $H = 41.28$, $df = 5$, $p < 0.001$, Year 3: $H = 208.92$, $df = 2$, $p < 0.001$). Year 2 was the only year where a northern reference site (R2) was retrieved. In that year R2 was the second coldest site, after R1, although not significantly different from the four discharge sites (Figure 2.4). In years 1 and 2, the discharge sites were between 0.7°C and 1.2°C warmer than R1. The largest range in temperatures was seen in year 3, where all three sites were significantly different from each other, with D1 on average more than 3°C warmer than R1 (Figure 2.4).

In addition to warmer average water temperature in all three years, the discharge sites showed an increase in temperature fluctuation. The daily temperature range and the rate of change were significantly greater at these sites compared to R1 (Table 2.2). Again, this was particularly evident in year 3, where the daily temperature range at both discharge sites was more than 8 fold greater and the rate of change at D1 was 40 fold greater compared to R1. The daily maximum temperature was also assessed at each site. In year

1, temperature only exceeded 8°C at one time point (D2 on April 4). In year 2, early in the recording period, Site D1a reached as high as 10.7°C. By the beginning of January, no sites exceed 8°C with the exception of D1a on two occasions (January 4 and 18). In year 3, temperature at R1 never exceeded 4°C for the entire recording period. Site D2 remained below 8°C for most of winter and never exceeded 10°C. Site D1 exceeded 10°C on only one time point (March 15) and peaked at 10.1°C.

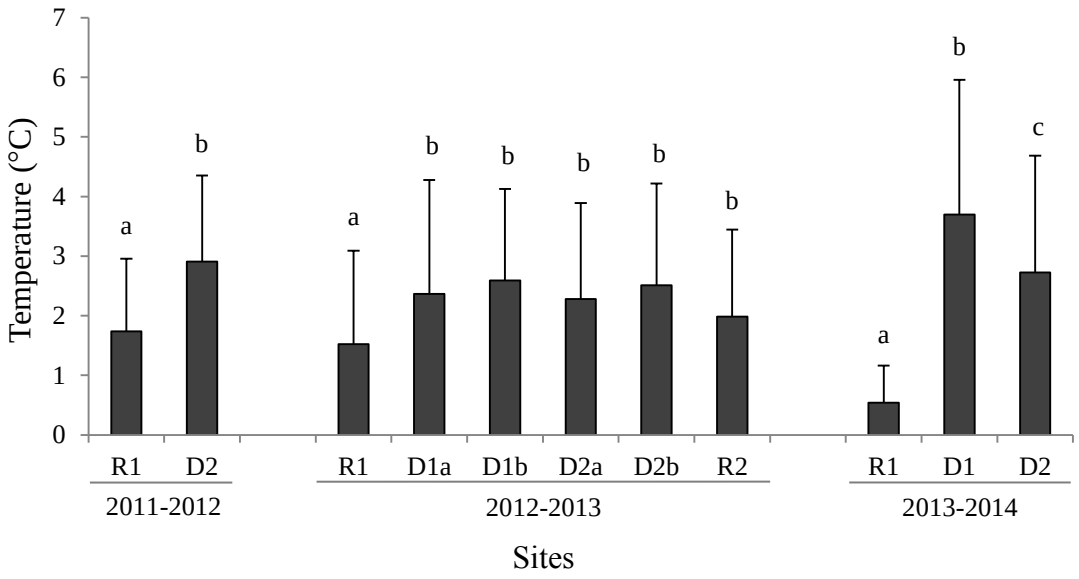

Figure 2.4. Mean site substrate temperature across all three study years. Mean temperature was calculated over the same time interval for each year (December 21 – March 16). Daily mean temperature was compared within years using a Kruskal-Wallis one-way ANOVA on ranks with Tukey's HSD test ($p < 0.05$). Letters denote statistical differences. Error bars represent SD.

In all three years, temperature loggers at R1 were placed in approximately the same location. The daily average temperature at this reference site differed significantly between the three years ($H = 63.53$, $df = 2$, $p < 0.05$). The coldest temperatures occurred

in year 3, where the mean was approximately 1°C colder than the two previous years (Figure 2.4). The daily range in temperature in year 3 was also half as much as in the other two years (Table 2.2).

Table 2.2. Temperature variation and predicted time to median hatch in lake whitefish. The range in temperatures was measured each day and averaged over the entire recording period. The rate of temperature change was taken between consecutive data points and averaged over the entire recording period. Median hatch times were predicted based on summation of incremental growth-at-temperature. Daily range and rate of change were compared within years using a Kruskal-Wallis one-way ANOVA on ranks with Tukey's HSD test. Letters denote statistical differences. Daily range and rate of change error represent 95% confidence intervals, median hatch error represents standard error of the estimate.

Site	Daily range (°C)	Rate of change (°C/hr)	Predicted median hatch (days)
Year 1: 2011-2012			
R1	1.0 ± 0.1^{a}	0.08 ± 0.01^{a}	131 ± 11.1
D2	1.4 ± 0.2^{b}	0.21 ± 0.01^{b}	123 ± 10.4
Year 2: 2012-2013			
R1	0.8 ± 0.1^{a}	0.15 ± 0.01^{a}	-
D1a	2.4 ± 0.4^{bc}	0.72 ± 0.03^{b}	-
D1b	2.0 ± 0.3^{bc}	0.52 ± 0.02^{b}	-
D2a	1.7 ± 0.2^{bc}	0.34 ± 0.01^{c}	-
D2b	2.5 ± 0.3^{b}	0.40 ± 0.01^{c}	122 ± 10.3
R2	1.7 ± 0.2^{c}	0.24 ± 0.01^{d}	133 ± 12.4
Year 3: 2013-2014			
R1	0.4 ± 0.1^{a}	0.10 ± 0.01^{a}	169 ± 14.2
D1	4.8 ± 0.3^{b}	4.38 ± 0.06^{b}	114 ± 9.6
D2	3.5 ± 0.3^{c}	0.56 ± 0.01^{c}	125 ± 10.6

2.4.2 Embryo data

Two chambers in years 2 and 3 were loaded with embryos and transported for deployment but then brought back to the laboratory for incubation. Survival in these chambers at hatch was greater than 97%. In year 1, weather conditions delayed retrieval until April 13, 2012, by which point all embryos had likely hatched and were able to swim free from the chambers. In year 2, four of the sites were retrieved on March 16, 2013 containing live embryos, for a total development time of 121 days from fertilization (Table 2.1). Several of the 5 chambers deployed at each site were damaged over the winter. The number of embryos retrieved per chamber varied, but was not significantly different by site ($F(2,5) = 1.87$, $p = 0.248$). The remaining two sites were retrieved on June 16, 2013 and contained no embryos. In year 3, D2 was retrieved on April 19, 2014 and contained live larval fish, for a total development period of 149 days from fertilization (Table 2.1). Chambers at that site were covered in a thin layer of algae preventing the hatched fish from swimming free. Due to ice cover, Sites R1 and D1 were only accessible for retrieval on May 19, 2014, and contained no embryos or larval fish. Equipment at R2 could not be located. In all, embryos from 4 sites in year 2 and hatched fish from 1 site in year 3 were recovered for comparison.

Morphometric measurements were performed on retrieved embryos and larval fish. In year 2, embryo body length was significantly larger ($F(3,313) = 15.36$, $p < 0.05$) at the three discharge sites compared to R1 (Figure 2.5A). In addition, yolk area was significantly smaller ($F(3,314) = 8.76$, $p < 0.05$) at D1b and D2a compared to R1 (Figure

2.5B). No significant difference was seen in eye diameter (data not shown). No difference in the frequency of developmental abnormalities (< 5%) was observed between sites. In year 3, average body length of the larval fish from D2 was 13.86 ± 0.54 mm, yolk area was 0.71 ± 0.13 mm^2 and eye diameter was 1.00 ± 0.05 mm.

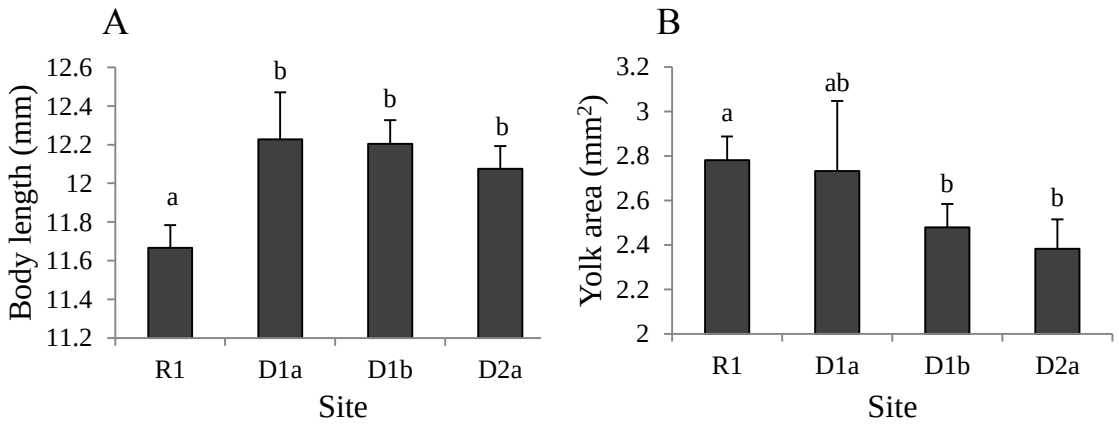

Figure 2.5. Morphometric measurements on lake whitefish embryos retrieved in year 2 (2012-2013). Dorsal body length was measured (A) and yolk area was calculated based on two perpendicular yolk diameters (B). Embryos were incubated for a total of 121 days (November 15, 2012 – March 16, 2013). Body length and yolk area were compared using a one-way ANOVA with Tukey's HSD test ($p < 0.05$). Letters denote statistical differences. Bars represent means ± 95% confidence intervals.

2.4.3 Development rate

To estimate the effects of temperature differences on development rate, a time to median hatch was calculated based on temperature data (Table 2.2). In years 1 and 2, the discharge sites had predicted hatch times that were approximately 10 days earlier than the reference sites. In year 3, the largest range was predicted between sites R1 and D1, with a difference of 55 days based on temperature data.

For sites in year 2 where live embryos were recovered, the percent of total time-to-hatch completed at retrieval was predicted using both temperature data and morphometric measurements. Both methods predicted the discharge sites were closer to hatch compared to R1. Based on temperature data, embryos at R1 were calculated to have completed 86.9 ± 10.2% of total time-to-hatch (Table 2.3). The three discharge sites were approximately 10% ahead, nearing 100% completion. Based on body length measurements, embryos at R1 had completed 90.1 ± 3.9% of total time-to-hatch at retrieval while the discharge sites had completed between 95 - 100% (Table 2.3). At each site, the development estimates based on data logger temperatures matched closely to the estimated development values calculated using morphometric measurements.

Table 2.3. Predicted percent time-to-hatch of lake whitefish embryos at the time of retrieval in 2012-2013. Percent time-to-hatch was estimated based on temperature data through summing incremental growth-at-temperature over each discrete recording time step and based on morphometric measurements by comparing retrieved embryo body lengths to development rate regression equations. Error represents standard error of the estimate.

Site	% time-to-hatch: temperature data	% time-to-hatch: morphometric measurements
R1	86.9 ± 10.2	90.1 ± 3.9
D1a	96.3 ± 10.2	98.6 ± 3.9
D1b	99.4 ± 10.2	98.2 ± 3.9
D2a	96.7 ± 10.2	96.1 ± 3.9

2.5 Discussion

Variable temperature environments resulting from a nuclear power plant thermal discharge had subtle but significant effects on lake whitefish embryonic development. Discharge sites had elevated temperatures, potentially advancing embryonic development, although likely below levels which would induce mortality or abnormalities. Similar in-situ systems have been used in other species to examine the effects of lake acidification (Kennedy 1980, Gunn and Keller 1984) and spawning habitat quality (Manny et al. 1989, Casselman 1995). The impacts of thermal discharges on development have been examined in-situ with round whitefish (*Prosopium cylindraceum)* embryos incubated near a nuclear generating station in Lake Ontario within gravel filled baskets. Lake temperatures near the discharge were on average 3°C warmer compared to reference sites, resulting in a hatch advance of up to 4 - 5 weeks (Griffiths 1987), similar to what is shown here for lake whitefish. In addition, an increase in mortality and egg strand disintegrations was seen in European perch (*Perca fluviatilis*) incubated near a nuclear power plant discharge in Sweden (Sandstrom et al. 1997). However, the impacts on a warm water developing species such as European perch may not accurately reflect impacts on lake whitefish or other species that develop throughout the winter. In addition, thermal discharges will have a greater impact on substrate temperature during the winter when lake temperature drops below 4°C, at which point a sinking thermal plume can occur because discharge water can be higher in density than ambient lake water (Raithby et al. 1988). The temperature effects seen on lake whitefish could have ecological consequences for embryonic development and post hatch survival.

In all years, substrate temperatures at the selected sites in front of Bruce Power were significantly elevated compared to the southern reference location. These temperature differences may partly be the result of natural variation in lake temperature due to bathymetric/topographic differences, with R1 being in a more sheltered location (Figure 2.2). Temperature patterns differed between the three study years, with year 3 significantly colder than the other two. Year 3 was a colder winter and it was the only year where Lake Huron completely froze over. Temperatures at R1 that year were below 0.5°C for most of the recording period and showed less variation, likely due to ice cover. Year 3 also had the largest temperature difference between the reference and discharge sites, suggesting that potential thermal impacts may be greater during a colder winter. However, sites D1 and D2 were located closer to the discharges in year 3 compared to the other two (Figure 2.2), which would have contributed to this temperature difference.

Based on temperature data no significant increase in mortality would be expected at any of the sites. Temperature effects on lake whitefish embryonic development have been examined in the laboratory using a variety of flow through, air bubbled or static water systems. Embryos raised under constant temperatures had optimal survival between 0.5°C and 6°C (Price 1940, Brooke 1975, Eme et al. 2015, Mueller et al. 2015). The average site temperatures, which ranged from 0.5°C to 3.7°C (Figure 2.4), were all within the optimal range for lake whitefish development. Periodic temperature spikes were seen, particularly at sites located close to the discharges; however, these rarely exceeded 8°C. Only twice did temperatures exceed 10°C, for between 5 and 30 minutes, up to a maximum 10.7°C. Both Griffiths (1979) and Patrick et al. (2013) incubated embryos in a constant flow

through setup using naturally varying Lake Ontario water, and found that periodic or continuous temperature increases above ambient reduced the time to hatch. Developing embryos were able to tolerate transient increases in temperature up to 10°C with no significant effects on survival (Griffiths 1979). Less frequent heat shocks, of 3°C one hour per week, did not impact survival, time to hatch, or pre-hatch embryonic size for fish incubated at 2 or 5°C (Lee et al., McMaster University, submitted manuscript).

The percentage of live embryos remaining in each of the chambers in year 2 ranged from 12% up to 75%, with no significant difference between sites. Several of the chambers were retrieved with small cobble and silt having penetrated the mesh and some mortality was likely caused by mechanical damage to the embryos. Griffiths (1990) found a correlation between sediment accumulation and round whitefish embryonic mortality during in-situ incubation. Natural mortality is expected to be low since chambers were loaded at the eyed stage, which typically experiences minimal mortality compared to that observed in earlier stages of development (Brooke 1975, Griffiths 1979). Survival in the laboratory raised embryos from the undeployed chambers was greater than 97%, indicating that transportation and housing overnight during deployment did not meaningfully affect survival. The low rate of developmental abnormalities seen in the retrieved embryos was consistent with what has previously been shown for lake whitefish raised at similar temperatures (Brooke 1975, Mueller et al. 2015).

In addition to mortality, some of the empty cells in retrieved chambers could be due to hatching. Based on development rate calculations, several of the sites would have been

within the hatch window at the time of retrieval. A large enough mesh size was chosen to allow free water flow; however, once hatched the larval fish were small enough to swim free from the chambers. The design of chambers necessitated that they be retrieved prior to hatch, which prevented a direct measure of when embryos would have hatched. Therefore, in chambers that were recovered with live embryos, the percent time-to-hatch completed at retrieval was predicted based on temperature data as well as morphometric measurements.

Although the average temperature at all sites was within the optimal range for lake whitefish, the elevated temperatures recorded at sites close to the discharge could accelerate the rate of embryonic development. Discharge sites were predicted based on temperature data to advance hatch by up to 7 weeks in year 3, where the average difference was approximately 3°C (Table 2.2). However, actual differences in hatching times will likely be less due to the triggering of hatching from seasonal warming from spring temperatures. Griffiths (1979) also predicted a hatch difference of 7 weeks in lake whitefish resulting from a 3°C temperature rise, though the actual recorded hatch difference was reduced to 3 weeks due to spring warming. The magnitude of temperature fluctuations was greater at the discharge sites likely reflecting the influence of the thermal plume. As water currents cause the plume to shift or as the effluent temperature changes due to plant operations, temperatures close to the discharge will fluctuate. This was especially evident in year 3, where D1 experienced an average daily range of almost 5°C even with full lake ice cover (Table 2.2). An increase in temperature fluctuations will influence embryonic development rates. Lake whitefish and other poikilotherms follow

an accelerating exponential growth relationship with temperature (Sharpe and DeMichele 1977). According to Jensen's inequality, an increase in temperature variation will result in growth rates that are higher than would be predicted based solely on average temperature (Ruel and Ayres 1999). Therefore development rate at the discharge sites would be accelerated both by a greater average temperature and by increased temperature fluctuation.

Temperature data were used to predict the percent time-to-hatch completed at retrieval using the incremental growth-at-temperature method (Alderdice and Velsen 1978). Previous growth rate calculations for lake whitefish have used the degree-day method (Price 1934). However, this method relies on the assumption of linear scaling of growth rate with temperature. In year 2, embryos at the three discharge sites were predicted to be approximately 10% closer to hatch compared to the southern reference (Table 2.3). To accurately predict development rate, laboratory temperature data were used from the time of fertilization until deployment, after which lake substrate temperature data were used. While in the lab, all embryos were raised at a constant 5°C. If embryos had been incubating in the lake from the time of fertilization, assuming lake temperature at each site followed the same trend, differences in development rate would likely be greater than predicted here.

The advanced development predicted from the in-situ thermal data was supported by morphometric measurements obtained in year 2. Embryos retrieved from site R1, where temperatures were consistently 0.5 – 1.5°C colder, were smaller in body length with a

larger yolk (Figure 2.5). All retrieved embryos were in the late stages of development, at which point all major developmental features had already formed and embryos were only increasing in size (Sreetharan et al. 2015). Therefore embryos were aged based on quantitative size measurements instead of morphological features. Comparing measurements to laboratory raised embryos, the percent time-to-hatch matched closely to what was predicted based on thermal data, with sites D1a and D1b up to 8% advanced (Table 2.3). Comparing percent time-to-hatch based on body length relies on the assumption that embryos are the same size at hatch regardless of incubation temperature. Lake whitefish are generally larger at hatch when raised at colder temperatures; however, within the temperature range of the 4 retrieved sites (between 1.5 and 2.5°C), a minimal difference of only 0.1-0.2 mm at hatch would be expected (Brooke 1975) and thus, size can be used to predict hatching. Overall, morphometric measurements predict that embryos at the discharge sites would hatch earlier compared to reference sites.

An advanced development rate and subsequent earlier hatch could impact larval survival. Post hatch, larval lake whitefish fish remain in shallow nearshore water for several months before migrating to deeper water later in the summer (Hart 1931). During that time they switch to exogenous feeding on zooplankton, including copepods, cladoceran as well as macroinvertebrates such as chironomidae (Johnson et al. 2009). Growth and survival of larval fish has been positively correlated to zooplankton density (Freeburg et al. 1990, Brown and Taylor 1992, Hoyle et al. 2011). Earlier hatching could result in trophic mismatch through a switch to exogenous feeding at a time when prey abundance is low. On the other hand, fish hatching earlier tend to have a larger yolk, meaning they

could potentially survive longer before requiring exogenous food sources. In addition, prey abundance could also be advanced by the thermal plume consequently making food more abundant to larvae advanced in hatch timing. Further work is needed to identify temporal patterns in prey availability during the spring hatching period.

Due to ice cover in year 3, live lake whitefish were only retrieved from D2, all of which had already hatched. Based on temperature at that site embryos were predicted to be 24 days post hatch. Brown and Taylor (1992) measured the rate of endogenous larval growth post hatch in Lake Whitefish before the switch to exogenous feeding. They found an average body length at hatch of 11.05 mm for live fish, and a larval growth rate of 0.01, measured as the change in natural log of body length over time. Based on the average size of our retrieved embryos of 13.86 mm, this growth rate model predicts that the embryos were retrieved 23 days post hatch. In both year 2 and 3, percent development calculations based on temperature data match closely to what was predicted based on embryo morphometric measurements (Table 2.3), highlighting the accuracy of the incremental growth-at-temperature methods for predicting development rate in fluctuating temperatures.

The deployment depth of 5 - 10 m was chosen as it falls within the natural range for lake whitefish spawning (Hart 1931) while still being sufficiently deep to avoid ice scour. Across the three years a total of 12 chain systems were deployed, of which all but one (R2 in year 3) were successfully recovered. Considering the systems were deployed within a high energy environment in Lake Huron for more than three months during the winter, a

retrieval rate of greater than 90% was remarkably high. Thermal monitoring was conducted at similar locations in front of Bruce Power during the winter of 2004-2005. In total, 32 temperature loggers were deployed attached to 1,100 kg concrete blocks, of which only 16 were successfully recovered (Bruce Power 2005). In year 2, despite retrieving all the systems, several of the individual chambers were damaged. Out of the 30 chambers deployed, 9 were retrieved with the outer plastic grid and mesh missing. This was likely caused by failure of the hardware holding the three chamber pieces together. The chambers were designed using nylon hardware as it had previously been shown to withstand the cold lake temperature better compared to stainless steel (Manny et al. 1989). In year 3, cable ties were added to each chamber for additional support. In that year, at each of the three retrieved sites, all 7 of the chambers were recovered fully intact. The challenge however was timing retrieval of the systems in order to recover live embryos due to ice cover and weather conditions each spring.

Overall, we present here an analysis of different temperature environments on lake whitefish embryonic development in-situ. Embryos were successfully incubated throughout the winter in Lake Huron using custom built incubation chambers. Chambers deployed in the vicinity of Bruce Power showed elevated and more variable temperatures compared to the reference locations, likely resulting from the thermal discharges. These temperature increases were below thresholds for embryonic mortality or developmental abnormalities but may result in accelerated development leading to an advanced hatch. This was supported through growth rate modeling of temperature data as well as

morphometric staging of in-situ incubated embryos. Further work is needed to determine if this potential hatch advance could result in any impact on larval growth and survival.

2.6 Acknowledgements

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Chapter 3

Developmental effects of morpholine and sodium hypochlorite, two industrial cooling water chemicals, on lake whitefish (*Coregoneus clupeaformis*)

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3.1 Abstract

Chemicals used in the prevention of corrosion and biofouling may be released into the environment via industrial cooling water discharges. We assessed the effect of two commonly used chemicals, morpholine and sodium hypochlorite, on embryonic development in lake whitefish (*Coregonus clupeaformis*). Whitefish embryos were exposed chronically, beginning at fertilization or at the eyed stage, and continuing until hatch. Chronic morpholine exposure induced mortality, with an $LC_{50/hatch}$ of 219 ± 54 mg/L when exposure began at fertilization and increased to 674 ± 12 mg/L when exposure began at the eyed stage, suggesting that embryos are more sensitive to morpholine prior to the eyed stage. Chronic morpholine exposure advanced hatching by up to 30% and the early hatching embryos were up to 10% smaller in body length at hatch. A decrease in yolk conversion efficiency was observed in embryos exposed to concentrations of 1000 mg/L morpholine. Acute 96 hour exposures were examined at four development stages with minimal effects up to and including the highest tested concentration (1000 mg/L). pH controls showed no significant effect on survival or development rate, but did alter size at hatch, suggesting that observed morpholine effects are not solely pH driven. Most of the effects from morpholine exposure manifested close to hatch, possibly reflecting changes in chorion permeability near the end of embryonic development. Sodium hypochlorite affected survival only in chronic exposure from fertilization, with a total residual chlorine $LC_{50/hatch}$ of 0.097 ± 0.007 mg/L.

3.2 Introduction

Cooling water discharges from industrial processes represent a potential environmental risk to aquatic biota. Many thermal power plants rely on once-through cooling systems, which use natural bodies of water to remove waste heat. In addition to the potential for thermal pollution, these effluents may also contain chemicals added to steam and cooling water systems to reduce biofouling and corrosion. The biological effects from chemical releases can be of particular concern during more sensitive early life stages. Fish embryos developing near cooling water discharge points have the potential to be exposed over the course of embryogenesis. It is therefore important to understand how chemical exposure can affect survival and development in species that spawn near industrial discharge sites.

Morpholine is a heterocyclic amine that is added to steam systems to prevent corrosion by neutralizing dissolved carbon dioxide and other acidic contaminants. In mammals, morpholine has been shown to act as a respiratory tract irritant when ingested or inhaled (Conaway et al. 1984, Harrison et al. 1988). The effects of morpholine on fish have only been examined in terms of survival following acute exposures, generally as part of a broader toxicity study looking at a suite of compounds. In mature adult fish, 96 hour LC_{50} values range from 200-400 mg/L depending on the species (Dawson et al. 1975-76, Brandão et al. 1992). Less is known about the effects of chronic exposures to morpholine, the impacts on developmental stages, or the biological effects apart from mortality such as development rate and growth.

Sodium hypochlorite is widely used in cooling water systems as an anti-fouling agent. The majority of biocidal action is a result of hypochlorous acid, formed when sodium hypochlorite dissociates in water (Fukuzaki 2006). Chlorination is normally measured as a concentration of total residual chlorine (TRC); the sum of free chlorine (hypochlorous acid, chlorine and hypochlorite ion) and combined chlorine (chloramines). Chlorine induced mortality in adult fish is thought to result from hypoxia caused by damage to gill structure (Bass et al. 1977). Less is known about the effects on developing embryos. Toxicity studies have identified a 10 fold range in LC_{50} values from 0.04 to 0.4 mg/L, suggesting a large species specific effect (Middaugh et al. 1977, Morgan and Prince 1977). There is evidence to suggest that embryos may be more resistant to chlorine exposure compared to free swimming fish due to protection from the chorion (Middaugh et al. 1978).

Lake whitefish (*Coregonus clupeaformis*) are a cold water coregonid native to the northern regions of North America including all the Great Lakes. They are an important commercial catch within the Great Lakes Basin (Ebener et al. 2008) and have a high ecological and cultural value. Mature fish spawn in late fall in shallow water depths normally less than 10 m (Hart 1931). Embryos develop throughout the winter and hatch the following spring as lake temperatures increase, generally coincident with ice break-up. Incubation times are temperature dependent, exceeding 180 days when raised at a constant 0.5°C (Brooke 1975). These slow development rates create the potential for prolonged exposure to environmental stressors and large accumulated doses.

The objective of this study was to determine the effects of morpholine and sodium hypochlorite on lake whitefish embryonic development. Embryos were subjected to acute exposures at four developmental time points and chronic exposures from fertilization or from the eyed stage to hatching. The effects of these compounds were evaluated based on survival, the duration of development, hatch dynamics and size-at-hatch.

3.3 Materials and methods

3.3.1 Embryo collection

Lake whitefish embryos were obtained through in-vitro fertilization over three consecutive years. Spawning adult fish were gillnetted in eastern Lake Huron (44°42’37.74”N, 81°18’38.94”W) on November 15, 2012, November 21, 2013 and November 29, 2014 (Ontario Ministry of Natural Resources scientific fishing permit to J.Y.W.). Eggs were stripped from multiple females, mixed with milt from multiple males, dry fertilized for 5 minutes and then wet fertilized with lake water for 5 minutes. Embryos were transferred to 1.5 L plastic jars containing a 50:50 ratio of embryos:lake water. Embryos were disinfected with Ovadine® (Syndel Laboratories Limited) for 30 minutes at a concentration of 5 ml Ovadine per 1 L lake water. Embryos were then washed twice and jars were filled with cold lake water before being transported back to the laboratory on ice.

3.3.2 Laboratory rearing

Embryos were raised in custom built incubation units housed within modified chromatography refrigerators (Mitz et al. 2014). Initially, embryos were housed in upwelling hatching jars supplied with recirculated, carbon and UV filtered municipal water, at a nominal temperature of 5°C (4.9 ± 0.2°C). Individual experiments were run in petri dishes (100 mm x 20 mm) filled with dechlorinated municipal water. Petri dishes were loaded with 50 ± 2 embryos per dish. Separate control dishes were run with each treatment group, matching the water change and temperature regimes of corresponding treated dishes.

Embryos were exposed chronically to laboratory grade morpholine (Sigma-Aldrich) or sodium hypochlorite (Sigma-Aldrich), beginning at fertilization or at the eyed stage, and continuing until hatch. At fertilization (Day 1), embryos were transferred from hatching jars to petri dishes containing morpholine (1, 10, 100, 500, 1000, 5000 mg/L) or sodium hypochlorite (Nominal concentration: 0.0001, 0.001, 0.01, 0.05, 0.1, 0.5 mg/L TRC, measured concentration: 0.0001, 0.0012, 0.013, 0.06, 0.12, 0.45 mg/L TRC). At the eyed stage (Day 30), a second set of embryos were transferred from hatching jars to petri dishes containing morpholine (10, 100, 500, 1000, 5000 mg/L) or sodium hypochlorite (0.001, 0.01, 0.05, 0.1, 0.5 mg/L TRC). Day 30 was chosen based on daily visual inspection of embryos to assess when they had reached the eyed stage (Sreetharan et al. 2015). The mean temperature in petri dishes during chronic exposures was 5.1 ± 0.3°C. Exposures from fertilization and from eyed stage were completed in triplicate or duplicate

dishes respectively, and the whole experiment was replicated over two years (n = 6 replicate dishes or 300 total embryos from fertilization, n = 4 replicate dishes or 200 total embryos from eyed stage). Due to higher natural mortality during the first month of development, dishes loaded from fertilization were completed in triplicate in order to improve statistical resolution. Water was changed once per week on all control and treated dishes.

To test the effect of elevated pH levels, embryos were chronically incubated in NaOH solutions from fertilization and from the eyed stage, continuing until hatch. Solutions of pH 8, 8.5, 9 and 10 were prepared from a 0.1 M NaOH stock solution (EMD) and verified using a digital pH meter (Mettle Toledo EL20). Embryos were transferred from hatching jars to petri dishes containing NaOH at fertilization (Day 1) and at the eyed stage (Day 30). Each concentration was performed in triplicate dishes over one field season (n = 150 embryos). Dishes were incubated at a matching water temperature to chronic morpholine (5.1 ± 0.3°C). Water was exchanged every three days on all control and treated dishes, which was more often than chronic morpholine trials, in order to maintain pH levels to those that were similar to the morpholine treatments.

For acute exposures, embryos were transferred from hatching jars to dishes at fertilization (Day 1), gastrulation (Day 7), closure of the blastopore (Day 15) and organogenesis(Day 30, Sreetharan et al. 2015) and incubated in water containing morpholine (1, 10, 100, 500, 1000 mg/L) or sodium hypochlorite (0.0001, 0.001, 0.01, 0.05, 0.1 mg/L TRC) for 96 hours. After 96 hours, the treatment water was removed and embryos were incubated in

normal dechlorinated water for the remainder of development. Water was changed once per week on all control and treated dishes. Acute exposures were performed at 2.9 ± 0.2°C. Each concentration was run in duplicate over one field season (n = 100 embryos). Different temperatures were used for the chronic and acute trials in order to stagger hatching and mortality and avoid simultaneous hatching of excessively large numbers of embryos. Both 3°C and 5°C are within the optimal range for lake whitefish development and natural mortality rates are similar at both temperatures (Price 1940, Brooke 1975).

3.3.3 Embryo endpoints

Dishes from all treatment groups were checked daily and dead or hatched embryos were removed and recorded. Daily mortality in each dish was used to generate survival curves, and calculate the cumulative percent mortality at hatch and the time to 50% mortality. Hatching in each dish was assessed based on the time to median hatch and the hatch duration (time between the first and last hatch).

Hatched fish were fixed in 10% neutral buffered formalin for 1 week and then transferred to 50% ethanol. All hatchlings were imaged and measured using a Zeiss AXIO Zoom V16 microscope equipped with a CANON SL6 digital camera. A dorsal image was used to measure total body length and a lateral image was used to measure yolk diameter. A subset of embryos (20 per concentration randomly selected amongst dishes) was also fixed from the chronic morpholine exposures on day 80 post fertilization (pre-hatch stage, Sreetharan et al. 2015). Fixed embryos were dechorionated, following which body length and yolk diameter was measured as described for hatchlings.

Following imaging, the embryo or hatchling body was dissected from the yolk and specimens were dried separately in a 70°C oven for 24 hours. Yolk-free body and yolk dry weights were measured the following day (± 0.01 mg, Mettler-Toledo XA105DU). A subset of 20 control embryos was fixed on Day 1 post fertilization (dpf) and weighed. A yolk conversion efficiency (YCE) was calculated according to the equation:

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

3.3.4 Water chemistry

The pH was measured in morpholine and NaOH dishes using a benchtop pH meter (Mettler Toledo EL20). pH values were recorded up to 7 days post preparation for morpholine and 3 days post preparation for NaOH, corresponding to the longest period between water changes. pH was measured in 4 independent replicates.

TRC concentration in sodium hypochlorite dishes was measured using N,N diethyl-p-phenylene diamine powder (Hatch). Absorbance was measured at 525 nm using a spectrophotometer and compared to a standard curve.

3.3.5 Statistical analysis

Statistical analysis was conducted using Sigmaplot V11.0. LC_{50} values were calculated at hatch based on probit analysis with percent mortality corrected for control mortality using the Schneider-Orelli method (Schneider-Orelli 1947). The percent mortality, time to 50% mortality, time to 50% hatch and the hatch duration between replicate dishes were

compared using one-way ANOVA followed by Tukey's HSD test. Morphometric measurements were compared using a non-parametric Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test, because data was not normally distributed. Embryo weights and YCE were log transformed and compared using a one-way ANOVA followed by Tukey's HSD test.

3.4 Results

3.4.1 Water chemistry

Morpholine increased pH at all concentrations on day 0, reaching as high 10.48 ± 0.12 at 5000 mg/L (Table 3.1). The pH decreased in all dishes over the course of 7 days, including the dechlorinated control water, by as much as 1 pH unit. The pH in NaOH dishes decreased more rapidly than morpholine. The largest change corresponded to the highest concentration of NaOH where pH decreased from 9.96 ± 0.13 to 8.28 ± 0.38 over the course of 3 days (Table 3.1).

3.4.2 Chronic morpholine

In control dishes where embryos were incubated from fertilization, the majority of mortality (approximately 30%) occurred within the first month of development (Figure 3.1A). Mortality then remained low until close to hatch when it increased again. Likewise, in the controls dishes where the embryos were incubated from the eyed stage, mortality increased near hatch (Figure 3.1B).

Table 3.1 pH measurements in morpholine and NaOH treated water. pH was tested up to 7 days post-mix for morpholine and 3 days for NaOH, the longest period between water changes. Values represent the mean of four individual replicates ± SD.

	pH			
	Day 0	Day 1	Day 3	Day 7
Morpholine				
0 mg/L	7.59 ± 0.08	7.35 ± 0.19	7.38 ± 0.25	7.34 ± 0.28
10 mg/L	8.09 ± 0.45	7.87 ± 0.29	7.62 ± 0.43	7.57 ± 0.54
100 mg/L	9.20 ± 0.04	8.50 ± 0.16	8.16 ± 0.29	8.08 ± 0.33
1000 mg/L	9.78 ± 0.14	9.07 ± 0.05	8.84 ± 0.05	8.82 ± 0.06
5000 mg/L	10.48 ± 0.12	9.87 ± 0.08	9.76 ± 0.13	9.61 ± 0.28
NaOH				
pH 8.0	8.02 ± 0.23	7.88 ± 0.06	7.68 ± 0.24	-
pH 8.5	8.54 ± 0.37	8.34 ± 0.45	7.93 ± 0.41	-
pH 9.0	9.19 ± 0.03	8.87 ± 0.22	8.01 ± 0.12	-
pH 10.0	9.96 ± 0.13	9.29 ± 0.15	8.28 ± 0.38	-

Embryos chronically incubated in morpholine from fertilization or from the eyed stage had a significant increase in mortality at concentrations of 1000 and 5000 mg/L (Figure 3.1, Fertilization: $F(3,32) = 8.77$, $p < 0.001$, Eyed: $F(5,18) = 123.95$, $p < 0.001$). Embryos were more sensitive from fertilization where both of these concentrations resulted in complete mortality. From the eyed stage however, approximately 25% of embryos survived at the 1000 mg/L concentration. A non-significant increase in mortality was also seen at the 500 mg/L concentration incubated from fertilization, which was not observed when incubated from the eyed stage. The majority of mortality occurred while embryos were still within the chorion. A small percentage (less than 4% within each dish) were free from the chorion when mortality was observed. It could not be determined whether mortality occurred post-hatch or whether the chorion degraded after embryo mortality. The $LC_{50/hatch}$ for exposure from fertilization was 219 ± 54 mg/L and from the eyed stage

674 ± 12 mg/L. Incubation in NaOH up to a pH of 10, from fertilization or from the eyed stage, had no significant impacts on embryonic survival (Supplemental Table S3.1)

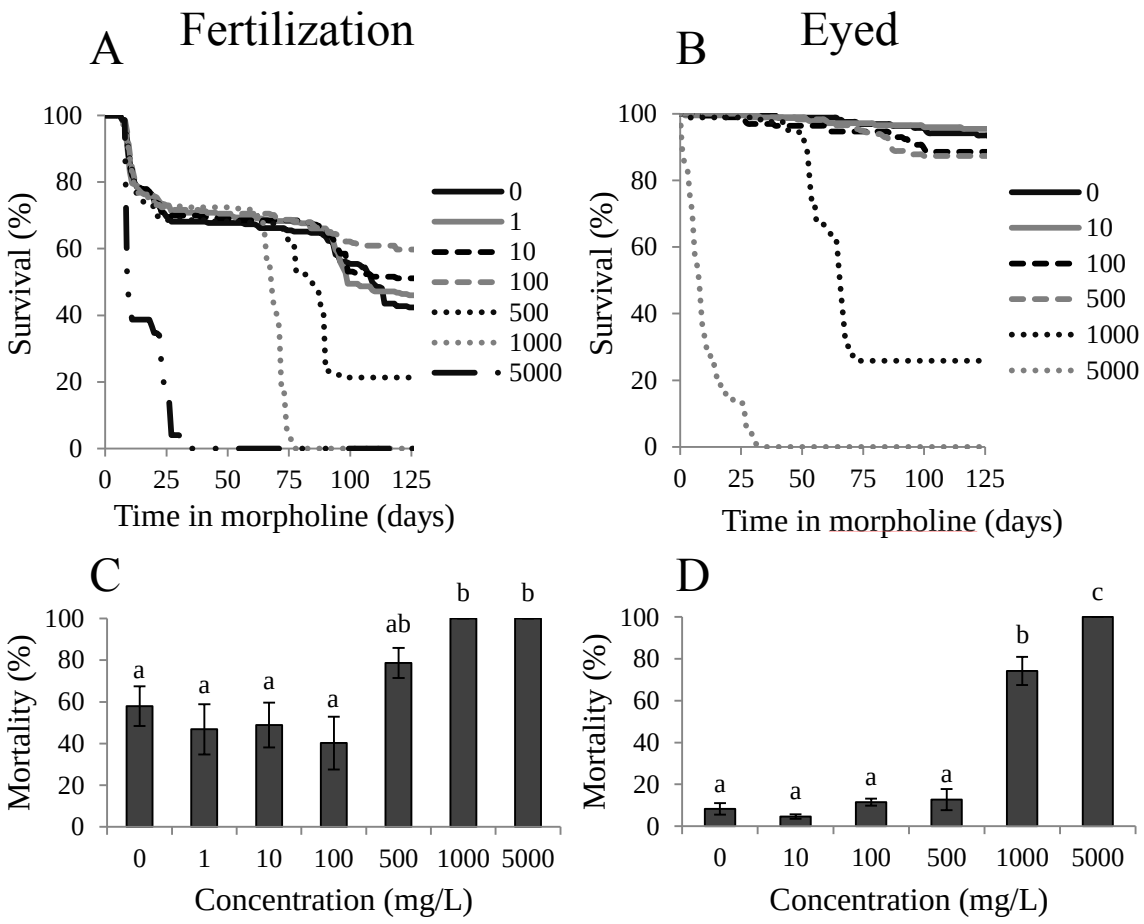

Figure 3.1. Embryonic survival following chronic morpholine exposure. Percent survival was measured as a function of days in morpholine from fertilization (A) and from the eyed stage (B). The total percent mortality was calculated at hatch (C,D). Percent mortality within each replicate dish was compared using a one-way ANOVA with Tukey's HSD test. Letters denote statistical differences. Bars represent means ± SE.

Morpholine induced mortality did not occur gradually over the course of exposure, but rather occurred in shorter windows at certain points during development (Figure 3.1A,B). At 5000 mg/L, mortality occurred soon after exposure began, within the first 10 days (Table 3.2). At 500 and 1000 mg/L there was a latency period between the beginning of exposure and mortality of 61 days and 86 - 88 days respectively (Table 3.2). The timing of mortality was normalized to controls by converting the day post fertilization to a percent of total time-to-hatch in control dishes (101 days to hatch in control dishes for exposure from fertilization and 135 days to hatch for exposure from the eyed stage). At 500 mg/L mortality occurred between 84 - 85% of development in control dishes and at 1000 mg/L mortality occurred between 68-71% (Table 3.2).

Table 3.2. Mortality and hatch timing following chronic morpholine exposure from fertilization and from the eyed stage. The time to 50% mortality and hatch within each replicate dishes was recorded in days post fertilization and days in morpholine. The timing of mortality and hatch was normalized to controls by converting the day post fertilization to a percent of total time-to-hatch in control dishes. Values represent means ± SD.

	50% Mortality			50% Hatch		
Concentration (mg/L)	Time post fertilization (days)	Time in morpholine (days)	Percent of control time-to-hatch	Time post fertilization (days)	Time in morpholine (days)	Percent of control time-to-hatch
Fertilization						
500	86.0 ± 6.9	86.0 ± 6.9	85.1 ± 6.9	88.0 ± 2.9	88.0 ± 2.9	87.1 ± 2.9
1000	69.5 ± 2.6	69.5 ± 2.6	68.8 ± 2.6	-	-	-
5000	9.7 ± 1.2	9.7 ± 1.2	9.6 ± 1.1	-	-	-
Eyed						
500	116.0 ± 1.4	87.0 ± 1.4	84.1 ± 1.0	116.3 ± 6.0	86.2 ± 5.9	86.1 ± 4.5
1000	95.5 ± 0.6	59.8 ± 7.8	70.8 ± 1.9	91.0 ± 1.4	61.0 ± 0.8	67.4 ± 1.1
5000	38.5 ± 0.6	8.3 ± 1.7	28.5 ± 0.8	-	-	-

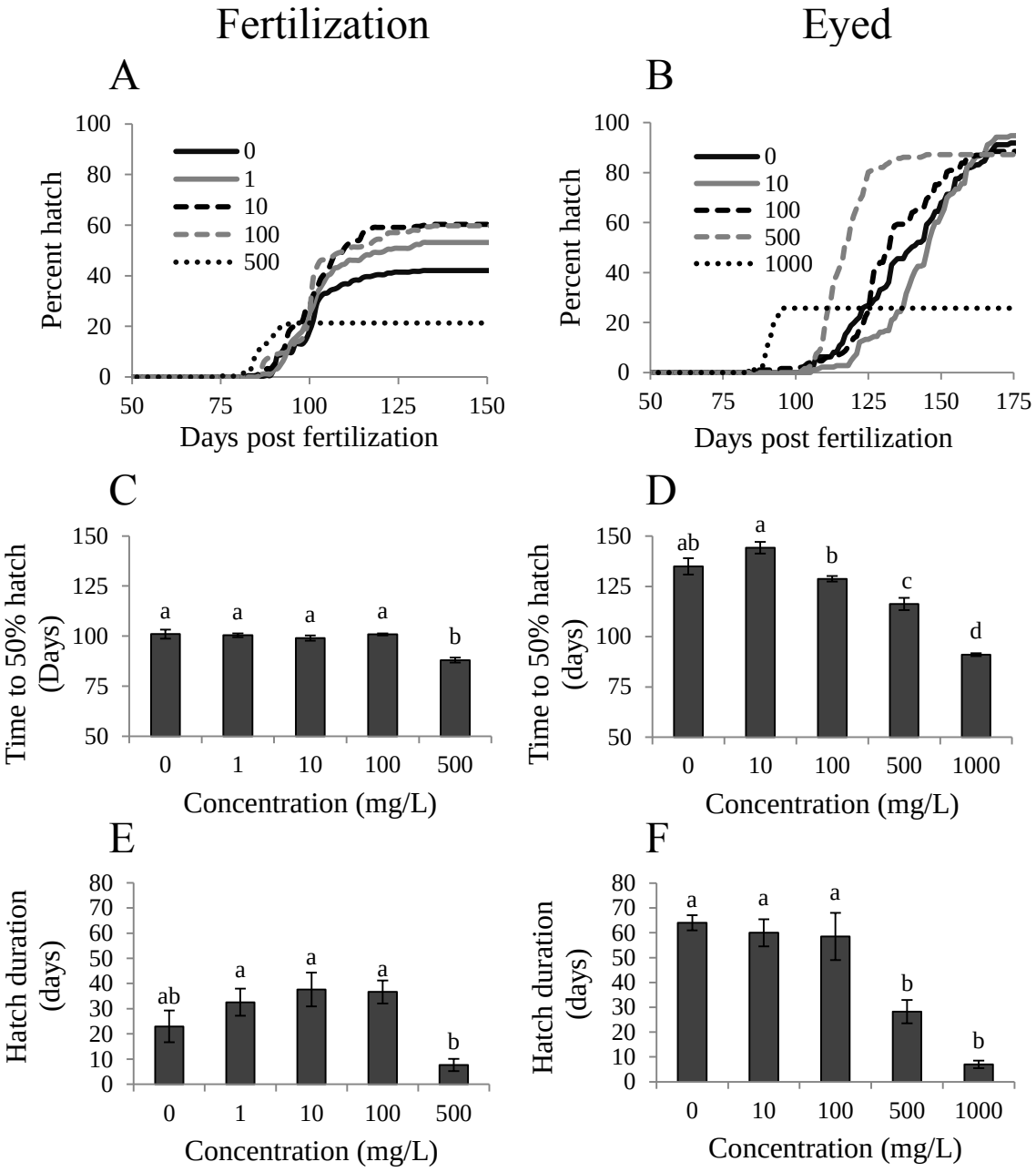

Figure 3.2. Hatch dynamics in embryos exposed to chronic morpholine. The cumulative percent hatch was measured as a function of days post fertilization following exposure from fertilization (A) and the eyed stage (B). Percent hatch was calculated by comparing the number of live hatches to the total number of embryos (live hatch or dead) within each dish. The time to median hatch (C,D) and the hatch duration, measured as the time between the first and last hatch (E,F), were calculated for each dish. Median hatch and hatch duration within each replicate dish were compared using a one-way ANOVA with Tukey's HSD test. Letters denote statistical differences. Bars represent means ± SE.

Control embryos incubated in dishes from fertilization had a shorter time to hatch (101 days vs 135 days) and shorter hatch duration (23 days vs 64 days) compared to embryos raised in dishes initiated at the eyed stage (Figure 3.2). Chronic morpholine exposure altered the timing and duration of hatching. Concentrations of 500 and 1000 mg/L resulted in a shorter time to 50% hatch compared to controls (Figure 3.2C,D, Fertilization: $F(4,22) = 13.88$, $p < 0.001$, Eyed: $F(4,15) = 58.66$, $p < 0.001$). At 500 mg/L, a similar percent reduction in the time-to-hatch was observed following exposure from fertilization and from the eyed stage (13% and 14% respectively, Table 3.2). When measured as a percent of control time-to-hatch, the advanced hatch coincided closely with the timing of morpholine induced mortality (Table 3.2). At the eyed stage, concentrations of 500 mg/L and 1000 mg/L also shortened the hatch duration by 56% and 89% respectively (Figure 3.2F, $F(4,15) = 20.26$, $p < 0.001$). Incubation in NaOH up to a pH of 10, from fertilization or from the eyed stage, had no significant impacts on hatch timing (Supplemental Table S3.1).

On day 80 post fertilization (pre-hatch stage), no significant difference was seen in body or yolk size for embryos exposed to morpholine from fertilization (Supplemental Table S3.2). With morpholine exposure from the eyed stage, on day 80 post fertilization, a reduction in yolk-free body weight was observed, but there were no differences in body length, yolk weigh or yolk diameter (Supplemental Table S3.3). Morphometric differences were observed at hatch. Chronic morpholine concentrations of 500 and 1000 mg/L resulted in hatchlings with significantly smaller body lengths (Figure 3.3, Fertilization: $H = 108.84$, $df = 4$, $p < 0.001$, Eyed: $H = 152.83$, $df = 4$, $p < 0.001$). Yolk

diameter did not differ from controls at these concentrations (Figure 3.3, Fertilization: H = 17.94, df = 4, p = 0.001, Eyed: H = 27.5, df = 4, p < 0.001). The exposure of embryos from fertilization to the lowest concentrations of 1 and 10 mg/L resulted in a significant increase in body length and a smaller yolk diameter (Figure 3.3A,C) compared to the other treatment groups and controls.

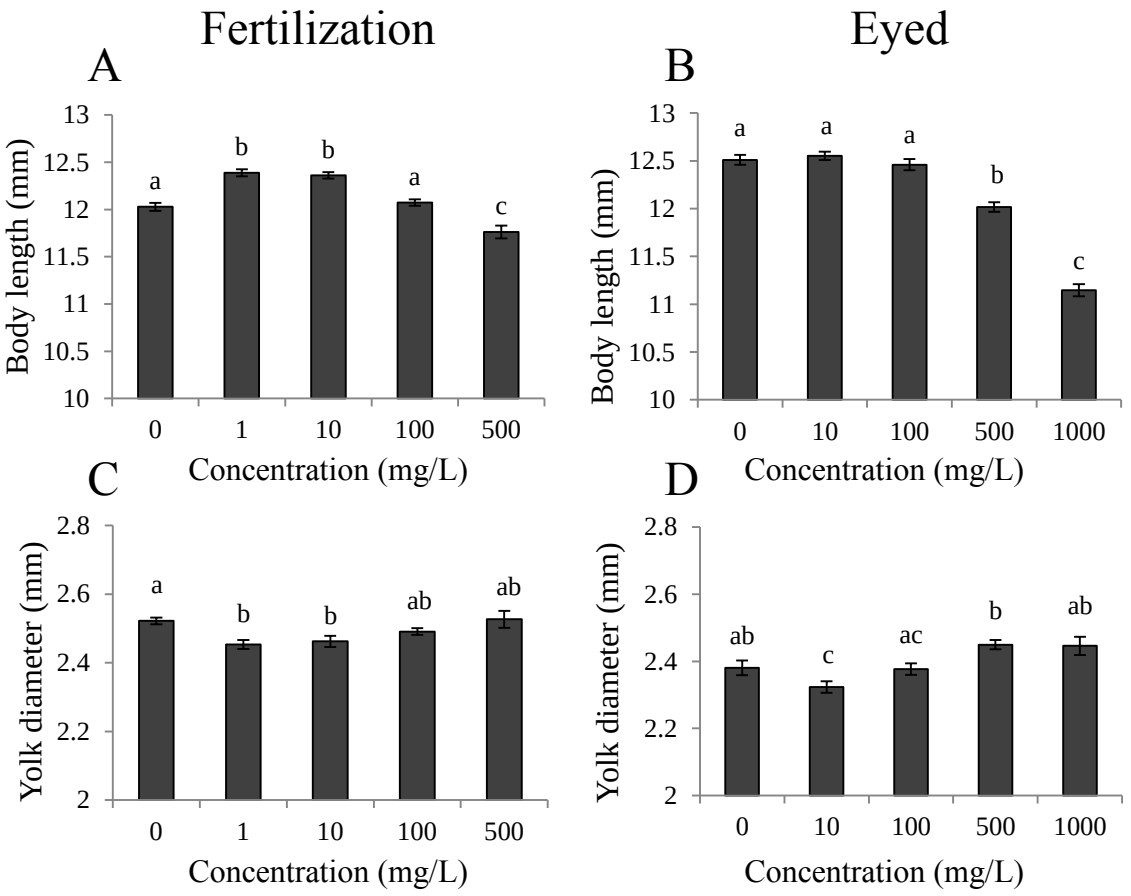

Figure 3.3. Morphometric measurements on preserved hatchlings following chronic morpholine exposure. Body length (A,B) and yolk diameter (C,D) were measured on embryos exposed from fertilization or the eyed stage. Measurements were compared using a Kruskal-Wallis one-way ANOVA on ranks with Dunn's test. Letters denote statistical differences. Bars represent means ± SE.

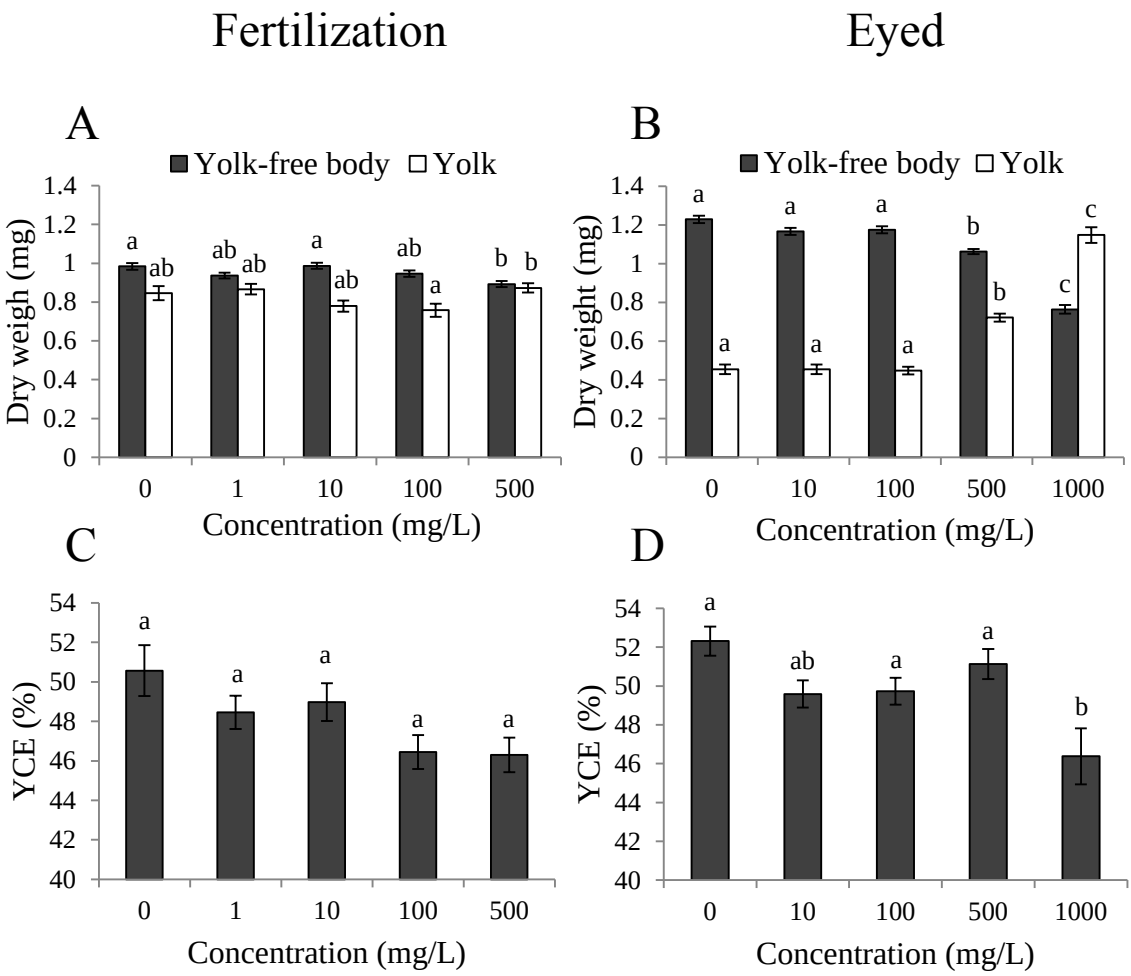

Figure 3.4. Dry weight measurements on preserved hatchlings. Yolk-free body and yolk weights were measured following chronic morpholine exposure from fertilization (A) and from the eyed stage (B). A yolk conversion efficiency (YCE) was calculated based on body and yolk weights (C,D). Dry weights and YCE were log transformed and compared using a one-way ANOVA with Tukey's HSD test. Letters denote statistical differences. Bars represent means ± SE.

Yolk-free body mass followed the same trend as body length and was significantly smaller at 500 and 1000 mg/L (Figure 3.4A,B, Fertilization: $F(4,197) = 5.77$, $p < 0.01$, Eyed: $F(4,296) = 84.18$, $p < 0.001$). Yolk dry weight was larger in embryos exposed from the eyed stage at 500 mg/L and 1000 mg/L (Figure 3.4B, $F(4,296) = 42.69$, $p < 0.001$),

but was not significantly different from controls in embryos exposed from fertilization (Figure 3.4A, $F(4,197) = 3.55$, $p = 0.008$). A reduction in YCE was also seen following exposure to 1000 mg/L from the eyed stage (Figure 3.4D, $F(4,296) = 5.90$, $p < 0.001$). A small number of developmental abnormalities (less than 5%) was observed in all control and treated dishes.

Incubation in NaOH impacted size at hatch. Following exposure from fertilization, body length at hatch at pH 8 and 8.5 was significantly greater than controls while at a pH of 10 body length was smaller (Figure 3.5A, $H = 104.12$, $df = 4$, $p < 0.001$). Following exposure from the eyed stage, all NaOH treatments increased body size at hatch (Figure 3.5B, $H = 43.99$, $df = 4$, $p < 0.001$). Yolk diameter was consistently smaller in NaOH exposure from fertilization and from the eyed stage with elevated pH (Figure 3.5C,D, Fertilization: $H = 13.44$, $df = 4$, $p = 0.009$, Eyed: $H = 29.62$, $df = 4$, $p < 0.001$).

3.4.3 Chronic sodium hypochlorite

Chronic hypochlorite exposure from fertilization resulted in a significant increase in mortality only at concentrations of 0.5 mg/L of TRC (Figure 3.6, $F(5,13) = 6.97$, $p = 0.002$). The $LC_{50/hatch}$ for exposure from fertilization was 0.097 ± 0.007 mg/L. Mortality occurred gradually throughout development in contrast to morpholine exposure. Embryos exposed at the eyed stage were more resistant, showing no significant increase in mortality up to the highest concentration of 0.5 mg/L (Supplemental Table S3.4). Exposure from fertilization or the eyed stage did not affect hatch timing or morphometric measurements (Supplemental Table S3.4, S3.5).

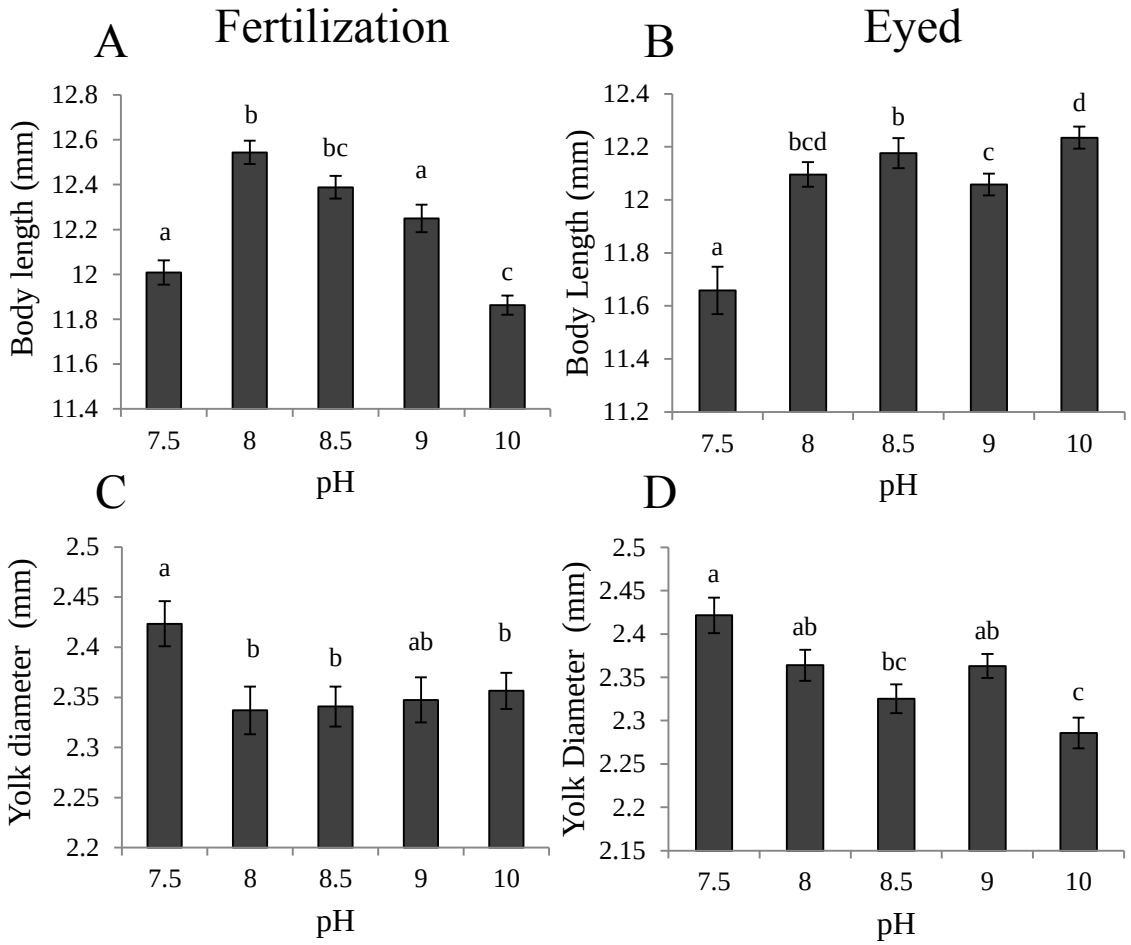

Figure 3.5. Morphometric measurements on hatchlings following chronic incubation in NaOH. Body length (A,B) and yolk diameter (C,D) were measured on fish exposed chronically to elevated pH from fertilization or from the eyed stage. Dechlorinated water (pH=7.5) was used as a control. Measurements were compared using a Kruskal-Wallis one-way ANOVA on ranks with Dunn's test. Letters denote statistical differences. Bars represent means ± SE

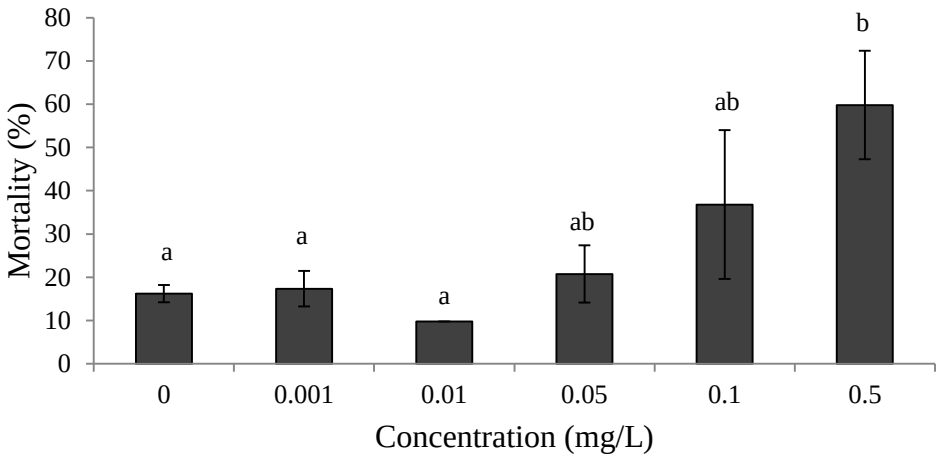

Figure 3.6. Cumulative percent mortality at hatch following sodium hypochlorite exposure from fertilization. Sodium hypochlorite was measured as a concentration of total residual chlorine. Percent mortality within each dish was compared using a Kruskal-Wallis one-way ANOVA on ranks with Dunn's test. Letters denote statistical differences. Bars represent means ± SE.

3.4.4 Acute exposure

Acute exposures (96 hours) had minimal effect on development. Acute morpholine exposures of up to 1000 mg/L beginning on day 1, 7, 15 and 30 had no significant impact on survival or hatch dynamics (Supplemental Table S3.6, S3.7). Similar to the chronic exposures, an acute exposure of 500 mg/L or 1000 mg/L beginning on day 1 resulted in a decrease in body length at hatch (Figure 3.7, $H = 69.39$, $df = 6$, $p < 0.01$) and an increase in yolk diameter ($H = 76.85$, $df = 6$, $p < 0.01$). However, no change in morphometric measurements was seen with exposure to these doses at any later time point (Supplemental Table S3.8, S3.9). Sodium hypochlorite exposure up to 0.1 mg/L had no significant effect on survival, hatch dynamics or morphometric measurements with acute exposures (Supplemental Table S3.10 - S3.13).

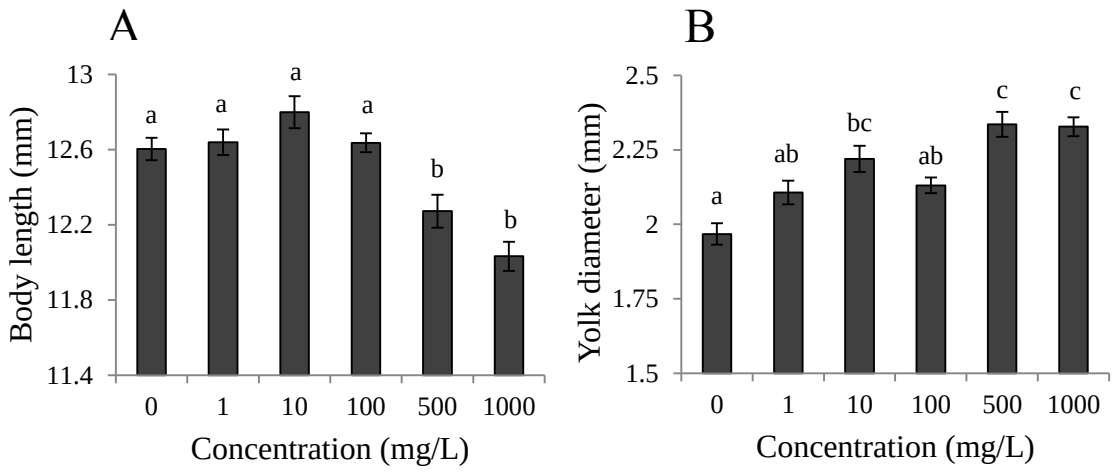

Figure 3.7. Morphometric measurements on hatchling body length (A) and yolk diameter (B) following acute (96 hour) morpholine exposure on day 1. Measurements were compared using a Kruskal-Wallis one-way ANOVA on ranks with Dunn's test. Letters denote statistical differences. Bars represent means ± SE.

3.5 Discussion

We have described for the first time the biological effects of morpholine, an industrial cooling water additive, on embryonic development in fish. Lake whitefish embryos were found to be resistant to morpholine exposure. Survival to hatch was not impacted by acute exposures. Mortality did occur following chronic exposure, but only at concentrations of 500 mg/L or above. Chronic exposures influenced the timing of hatch and hatchling size, with exposed embryos hatching earlier and smaller compared to controls. Embryos were minimally affected by sodium hypochlorite and elevated pH associated with NaOH.

The majority of natural mortality occurred within the first month of development. Once embryos reached the eyed stage mortality remained minimal until close to hatch, which is typical in lake whitefish (Price 1940, Brooke 1975, Mueller et al. 2015, Lee et al.,

McMaster University, submitted manuscript). A difference in hatch timing was observed in control dishes from chronic morpholine trials. Embryos incubated in petri dishes from fertilization had a time to median hatch of approximately 100 days (Figure 3.2A), consistent with what has been shown by others following incubation at a constant 5°C (Brooke 1975, Mueller et al. 2015, Sreetharan et al. 2015). Embryos incubated from the eyed stage, however, had a time to median hatch of 135 days (Figure 3.2B). These embryos were incubated in upwelling hatching jars until the eyed stage when they were then transferred to dishes. Embryos were transferred at this point, after the majority of natural mortality, in order to generate a larger sample size on which to examine the effects of morpholine. Water temperature in dishes and jars was closely monitored and remained at 5°C so temperature did not contribute to a longer development period. Embryos incubated in jars typically hatch earlier compared to petri dishes (Mitz, McMaster University, unpublished data), however embryos which were exposed to morpholine beginning at the eyed stage were in hatching jars for longer, so differences in rearing apparatus likely did not contribute to a longer development period. The stress of transferring embryos from the hatching jar, in which they were continuously moving, to static water dishes could have induced a temporary delay in development. Transferring embryos from continual movement to static water has been shown to influence development by prematurely triggering hatch (Yamamoto et al. 1979), the opposite of what was observed in this study. However, Yamamoto et al. (1979) transferred embryos close to the natural hatching window and the physiological response to the stress of moving may manifest differently earlier in development.

Exposure to morpholine in fish has previously only been examined through its impacts on survival. Acute 96 hour LC_{50} values have been reported as 350 mg/L in bluegill (*Lepomis macrochirus,* Dawson et al. 1975-76), 400 mg/L in tidewater silversides (*Menidia peninsulae,* Dawson et al. 1975-76) and 262 mg/L in golden orfe (*Leuciscus idus,* Brandão et al. 1992). Toxicity studies have all focused on the effects in adult fish and we found no published data for embryonic exposures. Lake whitefish embryos in this study were exposed acutely for 96 hours on day 1, 7, 15, and 30 post fertilization. These time points were selected as they pertain to the major developmental landmarks; cleavage, gastrulation, closure of the blastopore and organogenesis (Sreetharan et al. 2015). At each of these time points embryos showed high tolerance to acute exposures compared to adult fish of other species, with no excess mortality up to the highest tested concentration of 1000 mg/L.

Embryonic mortality did occur following chronic exposure. The highest dose of 5000 mg/L produced almost immediate and total mortality. Embryos exposed to 500 and 1000 mg/L were able to survive for the majority of development and excess mortality occurred close to hatch. An increase in mortality close to hatch can occur naturally (Price 1940, Brooke 1975) and a similar increase was seen in control dishes in this study (Figure 3.1A). The hatching and pre-hatching stages are known to be especially sensitive to chemical stress (Cleveland et al. 1986, Kazlauskiene and Stasiunaite 1999). During hatching, enzymes are released which break down the inner chorion membrane (Yamamoto et al. 1979). Chorion softening could result in increased permeability to morpholine, which would explain why embryos were resistant to acute but not chronic

exposures and why mortality in chronic exposures only occurred close to hatch, with the exception of the 5000 mg/L treatment. Further work is required to look at chorion permeability to morpholine and possible changes throughout development.

Morpholine concentrations of 500 and 1000 mg/L resulted in a shorter incubation period and smaller fish at hatch. Changes in hatch timing and size have been observed following incubation in various other chemicals and heavy metals (Rombough and Garside 1982, Woodworth and Pascoe 1982, Klein-MacPhee et al. 1984, Somasundaram et al. 1984, Scudder et al. 1988). Advanced hatch could be the result of premature triggering of hatching. It could also be due to changes in embryo growth or development rate. Embryos which were fixed on Day 80 showed no significant difference in body or yolk size, suggesting that morpholine did not increase growth rate and that earlier hatching was the result of premature hatch triggering. The absence of an increase in developmental abnormalities also supports that notion that morpholine is mainly impacting embryos at hatch and not continuously throughout development.

An advanced hatch could be caused by the premature release of hatching enzymes from the embryo in response to chemical exposure. A variety of different stressors including heat (Brooke 1975), mechanical agitation (Griem and Martin 2000) and hypoxia (Petranka et al. 1982) have been reported to trigger hatch through the release of hatching enzymes. Alternatively, early hatching could result from the physical break down of the chorion by morpholine. Alkaline solutions have been shown to degrade the inner chorion membrane (Nakano 1956). Weakening of the chorion by morpholine would enable

embryos to break free during normal body movement. Morpholine exposure also reduced the hatch window. Lake whitefish naturally have a large hatching window, particularly at colder temperatures, but hatching would be expected to be more synchronized when prematurely triggered (Brooke 1975). Collectively, this suggests that morpholine exposed embryos were prematurely hatching but the exact mechanism (e.g. stress induced, chemical effects on chorion strength) remains unclear.

The majority of morpholine exposed embryos were smaller at hatch and had a correspondingly larger yolk, indicative of earlier hatching embryos (Brooke 1975). The highest morpholine dose with embryos surviving to hatch, 1000 mg/L, resulted in a decrease in YCE. YCE is a common metric used to examine temperature effects in ectotherms (Lasker 1962, Heming 1982, Mueller et al. 2015). A decrease in YCE has been observed in Atlantic salmon (*Salmo salar*) following cadmium exposure (Peterson 1983). A decrease in YCE following morpholine exposure may be the result of energy being diverted away from growth towards repair and maintenance. Alternatively, it could be the result of changes in hatch timing; the efficiency of converting lipid and protein stores into body mass is not constant throughout development due to increased allocation of energy to maintenance with larger body size, and embryos could be hatching at a time where YCE is naturally lower.

The only significant effect from acute exposure to morpholine occurred following a 96 hour exposure beginning on day 1, where embryos were smaller with larger yolks compared to controls. No difference was seen in the time to hatch indicating that

differences in embryo size are not due to embryos hatching earlier. Size heterogeneity could be attributed to changes in sensitivity throughout development. Embryos are known to be sensitive to stressors close to fertilization, in addition to sensitivity close to hatch (Morgan and Price 1977, Nebeker et al. 1985). As with the chronic exposures, this could also be the result of changes to the chorion structure. In whitefish eggs, the chorion is not fully hardened until up to 2 days post fertilization (Zotin 1958), and may therefore be more permeable to morpholine on the first few days post fertilization, compared to the other three acute time points.

Morpholine is added to steam and cooling water systems to reduce corrosion through increasing pH levels. Incubating embryos in basic conditions using NaOH did not impact survival, suggesting that the effects seen with chronic morpholine exposure are the result of the morpholine compound itself instead of pH. However, pH levels in NaOH treatments fluctuated more than those in morpholine water (Table 3.1). The water in NaOH dishes was changed more frequently (3 days compared to 7 days) to minimize these effects. NaOH incubated embryos were larger at hatch, with the exception of those exposed to a pH 10 from fertilization which were smaller. Hatch timing was not significantly altered, indicating that size differences are due to NaOH stimulating or suppressing embryo growth. Basic pH has been reported to impact survival and hatch dynamics, but not embryo size or growth rate. Gao et al. (2011) observed mortality at a pH of 11 and 12 in Far Eastern catfish (*Silurus asotus*). Additionally, an advanced hatch and shorter hatch duration occurred at pH 8, 9 and 10, similar to what we observed in lake whitefish incubated in elevated morpholine concentrations. In brook trout (*Salvelinus*

fontinalis), a pH between 8 and 9 resulted in increased mortality, with newly fertilized embryos more sensitive compared to those at the eyed stage (Krishna 1953). Conversely, no change in survival or embryo size occurred in common carp (*Cyprinus carpio*) embryos at a pH of 9 (Ghillebaert et al. 1995).

The only significant biological effect observed from sodium hypochlorite exposure in Lake Whitefish was mortality, which occurred following chronic incubation from fertilization at the highest concentrations tested. The observed LC_{50} for lake whitefish embryos was similar to what has previously been reported in striped bass (*Morone saxatilis*), where no embryos survived at a concentration of 0.21 mg/L and 96 hour LC_{50} values ranged from 0.04 - 0.07 mg/L (Middaugh 1977). Morgan and Price (1977) observed higher 96 hour LC_{50} values on eggs from 5 marine species of between 0.2 and 0.4 mg/L, with increasing resistance throughout development and slightly smaller fish at hatch. Both of these studies however report on 96 hour LC_{50} values compared to $LC_{50/hatch}$ reported here for lake whitefish. Mortality in lake whitefish from chronic exposure occurred gradually throughout development and no mortality was observed after 96 hours up to the highest concentration of 0.5 mg/L. LC_{50} values in other species would likely be lower if mortality was examined up to the hatching stage. Mortality in adult fish is thought to be caused by hypoxia resulting from gill damage and reduced oxygen carrying capacity (Brungs 1973). Developing embryos have been shown to be more resistant to chlorine compared to adult fish, likely due to protection from the chorion (Middaugh et al. 1978).

The observed lethal levels in lake whitefish following exposure to morpholine and sodium hypochlorite are above environmental concentrations. Industrial discharge limits are on the order of 10 mg/L for morpholine and 0.01 mg/L for TRC from sodium hypochlorite (Bruce Power 2005, US FDA 2014). No sublethal effects were seen at these levels for sodium hypochlorite. Chronic incubation in morpholine at 1 and 10 mg/L however did have a slight impact on size at hatch, where embryos were larger in body length compared to controls with a smaller yolk diameter. Body size differences at hatch could have an effect, positive or negative, on larval survival by altering their ability to forage for food or avoid predation. Yolk size differences will alter the length of time larval fish can survive before switching to exogenous feeding.

Overall, chronic morpholine exposure was found to affect survival, hatch timing, hatchling size and metabolic efficiency in lake whitefish. This study represents the first time that the effects of morpholine, a chemical added to industrial cooling water systems, has been examined on embryonic development in fish. The majority of observed effects occurred in the late stages of development close to hatch. This is likely the result of structural changes in the chorion, which protects the embryo from toxic effects during earlier developmental stages. Comparable results to morpholine exposure were not seen in embryos incubated in NaOH, suggesting that the impacts of morpholine are not pH driven. Lake whitefish embryos were also found to be resistant to sodium hypochlorite, with minimal mortality following chronic exposures and no impacts following acute exposures.

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3.8 Supplemental tables

Table S3.1. Percent mortality and hatch timing following chronic NaOH exposure from fertilization and from the eyed stage. Percent mortality and hatch timing within each dish was compared using a one-way ANOVA with Tukey's HSD test. Letters denote statistical differences (Fertilization mortality: p = 0.845, Eyed mortality: p = 0.89, Fertilization hatch: p = 0.778, Eyed hatch: p = 0.452). Values represent means ± SE.

	Percent mortality		Median hatch (days)	
pH	Fertilization	Eyed	Fertilization	Eyed
7.5	32.45±13.76	59.72±10.56	101±1	100±1
8	39.08±16.93	43.05±20.55	101±4	97±5
8.5	30.62±25.46	39.08±28.73	102±2	96±5
9	41.52±14.67	38.30±25.41	103±1	103±1
10	18.14±19.29	31.84±18.33	104±1	102±2

Table S3.2. Morphometric measurements and weights on fixed embryos (day 80 post fertilization) exposed chronically to morpholine from fertilization. Body length and yolk diameter were compared using a Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test. Dry weights and yolk conversion efficiency (YCE) were log transformed and compared using a one-way ANOVA with Tukey's HSD test. (Body length: p = 0.074, Yolk diameter: p = 0.652, Yolk-free body weight: p = 0.134, Yolk weight: = 0.593, YCE: p = 0.184). Values represent means ± SE.

Concentration (mg/L)	Body length (mm)	Yolk diameter (mm)	Yolk-free body dry weight (mg)	Yolk dry weight (mg)	Yolk conversion efficiency
0	10.68±0.10	1.89±0.05	0.94±0.03	1.09±0.05	55.25±1.91
1	10.36±0.09	1.94±0.04	0.86±0.02	1.11±0.03	50.48±1.20
10	10.67±0.11	1.90±0.04	0.91±0.02	1.04±0.03	51.55±1.34
100	10.50±0.13	1.96±0.03	0.90±0.02	1.13±0.04	54.10±1.62
500	10.55±0.12	1.92±0.03	0.92±0.02	1.14±0.06	56.62±3.12

Table S3.3. Morphometric measurements and weights on fixed embryos (day 80 post fertilization) exposed chronically to morpholine from the eyed stage. Body length and yolk diameter were compared using a Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test. Dry weights and yolk conversion efficiency (YCE) were log transformed and compared using a one-way ANOVA with Tukey's HSD test. Letters denote statistical differences (Body length: $p = 0.067$, Yolk diameter: $p = 0.013$, Yolk-free body weight: $p < 0.001$, Yolk weight: $p = 0.405$, YCE: $p = 0.073$). Values represent means ± SE.

Concentration (mg/L)	Body length (mm)	Yolk diameter (mm)	Yolk-free body dry weight (mg)	Yolk dry weight (mg)	Yolk conversion efficiency
0	10.22±0.17	1.94±0.04	0.85±0.02[a]	1.17±0.04	52.95±2.10
10	10.34±0.11	2.11±0.03	0.83±0.02[a]	1.22±0.03	52.74±1.70
100	10.04±0.11	1.98±0.05	0.74±0.02[bc]	1.26±0.06	49.25±2.56
500	10.16±0.14	1.99±0.04	0.80±0.02[ab]	1.16±0.03	49.06±1.77
1000	9.88±0.14	1.94±0.04	0.72±0.02[c]	1.24±0.03	45.83±1.63

Table S3.4. Percent mortality and hatch timing following chronic sodium hypochlorite exposure from fertilization and from the eyed stage. Sodium hypochlorite was measured as a concentration of TRC. Percent mortality and hatch timing within each dish was compared using a one-way ANOVA with Tukey's HSD test. Letters denote statistical differences (Fertilization mortality: $p = 0.02$, Eyed mortality: $p = 0.112$, Fertilization hatch: $p = 0.376$, Eyed hatch: $p = 0.187$). Values represent means ± SE.

Concentration (mg/L)	Percent mortality		Median hatch (days)	
	Fertilization	Eyed	Fertilization	Eyed
0	16.20±1.98[a]	3.70±1.07	106±5	138±1
0.0001	14.23±6.22[a]	-*	104±3	-*
0.001	17.33±4.10[a]	6.35±0.47	103±1	138±1
0.01	9.80±0.12[a]	1.04±1.04	101±1	143±2
0.05	20.73±6.63[ab]	3.39±1.26	102±1	139±7
0.1	36.80±17.20[ab]	2.00±0.04	100±1	143±1
0.5	59.82±12.51[b]	6.63±2.46	104±2	152±1

* concentrations not tested

Table S3.5. Morphometric measurements on hatchlings following chronic sodium hypochlorite exposure from fertilization and from the eyed stage. Sodium hypochlorite was measured as a concentration of TRC. Measurements were compared using a Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test. Letters denote statistical differences (Fertilization body length: $p = 0.215$, Eyed body length: $p = 0.01$, Fertilization yolk diameter: $p = 0.121$, Eyed yolk diameter: $p = 0.011$). Values represent means ± SE.

Concentration (mg/L)	Body length (mm)		Yolk diameter (mm)	
	Fertilization	Eyed	Fertilization	Eyed
0	12.13±0.05	12.23±0.05^{ab}	2.36±0.02	2.35±0.03^{ab}
0.0001	12.08±0.08	-*	2.30±0.04	-*
0.001	12.09±0.05	12.33±0.05^{ab}	2.39±0.02	2.31±0.03^{ab}
0.01	12.18±0.01	12.36±0.05^{a}	2.18±0.01	2.25±0.03^{a}
0.05	12.23±0.13	12.15±0.05^{b}	2.14±0.03	2.29±0.02^{ab}
0.1	12.13±0.06	12.25±0.04^{ab}	2.30±0.02	2.32±0.02^{ab}
0.5	12.12±0.12	12.19±0.05^{ab}	2.27±0.04	2.39±0.03^{b}

* concentrations not tested

Table S3.6. Percent mortality at hatch following acute (96 hour) morpholine exposure on day 1, 7, 15 and 30 post fertilization. Percent mortality within each dish was compared using a one-way ANOVA with Tukey's HSD test (Day 1: $p = 0.111$, Day 7: $p = 0.338$, Day 15: $p = 0.152$, Day 30: $p = 0.263$). Values represent means ± SE.

Concentration (mg/L)	Percent mortality			
	Day 1	Day 7	Day 15	Day 30
0	61.3±2.9	63.7±1.7	54.5±5.5	4.0±0.1
1	68.5±3.6	74.5±1.0	62.6±1.4	2.0±0.7
10	51.3±2.8	68.4±5.6	68.8±2.1	1.0±0.9
100	60.3±3.1	63.9±9.0	51.5±2.5	5.1±1.0
500	53.4±8.1	62.8±0.3	54.4±6.4	2.1±2.0
1000	53.0±3.0	67.2±2.8	48.7±7.5	2.0±0.1

Table S3.7. Time to 50% hatch following acute (96 hour) morpholine exposure on day 1, 7, 15 and 30 post fertilization. Median hatch within each dish was compared using a one-way ANOVA with Tukey's HSD test (Day 1: p = 0.019, Day 7: p = 0.675, Day 15: p = 0.456, Day 30: p = 0.200). Values represent means ± SE.

Concentration (mg/L)	Median hatch (days)			
	Day 1	Day 7	Day 15	Day 30
0	138±2	138±3	143±2	141±1
1	145±3	138±3	137±4	132±3
10	150±2	137±4	141±7	131±1
100	151±2	143±2	146±1	142±1
500	140±1	141±1	141±1	138±1
1000	137±1	141±6	143±2	136±6

Table S3.8. Body length measurements on hatchlings following acute (96 hour) morpholine exposure on day 1, 7, 15 or 30 post fertilization. Measurements were compared within each day using a Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test. Letters denote statistical differences (Day 1: p<0.01, Day 7: p = 0.167, Day 15: p = 0.003, Day 30: p<0.001). Values represent means ± SE.

Concentration (mg/L)	Body length (mm)			
	Day 1	Day 7	Day 15	Day 30
0	12.60±0.06^{a}	12.43±0.16	12.65±0.08^{a}	12.06±0.05^{a}
1	12.64±0.09^{a}	12.66±0.14	12.86±0.07^{ab}	12.08±0.06^{a}
10	12.80±0.05^{a}	12.57±0.16	13.13±0.09^{b}	12.10±0.08^{a}
100	12.64±0.09^{a}	12.70±0.13	12.73±0.05^{a}	12.23±0.04^{b}
500	12.27±0.08^{b}	12.76±0.11	12.82±0.06^{ab}	12.07±0.04^{a}
1000	12.03±0.10^{b}	12.70±0.13	12.77±0.08^{ab}	12.09±0.06^{ab}

Table S3.9. Yolk diameter measurements on hatchlings following acute (96 hour) morpholine exposure on day 1, 7, 15 or 30 post fertilization. Measurements were compared within each day using a Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test Letters denote statistical differences (Day 1: p<0.001, Day 7: p = <0.001, Day 15: p = 0.031, Day 30: p=0.083). Values represent means ± SE.

Concentration (mg/L)	Yolk diameter (mm)			
	Day 1	Day 7	Day 15	Day 30
0	1.97±0.04[a]	2.28±0.03[a]	2.36±0.04[ab]	2.43±0.03
1	2.11±0.04[ab]	2.07±004[b]	2.38±0.04[ab]	2.49±0.04
10	2.22±0.03[bc]	2.15±0.06[ab]	2.23±0.05[a]	2.52±0.03
100	2.13±0.04[ab]	2.04±0.03[b]	2.42±0.03[ab]	2.47±0.03
500	2.34±0.03[c]	2.05±0.03[b]	2.36±0.03[ab]	2.54±0.02
1000	2.33±0.03[c]	2.04±0.04[b]	2.42±0.03[b]	2.52±0.02

Table S3.30. Percent mortality at hatch following acute (96 hour) sodium hypochlorite exposure on day 1, 7, 15 and 30 post fertilization. Percent mortality within each dish was compared using a one-way ANOVA with Tukey's HSD test (Day 1: p = 0.554, Day 7: p = 0.870, Day 15: p = 0.755, Day 30: p = 0.830). Values represent means ± SE.

Concentration (mg/L)	Percent mortality			
	Day 1	Day 7	Day 15	Day 30
0	73.5±1.9	68.7±3.3	52.6±3.6	2.0±1.9
0.0001	65.0±5.6	71.4±2.0	60.0±10.0	3.5±1.0
0.001	70.0±2.6	66.8±3.6	64.9±3.1	5.0±2.0
0.01	66.4±1.3	68.4±9.2	60.4±10.4	4.0±2.1
0.05	69.5±2.2	73.1±1.4	53.1±13.6	4.1±3.1
0.1	72.0±5.2	61.5±12.5	61.6±10.7	3.1±2.2

Table S3.41. Time to 50% hatch following acute (96 hour) sodium hypochlorite exposure on day 1, 7, 15 and 30 post fertilization. Median hatch within each dish was compared using a one-way ANOVA with Tukey's HSD test (Day 1: $p = 0.849$, Day 7: $p = 0.961$, Day 15: $p = 0.126$, Day 30: $p = 0.165$). Values represent means ± SE.

Concentration (mg/L)	Median hatch (days)			
	Day 1	Day 7	Day 15	Day 30
0	134±2	136±5	146±5	140±2
0.0001	133±1	137±7	147±1	143±1
0.001	130±6	142±1	143±3	140±1
0.01	134±1	136±2	132±1	138±1
0.05	134±3	137±3	142±3	145±3
0.1	136±3	138±5	138±3	141±1

Table S3.52. Body length measurements on hatchlings following acute (96 hour) sodium hypochlorite exposure on day 1, 7, 15 or 30 post fertilization. Measurements were compared within each day using a Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test. Letters denote statistical differences (Day 1: $p = 0.171$, Day 7: $p = 0.376$, Day 15: $p = 0.001$, Day 30: $p = 0.004$). Values represent means ± SE.

Concentration (mg/L)	Body length (mm)			
	Day 1	Day 7	Day 15	Day 30
0	12.69±0.05	12.54±0.16	12.64±0.07^{ab}	12.08±0.05^{ab}
0.0001	12.62±0.07	12.56±0.18	12.82±0.08^{a}	12.11±0.08^{ab}
0.001	12.53±0.08	12.36±0.19	12.75±0.07^{ab}	12.21±0.04^{ab}
0.01	12.50±0.08	12.28±0.17	12.44±0.09^{b}	12.02±0.07^{a}
0.05	12.68±0.08	12.46±0.16	12.46±0.08^{b}	12.25±0.05^{b}
0.1	12.44±0.11	12.51±0.17	12.46±0.08^{b}	12.03±0.06^{a}

Table S3.63. Yolk diameter measurements on hatchlings following acute (96 hour) sodium hypochlorite exposure on day 1, 7, 15 or 30 post fertilization. Measurements were compared within each day using a Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test. Letters denote statistical differences (Day 1: $p = 0.023$, Day 7: $p < 0.001$, Day 15: $p = 0.376$, Day 30: $p < 0.001$). Values represent means ± SE.

Concentration (mg/L)	Yolk diameter (mm)			
	Day 1	Day 7	Day 15	Day 30
0	1.97±0.04[a]	2.46±0.03[a]	2.45±0.03	2.12±0.04[a]
0.0001	2.02±0.04[ab]	2.43±0.02[a]	2.48±0.04	2.09±0.03[a]
0.001	2.09±0.03[ab]	2.42±0.03[a]	2.43±0.04	2.06±0.04[a]
0.01	2.11±0.03[b]	2.34±0.04[ab]	2.47±0.04	2.15±0.03[a]
0.05	2.02±0.04[a]	2.25±0.04[b]	2.48±0.03	2.33±0.03[b]
0.1	2.00±0.03[a]	2.27±0.03[b]	2.53±0.04	2.35±0.03[b]

Chapter 4

Growth and development effects from chronic and acute ionizing radiation exposure in lake whitefish (*Coregonus clupeaformis*) embryos

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4.1 Abstract

Ionizing radiation is known to impact development during early life stages. Lake whitefish (*Coregonus clupeaformis*) represent a unique model organism for examining such effects. Embryos develop slowly (up to 180 days-to-hatch under natural thermal regimes) allowing for extended exposure to chronic low doses or the ability to accurately target specific development stages. Additionally, embryonic development in oviparous ectotherms enables a direct measure of growth efficiency through quantifying yolk utilization and body size. We investigated the effects of both chronic and acute ionizing radiation exposure on lake whitefish development. Embryos were exposed to acute ^{137}Cs gamma rays at five time points corresponding to major developmental stages, with doses ranging from 0.008 to 15.5 Gy. Additionally, embryos were exposed to chronic doses of gamma rays within a custom built irradiator at dose rates between 0.06 and 4.4 mGy/day and total doses at hatch ranging from 10 to 664 mGy. Embryos showed a high resistance to acute exposures with an $LD_{50/hatch}$ of 5.0 ± 0.7 Gy immediately post fertilization increasing to 14.2 ± 0.1 Gy later in development. Chronic irradiation at all dose rates caused growth stimulation with embryos being up to 60% larger during development compared to non-irradiated controls. Embryos exposed to chronic irradiation also hatched significantly earlier. The effects of combined stressors were examined by administering a thermal or chemical stress prior to a high dose acute radiation exposure. Incubation in morpholine for 96 hours produced a synergistic effect on radiation induced mortality, while a 2 hour heat shock of 3 or 9°C, given 6 hours prior to irradiation, induced an adaptive response where radiation effects were diminished.

4.2 Introduction

Early development is one of the most sensitive stages to ionizing radiation exposure. During embryogenesis, particularly close to fertilization, the high proportion of actively dividing cells increases the risk of cell death and embryonic mortality (De Santis et al. 2007). Malformations and developmental abnormalities can occur during organogenesis when major internal organ systems are forming (De Santis et al. 2007). There is increasing concern over developmental effects of ionizing radiation exposure and the potential impacts that early exposures can have on later life stages.

Limited epidemiological data exists on whole-organism effects from radiation exposure in the low dose region (below 100 mGy). There is growing experimental data to suggest that linear extrapolation from higher doses overestimates risk and that dose response curves follow a sublinear, threshold or hormetic response. The hormetic model had been demonstrated following chemical and radiological stressors, where a stimulatory effect such as increased growth, survival or fecundity is observed at low doses compared to the inhibitory effects at high doses (Calabrese and Baldwin 2000). Proposed molecular mechanisms include the initiation of DNA repair, bystander signaling, stress response induction and free radical scavenging (Jolly and Meyer 2009). However, there is evidence to suggest that initiation of the hormetic response may come at a cost to other physiological processes (Hercus and Loeschcke 2001, Saul et al. 2013, Costantini et al. 2014).

A related phenomenon to hormesis is the adaptive response, where a low dose stress can protect cells or whole-organisms from a future high dose exposure (Tang and Loke 2015). A variety of different stressors have been shown to induce an adaptive response to reduce damage from high dose ionizing radiation, including low dose radiation (Cassidy et al. 2007, Choi et al. 2010), thermal stress (Shen et al. 1991, Boreham and Mitchel 1994) or oxidative chemical compounds (Laval 1988). Similar cellular and molecular mechanisms are thought to underlie the adaptive response as are responsible for hormesis (Tang and Loke 2015).

Embryonic development in fish represents an excellent model for examining whole-organism effects of ionizing radiation. Fish are oviparous ectotherms, which means a direct measure of metabolic efficiency is possible through quantifying yolk utilization and body size. Developing embryos are isolated within an egg shell where all potential energy reserves are contained within their yolk. As ectotherms, a large proportion of yolk lipids and proteins are converted to growth as opposed to thermoregulation. Lake whitefish (*Coregonus clupeaformis*) are a particularly good model species to test various dose response models. They have a long embryonic development period of close to 200 days when raised at cold temperatures (Brooke 1975). This slow development rate allows for the accurate targeting of specific developmental stages for acute exposures as well as enabling extended exposure to low dose chronic radiation.

Aquatic biota are considered to be more radioresistant compared to humans and other mammals and therefore substantially higher recommended dose limits of 400 μGy/hr (3.5

Gy/yr) have been established, compared to 50 mSv/yr in humans (IAEA 1992). The effects of acute radiation exposure on embryonic development in fish have been characterized mainly using external photon irradiation. Sensitivity decreases throughout embryogenesis and LD_{50} values can be more than 10 fold greater close to hatch compared to fertilization (Ward et al. 1971, Wadley and Welander 1971). Few studies have examined the effects of chronic low dose radiation exposure on developing embryos and most have assessed impacts using less sensitive endpoints such as embryonic mortality and reproductive capacity post hatch (Real et al. 2004). The effects from acute and chronic exposures can differ drastically and limited data exists directly comparing dose rate differences within the same species from matching radiation qualities.

The purpose of this study was to examine the effects of both acute and chronic radiation on embryonic development in lake whitefish. For acute radiation exposures, embryos were irradiated with a range of low to high doses at five critical developmental time points. A chronic irradiator was designed specifically for exposing fish embryos to low dose radiation throughout embryogenesis. Gamma rays from ^{137}Cs were used for acute and chronic exposures allowing for a direct comparison between differing dose rates. In addition to survival, embryos were monitored for development rate and size to provide insight into how both low and high doses impact embryonic growth and metabolic efficiency. Finally, embryos were subjected to thermal or chemical stress prior to acute radiation to test the effects of dual stressors.

4.3 Methods

4.3.1 Embryo collection

Lake whitefish embryos were in-vitro fertilized in the fall of three consecutive years (November 15, 2012, November 21, 2013 and November 30, 2014). Adult fish were gillnetted in Eastern Lake Huron (N 44.7094, W 81.3125, Ontario Ministry of Natural Resources scientific fishing permit to J.Y.W.). Eggs were stripped from multiple spawning females and combined with milt from multiple males. Eggs were dry fertilized for 5 minutes, then wet fertilized with lake water for 5 minutes, then transferred to 1.5 L jars at a 50:50 ratio of embryos:lake water. Ovadine® (Syndel Laboratories Limited) was added to each jar for 30 minutes to disinfect embryos at a concentration of 5 ml Ovadine per 1 L lake water. After 30 minutes, Ovadine was removed, embryos were washed twice with lake water, and then jars were filled full with lake water and transported back to the laboratory on ice.

4.3.2 Embryo rearing

Embryos were initially raised in upwelling hatching jars within a custom designed recirculating filtered water system (Mitz et al. 2014). Individual experiments were run in petri dishes containing dechlorinated municipal water. Petri dish water was changed twice per week for the first month of development and then reduced to once per week after the majority of natural mortality had occurred. Dishes were checked daily and dead or hatched embryos were removed. Hatching jar and petri dish water temperature was continuously monitored using data loggers (Onset HOBO).

Embryos in hatching jars were incubated at 5°C, which was the lowest temperature that circulating water could maintain (Mitz et al. 2014). Embryos given acute exposures were incubated on the shelf of the refrigeration units, where static water temperature was 3°C. Embryos irradiated with chronic exposures were also incubated at 3°C to match acute exposures. Combined stressor experiments were run in a separate unit at 2°C. A colder temperature was used for combined stressor experiments so that embryos could be given a 9°C heat shock while not allowing the absolute temperature to rise above 11°C to potentially lethal levels.

4.3.3 Acute irradiations

Embryos were given acute exposures of 662 keV gamma rays using a ^{137}Cs source. Irradiations were given on day 1, 7, 15, 30 and 60 post fertilization, corresponding to cleavage, gastrulation, closure of the blastopore, organogenesis and fin flutter stages respectively (Sreetharan et al. 2015). Embryos were transferred from hatching jars and irradiated in 12.5 cm^2 vented cap cell culture flasks on ice. Doses ranged from 0.008 ± 0.001 Gy to 15.5 ± 2.0 Gy, all at a dose rate of 0.39 ± 0.05 Gy/min. Doses were verified using thermoluminescent dosimeters (Mirion Technologies). At each dose, 50 embryos were irradiated per flask in duplicate or triplicate flasks. Following irradiation, embryos were transferred to petri dishes until hatch.

4.3.4 Chronic irradiations

Embryos were exposed throughout embryogenesis to chronic ^{137}Cs gamma irradiation using a custom designed irradiator (Figure 4.1). The irradiator was designed to fit within a

reinforced 2 door chromatography refrigerator. The containment unit was constructed from five 1/4" lead sheets to provide adequate shielding to the exterior of the unit. A 37 MBq ^{137}Cs source (J.L. Shepherd & Associates) was housed in the bottom of the unit, directed upwards towards a series of 6 shelves (Figure 4.1 A,B). The lowest shelf was loaded with one 15 cm diameter petri dish containing 100 embryos, due to the narrow beam window close to the source. The other 5 shelves were loaded with five 10 cm petri dishes, each containing 50 embryos. A lead slide made from five 1/4" sheets was used to block the beam window for when entry into the unit was required (Figure 4.1C). Dose rates were calculated using thermoluminescent dosimeters (Table 4.1, Mirion Technologies). Four dosimeters were placed on each shelf to record lateral spatial variation in dose rate. Five control dishes were placed outside of the lead box but within the same refrigerator. Embryos were loaded into the irradiator immediately once returned to the laboratory, approximately 7 hours post fertilization. The average temperature within the containment unit was 2.76 ± 0.25°C (mean ± SD), almost identical to the outside of the unit where control dishes were kept, which was 2.73 ± 0.25°C.

Figure 4.1. Design of chronic irradiator. A) Containment unit with door open and 6 shelves to hold dishes containing embryos. 1. Cover plate over source compartment. B) Bottom of unit with source compartment cover plate removed. 2. Lead pig containing 37 mBq ^{137}Cs source directed upwards. C) Lead slide on right side of unit to block beam window during entry into unit. Slide is shown in "open" position.

Table 4.1. Chronic irradiator dose measurements. Dose rates were measured on each shelf using thermoluminescent dosimeters. Cumulative exposure was calculated to day 75 and day 122, when embryos were fixed. Cumulative exposure at hatch was calculated using the median hatch date for embryos on each shelf. Error represents the range in dose rates based on measured lateral spatial variation on each shelf.

Distance from source (cm)	Dose rate (mGy/day)	Cumulative exposure: day 76 (mGy)	Cumulative exposure: day 122 (mGy)	Cumulative exposure: hatch (mGy)
81.44	0.06±0.01	4.72±1.03	7.58±1.66	9.53±2.22
67.15	0.11±0.02	8.21±1.29	13.18±2.08	16.57±2.77
52.86	0.19±0.03	14.48±2.59	23.25±4.15	29.40±5.78
38.58	0.39±0.04	29.35±3.10	47.12±4.98	59.71±7.52
24.29	1.05±0.09	79.74±7.11	128.00±11.42	160.52±17.28
10.01	4.40±0.78	334.54±59.35	537.03±95.27	664.69±117.92

4.3.5 Combined stressors

Embryos were exposed to an acute thermal or chemical (morpholine) stress prior to a high dose irradiation. Developing embryos could be exposed to these two stressors during natural development as a result of industrial discharges. Heat shock temperatures and morpholine concentrations were chosen to be at, or slightly above environmentally relevant levels. Combined stressor experiments were run during the first 2 weeks of development when embryos were more sensitive to radiation exposure. Combined stressor embryos were incubated in duplicate petri dishes.

On day 5 post fertilization (gastrulation), embryos were transferred from hatching jars to petri dishes at 2°C. After 48 hours in dishes, embryos were exposed to a transient heat shock of 3, 6 or 9°C for 2 hours. Water was removed from the dishes and pre heated water was added. After heat shock, dishes were transferred back to 2°C and water was

allowed to gradually cool. Embryos were irradiated with 7.75 Gy as described for acute irradiations at two separate time points. One set of embryos was irradiated 6 hours post heat shock and a second set irradiated 24 hours post heat shock. Post irradiation, embryos were transferred back to petri dishes until hatch.

On day 12 post fertilization (closure of the blastopore), embryos were transferred from hatching jars to petri dishes. After 48 hours, petri dish water was changed and morpholine treated water was added at concentrations of 10 or 100 mg/L. Embryos were incubated in morpholine for 96 hours and then irradiated with 3.88 or 7.75 Gy as described for acute irradiations, while maintained in morpholine treated water. Post irradiation, embryos were removed from morpholine and transferred back to petri dishes containing normal dechlorinated water until hatch.

4.3.6 Embryo endpoints

All live hatches from chronic and acute exposures were fixed in 10% neutral buffered formalin for one week then transferred to 70% ethanol for long term storage. A subset of embryos exposed to chronic radiation (25 per shelf) was fixed on day 76 and day 122 post fertilization. Hatchlings and dechorionated embryos were imaged, from which morphometric measurements were taken. Total body length was measured from a dorsal image and two perpendicular yolk diameters were measured from a lateral image. A yolk area was predicted based on the two measured diameters.

Embryo and hatchling weights were measured on chronic irradiated embryos. Post imaging, the embryo or hatchling was dissected apart from the yolk. Samples were dried

overnight in a 70°C oven and a yolk-free body and yolk dry weight was measured (Mettler-Toledo XA105DU, ±0.01 mg). A set of 25 embryos were also fixed and weighed on day 1 post fertilization, from which a yolk conversion efficiency (YCE) was calculated according to the equation:

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

4.3.7 Statistical analysis

Statistical analysis was conducted using Sigmaplot V11.0. Percent mortality and time to hatch was compared between replicate dishes using one-way ANOVA followed by Tukey's HSD test. LD_{50} values were calculated based on probit analysis with percent mortality corrected for control mortality using the Schneider-Orelli method (Schneider-Orelli 1947). Morphometric measurements were compared using a non-parametric Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test, because data was not normally distributed. Chronic irradiation weights and YCE were log transformed and compared using a one-way ANOVA followed by Tukey's HSD test. Combined stressor experiments were analyzed using a two-way ANOVA followed by Tukey's HSD test.

4.4 Results

4.4.1 Acute exposures

Embryos irradiated on day 1 had a significant reduction in the percent live hatch with exposure to 3.88 Gy and 100% mortality with exposure to 7.75 and 15.51 Gy (Figure 4.2A, $F(6,14) = 40.81$, $p < 0.001$). Mortality was elevated following irradiation on day 7, 15 and 30 with exposure to 7.75 Gy or greater (Day 7: $F(6,7) = 91.00$, $p < 0.001$, Day 15: $F(6,7) = 10.47$, $p = 0.003$, Day 30: $F(6,7) = 790.78$, $p < 0.001$). However, on day 60 there was no significant increase in mortality up to the highest tested dose ($F(5,6) = 0.91$, $p = 0.531$). Embryos become more resistant to radiation throughout development. $LD_{50/hatch}$ values increased from 5.0 ± 0.8 Gy on day 1 up to 14.2 ± 0.1 Gy on day 30 (Figure 4.2B). Increasing $LD_{50/hatch}$ values followed a logarithmic relationship up to 30 days post fertilization. This relationship likely differs later in embryogenesis. Extrapolation to day 60 predicted an $LD_{50/hatch}$ of approximately 16 Gy, however, no increase in mortality occurred at that time point up to the highest tested dose of 15.51 Gy.

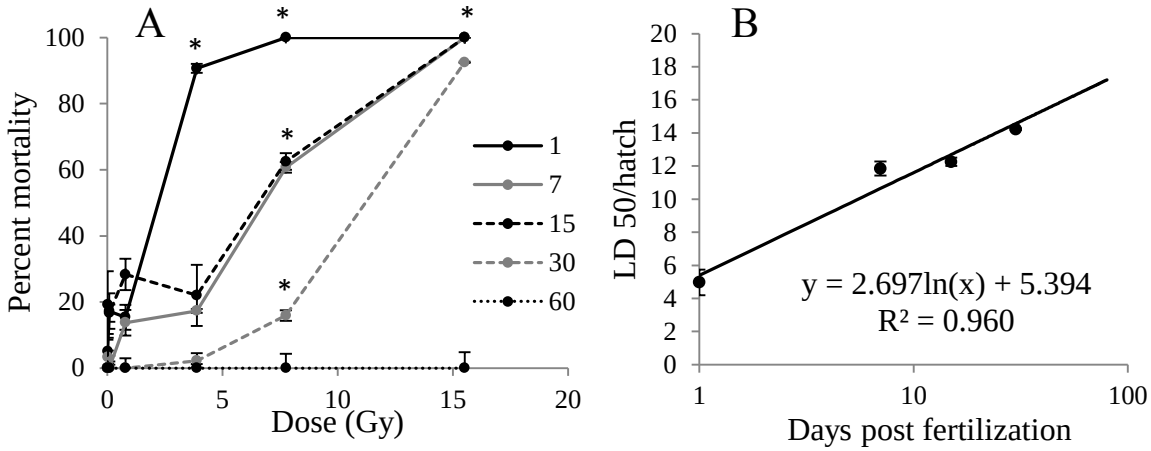

Figure 4.2. Embryonic mortality from acute radiation exposure. A) Cumulative percent mortality at hatch following irradiation on day 1, 7, 15, 30 or 60 post fertilization. To eliminate differences in natural mortality rates, percent mortality in control dishes was subtracted from each treatment dish. Percent mortality between replicate dishes was compared using a one-way ANOVA with Tukey's HSD test. * represents a significant difference from same day controls. Data points represent means ± SE. B) The LD_{50} at hatch increased logarithmically with embryo age at irradiation. Data points represent the mean of replicate dishes. Error was calculated based on the standard error in LD_{50} probit analysis.

There was a dose dependent latency period between radiation exposure and the timing of induced mortality. Following a 7.75 and 15.51 Gy irradiation on day 1, mortality occurred soon after exposure with a median time of 21 days. Mortality from 3.88 Gy however did not occur until day 129. On day 7, 15 and 30, the time to 50% mortality following 15.51 Gy was similar at all three time points (median of 87 days post irradiation) and occurred earlier than at 7.75 Gy (median of 114 days post irradiation).

Sublethal radiation exposure did not impact the timing or duration of hatching. Regardless of whether radiation exposure induced mortality, hatching amongst the surviving embryos began at the same time as controls and followed a similar rate.

However, at doses where there was a significant increase in mortality, the hatching window was shortened since there were fewer embryos surviving to hatch. A small non-significant increase in developmental abnormalities occurred following 7.75 Gy on day 7, 15 and 30. The largest occurred following irradiation on day 30, but was still less than 10%. At all doses below 7.75 Gy, the rate of developmental abnormalities was less than 5%.

A reduction in size at hatch occurred following irradiation, but only at doses where radiation induced mortality was observed (Figure 4.3). Hatchling body length was significantly smaller following 3.88 Gy on day 1 ($H = 17.55$, $df = 4$, $p = 0.002$), 7.75 Gy on day 7 ($H = 34.17$, $df = 5$,$p < 0.001$) and 15.51 Gy on day 30 ($H = 39.70$, $df = 6$, $p < 0.001$). Interestingly, a significant increase in body length did occur following a dose of 3.88 Gy on day 15 ($H = 22.33$, $df = 5$, $p < 0.001$) and day 30 (Figure 4.3). No change in body length occurred when embryos were irradiated on day 60 ($H = 7.72$, $df = 5$, $p = 0.173$). The yolk area did not differ significantly from controls at any of the doses or time points tested (data not shown).

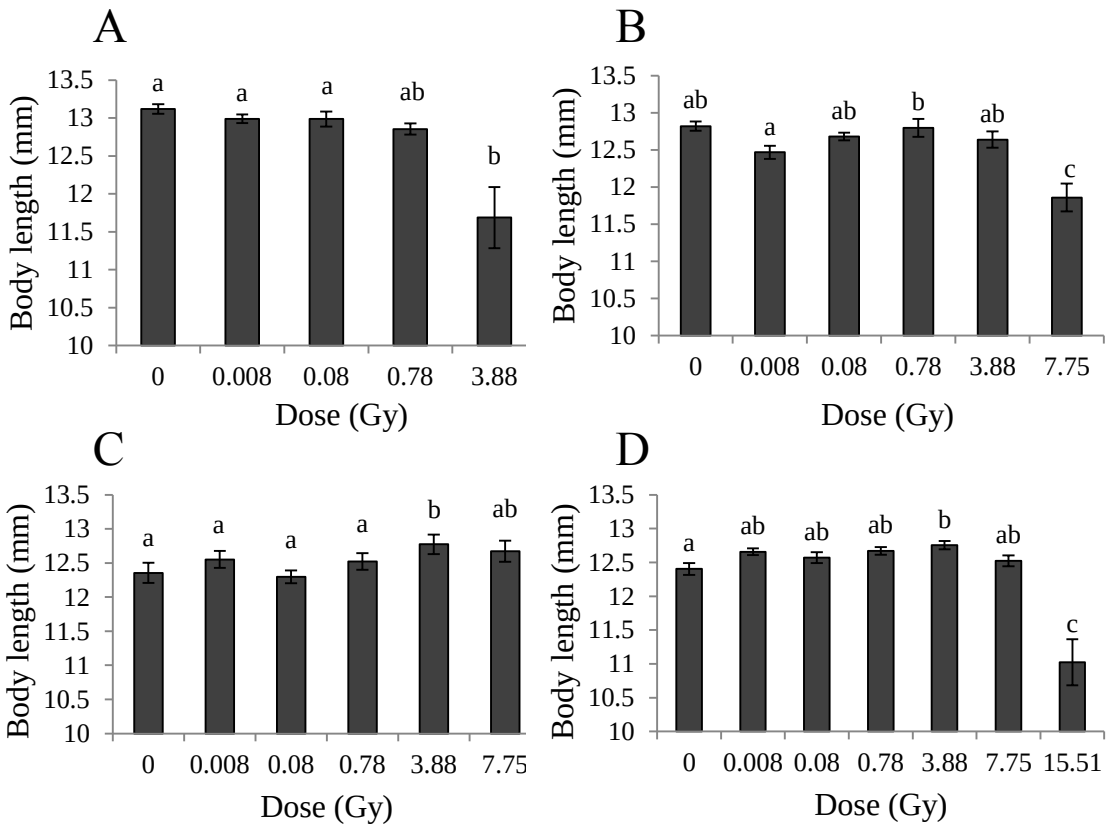

Figure 4.3. Body length measurements on preserved hatchlings following acute radiation exposure on day 1 (A), day 7 (B), day 15 (C) and day 30 (D) post fertilization. No embryos survived to hatch following 7.75 Gy on day 1 and 15.51 Gy on day 1, 7, and 15. Measurements were compared using a Kruskal-Wallis one-way ANOVA on ranks with Dunn's test. Letters denote statistical differences. Bars represent means ± SE.

4.4.2 Chronic exposure

A temperature increase occurred on all chronic irradiated and control dishes on day 161 post fertilization caused by ice buildup on the compressor of the refrigeration unit. Temperature within the unit rose to 14°C which resulted in a triggering of hatch in all embryos which had not already hatched naturally. The temperature rise did not induce any mortality.

No significant change in embryonic survival-to-hatch occurred at any of the six dose rates (Table 4.2, $F(5,23) = 0.594$, $p = 0.705$). Chronic irradiation did result in a change in hatch dynamics. At the time of compressor failure, more than 50% of the embryos in irradiated dishes had already hatched and therefore an accurate time to median hatch could still be calculated. The control dishes however only had a small percentage of embryos hatched, or no embryos hatched, when the temperature spike occurred. A conservative comparison was made by using day 161 as the date of initial hatch or median hatch in dishes which had not reached that stage. Chronic irradiation resulted in an earlier first hatch (Table 4.2, $F(5,23) = 6.531$, $p < 0.001$) and a shorter time to median hatch ($F(5,23) = 8.860$, $p < 0.001$). If the equipment failure had not occurred, then this difference would have been more pronounced.

Table 4.2. Percent mortality and hatch timing in chronic irradiated embryos. Cumulative percent mortality was recorded at hatch. First hatch and median hatch was measured in days post fertilization (dpf). Percent mortality and hatch timing were compared using a one-way ANOVA with Tukey's HSD test. Letters denote statistical differences. Values represent the means of individual replicate dishes ± SE.

Dose rate (mGy/day)	Percent mortality	First hatch (dpf)	Median hatch (dpf)
0	16.36±2.88	155±3[a*]	161[a*]
0.06	13.97±3.36	134±3[b]	153±1[b]
0.11	16.26±1.49	137±3[b]	153±1[b]
0.19	14.64±2.22	140±2[b]	154±1[b]
0.39	11.74±1.38	141±2[b]	154±1[b]
1.05	15.56±1.57	140±1[b]	153±1[b]
4.40	15	132[b]	151[b]

*****The first hatch and median hatch was advanced in control dishes due to compressor failure. At the time of compressor failure, irradiated dishes were already past median hatch.

A small number of developmental abnormalities (less than 3%) were observed in embryos and hatches. On day 76 and day 122, chronically irradiated embryos had a significantly larger body length (Figure 4.4A,C, Day 76: $H = 65.93$, $df = 6$, $p < 0.001$, Day 122: $H = 65.93$, $df = 6$, $p < 0.001$) and yolk-free body weight (Figure 4.5A,C, Day 76: $F(6,140) = 22.87$, $p < 0.001$, Day 122: $F(6,147) = 3.88$, $p = 0.001$). Yolk dry weights were correspondingly smaller in irradiated embryos (Figure 4.5A,C, Day 76: $F(6,147) = 7.481$, $p < 0.001$, Day 122: $F(6,146) = 4.65$, $p < 0.001$) although this was not reflected in yolk area measurements (Figure 4.4B,D, Day 76: $H = 17.802$, $df = 6$, $p = 0.007$, Day 122: $H = 15.13$, $df = 6$, $p = 0.019$). The increase in size was more pronounced on the day 76 time point. Body weights were up to 60% larger relative to controls (Figure 4.5A), compared to only 13% larger on day 122 (Figure 4.5C). Similarly, embryo body length was up to 15% larger on day 76 compared to 10% larger on day 122 (Figure 4.4A,C). Interestingly, the percent increase in body weight was much larger than the increase in body length (60% vs 15%). No significant difference was seen in YCE on day 76 or day 122 (Figure 4.5B,D). No dose response relationship was observed in embryo morphometric or dry weight measurements, although the highest dose rate of 4.40 mGy/day did have the most pronounced effect on several of the measurements (Figure 4.4, 4.5).

A different trend was observed at hatch. Irradiated hatchlings had a significantly smaller yolk-free body weight (Figure 4.5E, $F(6,1074) = 20.64$, $p < 0.001$). Embryo body length however was larger compared to non-irradiated controls (Figure 4.4E, $H = 54.45$, $df = 6$, $p < 0.001$). Both yolk area and yolk weight were significantly larger (Figure 4.4F, 4.5E, H

= 194.47, df = 6, $p < 0.001$, $F(6,1074) = 30.41$, $p < 0.001$) . No significant trend was observed in YCE at hatch (Figure 4.5F, $F(6,1038) = 6.54$, $p < 0.001$).

4.4.3 Combined stressors

Radiation exposure of 7.75 Gy, regardless of the heat shock, induced mortality (Figure 4.6A,B, 6 hour: $F(1,8) = 148.10$, $p < 0.001$, 24 hour: $F(1,8) = 201.25$, $p < 0.001$). Heat shock alone did not impact survival. A 3°C and 9°C heat shock resulted in a significant reduction in radiation induced mortality when embryos were irradiated 6 hours post heat shock (Figure 4.6A, $F(3,8) = 4.453$, $p = 0.041$). The percent mortality was reduced by up to 25% with a 3°C heat shock. However, by 24 hours none of the heat shock temperatures significantly altered radiation induced mortality (Figure 4.6B, $F(3,8) = 0.50$, $p = 0.69$). Heat shock did not affect the length of development or hatching dynamics (data not shown).

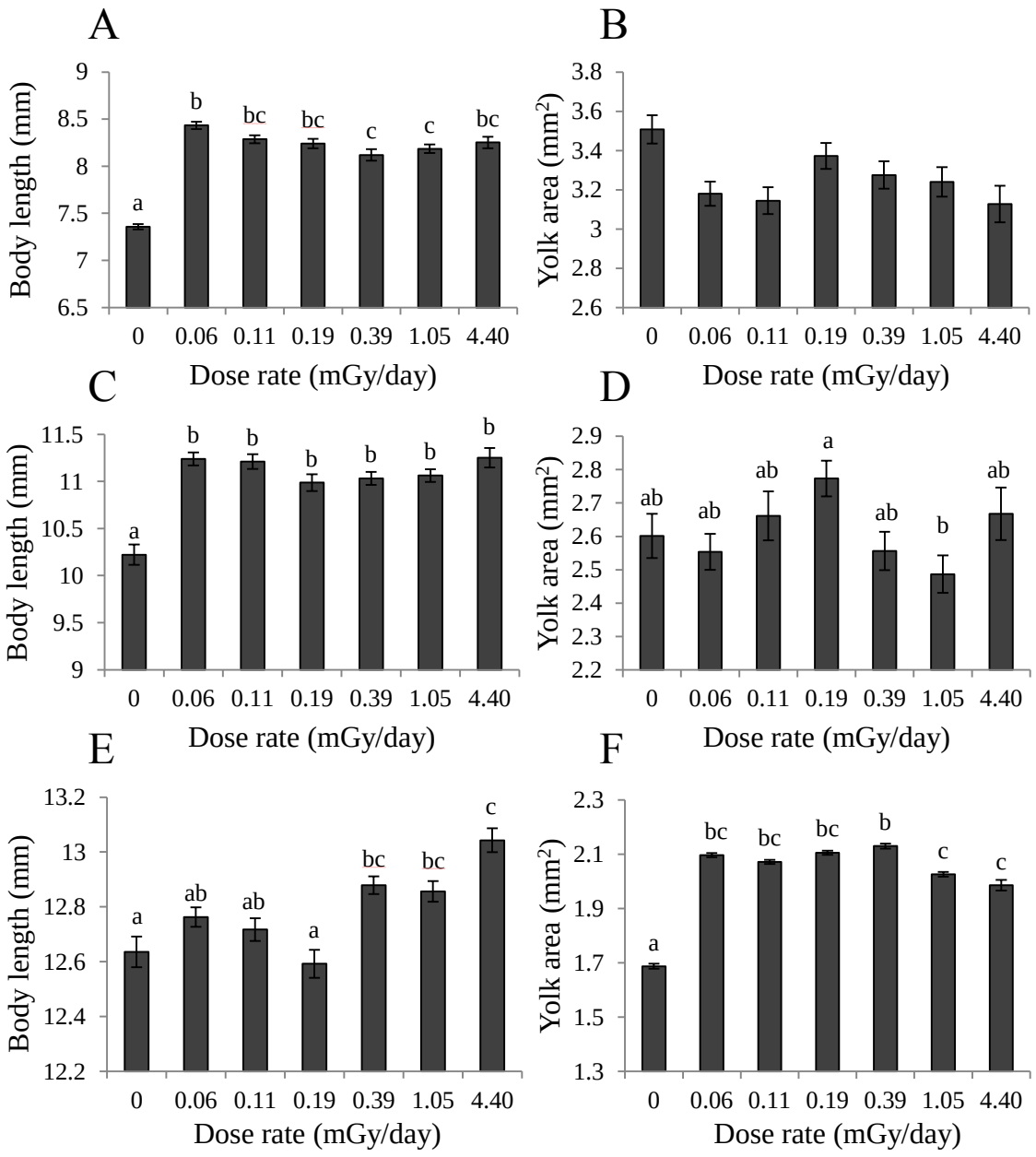

Figure 4.4. Morphometric measurements on chronic irradiated embryos and hatchlings. Body length and yolk area were measured on preserved embryos on day 76 (A,B), day 122 (C,D) and hatchlings (E,F). Measurements were compared using a Kruskal-Wallis one-way ANOVA on ranks with Dunn's test. Letters denote statistical differences. Bars represent means ± SE.

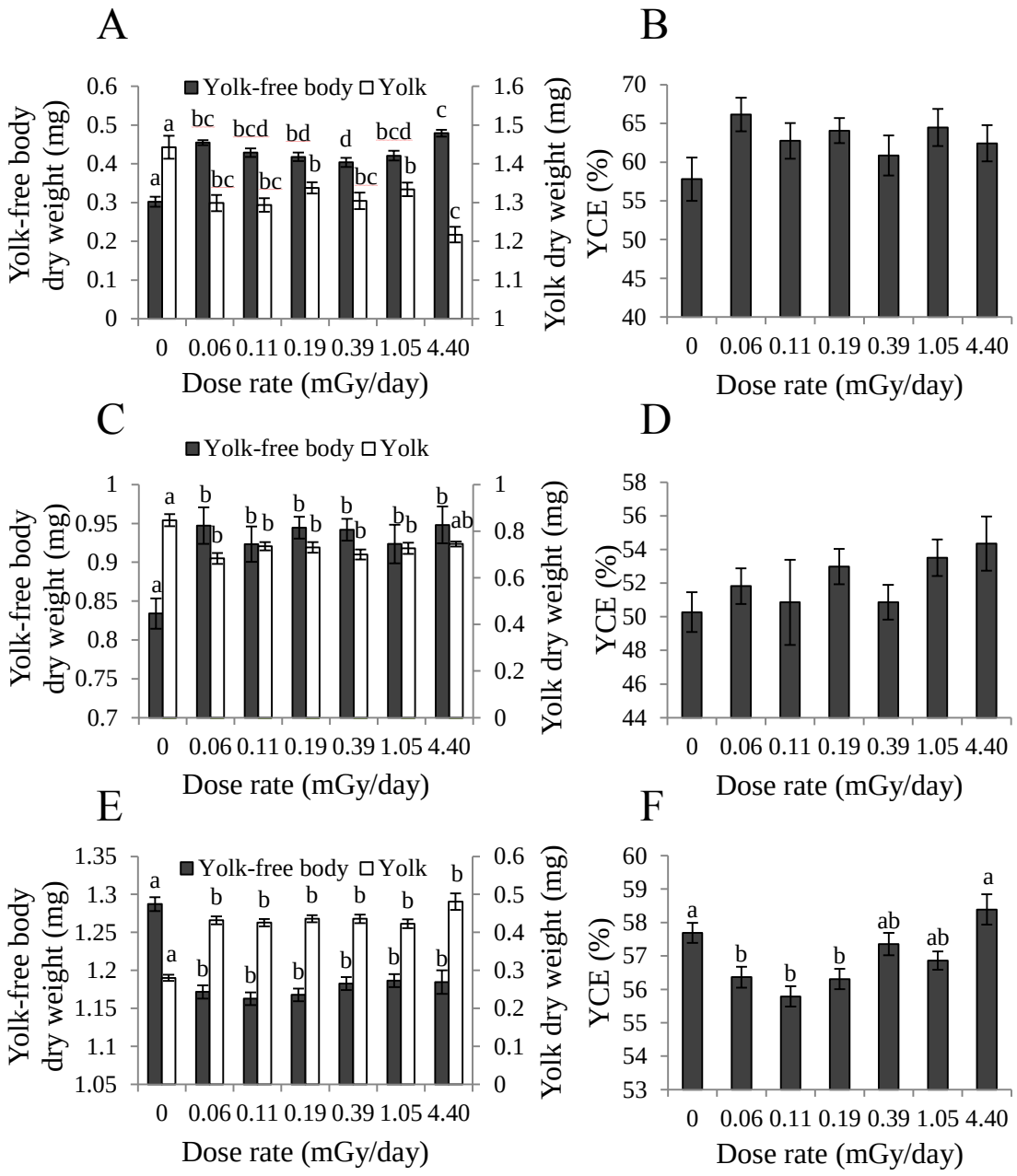

Figure 4.5. Dry weight measurements on chronic irradiated embryos and hatchlings. Yolk-free body weight, yolk weight and yolk conversion efficiency (YCE) were measured on preserved embryos on day 76 (A,B), day 122 (C,D) and hatchlings (E,F). Dry weights and YCE were log transformed and compared using a one-way ANOVA with Tukey's HSD test. Letters denote statistical differences. Bars represent means ± SE.

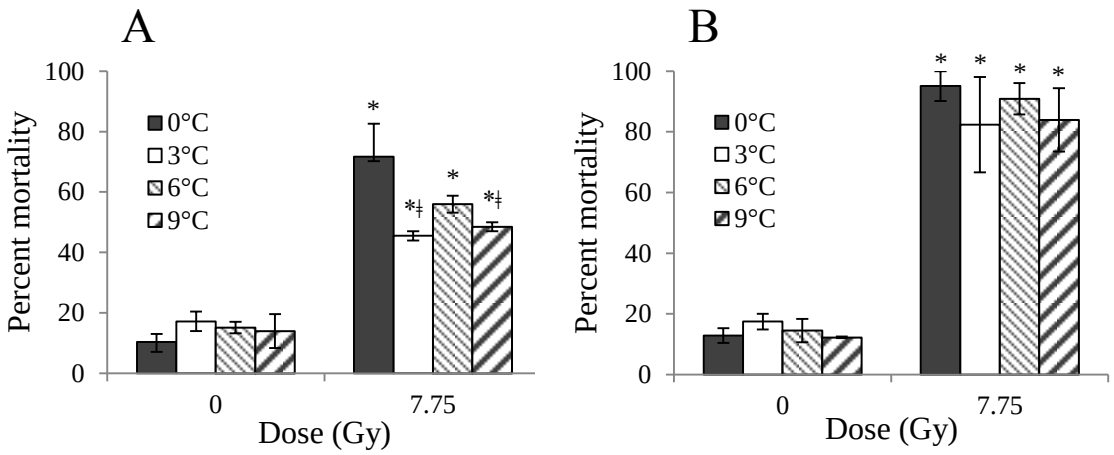

Figure 4.6. Percent mortality following combined heat shock and radiation exposure. Embryos were given a 2 hour heat shock of 3, 6 or 9°C, then irradiated 6 (A) or 24 (B) hours post heat shock. Percent mortality in replicate dishes was compared using a two-way ANOVA with Tukey's HSD test. * represents a significant difference from matching non-irradiated heat shock embryos. ǂ represents a significant difference from non-heat shock irradiated embryos. Bars represent means ± SE.

Morpholine exposure resulted in a synergistic response in percent mortality (Figure 4.7). A dose of 7.75 Gy increased mortality at all concentrations ($F(2,9) = 88.08$, $p < 0.001$). Morpholine alone at 10 or 100 mg/L did not induce an increase in mortality above controls ($F(2,9) = 3.11$, $p = 0.094$). A concentration of 10 mg/L did not alter radiation induced mortality, however exposure to 100 mg/L morpholine resulted in a significant increase in mortality after irradiation ($F(4,9) = 5.80$, $p = 0.014$). Morpholine exposure did not affect the length of development or hatching dynamics (data not shown).

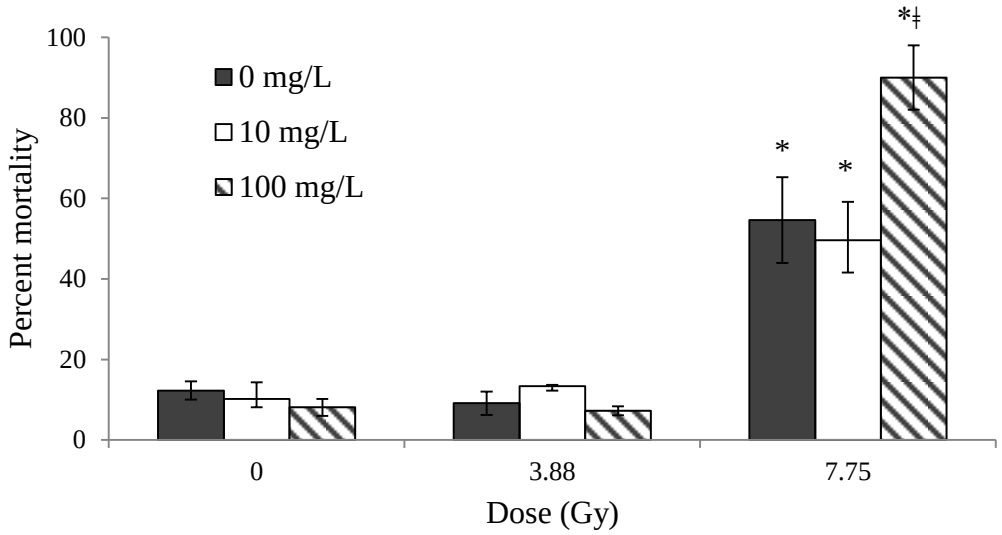

Figure 4.7. Percent mortality following combined morpholine and radiation exposure. Embryos were incubated in morpholine concentration of 10 or 100 mg/L for 96 hours prior to irradiation. Percent mortality in replicate dishes was compared using a two-way ANOVA with Tukey's HSD test. * represents a significant difference from matching concentration at 0 and 3.88 Gy. ǂ represents a significant difference from 0 and10 mg/L irradiated embryos. Bars represent means ± SE.

4.5 Discussion

Lake whitefish were used as a model organism for examining the effects of acute and chronic gamma irradiation and combined stressors (prior heat shock or morpholine exposure with irradiation) on embryonic development. We observed a hormetic stimulation in growth following low dose chronic irradiation. A slight growth enhancement was also observed following sublethal acute doses of 3.88 Gy delivered on day 15 and 30 post fertilization. Growth stimulation following low dose radiation exposure has not previously been demonstrated in fish, but had been shown in other cell culture and whole organism models (Miller and Miller 1987, Li et al. 2004, Liang et al.

2011, Stark et al. 2015). Additional hormetic effects, apart from growth, have been observed in developing fish embryos. Zebrafish (*Danio rerio*) were more resistant to starvation post hatch when embryos were exposed throughout development to external gamma rays between 1 and 1000 mGy/day (Simon et al. 2011). Chronic exposures of 4 mGy/day during embryogenesis in chinook salmon (*Oncorhynchus tshawytscha*) resulted in mature females producing more viable eggs (Donaldson and Bonham 1970). Multigenerational hormetic effects have been shown in mosquitofish (*Gambusia affinis*) where brood size was enhanced following exposure to radioisotope contaminated water at a dose rate of 109 mGy/day (Baylock 1969).

The stimulation of growth in chronically irradiated embryos was more pronounced earlier in development. On day 76, embryos were up to 60% larger in mass compared to only 13% on day 122. This suggests that hormetic effects from radiation occur closer to fertilization and diminish near hatch. Miyachi et al. (2003) found a similar effect on hatch timing in zebrafish following low dose x-irradiation. An acute dose given during the blastula period advanced hatch, but radiation had no effect when delivered later in development during gastrulation or segmentation. The percent change in body dry weight in lake whitefish was much greater than the percent change in body length, indicating that growth stimulation is resulting in a greater increase in lateral thickness compared to embryo length.

Radiation hormesis is thought to be the result of multiple interrelated cellular events including the up regulation of stress response proteins, DNA repair mechanisms,

bystander effects, and free radical induction (Jolly and Meyer 2009). Initiation of these responses could be metabolically costly to organisms and they are therefore not constitutively active under normal conditions (Tomanek 2010). As such, it is thought that a hormetic response could come at a cost to other physiological processes (Hercus and Loeschcke 2001, Saul et al. 2013, Costantini et al. 2014). However, chronically irradiated lake whitefish were equally efficient at converting yolk potential energy into body mass compared to non-irradiated controls. This demonstrates that the hormetic growth stimulation may not necessarily come at a cost to metabolic efficiency, at least not in a developmental context for fish.

Low dose chronic radiation resulted in a shorter time-to-hatch. Unlike with high acute doses, chronic irradiation did not increase mortality, so changes in hatch timing are not the indirect effect of embryo death. An advanced hatch from radiation exposure has previously been observed in fish. Zebrafish embryos hatched earlier following a single acute x-ray dose of 0.025 Gy delivered early in development (Miyachi et al. 2003) and following chronic exposure to gamma rays throughout development (Simon et al. 2011). Chronic irradiation in zebrafish at dose rates between 1 and 1000 mGy/day resulted in a shorter time-to-hatch but no dose response was observed (Simon et al. 2011), similar to what was found in this study for lake whitefish.

The advance in hatch is likely why embryos had a smaller body weight at hatch, despite demonstrating stimulated growth earlier in development. Although body weight was smaller at hatch compared to controls, embryos were slightly larger in length. Differences

in hatch timing have a greater effect on body weight compared to length. Embryos grown at warmer temperatures will hatch earlier and are smaller at hatch. A rearing temperature of 8°C compared to 2°C results in embryos which are almost 50% smaller in body weight at hatch (Mueller et al. 2015), but are less than 10% smaller in body length (Brooke 1975). The early hatch following chronic irradiation was large enough to overcome the growth stimulation in body weight from earlier in development, but was not large enough to reverse the trend in body length.

Mortality was observed following acute exposures. Embryos were most sensitive immediately post fertilization and then became more resistant later in development. The specific time points for irradiation were chosen as they pertain to major developmental landmarks; newly fertilized/cleavage (day 1), gastrulation (day 7), closure of the blastopore (day 15), organogenesis (day 30) and post organogenesis/fin flutter, where the majority of development had finished and embryos were only undergoing growth (Day 60, Sreetharan et al. 2015). Lake whitefish were more resistant compared to other related species. On day 1, lake whitefish had an $LD_{50/hatch}$ of approximately 5 Gy. Welander (1954) found an LD_{50} for x-rays in rainbow trout (*Oncorhynchus mykiss*) of 0.7 Gy when irradiated immediately post fertilization, increasing to 6 Gy at the germ ring stage. Additional LD_{50} values following irradiation at fertilization have been reported as 3 Gy in chinook salmon (*Oncorhynchus tshawytscha*, Wadley and Welander 1971) and 0.9 Gy in plaice (*Pleuronectes platessa*, Ward et al. 1971). One potential reason why lake whitefish are more resistant is their slow development rate. When grown at 3°C the incubation

period is approximately 140 days (Brooke 1975, Griffiths 1979, Mueller et al. 2015). This slower development would allow more time for repair of potentially lethal damage.

Mortality from acute exposures was modified by both thermal and chemical stress. A priming heat shock reduced mortality when delivered 6 hours prior to irradiation, but had no effect when delivered 24 hours prior. A heat shock induced adaptive response has previously been shown to protect against radiation induced mortality in yeast (Mitchel and Morrison 1982, Boreham and Mitchel 1994), cell culture (Shimada 1985, Boreham et al. 1997) and mice (Shen et al. 1991). Heat shock adaptive responses are thought to be partly due to the induction of heat shock proteins (HSP, Boreham and Mitchel 1994). The increase in HSP expression post heat shock is transient and a return to baseline levels after 24 hours could explain why no response was seen at that time point. In lake whitefish, a similar 2 hour heat shock of 9°C has been shown to increase expression of HSP70 mRNA as early as 2 hours post thermal stress (Stefanovic et al., University of Regina, unpublished data). However, no HSP induction was seen following a 3°C and 6°C thermal stress. Additionally, HSP levels following 9°C persisted up to 36 hours post heat shock, whereas the adaptive response in lake whitefish was not present at 24 hours. Stefanovic et al. measured HSP expression in lake whitefish on day 102 post fertilization though, whereas embryos in this study were heat shocked and irradiated on day 7 post fertilization. The magnitude and timing of the stress response in fish is known to change throughout development (Takle et al. 2005). In rainbow trout cells, HSP expression was increased 6 hours after a 6°C heat shock and diminished back down to near baseline levels after 24 hours (Mosser et al. 1986).

Morpholine was chosen as a chemical stressor because it is a common additive in industrial cooling water systems. Morpholine is a neutralizing amine that is added to steam systems to prevent corrosion through increasing pH. Lake whitefish spawn in shallow water in regions which could be impacted by industrial discharges, and therefore embryos could potentially be exposed during development. Environmentally relevant concentrations of 10 and 100 mg/L were selected for exposures (US FDA 2014). Morpholine on its own did not impact survival. When embryos were exposed prior to and during irradiation, it produced a synergistic effect; increasing the percent mortality above what was observed with radiation alone. The increase in mortality could be due to pH changes. At morpholine concentrations of 100 mg/L, pH levels are approximately 9.4 (IJT 1989). A more basic environment has been shown to reduce the duration of cell cycle delays following irradiation, which subsequently decreases the time available for repair of DNA damage and increases cell death (Park et al. 2003).

Hatch dynamics were only impacted at acute doses where radiation exposure induced mortality. Following 7.75 and 15.51 Gy, the rate of hatching was the same as controls but all surviving embryos were in the early portion of the natural hatching window. This early and shorter hatch window is partly the reason for body length differences at hatch. On average, surviving irradiated embryos hatched earlier and were therefore smaller compared to controls. Size differences are also likely due to the direct effects of high dose radiation inducing cell death.

A small increase in developmental abnormalities was seen in hatchlings following acute exposures, the highest percentage of which occurred on day 30 irradiated embryos. Day 30 in lake whitefish corresponds to organogenesis (Sreetharan et al. 2015), which is known to be a sensitive time in development for abnormalities as all major organ systems are forming (De Santis et al. 2007). In aquatic species, abnormalities have been observed following both acute and chronic irradiation (Baylock 1969, Hyodo-Taguchi et al. 1973).

Overall, both chronic and acute radiation impacted lake whitefish embryo development rate, size and hatch dynamics. Using a specially designed irradiator, embryos were exposed to low dose gamma rays for the entire development period of approximately 5 months. We demonstrated for the first time that chronic irradiation can stimulate growth during embryogenesis in fish and that this hormetic response does not come at a cost to metabolic efficiency. Chronic exposure also resulted in earlier hatching embryos compared to non-irradiated controls. With acute exposure, lake whitefish were more resistant to radiation induced mortality compared to related species, with up to 100% survival following a 15.5 Gy exposure. Survival from acute exposure was impacted by both priming thermal and chemical stress. A thermal stress delivered 6 hours post heat shock induced an adaptive response, which was absent at 24 hours. Chemical exposure to morpholine however produced the opposite effect by increasing embryonic mortality.

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Chapter 5

Discussion and conclusions

5.1 Main findings

The goal of this thesis was to investigate potential impacts on embryonic development in lake whitefish from exposure to various stressors associated with industrial discharges. Thermal power generation relying on once through cooling releases warmer water back into the environment after condensing turbine steam. Cooling water discharges can also contain chemicals such as morpholine and sodium hypochlorite used to prevent corrosion and biofouling. Additionally, nuclear power plants may release low levels of ionizing radiation. The three data chapters in this thesis addressed each one of these stressors individually; the third data chapter includes combined stressor effects.

Chapter 2 examined the effects of temperature differences during in-situ in lake incubation. Thermal impacts on lake whitefish development have been widely studied in the laboratory, but few studies have examined temperature differences during natural in-lake development. Embryos were successfully incubated near discharge sites in Lake Huron throughout the winter and retrieved over 4 months later the following spring. Both morphometric measurements and retrieved temperature logger data suggested that embryos incubated at discharge sites developed at slightly warmer temperatures and were more advanced in development compared to embryos from reference locations.

Chapter 3 focused on the impacts of both chronic and acute exposure to morpholine and sodium hypochlorite. The effects of chronic morpholine exposure were most apparent near the hatching stage. The timing of mortality coincided with the premature triggering

of hatching. Embryos were also smaller at hatch. Sodium hypochlorite had minimal effects on development, apart from mortality at high chronic concentrations.

Chapter 4 examined radiological effects on development. Embryos were exposed to both acute and chronic doses of ^{137}Cs gamma rays. High acute doses resulted in increased mortality and embryos become more resistant as development progressed. Chronically, low dose exposure had a stimulatory effect on growth and embryos hatched earlier compared to non-irradiated controls. A prior heat shock produced an adaptive response making embryos radiation resistant when combined with acute radiation, while exposure to morpholine prior to radiation produced a sensitizing synergistic response.

5.2 Sub lethal growth effects

A common underlying theme from the results in all three data chapters is how sublethal exposures impact size. Elevated temperature, morpholine exposure and chronic irradiation all resulted in changes in embryo body length and/or weight. Size changes could be the result of differences in development rate; embryos which are further ahead in development are generally larger. Conversely, embryos could be progressing through development at the same rate but are growing more efficiently and are therefore larger at any one stage of development. Accelerated development rate and growth stimulation are likely both involved in the increased size observed following these three different types of exposures.

Embryos incubated in front of Bruce Power were continually exposed to slightly warmer temperatures, which are known to advance development rate in lake whitefish (Price

1940, Brooke 1975, Griffiths 1979, Mueller et al. 2015). Therefore, the larger body length observed in retrieved embryos was likely due to an acceleration of development. Chronically irradiated embryos were larger in length and weight when measured on day 76 and day 122 post fertilization. The size enhancement in irradiated embryos could be due to accelerated development or enhanced growth. Once embryos reach the midpoint of the total time-to-hatch, most of the major organ systems and developmental features have formed (Sreetharan et al. 2015). The latter half of time-to-hatch is mainly just growth. During this time there are very few distinct development landmarks to identify if embryos are at the same development stage. Morpholine concentrations of 1 and 10 mg/L resulted in embryos which were significantly larger at hatch. No differences were seen in the timing of hatch, suggesting that size effects were not due to changes in development rate, but that these concentrations were stimulating growth.

A hormetic stimulation in growth has been demonstrated in other organisms following both chemical and radiological exposures (Calabrese and Baldwin 2000), but has never been observed during embryonic development in fish. We demonstrate here that both low dose chronic irradiation and chronic morpholine exposure can increase size in lake whitefish. Incubation in chronic morpholine from fertilization resulted in a characteristic hormetic j-shaped curve, where low concentrations produced larger hatchling than controls, compared to smaller hatches at high concentrations. In addition, we demonstrate with irradiated embryos that this hormetic stimulation does not come at a cost to metabolic efficiency.

5.3 Environmental relevance

The results of this thesis suggest that embryonic survival would not be directly impacted by thermal, chemical or radiological exposure at environmental levels. Recorded temperatures in the vicinity of Bruce Power near thermal discharge channels were well below lethal levels for lake whitefish. Average site temperatures were between 0.5°C - 3.7°C which is within the optimal range of 0.5°C - 6°C for lake whitefish development (Price 1940, Brooke 1975, Griffiths 1979). A significant increase in mortality from morpholine occurred following a 500 mg/L chronic exposure while sodium hypochlorite exposure impacted survival at 0.5 mg/L. Both of these concentrations are at least one order of magnitude greater than the Bruce Power discharge limits of 25 mg/L for morholine and 0.01 mg/L for TRC from sodium hypochlorite (Bruce Power 2005). Ionizing radiation delivered at the most sensitive point in development only induced mortality at 3.88 Gy. Environmentally, embryos would experience a combination of internal and external exposures from discharged radionuclides. Equating radiological releases to a dose rate depends on the uptake and retention of each isotope. At CANDU nuclear power plants, the largest radionuclide release is tritium. Levels of tritium in the discharge are still well below regulatory limits of 7000 Bq/L (Bruce Power 2014). Internal dose rates from tritium have been calculated in developing embryos of other fish species (Etoh and Hyodo-Taguchi 1983). A concentration of 7000 Bq/L of tritium would result in a dose rate on the order of several nGy per day, which is well below any lethal levels observed in lake whitefish.

Although survival would likely not be impacted, subletal effects were observed at environmentally relevant temperatures, morpholine concentrations and radiological doses. Both temperature and radiation resulted in an advanced hatch. An earlier hatch was modelled and predicted based on temperature data at all sites in front of Bruce Power and was supported by morphometric measurements. All chronic radiation doses, as low as 0.008 mGy/day, resulted in an earlier hatch compared to non-irradiated controls. Morpholine concentrations of 1 or 10 mg/L increased larval size at hatch. These changes could affect post hatch survival, positively or negatively, depending on predator and prey availability at the time of advanced hatching, and the impacts of size differences on larval behavior and fitness.

5.4 Lake whitefish

When compared to related aquatic species, lake whitefish were more resistant to chemical and radiological stressors. Lethal values for acute radiation exposure were above what has been reported for embryonic development in other species. No mortality was seen in acute morpholine or sodium hypochlorite treatments up to the highest tested concentrations. One likely reason for increased resistance is the prolonged development period in lake whitefish. A slow development rate allows time for repair of potentially lethal damage and short duration exposures of 96 hours only impact a small percentage of the total development period.

The results of Chapters 3 and 4 highlight interesting differences in stages where developing embryos are most sensitive to chemical or radiological exposure. Embryonic

mortality from radiation was highest when delivered immediately post fertilization and decreased later in development. Sensitivity to morpholine exposure on the other hand was highest at hatch. The higher resistance to chemical exposure early in development is likely due to changes in chorion permeability. The developing embryos may well be more sensitive to mopholine early in development, but because of chorion protection they are not directly exposed to it until closer to hatch. The chorion provides little protection however against external gamma irradiation. Photons are only very minimally attenuated by the thin egg shell, therefore radiation sensitivity is highest early in development when there are fewer, more actively dividing cells.

The results of this thesis are not only applicable to lake whitefish but are relevant to embryonic development in other aquatic species. The sensitivity to each individual stressor may vary between species but the overall response will likely be similar. Lake whitefish are a good model organism. Embryos are easy to rear in large numbers under minimal equipment and space requirements. The chorion is relatively transparent allowing direct visualization of the developing embryo. They are useful for studying the effects of low dose chemical or radiological stressors due to their slow development rate, thereby allowing extended exposure and large accumulated doses. The growth stimulation seen in lake whitefish may not have been observed in more commonly studied faster developing species such as zebrafish (Kimmel et al. 1995). However, there are some challenges in working with lake whitefish. Adult fish only spawn in the late fall, meaning that embryonic experiments can only be conducted once per year. Although a long development period is useful for certain exposures, it means that experiments looking at

the entire embryogenesis can take up to 6 months to complete. There are few commercial sources of lake whitefish embryos and collection in late fall can be difficult and expensive due to weather conditions. Lastly, lake whitefish take several years to reach sexual maturity (Hart 1931) making it difficult to examine multigenerational effects.

5.5 Embryo rearing

In the initial planning of experiments, logistical challenges were encountered with how to successfully raise embryos. Lake whitefish had previously only been raised in large scale apparatus using continuous water flow through or aeration (Price 1940, Brooke 1975). These types of setups were not convenient for examining the effects of multiple chemical or radiological exposures. Conventional systems required too much space for a high number of replicates and chemical exposures necessitated that different concentration be run on separate water supplies. To overcome these limitations, several unique systems were custom designed for lake whitefish rearing. In the laboratory, a small scale hatchery was built within a 2 door chromatography refrigerator, incorporating both upwelling hatching jars on a recirculated filtered water supply and shelf space for static water petri dish rearing (Mitz et al. 2014, Appendix A). Using these systems, over the course of four years, close to half a million lake whitefish embryos were raised in hatching jars and several hundred individual petri dishes.

The success of petri dish rearing enabled the construction of a chronic irradiator. The irradiator needed to fit within a refrigerator to maintain temperature and required enough shielding to eliminate leakage of gamma rays, both of which placed restriction of the size

and weight of the containment unit. The ability to raise embryos in petri dishes allowed an adequate number of replicate embryo groups to be raised at each dose rate, which could not have been achieved with large scale hatching jar rearing. Also, petri dishes removed the need to have a continuous flow of water in close proximity to a radiation source.

In addition to the laboratory setups, an incubation chamber system was constructed for in lake in-situ rearing. The chain and anchor system was designed to minimize movement throughout the winter due to wave action and ice scour, and to be easily retrieved the following spring. Over the course of three years, 90% of the systems deployed were successfully recovered. The design of the chambers themselves was modified throughout the course of the study and by year 3 all chamber were recovered intact and undamaged.

An important finding from Chapter 2 is the accuracy of laboratory rearing to describe natural in-lake development. The prediction of time-to-hatch based on data logger temperatures matched remarkably close to predictions based on morphometric measurements. Temperature modelling was based on growth rate curves generated by Brooke (1975), Griffiths (1979) and Mueller et al. (2014). All three of these studies measured time-to-hatch at various temperatures in laboratory reared embryos. When this laboratory based temperature model was applied to field temperature data, it accurately described development in retrieved lake whitefish embryos incubated in-situ.

Chemical and radiological stressors were examined using various doses, time points and incorporating both chronic and acute exposures. Conducting these types of experiments

in-situ is virtually impossible and therefore controlled laboratory rearing needed to be used. The findings from Chapter 2 give confidence that laboratory results closely describe what would occur during in-lake development.

5.6 Future directions

The work in this thesis focused solely on the impacts of thermal, chemical and radiation exposure on embryonic development. The endpoint in all experiments was hatching, at which point surviving fish were fixed for morphometric analysis. The effects of these stressors on lake whitefish are still largely unknown during later life stages. Adult lake whitefish spend most of their life in cold deep water and are therefore not likely to be directly influenced by cooling water discharges during the adult stage. Newly hatched fish however reside in shallow water nursery beds for several months before moving into deeper water later in the summer (Hart 1931). Therefore, like embryos, larval fish could be subjected to these same environmental stressors. Thermal stress may not have as great a potential impact since larval fish in general prefer warmer temperatures compared to embryos (Edsall 1999) and they are mobile and can avoid sub-optimal conditions. The results of Chapter 3 though suggest that lake whitefish embryos are resistant to morpholine and sodium hypochlorite due to protection from the chorion. It may be the case then that newly hatched fish are more sensitive to chemical exposure compared to developing embryos.

Another avenue for future research, which would provide an ecological context for the results in this thesis, is the impacts that differences in body and yolk size at hatch have on

behavior, survival and fitness. All three of the stressors examined here impacted embryo size. Fish that are larger or smaller at hatch could impact the ability to avoid predation or to forage for food. Changes in yolk size will alter how long larval fish can survive before switching to exogenous feeding. In addition to size changes, differences in hatch timing were observed with each of the stressors. In order to understand what the potential effects of hatch differences are, temporal patterns in the abundance of larval lake whitefish prey and predators need to be identified.

A topic that was addressed in Chapter 4 was the impact of combined stressors. From an environmental perspective, organisms are continuously exposed to multiple stressors. With the exception of controlled laboratory rearing, single isolated exposures rarely occur. The dual stressor results from Chapter 4 show that when combined, exposures can have complex interactions. Both synergistic and adaptive results were seen when combining heat or chemical exposure with radiation. These results also demonstrate that timing of priming and challenge doses are important, as heat shock produced an adaptive response at the 6 hour time point but no effect at 24 hours. In order to properly design experiments looking at combinations of exposures, a thorough understanding is needed of the impacts of the stressors individually. The results of this thesis lay the framework for future experiments looking at multiple combinations of thermal, chemical and radiological stress. Additional time points could be examined between heat shock and radiation exposure to identify the peak adaptive response and the time course over which it diminishes. Experiments can also be conducted examining the effects of combined chronic and acute exposures. Hormetic effects were observed from both chronic radiation

and chronic morpholine, and it would be interesting to see how these low dose chronic exposures could alter the response to an acute stressor.

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Appendix A

A self-contained, controlled hatchery system for rearing lake whitefish embryos for experimental aquaculture

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A.1 Abstract

A self-contained, small-scale research hatchery was constructed in a modified chromatography refrigerator equipped with a filtered and UV-sterilized water recirculation system. Lake whitefish embryos were raised in conventional upwelling hatching jars, in dishes with a continuous slow “drip feed”, and using a variety of static water incubation systems in petri dishes and multiwell plates. The optimal rearing density for petri dishes was found to be 50 embryos per dish, with weekly water changes. The highest survival in multiwell plates was seen in the 6 and 24 well sizes. Survival rates in most multiwell plates and petri dishes, as well as in the hatching jar incubators, was between 40 – 60%, which is in line with survival rates seen in commercial large scale rearing. Overall, these techniques permitted the rearing of large numbers of whitefish in separate batches and under controlled conditions, while greatly reducing space requirements and material costs. Our system is well suited for research and other situations requiring the temperature controlled rearing of embryos on a small scale.

A.2 Introduction

Considerable research and experimentation has been devoted to the development of fish rearing techniques for fisheries enhancement, restoration, and aquaculture. Many of the well-established rearing methods are designed to produce large numbers of similarly sized healthy fingerlings suitable for culture or stocking. Production of fish for research purposes encompasses a somewhat different suite of requirements, including the ability to conduct a large number of experimental trials under controlled temperature and water quality conditions. Similarly, it may be desirable to simultaneously raise multiple genetic lines of rare or endangered species in distinct batches. These requirements have spurred a number of innovative techniques including small scale incubation methods (Wedekind et al. 2001, Wedekind and Müller 2004, Barnes and Durben 2008), in-situ incubators (Gunn and Keller 1984, Manny et al. 1989), and even apparatus for rearing single embryos (Bardega and Luczynski 2007).

To test the impacts of multiple stressors (thermal, chemical, and radiological) on lake whitefish (*Coregonus clupeaformis*) embryos at different stages of embryogenesis, we required a closed and controlled hatchery system with a small foot print. Exposure to radioisotopes necessitated that embryos be housed in a commissioned radioisotope laboratory rather than an aquatics facility. To accommodate the chemical and radiological exposures, as well as the space constraints, we built a closed recirculating system that uses dechlorinated municipal water and is able to maintain low stable incubation temperatures.

A.3 Methods

A.3.1 Apparatus

To house developing embryos, we built two self-contained hatchery units (Figures A.1 and A.2). The basis of each unit was a 1.33 m^3 capacity VWR GDM-47 2 Door Chromatography Refrigerator with a Dixel XR02CX Digital Temperature Controller. The interior components of the system comprised a custom fabricated glass aquarium as a lower reservoir (130 L nominal capacity) with a single bulkhead fitting to magnetically coupled pumps mounted on the exterior of the refrigerator unit (combined capacity ca. 30 L/min). Two pumps were plumbed to provide redundancy in the event of a mechanical failure. Water from the lower reservoir was pumped through a media filter (i.e. carbon) and UV sanitization unit to an upper reservoir constructed of 75 mm diameter potable water grade PVC pipe with a vented overflow return to the lower reservoir to maintain constant and stable water pressures. Water from the upper reservoir was directed through two 50 mm diameter PVC manifolds supplying controlled amounts of water to individual drip fed Petri dish incubators and to a bank of seven upwelling hatching jar incubators (J32 Mini Hatching Jar, 300 mm high x 150 mm diameter; cap. maximum of 10,000 embryos per jar; Aquatic Eco-systems Incorporated) outletting to the lower reservoir. Temperature monitoring was provided using the factory installed temperature controller and Schlumberger DI 501 Mini-Diver loggers (accurate to 0.01°C) installed in the upper and lower reservoirs and connected to a remote computer using direct-read cables. Units

were supplied with power by duplex outlets connected to the building emergency backup power system.

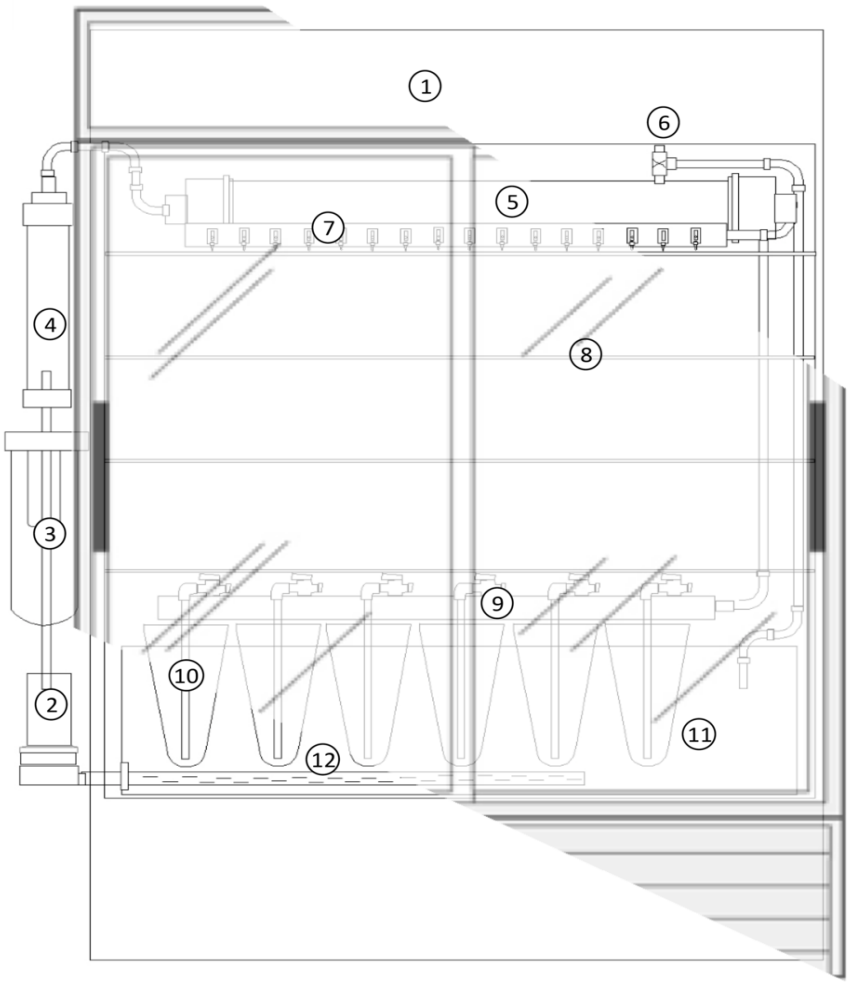

Figure A.1. Embryo rearing apparatus. 1. VWR GDM-47 2 Door Chromatography Refrigerator; 2. 2 x Little Giant® PM3, 50 W magnetically coupled pumps; 3. 14-GCB2-10 Giant Carbon Block Filter; 4. 25 W EU25-U Emperor UV Sterilizer; 5. 75 mm diameter PVC reservoir ; 6. Vented overflow return; 7. 50 mm dia. PVC header with approx 20 valved discharge ports; 8. Wire shelves for micro-incubators; 9. 50 mm dia. PVC header with eight valved ports; 10. Hatching jar incubators; 11. Lower reservoir; 12. Filtered intake.

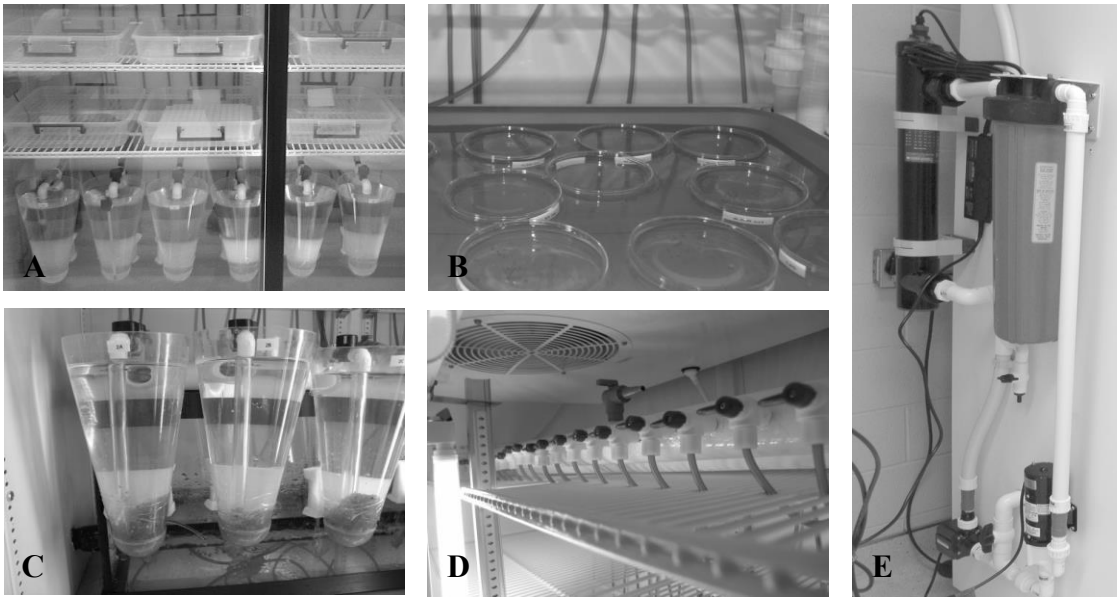

Figure A.2. Detailed view of embryo incubator apparatus components. (A) Incubator trays and upwelling incubators (B) Static water Petri dish incubators on the shelves of the unit (C) Mini-hatch jar incubators (D) Vented manifold for the distribution of reservoir water to various incubators (E) Pumps and filters mounted on the exterior of the cooler units.

Flow to the hatching jars was controlled by a dual valve system driven by the head pressure from the elevated reservoir. Flow rates to each hatching jar could be controlled between 0.1 and 3 L/min. In addition to the hatching jar incubators, micro-incubators were placed on the cooler shelves. These included drip feed incubation, and static water incubation using petri dishes and multiwell plates. The smaller drip-feed lines were controlled via a single valve for a slow drip to a maximum of 150 ml/min. The constant pressure provided by the elevated reservoir gave stable flows over all ranges but the use of T-connector to divide the lines between two micro-incubators reduced flow stability as flow changed from one branch to another in an erratic manner. The latter could be remedied by placing a valve downstream of the T-connector for each micro-incubator.

A.3.2 Embryos

Lake whitefish embryos were produced via in vitro fertilization from gametes collected from Lake Simcoe (2011-2012) and Lake Huron (2012-2013). Lake Simcoe embryos were provided by the Ontario Ministry of Natural Resources at the eyed embryo stages. Lake Huron whitefish were gillnetted on November 15, 2012 between 3 and 6 m of ca. 7°C water south of Whitefish Island (44°42'37.74"N, 81°18'38.94"W). Eggs were stripped from 18 females, mixed with milt from 27 ripe males, allowed to harden, and then disinfected with Ovadine® (5 ml/L, Syndel Laboratories Limited) prior to being transported in 1.5 L plastic jars by truck to the rearing laboratory. All animal procedures were completed with approval from McMaster University's Animal Ethics Review Board.

A.3.3 Experimental design

Lake whitefish embryos were reared in both constant flow through hatching jar incubators as well as petri dish and multiwell plate static water incubators, from fertilization until hatch. Embryos measured approximately 3 mm in diameter, equating to 38 embryos per ml. In each refrigeration unit, 7 hatching jars were filled half full (5,000 embryos each, 130 ml) and maintained at a flow rate sufficient to impart a gentle rolling action to the eggs (typically about 1 L/min). Petri dishes (20 x 100 mm) and varying sized multiwell plates (6, 12, 24, 48 and 96 well) were used to examine the effects of embryo loading density. Petri dishes were loaded with Lake Simcoe embryos, between 20 and 180 per dish, to determine the percent viable hatch from the eyed stage. Dishes received either

minimal water changes, regular weekly water changes or were fed with the continuous drip feed system. Multiwell plates were loaded with Lake Huron embryos from fertilization, with a single embryo per well (Table A.1). Significant differences in embryonic survival were analyzed using a t-test (SigmaPlot 11.0).

Table A.1. Surface area and volumes for different still-water incubation methods.

Rearing dish	Embryo density	Surface area (cm^2 per embryo)	Volume (ml per embryo)	Wetted surface (cm^2 per embryo)
20 x 100 mm	25 per dish	3.14	6.28	8.17
20 x 100 mm	50 per dish	1.57	3.14	4.08
6 well plate	1 per well	9.60	15.5	35.35
12 well plate	1 per well	3.80	6.00	18.29
24 well plate	1 per well	2.00	3.50	12.88
48 well plate	1 per well	0.75	1.40	7.33
96 well plate	1 per well	0.32	0.37	3.42

Reservoir water was changed at a rate of approximately 10 L per day, or 8 % of total volume. In static water dishes, water was changed by gently spilling standing water from the petri dish and replacing with fresh water from the incubator reservoir, decanting again and replenishing water levels to about 1.5 cm depth (3/4 full). Water changes were carried out weekly and after removal of mortalities or hatched larvae. Water changes were less frequently carried out for the multiwell plates owing to their generally lower loading. Water was tested on a daily basis to monitor pH, ammonia, nitrite and nitrate levels (API freshwater test kit). In addition a full water analysis (AGAT Laboratories) was performed to test for anions, metals, conductivity, dissolved solids and alkalinity. Oxygen

concentration was determined in the jars and dishes using an O_2 microelectrode (Microelectrodes Incorporated MI-730).

The embryos were reared at temperatures of between 2 and 6°C. The unit air temperature as well as the reservoir water temperature were recorded at 1 minute time intervals throughout the development period to determine the temperature stability of circulating and static water incubation methods. Reservoir temperature was measured using built in Schlumberger DI 501 Mini-Diver loggers and air temperature was monitored using Onset HOBO Pendant (UA-002) loggers placed on the cooler shelves.

A.4 Results and discussion

A.4.1 Loading density

Survival in hatching jars was similar between both units, and was approximately 50% from fertilization to hatch. This is comparable to survival rates observed in similar lake whitefish studies (Price 1940, Brooke 1975) as well as the 30-50% survival from fertilization to hatch over the last three years at the White Lake Hatchery, which uses conventional intensive culture methods (J. Brumpton, Ontario Ministry of Natural Resources, personal communication, 2014). The majority of mortalities occurred early in development. Most embryos reaching the eyed stage survived until hatch, which is consistent for lake whitefish (Brooke 1975). Rearing embryos in multiwell plates allowed us to observe development in single isolated embryos, without them being impacted by

mortality or hatching events from neighboring embryos in the same dish. The 24 and 48 well plates were of most interest as they provided a large enough sample size per plate, while still allowing ample water volume per embryo. The 24 well plates showed a significantly higher survival rate at the eyed stage compared to the 48 well plates (Figure A.3, $p = 0.043$). Embryos were also raised in 6, 12 and 96 well plates, with 6 showing the highest survival and 12 showing the lowest, although this was not analyzed statistically. The hatching rate was found to be suppressed in 96-well plates compared to plates having a greater volume per well (data not shown). In petri dishes, constant flow drip feed had the greatest survival rates at higher loading densities. Static water dishes had survival rates greater than 80% when loaded with 50 or fewer eyed embryos with weekly water changes (Figure A.4). Minimal water changes led to poor survival in dishes with greater than 30 embryos. Although Wedekind et al. (2007) reported loading densities as high as 125 embryos (3.3 ml) per petri dish, we found the optimal loading density with weekly water changes to be 50 embryos (1.3 ml). Higher loading densities, similar to those reported by Wedekind et al. (2007), required more frequent water exchange and were accompanied by a marked increase in hatch rate. A similar increase in hatching rate with high embryo loading density was reported by Barnes and Durben (2008) for rainbow trout (*Oncorhynchus mykiss*) and splake (*Salvelinus namaycush* X *Salvelinus fontinalis*).

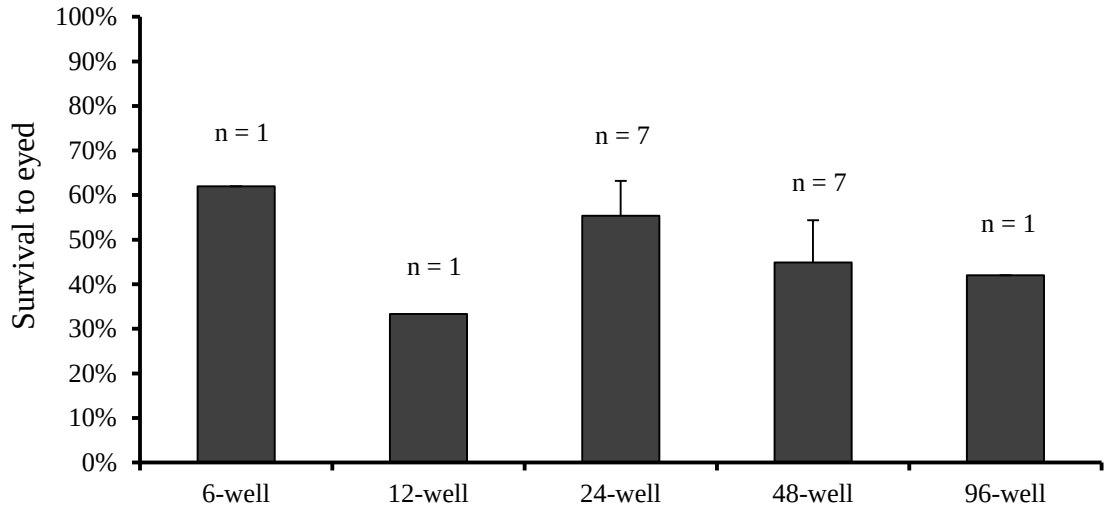

Figure A.3. Percent lake whitefish embryo survival at eyed stage in various size multiwell plates. Embryos were produced from Lake Huron gametes and incbated in multiwell plates from fertilization until the eyed stage. Embryos in 24 well plates had significantly higher survival that in 48 well plates ($p = 0.043$). n = number of plates used, error bars represent SD.

The multiwell plates became visibly coated with a bacteria/fungi film after about 6 weeks. Multiwell plates generally have a higher surface area of wetted plastic surface per embryo than the petri dishes (Table A.1). For this reason, water changes for the multiwell plates should include transferring the embryos to a new cleaned and disinfected multiwell plate rather than simply replacing the water.

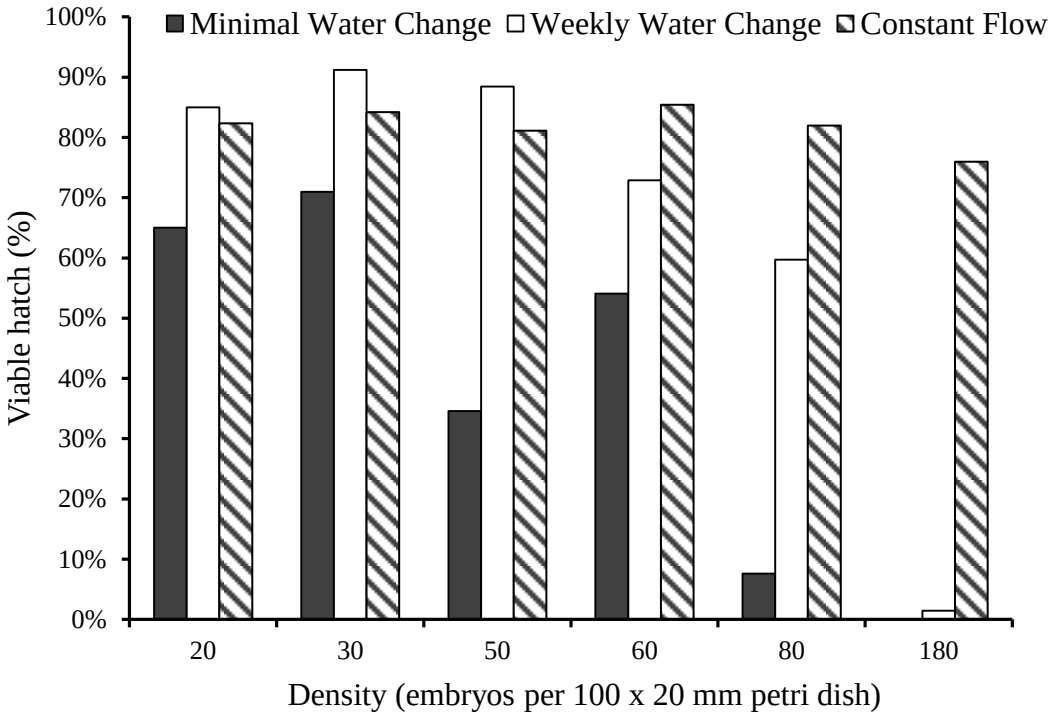

Figure A.4. Percent viable hatch of lake whitefish embryos in 100 x 20 mm petri dishes with various loading densities. Dishes were loaded with Lake Simcoe embryos at the eyed stage. Dishes received either minimal water changes, weekly water changes or were fed with a constant water flow through a drip feed system at 150 ml/min. Bars represent the percent hatch from 1 dish (n=1).

A.4.2 Temperature stability

Refrigerators operate by the compression and subsequent evaporation of a refrigerant. Since the temperature of the evaporator is below 0°C, it is susceptible to ice accumulation, particularly in humid environments (Lawrence and Evans 2008). Over time, the frost accumulation may become sufficient to cause the loss of cooling capacity. Our units were able maintain stable air temperatures as low as 2°C when used within their

normal operational limits and with regular defrosting cycles of short duration. This defrost cycle resulted in low amplitude temperature oscillations in the circulating water as shown on Figure A.5. The amplitude of these fluctuations, and the difference in temperature between the reservoirs and the cooler shelves can be modified by shorter defrost cycles, increased use of insulation, especially between the bottom of the cooler and the lower reservoir, and by increasing water volume in the reservoir. We found that a fluctuation of ± 0.2°C was acceptable for our purposes; still water incubation in petri dishes required the use of a water bath to dampen temperature fluctuations. The units were tested below their normal operational limit. Although we were able to maintain stable air temperatures lower than 1°C for several weeks, ice accumulation eventually caused cooling system failure and a spike in temperatures.

A.4.3 Water quality

Good water quality was achieved at loadings of up to 50,000 embryos (ca. 1.3 L) per incubator unit, except during the early stages of embryonic development and hatching, which caused elevated levels of ammonia. Although ammonia-induced mortalities were low, levels of 1 to 2 mg/L NH_3 occurred during hatching unless the water replacement rate was increased to 25% per day or more. pH values remained stable throughout development between 8 – 8.5. Dissolved solids were measured between 400 – 450 mg/L and alkalinity (as $CaCO_3$) between 150 – 250 mg/L.

Oxygen levels ranged from 70 to 90% of saturation throughout embryogenesis. This level of oxygenation is sufficient to ensure no adverse effects (Silver et al. 1967, Czerkies et al. 2002).

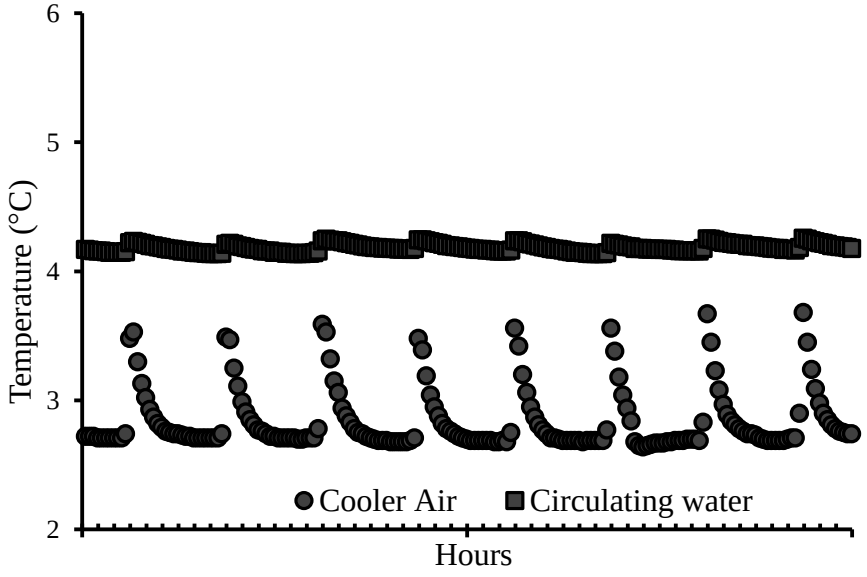

Figure A.5. Temperature fluctuations in circulating reservoir water and refrigerator air due to defrost cycling.

A.4.4 Conclusion

We report on the design and construction of a cost-effective, self-contained hatchery system for lake whitefish, with reliable temperature control and a small foot print. The unit makes use of a closed water recirculation system with filtration and UV sterilization that allows for multiple configurations of flowing and static water incubation methods. Our system is relatively inexpensive and easy to construct, and offers a novel approach for laboratories lacking conventional aquaculture facilities. This system was designed for

rearing of lake whitefish embryos, however the technology is likely transferable to other cold water spawning fish in the same or other families. Higher rearing temperatures or larger embryos, characteristic of the non coregonid salmonids, would likely require a lower loading density compared to those described here.

A.5 Acknowledgements

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