Early Life Stage Development and Biomarkers of Polycyclic Aromatic Compound Exposure in Lake Trout Fry From a Lake Used to Conduct Model Oil Spills

by

Cody Jackson

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Biological Sciences

University of Manitoba

Winnipeg

Copyright © 2022 by Cody Jackson

GENERAL ABSTRACT

In the summers of 2018 and 2019, controlled and contained spills of diluted bitumen (dilbit) were introduced into enclosures installed in Lake 260 at the IISD-Experimental Lakes Area (IISD-ELA). These additions were performed to examine the fate and behavior of oil in freshwater systems and to compare the efficacy of selected cleanup techniques. The potential for impacts to early development of lake trout arising from exposure to residual oil was examined by comparing the health of early life stages of lake trout (*Salvelinus namaycush*) obtained from the lake where oil studies were conducted to those from three reference lakes. Eggs were collected from fish in the four study lakes (n = 3-7) and reared in both an *in-situ* and laboratory environment. Eggs that were reared *in-situ* were assessed for hatching success, with no significant differences being observed between lakes. In the laboratory, fish were reared until swim-up and a subsample of fry from each lake were evaluated for deformities, with no increase in deformity rates observed in the study lake, compared to fry originating from the reference lakes. Fertilization success, egg mortality, time to 50% hatch, hatching success and growth rates were also similar among lakes. We also evaluated PAC accumulation in unfertilized eggs as well as molecular and biochemical markers of PAC exposure in swim-up fry. Polycyclic aromatic compounds were detected in freshly spawned eggs, however concentrations were lowest in samples originating from Lake 260, indicating that the PACs were unlikely to be related to the dilbit additions to enclosures. Cytochrome P4501A (*cyp1a*) induction, as well as total glutathione concentrations and the ratio of its oxidized and reduced forms were measured in whole swim-up fry and levels were found to be similar between the study lakes. Results from this study indicate that the efficacy of containment and contingency measures from the 2018 and 2019 model oil

spill studies at the IISD-ELA were effective. This research contributes to the growing body of literature examining the lower threshold of PAC toxicity in early life stage salmonids and highlights maternal transfer of PACs as a potential route of exposure in these fishes.

ACKNOWLEDGEMENTS

This project wouldn't have been possible without the help of so many great people. Only one month into my program, I began collecting lake trout eggs for my study at ELA. What initially seemed like an overwhelming task was quickly made easy thanks to the help of Fish Biologists, Lee Hrenchuk and Lauren Hayhurst. Lee and Lauren assisted with egg collection at every step and answered all my questions. A special thanks goes out to Lauren Timlick and Marco Haust for assisting with my field work for this study as well.

The laboratory component of this study wouldn't have been possible without the help of Kerry Wautier and A.J. Chapelsky. Kerry and A.J. facilitated the incubation of the lake trout eggs at DFO and were in the lab every weekend to remove moralities. I can't thank you guys enough for all your help during those five months.

Thank you to Ken Jeffries and Jay Treberg, for assisting with my glutathione and *cyp1a* assays, and to Theresa Mackey for all her help running my samples and editing my methods. To Lisa Peters and everyone at the COGRAD lab at the University of Manitoba, thank you for the assistance running my egg PAC assay.

To my good buddy and field partner, Adam Scott. Adam not only assisted with my initial egg collection but was present for every trip to check on my *in-situ* incubators at ELA. This meant nearly freezing his hands off to paddle out to our sampling locations in a particularly miserable late November trip. Hopefully the ice fishing trip on Roddy in March made up for that, pal.

A special thank you is in order for my fearless advisor, Vince Palace. I honestly couldn't have asked for a more knowledgeable and supportive advisor than Vince. Through every twist and turn, Vince was there to lend a helping hand (in the lab and the field), answer every question,

and calm my nerves when something didn't go as planned. Vince, your mentorship has meant the world to me – I look forward to many more rounds of golf in the coming years!

To my family and my partner, Camila, thanks for putting up with my crazy career choice that has led me to live in the wilderness more often than not. You have all been so supportive of my passion for science and the outdoors and I'll forever be grateful for that.

Lastly, I wanted to thank all the special people I met while working at ELA. There are too many to name here, but you know who you are. Summers spent at camp were some of the best of my life, and memories I'll cherish forever.

TABLE OF CONTENTS

GENERAL ABSTRACT ..... *i*
ACKNOWLEDGEMENTS ..... *iii*
CHAPTER 1 ..... *1*
GENERAL INTRODUCTION 2
DILUTED BITUMEN AND POLYCYCLIC AROMATIC COMPOUNDS 4
BOREAL AND FOReSt PROJECT OVERVIEW 12
STUDY AREA AND SPECIES 14
RESEARCH QUESTIONS AND OBJECTIVES 17
STUDY SIGNIFICANCE 18
CHAPTER 2 ..... *20*
ABSTRACT 21
INTRODUCTION 22
METHODS AND MATERIALS 26
RESULTS 37
DISCUSSION 41
CONCLUSION 50
TABLES AND FIGURES 52
CHAPTER 3 ..... *70*
ABSTRACT 71
INTRODUCTION 72
METHODS AND MATERIALS 77
RESULTS 89
DISCUSSION 91
CONCLUSION 98
TABLES AND FIGURES 100
CHAPTER 4 ..... *105*
REFERENCES *109*
APPENDIX A: ADDITIONAL FIGURES AND TABLES *125*
APPENDIX B: DEFORMITY RUBRIC *144*
APPENDIX C: STATISTICAL ANALYSIS *156*

LIST OF FIGURES

FIGURE 2.1. SCOTTY-JORDAN INCUBATORS. PICTURED ABOVE IS AN ASSEMBLED UNIT CONTAINING FIVE INDIVIDUAL TRAYS. 28
FIGURE 2.2. (A) VERTICAL INCUBATORS (HEATH TRAYS) USED FOR INCUBATIONS AT THE FRESHWATER INSTITUTE IN WINNIPEG, MANITOBA. (B) OVER-HEAD VIEW OF LAKE TROUT EGGS DIVIDED UP INTO 12 COMPARTMENTS OF A HEATH TRAY. 30
FIGURE 2.3. FLOW CHART DEPICTING EGG/FRY SAMPLING FOR THE STUDY. 32
FIGURE 2.4. CONDITION FACTOR AMONGST YOLK-SAC FRY (~550 ATU, LEFT) AND SWIM-UP FRY (~800 ATU, RIGHT) ORIGINATING FROM THE FOUR STUDY LAKES. 54
FIGURE 2.5. LENGTH AMONGST YOLK-SAC FRY (~550 ATU, LEFT) AND SWIM-UP FRY (~800 ATU, RIGHT) ORIGINATING FROM THE FOUR STUDY LAKES. 54
FIGURE 2.6. WEIGHT AMONGST YOLK-SAC FRY (~550 ATU, LEFT) AND SWIM-UP FRY (~800 ATU, RIGHT) ORIGINATING FROM THE FOUR STUDY LAKES. 55
FIGURE 2.7. YOLK VOLUME AMONGST YOLK-SAC FRY AT ~550 ATU DEVELOPMENT AND ORIGINATING FROM THE FOUR STUDY LAKES. 56
FIGURE 2.8. PERCENT HATCHING SUCCESS IN SCOTTY-JORDAN INCUBATORS AMONGST THE FOUR STUDY LAKES. 57
FIGURE 2.9. PERCENT HATCHING SUCCESS IN SCOTTY-JORDAN INCUBATORS FROM LAKE 260 AND THE THREE REFERENCE LAKES (LAKE 223, LAKE 375, LAKE 378) EXCLUDING A POTENTIAL OUTLIER IN LAKE 375 (LT2). 58
FIGURE 2.10. PERCENTAGE OF UNFERTILIZED EGGS IN HEATH TRAYS, AMONGST THE FOUR STUDY LAKES. 59
FIGURE 2.11. PERCENTAGE OF MORTALITY PRE-HATCH IN HEATH TRAYS, AMONGST THE FOUR STUDY LAKES. 60
FIGURE 2.12. PERCENTAGE OF SUCCESSFUL HATCHES IN HEATH TRAYS, AMONGST THE FOUR STUDY LAKES. 61
FIGURE 2.13. ACCUMULATED THERMAL UNITS (ATU) TO 50% HATCH. 62
FIGURE 2.14. PERCENTAGE OF MORTALITY IN HATCHED FRY, EXPRESSED AS A TOTAL ACROSS THE THREE HEATH TRAYS. 63
FIGURE 2.15. PERCENTAGE OF SEVERE EDEMA IN HATCHED FRY, EXPRESSED AS A TOTAL ACROSS THE THREE HEATH TRAYS. 64
FIGURE 2.16. TOTAL GSI SCORE AMONGST YOLK-SAC FRY (~550 ATU) ORIGINATING FROM THE FOUR STUDY LAKES. 65
FIGURE 2.17. TOTAL GSI SCORE AMONGST SWIM-UP FRY (~800 ATU) ORIGINATING FROM THE FOUR STUDY LAKES.. 66
FIGURE 2.18. PROBABILITIES OF VARIOUS GSI SCORES AMONG YOLK-SAC FRY (~550 ATU) FROM THE FOUR STUDY LAKES, PREDICTED FROM AN ORDINAL LOGISTIC REGRESSION MODEL.. 67

FIGURE 2.19. PROBABILITIES OF VARIOUS GSI SCORES AMONG SWIM-UP FRY (~800 ATU) FROM THE FOUR STUDY LAKES, PREDICTED FROM AN ORDINAL LOGISTIC REGRESSION MODEL.. 68
FIGURE 2.20. PERCENT DIFFERENCE BETWEEN SCORING ITERATIONS FOR YOLK-SAC FRY (~550 ATU, RED) AND SWIM-UP FRY (~800 ATU, BLUE).. 69
FIGURE 3.1. TOTAL (LEFT) AND ALKYLATED (RIGHT) POLYCYCLIC AROMATIC COMPOUND CONTENTS (NG/G WET WT.) AMONGST UNFERTILIZED EGGS ORIGINATING FROM THE FOUR STUDY LAKES.. 100
FIGURE 3.2. POLYCYCLIC AROMATIC COMPOUND CONTENT (NG/G WET WT.) AMONGST UNFERTILIZED EGGS ORIGINATING FROM THE FOUR STUDY LAKES.. 101
FIGURE 3.3. CYP1A GENE EXPRESSION AMONGST SWIM-UP FRY (~800ATU) ORIGINATING FROM THE FOUR STUDY LAKES AT THE IISD-EXPERIMENTAL LAKES AREA.. 102
FIGURE 3.4. TOTAL GLUTATHIONE CONTENTS (PMOL/MG WET WT) AMONGST SWIM-UP FRY (~800ATU) ORIGINATING FROM THE FOUR STUDY LAKES.. 103
FIGURE 3.5. RATIO OF REDUCED (GSH) TO OXIDIZED (GSSG) GLUTATHIONE AMONGST SWIM-UP FRY (~800ATU) ORIGINATING FROM THE FOUR STUDY LAKES..... 104

LIST OF TABLES

TABLE 2.1. WEIGHT, LENGTH, CONDITION FACTOR, EGG DIAMETER, EGG NUMBER/GRAM BODY WEIGHT AND EGG COLOUR FROM ADULT FEMALE FISH COLLECTED IN THE FOUR STUDY LAKES..... 52
TABLE 2.2. WEIGHT, LENGTH, AND CONDITION FACTOR FROM ADULT MALE FISH COLLECTED IN THE FOUR STUDY LAKES..... 52
TABLE 2.3. FREQUENCY AND MEAN SEVERITY (± SE) OF DEFORMITY SCORES FROM YOLK-SAC FRY (~550 ATU) IN THE FOUR STUDY LAKES AND THE WAF EXPOSED GROUP. 53
TABLE 2.4. FREQUENCY AND MEAN SEVERITY (± SE) OF DEFORMITY SCORES FROM SWIM-UP FRY (~800 ATU) IN THE FOUR STUDY LAKES, PLUS THE WAF EXPOSED GROUP. 53
TABLE 3.1. PAC COMPOUNDS INVESTIGATED IN LAKE TROUT EGGS..... 83
TABLE 3.2. PRIMER SEQUENCES FOR QUANTITATIVE PCR IN LAKE TROUT (SALVELINUS NAMAYCUSH)..... 85

CHAPTER 1

INTRODUCTION AND BACKGROUND

GENERAL INTRODUCTION

In Canada, and across the globe, oil production as a source of energy remains an important means of economic development, accounting for the largest share of global primary energy consumption (Statistical Review of World Energy, 2021). Pipelines are known to be the safest mode of transporting Canada's oil to markets; with rail transport being ~4.5 times more likely to experience a spill compared to pipelines when volumes of oil are normalized to the distances transported for both modes (Green and Jackson, 2015). Still, the threat of a spill from all modes of transportation remains. The public's resistance to the construction of new pipelines has been well publicized and continues to be a contentious issue in Canadian politics (Hoberg, 2016). First Nations and environmental groups have been vocal in expressing concern that construction of new pipelines will promote the expansion of the Alberta Oil Sands Region, which would further damage the surrounding environment. Construction of new pipelines themselves would inevitably also lead to increased spill incidence (Hoberg, 2016). In 2017, the International Institute for Sustainable Development-Experimental Lakes Area (IISD-ELA) embarked on a multi-year project to improve understanding of these issues with the goal of determining the best methods for remediating freshwater ecosystems affected by spills of heavy crude oil.

As one of the most commonly transported oil products, diluted bitumen was selected for use in these studies. In the summers of 2018 and 2019, controlled and contained spills of dilbit were introduced into enclosures installed in Lake 260 at the IISD-ELA. These additions were performed to examine the fate and behavior of the commonly transported oil mixture in freshwater systems and to compare the efficacy of selected cleanup techniques as part of the Boreal-lake Oil Release Experiment by Additions to Limnocorals (BOREAL) (Rodriguez-Gil et

al., 2021) and Freshwater Oil-Spill Remediation Study (FOReSt) studies, respectively. Even though both projects established rigorous environmental protection and contingency plans prior to the studies, additional monitoring for potential dispersion of oil constituents into the general lake environment was required as part of the authorization permits issued for the experiments.

IISD-ELA is a research station located in Northwestern Ontario – approximately 52 km east southeast from Kenora, Ont., and is comprised of 58 lakes of varying sizes. The site was opened in 1968, to study the impact of algal blooms on freshwater lakes, and to model processes occurring in the Great Lakes at the time (Stokstad, 2008). Wally E. Johnson and Jack Vallentyne are credited with establishing the research station, which has the capacity to carry out whole ecosystem studies, making it a truly unique facility globally. The data collected from IISD-ELA has been highly influential in policy decisions, dating back to the 1980's (Stokstad, 2008). Among the most notable include works examining the contribution of anthropogenic phosphorus and nitrogen in algal blooms (Findlay and Kasian, 1987) and early research on the impacts of acid rain in freshwater lakes (Schindler et al., 1980). More recently, researchers at the Experimental Lakes Area carried out a host of studies examining mercury loading and cycling in the environment (Kelly et al., 1997; Bodaly et al., 2004; Sandilands et al., 2008; Blanchfield et al., 2021), examined the impacts of artificial estrogen in wastewater arising from the use of hormonal birth control (Kidd et al., 2007), and have investigated the effects of climate change and subsequent increases in water temperatures on lake trout habitat availability (Guzzo and Blanchfield, 2017). The impacts of the BOREAL and FOReSt studies will continue to be monitored for at least a decade after the initial model spills (V. Palace, pers. comm.).

The current study examined the potential impacts of residual oil from model spills, conducted on Lake 260 at the IISD-ELA, on the development of early life stages of lake trout

(*Salvelinus namaycush*). Lake trout are cold water fishes belonging to the family Salmonidae. Salmonids are among the most sensitive fishes to potential developmental effects arising from exposure to dioxin-like compounds found in oil (Elonen et al., 1998). Comparisons were made between eggs originating from Lake 260 and those originating from three other IISD-ELA reference lakes (Lakes 223, 375, 378) that did not receive oil. The goal was to assess if there have been any significant impacts to lake trout embryonic or larval development in Lake 260. Markers of oil exposure and oxidative stress were also investigated in the developing embryos (Cytochrome P450 1A, glutathione) as additional indicators of potential chronic effects. Results from this study will satisfy a requirement set out in the *Statements of Authorization* from the Ontario Ministry of Environment and Climate Change (OMECC) for both the BOREAL and FOReSt projects.

DILUTED BITUMEN AND POLYCYCLIC AROMATIC COMPOUNDS

Composition and Physical Properties

Diluted bitumen is a mixture of bitumen (70-80%), the primary product mined from Alberta's oil sands region, and natural gas condensates (20-30%) (Crosby et al., 2013). Due to the high viscosity of bitumen alone, it cannot be readily transported through pipelines to refineries. To solve this problem, less viscous constituents (i.e. natural gas condensate) are added to the bitumen to allow for easier flow through these pipelines (Dew et al., 2015). Bitumen itself is a form of crude oil which has biodegraded over time. As a result, many of the low molecular weight compounds that were once a part of its chemical make-up have dissipated and the result is a mixture of high molecular weight n-alkanes, alkylated polycyclic aromatic compounds (PAC), as well as resins, asphaltenes and heavy metals. The diluents added to bitumen to reduce its

viscosity to facilitate transport through pipelines consist of low molecular weight saturates, and volatile organic compounds such as benzene, toluene, ethylbenzene, and xylene (reviewed in Lee et al., 2015).

In the event of a spill, the behaviour of dilbit can vary significantly with changing environmental conditions. This process, known as "weathering", is due to the evaporation, photo-oxidation, emulsification, dispersion, biodegradation, and sedimentation that will occur when dilbit is exposed to the environment (reviewed in Lee et al., 2015). Considering changes in the chemical composition of dilbit are crucial to understanding how it will interact with freshwater ecosystems. Multiple factors, such as wind, rain (i.e. increased mixing into the water column) and exposure to sunlight (i.e. photo-oxidation), can influence how dilbit weathers after a spill, and ultimately how it will affect the ecology of an impacted area (reviewed in Lee et al., 2015).

The density of oil immediately post-spill and after weathering has implications for clean-up operations in freshwater environments. When initially spilled, Cold Lake blend dilbit – the highest volume dilbit type transported in Canada – has a density less than that of fresh water at 15°C (23° API gravity) (reviewed in Lee et al., 2015). After weathering, the lower molecular weight components of oil will dissipate, and its density is likely to become greater than fresh water and sink, however this phenomenon was only recently confirmed to occur (reviewed in Lee et al., 2015; Stoyanovich et al., 2019). The behaviour of oil after weathering was investigated in a recent study conducted at the IISD-ELA, which examined the fate of diluted bitumen in a simulated freshwater spill (Stoyanovich et al., 2019). As the lighter ends of the oil volatilized, the density of the dilbit was greater than water and the dilbit submerged to the bottom of the experimental mesocosms.

Effects of PACs on Fish Embryos and Larvae

Previous studies have shown that salmonids are particularly sensitive to oil constituents (Le Bihanic et al., 2014; Alderman et al., 2018; Berube et al., 2022; Rigaud et al., 2022). Polycyclic aromatic compounds are the main drivers of this toxicity, and as such are often the primary focus of studies examining the effects of oil on early life stage fish. Rainbow trout (*Oncorhyncus mykiss*) embryos exposed to environmentally relevant total PAC (TPAC) concentrations as low as 0.7 μg/g in sediment showed evidence of developmental defects (Le Bihanic et al., 2014). In a similar study, researchers examined PAC toxicity in the sockeye salmon (*Oncorhynchus nerka*) embryos exposed to water soluble fractions of dilbit containing 35 μg/L TPAC (Alderman et al., 2018). At this concentration, fry showed an increased incidence of developmental deformities compared to reference fish (Alderman et al., 2018). More recently, early life stage rainbow trout were studied to compare the effects of low exposures to unconventional (i.e. dilbit) and conventional oils (2.4 – 17.5 μg/L) (Berube et al., 2022). Higher dose exposures to both types of oil resulted in hatching delays and significant mortality, while dilbit exposed fish also experienced increases in malformation rates. While model salmonid species such as rainbow trout have been more frequently investigated in toxicological studies, attention has recently been paid to sensitivity differences within this family of fishes. Rigaud et al. (2022) determined that brown trout (*Salmo trutta*) were more sensitive to retene PAC exposure than rainbow trout. When exposed 320 μg/L of retene, a three-ringed alkylated PAC, over a period of 15 days, brown trout experienced higher mortality rates. When exposed to lower concentrations of the compound (10 or 100 μg/L) brown trout showed an increased incidence of pericardial edemas and hemorrhages, effects characteristic of blue-sac disease (BSD). Blue-sac disease is a syndrome which has long been known to manifest itself as edemas and cranial

deformities in salmonid fry exposed to retene (Brinkworth et al., 2003). The signs of BSD can be wide ranging, with other potential effects such as cardiotoxicity, ocular and spinal deformities as well as degeneration of muscle somites, peripheral neurons and endothelial cells. Changes in behaviour and swimming ability can also result secondarily from impacts to the swim bladder and fin erosion as a result of BSD (reviewed in Lee at al., 2015).

In some cases, the initial response to PAC exposure in early life stage fish can only be detected by measuring well-established biomarkers. Elevated levels of these biomarkers can indicate cellular level changes which can precede effects on growth, disease, and survival (reviewed in Lee et al., 2015). For normal embryo development to occur, a multitude of genes require activation in a specific sequence and exposure to PACs is known to interfere with this tightly regulated process (reviewed in Lee et al., 2015). This high degree of sensitivity has led researchers to rely on biomarkers such as cytochrome P450 (*cyp1a* gene/CYP1A protein) and glutathione redox status as a means of early detection when exposure to xenobiotics such as PACs is thought to have occurred (reviewed in Sarasquete and Segner, 2000; reviewed in Santana et al., 2018). *cyp1a* has been utilized to detect PAC exposure in salmonid species at retene concentrations as low as 3.2 µg/L, while significant changes to glutathione redox status have been observed in 320 µg/L retene exposures (Rigaud et al., 2022; Bauder et al., 2005)).

Upon introduction to the organism, often via the gills or from a food source, PACs can freely cross the lipid composed cell membrane due to their hydrophobic structure. Once internalized, certain PACs can bind to the aryl hydrocarbon receptor (AhR) in the cytosol (Tuvikene, 1995). Upon binding, a subunit of the AhR migrates to the nucleus to initiate the transcription of the *cyp1a* gene, which is subsequently translated to the CYP1A protein, a unique enzyme capable of catalyzing a multitude of reactions, as part of "phase I" of the organism's

detoxification response. Among them are hydrolyzation, oxidation and reduction reactions for several types of substrates (Santana et al., 2018). These reactions can convert lipophilic hydrocarbons to more hydrophilic metabolites, enabling excretion through bile or in urine (reviewed in Dearnley et al., 2020). When *cyp1a* is induced for prolonged periods, the monooxygenation reactions it catalyzes are known to lead to an overabundance of reactive oxygen species (ROS), overwhelming antioxidant capacity that normally maintains the cellular redox environment (Bauder et al., 2005). Elevated levels of ROS in the cell cause damage to DNA, proteins, and lipids (Birnie-Gauvin et al., 2017)

In addition to the effects related to ROS, it is primarily the metabolites produced from the parent PAC compounds which can cause toxicity leading to syndromes such as BSD. The biotransformation of parent compounds to a more electrophilic metabolite can result in covalent bonding to DNA, RNA, and proteins, impairing proper cellular function (Tuvikene, 1995; Franco and Lavado 2019). While not all PACs will induce *cyp1a* gene expression – due to variable binding affinity for the AhR or an inherent recalcitrance – all PACs can be oxygenated by CYP1A proteins for excretion (Barron et al., 2004; reviewed in Lee et al., 2015; Lindberg et al., 2017). Most oil mixtures will contain PACs that will induce the *cyp1a* gene and production of the CYP1A enzyme and in some instances, the metabolite produced can be excreted after modification in this initial detoxification step (i.e. “phase I”). Quite often, subsequent reactions are required to eliminate the xenobiotic from the system, including “phase II” enzymes that bind the transformed metabolite to large polar molecules, referred to as conjugation reactions, so that the entire complex can be more readily excreted in aqueous media (Hodson et al., 2007; reviewed in Lee et al., 2015).

Phase II reactions involve cytosolic scavengers and various antioxidant enzymes as part of the detoxification response in fish (Santana et al., 2018). These enzymes can be upregulated by transcriptional activation (often via AhR) and are required to prevent further cellular damage (Regoli and Giuliani, 2014). Glutathione (GSH) is a cellular antioxidant that can interact with ROS and in doing so is converted to its oxidized form, glutathione disulfide (GSSG). This process can be catalyzed by enzymes such as glutathione-S-transferases (GST) and glutathione peroxidase (GPx), thus converting more harmful hydroperoxides to water (Massarsky et al., 2017). GSSH is then regenerated back to GSH by the enzyme glutathione reductase (GR), using NADPH as an electron donating co-factor in the reaction. This balance between oxidized (~5%) and reduced glutathione (~95%) is crucial for maintaining cellular function in fish (Regoli and Giuliani, 2014; Bauder et al., 2005; Leggatt, 2006). As such, the ratio of reduced to oxidized glutathione can be a useful indicator of oxidative stress in an organism, with an increase in oxidized glutathione being indicative of an organism experiencing either acute or chronic cellular stress (Bauder et al., 2005). GSH is also a component of the conjugation reactions involving partially metabolized PACs from phase I reactions. These subsequent reactions produce a non-toxic polar compound that can be effectively excreted by the organism, thus eliminating the xenobiotic from the system (Regoli and Giuliani, 2014).

Other notable toxic responses to PAC exposure in fish embryos and larvae include cardiotoxicity and impaired eye development. Previous studies have shown that environmentally relevant concentrations of PACs can negatively impact heart development in salmonids by triggering hypertrophy in the spongy myocardium, while retene exposure can possibly alter Wnt and NF-κB signalling, impacting cell adhesion and motility in developing rainbow trout (Incardona et al., 2015; Vehniäinen et al., 2016). Development may appear to proceed normally

in fish embryos exposed to tricyclic PACs but heart defects that will impair fitness can develop later in life (Hicken et al., 2011). A primary consequence of diminished heart function in juvenile and adult fish is a reduction in swimming ability, which can increase the risk of predation, while also limiting the capacity for prey capture. Indirect effects such as a reduction in basal metabolism and growth due to poor cardiac function can also negatively impact fitness (Incardona et al., 2015). The mechanism for this toxicity has been shown to have two distinct pathways which are generally classified as either AhR-dependent or AhR-independent (Incardona, 2017). Cardiac morphology is impacted before function is affected in AhR-dependent mechanisms, whereas the reverse is true with AhR-independent mechanisms (Incardona, 2017). Cardiac looping (i.e. formation of cardiac chambers) and cardiomyocyte proliferation can be adversely affected before any observable changes in function can be detected in AhR-dependent toxicity, while changes in contractability and arrythmia are typically detected prior to changes in form when toxicity is independent of the AhR (Incardona, 2004; Incardona, 2017; Marris et al., 2020). This differing response is due to the variability of binding affinity to the AhR in the numerous PAC structures (Barron et al., 2004). In short, AhR-independent cardiotoxicity results from non-alkylated tricyclic PACs interacting directly with intracellular proteins that regulate excitation contraction coupling and action potentials, which can negatively affect intracellular calcium signaling (Brette et al., 2017). Conversely, AhR-dependent cardiotoxicity is a by-product of the dimerization of certain PACs with the AhR, which can initiate the induction of genes that target cardiovascular tissue (Vehniäinen et al., 2016). When early life stage fish are exposed to complex mixtures of PACs, such as those found in dilbit or crude oil, it is likely that both mechanisms contribute to this toxic response (Incardona, 2017).

In eyed fishes, impairment of visual structures inevitably leads to reduced fitness. In developing fish, adequate visual stimuli are required to initiate proper feeding behaviour and evade predation (Carvalho et al., 2004). It has been suggested that eye development is tightly linked to proper cardiac function in larval zebrafish (*Danio rerio*). Exposure to a water accommodate fraction (WAF) of crude oil resulted in reduced eye development, and it was hypothesized that the mode of action of this malformation may have resulted from poor cardiac development, in addition to direct effects on the eye itself, however the exact mechanism of these effects remains unknown (Magnuson et al., 2020). Furthermore, PACs have been shown to adversely affect eye development in larval white sucker (*Catostomus commersonii*) and fathead minnows (*Pimephales promelas*), with poor retinal differentiation, unilateral or bilateral micropthalmia, patent optic fissures and dysphasic retinas being observed (Colavecchia et al., 2007). An increase in eye deformities has been correlated with an increase in *cyp1a* induction in these species, although researchers noted varying degrees of sensitivity to PACs, suggesting a species-specific response (Colavecchia et al., 2007). More recent studies have pointed to several genes not associated with the AhR mediated pathway (i.e. *cyp1a* induction) of toxicity as being downregulated after exposure to PAC in larval mahi-mahi (*Coryphaenidae hippurus*). For example, the transcripts for proteins involved in the visual transduction cascade (*rho*), membrane hyperpolarization – leading to the release of neurotransmitters (*gnat2, pde6g*) and the recovery of light from the photolyzed rhodopsin (*grk7, regs9*) were all downregulated in mahi-mahi larvae exposed to Deepwater Horizon contaminated water (Xu et al., 2016). Eye development has yet to be thoroughly examined in salmonids exposed to PACs, although AhR mediated effects on ganglion cell densities in the retina have been observed after exposure to 2,3,7,8-tatrachlorodibenzo-p-dioxin (TCDD) in rainbow trout fry (Carvalho et el., 2004).

While the main routes of exposure to PACs in fish is often through trophic transfer or environmental uptake, maternal transfer to embryos through the yolk can also be significant (reviewed in Lee et al., 2015). Maternal exposures to PAC mixtures could lead to behavioural disruptions and bradycardia in zebrafish larvae, however no firm conclusions could be made attributing the PAC exposure to these teratogenic effects (Perrichon et al., 2015; Vignet et al., 2015). Decreased hatching success and an increase in deformities in fathead minnow embryos maternally exposed to anthracene, a three-ringed PAC, have been noted (Tilghman Hall and Oris, 1991). Most recently, maternal transfer has been demonstrated as a potential route of exposure in northern pike (*Esox lucius*) (Sundberg et al., 2007) and in pacific herring (*Clupea pallasii*) (West et al., 2014). To our knowledge, there is no known study examining maternal PAC transfer in salmonids, making it an important area of further research. Adult lake trout in Lake 260 had upregulated *cyp1a3* in the epidermal mucus following the simulated oil spill in 2019, indicating that an exposure to PACs may have occurred (Andrzejczyk, 2020). PACs transferred to eggs of fish from this study could have implications for lake trout population recruitment in Lake 260.

BOREAL AND FOReSt PROJECT OVERVIEW

Two seperate large scale oil spill studies were conducted in Lake 260 at the IISD-ELA from 2018-2020. These two separate studies addressed different aspects of oil behaviour and remediation and used different exposure methods. Brief descriptions of both projects are provided here to provide context for the analysis of potential effects in resident lake trout from Lake 260.

BOREAL Project

The BOREAL project applied dilbit into seven (7) individual 10m diameter octagonal mesocosms approximately 1.6m deep. The amounts of dilbit applied ranged between 1.5 and 179.8L, reflective of a range of oil:water ratios corresponding to the 75th to the 100th percentile of historic spills in North America over a decade (Rodriguez-Gil et al., 2021). Mesocosms consist of a floatation collar that supports an impermeable polyethylene curtain sealed to the sediments with a continuous row of sandbags. This isolates a column of water and the underlying sediments as a model ecosystem for the rest of the lake. In this case, they also served to contain the applied oil in relatively small areas for a period of 70d. However, in the fall of 2018 and just prior to the lake freezing, the flotation collars of the BOREAL enclosures were removed, and the curtains were cut approximately 1m beneath the water surface. As a result, the bulk of oil which had sunk to the sediment, was then exposed to the open water in Lake 260 for a duration of approximately 10 months before it was eventually removed in August 2019.

FOReSt Project

The FOReSt project may also have contributed oil contamination to Lake 260, but the volumes used for this project were much less than for the BOREAL project. In 2018 and 2019 this project used mesocosm structures (10m X 5m) modified to straddle the shoreline environment to examine the effectiveness of various cleanup techniques for small model spills (1-1.5L) applied at the shoreline. In 2018 the FOReSt project applied a total of ~3kg of crude oil to two (2) enclosures. These enclosures remained intact until oil could be removed from the shoreline in the fall of 2018, just prior to lake freezing. In 2019 the FOReSt program applied crude oil to 14 enclosures (~1kg each) and after free floating oil was removed 72h later, the

remaining oil (~90-95% of the initial mass) that was left in place over the winter of 2019 to facilitate additional study of bacterial degradation during the winter months. The enclosures froze in place and continued to effectively contain the remaining oil until they were dismantled in the spring of 2021. An increase in total PAC concentrations in the "lake reference" sampling location immediately after the contained spills indicates that there may have been some water exchange between the enclosed environments and the lake (V. Palace, pers. comm.).

STUDY AREA AND SPECIES

Study Lakes

Lake 260 was chosen as the site of 2018 BOREAL and 2019 FOReSt projects due to its proximity to the field station, and due to several limnological features. The lake is oligotrophic, and hydrologically isolated from the multitude of lakes in the area (Brunskill and Schindler, 1971). Lake 260 is a small lake, a common feature of all the IISD-ELA lakes, with a surface area of 34 ha, a maximum depth of 14.4 m and a volume of 17.64 x 10^5 m^3. The lake is composed of three main types of substrate (sand/organic, sand/cobble, and wetland/peat), making it a suitable study lake to test the response of different shoreline types to the various cleanup techniques utilized in the FOReSt project. The climate in the area is classified as "continental", with the region experiencing mean total annual precipitation of 705 mm, mean daily summer temperatures ranging from +4.7 to +19.4°C and mean daily winter temperatures ranging from -15.6 to +3.6°C (Emmerton et al., 2019).

Lakes 233, 375 and 378 were selected as reference lakes for the present study due to their similarity in limnological parameters to those of Lake 260, as well as their composition of fish species and relatively pristine nature. All three lakes contain healthy, spawning populations of

lake trout, however it should be noted that two of these lakes have been used for whole-lake manipulation studies in the past. Lake 223 was the site of an acidification experiment from 1976 – 1978, whereby sulfuric acid was added to the lake to simulate the effects of acid rain (Schindler et al., 1980). Lasting effects of this study include the extirpation of an important food source for lake trout, *Mysis diluviana* (Milling, 2020). The rapid decline of Mysis contributed to a reduction in both mean condition factor and species abundance of lake trout by 1986, eight years after acid addition was terminated. However, fish health and lake pH have rebounded to pre-study status in subsequent years (Mills et al., 2000). In 2003, an aquaculture cage containing rainbow trout (*Oncorhynchus mykiss*) was deployed in Lake 375 to examine the effects of the increased nutrient load on the algal and bacteria communities of the lake (Findlay et al., 2009). The findings from this study included a reduction in overall water quality, and a nearly 50% decline in the native lake trout population within two years of study completion (Findlay et al., 2009; Rennie et al., 2019). Lake trout size at maturity remained below pre-study levels as late as 2016-17, but the lake is considered to have returned to pre-manipulation condition (Rennie et al., 2019). Lake 378 has yet to be the site of a whole-ecosystem study at IISD-ELA, and as such very little baseline data exists for lake trout abundance and morphometrics.

Lake trout (Salvelinus namaycush)

Lake trout are an iteroparous, cold water inhabiting fish species that spawn on rocky shoals in the fall (Callaghan et al., 2016). They are widely distributed across northern Canada, preferring low nutrient, deep water basins that meet a narrow oxythermal range year-round (Gunn 1995; Guzzo and Blanchfield, 2017). There can be a great degree of variability in growth rate and age of maturation among lake trout depending on food availability and type (Redick,

1967, Rawson, 1961). Lake trout diet varies seasonally, particularly when dimictic lakes become thermally stratified in late spring (Redick, 1967). Prey fish can often remain in the epilimnion and are therefore separated from lake trout as they migrate to deeper, cooler waters. Typically, larger fish tend to be piscivorous, while smaller fish feed on either plankton or small minnows. Young lake trout are particularly dependent on freshwater shrimp (*Mysis relicta*), although they typically become less reliant on this food source after reaching 14 inches length (Redick, 1967).

The spawning behaviour of lake trout is different from other salmonids because it occurs at night (Gunn, 1995). Additionally, there has been no observed aggression between males during spawn, and no sexual dimorphism, which is likely related to this unique spawning behaviour. They prefer temperatures between 8-14°C for spawning, while other factors such as sunlight, wind direction, and rate of temperature decline have been shown to influence both the timing and duration of the spawn (Gunn, 1995). The spawning grounds are made up of clean pebbles and cobble, rubble, angular rocks and some larger boulders (Callaghan et al., 2016). Lake trout are very selective of cobble size and type, as it is crucial for egg survival (Callaghan et al., 2016). Eggs require sufficient space to allow for sufficient oxygen exchange, while also remaining adequately hidden to avoid predation (Callaghan et al., 2016; Gunn, 1995).

Hatching time for salmonid eggs is largely dependent on, although not directly proportional to, incubation temperature (Brannon, 1987). Embryo and fry development in fish is typically measured in "accumulated thermal units" (ATU), by summing the mean daily water temperature. Eggs incubated at temperatures <5°C will develop at fewer ATU than those at higher temperatures (Brannon, 1987). This adaptation is crucial for ensuring a stable hatching phenology for eggs spawned later in the breeding season (Brannon, 1987).

RESEARCH QUESTIONS AND OBJECTIVES

Research Question 1: Is there a difference between the health of eggs and fry from Lake 260 and the three reference lakes (Lakes 223, 375, 378) related to the presence of residual oil constituents?

Objectives:

1. Rear eggs obtained from fish captured in a lake where oil spill studies were conducted and in three similar reference lakes at the IISD-ELA using *in-situ* Scotty-Jordan incubators.
2. Rear eggs obtained from fish captured in a lake where oil spill studies were conducted and in three similar reference lakes at the IISD- ELA using *ex-situ* Heath tray incubators in a laboratory setting.
3. Sub-sample eggs and fry from *in-situ* and *ex-situ* exposures at various developmental milestones, to assess morphometrics (egg size & colour, ATU to 50% hatch, length & weight of hatched fry), fertilization success, hatching rates, mortality, and the presence of deformities.

Null hypothesis: There will be no significant difference in morphometrics, deformity rates, fertilization success, hatching success or mortality in eggs/fry from Lake 260, where oil spill studies were conducted, and eggs/fry from the three reference lakes.

Research Question 2: Is there evidence of exposure to PACs or increased oxidative stress in Lake 260 fry related to the presence of residual oil constituents, compared to the three reference lakes (Lakes 223, 375, 378)?

Objectives:

1. Rear eggs obtained from fish captured in a lake where oil spill studies were conducted and in three similar reference lakes at the IISD- ELA using *ex-situ* Heath tray incubators in a laboratory setting.
2. Sub-sample unfertilized eggs, to measure PAC content.
3. Sub-sample fry at swim-up, to assess *cyp1a* induction, total glutathione (TGSH) and the ratio of reduced to oxidized glutathione (GSH/GSSG).

Null hypothesis: There will be no significant difference in levels of PACs, *cyp1a* induction, TGSH and GSH/GSSG in eggs/fry from Lake 260 and those from the three reference lakes.

STUDY SIGNIFICANCE

While large oil-spills will always catch the public's attention, smaller spills which can also potentially have lasting effects on the ecosystems they disrupt (Moroni, 2019). It is imperative to understand the toxicity thresholds of oil exposure in a species such as lake trout, which is of great importance commercially, recreationally and as a food source for many indigenous communities (Callaghan et al., 2016). Few studies have compared the development of salmonids exposed to extremely low levels of PACs using both a controlled laboratory study and *in-situ* experiments. Laboratory based studies can control for factors such as temperature, predation, and oxygen levels, while the *in-situ* study provides the "real-world" scenario where eggs are reared and sampled in the lake from which they originated. Together, the two studies

can more comprehensively examine the impacts of residual oil on reproduction of lake trout in Lake 260.

Recent projections estimate that oil production from the Alberta Oil Sands Region will continue to increase by 2% annually from 2022 onward, with nearly 1.7 million barrels per day of additional oil being extracted by 2035 (CAPP, 2019). Although dips in oil prices and demand have been observed globally – largely due to the COVID-19 pandemic – it is expected that the industry will recover (Jefferson 2020). The continued investment in oil export infrastructure by the Canadian government will inevitably lead to higher potential for spills occurring in the boreal ecozone, home to various salmonid species, including lake trout (Corkal, 2021). By studying lake trout at a sensitive early life stage (fertilization to swim-up fry), this study provides an excellent opportunity to investigate deleterious effects resulting from exposure to low concentrations of PACs (Finn, 2007).

Researchers have utilized the facilities at IISD-ELA to conduct a wealth of "whole-lake" manipulation studies over their 50-plus year history – the results of which have contributed immensely to advancements in freshwater ecology and updates to government policy. The suite of oil-spill studies in Lake 260 from 2017-2019 have the potential to enhance scientific knowledge on the environmental impacts of freshwater spills, and to assess novel remediation methods. If we fail to reject our null hypothesis, this study will contribute to the fulfillment of IISD-ELA's mandate to impose minimal harm on the ecosystem, and ultimately return the study lake to its natural state.

CHAPTER 2

EARLY LIFE STAGE DEVELOPMENT IN LAKE TROUT FROM A LAKE USED TO CONDUCT MODEL OIL SPILLS

ABSTRACT

Two experiments involving the addition of diluted bitumen (dilbit) into contained enclosures were performed in Lake 260 at the IISD-Experimental Lakes Area in 2018 and 2019. To assess the effects on resident lake trout (*Salvelinus namaycush*), we collected eggs and milt from adults in the fall of 2019 and examined early life stage development of fertilized eggs, compared to lake trout collected from three unimpacted reference lakes. A subsample of eggs were reared *in-situ* in the four study lakes using Scotty-Jordan incubators to evaluate hatching success and the remainder of the eggs were transported to a laboratory where they were reared in health trays until swim-up. The laboratory experiment permitted the evaluation of several additional parameters of interest, including: fertilization success, egg mortality, time to 50% hatch, hatching success, growth rates and deformity rates. Polycyclic aromatic compounds (PACs), the primary toxic components of oil, are known to impact early life stage fish development at low concentrations. Because PAC concentrations only reached ~2 μg/L in the general lake environment immediately after dilbit addition in 2019, but quickly returned to pre-addition levels (~0.1 μg/L), we hypothesized that there would be no teratogenic effects to the lake trout in Lake 260. Correspondingly, we observed no significant differences in the parameters we investigated between lake trout originating from Lake 260, and those from the three reference lakes.

INTRODUCTION

Polycyclic aromatic compounds (PAC) are ubiquitous environmental contaminants, often released through anthropogenic activity, such as the combustion of fossil fuels, as an effluent from pulp and paper mills or from oil spills occurring from pipeline, train, or vehicle transport (reviewed in Lee et al., 2015). The continued demand for petroleum products, and further development of pipeline infrastructure necessary to meet global energy requirements will inevitably pose a threat to freshwater ecosystems in the vicinity of these transportation routes (Corkal, 2021). For example, exposure to low concentrations of PACs has been shown to cause a wide variety of biological effects in several marine and freshwater fish species – including developmental abnormalities (Vignet et al., 2019), genetic damage (Carls et al., 1999), behavioural changes (Cresci et al., 2020), and increased mortality (Le Bihanic et al., 2014). Still, much of the scientific literature in the past few decades has focused on impacts to marine species. This discrepancy was highlighted in the 2015 Royal Society of Canada Report, which addressed the need for increased research on the effects of PACs released into freshwater environments (reviewed in Lee et al., 2015).

Diluted bitumen (dilbit) and synthetic crude oil (SCO) are currently two of the most popular petroleum products transported in Canada (Yang et al., 2021). As such, the fate and toxicity of these products in freshwater has recently received greater attention from the scientific community (Stoyanovich et al., 2021; Rodriguez-Gil et al., 2021; Yang et al., 2021). PACs make up a large class of compounds, consisting of thousands of congeners (Hodson et al., 2020). They can vary in number of aromatic rings, as well as in the presence of hydrocarbon side chains (i.e. alkylation), which have been shown to increase their toxicity (reviewed in Hodson, 2017). Alkylated 2-4 ringed structures typically make up 80-95% of the total PACs in petrogenic oil

mixtures (Hodson et al., 2020; Wang et al., 2003). Of these alkylated PAC compounds, it is primarily the 3-5 ringed structures that have been shown to cause chronic toxicity in early life stage fish, largely due to their resistance to weathering and capacity to freely cross cellular membranes (reviewed in Lee et al., 2015). Upon entry into the cell, it is often the secondary metabolites produced through enzymatic reactions that exert most of the toxicity at this sensitive life stage (Hodson et al., 2007).

Embryonic fish are especially sensitive to PAC exposure at two developmental milestones. First, during cleavage, early cell divisions and complex signalling determine the dorsal and ventral axis of the developing embryo. Exposure to PACs interferes with the Wnt/B-catenin pathway, which is necessary for the establishment of this axis. Later in development, as the cardiovascular system begins to develop, PACs can interfere with potassium channels and calcium signalling, crucial elements of proper cardiac function (Honda and Suzuki, 2020). Impairments to cardiac function during this critical developmental stage is the precursor to spinal, craniofacial and edema related deformities, due to organism's inability to efficiently circulate oxygenated blood (Incardona and Scholz, 2016). Some PACs have a greater effect on development after hatch (i.e. when the protective chorion is removed). In rainbow trout (*Oncorhynchus mykiss*) eggs exposed to retene, a 3-ringed alkyl PAC, rates of deformities increased substantially after hatching, suggesting that that post-hatch fry may be the best indicator of any phenotypic changes resulting from PAC exposure (Brinkworth et al., 2003).

Salmonids reside and spawn in oligotrophic freshwater environments across much of the Northern Hemisphere, where their proximity to oil transportation routes puts many species at risk of exposure to these toxic PACs (Finn, 2007). Researchers have drawn particular attention to the sensitivity of salmonids to xenobiotic exposure at their early life stages, where it has been shown

that very low concentrations of PACs in the aquatic environment can lead to deleterious effects, impacting proper development (Rigaud et al., 2022; Berube et al., 2022). While free swimming fish can be exposed to these compounds through their diet or through uptake through the gills, embryos– protected by the chorion but immobile and thus unable to flee the source of contamination – are susceptible to dissolved PACs though direct contact with the eggs, or possibly through maternal transfer to the yolk (Carls et al., 2008, reviewed in Lee at al., 2015). PACs can be metabolized and excreted by salmonids, and therefore are not thought to bioaccumulate to the extent of toxicants such as organochlorines or selenium (Collier et al., 2013; Guiney et al., 1996; Covington et al., 2018). However, some studies have shown the potential for PACs to be transferred maternally to the eggs in freshwater fish species such as northern pike (*Esox lucius*) (Sundberg et al., 2007), and marine fish species such as pacific herring (*Clupea pallasii*), winged lanternfish (*Lampanyctus alatus*) and striped rockfish (*Sebastes elongatus*) (West et al., 2014; Romero et al., 2018).

Rainbow trout are most often used as a model salmonid species for chronic early life stage toxicity tests (ECCC, 1998). Lake trout (*Salvelinus namaycush*) are a closely related but rarely studied species, therefore baseline survival and deformity rates have only seldom been examined (Werner et al., 2006; McGurk et al., 2006). It is necessary to gain a better understanding of the effect of PAC exposure in lake trout at these sensitive early life stages due to their popularity for commercial and recreational fisheries, as well as being a staple food source for some indigenous communities (Callaghan et al., 2016). Importantly, lake trout are often a top predator in the freshwater ecosystems they inhabit (McDonald et al., 1996; Hansen and Bronte, 2019). Furthermore, the unique spawning strategy of lake trout compared to many other salmonids (i.e. in lakes rather than flowing streams) could make them more at risk of oil

exposure in the event of a spill due to their proximity to shore, coupled with the potential absence of flow in the lakes they inhabit. As a cold-water inhabiting stenotherm, lake trout are already threatened by increasing water temperatures and hypolimnetic anoxia associated with climate change across much of their native range (Guzzo and Blanchfield, 2017). The anthropogenic release of xenobiotics such as PACs into freshwater environments will only exacerbate the ever-evolving threat facing lake trout in the coming decades, therefore the current lack of knowledge on how PACs influence lake trout at the especially sensitive developmental stage creates a critical gap in scientific knowledge needed for protecting this species.

Few studies have run parallel laboratory and in-situ components to test survival and development in early life stage fish (Marlatt et al., 2016). The benefit of such an experiment is that the *in-situ* component better replicates a natural rearing environment, where eggs develop in their native habitat. Conversely, the laboratory component of the study allows for more control of extrinsic factors such as oxygen saturation and predatory fungus that can quickly infect a batch of eggs. The potential effects of PACs on early life stages of lake trout were examined herein using *in-situ* and laboratory exposures. For the *in-situ* exposure, lake trout eggs were obtained from a lake at the International Institute for Sustainable Development-Experimental Lakes Area (IISD-ELA) where model oil spills have been conducted and where low concentrations of residual PACs may be present. Previous evidence of PAC exposure and accompanying changes in gene expression have been detected in the mucus of adult lake trout from in the study lake (Andrzejczyk, 2020). Eggs were reared in incubators placed on spawning shoals and checked at various milestones of development to remove mortalities. Sub-sets of eggs obtained from fish used for the *in-situ* exposure were also transported and reared in the laboratory. Hatching success was determined in the *in-situ* component of the study and

fertilization success, egg mortality, hatching success, time to 50% hatch, morphometrics (i.e. weight, length, yolk volume) and deformity rates in yolk-sac (i.e. alevin) and swim-up fry were assessed in the laboratory study. Because only negligible petrogenic PAC concentrations (<0.1 µg/L) were present in the study lake environment at the time of spawning (reviewed in Hodson, 2017), it was hypothesized that there would be no biologically relevant difference in the aforementioned parameters between the lake trout in Lake 260 and those in three uncontaminated reference lakes (Lakes 223, 375, and 378). This study was designed to test for intergenerational legacy contamination effects in these fish, which while assumed to be negligible, have rarely been tested.

METHODS AND MATERIALS

Adult Fish Collection

In the fall of 2019, eggs were collected from seven adult female lake trout in each of Lakes 260 and 223, and from three female lake trout in each of Lakes 378 and 375 at the IISD – ELA in Northwestern Ontario, Canada. The fish were captured using either trap nets or short set gill nets (20-30 mins) and transported in plastic tubs containing aerated lake water to shore. Once brought to shore, fish designated as "ripe and running" were selected for the study (i.e. the first seven fish that were observed to be in spawning condition) and were anesthetized with 0.1 g/L tricaine methanesulfonate (MS-222) until movement ceased. Female fish were weighed, measured, and then eggs were collected by gently massaging the abdomen of the fish to express eggs. Eggs from each female were collected in separate ZipLoc™ bags, the head space was filled with oxygen and the bags were sealed and stored on ice in coolers for transportation to the Freshwater Institute in Winnipeg, Manitoba for fertilization. Milt was collected in a similar

fashion to egg collection from approximately 3-7 males in one bag per lake and kept on ice until fertilization. Egg diameter for each female was measured in a subsample of 5 eggs using calipers, and the number of eggs in each clutch was estimated by weighing a sample of 30 eggs using an analytical balance that was precise to ± 0.1g and extrapolating the number of total eggs based in the weight of the entire clutch. Lastly, egg colour was subjectively measured by comparing colour to a colour gradient (Figure A.9) to determine approximate carotenoid content, which can be used to infer antioxidant levels in the yolk (Palace and Werner., 2006; Tyndale et al., 2008). Eggs expressed from each female were then subdivided into two batches, with ~200 being removed from the bags for the *in-situ* experiment, and the remainder designated for the lab-based study (see Table A.1 for adult fish sampling and egg deployment dates).

Egg and Larvae Rearing

In-situ incubations

Once the milt and eggs were collected, approximately 200 eggs were removed from each clutch and dry fertilized in individual stainless-steel bowls following the procedures previously described by Werner et al. (2006). The dry fertilization technique comprised of mixing the eggs with 100 µL milt from 3-7 males using a sterilized goose feather to stir (i.e. no water involved in the initial step). After the milt was adequately stirred so as to make contact with the entirety of the eggs, lake water was poured into the bowls to cover the eggs and activate the sperm to allow for fertilization to occur, and the eggs were stirred again using a sterilized goose feather. After a minute, approximately 5 times the volume of water was added to the bowl and the eggs were set aside to water harden for 5 minutes. Once water hardened, the eggs were deployed in Scotty-Jordan incubators (Scotty; Sidney, B.C.), with each tray accommodating 200 eggs from a given female, each egg held in a separate compartment. These trays were then deployed at

approximately 2 metres depth in known spawning locations at each of the lakes (see Figures A.3 – A.6 in Appendix A). The trays were equipped with two HOBO loggers (Onset Computer Corp.; Bourne, MA) affixed to each tray to measure temperature throughout the study period. Hatching success was determined at the end of the study, as the Scotty-Jordan incubators are designed to allow hatched fry to swim out of the incubator and into the lake. In accordance with the number of female fish captured in the four lakes, seven trays were deployed in Lakes 260 and 233, while three trays were deployed in Lakes 378 and 375.

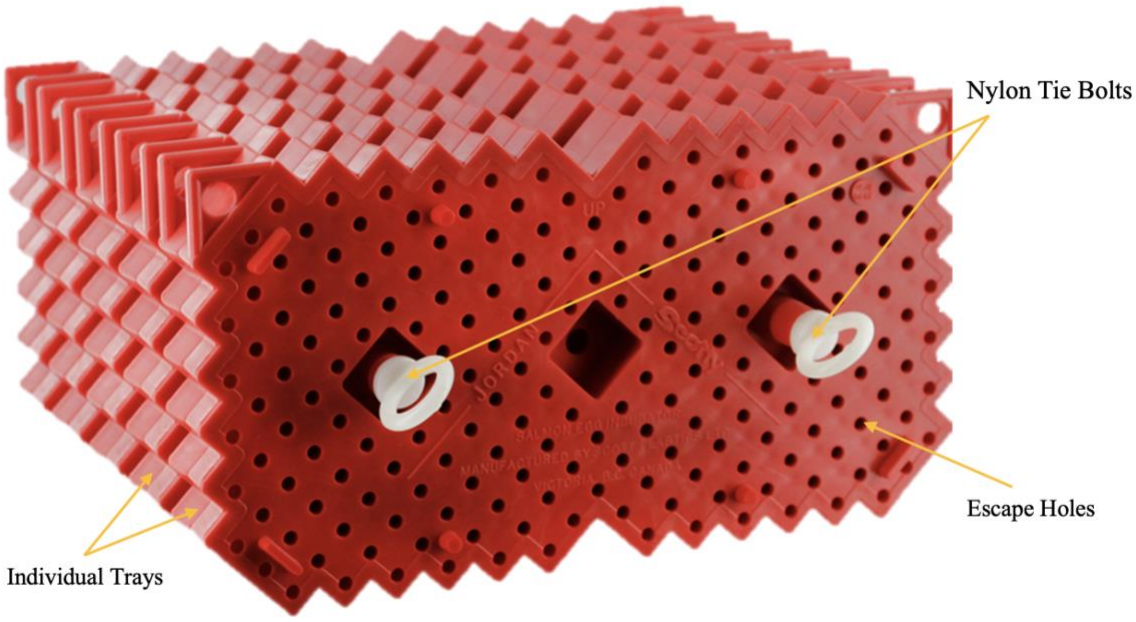


FIGURE 2.1. Scotty-Jordan incubators. Pictured above is an assembled unit containing five individual trays (Image from www.scotty.com).

Laboratory incubations

The remaining eggs (i.e. after the 200 were removed for the *in-situ* incubations) and milt collected from each lake were packed in coolers with ice and transported from IISD-ELA to the Freshwater Institute in Winnipeg, Manitoba in sealed plastic bags filled with oxygen. The time

from egg collection onshore until fertilization in the lab was less than 48 hours for all batches. These eggs were dry fertilized in the lab using exactly the same procedures as those in the field except 200 µL of milt was used for every 200 eggs to improve the chances of fertilization. After water hardening, the eggs were incubated in separate compartments of a commercial incubator containing Heath trays, each receiving 6L/min of dechlorinated Winnipeg tap water (8.5±1°C), employing the method described by Werner et al. (2006). Each lake trout had its eggs placed in a designated compartment of the tray, with some fish requiring two compartments to incubate all the eggs in a monolayer. Dividers were made from plexiglass and fixed in place using 100% silicone.

A subsample of ~25 eggs from each fish from Lake 260 (n=7) were pooled and then exposed collectively to a water accommodated fraction (WAF) containing a 1:100 dilution of crude oil during water hardening for 60 minutes to act as a positive control (detail in WAF preparation below). These eggs were rinsed in clean water after the exposure and placed in a dedicated compartment. Eggs were checked daily for the presence of mortalities, which were removed and placed in ovadine solution and subsequently disposed. Removing mortalities frequently is critical to limit the possibility of predatory fungus from infecting a batch of eggs (Werner et al., 2006).

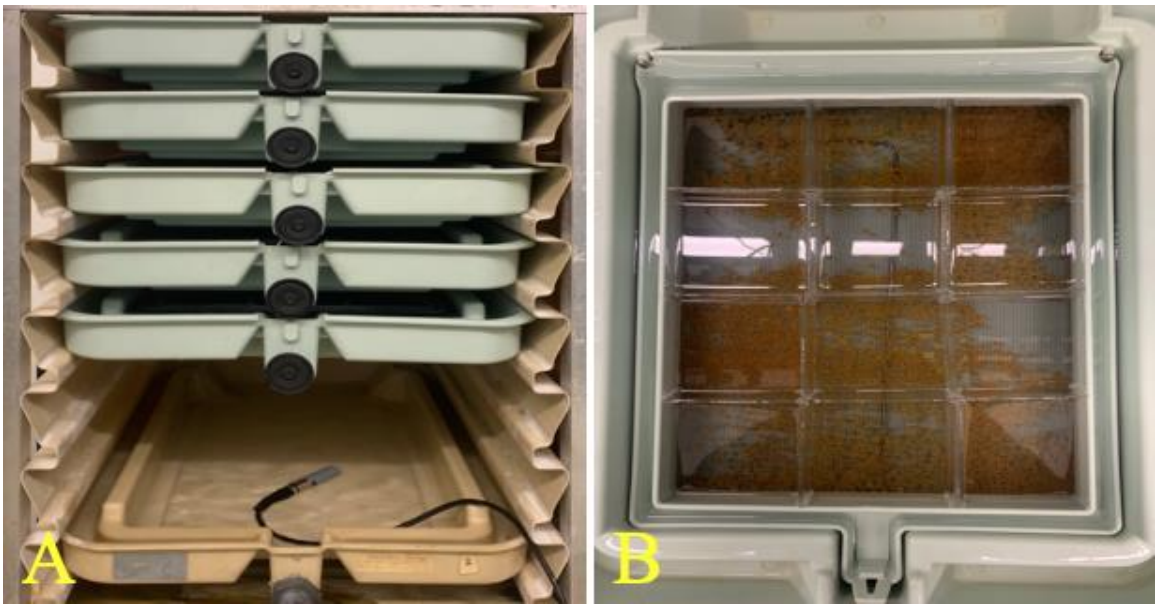


FIGURE 2.2. (A) Vertical incubators (Heath trays) used for incubations at the Freshwater Institute in Winnipeg, Manitoba. (B) Over-head view of lake trout eggs divided up into 12 compartments of a Heath tray.

Water Accommodated Fraction (WAF) Preparation

Preparation of the WAF was conducted using reverse osmosis filtered dechlorinated City of Winnipeg tap water, in accordance with the DFO Canadian Science Advisory Secretariate Report (2017). A final concentration of 1:100 (w/v of heavy crude) was achieved by mixing the oil and water at room temperature (20-22°C) for 24 hours at a vortex depth of 20-25% the total water depth. This oil to water ratio corresponds to ~0.2 µg/L TPAC (Figure A.2). One hundred (100) mL of WAF was transferred into an amber bottle for transportation to the DFO lab, where it was used to expose a subset of Lake 260 eggs during water hardening as described above.

Temperature Unit Calculations

Two HOBO loggers were attached to the Scotty-Jordan incubators in each lake to track temperature at 5-minute intervals over the course of the experiment. Malfunctions occurred in the loggers in lake 375, with data being lost or corrupted until the first sampling period.

Temperature during this time period was interpolated based on a linear change in temperature between a reading at the time of deployment and the temperature at the first sampling event.

In the laboratory, one HOBO logger was used to collect temperature data at 15-minute intervals. The loggers were located ~30 cm beneath the heath trays where water collection occurred before drainage from the incubator. The potential for a temperature gradient between the first tray and the collection tray was assessed at the time of deployment, and no detectable difference in temperature was observed. Accumulated thermal units (ATU) were determined as the sum of mean daily temperature since fertilization.

Embryo Sampling

In the lab, eggs/fry were subsampled pre-fertilization, and at approximately 200, 300, 400, 550 and 800 accumulated thermal units (ATU), representing developmental milestones in the trout embryos/fry. *In-situ*, eggs/fry were sampled at approximately 200, 300 and 400 ATUs. Due to logistical difficulties caused by the COVID-19 pandemic, only hatched fry were analyzed for deformities. At sampling time, 10 fry from each incubator/compartment were preserved in Davidson's fixative (2 parts 37% formaldehyde w/v with 5-10% methanol v/v, 3 parts 95% ethanol, 1 part glacial acetic acid, 3 parts distilled water) for deformity analysis. Hatched fry were euthanized with a lethal dose of pH buffered MS-222 (0.4g/L) until movement ceased. Where limited number of eggs remained or if substantial mortality had occurred, sample number was adjusted accordingly. Dead eggs were enumerated and removed daily in the lab and at each sampling event in the lake so that percent fertilization, hatching success, egg mortality could be determined. Egg mortalities prior to 14d post fertilization were assumed to be unfertilized (Werner et al., 2006). Additionally, all non-eyed eggs were removed 16 days post fertilization to

determine total fertilization success. Removal of these eggs occurred prior to the 200 ATU sampling.

A small proportion of hatched fry moved between compartments in the Heath trays. Affected compartments included eggs from Lake 260 and a separate tray from Lake 375 and half the eggs from Lake 223 (LT # 4-7). Counts taken pre-hatch were used to enumerate eggs in various compartments in these two trays. Percent fry mortality and severe edema (i.e. observable to the naked eye) between compartments was excluded from statistical analysis due to some uncertainty regarding where the fry originated in these trays, however total proportions for these parameters amongst trays were calculated.

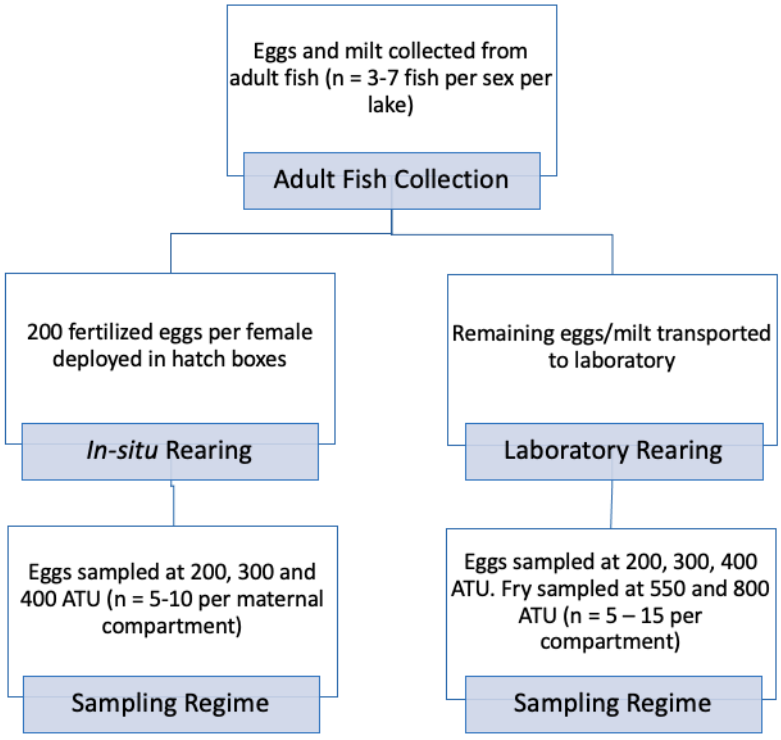


FIGURE 2.3. Flow chart depicting egg/fry sampling for the study. In the lab study, eggs and fry from a single maternal female were divided into two compartments when the number of eggs exceeded a monolayer.

Deformity Analysis

A subsample of fry (n = 5-15 per compartment) at two separate time points (550 ATU and 800 ATU) were haphazardly sampled, preserved in Davidson's fixative and analyzed for deformities. Preserved fish were photographed using a Leica EZ4W dissecting scope at 8x magnification, with fish consistently photographed on the same lateral side. Two photos were required per specimen in order to capture the anterior and posterior ends of the fry in the field of view. Photos were subsequently amalgamated into one reconstructed image using an online software tool prior to scoring (https://www.quickpicturetools.com/en/combine_images/).

Deformities were scored in four categories: edema, skeletal, craniofacial and finfold, with a severity index (0-3) for each (see Appendix B for detailed rubric based on Holm et al. (2003); Rudolph (2006); and Covington et al., (2018)). To ensure that the fish were scored objectively, sample sketches depicting criteria for each deformity versus normal fry were created (Elise Epp, International Institute for Sustainable Development, see Appendix B). The sketches represent all severity levels of the four main categories of deformities and contain sub-categories in some cases. Finfold deformities were scored by assessing the absence or reduction in any fins. Edema examined the presence of fluid build-up in the ventral/yolk region. Skeletal deformities contained three subcategories: lordosis, kyphosis and scoliosis, although scoliosis was rarely scored due to the possibility of fixation artefact. Craniofacial deformities were categorized as either jaw, snout or eye deformities. An annotating software (JMP) was used to determine length and area ratios in the snout and edema measurements (see Appendix B) in order to provide an objective method to score the fish in accordance with the definitions set out in the rubric.

After the initial scoring was completed, photos were re-randomized and scored a second time by the same scorer to assess the consistency and objectivity in the rubric (Janz and Muscatello, 2008).

Fish sampled for deformity analysis were also measured and weighed. Fish with severe spinal deformities were omitted from mean length calculations because length could not be accurately measured. Additionally, fish with severe edema were not included in mean weight calculations because of the expected large mass contribution from accumulated fluid. Yolk volume was determined in yolk-sac fry according to the method described by Westernhagen et al. (1981), by measuring the large (a) and small (b) diameter of the yolk sac and using the formula: $V\ (mm^3) = 4/3\ \pi\ b^2a$.

Statistical Analysis

Statistical differences in parameters for adult fish (length, weight, egg diameter, egg colour) in the four study lakes were examined using linear regression, or Kruskall-Wallis if data failed to meet the assumptions of a normal distribution ($\alpha < 0.05$). The linear regression formula utilizes dummy coding (0 or 1) to model the explanatory variable of "lake origin" and a random error term (ϵ). The expected value (Y) is predicted by the following linear regression formula, with the intercept ($\beta 0$) representing the predicted mean value for Lake 260:

$$Y = \beta 0 + \beta 1*\text{Lake223} + \beta 2*\text{Lake375} + \beta 3*\text{Lake378} + \epsilon$$

Model fit to the normal distribution was assessed using Q-Q plots, a probability plot that compares model residuals along theoretical quantiles to the model fit. For each model, normality of the residuals was verified using a Shapiro-Wilk test ($\alpha = 0.05$), and homogeneity of variance across groups (i.e. lake) was tested with a Levene's test ($\alpha = 0.05$). If the model residuals failed tests of normality, the response data was log transformed. The removal of outliers was

considered if standardized residuals exceeded "3". Model significance was assessed by evaluating the *F*-statistic and *p*-value of the linear regression models ($\alpha = 0.05$). Differences in condition factor in the adult fish were assessed with an analysis of covariance (ANCOVA) to determine differences in weight, when controlling for length ($\alpha = 0.05$) (Janz 2008). Several correlation analyses were performed to assess the relationship between various metrics in the maternal fish vs. the eggs (i.e. fork length vs. expressed egg number, weight vs. expressed egg number, fork length vs. egg diameter, weight vs. egg diameter) and in the hatched fry vs the eggs (i.e. alevin length vs. egg diameter, alevin weight vs. egg diameter, alevin yolk volume vs. egg diameter, swim-up fry length vs. egg diameter, swim-up fry weight vs. egg diameter) and to assess a correlation between percent egg mortality and number of eggs expressed/gram maternal female body weight. These results are tabulated in Appendix A.

Percentage hatching success was compared amongst study lakes from the *in-situ* experiment using a binomial generalized linear model (GLM) which computes predicted probabilities of a binary outcome. The log odds are predicted by the following logistic regression equation, with a categorical dummy coded variable representing "lake origin" and Lake 260 represented by the intercept term ($\beta 0$):

$$\log\left(\frac{\mathrm{P(y=1)}}{\mathrm{P(y=0)}}\right) = \beta 0 + \beta 1 * \mathrm{Lake223} + \beta 2 * \mathrm{Lake375} + \beta 3 * \mathrm{Lake378} + b + \epsilon$$

Observation level random effects (b) were modelled in these analyses to account for variability between compartments within the same lake (i.e. maternal effects or positioning of incubators in water). For the lab component of the study, percent unfertilized eggs, percent egg mortality, and percent hatch fail and hatching success were compared across study lakes using the same binomial GLM formula as the *in-situ* hatching success data, with the only notable change being a random effect assigned to compartments by maternal origin. The addition of the random effect

accounts for non-independence that exists between batches from the same maternal female (i.e. some egg batches were divided into two compartments). Half-normal plots were used to assess goodness-of-fit and the presence of outliers for all binomial models, by comparing the actual data distribution to a simulated "envelope" of expected values (Moral et al. 2017). Likelihood ratio tests (LRT) were conducted to assess the significance of "lake origin" as a predictor by comparing the full model to the null (intercept only) model. Chi-squared test statistics were computed by comparing log likelihoods of model fit (df = difference in number of parameters between the two models), and the null hypothesis (H_0 = lake as a predictor does not improve model fit) was rejected at $\alpha < 0.05$ (Hothorn 2021). Accumulated thermal units to 50% hatch in rearing compartments was compared between lake origin in the lab component of the study using a non-parametric Kruskall-Wallis test ($\alpha = 0.05$).

Total graduated severity index (GSI) scores were compared in fry between study lakes at the alevin stage and at swim-up using an ordinal logistic regression model (Christensen 2019). This modelling technique utilizes a "logit" link function to compute predicted probabilities (± 95% confidence interval) of an ordered response variable across levels of a categorical predictor variable (lake origin) using the following equation:

$$logit[P(Y \leq j)] = \alpha j - \beta 1 * \text{Lake223} + \beta 2 * \text{Lake375} + \beta 3 * \text{Lake378} + \epsilon ij$$

In this model, j represents the three levels of the ordered factor (GSI score = 1, 2, ≥3), and αj represents the corresponding intercept term (corresponds to Lake 260 predicted probability) at those levels. Variables representing lake origin were dummy coded (0 or 1). Model fit and the presence of outliers was examined using Q-Q plots from the 'sure' package in R (Greenwell et al., 2017). The GSI score data was transformed to convert all total scores greater than "2" to a single factor (≥3). This level designation is somewhat arbitrary but represents either a

combination of three mild deformities, a mild and moderate deformity in two categories, or a severe deformity in a particular category. Graduated severity index scores of "1" or "2" represent either a single or combination of mild deformities or a moderate deformity in one of the categories and are less likely affect fitness (Covington et al. 2018). Random effects were not included in modelling of deformity data, due to an insufficient variance between maternal groups (i.e. several maternal groups that had no visible deformities). Likelihood ratio tests were used to assess the overall effect of "lake origin" as a predictor for total score by comparing the full model (includes lake origin) to the null model (intercept only) (Hothorn 2021). All statistical analysis was performed in R (R Core Team, 2021). Model parameters are summarized in Appendix C1.

RESULTS

Adult Fish Characteristics

Lake origin was not a significant predictor of differences in fork length and weight amongst the female fish collected from the four study lakes ($F(3,16) = 0.57$, p-value = 0.64; $F(3,16) = 1.10$, p-value = 0.38, respectively). Similarly, mean condition factor was not significantly different ($F(3,15) = 2.34$, p-value = 0.12), with mean ± SE values ranging from 1.06 ± 0.04 – 1.16 ± 0.02 among the study lakes. Egg volume, measured as the number of eggs expressed per gram of female body weight, was similar between females collected from the study lakes ($F(3,16) = 0.57$, p-value = 0.64).

Mean fork length and weight was similar in male fish between lakes ($F(3,17) = 1.88$, p-value = 0.17; $F(3,17) = 0.84$, p-value = 0.49, respectively). Additionally, differences in mean

condition factor (1.02 ± 0.03 – 1.12 ± 0.03) amongst the male fish were determined to be non-significant ($F(3,16) = 0.91$, p-value = 0.46). All metrics are summarized in Tables 2.1 and 2.2.

Hatching and Mortality

For the *in-situ* component of the study, hatching success was most variable between clutches of eggs in Lakes 223 and 375. In Lake 223, percent hatch ranged from 32-97%, while in Lake 375, the range was 5-67%. Lakes 260 and 378 had more consistent hatching success between clutches, with a percent hatch ranging from 70-88% in Lake 260 and 79-86% in Lake 378. There was a significant difference in hatch rate between fry in Lake 260 and those in Lake 375 ($z = -2.20$, p-value = 0.03) (Figure 2.8). A subsequent model excluding a potential outlier in Lake 375 (LT2) showed that mean hatching success was similar between Lake 260 and the three reference lakes (pooled) (χ^2 (1) = 38, p-value = 0.54) (Figure 2.9).

In lab reared fish, differences in the percentage of eggs that were unfertilized were non-significant across all four study lakes (χ^2 (3) = 5.13, p-value = 0.16) (Figure 2.10). Egg mortality, deemed as fertilized eggs that died prior to hatch, was similar between lakes with mean proportions varying between 4.9 – 8.7% (χ^2 (3) = 1.65, p-value = 0.65) (Figure 2.11). Percent hatching success was found to be similar across the four lakes in lab reared fish after removing an extreme outlier from Lake 223 with very low hatch success (LT6, 10.59%) (χ^2 (3) = 1.40, p-value = 0.71) (Figure 2.12).

Accumulated thermal units to 50% hatch was significantly less in fry from Lake 260, compared to the three reference lakes (L260 - L223: p-value < 0.001; L260 - L375: p-value < 0.001; L260 - L378: p-value < 0.001). ATU to 50% hatch was similar amongst batches originating from the three reference lakes (p-value = 1.0) (Figure 2.13). Percent fry mortality was

less than 2.5% across all trays (Figure 2.11), and severe edema was observed in less than 1% of hatched fry (Figure 2.15).

Egg, Larval and Juvenile Characteristics

There were no significant differences in mean diameter and colour amongst eggs collected from maternal female fish in the four study lakes ($F(3,16) = 0.52$, p-value = 0.68; $H(3) = 5.59$, p-value = 0.13, respectively). Mean ± SE egg diameter was smallest in Lake 378 (4.92 ± 0.08 mm) and largest in Lake 375 (5.33 ± 0.17 mm). Eggs sampled from Lake 223 were more pale in colour, on average (9.1 ± 1.3), while eggs from Lake 378 were generally darker in colour (5.0 ± 0.9). Mean ± SE values for each lake are shown in Table 2.1.

Condition factor was significantly higher in yolk-sac fry (~550 ATU) from Lake 378 compared to those from Lake 260 (t (16) = 5.02, p-value < 0.001), but similar between fry from Lake 260 and those from Lakes 223 and 375 (t (16) = 0.47, p-value = 0.64; t (16) = 1.30, p-value = 0.21, respectively) (Figure 2.4). Conversely, yolk-sac fry from Lake 378 were significantly shorter than those from Lake 260 (t (16) = -3.65, p-value = 0.002), while fry from Lakes 223 and 375 were comparable in length to those from Lake 260 (t (16) = -0.86, p-value = 0.40; t (16) = -1.73, p-value = 0.10, respectively) (Figure 2.5). Mean weight (Figure 2.6) and yolk volume (Figure 2.4) were similar among yolk-sac fry from the four study lakes ($\chi^2(3) = 1.29$, p-value = 0.73; $\chi^2(3) = 0.92$, p-value = 0.82).

Fry sampled at swim-up (~800 ATU) from Lake 223 had similar mean condition factor to those from Lake 260 (t (16) = 1.79, p-value = 0.09), whereas fry from Lakes 375 and 378 had a higher mean condition factor than those from Lake 260 (t (16) = 2.37, p-value = 0.03; t (16) = 4.11, p-value < 0.001, respectively) (Figure 2.1). Mean length was again comparable between fry from Lakes 260 and 223 (t (16) = -1.06, p-value = 0.30), and between Lakes 378 and 260 (t (16)

= -2.11, *p*-value = 0.05). Fry sampled from Lake 375 were shorter, on average than those from Lake 260 (t (16) = -2.87, *p*-value = 0.01) (Figure 2.5). Mean weight was similar in swim-up fry amongst the four study lakes ($\chi^2(3) = 2.86$, *p*-value = 0.41) (Figure 2.6).

Deformity Analysis

Frequency and mean total graduated severity index (GSI) scores for fry from the four study lakes are shown in Tables 3 and 4. Model predictions from an ordinal logistic regression indicated that lake origin was a significant predictor of total GSI score post-hatch (~550 ATU) and at swim-up (~800 ATU) (χ^2 (3) = 43.30, *p*-value < 0.001; χ^2 (3) = 16.93, *p*-value < 0.001, respectively).

In fry examined post-hatch (alevin), those originating from Lakes 375 (z = 5.91, *p*-value < 0.001) and 378 (z = 2.26, *p*-value = 0.02) were more likely to be assigned a GSI score >0 than those from Lake 260. Predicted GSI scores were similar in fry analyzed from Lake 223 and Lake 260 (z = 1.039, *p*-value = 0.30) (Figure 2.18). The percentage of fry with a total GSI score of "0" ranged from 33% (Lake 375) to 82% (Lake 260). Severe deformities (total GSI ≥ 3) were present in only a small proportion of fish per lake (2 - 5%) (Figure 2.13).

At swim-up, fry from lake 260 were most likely to have no visible deformities (i.e. total GSI score of "0"). Model predictions indicated that fry from Lakes 223, 375 and 378 had a greater probability of receiving a GSI score >0 (z = 2.29, *p*-value = 0.02; z = 3.50, *p*-value < 0.001; z = 3.27, *p*-value = 0.001, respectively) although there was greater uncertainty in model estimates for these lakes (Figure 2.17). The percentage of swim-up fry scored with no deformities was highest in Lake 260 (91%), while fry originating from Lake 375 had the smallest percentage scoring "0" (71%). Analogous to fry scored at the alevin stage, very few swim-up fry originating from any the study lakes received GSI scores ≥ 3 (0 - 5%) (Figure 2.19).

Additional fish from Lake 260 that were exposed to a crude oil WAF are included in these tables/figures but were excluded from statistical analysis due to insufficient sample size. Percent frequency and severity of deformities in the WAF exposed sample was comparable to samples from the four study lakes, with no observable increase in deformity rates (Tables 2.3 and 2.4)

Percent differences between the two scoring iterations are shown in Figure 2.20. At the yolk-sac fry stage, re-scoring of all deformity types varied by less than 5% (0 – 4.6%) except for finfold deformities, which were different by 16.1%. Re-scoring at the swim-up stage was more consistent, with less than 5% difference between scores for all deformity types (0 – 4.3%).

DISCUSSION

The aim of this study was to assess the potential impact of previous model oil spill studies conducted in Lake 260 at the IISD – ELA on early life stage development in the resident lake trout population. Fry originating from Lake 260 and reared in the lab exhibited no phenotypic or developmental abnormalities relative to fish from reference lakes as a result of potential oil exposure. Adult fish collected in the fall of 2019 to obtain eggs and milt were similar in size and condition, reducing these as potential confounding variables on early life stage development. For example, increases in maternal length have been associated with increased juvenile fitness in wild sockeye salmon (*Oncorhynchus nerka*) (Braun et al., 2013). To further confirm homogeneity in egg condition, a subsample of eggs from each female were measured and assessed for colour as an indicator of carotenoid pigment content. Egg size was similar between lakes, while colour, although not significantly different between lakes, was more variable (see Figure A.9 for colour chart, and Table 2.1 for Mean ± SE colour in each lake). Egg

carotenoid content is primarily determined by maternal diet and is thought to be a potential predictor of egg survival in salmonid eggs experiencing oxidative stress (Palace and Werner 2006; Tyndale et al., 2008). Eggs with increased carotenoid content are thought to have a better chance at survival due to improved immune function and antioxidant reserves during sensitive early life stages. This reserve may also "prime" the organism to better incorporate antioxidants from their diet later in life (Tyndale et al. 2008). In our study, differences in egg colour were not correlated with egg mortality (Figure A.20), likely indicating that eggs from the four study lakes were not experiencing elevated oxidative stress. Thus, differences in carotenoid content ultimately played no role in survival to hatch.

Differences in egg diameter were generally positively correlated with length, weight, and yolk volume of hatched fry in most lakes (Figures A.14 – A.18). Salmonid species, including rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and sockeye salmon (*Oncorhynchus nerka*), consistently produce larger fry from larger eggs (Springate and Bromage 1985; Ojanguren et al., 1996; Braun et al., 2013). Nonetheless, differences in mean length, weight and condition factor among alevin and fry originating from the four study lakes were generally negligible. The slight mean differences in these metrics between fry in Lake 260 and those from other lakes reinforced the need for a study design involving more than one reference lake for comparison, due to natural variability. Fry from Lake 260 were comparable in length, weight, condition factor and yolk volume after hatch to one or more of the three reference lakes, and in many cases showed signs of increased fitness at hatch, relative to fry from the reference lakes. It has long been known that there is a positive correlation between fry size at emergence and fitness in salmonids (Ojanguren et al., 1996). The reasons for this are primarily due to a reduced threat of being consumed as prey at a larger size (i.e. too large for certain predators to

consume the fry), and improved swimming performance at a larger size (i.e. greater chance at evading predators). In addition, larger fry have an increased availability of food options, due to their proportionally larger jaw size (Miller et al., 1988). Low dose exposures to PACs during early development can reduce growth rates in some fish species. Early life stage fathead minnows, exposed to 12.5 µg/L fluoranthene had reduced growth rates after hatch, compared to controls or those exposed to 6 µg/L (Schuler et al., 2007). Moreover, pink salmon fry sampled from contaminated waters after the Exxon Valdez oil spill and reared in a laboratory environment had depressed growth compared to those collected from unexposed reference sites (Wilette 1996, Rice et al., 2001). While previous studies have reported links between PAC exposure and altered length and weight, concentrations in this study were comparatively low and may not have reached a threshold for these effects (Figure Al.1).

Due to limitations imposed by the study design, percent hatch was the only relevant metric that could be measured from the *in-situ* component of the study. While we were unable to determine the condition of fry that swam out of the hatch boxes, knowledge of the number present at the beginning of the study, and removal of mortalities at four time points allowed for an accurate calculation of percent hatch. Elimination of the outlier in Lake 375 (LT2) (potentially due to a fungal infection), and subsequent pooling of mean hatch success between the three reference lakes yielded comparable mean hatch to what was observed in Lake 260 (Figure 2.9). Mean percent survival ± SD in the reference lakes (77.3 ± 18.7 %) and in Lake 260 (76.2 ± 8.0 %) were comparable to the reference results (82.0 ± 8.1 %) from a previous *in-situ* study examining early life stage development in cutthroat trout (Chalmers et al., 2014). Furthermore, reference site percent hatch success in a separate hatch box study – also examining early life stage cutthroat trout – was far less (67%) than what was observed in the present study,

although the sample size at their reference site was smaller, increasing the likelihood of random error in the measurements (4 hatch boxes, 30 eggs per box) (Marlatt et al., 2016). The authors noted that leeches were observed at the reference site and may have contributed to a higher mortality than usual. There was no evidence of predation in the Scotty-Jordan incubators throughout this study. PAC exposure has been shown to negatively impact hatching success in several early life stage fish species, but most studies have been laboratory based and exposures are often limited to a single PAC compound of interest (Hose et al., 1982; Turcotte, 2008; Mu et al., 2012). Dilbit and crude oil contain 1000s of PAC congeners, due to the various modes of alkylation and substitution of the parent compounds. More recent research has emphasized the importance of investigating the interactive and potentially additive toxicity of these complex PAC mixtures, and as such, it is generally understood that lower concentrations of TPAC (~0.1 µg/L) can have a greater toxic effect in early life stage fish than a single compound alone (reviewed in Hodson, 2017). Total PAC concentrations in the surface waters of Lake 260 were 2295 ng/L 5 days after oil addition (June 26, 2019) but declined to 65 ng/L 23 days prior to egg collection (September 16, 2019) (Figure A.1). It is likely that eggs deployed in Scotty-Jordan incubators were exposed to PAC concentrations similar to those measured in mid-September– approximately half the hazardous threshold concentration for early life stage fish indicated by Hodson (2017). It is worth noting that 92% of the PACs measured in mid-September in 2019 were two or three ringed structures, an indication of petrogenic origin (Sakari 2012). Ideally, hatched fry would have been sampled from the incubators in all study lakes and assessed for morphological abnormalities, in a similar fashion to those assessed in the lab component of this study. Doing so would have increased the confidence in our conclusions regarding early life stage lake trout development in Lake 260. Hatching success – while still a valuable metric – is

ultimately insufficient to make any firm conclusions regarding the sublethal impact of potential residual oil on the developing embryos/fry.

Rearing separate batches of eggs, originating from each study lake, in a lab environment permitted the evaluation of several additional metrics. The number of unfertilized eggs, egg mortality, hatching success and accumulated thermal units to 50% hatch among batches were all monitored in eggs reared in Heath trays until "swim-up" at ~800 ATU. The percentage of unfertilized eggs was similar among clutches originating from the four study lakes, and the eggs exposed to the WAF during water hardening. There was a far greater proportion of unfertilized eggs observed in this study than was seen in a similar previous study conducted by Werner et al. (2006). Authors of the previously mentioned study collected eggs from Lake 260 and Lake 442 at the IISD - ELA, fertilized and reared them in a similar fashion to the method described here. Fertilization success approached 100% in the Werner et al. (2006) study for all clutches collected over a five-year time span. It is not clear why the eggs in this study were not dry fertilized with the same success, but the lack of differences among lakes confirms that it likely is not related to potential oil exposure in Lake 260 (Figure 2.10). Percent egg mortality (pre-hatch) and hatching success were similar across all study lakes but differed from values observed in comparable lab based salmonid early life stage studies. Le Bihanic et al. (2014) reported embryonic mortality of $2.0 \pm 2.8\%$ in reference group rainbow trout and observed a hatching rate of $94.0 \pm 2.8\%$. Similarly, Werner et al. (2006) observed 94% hatching success in lake trout eggs reared in Heath trays, while percent mortality was more variable, ranging from ~7-38%. In the present study, we observed mean mortality rates ranging from 4.9 – 8.7% and mean hatching success ranging from 79 – 85.5%, among the four study lakes. Given the similarity in these metrics between lakes, potential maternal exposure to oil was not a factor in any increased mortality or decreased

hatching success. The differences in mortality and hatch success compared to other similar studies most likely reflects a difference in rearing protocol. Exposure to light, increased handling during the eye up period or a lack or egg disinfection can all negatively impact egg survival, and there is potential for differences in these factors between the studies (Leitritz, 1959). Eggs were not disinfected at any point; were handled at all points throughout the study to remove dead eggs; and fluorescent lighting was present when picking for mortalities – any of which could have contributed to the differences in percent hatching and egg mortality observed between our study an those previous. The percent mortality of hatched fry amongst rearing trays was consistent with metrics observed in reference fish by Le Bihanic et al. (2014), however in their study, exposure to low concentrations of PACs did not appear to influence fry survival. Severe edema was observed in a small proportion of fry amongst the three rearing trays (<1% in each tray) and was comparable or less than background rates typically reported in hatchery reared salmonids (Carls et al., 2010; Le Bihanic et al., 2014). While fry from Lake 260 required fewer mean ATUs to reach 50% hatch than those from the reference lakes (i.e. earlier hatch), exposure to PACs is more often associated with delays in development and hatching (Berube et al., 2022). Additionally, ATU to 50% hatch was consistently within the range of expected values for hatchery reared lake trout amongst all compartments (Guiney et al., 1997).

Several additional morphological abnormalities were investigated in a subsample of fry at two developmental stages: the alevin (550 ATU), and the swim-up fry (800 ATU). Most studies assessing early life stage salmonid deformities have focused on the swim-up stage, but alevin have also been examined (Carls and Thedinga, 2010). At both stages, fry from Lake 260 had the greatest frequency of zero scores (i.e. no deformity) (Figures 2.16 and 2.17). Correspondingly, predicted model probabilities of a GSI score of zero were highest in Lake 260 fish at both

developmental stages (Figures 2.18 and 2.19). It should be noted that a GSI score of "1" denotes a minor deformity and is close to a score of "0" in this analysis. For this reason, Covington et al. (2018) grouped GSI scores of "0" and "1". Alternatively, Janz et al. (2010) recommend using a frequency approach, noting the presence or absence of various deformity types as a more repeatable method for scoring developing fry. We decided that due to the uncertainty surrounding fish with mild deformities, it was best to score fry for severity of deformity, while also acknowledging the difficulty in assessing a score of "1" for a given category. We did not group scores of "0" and "1" together, but acknowledge the small difference in these scores supports that the incidence of severe deformities in the fry from this study is extremely low. Lake 260 fish showed no phenotypic effects of exposure to oil constituents via maternal transfer. Previous studies have shown that exposure to low levels of PACs in maternal fathead minnows and zebrafish can lead to an increased incidence of deformities in the F1 generation, but it has yet to be shown to occur in salmonids (Tilghman Hall and Oris, 1991; Corrales et al., 2014).

While no phenotypic effects were detected, adult lake trout from Lake 260, sampled in 2019, did have upregulated *cyp1a3* in mucus samples collected after oil addition – an indicator of PAC exposure – albeit to a non-statistically significant extent (1.2 fold) (Andrzejczyk, 2020). The upstream effects of *cyp1a* induction (i.e. activation of the aryl hydrocarbon receptor) have been shown to yield both oestrogenic or anti-estrogenic effects in fish, leading to potential reproductive impairment (Navas and Segner, 1998; Valdehita et al., 2012). In June of 2019, female lake trout in Lake 260 may have been exposed to the residual oil from one of the model oil spill studies (the FOReSt project), potentially impacting egg development via hormonal disruption or by depositing PACs absorbed through the gills or through their diet into the lipid rich yolk. Since the developing fry raised in the laboratory were never exposed to the water in

their lake of origin, maternal transfer of PACs to the yolk was the only potential pathway of exposure for fry from Lake 260. Several studies have examined the effects of PAC exposure on early life stage salmonids, due to their high sensitivity to the compounds. Le Bihanic et al. (2014) exposed rainbow trout to two model PACs, fluoranthene and benzo[*a*]pyrene (0.3 µg/g dw from spiked gravel), as well as three different PAC fractions including a pyrolytic PAC fraction, and two petrogenic fractions (3000, 10 000 ng/g dw from spiked gravel). They observed significant increases in larval abnormalities among fry exposed to the fluoranthene and benzo[*a*]pyrene spiked gravel, particularly in the form of edema and skeletal deformities. In fry exposed to the petrogenic fractions containing 3000 ng/g TPAC, between 81.7 – 95.6 % of larvae were deformed. In an earlier study, researchers observed that early life stage rainbow trout displayed a dramatic increase in the incidence of blue sac disease (i.e. edema) when exposed to retene (9-32 µg/L) near hatch, while eggs exposed before hatch and transferred to clean water showed a less pronounced response (Brinkworth et al., 2003).

In the present experiment, eggs exposed to a conventional heavy crude WAF containing ~0.2 µg/L TPAC during water hardening were included as a positive control. These fry showed no increase in the frequency of deformities, compared to the unexposed fry. The lack of a teratogenic effect in these fry could be explained by the induction of *cyp1a* after liver formation, facilitating successful metabolism of PACs and the subsequent contribution of maternally deposited antioxidants in eliminating harmful ROS produced from the PAC metabolism (Brinkworth et al., 2003; Perrichon et al., 2016). A more likely explanation is that some combination of PAC concentration in the WAF and duration of exposure were insufficient to yield a phenotypic effect. Rigaud et al. (2020) exposed rainbow trout eyed embryos to three model PACs (retene, pyrene and phenanthrene) at nominal concentrations ranging from 32 – 100

µg/L until just after hatch (alevin). These exposure concentrations – two orders of magnitude greater than the conventional heavy crude WAF used for exposure in our study – caused no observed changes in deformity rates, compared to the controls. It's worth noting that eyed embryos were used to conduct the previously mentioned study, whereas our exposure occurred during water hardening, and only lasted one hour.

The timing of exposure has been shown to yield different effects in early life stage salmonids, so direct comparisons between studies are ultimately difficult (Brinkworth et al., 2003). At both developmental stages assessed in this study, GSI scores >1 were rarely observed, meanwhile baseline deformity rates in lab reared trout are ~5% (Janz et al., 2010). In Lake 260 fry, GSI scores >1 were observed in 4% of fry at the alevin stage and 5% of fry at swim-up. These rates were among the lowest in the four study lakes, further confirming the lack of any PAC related phenotypic effect in these early life stage lake trout.

To assess the objectivity of the deformity scoring rubric, pictures were blindly re-randomized and subsequently re-scored. All fry were re-scored to assess whether the difference between scores was less than 10%, a common threshold that indicates reproducibility in the rubric (Janz and Muscatello, 2008). For all deformity types, apart from the finfold category at the alevin stage, percent difference was less than 5% (Figure 2.20). Finfold deformities were particularly difficult to score at this stage due to the translucence of fins, their relatively small size, and lack of differentiation. Moreover, folding, and possible damage due to fixation made it challenging to properly assess whether a reduction in fin size was the result of a true deformity. Finfold deformities were among the most prevalent deformity in fry from all lakes, but again these results should be interpreted with caution.

Analysis of deformities in developing salmonid fry is a sensitive indicator of potential effects from exposure to PACs (Le Bihanic et al., 2014). Using an objective scoring technique has several advantages, but some limitations must be addressed. Firstly, fry were scored based on photos from one lateral angle making it difficult or impossible to fully assess craniofacial (hidden eye, ventral portions of the jaw) and finfold (hidden pectoral fin) deformities. Future studies should ensure that several angles of the fry are photographed to allow for a more complete analysis of these deformity types. Furthermore, the preservation of fry in Davidson's fixative led to spinal bends resembling scoliosis (fry likely bent in the curved bottom of Falcon tubes), damaged fins (pectoral, dorsal and caudal) and punctured yolk sacs on some occasions. While not always practical, it is recommended that similar deformity analyses be conducted on recently euthanized fry to minimize artefacts that can complicate the experiment (i.e. distinguishing between a fixation artefact vs. a true deformity). At minimum, fry should be preserved in a container with a flat bottom surface, to reduce the chance of non-deformity spinal bends.

CONCLUSION

Results from this study indicate that potential exposure to PACs in the adult lake trout from Lake 260 was too low to impact reproduction. Although PAC concentrations were elevated in a water sampling location outside of the enclosures for a short time after oil addition to the enclosures in 2019, concentrations quickly returned to pre-addition levels as compounds were either naturally degraded, volatilized, or were diluted into the lake. Lake trout eggs spawned from fish in Lake 260 after model oil spill studies in 2018 and 2019 model oil spill studies showed no evidence of differences in fertilization success, hatching abnormalities, phenotypic

abnormalities or mortality compared to eggs spawned from fish originating from three unimpacted IISD-ELA reference lakes. A subsample of eggs were intentionally exposed to a crude oil WAF containing ~0.2 µg/L of PACs during water hardening to serve as a positive control for the laboratory study. These fish showed no apparent change in the aforementioned parameters, highlighting the importance of exposure timing and duration if attempting to elicit a phenotypic effect in early life stage salmonids. These findings underscore the efficacy of containment and contingency measures put in place at IISD-ELA to prevent diluted bitumen from negatively impacting the ecology of Lake 260 resulting from the 2018 and 2019 model oil-spill studies. We recommend the utilization of similar containment methods for future enclosure studies examining a harmful contaminant that is unsuitable for a whole-lake study.

TABLES AND FIGURES

TABLE 2.1. Weight, length, condition factor, egg diameter, egg number/gram body weight and egg colour from adult female fish collected in the four study lakes. Data are expressed as Mean ± standard error. See Appendix A for Colour scale. No statistical differences were noted compared to Lake 260 fish.

Lake	N	Weight (g)	Fork Length (mm)	Condition Factor (K)	Egg Diameter (mm)	Eggs/g Bdwt	Colour
260	7	817 ± 43[a]	417 ± 9	1.12 ± 0.02	5.21 ± 0.21	0.75 ± 0.1	6.9 ± 0.2
223	7	741 ± 26	403 ± 8	1.13 ± 0.04	5.14 ± 0.14	0.74 ± 0.06	9.1 ± 1.3
375	3	758 ± 15	416 ± 8	1.06 ± 0.04	5.33 ± 0.17	0.94 ± 0.06	7.3 ± 1.2
378	3	817 ± 52	412 ± 10	1.16 ± 0.02	4.92 ± 0.08	0.78 ± 0.03	5.0 ± 0.9

TABLE 2.2. Weight, length, and condition factor from adult male fish collected in the four study lakes. Data are expressed as Mean ± standard error. No statistical differences were noted compared to Lake 260 fish.

Lake	N	Weight (g)	Fork Length (mm)	Condition Factor (K)
260	7	754 ± 36	420 ± 10	1.02 ± 0.03
223	8	713± 28	402 ± 5	1.10 ± 0.02
375	4	673 ± 26	398 ± 6	1.06 ± 0.03
378	2	702 ± 85	396 ± 12	1.12 ± 0.03

TABLE 2.3. Frequency and mean severity (± SE) of deformity scores from yolk-sac fry (~550 ATU) in the four study lakes and the WAF exposed group. See Appendix B for deformity rubric.

Lake	260 WAF	260	223	375	378
Frequency of deformity (%)					
Edema	10.0	0	0	3.3	0
Skeletal	0	6.9	10.9	20.0	7.1
Craniofacial	10.0	3.9	5.9	25.0	10.7
Finfold	0	9.8	9.9	30.0	23.2
Severity of deformity (score)					
Edema	0.1 ± 0.1	0	0	0.03 ± 0.02	0
Skeletal	0	0.11 ± 0.05	0.17 ± 0.05	0.23 ± 0.06	0.11 ± 0.06
Craniofacial	0.2 ± 0.2	0.08 ± 0.04	0.06 ± 0.02	0.35 ± 0.09	0.11 ± 0.04
Finfold	0	0.11 ± 0.03	0.1 ± 0.03	0.35 ± 0.07	0.25 ± 0.06

TABLE 2.4. Frequency and mean severity (± SE) of deformity scores from swim-up fry (~800 ATU) in the four study lakes, plus the WAF exposed group. See Appendix B for deformity rubric.

Lake	260 WAF	260	223	375	378
Frequency of deformity (%)					
Edema	0	1.3	1.9	0	0
Skeletal	0	1.3	1.9	4.8	0
Craniofacial	6.7	8.3	10.2	11.1	26.9
Finfold	0	1.9	7.4	17.4	0
Severity of deformity (score)					
Edema	0	0.01 ± 0.01	0.04 ± 0.03	0	0
Skeletal	0	0.04 ± 0.03	0.06 ± 0.04	0.11 ± 0.06	0
Craniofacial	0.7 ± 0.7	0.15 ± 0.06	0.16 ± 0.07	0.11 ± 0.04	0.34 ± 0.08
Finfold	0	0.04 ± 0.02	0.08 ± 0.03	0.22 ± 0.07	0

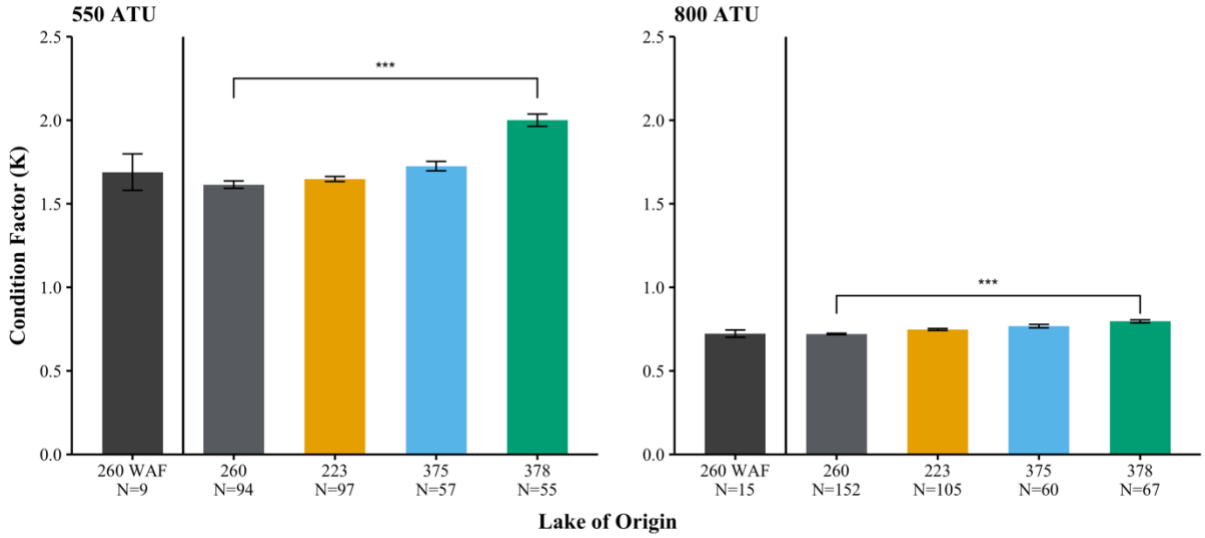


FIGURE 2.4. Condition factor amongst yolk-sac fry (~550 ATU, left) and swim-up fry (~800 ATU, right) originating from the four study lakes. Fry from Lake 260 exposed to WAF during water hardening are included for visual comparison but excluded from statistical analysis. Data are expressed as Mean ± standard error (Statistical differences denoted by * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Sample size is shown below each respective lake.

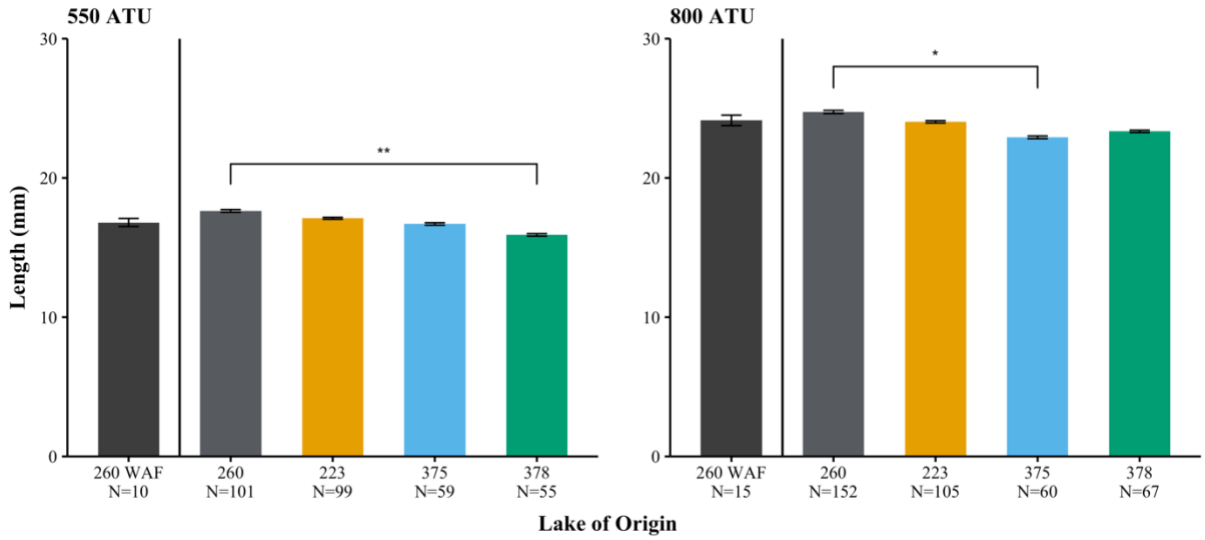


FIGURE 2.5. Length amongst yolk-sac fry (~550 ATU, left) and swim-up fry (~800 ATU, right) originating from the four study lakes. Fry from Lake 260 exposed to WAF during water hardening are included for visual comparison but excluded from statistical analysis. Data are expressed as Mean ± standard error (Statistical differences denoted by * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Sample size is shown below each respective lake.

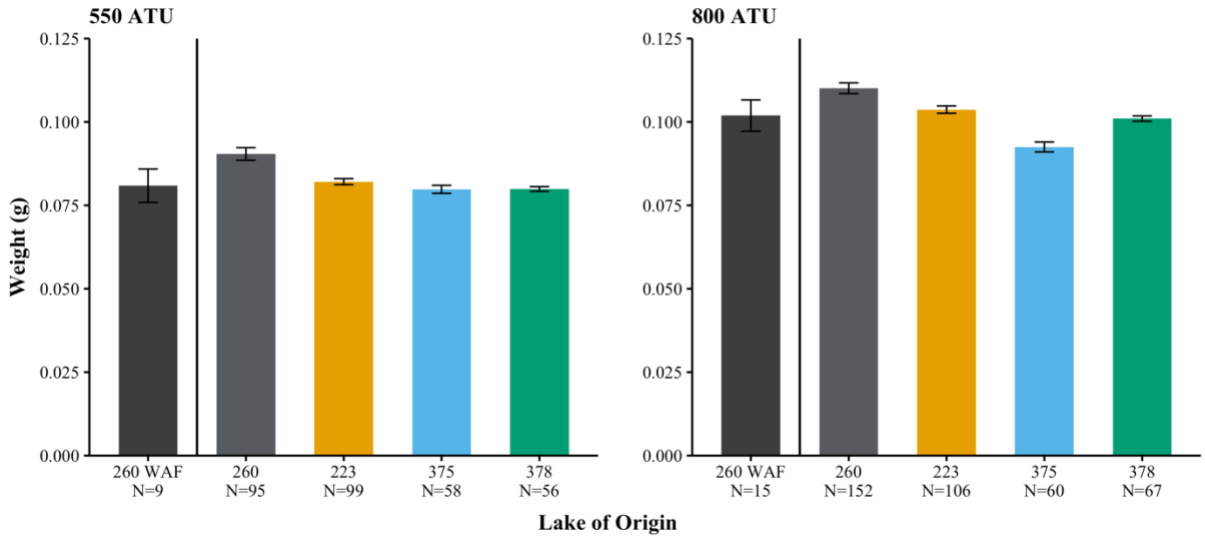


FIGURE 2.6. Weight amongst yolk-sac fry (~550 ATU, left) and swim-up fry (~800 ATU, right) originating from the four study lakes. Fry from Lake 260 exposed to WAF during water hardening are included for visual comparison but excluded from statistical analysis. Data are expressed as Mean ± standard error. No statistical differences were noted compared to Lake 260 fry. Sample size is shown below each respective lake.

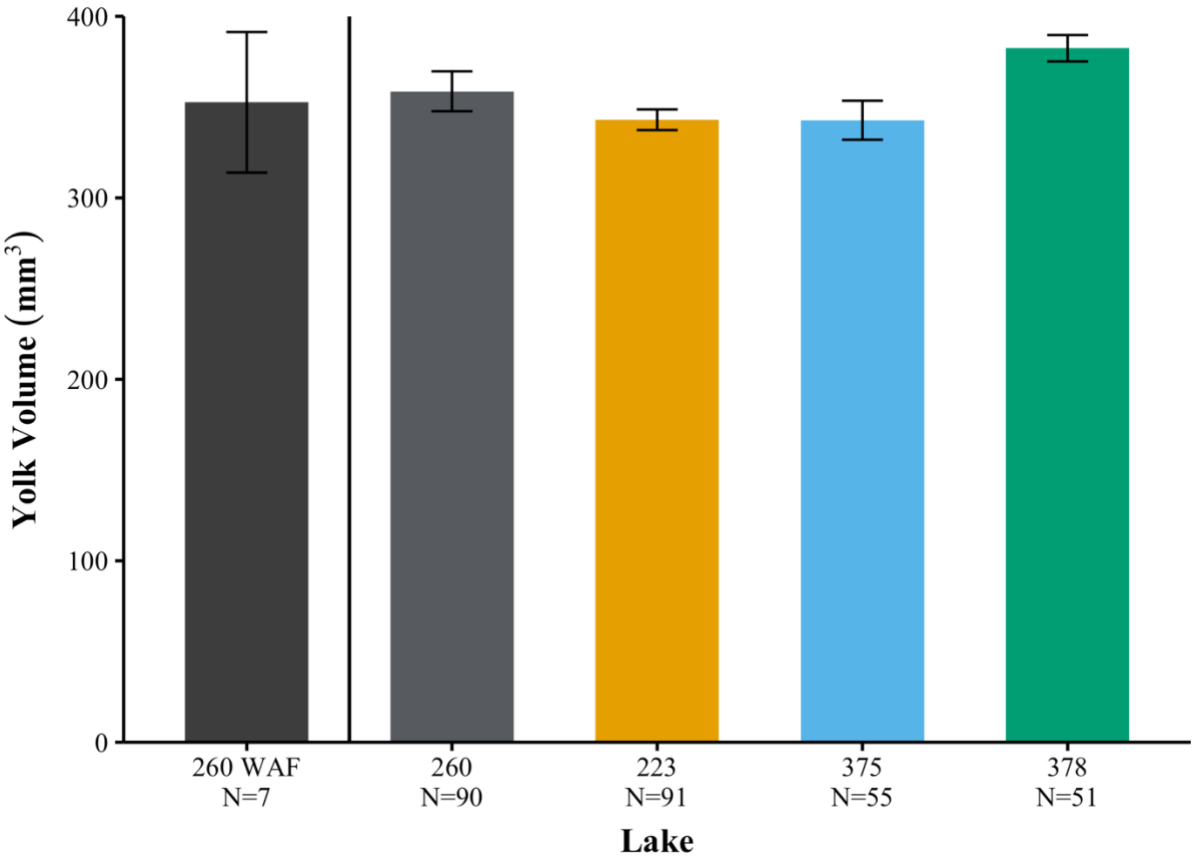


FIGURE 2.7. Yolk volume amongst yolk-sac fry at ~550 ATU development and originating from the four study lakes. Fry from Lake 260 exposed to WAF during water hardening are included for visual comparison but excluded from statistical analysis. Data are expressed as Mean ± standard error. No statistical differences were noted compared to Lake 260 fry. Sample size is shown below each respective lake.

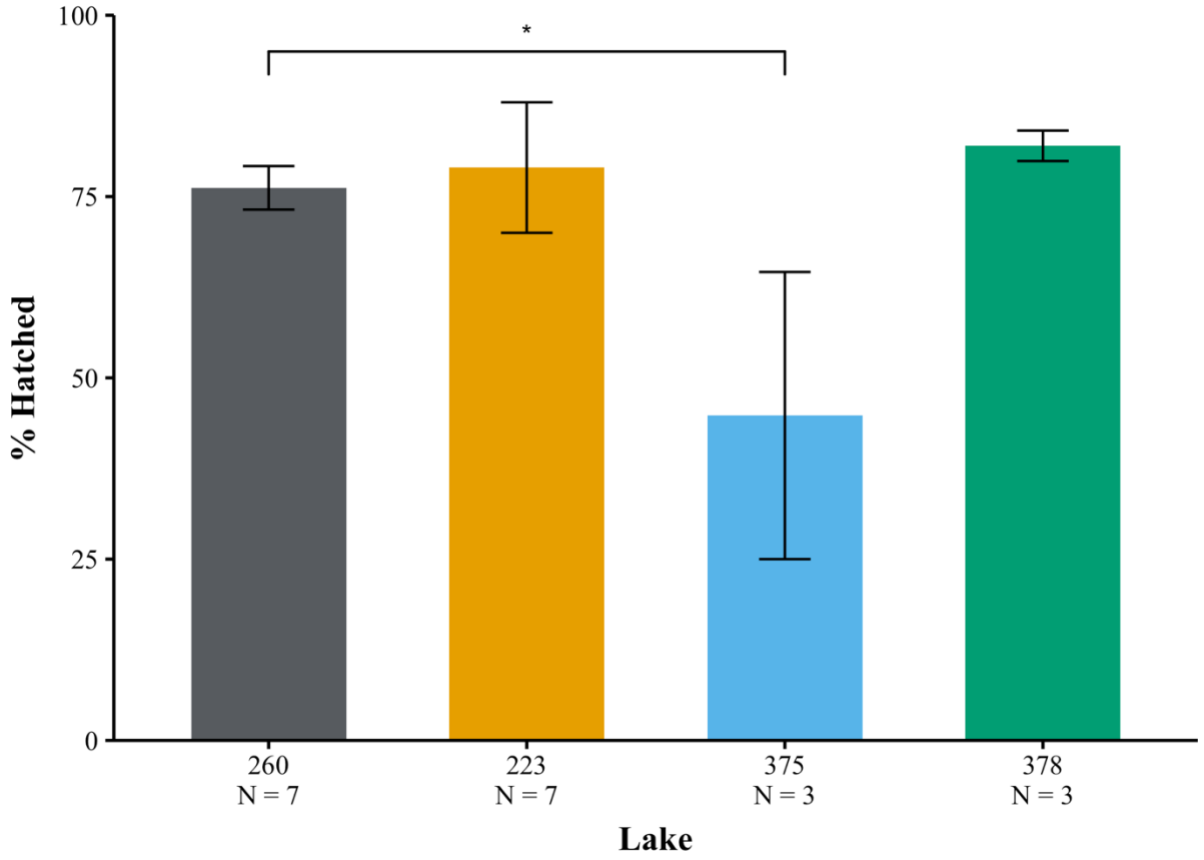


FIGURE 2.8. Percent hatching success in Scotty-Jordan incubators amongst the four study lakes. Data are expressed as Mean ± standard error (Statistical differences from Lake 260 are denoted by * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Sample size is shown below each respective lake.

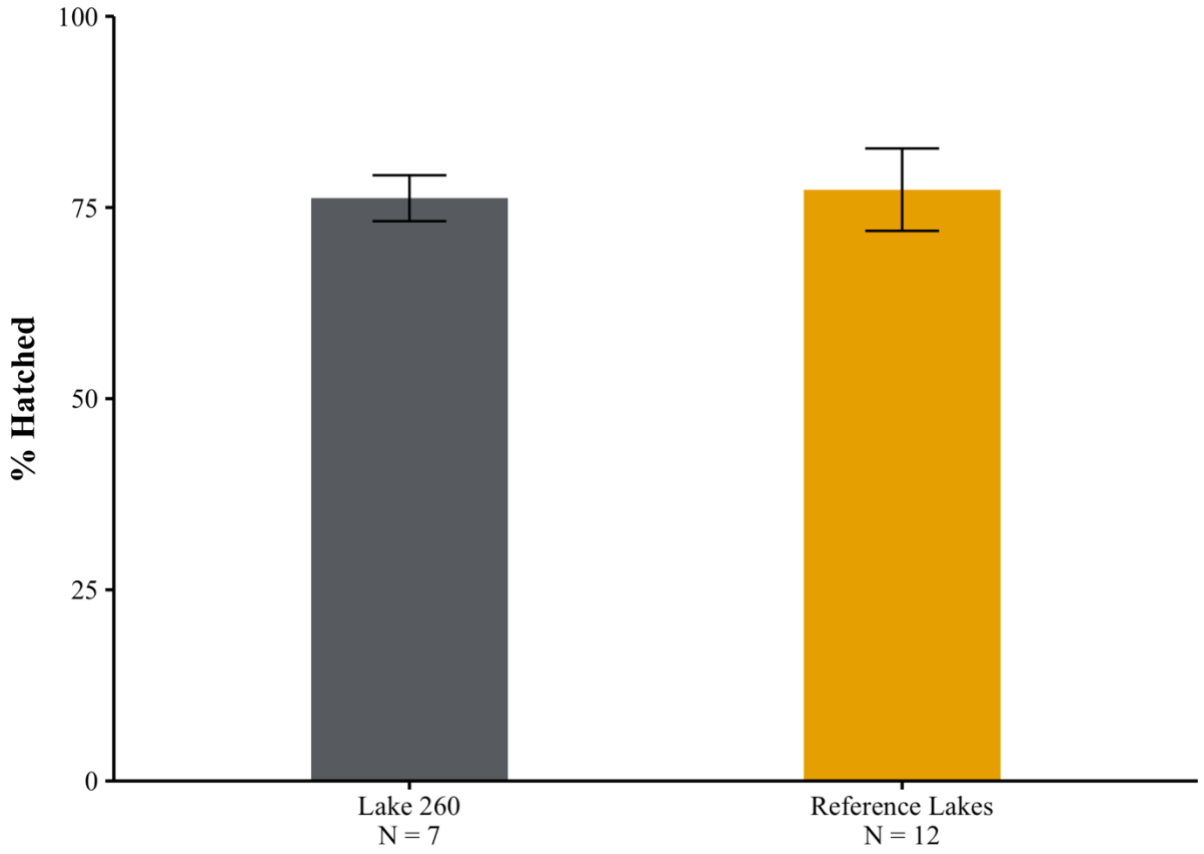


FIGURE 2.9. Percent hatching success in Scotty-Jordan incubators from Lake 260 and the three reference lakes (Lake 223, Lake 375, Lake 378) excluding a potential outlier in Lake 375 (LT2). No statistical differences were noted compared to Lake 260 fry. Sample size is shown below each respective lake.

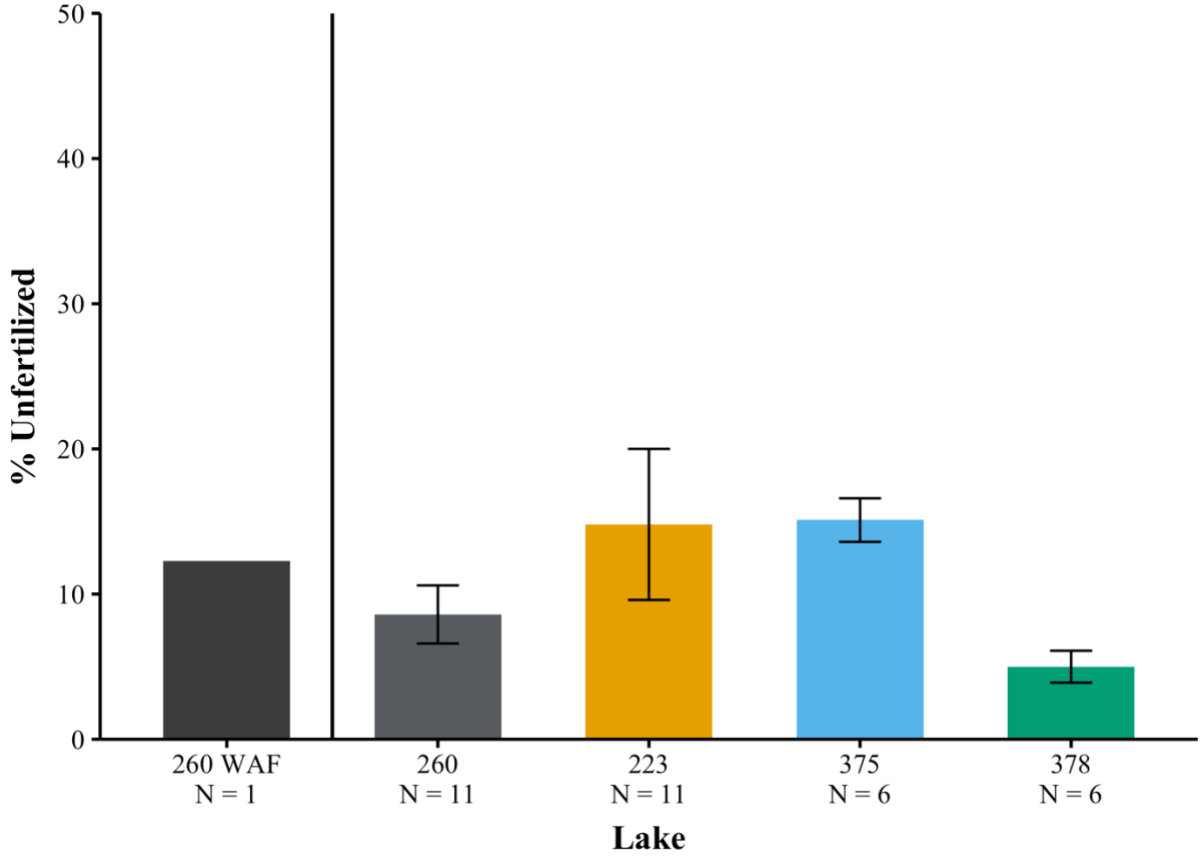


FIGURE 2.10. Percentage of unfertilized eggs in heath trays, amongst the four study lakes. Fry from Lake 260 exposed to WAF during water hardening are included for visual comparison but excluded from statistical analysis. No statistical differences were noted compared to Lake 260 fry. Sample size is shown below each respective lake.

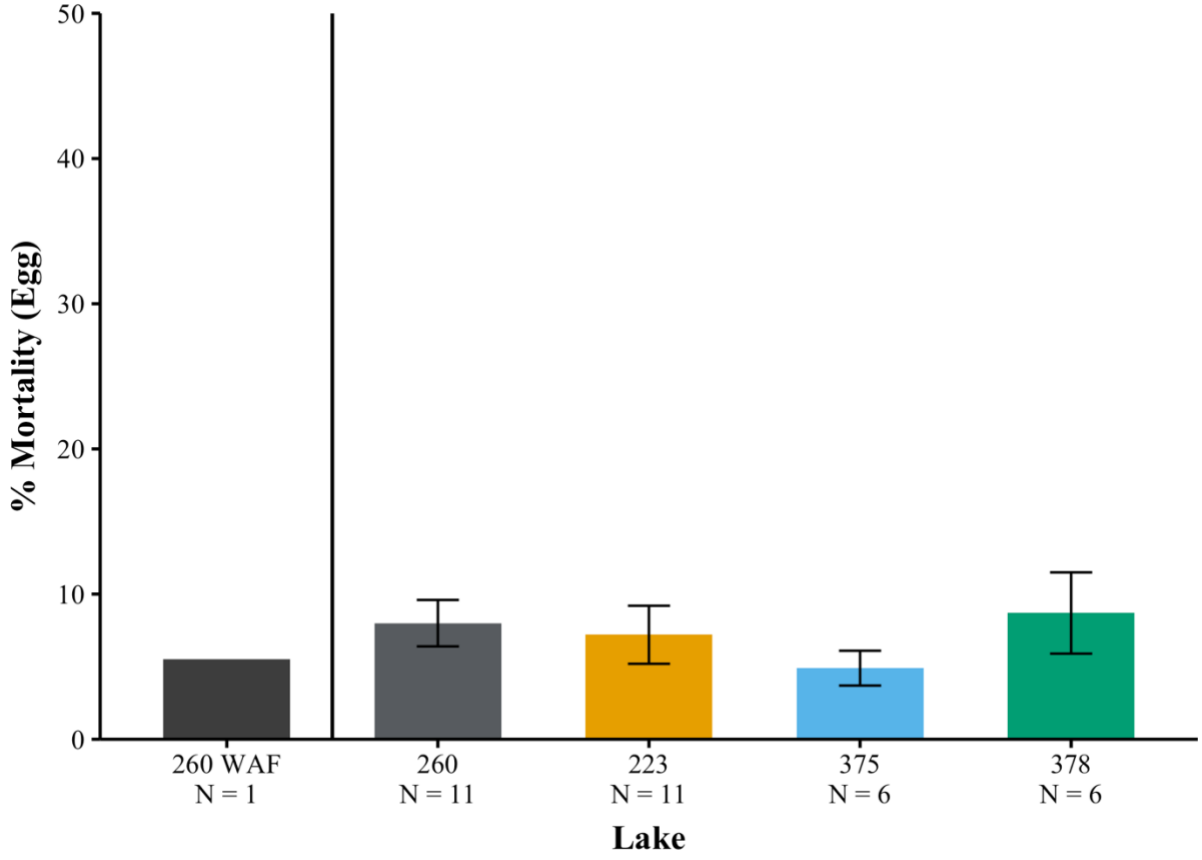


FIGURE 2.11. Percentage of mortality pre-hatch in heath trays, amongst the four study lakes. Fry from Lake 260 exposed to WAF during water hardening are included for visual comparison but excluded from statistical analysis. No statistical differences were noted compared to Lake 260 fry. Sample size is shown below each respective lake.

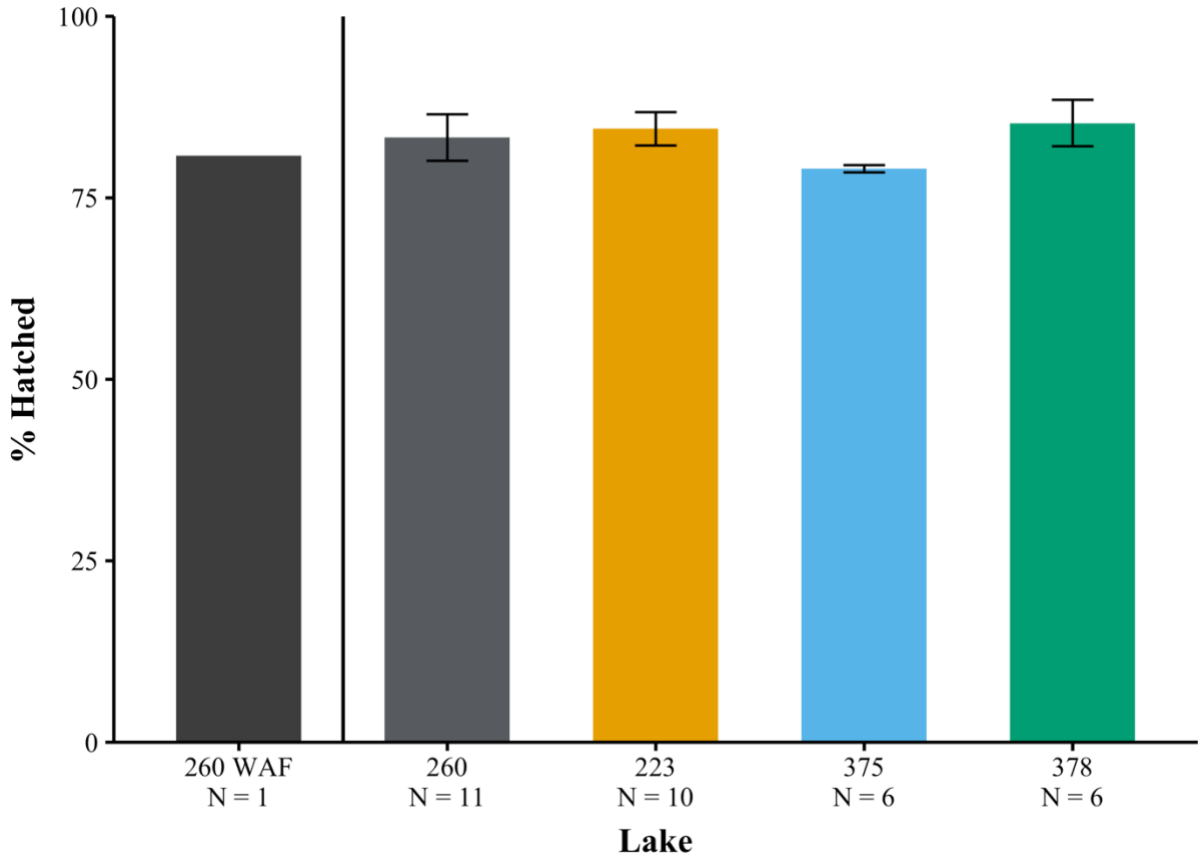


FIGURE 2.12. Percentage of successful hatches in heath trays, amongst the four study lakes. Fry from Lake 260 exposed to WAF during water hardening are included for visual comparison but excluded from statistical analysis. No statistical differences were noted compared to Lake 260 fry. Sample size is shown below each respective lake.

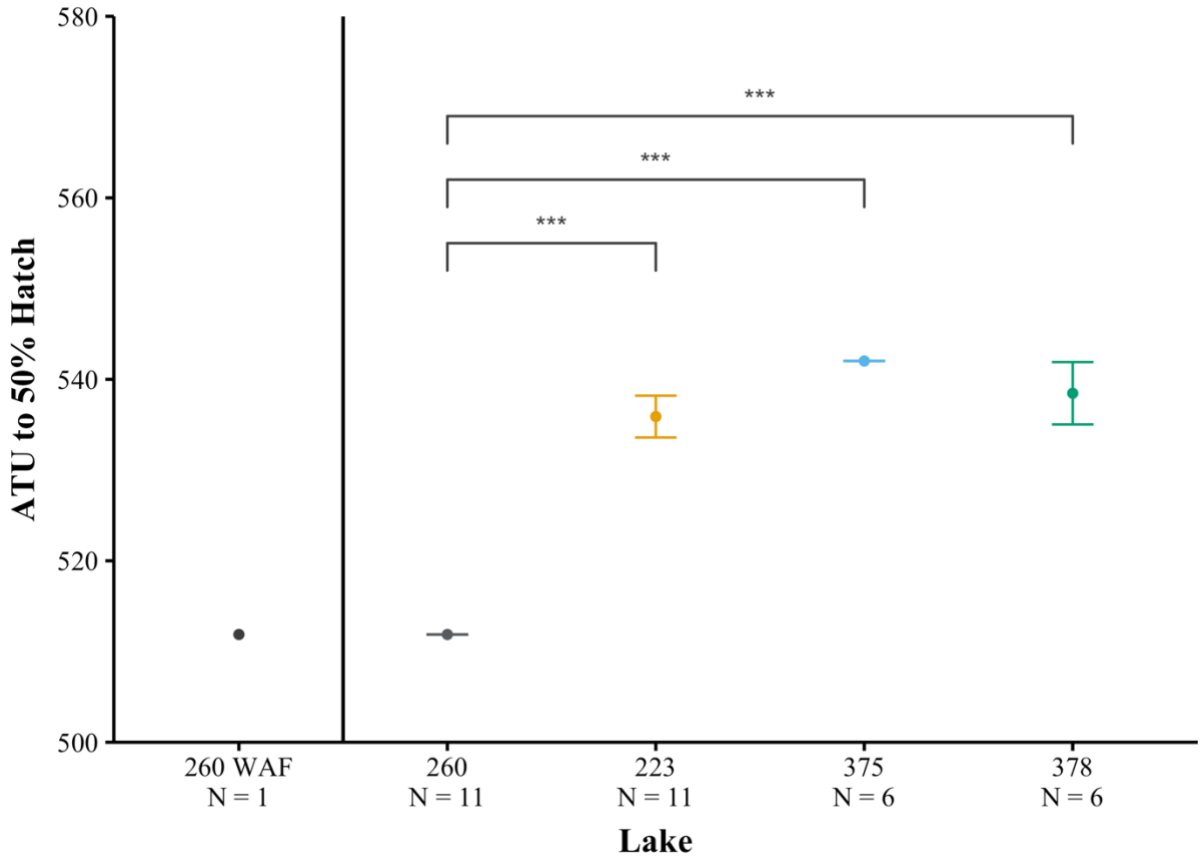


FIGURE 2.13. Accumulated thermal units (ATU) to 50% hatch. Compartment from Lake 260 exposed to WAF during water hardening are included for visual comparison but excluded from statistical analysis. Points represent Mean ± standard error. Note y-axis origin at 500 ATU. Sample size is shown below each respective lake.

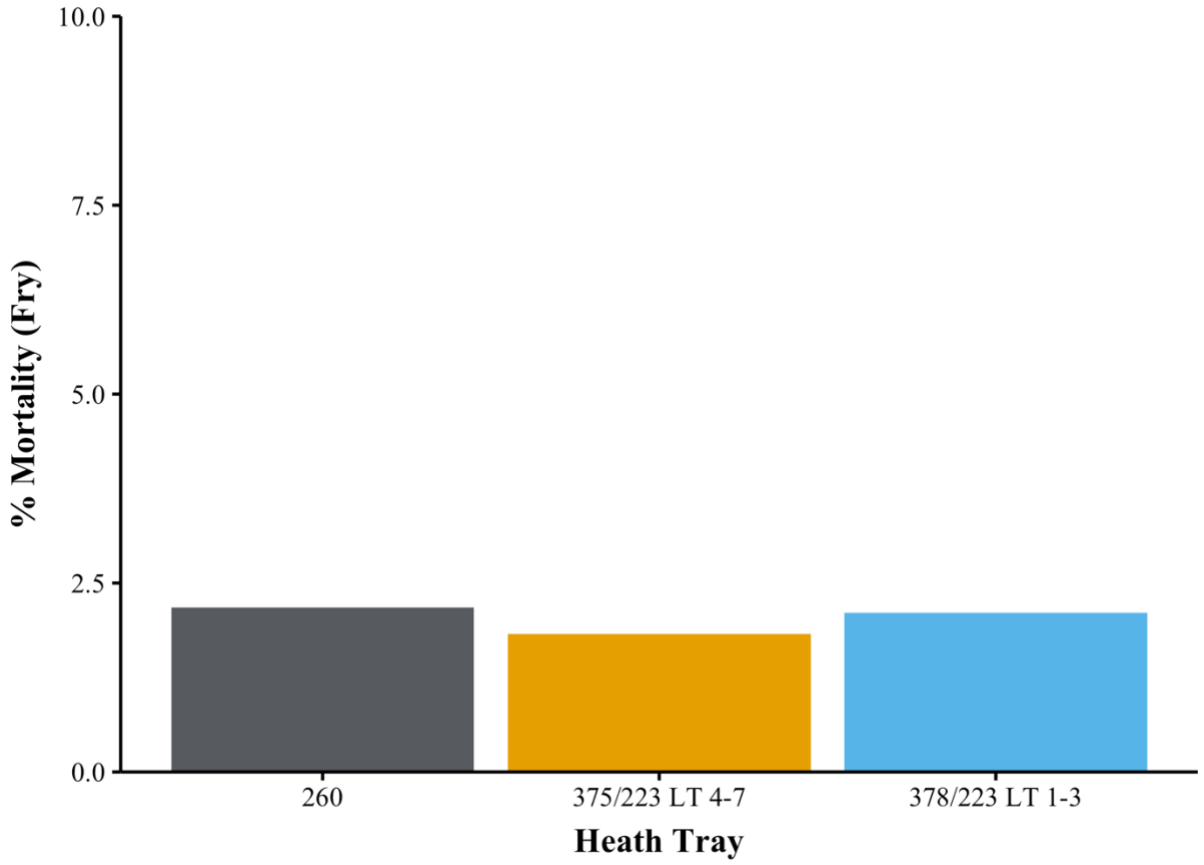


FIGURE 2.14. Percentage of mortality in hatched fry, expressed as a total across the three Heath trays. Tray “260” includes the subsample of fry exposed to crude oil WAF.

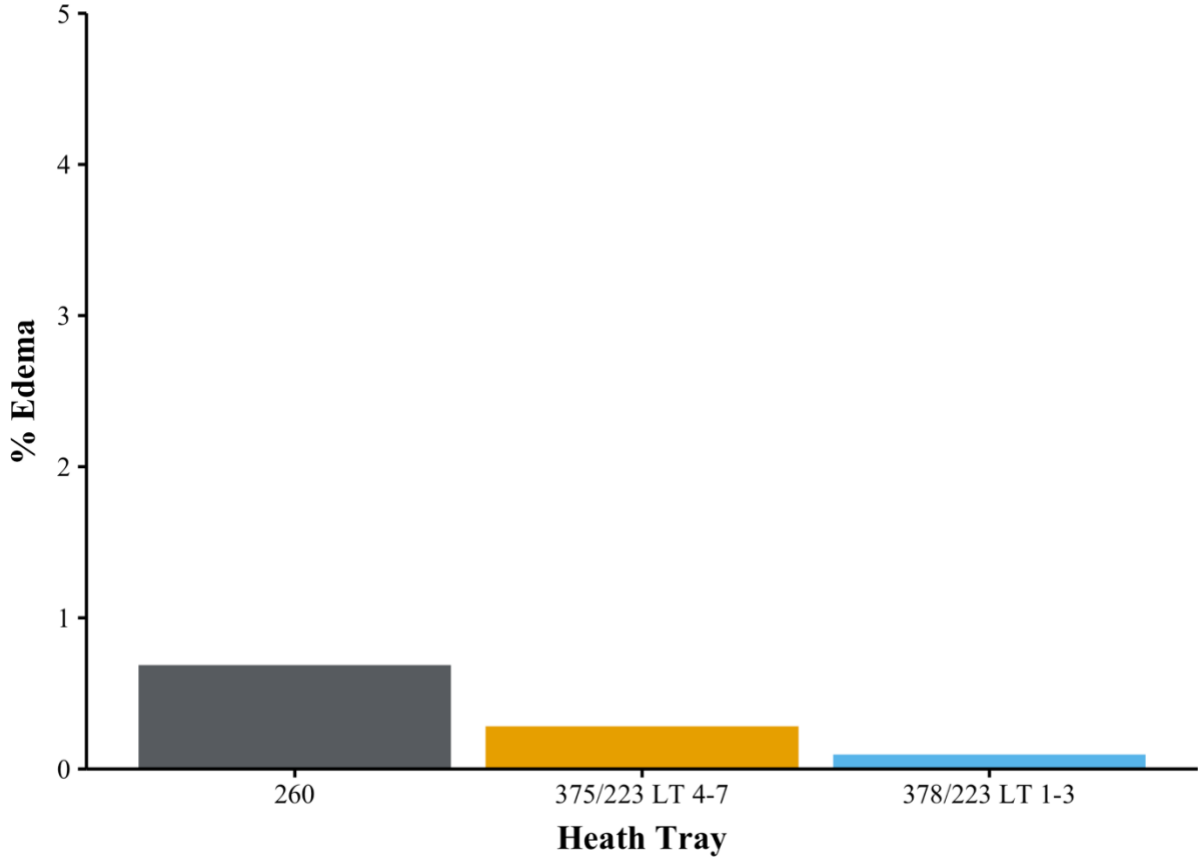


FIGURE 2.15. Percentage of severe edema in hatched fry, expressed as a total across the three Heath trays. Tray “260” includes the subsample of fry exposed to crude oil WAF.

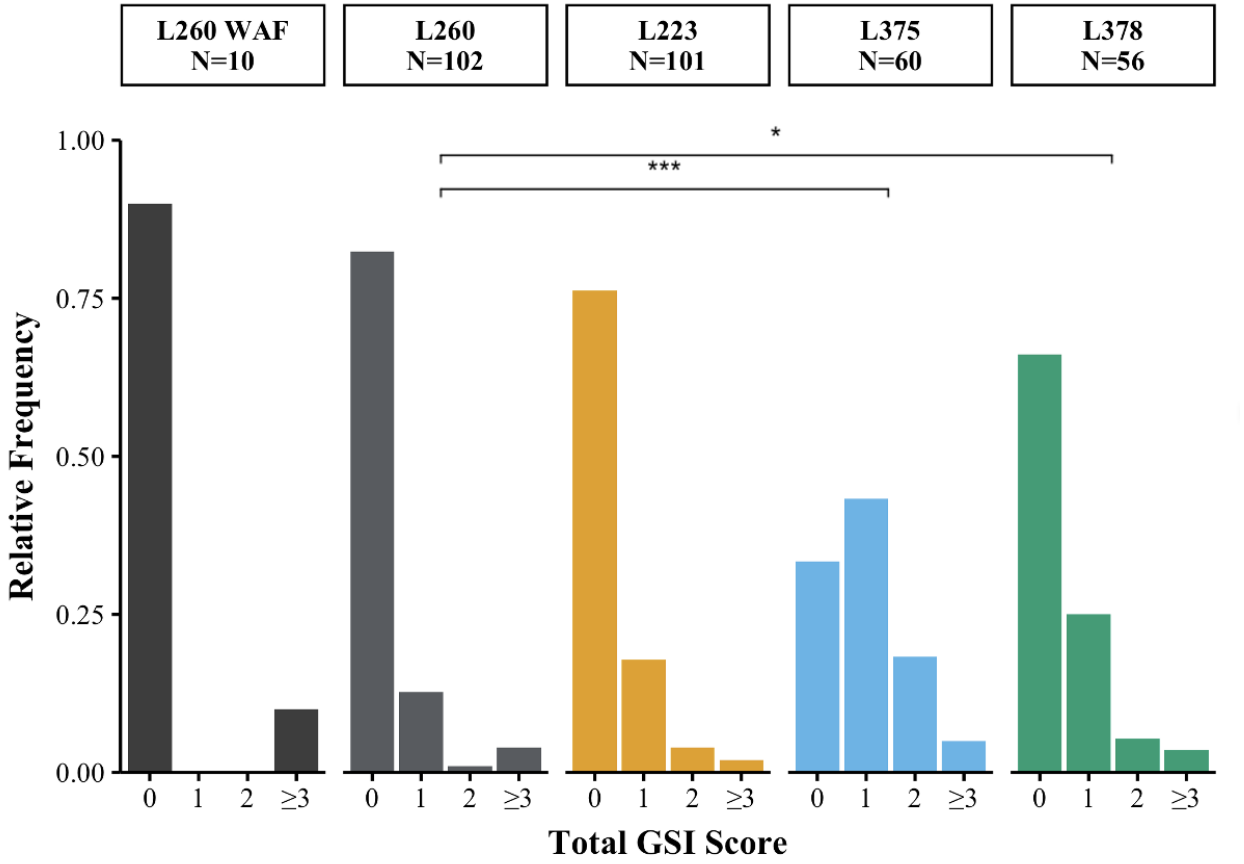


FIGURE 2.16. Total GSI score amongst yolk-sac fry (~550 ATU) originating from the four study lakes. Fry from Lake 260 exposed to WAF during water hardening are included for visual comparison but excluded from statistical analysis. Bars represent relative frequencies for a given lake (Statistical differences from Lake 260 are denoted by * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Sample size is shown below each respective lake.

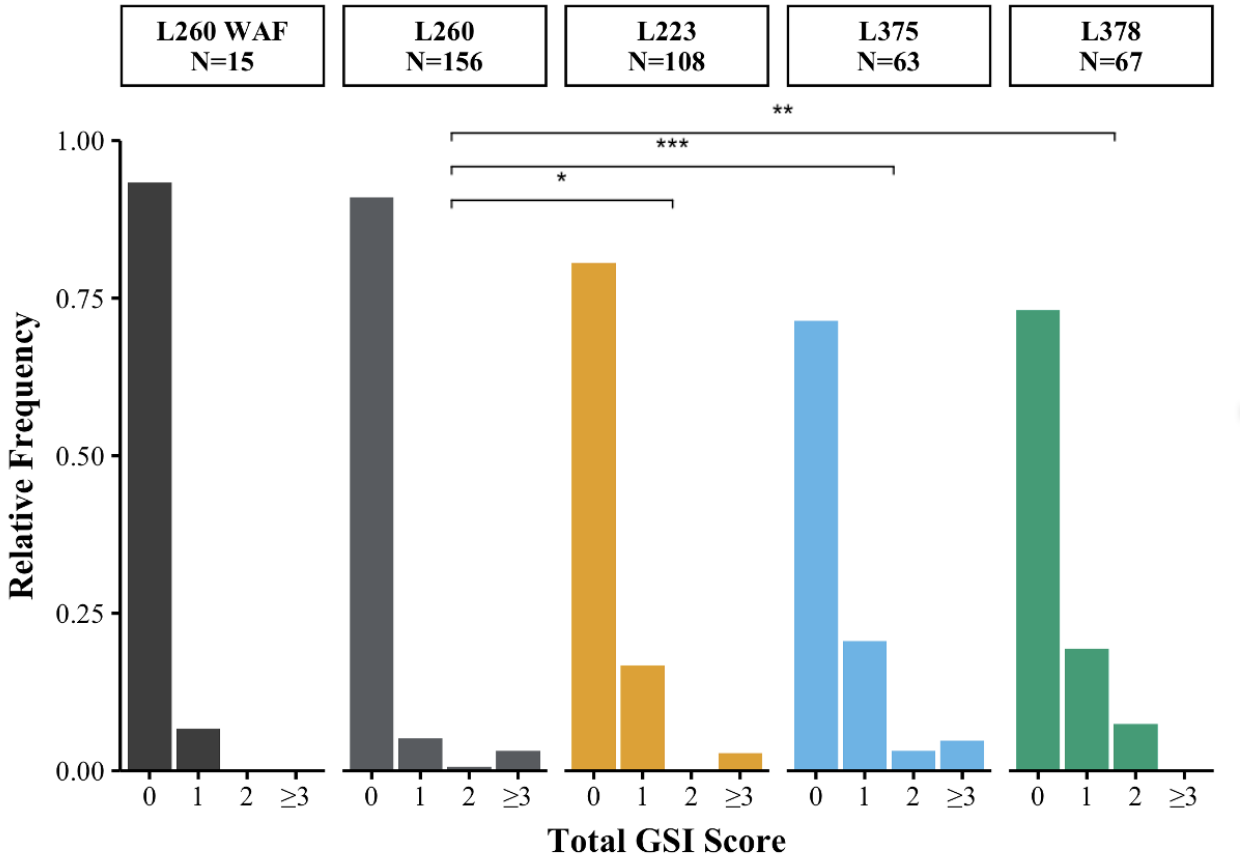


FIGURE 2.17. Total GSI score amongst swim-up fry (~800 ATU) originating from the four study lakes. Fry from Lake 260 exposed to WAF during water hardening are included for visual comparison but excluded from statistical analysis. Bars represent relative frequencies for a given lake (Statistical differences from Lake 260 are denoted by * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Sample size is shown below each respective lake.

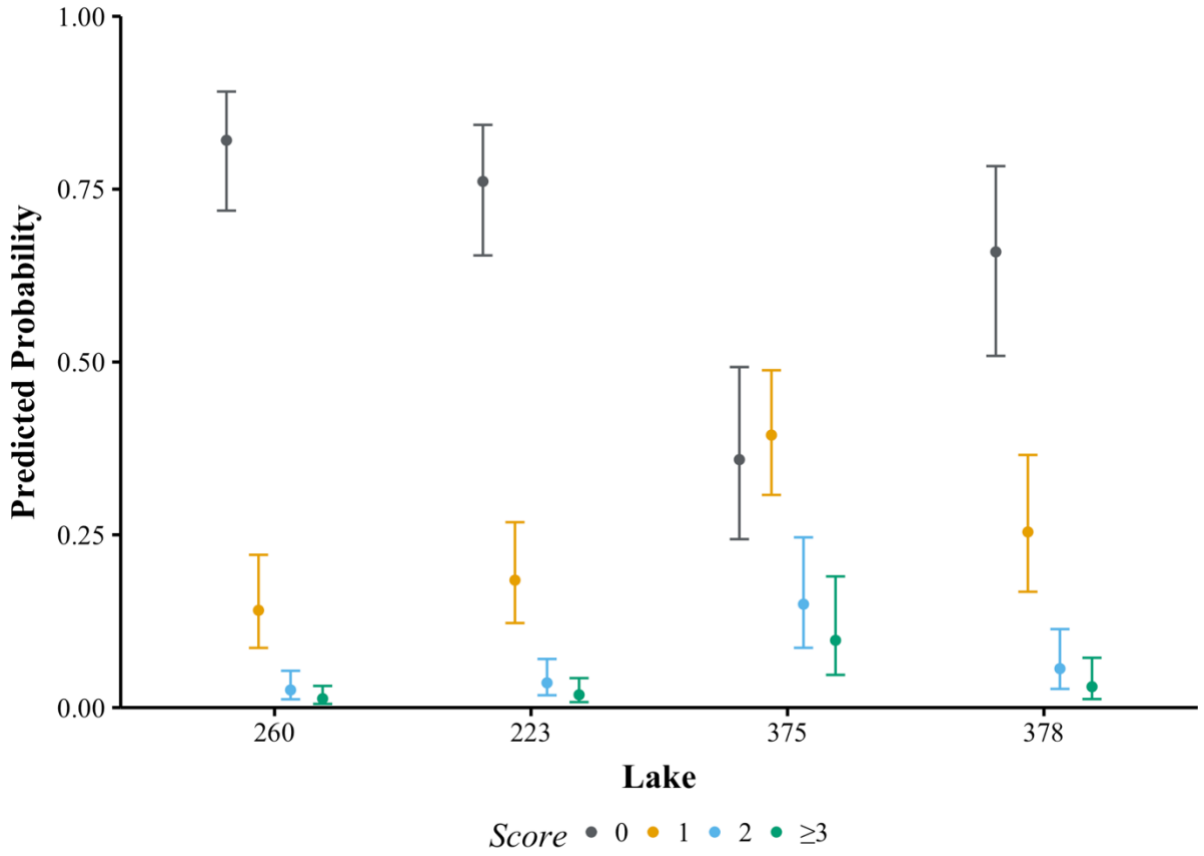


FIGURE 2.18. Probabilities of various GSI scores among yolk-sac fry (~550 ATU) from the four study lakes, predicted from an ordinal logistic regression model. Error bars represent 95% confidence limits about their means.

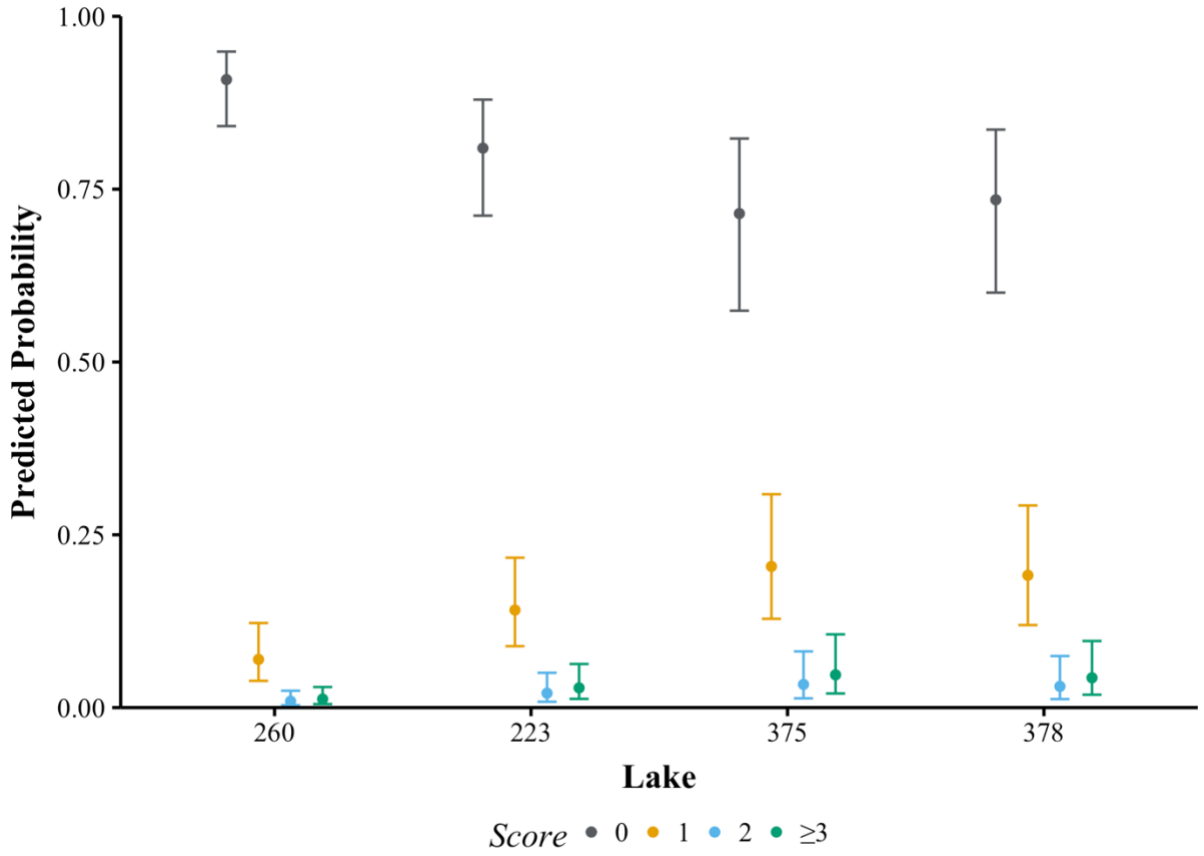


FIGURE 2.19. Probabilities of various GSI scores among swim-up fry (~800 ATU) from the four study lakes, predicted from an ordinal logistic regression model. Error bars represent 95% confidence limits about their means.

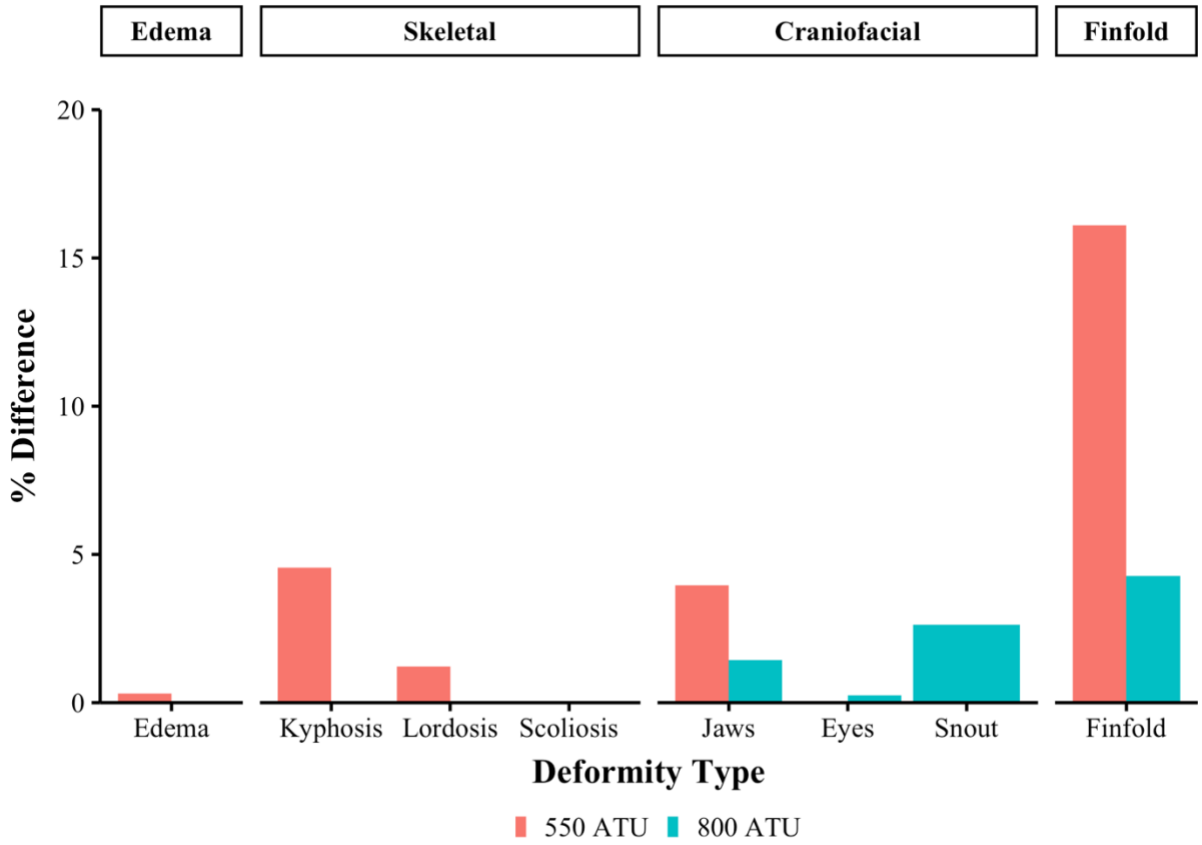


FIGURE 2.20. Percent difference between scoring iterations for yolk-sac fry (~550 ATU, red) and swim-up fry (~800 ATU, blue). Difference is indicative of an overall change in score for a given category for a particular fry, not the magnitude of difference. The "snout" subcategory was not evaluated in yolk-sac fry.

CHAPTER 3

BIOMARKERS OF OXIDATIVE STRESS AND POLYCYCLIC AROMATIC COMPOUND EXPOSURE IN LAKE TROUT FRY FROM A LAKE USED TO CONDUCT MODEL OIL SPILLS

ABSTRACT

Lake trout (*Salvelinus namaycush*) eggs originating from Lake 260 at the IISD-Experimental Lakes Area (IISD-ELA) – a lake used to conduct model oil spills in contained enclosures in 2018 and 2019 – and those from three reference lakes were assessed for polycyclic aromatic compounds (PACs) that would likely have been transferred maternally from the adult fish to the lipid rich yolk. Additionally, biomarkers of PAC exposure (*cyp1a* induction, glutathione) were investigated to determine if the compounds were being metabolized or causing oxidative stress in "swim-up" fry that were reared in a laboratory environment. For all analyses, lake trout eggs/fry originating from Lake 260 were compared to those originating from three unimpacted reference lakes the IISD-ELA. While maternal transfer of PACs has been demonstrated for some species, it has not yet been specifically examined in salmonids. Early life stages of salmonids are known to be especially vulnerable to PAC exposure. Eggs originating from all four study lakes contained quantifiable levels of PACs, however concentrations measured in eggs from Lake 260 were among the lowest and are likely to have originated from natural processes (i.e. incomplete combustion of organic material during forest fires; synthesized by algae and phytoplankton during slow conversion of organic materials) rather than the diluted bitumen added to the enclosures in Lake 260. Moreover, fry from Lake 260 showed no evidence of increased *cyp1a* induction and there was no biologically significant change in glutathione concentrations or to the glutathione redox cycle that would be indicative of oxidative stress. Model oil spill cleanup, environmental protection measures and low residual oil were not sufficient to affect resident lake trout in Lake 260.

INTRODUCTION

The growing demand for fossil fuels globally presents risks associated with spills for aquatic species that are situated along its transportation routes (CAPP, 2019). Pipeline and rail are the primary means of transport for un-refined oil products, where accidental spills have a history of causing impacts to aquatic environments (de Santiago-Martin et al., 2015; Khearaj, 2020). Most of the scientific literature concerning oil-spills over the past few decades has focused on marine and coastal spills (reviewed in Lee et al., 2015). Recently, the fate and effects of oil introduced to freshwater environments has received more attention (Rodriguez-Gil et al., 2021). Early life stage fishes inhabiting both marine and freshwater environments are known to be especially sensitive to oil constituents, particularly 3-5 ringed alkylated polycyclic aromatic compounds (PACs). Exposures to these components of crude oil and diluted bitumen can induce *cyp1a* genes in early life stage freshwater fish at "parts per billion" concentrations (Rigaud et al., 2022). The CYP1A enzyme, produced upon activation of the *cyp1a* gene, is a primary component of "phase I" of the metabolism of PACs in fish. However, its prolonged activity is known to initiate the overproduction of reactive oxygen species (ROS), resulting in increased lipid peroxidation rates and blue-sac disease (BSD), which includes characteristic accumulation of fluid surrounding the yolk sac (Brinkworth et al., 2003; Bauder et al., 2005; Carls et al., 2009; Madison et al., 2015). During the second stage of detoxification, glutathione (GSH) can bind PAC metabolites as part of "phase II" conjugation reactions and is also a key component of the cells antioxidant defense system, capable of neutralizing ROS (Santana et al., 2018). While *cyp1a* induction is frequently assessed to determine PAC exposure in early life stage fish due to its high sensitivity, GSH is less commonly investigated in such studies (Santana et al., 2018).

Cytochrome P450 1A (CYP1A) is part of a larger group of heme-thiolate monooxygenase proteins and is ubiquitous throughout the animal kingdom (Stagg et al., 2016; Chu et al., 2019). Its *de novo* synthesis can be induced through the binding of certain planar PACs (3 or more rings) to the intracellular arylhydrocarbon-receptor (AhR) protein (reviewed in Hodson, 2017). The role of CYP1A proteins is to catalyze mono-oxygenation reactions as part of the "phase I" detoxification response (reviewed in Sarasquette and Segner, 2000). The mode of action involves the addition of a polar moiety to the PAC, which reduces its lipophilicity to enable excretion from the cell, but can occasionally result in the formation of metabolites that are more toxic to the organism than the parent compound (reviewed in Sarasquette and Segner, 2000; reviewed in Hodson, 2017). Furthermore, certain PAC molecules bind more tightly to the enzyme, which can increase the potential for the uncoupling of election transfer and oxygen reduction, increasing the production of reactive oxygen species (Schlezinger et al., 2006). Reactive oxygen species can inhibit the AhR mediated induction of *cyp1a*, decreasing the organism's capacity to metabolize and excrete the harmful PACs, while also damaging other proteins, lipids, and DNA within the cell (Regoli and Giuliani 2014). The role of *cyp1a* in the detoxification and metabolism of xenobiotics is complex – although its induction confers protection, it can also contribute to toxicity.

Glutathione is a non-enzymatic component of the oxidative stress response (Massarsky et al., 2017). In conjunction with certain catalytic enzymes, glutathione can reduce ROS by donating a hydrogen to a receptor molecule, yielding glutathione disulfide (GSSG) and compounds such as water or alcohols, thereby decreasing the likelihood of cellular damage to the organism (Hellou et al., 2012). The oxidized GSSG – comprised of two glutathione molecules linked together by a disulfide bond – is then recycled back to GSH in a reaction catalyzed by

glutathione reductase. Under basal aerobic conditions these reactions occur in a perpetual cycle that maintains homeostatic cellular redox status (Regoli and Giuliani 2014). Previous studies have reported that GSSG comprises approximately 5% of the total cellular glutathione in most fish species (Leggatt, 2006). If a fish is experiencing oxidative stress, the proportion of GSSG can be severalfold higher (10-50%) (Bauder et al., 2005; Leggatt, 2006). GSH is also part of "phase II" of the detoxification response, binding "phase I" PAC metabolites through catalyzed conjugation reactions (Hellou et al., 2012). The resultant product of these reactions is a more polar, and thus more hydrophilic molecule, which can be efficiently excreted from the cell and out of the organism via bile or urine (Regoli and Giuliani 2014).

The disparity in sensitivity to PAC exposure between early life stage and adult fishes is best explained by (1) an underdeveloped antioxidant defense system (i.e. finite, maternally derived supply deposited into the egg), (2) the inability to synthesize RNA to produce enzymes associated with antioxidant defense and metabolism until the blastula stage, (3) continuous catabolism during embryonic development, and (4) tightly regulated gene activation to ensure proper early development (Hodson et al., 2007; reviewed in Lee et al., 2015; Berube et al., 2022). Moreover, salmonids are known to be especially sensitive to such contaminants (Elonen et al., 1998). Increased rates of BSD, a syndrome characterized by yolk-sac or pericardial edema, hemorrhaging and early mortality have frequently been attributed to low PAC exposures during early life stages in this family of fishes (Hose et al., 1984; Le Bihanic et al., 2014; Rigaud et al., 2022). Salmonid reproduction strategies can vary considerably, with some species being semelparous, iteroparous, potamodromous and anadromous (Hutchings and Morris 1985; Finn, 2007). Lake trout (*Salvenlinus namaycush*) are an iteroparous freshwater species that have yet to receive significant attention in toxicological studies. Spawning occurs in the fall, as fish

congregate in around rocky shoals at night (Marsden et al., 1995; Finn, 2007). In the event of an oil-spill in proximity to their spawning habitat, there are several defense systems in place to protect the developing embryos. Among them are the chorion and vitelline membrane, which are composed primarily of glycoproteins (Finn, 2007). These protective layers can prevent the passage of some xenobiotics by either chemically binding or mechanically blocking entry into the yolk and developing embryo (Finn, 2007). Freshly spawned eggs are particularly susceptible to PAC contamination due to increased permeability of the chorion during water hardening. Permeability continues to decrease after fertilization up until the eyed stage of development (Valdebenito et al., 2021).

After hatch, fry are at an increased risk of exposure to PACs. Lacking the protective chorion, xenobiotics are free to enter the organism through the gills and skin (Brinkworth et al., 2003). Increases in *cyp1a* induction and BSD have been observed after hatch under consistent exposure conditions beginning from fertilization, likely related to the increased uptake of an AhR agonistic chemical (Brinkworth et al., 2003; reviewed in Hodson, 2017). These findings are in agreement with those from Bauder et al. (2005), which suggested that the metabolism of retene, a 3-ringed alkylated PAC (i.e. *cyp1a* induction), generates oxidative stress, causing deformities in early life stage salmonids. However, recent evidence has indicated that *cyp1a* induction after exposure to low concentrations of complex PAC mixtures does not always yield a phenotypic response, further emphasizing its usefulness as a biomarker of "exposure", rather than one of "effect" (Adams et al., 2014; Berube et al., 2022). These studies have highlighted the need to evaluate a variety of biomarkers to fully assess the impacts oil constituents on early life stage fishes.

Among the most understudied PAC exposure routes in fish is maternal transfer to eggs. Tilghman Hall and Oris (1991) reported that anthracene, a three-ring PAC, could be maternally transferred in fathead minnows (*Pimephales promelas*), leading to decreased survivorship in the F1 generation. More recently, studies have demonstrated the capacity for PACs to be offloaded into the yolk in freshwater species such as northern pike (*Esox lucius*) and zebrafish (*Danio rerio*) and marine species such as pacific herring (*Clupea pallasii*) (Sundberg et al., 2007; Vignet et al., 2015; West et al., 2014). Due to their rapid metabolism and excretion, more research is required to better understand transfer rates, and contaminant thresholds of common PAC mixtures that could pose a threat to reproduction in freshwater species (reviewed in Lee et al., 2015).

The current study was designed to assess biomarkers of oxidative stress and PAC exposure (total glutathione (TGSH), GSH/GSSG, *cyp1a* induction) in lake trout fry spawned from a lake used to conduct model oil spills at the IISD-Experimental Lakes Area (Lake 260). Comparisons were made to fry originating from three uncontaminated reference lakes. Additionally, unfertilized eggs originating from Lake 260 and three reference lakes were analyzed to examine the possibility of maternal offloading of PACs into the lipid rich yolk as a mechanism of exposure. Recent studies have reported differences in sensitivity to low PAC exposures among salmonids, highlighting the need to investigate the cellular response in an understudied species such as lake trout (Rigaud et al., 2022). Because only low PAC concentrations (<0.1 µg/L) were present in the study lake environment at the time of spawning (reviewed in Hodson, 2017), we hypothesized that there would be no significant difference in the aforementioned parameters between the lake trout in Lake 260 and those in the three reference lakes (Lakes 223, 375, and 378). In the event of an exposure, early detection of these biomarkers

could facilitate action to prevent more serious consequences, such as increases in malformations (i.e. BSD) and reductions in recruitment.

METHODS AND MATERIALS

Adult Fish Collection

In the fall of 2019, eggs were collected from seven adult female lake trout in each of Lakes 260 and 223, and from three female lake trout in each of Lakes 378 and 375 at the IISD – Experimental Lakes Area in Northwestern Ontario, Canada. The fish were captured using either trap nets or short set gill nets (20-30 mins) and transported in plastic tubs containing aerated lake water to shore. Once brought to shore, fish determined to be "ripe and running" were selected for the study (i.e. the first seven fish that were observed to be in spawning condition) and were anesthetized with 0.1 g/L tricaine methanesulfonate (MS-222) until movement ceased. Female fish were weighed, measured, and then spawned by applying gentle pressure to the abdomen of the fish to express eggs or milt. Eggs from each female were collected in separate ZipLoc™ bags, the head space was filled with oxygen and the bags were sealed and transported on ice in coolers for transportation to the laboratory for fertilization. Milt was collected in a similar fashion to egg collection from approximately 3-7 males in one bag per lake, and kept on ice until fertilization (see Table A.1 for adult fish sampling and egg deployment dates).

Egg and Larvae Rearing

The time from egg collection onshore until fertilization in the lab was less than 48 hours for all batches. Clutches of eggs were dry fertilized in in individual stainless-steel bowls. Dry fertilization comprised of mixing each clutch of eggs with 200 µL milt from 3-7 males using a sterilized goose feather to stir. After the milt was adequately stirred to make contact with the

entirety of the eggs, water was poured into the bowls to just cover the eggs. This process activates the sperm to allowed fertilization to occur. A sterilized goose feather was used to briefly stir the eggs to ensure that the activated sperm contacted all of the eggs and to prevent adhesion to the bowl or aggregation of eggs. After 1 minute, approximately 5 times the volume of water was added to the bowl and the eggs were set aside to water harden for 5 minutes. After water hardening, the eggs were incubated in separate compartments of a commercial incubator containing Heath trays, each receiving 6L/min of dechlorinated Winnipeg tap water (8.5±1°C), employing the method described by Werner et al. (2006). Each lake trout had its eggs placed in a designated compartment of the tray, with some fish requiring two compartments to incubate all the eggs in a monolayer. Dividers were made from plexiglass and fixed in place using 100% silicone.

A subsample of ~25 eggs from each fish from Lake 260 (n=7) were pooled and then exposed collectively to a water accommodated fraction (WAF) containing a 1:100 dilution of crude oil during water hardening for 60 minutes to act as a positive control. These eggs were rinsed several times in clean water after the exposure and placed in a dedicated compartment of the Heath Tray. Eggs were checked daily for the presence of mortalities, which were removed and placed in ovadine solution and subsequently disposed. Removing mortalities frequently is critical to limit the possibility of predatory fungus from infecting a batch of eggs (Werner et al., 2006).

Temperature Unit Calculations

One HOBO logger was used to collect temperature data at 15-minute intervals. The loggers were located ~30 cm beneath the heath trays where water collection occurred before drainage from the incubator. The potential for a temperature gradient between the first tray and

the collection tray was assessed at the time of deployment, and no detectable difference in temperature was observed. Accumulated thermal units (ATU) were determined as the sum of mean daily temperature in Celcius since fertilization.

Water Accommodated Fraction (WAF) Preparation

Preparation of the WAF was conducted using reverse osmosis filtered dechlorinated City of Winnipeg tap water, in accordance with the DFO Canadian Science Advisory Secretariate Report (2017). A final concentration of 1:100 (w/v of heavy crude) was achieved by mixing the oil and water at room temperature (20-22°C) for 24 hours at a vortex depth of 20-25% the total water depth. This oil to water ratio corresponds to ~0.2 µg/L TPAC (Figure A.2). One hundred (100) mL of WAF was transferred into an amber bottle for transportation to the DFO lab, where it was used to expose a subset of Lake 260 eggs during water hardening as described above.

Sampling Regimen

Eggs were subsampled pre-fertilization (10 eggs per maternal female) and frozen at -80°C. At swim-up (~800 ATU), 20 fry from each compartment were haphazardly sampled and euthanized with a lethal dose of pH buffered MS-222 (0.4g/L) until movement ceased. Fry were frozen in plastic falcon tubes (10 fry x 2 tubes per compartment) and stored at -80°C until further analysis. Where limited number of fry remained or if significant mortality had occurred, sample numbers were reduced accordingly. A small proportion of hatched fry moved between compartments in the Heath trays. Affected compartments included eggs from Lake 260 and a separate tray from Lake 375 and half the eggs from Lake 223 (LT # 4-7). As a result, there is a limited possibility that some sampled fry may be misidentified according to maternal/lake origin. However, fry from Lake 260 were isolated in a single hatching tray, therefore any fry movement could only have resulted in misidentified maternal origin.

Detection of Polycyclic Aromatic Compounds and Alkylated Isomers in Unfertilized Eggs

Parent and alkylated polycyclic aromatic compound (PAC) concentrations were determined in unfertilized lake trout eggs in accordance with the Centre for Oil and Gas Research and Development (COGRAD) micro-bead extraction and gas chromatography standard operating procedure. For each sample, 1-2 grams (±0.01 g) of tissue was weighed in 15 mL micro-bead extraction tubes (MBETs), containing 2.5 grams of 2.8 mm zirconium oxide. Samples were spiked with the recovery internal standard (RIS) (1 ng/µL) for a target final sample extract concentration of 100 pg/µL. The RIS consisted of d8-naphthalene, d8-acenaphthylene, d10-acenaphthene, d10-fluorene, d10-phenanthrene, d10-pyrene, d12-Benz(a)anthracene, d12-chrysene, d12-benzo(b)fluoranthene, d12-benzo(k)fluoranthene, d12-benzo(a)pyrene, d12-indeno(1,2,3-c,d)pyrene, d14-dibenzo(a,h)anthracene, and d14-benzo(g,h,i)perylene. In-house reference material made from frozen walleye (*Sander vitreus*) eggs (Gimli Fish Market, Winnipeg, MB) was spiked with a polycyclic aromatic hydrocarbon (PAH) native mix (NS) spiking solution (1 ng/µL) and an alkylated PAH NS spiking solution (1 ng/µL), for a target final sample extract concentration of 100 pg/µL (see Table 3.1 for complete list). Dichloromethane (DCM) was added to the MBETs and samples were homogenized using a sonicator (Bertin Technologies – Precellys Evolution, 6500 RPM, 3 cycles of 20 seconds, 120s pause). After homogenization, samples were centrifuge using a ThermoFisher Scientific Sorvall Legend Micro 21 (Speed: 5000 RPM/4694 XG; Temperature: 10°C; Time: 10 min). The supernatant extracts from the MBET were transferred to a 60 mL accelerated solvent extractor (ASE) vials. To rinse the MBETs, an additional 8 mL of DCM was added, vortexed for 1-minute, centrifuged with the same settings as above and transferred to the extract vials. After a second and final rinse, sodium sulfate was added to the DCM extracts and manually shaken to

remove any residual water. Once sufficiently dried, the sample was transferred to a fresh 60 mL vial using a borscillate glass pipette, rinsing the fresh vial three times with 2 mL hexanes each. Sample extract volume was adjusted to 10 mL using nitrogen evaporation. Nitrogen evaporation was achieved at level 4 using the hot bath.

To determine the percentage of lipids in the sample, 0.5 mL of the sample extract was weighed in aluminum weigh boats, left to evaporate in a fume hood, and then sample residue was weighed again. The following equation was used to determine the percentage of lipids:

$$\% \, lipid = \frac{\frac{(weight \; of \; lipid \; in \; 0.5 \; mL)}{0.5mL} \times 10 \; mL}{sample \; mass} \times 100$$

Samples were prepared for extraction by further reducing the sample extract volume to 2.5 mL by nitrogen evaporation. The extract was transferred to a 10 mL gel permeation chromatography (GPC) vial (2 x 1 mL DCM rinses) and DCM was added to reach a final volume of 5 mL. Samples were processed according to the following GPC parameters (Mobile Phase: DCM:Hexane (1:1); Flow Rate: 5 mL/min; Pressure: 7-9 psi; Dump Time: 20 min (0 – 140 mL); Collect Time: 36 min (140 – 320 mL)). Next, samples were reduced to 2 mL by roto-evaporation (Speed: 80). Dispersants were added to a round bottom beaker, along with 30 mL of HEX:DCM (30:70), and were gently swirled to mix. After resting for 30 minutes, samples were transferred through a pipette with glass wool and plunged into a 60 mL tube using 2 x 5 mL HEX:DCM (30:70) rinses. The target final volume of 200 µL was achieved by nitrogen evaporation, and all samples were spiked with the Instrument Performance Internal Standard (IPIS) (1 ng/µL x 20 µL) to yield a sample extract concentration of 100 pg/µL. Samples were stored at 4°C prior to GC analysis.

The native and d-labelled PACs and alkylated PACs in the samples were analyzed by GC-MS/MS, using an Agilent 7890 gas chromatographer coupled with a 7000C triple quadrupole mass spectrophotometer fitted with an electron ionization (EI) source. The carrier gas was Helium, with a flow rate of 1.2mL/min, and column was an Agilent J&W HP-5ms ultra inert column (30 m × 0.25 mm, 0.25 μm film thickness). Sample volumes of 1μl were injected by a PAL RSI 85 auto sampler at a temperature of 60°C. In-depth GC-MS/MS parameters used to analyze the 16 priority PACs and alkylated PACs in the eggs can be found in Idowu et al. 2018. The software used for quantification was MassHunter Workstation Software, Quantitative Analysis, Version B.07.01 SP1/Build 7.1.524.1 for QQQ (Agilent Technologies, Inc. 2015).

In cases where PAC concentrations were measured below the limit of quantification (LOQ), concentrations were assumed to be ½ LOQ, due to the assumption of a normal distribution in repeated measures of the sample.

TABLE 3.1. PAC compounds investigated in lake trout eggs.

Parent Compounds	Number of Rings	Alkylated Compounds	Number of Rings
Naphthalene	2	1-Methylnaphthalene	2
Acenapthene	3	2-Methylnaphthalene	2
Acenapthylene	3	C2 Naphthalene	2
Anthracene	3	C3 Naphthalene	2
Fluorene	3	C4 Naphthalene	2
Phenanthrene	3	1-Methylphenanthrene	3
Benzo[a]anthracene	4	2-Methylphenanthrene	3
Chrysene	4	1,7-Dimethylphenanthrene	3
Fluoranthene	4	1,8-Dimethylphenanthrene	3
Pyrene	5	2,6-Dimethylphenanthrene	3
Benzo[b]fluoranthene	5	3,6-Dimethylphenanthrene	3
Benzo[k]fluoranthene	5	C2 Phenanthrene	3
Benzo[a]pyrene	5	3-Methylphenanthrene	3
Dibenzo[a,h]anthracene	5	9/4-Methylphenanthrene	3
Benzo[g,h,i]perylene	6	C3 Phenanthrene	3
Indeno[1,2,3-c,d]pyrene	6	C4 Phenanthrene	3
		Dibenzothiophene	3
		C1 Dibenzothiophene	3
		C2 Dibenzothiophene	3
		C3 Dibenzothiophene	3
		C1 Fluorene	3
		C2 Fluorene	3
		Retene	3
		C1 Chrysene	4
		C3 Chrysene	4
		C1 Pyrene	4
		C2 Pyrene	4
		C1 Benzo[a]pyrene	5

CYP1A Quantitative PCR

This assay was adapted from the method described by Mackey et al. (2021). RNA from whole-body swim-up fry was extracted with a RNeasy Plus Mini Kit (Qiagen, Toronto, ON, Canada). Sample purity and concentrations (A_{260}/A_{280}, A_{260}/A_{230}) were determined using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and RNA integrity was investigated by electrophoresis. 1 μg of total RNA was reverse transcribed to form cDNA, using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). All assays were in accordance with manufacturers protocols.

All primers were designed using Geneious Prime® 2021.2.2 (Biomatters Ltd, Auckland, New Zealand) using sequences of multiple salmonid sequences including lake trout (GenBank® (Sayers et al., 2019), PhyloFish (Sutherland et al., 2019; http://phylofish.sigenae.org/index.html), and available transcriptomes)). Primer design was based off the consensus sequence across various the salmonid species. A primer was designed for cytochrome P450 1A (*cyp1a1*), a common biomarker of PAC exposure in fish (Brinkworth et al., 2003) (Table 3.2.1). Primers were designed for three reference genes, 60s ribosomal protein L7 and L13a (*rpl7* and *rpl13a*) and 40s ribosomal protein S9 (*rps9*) (Table 3.2). Standard curves were generated to assess primer efficiency using RNA pooled for six individuals. qPCR reaction mixtures included 1 μL of a 1:10 dilution of cDNA, 500 nmol/L forward and reverse primer, 6 μL of PowerUP SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific) and 4.8 μL RNase-free water. These reactions were run on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Life Technologies Corporation, Carlsbad, CA, USA). The stability of the three reference genes was assessed by integrating multiple computational programs (geNorm, Normfinder, BestKeeper, Delta-Ct method) and ranking the tested candidate genes using RefFinder (Xie et al., 2012). It

was determined that only two of the three reference genes were stable (*rps9*, *rpl13a*), thus, *cyp1a1* mRNA levels were normalized to the two reference genes using the $2^{-\Delta Ct}$ method (Livak and Schittgen, 2001).

TABLE 3.2. Primer sequences for quantitative PCR in lake trout (*Salvelinus namaycush*)

Gene name	**Gene Abbrieviation**	**Primer Sequence (5'–3')**	**Product size (bp)**	**Eff. (%)**
Cytochrome P450 1a	*cyp1a*	F: CAGTGGCAGGTCAACCATGA R: GACCGTTTCCTGAGTGCTGA	77	102
60s ribosomal protein L13a	*rpl13a*	F: CACTGGAGAGGCTGAAGGTG R: GTGGGCTTCAGACGGACAAT	103	108
40s ribosomal protein S9	*rps9*	F: TCTCCCTGCGTTCACCATAC R: GCCCTTCTTGGCATTCTTTC	67	109

Glutathione Assay

Total glutathione (TGSH) and glutathione disulfide (GSSG) concentrations were determined in whole-body swim-up fry preserved at -80°C based on a method adapted from Massarsky et al. (2017), and first described by Griffith (1980). Individual fish were homogenized in 1.2 mL 5% sulfosalisylic acid dihydrate using a sonicator (Bertin Technologies – Precellys Evolution, 6500 RPM, 3 cycles of 20 seconds) and then centrifuged at 5000 g for 3 minutes at room temperature using a ThermoFisher Scientific Sorvall Legend Micro 21.

This assay utilizes enzyme recycling to determine concentrations of GSH and GSSG. To assess GSH, 10 µL of supernantant was added to a 96-well microplate and mixed with 200 µL assay solution containing 0.7 mM 5,5'-dithiobis-(2-nitrobenzioc acid) (DTNB) and 0.3mM

NADPH. The reaction was initiated by adding 10 μL of 2 U/ml glutathione reductase (GR) solution. Absorbance values were monitored on the microplate spectrophotometer at 412 nm for 15 minutes. The reaction involves the reduction of DTNB to 5-thio-2-nitrobenzoic acid (TNB) and subsequent oxidation of glutathione in its reduced form (GSH) to GSSG. Glutathione disulfide is then cycled back to GSH by GR, with NADPH acting as a reducing agent. TNB is yellow in colour, and its rate of formation is proportional to the total glutathione in the sample, making 412 nm an appropriate wavelength to detect changes in absorbance. The linear reaction rate (absorbance/minute) was then compared to a standard curve, generated at known concentrations of TGSH (0 μM, 6.25 μM, 12.5 μM, 25 μM, 50 μM, 100 μM) to estimate the TGSH in individual samples.

To measure GSSG, 50 μL of the supernatant from each sample was mixed with 50 μL of 375 mM NaOH solution and 10 μL of 28 μL/mL 2-vinylpyridine (2VP) solution – a masking agent, that reacts with GSH to facilitate the enzyme recycling reaction described above for TGSH, but with only the GSSG in the sample available to reduce DTNB to TNB. The protocol described by Massarsky et al. (2017) used 125 mM NaOH solution to mix with the supernatant and 2VP, but pH measurements revealed that this concentration was insufficient to elevate the pH of the mixture to approximately neutral, where 2VP efficiently reacts with GSH (pH = 2.98) (Griffith, 1980). An NaOH concentration of 375 mM was determined to adequately elevate the pH in the mixture to allow the necessary reactions to occur (pH = 6.89). The mixture was left to incubate for 90 minutes at room temperature to facilitate the 2VP – GSH reaction. A standard curve was created for GSSG at known concentrations (0μM, 0.625 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM) and used to determine concentrations in the samples.

Two buffer solutions used to prepare solutions for this assay. KPB-100 contained 4 mL of 1000 mM KH_2PO_4, 6 mL of 1000 mM K_2HPO_4, 0.82 mL of 134 mM ethlylenediaminetetraacetic acid (EDTA) and was completed to 100 mL with Milli-Q water. KPB-500 contained 2 mL of 1000 mM KH_2PO_4 and 3 mL of 1000 mM K_2HPO_4, 0.074 mL of 134 mM EDTA and was completed to 10 mL with Milli-Q water. Buffer solutions were refrigerated (4°C) and refreshed weekly. KPB-100 was used to prepare the 375 mM NaOH solution, the assay solution, and the GR solution. KPB-500 was used to make the 2VP solution. All reagents for this assay were ordered fresh from Sigma-Aldrich (Sigma-Aldrich CO, USA).

Known concentrations of GSH and GSSG were analyzed in triplicate to construct standard curves daily and to ensure consistency and accurate measurement of reagents. Samples were run in duplicate. The absorbance was measured with a PowerWave XS2 microplate spectrophotometer from BioTek with the Gen5 software program. Wells were mixed for 5 seconds, and absorbance was measured at one-minute intervals for a total of 15 minutes. Slopes of standard curves and samples were calculated in R (R Core Team, 2021).

GSH concentrations were calculated according to the formula: GSH = TGSH – 2*GSSG (i.e. 2 GSH molar equivalents for each mole of GSSG).

Statistical Analysis

Linear regression was used to determine differences in total PACs (TPACs) and alkylated PACs in unfertilized eggs between the study lakes. The linear regression formula utilizes dummy coding (0 or 1) to model the explanatory variable of "lake origin" and a random error term (ϵ). The expected value (Y) is predicted by the following formula, with the intercept (β_0) representing the predicted mean value for Lake 260:

$$Y = \beta_0 + \beta_1 * \text{Lake223} + \beta_2 * \text{Lake375} + \beta_3 * \text{Lake378} + \epsilon$$

Model fit to the normal distribution was assessed using Q-Q plots, a probability plot that compares model residuals along theoretical quantiles to the model fit. For each model, normality of the residuals was verified using a Shapiro-Wilk test ($\alpha = 0.05$), and homogeneity of variance across groups (i.e. lake) was tested with a Levene's test ($\alpha = 0.05$). If the model residuals failed tests of normality, the response data was log transformed. The removal of outliers was considered if standardized residuals exceeded "3". In cases where PAC concentrations were "0", a constant of 0.1 was added to all response variables to facilitate a log transformation of the complete dataset. Model significance was assessed by evaluating the *F*-statistic and *p*-value of the linear regression models ($\alpha = 0.05$).

Differences in normalized *cyp1a* expression, total glutathione (TGSH) and the ratio of oxidized to reduced glutathione (GSSG/GSH) were assessed between study lakes using the following linear mixed modelling formula (Penheiro et al., 2021):

$$Y = \beta_0 + \beta_1 * \text{Lake223} + \beta_2 * \text{Lake375} + \beta_3 * \text{Lake378} + b + \epsilon$$

Lake 260 was assigned as the reference category (β_0), and *p*-values for comparisons with each of the three reference lakes (dummy coded) at $\alpha < 0.05$ were deemed significant. Likelihood ratio tests (LRT) were conducted to assess the significance of "lake origin" as a predictor by comparing the full model to the null (intercept + random effect only) model. Chi-squared test statistics were computed by comparing log likelihoods of model fit (df = difference in number of parameters between the two models), and the null hypothesis (H_0 = lake as a predictor does not improve model fit) was rejected at $\alpha < 0.05$ (Hothorn 2021). A random effect (*b*) of "maternal female origin" was included in both the full and null models to account for non-independence between the maternally derived groups. The intraclass correlation coefficient (ICC) was computed in the null (intercept and random effects only) model, to determine the percentage of

variability in the data explained to the grouping structure (i.e. random effect). This metric is calculated by dividing the between group variance (random intercept variance) by the total variance (sum of the between group variance and the residual variance) (Hox 2002). Model fit and the presence of outliers was assessed using Q-Q plots, and response data were log transformed in cases where the model residuals were not normally distributed. For each model, normality of the residuals was verified using a Shapiro-Wilk test ($\alpha = 0.05$), and homogeneity of variance across groups (i.e. lake) was tested with a Levene's test ($\alpha = 0.05$). All statistical analysis was performed using R (R Core Team, 2021).

RESULTS

Polycyclic Aromatic Compounds in Unfertilized Eggs

A combination of parent and alkyl PACs were detected in all samples of unfertilized eggs, at concentrations ranging from 2.5 – 42.4 ng/g wet wt. No clear pattern of ring composition (Figure 3.6) or proportion of alkylated compounds could be detected among the samples. There was no significant difference in total PAC or alkylated PAC (Figure 3.1) concentrations between the study lakes ($F(3,16) = 1.32$, p-value = 0.30; $F(3,16) = 2.90$, p-value = 0.07, respectively).

cyp1a gene expression

There were no differences in normalized *cyp1a* expression (log transformed) among swim-up fry from the four study lakes (Figure 3.3). Lake origin was also not a significant predictor of differences in *cyp1a* expression among fish sampled for this analysis ($\chi^2(3) = 2.28$, p = 0.52) and the ICC was calculated to be 0.61 in the null model (random effects only). Fry originating from LT3 in Lake 260 were a substantial outlier in terms of their *cyp1a* expression, compared to fry from the six other maternal fish in Lake 260. There was no indication that these

values resulted from technical error, and their exclusion from the dataset did not alter model predictions, therefore these data were retained in the final analysis.

Glutathione

There was no difference in mean total glutathione concentration (TGSH, pmol/mg) measured in whole-body swim-up fry originating from Lakes 260, 375, and 378 (260-375: t (14) = 1.57, p = 0.14; 260-378: t (14) = 0.16, p = 0.87) (Figure 3.4). Total glutathione concentration was significantly less in fry sampled from Lake 223, compared to those sampled from Lake 260 (t (14) = -2.21, p = 0.04). Percent differences in mean concentrations between Lakes 260 and 223 was 13.08%, whereas the percent difference in mean values ranged from 1.11% between Lakes 260 and 378 to 10.17% between Lakes 260 and 375. The intraclass correlation coefficient (ICC) was calculated to be 0.44 in the null (random effects only) model, indicating that 44% of the variability in TGSH can be explained by the grouping of fry by maternal female origin. Lake origin was not a significant predictor of the log transformed ratio of reduced to oxidized glutathione (GSH/GSSG) amongst fry from the four study lakes ($\chi^2(3) = 3.04$, p = 0.39) (Figure 3.5). Mean measured proportions ranged from 13.1 (± 0.7) in fry from Lake 223 to 19.7 (± 2.0) in fry from Lake 375. The ICC was calculated to be 0.51 in the null model.

DISCUSSION

In this study, lake trout eggs originating from Lake 260, a lake used for model oil spill studies at the IISD-Experimental Lakes Area, were reared until swim-up under optimal laboratory conditions. These fish showed no evidence of increased exposure to PACs, compared to those originating from three nearby reference lakes. In a parallel study from 2019, researchers observed that adult lake trout from Lake 260 exhibited evidence of *cyp1a3* upregulation in mucus compared to fish sampled from nearby reference lakes at the IISD-ELA (Andrzejczyk, 2020). As a result, maternal exposure could conceivably have occurred among lake trout from Lake 260. The maternal transfer of PACs to eggs is known to occur but has been rarely studied in freshwater species. Tilghman Hall and Oris (1991) reported maternal transfer of anthracene in fathead minnows exposed concentrations ranging from 6-20 µg/L over a 9-week period, with eggs originating from the highest maternal exposures showing reduced survival. Sundberg et al. (2007) found that hepatic DNA adducts in northern pike (*Esox lucius*) resulting from chronic PAC exposure were correlated with detection of PACs in their eggs. These researchers postulated that this mechanism might assist with detoxification, enabling the adults to survive a chronic exposure. More recently, Pollino et al. (2009) speculated that maternal transfer of naphthalene (2-ring, unsubstituted PAC abundant in crude oil) was responsible for increased deformities in the larval offspring of rainbowfish (*Melanotaenia fluviatilits*). Most of the research into maternal transfer of PACs in fish over the last decade has focused on marine species. For example, studies have traced the maternal contribution of PACs from contaminated sites in early life stage pacific herring (West et al., 2014), and several mesopelagic species (Romero et al., 2018). In laboratory studies examining zebrafish reproduction via maternal exposures to PACs, researchers noted behavioural abnormalities in the F1 generation, although

these effects could not be conclusively attributed to maternal transfer of PAC compounds to the eggs (Perrichon et al., 2015; Vignet et al., 2015).

To assess the potential for this mechanism of exposure in Lake 260, unfertilized eggs from all lakes were analyzed to assess the potential for maternal transfer of PACs from adult females in Lake 260 related to the model oil spill studies conducted in the lake. Although measured concentrations were low (1.1 - 42.4 ng/g wet wt), PACs were detected above the limit of quantification in all samples (n = 10 eggs pooled / female), across all lakes, confirming that maternal transfer is a potential route of PAC exposure in early life stage salmonids (Figure 3.1). Mean PAC concentration measured in eggs originating from Lake 260 was less than that of eggs from Lakes 223 and 375, indicating that the detected PACs likely were not associated with the model oil spill studies at IISD-ELA. To our knowledge, these results represent some of the first evidence of maternal transfer of PACs in a family of fish thought to be capable of efficiently metabolizing and excreting the compounds (Kennedy et al., 1990; Meador et al., 2006).

There was no clear pattern of PAC origin in the eggs, with a combination of parent and alkylated isomers, as well as both low (2-3 rings) and high (4+ rings) molecular weight compounds being observed across all lakes (Figures 3.1 and 3.2). Common diagnostic ratios were not useful in deducing PAC origin, due in part to the lack of detection of several key compounds used for the ratios, and within lake variability in the ratios that could be successfully calculated (Stogiannidis and Laane, 2015). The lake trout sampled from Lakes 260, 223, 375 and 378 could have been exposed to low concentrations of PACs of either pyrogenic (e.g. combustion of organic material) or biogenic (e.g. natural diagenetic transformation of biological material) origin that were unmetabolized and partitioned from the liver into the lipid rich yolk during vitellogenesis (Romero et al., 2018). Such phenomena have been observed in salmonids

for other lipophilic compounds such as polychlorinated biphenyls (PCBs) and dichlorodiphenyldichloroethylene (*p,*p'-DDE) (Miller, 1995).

Normalized *cyp1a* expression was examined in the present study as an additional metric to assess exposure to PACs in the larval lake trout. Several other studies have utilized similar methods to detect the metabolism of model PACs in early life stages of freshwater fish (Billiard et al., 1999; Hawkins et al., 2002; Brinkworth et al., 2003; Colavecchia et al., 2007). Recently, Berube et al. (2022) concluded that induction of *cyp1a* was a reliable biomarker of exposure to PACs in rainbow trout (*Oncorhynchus mykiss*) larvae exposed to complex mixtures of PAC compounds, from diluted bitumen and conventional heavy crude oil. However, these researchers noted that exposure timing was a key contributor to *cyp1a* induction. When eggs were exposed to PACs (1.84 ± 0.62 µg/L – 17.50 ± 1.53 µg/L) from the time of fertilization, transferred to clean water after hatch, and sampled at swim-up (i.e. when yolk sac was resorbed) *cyp1a* was not induced. Conversely, induction did occur in a dose dependent fashion when exposure began at hatch and lasted until swim-up. Because eggs used in this study were reared in clean water for the duration of the experiment, no additional exposure is likely to have occurred over the duration of the experiment. The results reported by Berube et al. (2022) indicate that it may not have been possible to detect the induction of *cyp1a* when the only possible exposure would have occurred prior to hatch. Nevertheless, our statistical model revealed that 61% of the variability in *cyp1a* induction across all lakes could be explained by maternal origin, indicating the potential for a genetic component of basal activity.

In addition to exposure timing, genetic factors can also play a role in *cyp1a* induction. Previous research has observed a refractory CYP1A response in early life stage killifish (*Fundulus heteroclitus*) inherited from the previous generation (Meyer et al., 2002). In this

study, CYP1A protein levels were measured in the livers of adult fishes, and in egg homogenates. It was hypothesized that the observed effects could be due to maternal deposition of mRNA related to CYP1A protein activity into the egg. While some adult lake trout sampled the Lake 260 in 2019 showed some evidence of *cyp1a3* upregulation in mucus (Andrzejczyk, 2020), induction of *cyp1a* regulated enzymatic activity or gene expression in other tissues was not examined so no firm conclusions can be made regarding the relationships to potential oil constituents in Lake 260.

At least one group of eggs from the present study were exposed to oil. A subsample of eggs from Lake 260 were exposed to a crude oil WAF (~0.2 µg/L, 60-min exposure) to serve as a positive control. Despite being exposed to ~0.2 µg/L TPAC there was no increase in *cyp1a* induction in swim-up fry, compared to fry from the four study lakes (Figure 3.3). The lack of an observable effect in this positive control group highlights the importance of exposure timing during development and should therefore be considered in future studies. As the liver develops, exposures to complex mixtures of PAC compounds, such as those found in dilbit or heavy crude oil are likely to induce *cyp1a*, which initiates "phase I" of their metabolism (Sarasquete and Segner 2000). Lake trout eggs are often deposited on rocky shoals near the shoreline and are thus particularly vulnerable to exposure to oil constituents, due to their immobility, finite supply of antioxidants and limited capacity to metabolize PACs (Callaghan et al., 2016; Brinkworth et al., 2003). Hatched salmonid fry that are near complete yolk sac resorption can move away from the site of contamination and have a more efficient xenobiotic metabolism system at this later stage of development (i.e. increased capacity to induce *cyp1a*) (Andreasen et al., 2002). Laboratory studies have observed *cyp1a* activity reverting to reference levels after a 14-day depuration period from PAC exposure (Avey et al., 2020). Consequently, fry sampled near a contaminated

site may not express the biomarker but could still be susceptible to chronic effects from an earlier exposure (Heintz et al., 2007; Hicken et al., 2011; Incardona et al., 2015). Despite these potentially confounding factors, the most likely conclusion regarding fry from Lake 260 is that exposure concentrations that existed due to maternal transfer were too low to elicit any effects.

Results from our glutathione assay were in agreement with those from the *cyp1a* analysis, supporting our claim that the fry from Lake 260 were not exposed to sufficient PAC concentrations to observe any deleterious effects. Previous research has drawn a link between *cyp1a* induction and oxidative stress in fish, with the overexpression of the gene playing a role in the increased formation of harmful ROS within the cell (Bauder et al., 2005; Crowe et al., 2014; Madison et al., 2015). Glutathione (GSH) acts as an intracellular "scavenger", capable of neutralizing these highly reactive and damaging compounds through a reaction that produces glutathione disulfide (GSSG) (Hellou et al., 2012). This antioxidant defence system is ubiquitous throughout the animal kingdom and is often characterized by the maintenance of a high GSH/GSSG ratio under basal aerobic conditions (Storey, 1996). In the present study, fry originating from Lake 260 had a high GSH/GSSG ratio that was comparable to fry sampled from two reference lakes (Lakes 223 and 378). Only fry originating from Lake 375 had a higher mean ratio, although differences between the lakes were not significant (Figure 3.5). The intraclass correlation coefficient (ICC) between of our statistical model indicated that 51% of the variability in GSH/GSSG could be explained by maternal origin. While previous studies have demonstrated the capacity for maternal diet to influence antioxidant content in the salmonid egg (Tyndale et al., 2008; Palace and Werner, 2006), research regarding maternal influence on glutathione in early life stage fish is needed (Wischhusen et al., 2019). In addition, the significance of ICC values less than 0.5 (50%) are difficult to interpret, due to the equal

contribution of variance partitioned to the intercept (maternal groups) and the residual (individual) (Liljequist et al., 2019). It is therefore unlikely that the ICC for the GSH/GSSG model is biologically meaningful.

A previous study examining the oxidative stress response in lake trout swim-up fry observed similar ratios for GSH/GSSG (95% confidence interval = ~14-35) in their reference exposure (Bauder et al., 2005). The mean GSH/GSSG ratio was at the low end of this approximate range in fry from Lake 223 (13.1 ± 0.7) but was similar in fry from the other three lakes (14.0 ± 2.1 – 19.7 ± 2.0). A subsample of Lake 260 fry exposed to a WAF of crude oil (n = 5) showed the lowest mean GSH/GSSG ratio (9.8 ± 0.3), although it is difficult to make a clear conclusion on the source of this difference, due to the lack of a corresponding change in *cyp1a* expression in this positive control group. Furthermore, the low sample size of this group decreases confidence in the accuracy of the measurements. Additional studies evaluating the GSH/GSSG ratio in juvenile salmonids reported similar values to the results herein. In rainbow trout fry fed for 4-weeks post yolk-sac resorption, GSH/GSSG ratios were determined to be 11.5 ± 0.2 (Zengin, 2021). In contrast, fry that were starved for 14-days to invoke the oxidative stress response saw a decrease in GSH concentrations by nearly -2-fold. Correspondingly, the ratio of GSH/GSSG fell to 3.62 ± 0.9 in these fry. Results such as these highlight the sensitivity this component of the oxidative stress response in early life stage fish and its utility as biomarker for early detection of physiological stress. The similarity in GSH/GSSG reported in reference fish to those in the present study support our conclusions that PAC exposure via maternal transfer was too low to cause any effects.

In addition to its function as part of the antioxidant defence system, GSH is involved in phase II of the biotransformation of PACs to facilitate their excretion (Santana et al., 2018). GSH

can react with PACs via conjugation reactions to further reduce their hydrophobicity, and consequently, reductions in cellular GSH concentrations are often associated with exposures to this class of compound (Santana et al., 2018). In our study, total glutathione concentrations in fry originating from Lake 260 were similar to those in fry from Lakes 375 and 378, while concentrations in fry from Lake 223 measured significantly less (Figure 3.3). Additionally, the ICC in this model indicated that only 44% of the variability in total glutathione could be explained by maternal origin. The significance of the ICC is difficult to interpret for the same reasons stated for GSH/GSSG.

There exists a paucity of studies measuring glutathione concentrations in early life stage fish, and in those that do, concentrations are typically measured "per gram of protein", as opposed to "per mg of tissue" in the present study. This difference in measurement technique makes comparison between these limited studies difficult, and largely uninformative. Bauder et al. (2005) reported mean total glutathione concentrations of ~330 pmol/mg wet wt (95% confidence interval = ~300-360 pmol/mg wet wt) in fry exposed to waterborne vitamin E, somewhat less than concentrations measured across the four study lakes herein (392.8 – 496.2 pmol/mg wet wt) (Figure 3.4). It is unclear why mean concentrations differed, however the increased concentrations of total glutathione observed in our study is a strong indication that the developing fry were of good health. In rare occurrences, exposure to model PACs has resulted in an increase in GSH concentrations in fish (Palanikumar et al., 2012). In the study, researchers observed that anthracene (3-ringed, unsubstituted PAC) exposure induced GSH production in milkfish fingerlings (*Chanos chanos*) in a dose dependent fashion. Conversely, exposure to benzo [a] pyrene (5-ringed, unsubstituted PAC) resulted in reductions of intracellular GSH. These conflicting results are best explained by the differences in the bioavailability of various

PAC compounds. In response to an exposure to anthracene, GSH production is induced, but it cannot be utilized in the conjugation reactions to facilitate excretion, whereas GSH can bind to benzo [a] pyrene metabolites and is thus depleted. Exposures to a broad range of PACs in early life stage fish are more often associated with decreases in intracellular glutathione, as the compound is incorporated in the conjugation reactions and ultimately excreted (Bauder et al., 2005; Madison et al., 2015; Yazdani, 2020). The increased GSH in our study compared to Bauder et al., (2005) is likely unrelated to PAC exposure, as evidenced by the results from the other analyses that were part of this study. Taken together, the results presented in this study indicate that maternal PAC concentrations in eggs originated from Lake 260 were insignificant, and ultimately yielded no detectable effects.

CONCLUSION

The findings from this study indicate that maternal transfer of PACs likely occurs in salmonids. However, concentrations measured in eggs from Lake 260 were too low to elicit an effect based on our measured endpoints and were ultimately the lowest among eggs collected from the four study lakes. *cyp1a* induction, a sensitive biomarker of exposure to PACs, was assessed in swim-up fry to detect the metabolism of PACs, but we observed no changes in induction levels compared to the three reference lakes. Similarly, the glutathione redox cycle was unchanged between lakes, and there was no evidence that fry originating from any of the four study lakes were experiencing oxidative stress. The mean total glutathione concentration in fry originating from Lake 223 was significantly less than that of fry from Lake 260, but these differences were deemed unlikely to be biologically significant or in any way related to exposure to PACs. These findings provide experimental evidence that the containment and contingency

measures put in place at IISD-ELA as part of the 2018 and 2019 model oil-spill studies prevented diluted bitumen from negatively impacting the ecology of Lake 260. Similar methods should be utilized in future enclosure studies examining a harmful contaminant that is unsuitable for a whole-lake study.

TABLES AND FIGURES

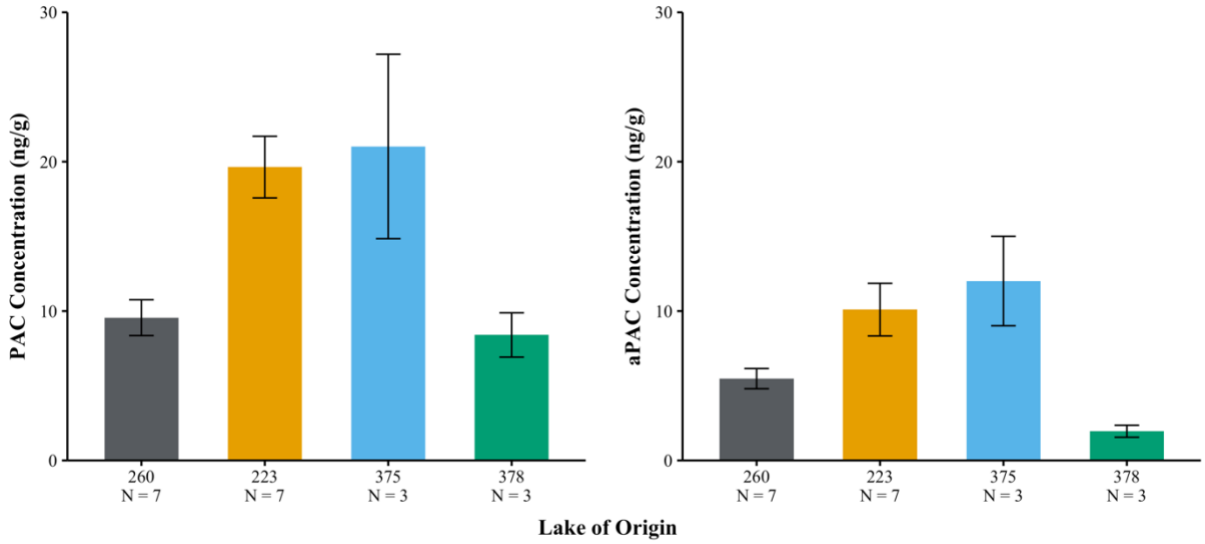


FIGURE 3.1. Total (left) and alkylated (right) polycyclic aromatic compound contents (ng/g wet wt.) amongst unfertilized eggs originating from the four study lakes. One sample consisted of a homogenate of 10 eggs per maternal female. Data are expressed as Mean ± standard error. No statistical differences were noted compared to Lake 260. Sample size is shown below each respective lake. Concentrations are not corrected for lipid content, however differences in lipid content between batches were found to be negligible.

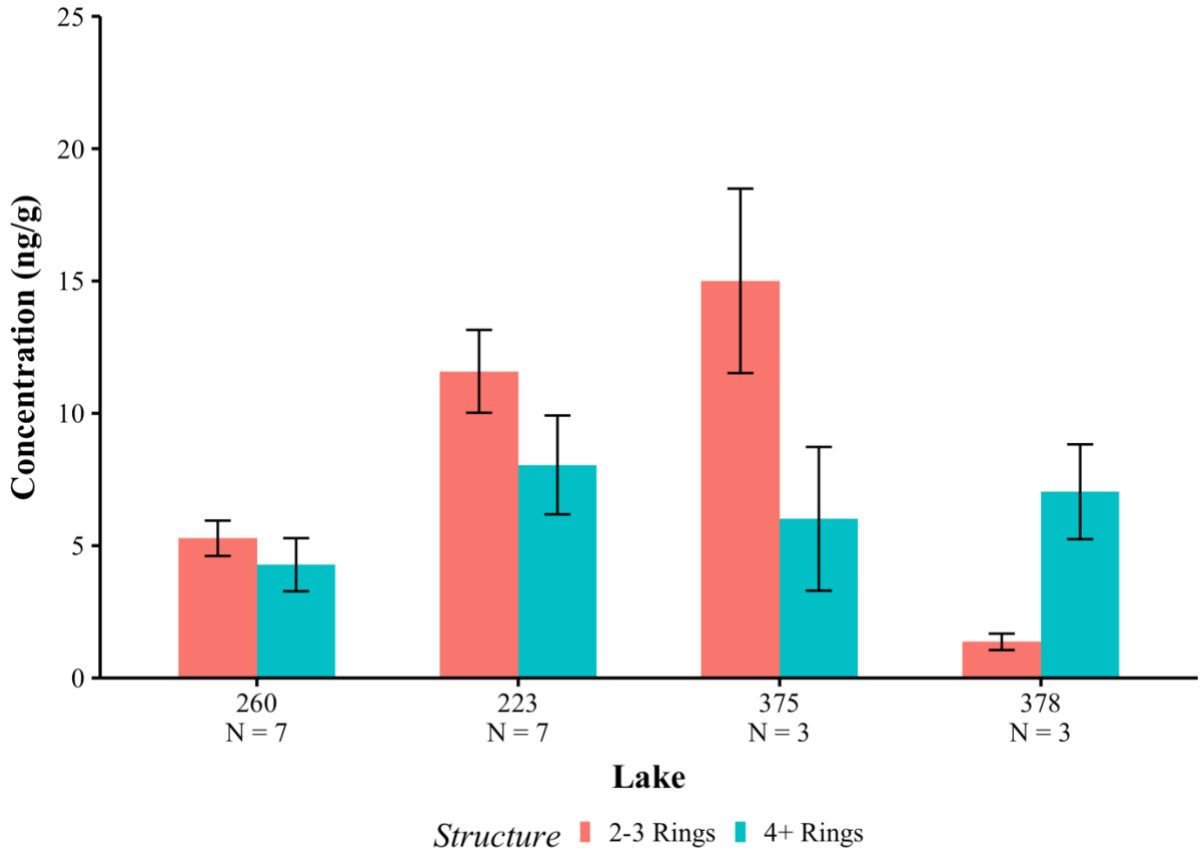


FIGURE 3.2. Polycyclic aromatic compound content (ng/g wet wt.) amongst unfertilized eggs originating from the four study lakes. One sample consisted of a homogenate of 10 eggs per maternal female. Data are expressed as Mean ± standard error. Sample size is shown below each respective lake. Concentrations are not corrected for lipid content, however differences in lipid content between batches were found to be negligible.

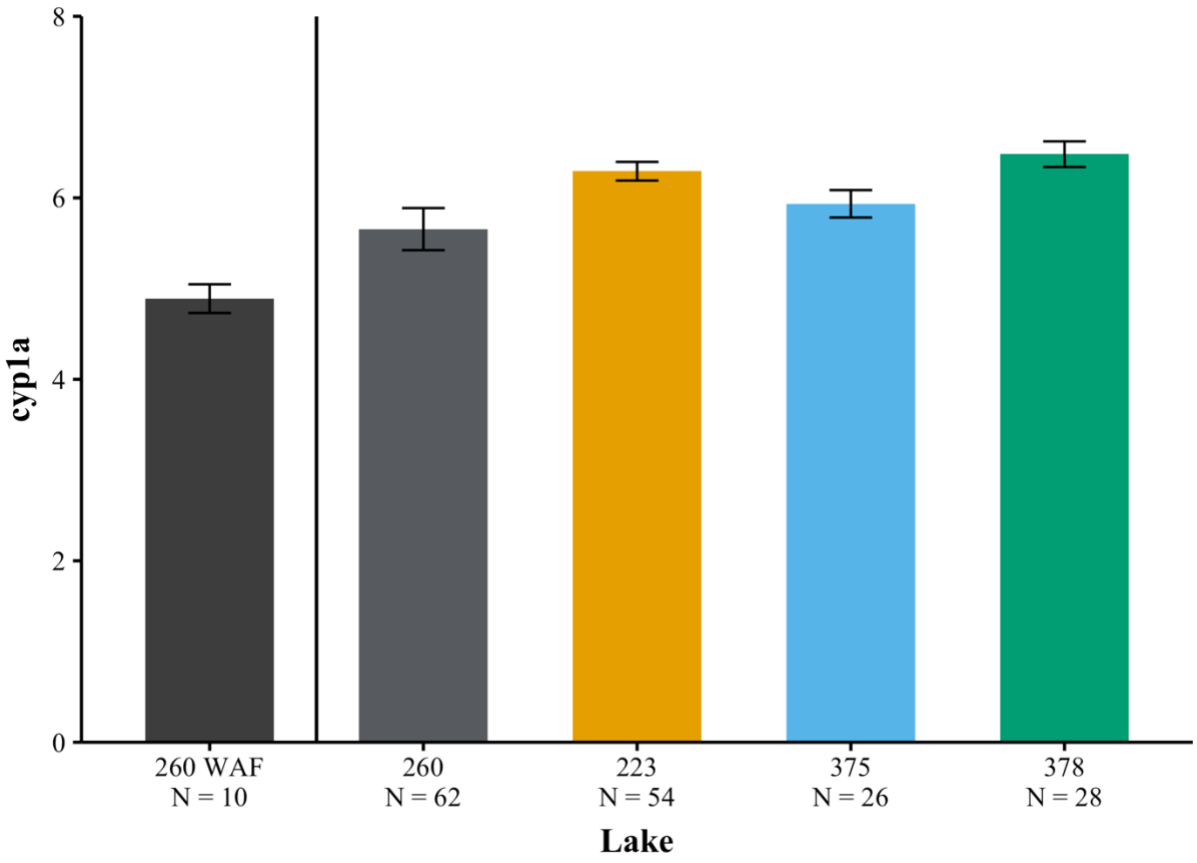


FIGURE 3.3. Relative *cyp1a* gene expression amongst swim-up fry (~800ATU) originating from the four study lakes at the IISD-Experimental Lakes Area. Fry from Lake 260 exposed to WAF during water hardening are included for visual comparison but excluded from statistical analysis. Data are expressed as Mean ± standard error. No statistical differences were noted compared to Lake 260. Sample size is shown below each respective lake.

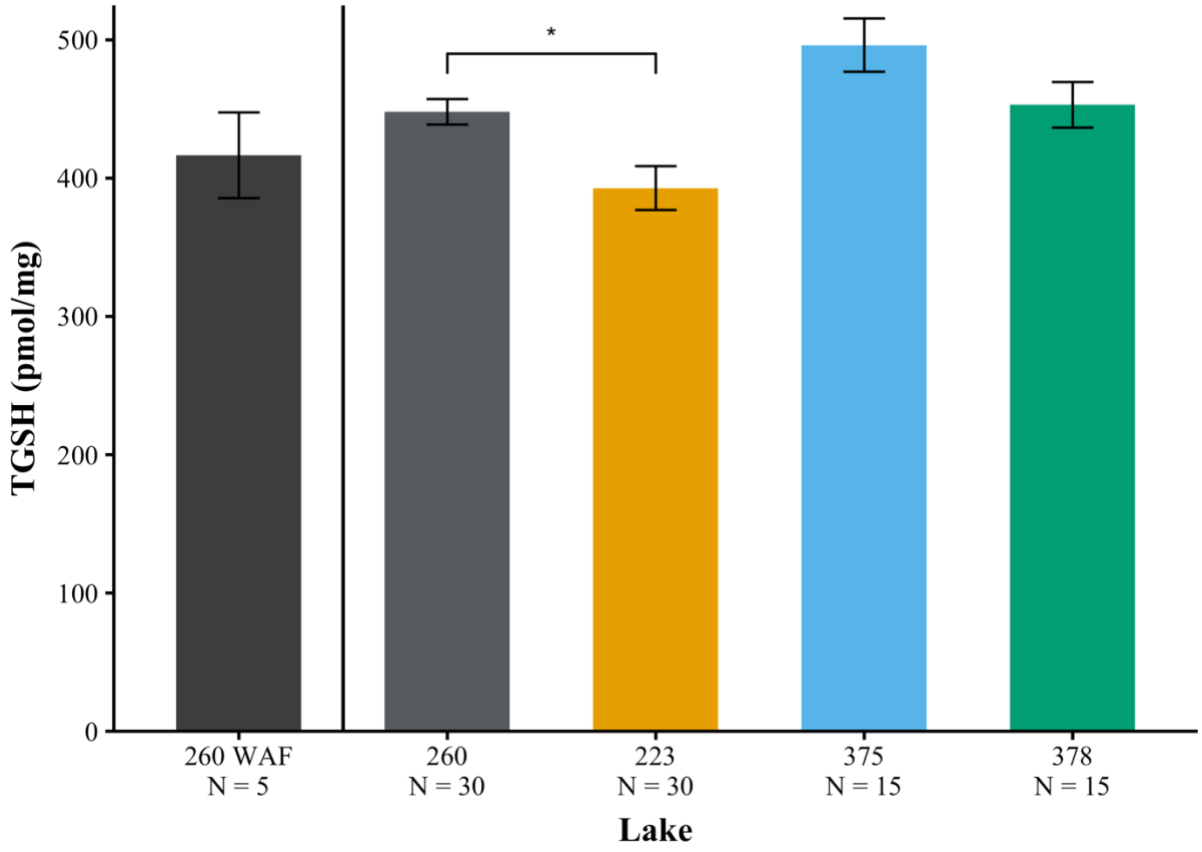


FIGURE 3.4. Total glutathione contents (pmol/mg wet wt) amongst swim-up fry (~800ATU) originating from the four study lakes. Fry from Lake 260 exposed to WAF during water hardening are included for visual comparison but excluded from statistical analysis. Data are expressed as Mean ± standard error (Statistical differences from Lake 260 denoted by* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Sample size is shown below each respective lake.

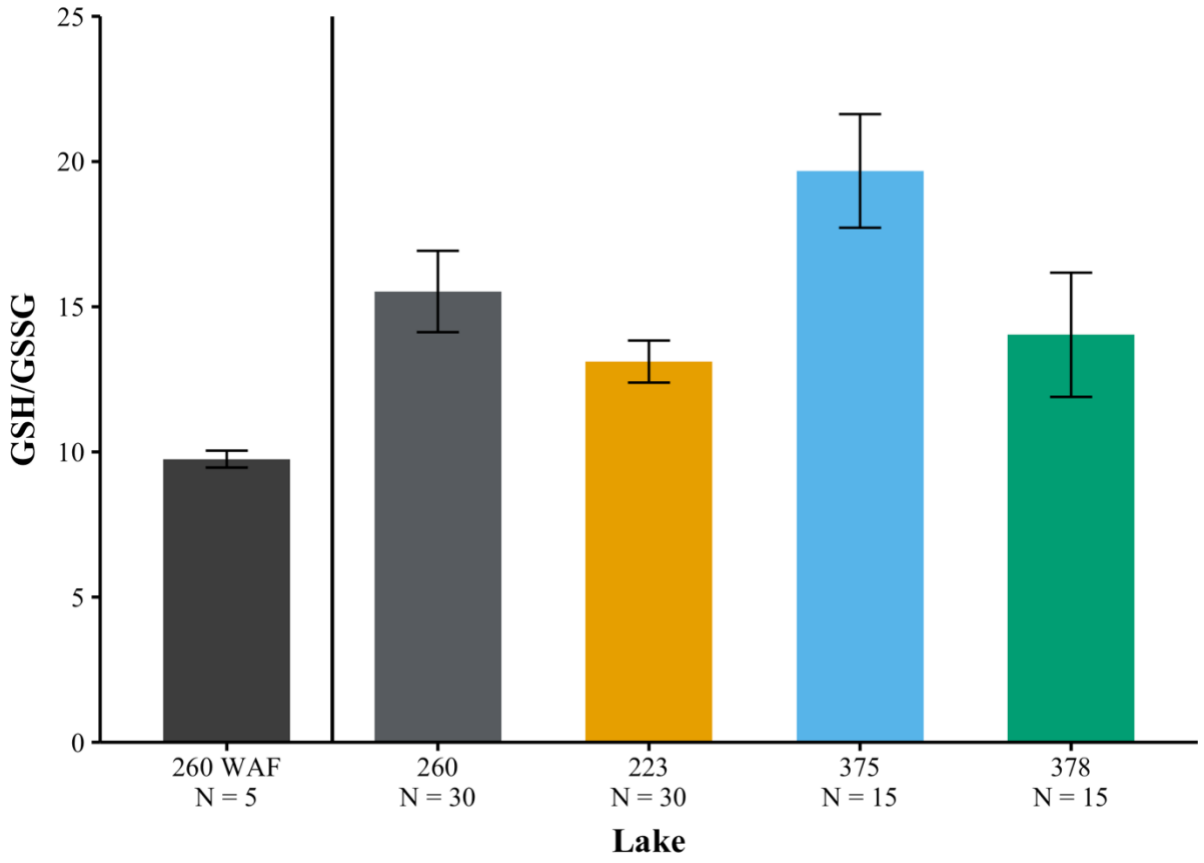


FIGURE 3.5. Ratio of reduced (GSH) to oxidized (GSSG) glutathione amongst swim-up fry (~800ATU) originating from the four study lakes. Fry from Lake 260 exposed to WAF during water hardening are included for visual comparison but excluded from statistical analysis. Data are expressed as Mean ± standard error. No statistical differences were noted compared to Lake 260. Sample size is shown below each respective lake.

CHAPTER 4

GENERAL CONCLUSION

Determining potential effects and recovery from freshwater oil spills was an important research objective identified by the Royal Society Report "*The Behaviour and Environmental Impacts of Crude Oil Released into Aqueous Environments*" (Lee et al. 2015). Several oil spill studies (Rodriguez-Gil et al., 2021; FOReSt studies, unpub.) conducted between 2018 and 2021 in Lake 260 at the IISD-ELA have examined this theme. The main objective of this thesis was to determine if the resident lake trout population in the study lake experienced any negative reproductive impacts resulting from exposure to residual oil after the model oil-spill studies. This analysis provides empirical evidence that early life stages of lake trout originating from Lake 260 were not exposed to polycyclic aromatic compound (PAC) concentrations sufficient to induce molecular or biochemical effects or to affect development. Specifically, among eggs/fry reared *in-situ* in Lake 260, hatching success was comparable to lake trout eggs/fry reared in three unimpacted IISD-ELA reference lakes.

When eggs/fry derived from the study lakes were reared in a laboratory environment, all metrics we investigated (fertilization success, egg mortality, hatching success, time to 50% hatch, growth rates, deformity rates) were comparable between Lake 260 and the three reference lakes. In addition to these phenotypic measures of fish health, we examined PAC accumulation in unfertilized eggs originating from the four study lakes and assessed biomarkers of PAC exposure (*cyp1a* induction, glutathione redox status) in swim-up fry reared in the laboratory. Concentrations of PACs were not elevated in the Lake 260 eggs compared with eggs from the other lakes and there was no significant induction of *cyp1a* or changes to glutathione levels in the fry during later stages of development.

Previous research has demonstrated that early life stage fishes are especially sensitive to PAC exposure (Billiard et al., 1999; Brinkworth et al., 2003; Barron et al., 2004; Colavecchia et

al., 2007). Salmonids, a family of fishes known to inhabit nutrient deficient habitats, have been shown to be among the most sensitive species to these types of compounds, with some salmonids exhibiting varying degrees of sensitivity compared to others (Le Bihanic et al., 2014; Alderman et al., 2018; Berube et al., 2022; Rigaud et al., 2022). The effects of PAC exposure to early life stage lake trout have been understudied, and as such, this study provided a unique opportunity to monitor the cumulative effects of two dilbit enclosure studies on the native lake trout population in Lake 260. Surface water samples collected from the general lake environment of Lake 260 in June of 2019 indicated that PAC concentrations rose to ~2 µg/L in the days immediately after oil addition to the contained enclosures, however PAC origin was not determined and levels quickly returned to those prior to oil addition. As a result, the adult lake trout sampled from Lake 260 experienced a limited window of increased exposure to PACs (Andrzejczyk, 2020). Once spawning occurred in the fall, the only potential route of exposure to the eggs would have been maternal transfer to the yolk. Polycyclic aromatic compound concentrations in the eggs, likely derived from maternal deposition during oogenesis, were too low to result in toxic effects according to the measured endpoints. In fact, eggs originating from Lake 260 had the lowest mean PAC concentration, compared to the those originating from the reference lakes, suggesting that the PACs detected in eggs from all lakes likely originated from natural processes (i.e. pyrogenic or biogenic origin) rather than from the dilbit (i.e. petrogenic origin) added to the enclosures. Bioaccumulation of PACs is generally limited in adult salmonids, but hydrophobic PACs can accumulate in tissues and become mobilized during oogenesis and be deposited with lipids in the developing eggs (Niimi and Palazzo, 1986; Nicolas, 1999; Sundberg et al., 2007). This mechanism may have implications for anadromous salmonid species that could be exposed to a marine spill, but travel great distances to cleaner fresh water for spawning. Further research

is required to determine whether maternal transfer of PACs alone can result in a toxic exposure to salmonid embryos.

Ultimately, the results from this study indicate that residual oil can be cleaned up or remediated using minimally invasive techniques without impacting the general lake environment. Exposures to PAC concentrations >0.1 μg/L have been deemed hazardous to early life stage fish development, mostly due to the paucity of research examining interactions between compounds that exist in complex mixtures such as diluted bitumen or crude oil (reviewed in Hodson, 2017). Although PAC concentrations of unknown origin appeared to exceed this threshold transiently at the reference sampling location after the addition of diluted bitumen to shoreline enclosures, results from our study indicate that there were no negative effects to lake trout reproduction. By examining the impacts to a species belonging to a sensitive family of fishes, at an especially sensitive life stage, lake trout were intended to serve as an indicator species, representative of the health of the Lake 260 ecosystem. Taken together, the results from this study suggest that adequate protections were put in place to prevent any significant oil contamination to Lake 260 beyond the contained enclosures. Our findings provide reassurance that the methodology utilized in the 2018 and 2019 model oil-spill studies were effective in preventing any harmful ecological impact to the general lake environment and could be used safely for similar enclosure studies at IISD-ELA.

REFERENCES

Alderman, S.L., Lin, F., Gillis, T.E., Farrell, A.P., and Kennedy, C.J. 2018. Developmental and latent effects of diluted bitumen exposure on early life stages of sockeye salmon (Oncorhynchus nerka). Aquat. Toxicol. **202**(May): 6–15. Elsevier. doi:10.1016/j.aquatox.2018.06.014.

Andreasen, E.A., Spitsbergen, J.M., Tanguay, R.L., Stegeman, J.J., Heideman, W., and Peterson, R.E. 2002. Tissue-specific expression of AHR2, ARNT2, and CYP1A in zebrafish embryos and larvae: Effects of developmental stage and 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure. Toxicol. Sci. **68**(2): 403–419. doi:10.1093/toxsci/68.2.403.

Andrzejczyk, N.E. 2020. Transcriptomics in Ecotoxicology: Characterizing the Effects of Contaminants and Environmental Parameters on Aquatic Populations.

Avey, S.R., Kennedy, C.J., Farrell, A.P., Gillis, T.E., and Alderman, S.L. 2020. Effects of diluted bitumen exposure on Atlantic salmon smolts: Molecular and metabolic responses in relation to swimming performance. Aquat. Toxicol. **221**(September 2019): 105423. Elsevier. doi:10.1016/j.aquatox.2020.105423.

Barron, M.G., Carls, M.G., Heintz, R., and Rice, S.D. 2004. Evaluation of fish early life-stage toxicity models of chronic embryonic exposures to complex polycyclic aromatic hydrocarbon mixtures. Toxicol. Sci. **78**(1): 60–67. doi:10.1093/toxsci/kfh051.

Bauder, M.B., Palace, V.P., and Hodson, P. V. 2005. Is oxidative stress the mechanism of blue sac disease in retene-exposed trout larvae? Environ. Toxicol. Chem. **24**(3): 694–702. doi:10.1897/04-23R.1.

Berube, R., Lefebvre-Raine, M., Gauthier, C., Bourdin, T., Bellot, P., Triffault-Bouchet, G., Langlois, V.S., and Couture, P. 2022. Comparative toxicity of conventional and unconventional oils during rainbow trout (Oncorhynchus mykiss) embryonic development: From molecular to health consequences. Chemosphere **288**. doi:10.1016/j.chemosphere.2021.132521.

Billiard, S.M., Querbach, K., and Hodson, P. V. 1999. Toxicity of retene to early life stages of two freshwater fish species. Environ. Toxicol. Chem. **18**(9): 2070–2077. doi:10.1897/1551-5028(1999)018<2070:TORTEL>2.3.CO;2.

Birnie-Gauvin, K., Costantini, D., Cooke, S.J., and Willmore, W.G. 2017. A comparative and evolutionary approach to oxidative stress in fish: A review. Fish Fish. **18**(5): 928–942. doi:10.1111/faf.12215.

Blanchfield, P.J., Rudd, J.W.M., Hrenchuk, L.E., Amyot, M., Babiarz, C.L., Beaty, K.G., Bodaly, R.A.D., Branfireun, B.A., Gilmour, C.C., Graydon, J.A., Hall, B.D., Harris, R.C., Heyes, A., Hintelmann, H., Hurley, J.P., Kelly, C.A., Krabbenhoft, D.P., Lindberg, S.E.,

Mason, R.P., Paterson, M.J., Podemski, C.L., Sandilands, K.A., Southworth, G.R., St Louis, V.L., Tate, L.S., and Tate, M.T. 2021. Experimental evidence for recovery of mercury-contaminated fish populations. Nature **601**(January). Springer US. doi:10.1038/s41586-021-04222-7.

Bodaly, R.A., Beaty, K.G., Hendzel, L.H., Majewski, A.R., Paterson, M.J., Rolfhus, K.R., Penn, A.F., St.Louis, V.L., Hall, B.D., Matthews, C.J.D., Cherewyk, K.A., Mailman, M., Hurley, J.P., Schiff, S.L., and Venkiteswaran, J.J. 2004. Experimenting with Hydroelectirc Reservoirs. Environ. Sci. Technol. **38**(18): 346A-352A.

BP Statistical Review of World Energy. 2021.

Brannon E.L. 1987. Mechanisms stabilizing salmonid fry emergence timing. In HD Smith, L Margolis, CC Wood, eds, Sockeye Salmon (Oncorhynchus nerka) Population Biology and Future Management. Canadian Special Publication of Fisheries and Aquatic Sciences,Department of Fisheries and Oceans, Canada, Ottawa, Ontario, Canada. Vol 96. pp. 102 –124

Braun, D.C., Patterson, D.A., and Reynolds, J.D. 2013. Maternal and environmental influences on egg size and juvenile life-history traits in Pacific salmon. Ecol. Evol. **3**(6): 1727–1740. doi:10.1002/ece3.555.

Brette, F., Shiels, H.A., Galli, G.L.J., Cros, C., Incardona, J.P., Scholz, N.L., and Block, B.A. 2017. A Novel Cardiotoxic Mechanism for a Pervasive Global Pollutant. Sci. Rep. **7**: 1–9. Nature Publishing Group. doi:10.1038/srep41476.

Brinkworth, L.C., Hodson, P. V., Tabash, S., and Lee, P. 2003. CYP1A induction and blue sac disease in early developmental stages of rainbow trout (oncorhynchus mykiss) exposed to retene. J. Toxicol. Environ. Heal. - Part A **66**(7): 627–646. doi:10.1080/15287390309353771.

Brunskill, G.J., and Schindler, D.W. 1971. Geography and Bathymetry of Selected Lake Basins, Experimental Lakes Area, Northwestern Ontario. J. Fish. Res. Board Canada **28**(2): 139–155. doi:10.1139/f71-028.

Callaghan, D.T., Blanchfield, P.J., and Cott, P.A. 2016. Lake trout (Salvelinus namaycush) spawning habitat in a northern lake: The role of wind and physical characteristics on habitat quality. J. Great Lakes Res. **42**(2): 299–307. doi:10.1016/j.jglr.2015.07.001.

CAPP. 2019. 2019 Crude Oil Forecast, Markets and Transportation.

Carls, M.G., Holland, L., Larsen, M., Collier, T.K., Scholz, N.L., and Incardona, J.P. 2008. Fish embryos are damaged by dissolved PAHs, not oil particles. Aquat. Toxicol. **88**(2): 121–127. doi:10.1016/j.aquatox.2008.03.014.

Carls, M.G., and Meador, J.P. 2009. A Perspective on the toxicity of petrogenic PAHs to developing fish embryos related to environmental chemistry. Hum. Ecol. Risk Assess. **15**(6): 1084–1098. doi:10.1080/10807030903304708.

Carls, M.G., Rice, S.D., and Hose, J.E. 1999. Sensitivity of fish embryos to weathered crude oil: Part I. Low-level exposure during incubation causes malformations, genetic damage, and mortality in larval pacific herring (Clupea pallasi). Environ. Toxicol. Chem. **18**(3): 481–493. doi:10.1897/1551-5028(1999)018<0481:SOFETW>2.3.CO;2.

Carls, M.G., and Thedinga, J.F. 2010. Exposure of pink salmon embryos to dissolved polynuclear aromatic hydrocarbons delays development, prolonging vulnerability to mechanical damage. Mar. Environ. Res. **69**(5): 318–325. Elsevier Ltd. doi:10.1016/j.marenvres.2009.12.006.

Carvalho, P.S.M., and Tillitt, D.E. 2004. 2,3,7,8-TCDD effects on visual structure and function in swim-up rainbow trout. Environ. Sci. Technol. **38**(23): 6300–6306. doi:10.1021/es034857i.

Chalmers, B., Elphick, J., Gilron, G., and Bailey, H. 2014. Evaluation of an in situ early life stage test with cutthroat trout, Oncorhynchus clarki, for environmental monitoring - A case study using mine effluent. Water Qual. Res. J. Canada **49**(2): 95–103. doi:10.2166/wqrjc.2013.047.

Christensen, R.H.B. 2019. ordinal—Regression Models for Ordinal Data. R package version 2019.12-10. : 54.

Chu, P., He, L., Zhu, D., Huang, R., Liao, L., Li, Y., Zhu, Z., and Wang, Y. 2019. Identification, expression and functional characterisation of CYP1A in grass carp (Ctenopharyngodon idella). Fish Shellfish Immunol. **95**(October): 35–43. Elsevier. doi:10.1016/j.fsi.2019.10.022.

Colavecchia, M. V., Hodson, P. V., and Parrott, J.L. 2007. The relationships among CYP1A induction, toxicity, and eye pathology in early life stages of fish exposed to oil sands. J. Toxicol. Environ. Heal. - Part A Curr. Issues **70**(18): 1542–1555. doi:10.1080/15287390701384726.

Collier, T.K., Anulacion, B.F., Arkoosh, M.R., Dietrich, J.P., Incardona, J.P., Johnson, L.L., Ylitalo, G.M., and Myers, M.S. 2013. Effects on Fish of Polycyclic Aromatic Hydrocarbons (PAHs) and Naphthenic Acid Exposures. *In* Fish Physiology, First Edit. Elsevier Inc. doi:10.1016/B978-0-12-398254-4.00004-2.

Corkal, V. 2021. Pipelines or Progress: Government support for oil and gas pipelines in Canada.

Corrales, J., Thornton, C., White, M., and Willett, K.L. 2014. Multigenerational effects of benzo[a]pyrene exposure on survival and developmental deformities in zebrafish larvae. Aquat. Toxicol. **148**: 16–26. Elsevier B.V. doi:10.1016/j.aquatox.2013.12.028.

Covington, S.M., Naddy, R.B., Prouty, A.L., Werner, S.A., and Lewis, M.D. 2018. Effects of in situ selenium exposure and maternal transfer on survival and deformities of brown trout (Salmo trutta) fry. Environ. Toxicol. Chem. **37**(5): 1396–1408. doi:10.1002/etc.4086.

Cresci, A., Paris, C.B., Browman, H.I., Skiftesvik, A.B., Shema, S., Bjelland, R., Durif, C.M.F., Foretich, M., Di Persia, C., Lucchese, V., Vikebø, F.B., and Sørhus, E. 2020. Effects of Exposure to Low Concentrations of Oil on the Expression of Cytochrome P4501a and Routine Swimming Speed of Atlantic Haddock (Melanogrammus aeglefinus) Larvae in Situ. Environ. Sci. Technol. **54**(21): 13879–13887. doi:10.1021/acs.est.0c04889.

Crosby, S., Fay, R., Groark, C., Kani, A., Smith, J., and Sullivan, T. 2013. Transporting Alberta's oil sands products: Defining the Issues and Assessing the Risks. doi:10.13140/2.1.1893.0240.

Crowe, K.M., Newton, J.C., Kaltenboeck, B., and Johnson, C. 2014. Oxidative stress responses of gulf killifish exposed to hydrocarbons from the Deepwater Horizon oil spill: Potential implications for aquatic food resources. Environ. Toxicol. Chem. **33**(2): 370–374. doi:10.1002/etc.2427.

de Santiago-Martín, A., Guesdon, G., Diaz-Sanz, J., and Galvez-Cloutier, R. 2015. Oil Spill in Lac-Mégantic, Canada: Environmental Monitoring and Remediation. Int. J. Water Wastewater Treat. **2**(1). doi:10.16966/2381-5299.113.

Dearnley, J.M., Killeen, C., Davis, R.L., Palace, V.P., and Tomy, G.T. 2020. Monitoring polycyclic aromatic compounds exposure in fish using biliary metabolites. Crit. Rev. Environ. Sci. Technol. **52**(4): 475–519. Taylor & Francis. doi:10.1080/10643389.2020.1825895.

Dew, W.A., Hontela, A., Rood, S.B., and Pyle, G.G. 2015. Biological effects and toxicity of diluted bitumen and its constituents in freshwater systems. J. Appl. Toxicol. **35**(11): 1219–1227. doi:10.1002/jat.3196.

DFO. 2017. A Framework for the Development of Standard Methods to Evaluate the Toxicity of Petroleum Hydrocarbons on Aquatic Organisms. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2017/053.

Di Toro, D.M., McGrath, J.A., and Stubblefield, W.A. 2007. Predicting the toxicity of neat and weathered crude oil: Toxic potential and the toxicity of saturated mixtures. Environ. Toxicol. Chem. **26**(1): 24–36. doi:10.1897/06174R.1.

ECCC. 1998. Biological test method: Toxicity tests using early life stages of salmonid fish (Rainbow trout). EPS 1/RM/28 Second Edition, July 1998. Method Development and Application Section, Environmental Technology Center. (https://www.canada.ca/en/environment-climate-change/services/wildlife-research-landscape-science/biological-test-method-publications/toxicity-rainbow-trout.html)

Elonen, G.E., Spehar, R.L., Holcombe, G.W., Johnson, R.H.J.N.E.Y.D., Fernandez, J.D., Erickson, R.J., Tietge, J.E., and Cook, P.M. 1998. Comparative toxicity of 2, 3, 7, 8-tetrachlorodi- benzo-p-dioxin to seven freshwater fish species during early life-stage development. Environ. Toxicol. Chem. **17**(3).

Emmerton, C.A., Beaty, K.G., Casson, N.J., Graydon, J.A., Hesslein, R.H., Higgins, S.N., Osman, H., Paterson, M.J., Park, A., and Tardif, J.C. 2019. Long-Term Responses of Nutrient Budgets to Concurrent Climate-Related Stressors in a Boreal Watershed. Ecosystems **22**(2): 363–378. Springer US. doi:10.1007/s10021-018-0276-7.

Findlay, D.L., and Kasian, S.E.M. 1987. Phytoplankton community responses to nutrient addition in Lake 226, Experimental Lakes Area, northwestern Ontario. Can. J. Fish. Aquat. Sci. **44**(Supplement 1): 35–46. doi:10.1139/f87-278.

Findlay, D.L., Podemski, C.L., and Kasian, S.E.M. 2009. Aquaculture impacts on the algal and bacterial communities in a small boreal forest lake. Can. J. Fish. Aquat. Sci. **66**(11): 1936–1948. doi:10.1139/F09-121.

Finn, R.N. 2007. The physiology and toxicology of salmonid eggs and larvae in relation to water quality criteria. Aquat. Toxicol. **81**(4): 337–354. doi:10.1016/j.aquatox.2006.12.021.

Franco, M.E., and Lavado, R. 2019. Applicability of in vitro methods in evaluating the biotransformation of polycyclic aromatic hydrocarbons (PAHs) in fish: Advances and challenges. Sci. Total Environ. **671**: 685–695. Elsevier B.V. doi:10.1016/j.scitotenv.2019.03.394.

Green, K.P., and Jackson, T. 2015. Safety in the Transportation of OIl and Gas: Pipelines or Rail? Fraser Res. Bull.: 1–14.

Greenwell, B., McCarthy, A., Boehmke, B., and Liu, D. (n.d.). Package “sure.” doi:10.1080/01621459.2017.1292915.

Griffith, O.W. 1980. Determination of Glutathione and Glutathione Disulfide Using Glutathione Reductase and 2-Vinylpyridine. Anal. Biochem. **106**: 207–212. doi:10.1248/bpb.33.556.

Guiney, P.D., Cook, P.M., Casselman, J.M., Fitzsimmons, J.D., Simonin, H.A., Zabel, E.W., and Peterson, R.E. 1996. Assessment of 2,3,7,8-tetrachlorodibenzo-p-dioxin induced sac fry mortality in lake trout (Salvelinus namaycush) from different regions of the Great Lakes. Can. J. Fish. Aquat. Sci. **53**(9): 2080–2092. doi:10.1139/cjfas-53-9-2080.

Guiney, P.D., Smolowitz, R.M., Peterson, R.E., and Stegeman, J.J. 1997. Correlation of 2,3,7,8-tetrachlorodibenzo-p-dioxin induction of cytochrome P4501A in vascular endothelium with toxicity in early life stages of lake trout. Toxicol. Appl. Pharmacol. **143**(2): 256–273. doi:10.1006/taap.1996.8051.

Gunn, J.M. 1995. Spawning Behavior of Lake Trout: Effects on Colonization Ability. J. Great Lakes Res. **21**(Supplement 1): 323–329. Elsevier. doi:10.1016/S0380-1330(95)71106-6.

Guzzo, M.M., and Blanchfield, P.J. 2017. Climate change alters the quantity and phenology of habitat for lake trout (Salvelinus namaycush) in small boreal shield lakes. Can. J. Fish. Aquat. Sci. **74**(6): 871–884. doi:10.1139/cjfas-2016-0190.

Hansen, M.J., and Bronte, C.R. 2019. Restoration of Lake Trout in Lake Superior through Interagency Cooperative Management. From Catastr. To Recover. Stories Fish. Manag. Success (September): 385+.

Hawkins, S.A., Billiard, S.M., Tabash, S.P., Brown, R.S., and Hodson, P. V. 2002. Altering Cytochrome P4501a Activity Affects Polycyclic Aromatic Hydrocarbon Metabolism and Toxicity in Rainbow Trout (Oncorhynchus Mykiss). Environ. Toxicol. Chem. **21**(9): 1845. doi:10.1897/1551-5028(2002)021<1845:acpaap>2.0.co;2.

Heintz, R.A. 2007. Chronic exposure to polynuclear aromatic hydrocarbons in natal habitats leads to decreased equilibrium size, growth, and stability of pink salmon populations. Integr. Environ. Assess. Manag. **3**(3): 351–363. doi:10.1897/1551-3793(2007)3[351:CETPAH]2.0.CO;2.

Hellou, J., Ross, N.W., and Moon, T.W. 2012. Glutathione, glutathione S-transferase, and glutathione conjugates, complementary markers of oxidative stress in aquatic biota. Environ. Sci. Pollut. Res. **19**(6): 2007–2023. doi:10.1007/s11356-012-0909-x.

Hicken, C.E., Linbo, T.L., Baldwin, D.H., Willis, M.L., Myers, M.S., Holland, L., Larsen, M., Stekoll, M.S., Rice, S.D., Collier, T.K., Scholz, N.L., and Incardona, J.P. 2011. Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. Proc. Natl. Acad. Sci. U. S. A. **108**(17): 7086–7090. doi:10.1073/pnas.1019031108.

Hoberg, G. 2016. Pipelines and the Politics of Structure: A Case Study of the Trans Mountain Pipeline George Hoberg Liu Institute for Global Issues UBC Prepared for delivery at the. Annu. Meet. Can. Polit. Sci. Assoc. Available from https://cpsa-acsp.ca/documents/conference/2016/Hoberg.pdf.

Hodson, P. V., Wallace, S.J., de Solla, S.R., Head, S.J., Hepditch, S.L.J., Parrott, J.L., Thomas, P.J., Berthiaume, A., and Langlois, V.S. 2020. Polycyclic aromatic compounds (PACs) in the Canadian environment: The challenges of ecological risk assessments. Environ. Pollut. **266**: 115165. Elsevier Ltd. doi:10.1016/j.envpol.2020.115165.

Hodson, P. V. 2017. The Toxicity to Fish Embryos of PAH in Crude and Refined Oils. Arch. Environ. Contam. Toxicol. **73**(1): 12–18. Springer US. doi:10.1007/s00244-016-0357-6.

Hodson, P. V., Qureshi, K., Noble, C.A.J., Akhtar, P., and Brown, R.S. 2007. Inhibition of CYP1A enzymes by α-naphthoflavone causes both synergism and antagonism of retene

toxicity to rainbow trout (Oncorhynchus mykiss). Aquat. Toxicol. **81**(3): 275–285. doi:10.1016/j.aquatox.2006.12.012.

Holm, J., Palace, V.P., Wautier, K., Evans, R.E., Baron, C.L., Podemski, C., Siwik, P., and Sterling, G. 2003. An assessment of the development and survival of wild rainbow trout (Oncorhynchus mykiss) and brook trout (Salvelinus fontinalis) exposed to elevated selenium in an area of active coal mining. : 17.

Honda, M., and Suzuki, N. 2020. Toxicities of polycyclic aromatic hydrocarbons for aquatic animals. Int. J. Environ. Res. Public Health **17**(4). doi:10.3390/ijerph17041363.

Hose, J.E., Hannah, J.B., DiJulio, D., Landolt, M.L., Miller, B.S., Iwaoka, W.T., and Felton, S.P. 1982. Effects of benzo(a)pyrene on early development of flatfish. Arch. Environ. Contam. Toxicol. **11**(2): 167–171. doi:10.1007/BF01054893.

Hose, J.E., Hannaht, J.B., Puffer, H.W., and Landolt, M.L. 1984. Histologic and skeletal abnormalities in benzo(a)pyrene-treated rainbow trout alevins. Arch. Environ. Contam. Toxicol. **13**(6): 675–684. doi:10.1007/BF01055930.

Hothorn, T., Zeileis, A., Farebrother, R.W., Cummins, C., Millo, G., and Mitchell, D. 2021. Package ‘ lmtest .’

Hox, J. 2002. Multilevel Analysis: Techniques and Applications.

Hutchings, J.A., and Morris, D.W. 1985. The Influence of Phylogeny, Size and Behaviour on Patterns of Covariation in Salmonid Life Histories. OIKOS **45**(1): 118–124.

Idowu, I., Francisco, O., Thomas, P.J., Johnson, W., Marvin, C., Stetefeld, J., and Tomy, G.T. 2018. Validation of a simultaneous method for determining polycyclic aromatic compounds and alkylated isomers in biota. Rapid Commun. Mass Spectrom. **32**(3): 277–287. doi:10.1002/rcm.8035.

Incardona, J.P. 2017. Molecular Mechanisms of Crude Oil Developmental Toxicity in Fish. Arch. Environ. Contam. Toxicol. **73**(1): 19–32. Springer US. doi:10.1007/s00244-017-0381-1.

Incardona, J.P., Carls, M.G., Holland, L., Linbo, T.L., Baldwin, D.H., Myers, M.S., Peck, K.A., Tagal, M., Rice, S.D., and Scholz, N.L. 2015. Very low embryonic crude oil exposures cause lasting cardiac defects in salmon and herring. Sci. Rep. **5**: 1–13. Nature Publishing Group. doi:10.1038/srep13499.

Incardona, J.P., Collier, T.K., and Scholz, N.L. 2004. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. Toxicol. Appl. Pharmacol. **196**(2): 191–205. doi:10.1016/j.taap.2003.11.026.

Incardona, J.P., and Scholz, N.L. 2016. The influence of heart developmental anatomy on cardiotoxicity-based adverse outcome pathways in fish. Aquat. Toxicol. **177**: 515–525. Elsevier B.V. doi:10.1016/j.aquatox.2016.06.016.

Janz, D., DeForest, D., Brooks, M., Chapman, P., Gilron, G., Hoff, D., Hopkins, W., McIntyre, D., Mebane, C., Palace, V., Skorupa, J., and Wayland, M. 2010. Selenium Toxicity to Aquatic Organisms. Ecol. Assess. Selenium Aquat. Environ.: 141–231. doi:10.1201/ebk1439826775-c6.

Janz, D., and Muscatello, J. 2008. Standard Operating Procedure for Evaluating Selenium-Induced Deformities in Early Like Stages of Freshwater Fish. *In* North Americ Metals Council - Selenium Working Group.

Jefferson, M. 2020. A crude future? COVID-19s challenges for oil demand, supply and prices. Energy Res. Soc. Sci. **68**.

Kelly, C.A., Rudd, J.W.M., Bodaly, R.A., Roulet, N.P., St.Louis, V.L., Heyes, A., Moore, T.R., Schiff, S., Aravena, R., Scott, K.J., Dyck, B., Harris, R., Warner, B., and Edwards, G. 1997. Increases in fluxes of greenhouse gases and methyl mercury following flooding of an experimental reservoir. Environ. Sci. Technol. **31**(5): 1334–1344. doi:10.1021/es9604931.

Kennedy, C.J., and Law, F.C.P. 1990. Toxicokinetics of selected polycyclic aromatic hydrocarbons in rainbow trout following different routes of exposure. Environ. Toxicol. Chem. **9**(2): 133–139. doi:10.1002/etc.5620090202.

Kheraj, S. 2020. A history of oil spills on long-distance pipelines in Canada. Can. Hist. Rev. **101**(2): 161–191. doi:10.3138/CHR.2019-0005.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., and Flick, R.W. 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. U. S. A. **104**(21): 8897–8901. doi:10.1073/pnas.0609568104.

Le Bihanic, F., Morin, B., Cousin, X., Le Menach, K., Budzinski, H., and Cachot, J. 2014. Developmental toxicity of PAH mixtures in fish early life stages. Part I: adverse effects in rainbow trout. Environ. Sci. Pollut. Res. **21**(24): 13720–13731. doi:10.1007/s11356-014-2804-0.

Lee, K., Boufadel, M., Chen, B., Foght, J., Hodson, P., Swanson, S., and Venosa, A. 2015. Behaviour and Environmental Impacts of Crude Oil Released into Aqueous Environments. *In* Royal Society of Canada. Available from https://www.rsc-src.ca/sites/default/files/pdf/OIW Report.pdf.

Leggatt, R.A. 2006. Glutathione in fish: transport, influence of temperature and growth rate, and interaction with the stress response. Available from https://open.library.ubc.ca/cIRcle/collections/ubctheses/831/items/1.0092848.

Leitritz, E. 1959. Trout and Salmon Culture (Hatchery Methods). State of California Department of Fish and Game. Fish Bulletin No. 107.

Liljequist, D., Elfving, B., and Roaldsen, K.S. 2019. Intraclass correlation – A discussion and demonstration of basic features. *In* PLoS ONE. doi:10.1371/journal.pone.0219854.

Lindberg, S.D., Jayasundara, N., Kozal, J.S., Leuthner, T.C., and Di Giulio, R.T. 2017. Resistance to polycyclic aromatic hydrocarbon toxicity and associated bioenergetic consequences in a population of Fundulus heteroclitus. Ecotoxicology **26**(3): 435–448. doi:10.1007/s10646-017-1775-6.Resistance.

Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods **25**(4): 402–408. doi:10.1006/meth.2001.1262.

Mackey, T.E., Hasler, C.T., Durhack, T., Jeffrey, J.D., Macnaughton, C.J., Ta, K., Enders, E.C., and Jeffries, K.M. 2021. Molecular and physiological responses predict acclimation limits in juvenile brook trout (Salvelinus fontinalis). J. Exp. Biol. **224**(16). doi:10.1242/jeb.241885.

Madison, B.N., Hodson, P. V., and Langlois, V.S. 2015. Diluted bitumen causes deformities and molecular responses indicative of oxidative stress in Japanese medaka embryos. Aquat. Toxicol. **165**: 222–230. Elsevier B.V. doi:10.1016/j.aquatox.2015.06.006.

Magnuson, J.T., Bautista, N.M., Lucero, J.A., Lund, A.K., Xu, E.G., Schlenk, D., Burggren, W.W., and Roberts, A.P. 2020. Exposure to Crude Oil Induces Retinal Apoptosis and Impairs Visual Function in Fish. Environ. Sci. Technol. **54**(5): 2843–2850. doi:10.1021/acs.est.9b07658.

Marlatt, V.L., Sherrard, R., Kennedy, C.J., Elphick, J.R., and Martyniuk, C.J. 2016. Application of molecular endpoints in early life stage salmonid environmental biomonitoring. Aquat. Toxicol. **173**: 178–191. Elsevier B.V. doi:10.1016/j.aquatox.2016.01.006.

Marsden, J.E., Perkins, D.L., and Krueger, C.C. 1995. Recognition of Spawning Areas by Lake Trout: Deposition and Survival of Eggs on Small, Man-Made Rock Piles. J. Gt. Lakes Res. **21**(1): 330–336.

Massarsky, A., Kozal, J.S., and Di Giulio, R.T. 2017. Glutathione and zebrafish: Old assays to address a current issue. Chemosphere **168**(919): 707–715. doi:10.1016/j.chemosphere.2016.11.004.

McDonald, M.E., Hershey, A.E., and Miller, M.C. 1996. Global warming impacts on lake trout in arctic lakes. Limnol. Oceanogr. **41**(5): 1102–1108. doi:10.4319/lo.1996.41.5.1102.

McGurk, M.D., Landry, F., Tang, A., and Hanks, C.C. 2006. Acute and chronic toxicity of nitrate to early life stages of lake trout (Salvelinus namaycush) and lake whitefish

(Coregonus clupeaformis). Environ. Toxicol. Chem. **25**(8): 2187–2196. doi:10.1897/05-270R.1.

Meador, J.P., Sommers, F.C., Ylitalo, G.M., and Sloan, C.A. 2006. Altered growth and related physiological responses in juvenile Chinook salmon (Oncorhynchus tshawytscha) from dietary exposure to polycyclic aromatic hydrocarbons (PAHs). Can. J. Fish. Aquat. Sci. **63**(10): 2364–2376. doi:10.1139/F06-127.

Meyer, J.N., Nacci, D.E., and Di Giulio, R.T. 2002. Cytochrome P4501A (CYP1A) in killifish (Fundulus heteroclitus): Heritability of altered expression and relationship to survival in contaminated sediments. Toxicol. Sci. **68**(1): 69–81. doi:10.1093/toxsci/68.1.69.

Miller, M.A. 1995. Maternal transfer of organochlorine compounds in lake superior siscowet (Salvelinus namaycush siscowet) to their eggs. Bull. Environ. Contam. Toxicol. **55**(1): 96–103. doi:10.1007/BF00212394.

Miller, T.J., Crowder, L.B., Rice, J.A., and Marschall, E.A. 1988. Larval size and recruitment mechanisms in fishes: toward a conceptual framework. Can. J. Fish. Aquat. Sci. **45**(9): 1657–1670. doi:10.1139/f88-197.

Milling, A. 2020. Impacts of Mysis diluviana extirpation on a whole lake ecosystem.

Mills, K.H., Chalanchuk, S.M., and Allan, D.J. 2000. Recovery of fish populations in Lake 223 from experimental acidification. Can. J. Fish. Aquat. Sci. **57**(1): 192–204. doi:10.1139/f99-186.

Moral, R.A., Hinde, J., and Demétrio, C.G.B. 2017. Half-normal plots and overdispersed models in R: The hnp package. J. Stat. Softw. **81**(10). doi:10.18637/jss.v081.i10.

Moroni, D., Pieri, G., and Tampucci, M. 2019. Environmental decision support systems for monitoring small scale oil spills: Existing solutions, best practices and current challenges. J. Mar. Sci. Eng. **7**(1). doi:10.3390/JMSE7010019.

Mu, J. li, Wang, X. hong, Jin, F., Wang, J. ying, and Hong, H. sheng. 2012. The role of cytochrome P4501A activity inhibition in three- to five-ringed polycyclic aromatic hydrocarbons embryotoxicity of marine medaka (Oryzias melastigma). Mar. Pollut. Bull. **64**(7): 1445–1451. Elsevier Ltd. doi:10.1016/j.marpolbul.2012.04.007.

Navas, J.M., and Segner, H. 1998. Antiestrogenic activity of anthropogenic and natural chemicals. Environ. Sci. Pollut. Res. **5**(2): 75–82. doi:10.1007/BF02986390.

Nicolas, J.M. 1999. Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants. Aquat. Toxicol. **45**(2–3): 77–90. doi:10.1016/S0166-445X(98)00095-2.

Niimi, A.J., and Palazzo, V. 1986. Biological half-lives of eight polycyclic aromatic hydrocarbons (PAHs) in rainbow trout (Salmo gairdneri). Water Res. **20**(4): 503–507. doi:10.1016/0043-1354(86)90200-9.

Ojanguren, A.F., Reyes-Gavilán, F.G., and Braña, F. 1996. Effects of egg size on offspring development and fitness in brown trout, Salmo trutta L. Aquaculture **147**(1–2): 9–20. doi:10.1016/S0044-8486(96)01398-1.

Palace, V.P., and Werner, J. 2006. Vitamins A and E in the maternal diet influence egg quality and early life stage development in fish: A review. Sci. Mar. **70**(SUPPL. 2): 41–57. doi:10.3989/scimar.2006.70s241.

Palanikumar, L., Kumaraguru, A.K., Ramakritinan, C.M., and Anand, M. 2012. Biochemical response of anthracene and benzo [a] pyrene in milkfish Chanos chanos. Ecotoxicol. Environ. Saf. **75**(1): 187–197. Elsevier. doi:10.1016/j.ecoenv.2011.08.028.

Perrichon, P., Akcha, F., Le Menach, K., Goubeau, M., Budzinski, H., Cousin, X., and Bustamante, P. 2015. Parental trophic exposure to three aromatic fractions of polycyclic aromatic hydrocarbons in the zebrafish: Consequences for the offspring. Sci. Total Environ. **524**–**525**: 52–62. Elsevier B.V. doi:10.1016/j.scitotenv.2015.04.018.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S., Van Willigen, B., Ranke, J., R-core, and Authors, E. 2021. Package ‘ nlme .’

Pollino, C.A., Georgiades, E., and Holdway, D.A. 2009. Physiological changes in reproductively active rainbowfish (Melanotaenia fluviatilis) following exposure to naphthalene. Ecotoxicol. Environ. Saf. **72**(4): 1265–1270. doi:10.1016/j.ecoenv.2009.01.012.

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rawson, D.S. 1961. The Lake Trout of Lac la Ronge, Saskatchewan. J. Fish. Res. Board Canada **18**(3): 423–462. doi:10.1139/f61-038.

Redick, R.R. 1967. A review of literature on lake trout life history with notes on Alaskan management. Alaska Dep. Fish Game Informational Leaflet. **111**: 1–19.

Regoli, F., and Giuliani, M.E. 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar. Environ. Res. **93**: 106–117. Elsevier Ltd. doi:10.1016/j.marenvres.2013.07.006.

Rennie, M.D., Kennedy, P.J., Mills, K.H., Rodgers, C.M.C., Charles, C., Hrenchuk, L.E., Chalanchuk, S., Blanchfield, P.J., Paterson, M.J., and Podemski, C.L. 2019. Impacts of freshwater aquaculture on fish communities: A whole-ecosystem experimental approach. Freshw. Biol. **64**(5): 870–885. doi:10.1111/fwb.13269.

Rice, S.D., Thomas, R.E., Carls, M.G., Heintz, R.A., Wertheimer, A.C., Murphy, M.L., Short, J.W., and Moles, A. 2001. Impacts to Pink Salmon Following the Exxon Valdez Oil Spill: Persistence, Toxicity, Sensitivity, and Controversy. Rev. Fish. Sci. **9**(3): 165–211. doi:10.1080/20016491101744.

Rigaud, C., Eriksson, A., Krasnov, A., Wincent, E., Pakkanen, H., Lehtivuori, H., Ihalainen, J., and Vehniäinen, E.R. 2020. Retene, pyrene and phenanthrene cause distinct molecular-level changes in the cardiac tissue of rainbow trout (Oncorhynchus mykiss) larvae, part 1 – Transcriptomics. Sci. Total Environ. **745**: 141031. Elsevier B.V. doi:10.1016/j.scitotenv.2020.141031.

Rigaud, C., Härme, J., and Vehniäinen, E.R. 2022. Salmo trutta is more sensitive than Oncorhynchus mykiss to early-life stage exposure to retene. Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. **252**(September 2021). doi:10.1016/j.cbpc.2021.109219.

Rodriguez-Gil, J.L., Stoyanovich, S., Hanson, M.L., Hollebone, B., Orihel, D.M., Palace, V., Faragher, R., Mirnaghi, F.S., Shah, K., Yang, Z., Black, T.A., Cederwall, J., Mason, J., Patterson, S., Timlick, L., Séguin, J.Y., and Blais, J.M. 2021. Simulating diluted bitumen spills in boreal lake limnocorrals - Part 1: Experimental design and responses of hydrocarbons, metals, and water quality parameters. Sci. Total Environ. **790**: 1–14. Elsevier B.V. doi:10.1016/j.scitotenv.2021.148537.

Romero, I.C., Sutton, T., Carr, B., Quintana-Rizzo, E., Ross, S.W., Hollander, D.J., and Torres, J.J. 2018. Decadal Assessment of Polycyclic Aromatic Hydrocarbons in Mesopelagic Fishes from the Gulf of Mexico Reveals Exposure to Oil-Derived Sources. Environ. Sci. Technol. **52**(19): 10985–10996. doi:10.1021/acs.est.8b02243.

Rudolph, B. 2006. The effects of selenium on westslope cutthroat trout reproduction and development.

Sakari, M. 2012. Depositional History of Polycyclic Aromatic Hydrocarbons: Reconstruction of Petroleum Pollution Record in Peninsular Malaysia. Org. Pollut. Ten Years After Stock. Conv. - Environ. Anal. Updat. doi:10.5772/31624.

Sandilands, K.A., Rudd, J.W.M., Kelly, C.A., Hintelmann, H., Gilmour, C.C., Tate, M.T., Rudd, J.W.M., Tate, M.T., Hintelmann, H., Dimock, B., and Harris, R. 2008. Application of Enriched Stable Mercury Isotopes to the Lake 658 Watershed for the METAALICUS Project , at the Experimental Lakes Area , Northwestern Ontario , Canada 2005 Canadian Technical Report of Fisheries and Aquatic Sciences 2597. Can. Tech. Rep. Fish. Aquat. Sci.: 48.

Santana, M.S., Sandrini-Neto, L., Filipak Neto, F., Oliveira Ribeiro, C.A., Di Domenico, M., and Prodocimo, M.M. 2018. Biomarker responses in fish exposed to polycyclic aromatic hydrocarbons (PAHs): Systematic review and meta-analysis. Environ. Pollut. **242**: 449–461. Elsevier Ltd. doi:10.1016/j.envpol.2018.07.004.

Sarasquete, C., and Segner, H. 2000. Cytochrome P4501A CYP1A in teleostean fishes. Sci. Total Environ. **247**: 313–332.

Sayers EW, Cavanaugh M, Clark K, Ostell J, Pruitt KD, Karsch-Mizrachi I. GenBank. Nucleic Acids Res. 2019 Jan 8;47(D1):D94-D99. doi: 10.1093/nar/gky989. PMID: 30365038; PMCID: PMC6323954.

Schindler, D.W., Wagemann, R., Cook, R.B., Ruszczynski, T., and Prokopowich, J. 1980. Experimental Acidification of Lake 223, Experimental Lakes Area: Background Data and the First Three Years of Acidification. Can. J. Fish. Aquat. Sci. **37**(3): 342–354. doi:10.1139/f80-048.

Schlezinger, J.J., Struntz, W.D.J., Goldstone, J. V., and Stegeman, J.J. 2006. Uncoupling of cytochrome P450 1A and stimulation of reactive oxygen species production by co-planar polychlorinated biphenyl congeners. Aquat. Toxicol. **77**(4): 422–432. doi:10.1016/j.aquatox.2006.01.012.

Schuler, L.J., Landrum, P.F., and Lydy, M.J. 2007. Response spectrum of fluoranthene and pentachlorobenzene for the fathead minnow (Pimephales promelas). Environ. Toxicol. Chem. **26**(1): 139–148. doi:10.1897/06-169R.1.

Springate, J.R.C., and Bromage, N.R. 1985. Effects of Egg Size on Early Growth and Survival. Aquaculture **47**(February): 163–172.

Stagg, R., Mcintosh, A., and Gubbins, M.J. 2016. Determination of CYP1A-dependent mono-oxygenase activity in dab by fluorimetric measurement of EROD activity in S9 or microsomal liver fractions. ICES Tech. Mar. Environ. Sci. (57): 21.

Stogiannidis, E., and Laane, R. 2015. Source Characterization of Polycyclic Aromatic Hydrocarbons by Using Their Molecular Indices: An Overview of Possibilities. In: Whitacre D. (eds) Reviews of Environmental Contamination and Toxicology. Reviews of Environmental Contamination and Toxicology. Springer, Cham. doi:https://doi-org.uml.idm.oclc.org/10.1007/978-3-319-10638-0_2.

Stokstad, E. 2008. Canada's Experimental Lakes. Science (80-.). **322**(5906): 1316–1319. doi:10.1108/eb053675.

Storey, K.B. 1996. Oxidative stress: Animal adaptations in nature. Brazilian J. Med. Biol. Res. **29**(12): 1715–1733.

Stoyanovich, S., Rodríguez-Gil, J.R., Hanson, M.L., Hollebone, B.P., Orihel, D.M., Palace, V.P., Faragher, R., Mirnaghi, F.S., Shah, K., Yang, Z., and Blais, J.M. 2021. Simulating diluted bitumen spills in boreal lake limnocorrals - part 2: Factors affecting the physical characteristics and submergence of diluted bitumen. Sci. Total Environ. **790**: 148580. Elsevier B.V. doi:10.1016/j.scitotenv.2021.148580.

Stoyanovich, S.S., Yang, Z., Hanson, M., Hollebone, B.P., Orihel, D.M., Palace, V., Rodriguez-Gil, J.L., Faragher, R., Mirnaghi, F.S., Shah, K., and Blais, J.M. 2019. Simulating a Spill of Diluted Bitumen: Environmental Weathering and Submergence in a Model Freshwater System. Environ. Toxicol. Chem. **38**(12): 2621–2628. John Wiley & Sons, Ltd. doi:10.1002/etc.4600.

Sundberg, H., Hanson, M., Liewenborg, B., Zebühr, Y., Broman, D., and Balk, L. 2007. Dredging associated effects: Maternally transferred pollutants and DNA adducts in feral fish. Environ. Sci. Technol. **41**(8): 2972–2977. doi:10.1021/es070073j.

Sutherland, B.J.G., Prokkola, J.M., Audet, C., and Bernatchez, L. 2019. Sex-specific co-expression networks and sex-biased gene expression in the salmonid Brook Charr Salvelinus fontinalis. G3 Genes, Genomes, Genet. **9**(3): 955–968. doi:10.1534/g3.118.200910.

Tilghman Hall, A., and Oris, J.T. 1991. Anthracene reduces reproductive potential and is maternally transferred during long-term exposure in fathead minnows. Aquat. Toxicol. **19**: 249–264.

Turcotte, D. 2008. Toxicity and metabolism of alkyl-polycyclic aromatic hydrocarbons in fish. *In* Methods. Available from http://qspace.library.queensu.ca/handle/1974/1382.

Tuvikene, A. 1995. Responses of fish to polycyclic aromatic hydrocarbons (PAHs). Ann. Zool. Fenn. **32**: 295–309.

Tyndale, S.T., Letcher, R.J., Heath, J.W., and Heath, D.D. 2008. Why are salmon eggs red? Egg carotenoids and early life survival of Chinook salmon (Oncorhynchus tshawytscha). Evol. Ecol. Res. **10**(8): 1187–1199.

Valdebenito, I., Figueroa, E., Valdebenito, M., and Paiva, L. 2021. Chorion Alterations in Eyed-Stage Salmonid Eggs Farmed in La Araucania, Chile: A Retrospective Study. Animals **11**(2427): 1–10.

Valdehita, A., Fernández-Cruz, M.L., Torrent, F., Sericano, J.L., and Navas, J.M. 2012. Differences in the induction of cyp1A and related genes in cultured rainbow trout Oncorhynchus mykiss. Additional considerations for the use of EROD activity as a biomarker. J. Fish Biol. **81**(1): 270–287. doi:10.1111/j.1095-8649.2012.03338.x.

Vehniäinen, E.R., Bremer, K., Scott, J.A., Junttila, S., Laiho, A., Gyenesei, A., Hodson, P. V., and Oikari, A.O.J. 2016. Retene causes multifunctional transcriptomic changes in the heart of rainbow trout (Oncorhynchus mykiss) embryos. Environ. Toxicol. Pharmacol. **41**: 95–102. Elsevier B.V. doi:10.1016/j.etap.2015.11.015.

Vignet, C., Frank, R.A., Yang, C., Wang, Z., Shires, K., Bree, M., Sullivan, C., Norwood, W.P., Hewitt, L.M., McMaster, M.E., and Parrott, J.L. 2019. Long-term effects of an early-life

exposure of fathead minnows to sediments containing bitumen. Part I: Survival, deformities, and growth. Environ. Pollut. **251**: 246–256. Elsevier Ltd. doi:10.1016/j.envpol.2019.05.007.

Vignet, C., Joassard, L., Lyphout, L., Guionnet, T., Goubeau, M., Le Menach, K., Brion, F., Kah, O., Chung, B.C., Budzinski, H., Bégout, M.L., and Cousin, X. 2015. Exposures of zebrafish through diet to three environmentally relevant mixtures of PAHs produce behavioral disruptions in unexposed F1 and F2 descendant. Environ. Sci. Pollut. Res. **22**(21): 16371–16383. doi:10.1007/s11356-015-4157-8.

Wang, Z., Hollebone, B.P., Fingas, M., Fieldhouse, B., Sigouin, L., Landriault, M., Smith, P., Noonan, J., Thouin, G., and Weaver, J.W. 2003. Characteristics of spilled oils, fuels, and petroleum products: 1. Composition and properties of selected oils. USEPA Publ. **EPA/600/R-**(July): 1–286. Available from http://nepis.epa.gov/Adobe/PDF../P1000AE6.pdf%5Cnpapers2://publication/uuid/504BA836-2847-4C34-9BF4-4DA351C932B6%5Cnpapers2://publication/uuid/C0AC5D53-502E-44D7-9EA1-4D8B00FD9C0C.

Werner, J., Palace, V.P., Wautier, K.G., Mills, K.H., Chalanchuk, S.M., and Kidd, K.A. 2006. Reproductive fitness of lake trout (Salvelinus namaycush) exposed to environmentally relevant concentrations of the potent estrogen ethynylestradiol (EE2) in a whole lake exposure experiment. Sci. Mar (October): 59–66.

West, J.E., O'Neill, S.M., Ylitalo, G.M., Incardona, J.P., Doty, D.C., and Dutch, M.E. 2014. An evaluation of background levels and sources of polycyclic aromatic hydrocarbons in naturally spawned embryos of Pacific herring (Clupea pallasii) from Puget Sound, Washington, USA. Sci. Total Environ. **499**: 114–124. Elsevier B.V. doi:10.1016/j.scitotenv.2014.08.042.

Westernhagen, H., and Rosenthal, H. 1981. On condition factor measurements in Pacific herring larvae. Helgoländer Meeresuntersuchungen **34**(3): 257–262. doi:10.1007/BF02074122.

Willette, M. 1996. Impacts of the Exxon Valdez oil spill on migration, growth, and survival of juvenile pink salmon in Prince William Sound. Am. Fish. Soc. Symp., 18: 33–550.

Wischhusen, P.P.C. 2020. Parental selenium and antioxidant status in fish. *In* Zootechny. Universite de Pau et des Pays de l'Adour.

Wischhusen, P., Parailloux, M., Geraert, P.A., Briens, M., Bueno, M., Mounicou, S., Bouyssiere, B., Antony Jesu Prabhu, P., Kaushik, S.J., Fauconneau, B., and Fontagné-Dicharry, S. 2019. Effect of dietary Se in rainbow trout (Oncorhynchus mykiss) broodstock on antioxidant status, its parental transfer and oxidative status in the progeny. Aquaculture **507**(December 2018): 126–138. Elsevier. doi:10.1016/j.aquaculture.2019.04.006.

Wu, D., Wang, Z., Hollebone, B., McIntosh, S., King, T., and Hodson, P. V. 2012. Comparative toxicity of four chemically dispersed and undispersed crude oils to rainbow trout embryos. Environ. Toxicol. Chem. **31**(4): 754–765. doi:10.1002/etc.1739.

Xie, F., Xiao, P., Chen, D., Xu, L., and Zhang, B. 2012. miRDeepFinder: A miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol. Biol. **80**(1): 75–84. doi:10.1007/s11103-012-9885-2.

Xu, E.G., Mager, E.M., Grosell, M., Pasparakis, C., Schlenker, L.S., Stieglitz, J.D., Benetti, D., Hazard, E.S., Courtney, S.M., Diamante, G., Freitas, J., Hardiman, G., and Schlenk, D. 2016. Time- and Oil-Dependent Transcriptomic and Physiological Responses to Deepwater Horizon Oil in Mahi-Mahi (Coryphaena hippurus) Embryos and Larvae. Environ. Sci. Technol. **50**(14): 7842–7851. doi:10.1021/acs.est.6b02205.

Yang, Z., Shah, K., Fieldhouse, B., Mirnaghi, F., Hollebone, B.P., Lambert, P., Goldthorp, M., Brown, C.E., and Yang, C. 2021. Characterization, occurrence and natural attenuation of spilled light synthetic crude oil in a boreal freshwater ecosystem. Fuel **285**(September 2020): 119276. Elsevier Ltd. doi:10.1016/j.fuel.2020.119276.

Yazdani, M. 2020. Comparative toxicity of selected PAHs in rainbow trout hepatocytes: genotoxicity, oxidative stress and cytotoxicity. Drug Chem. Toxicol. **43**(1): 71–78. doi:10.1080/01480545.2018.1497054.

Zengin, H. 2021. The effects of feeding and starvation on antioxidant defence, fatty acid composition and lipid peroxidation in reared Oncorhynchus mykiss fry. Sci. Rep. **11**(1): 1–13. Nature Publishing Group UK. doi:10.1038/s41598-021-96204-y.

APPENDIX A: ADDITIONAL FIGURES AND TABLES

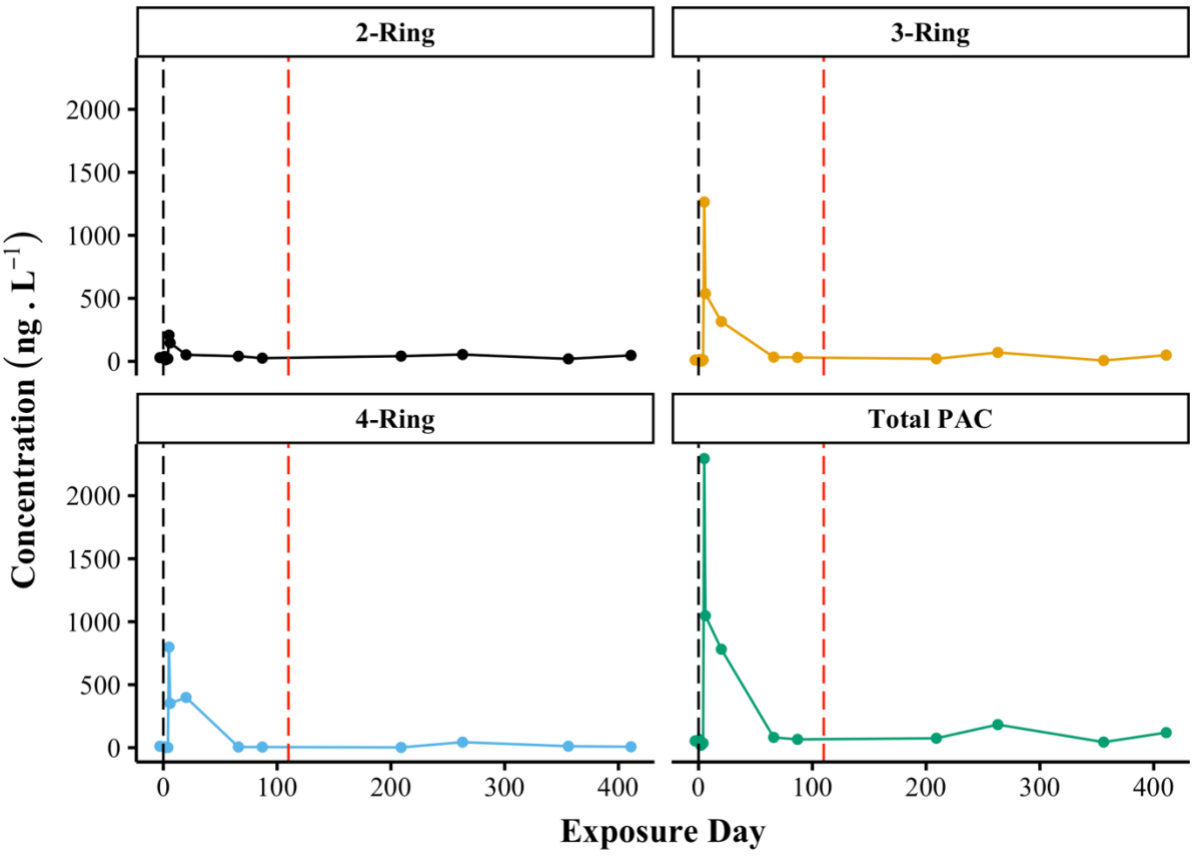


FIGURE A.1. Concentrations of polycyclic aromatic compounds (PACs) in the water of Lake 260 at the IISD – Experimental Lakes Area. The vertical black dashed line indicates oil addition date for model oil spill studies in the lake (June 21, 2019), and the vertical red dashed line indicates lake trout egg collection date (October 9, 2019).

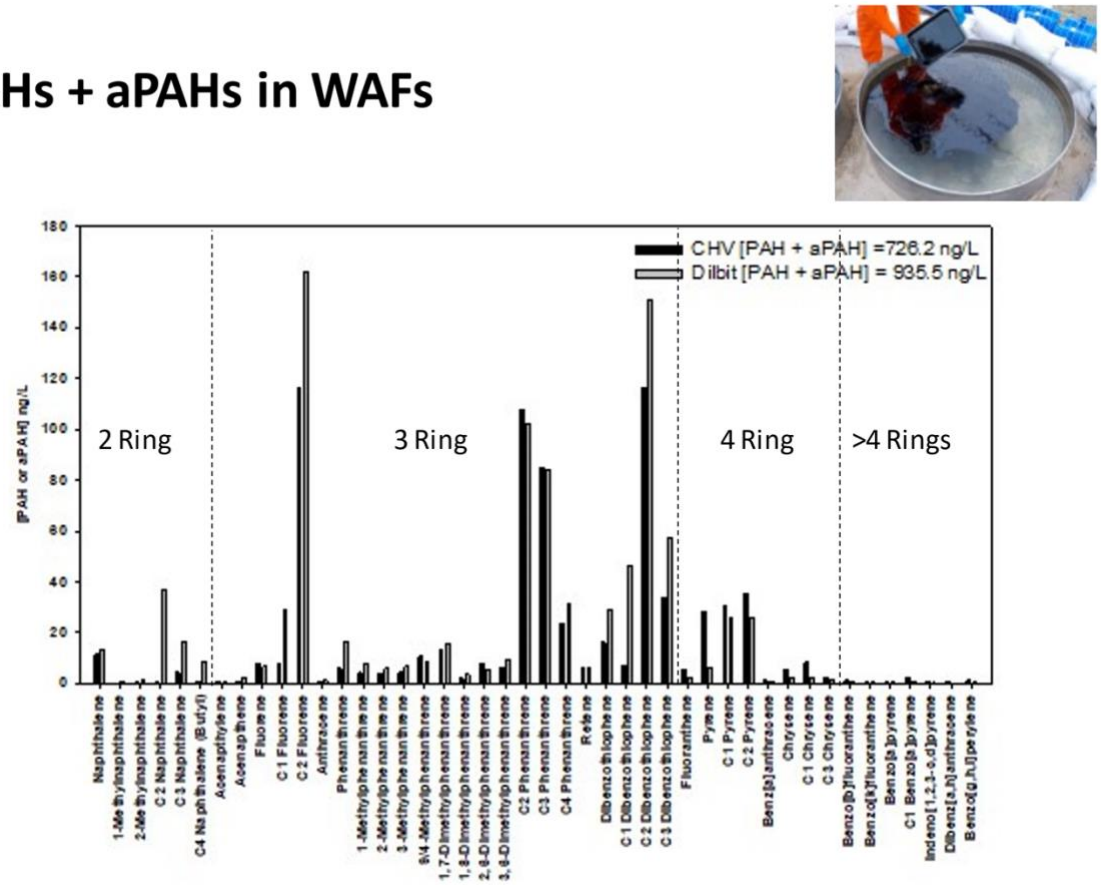


FIGURE A.2. Concentrations of polycyclic aromatic compounds (PACs) from a crude oil WAF (black) sampled from weathered oil (7 kg) on 200 L of water at the IISD-Experimental Lakes Area in 2018. PAC concentrations in the WAF (726.2 ng/L) were used to deduce the concentration of the WAF generated for this study (1:100 oil to water = ~0.2 µg/L PACs).

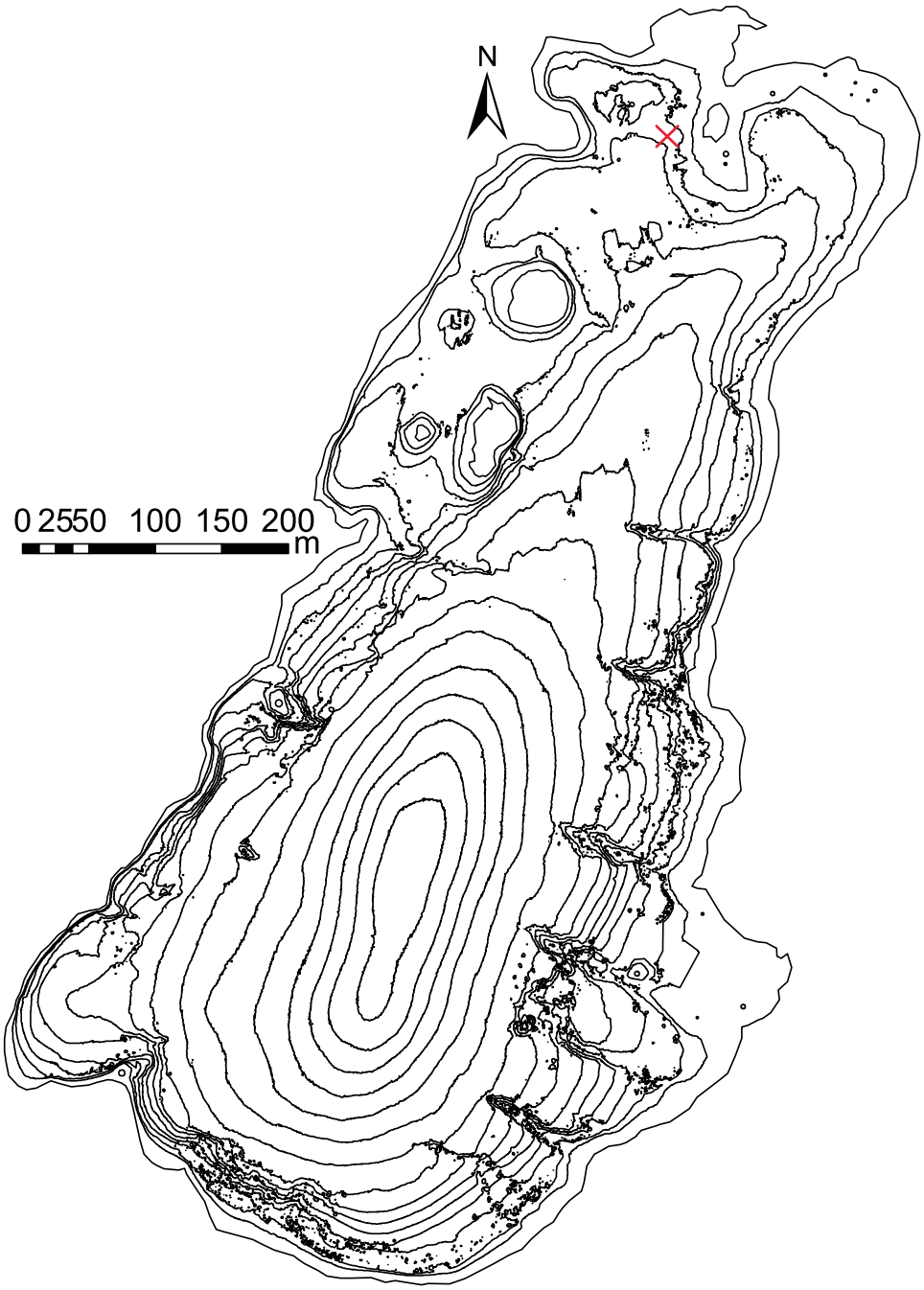


FIGURE A.3. Bathymetric map of Lake 260 at the IISD – Experimental Lakes Area. Contour lines represent one meter depth intervals. Hatch box (Scotty-Jordan incubator) deployment location denoted by red “X” (15U 444780m E 5505566m N).

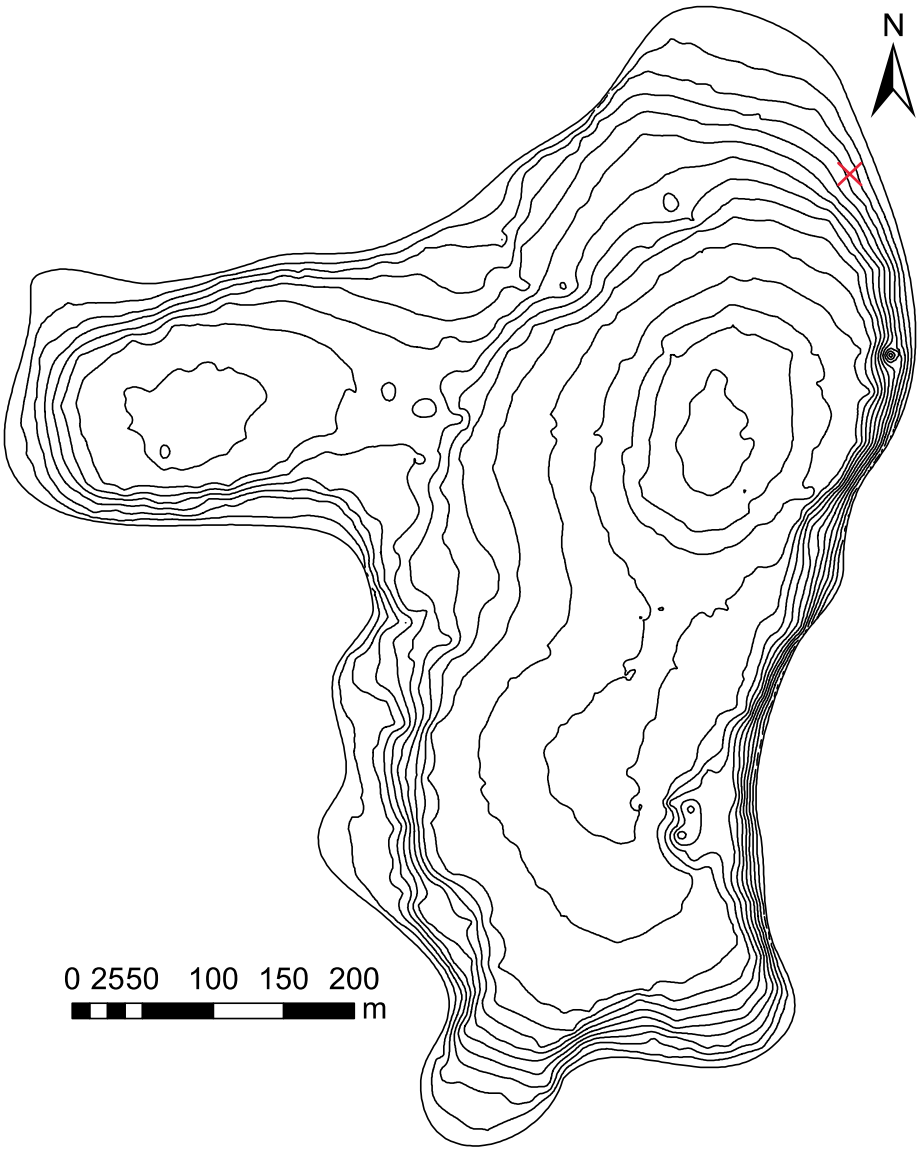


FIGURE A.4. Bathymetric map of Lake 223 at the IISD – Experimental Lakes Area. Contour lines represent one meter depth intervals. Hatch box (Scotty-Jordan incubator) deployment location denoted by red “X” (15U 0449120m E 5505596m N).

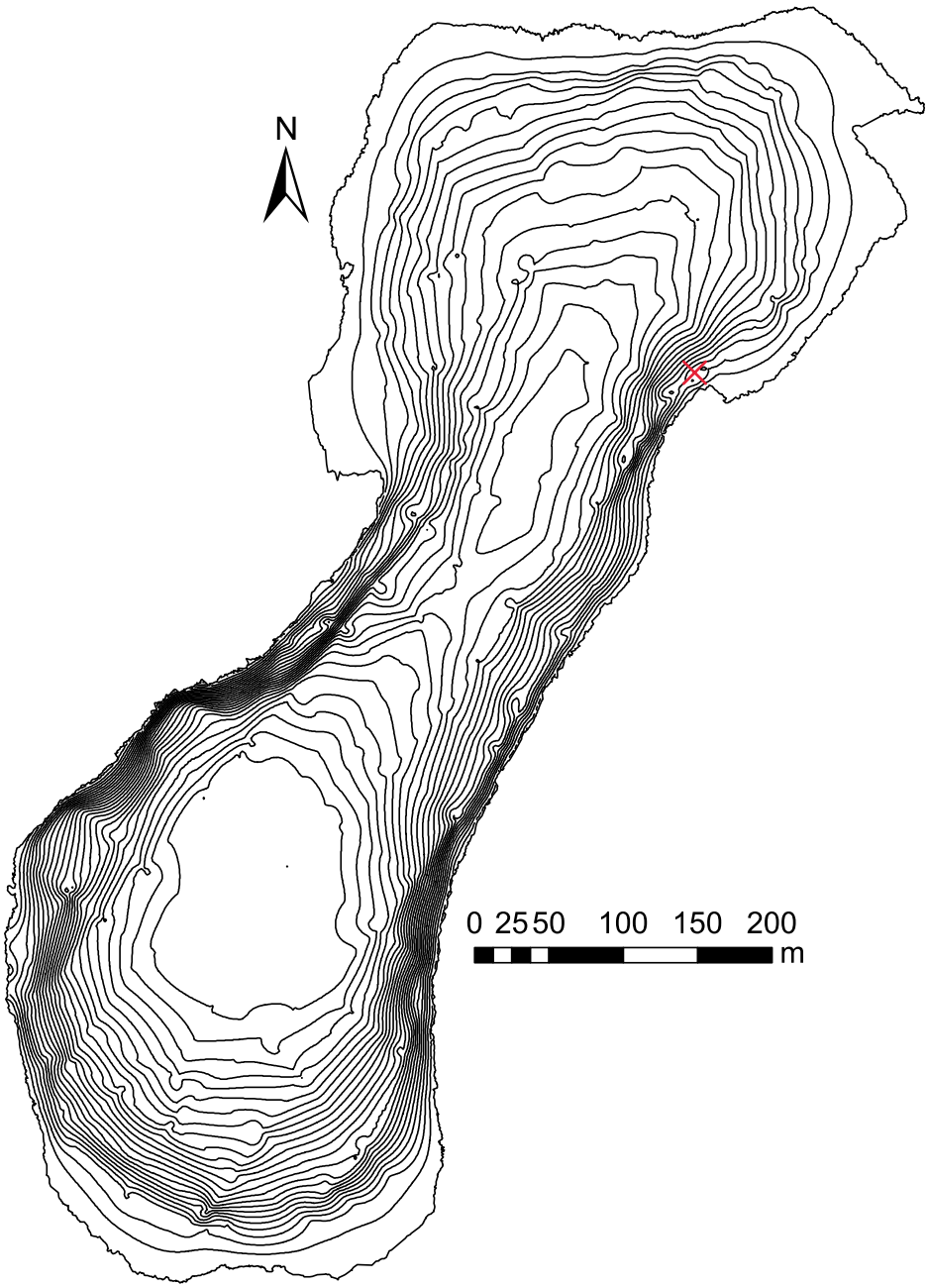


FIGURE A.5. Bathymetric map of Lake 375 at the IISD – Experimental Lakes Area. Contour lines represent one meter depth intervals. Hatch box (Scotty-Jordan incubator) deployment location denoted by red “X” (15U 0443389m E 5510703m N).



FIGURE A.6. Bathymetric map of Lake 378 at the IISD – Experimental Lakes Area. Contour lines represent one meter depth intervals. Hatch box (Scotty-Jordan incubator) deployment location denoted by red “X” (15U 0444278m E 5506543m N).

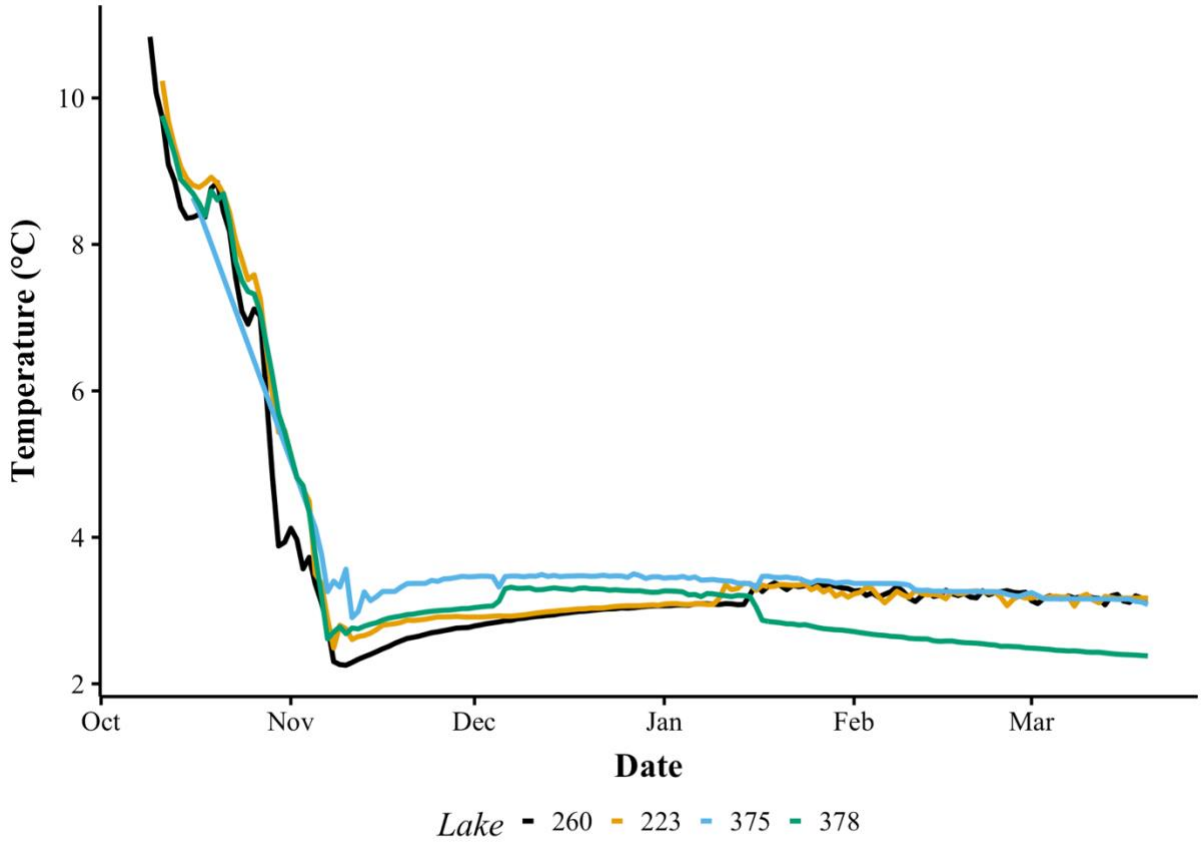


FIGURE A.7. Temperature changes in Lakes 260, 223, 375 and 378 at the IISD-Experimental Lakes Area from October 2019 – March 2020. Temperatures were recorded with a HOBO temperature logger, every 15 minutes. Daily temperatures were averaged to reduce noise in the data.

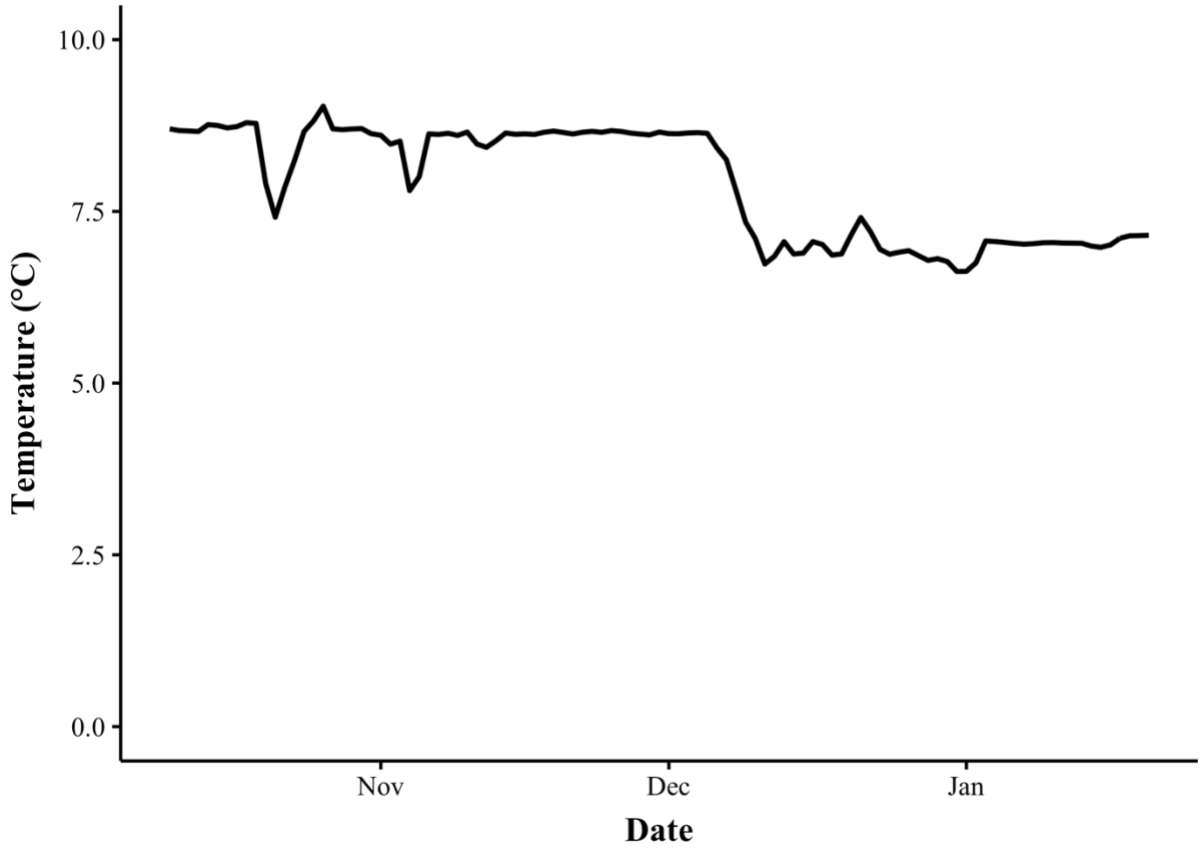


FIGURE A.8. Temperature changes in the Heath trays used to incubate lake trout eggs throughout the study period. Temperatures were recorded with a HOBO temperature logger, every 15 minutes. Daily temperatures were averaged to reduce noise in the data.

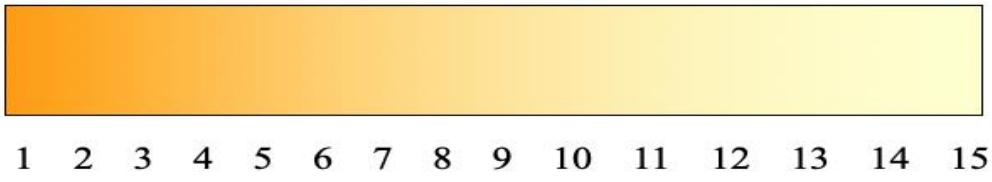


FIGURE A.9. Gradient used to estimate colour in lake trout eggs colour.

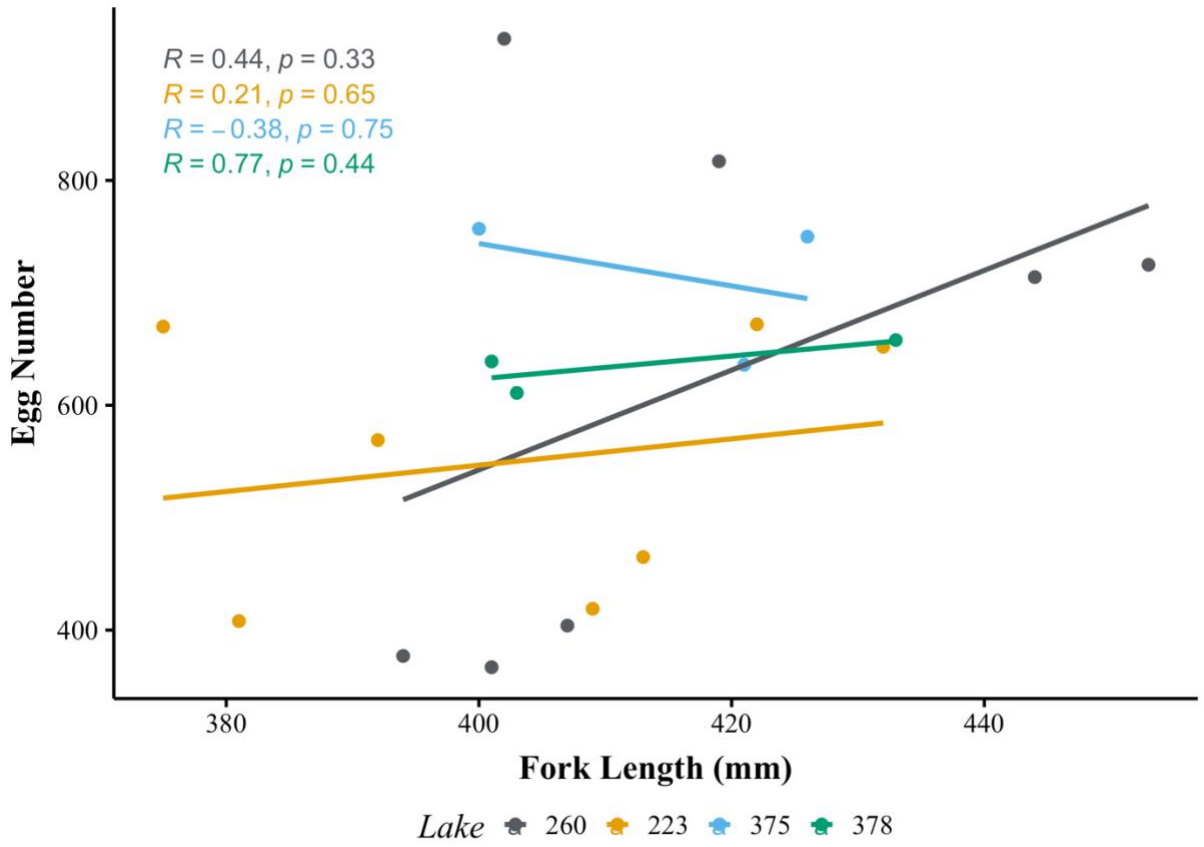


FIGURE A.10. Relationship between fork length of adult female lake trout and the number of eggs expressed from each fish for each of the study lakes. Pearson correlation and associated p-values for each lake are included in the matching colour to their corresponding lake ($\alpha = 0.05$).

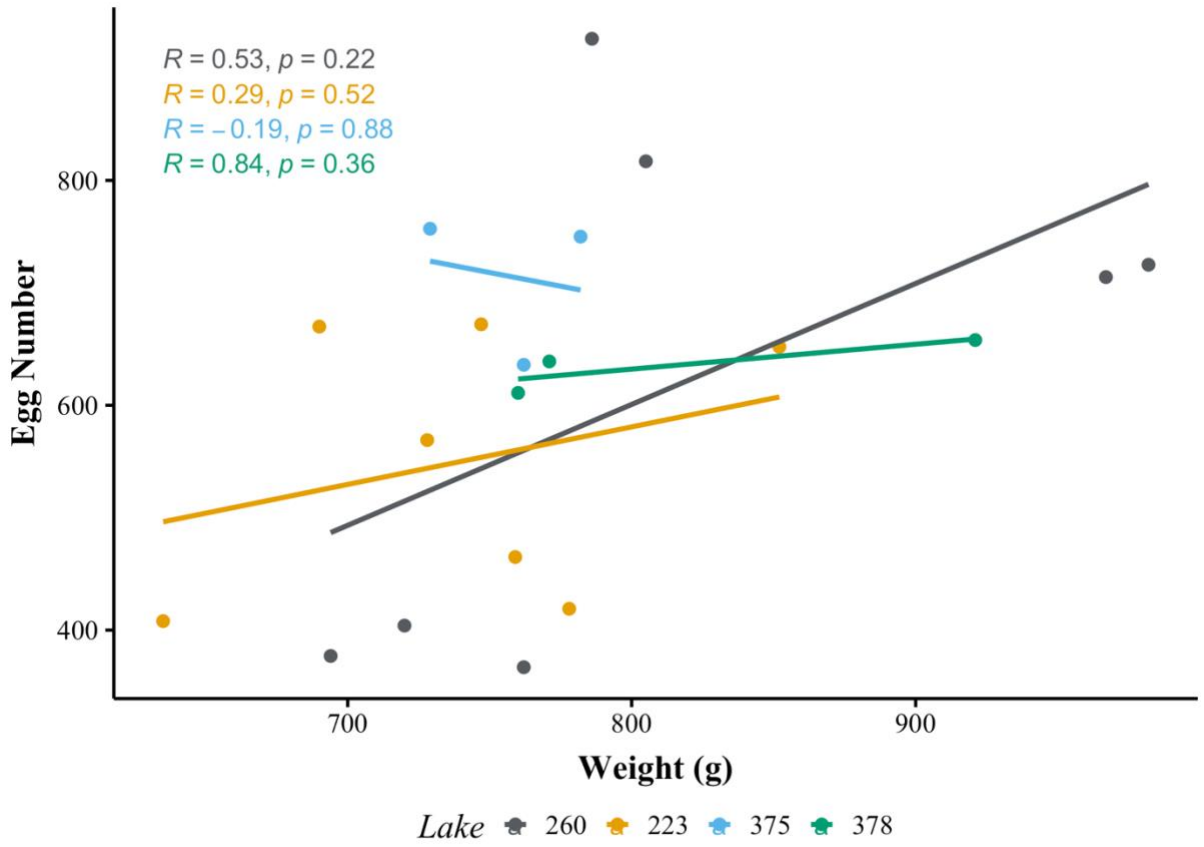


FIGURE A.11. Relationship between weight of the adult female lake trout and the number of expressed eggs from each fish for each of the study lakes. Pearson correlation and associated p-values for each lake are included in the matching colour to their corresponding lake ($\alpha = 0.05$).

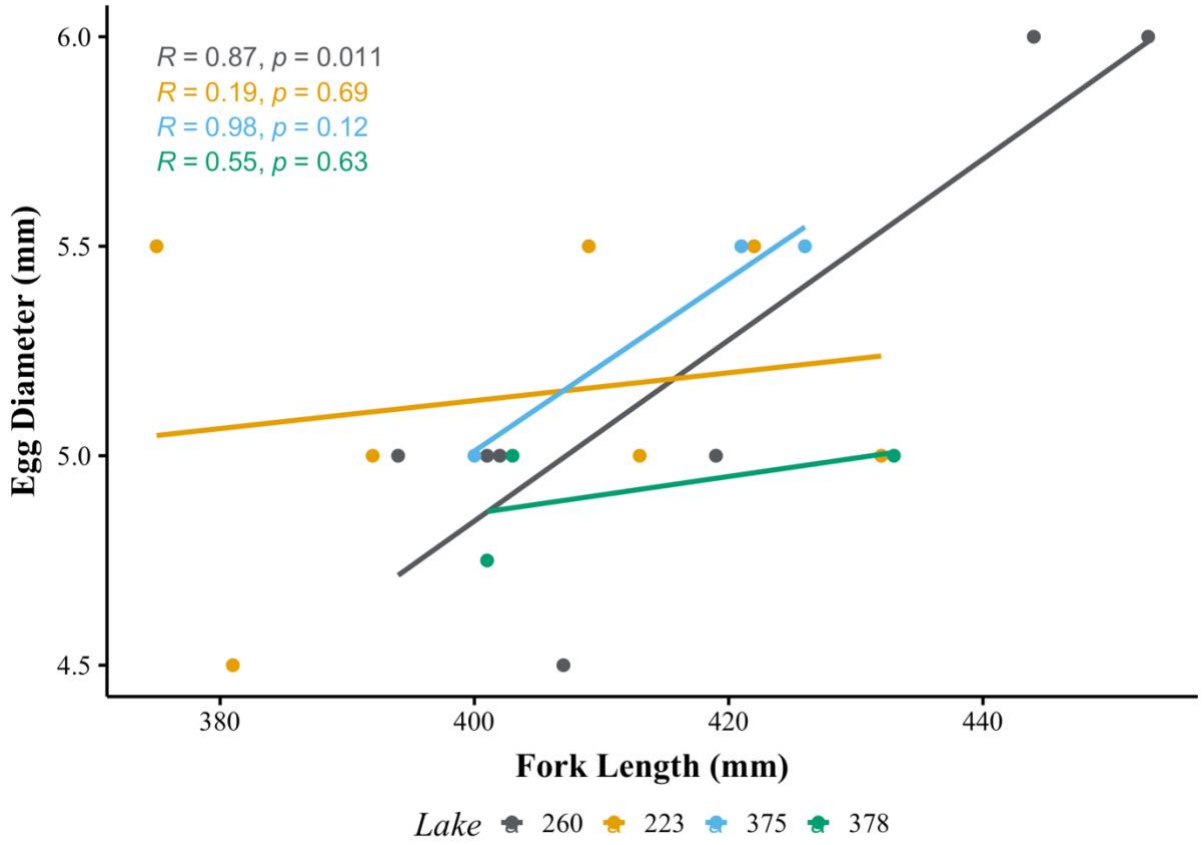


FIGURE A.12. Relationship between fork length of the adult female lake trout and egg diameter for each of the study lakes. Pearson correlation and associated p-values for each lake are included in the matching colour to their corresponding lake ($\alpha = 0.05$).

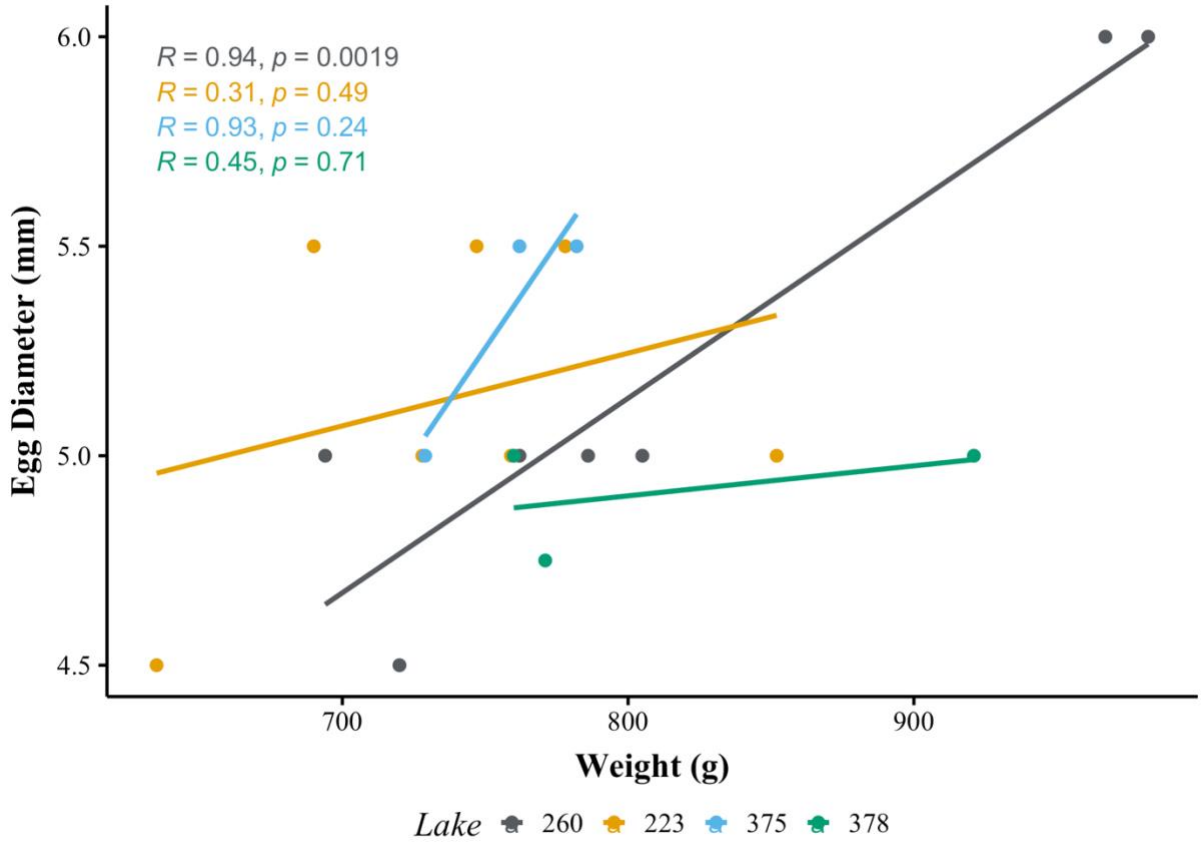


FIGURE A.13. Relationship between weight in the adult female lake trout and egg diameter for each of the study lakes. Pearson correlation and associated p-values for each lake are included in the matching colour to their corresponding lake ($\alpha = 0.05$).

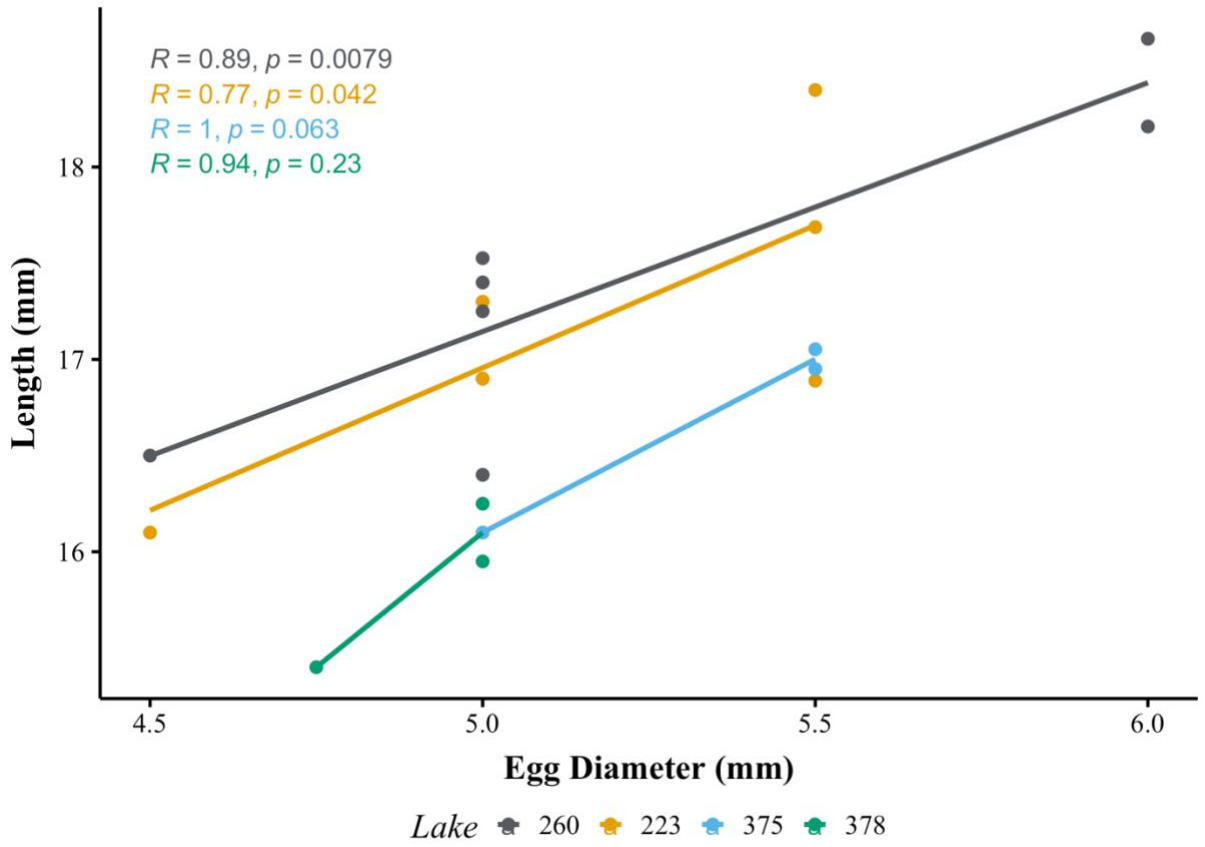


FIGURE A.14. Relationship between mean length in yolk-sac fry (~550 ATU) and egg diameter for each of the study lakes. Pearson correlation and associated p-values for each lake are included in the matching colour to their corresponding lake ($\alpha = 0.05$).

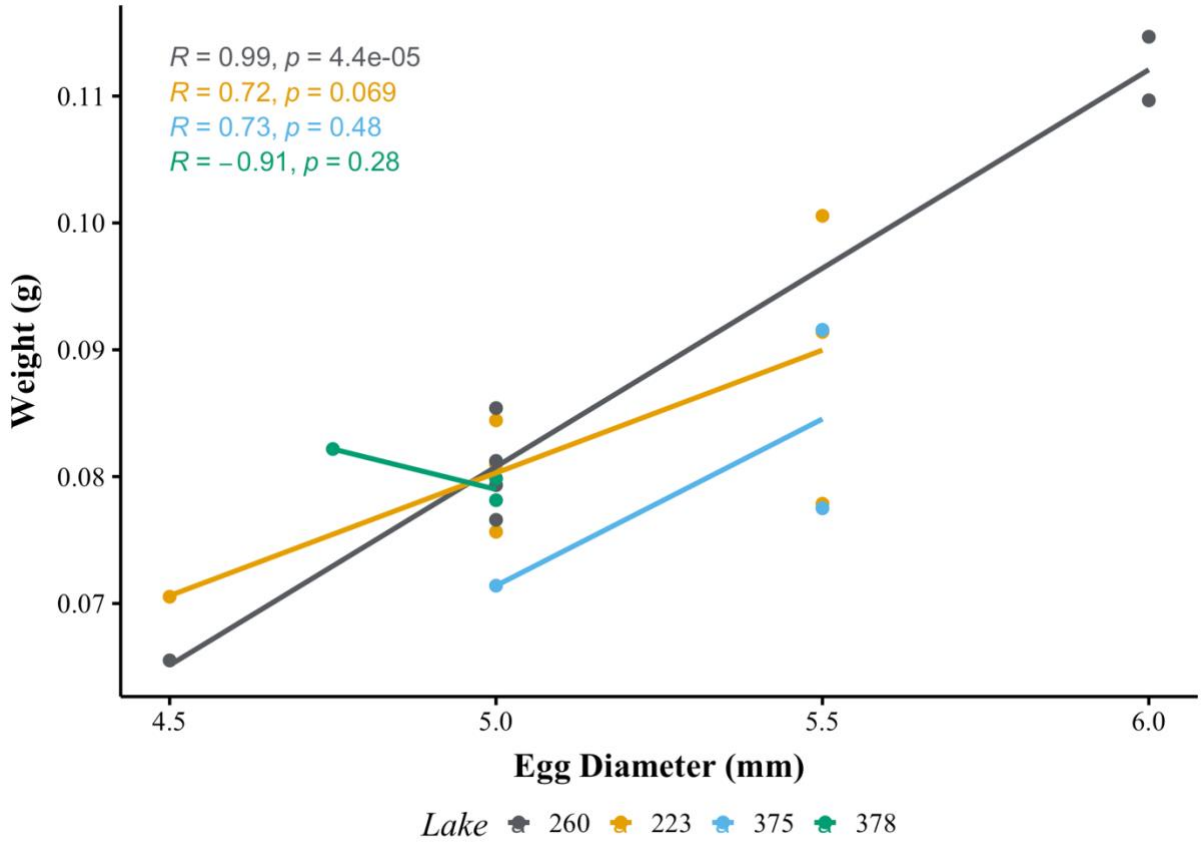


FIGURE A.15. Relationship between mean weight in yolk-sac fry (~550 ATU) and egg diameter in each of the study lakes. Pearson correlation and associated p-values for each lake are included in the matching colour to their corresponding lake (α = 0.05).

]

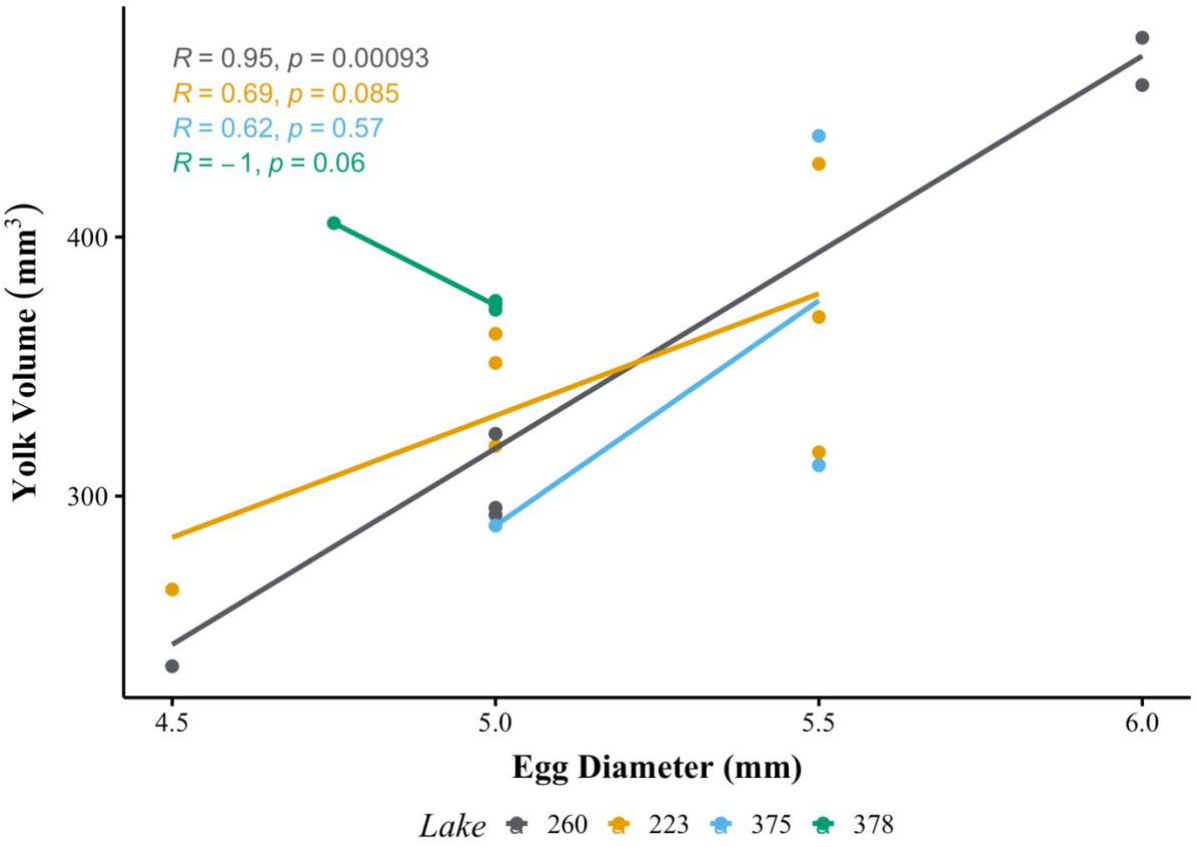


FIGURE A.16. Relationship between mean yolk volume in swim-up fry (~550 ATU) and egg diameter in each of the study lakes. Pearson correlation and associated p-values for each lake are included in the matching colour to their corresponding lake (α = 0.05).

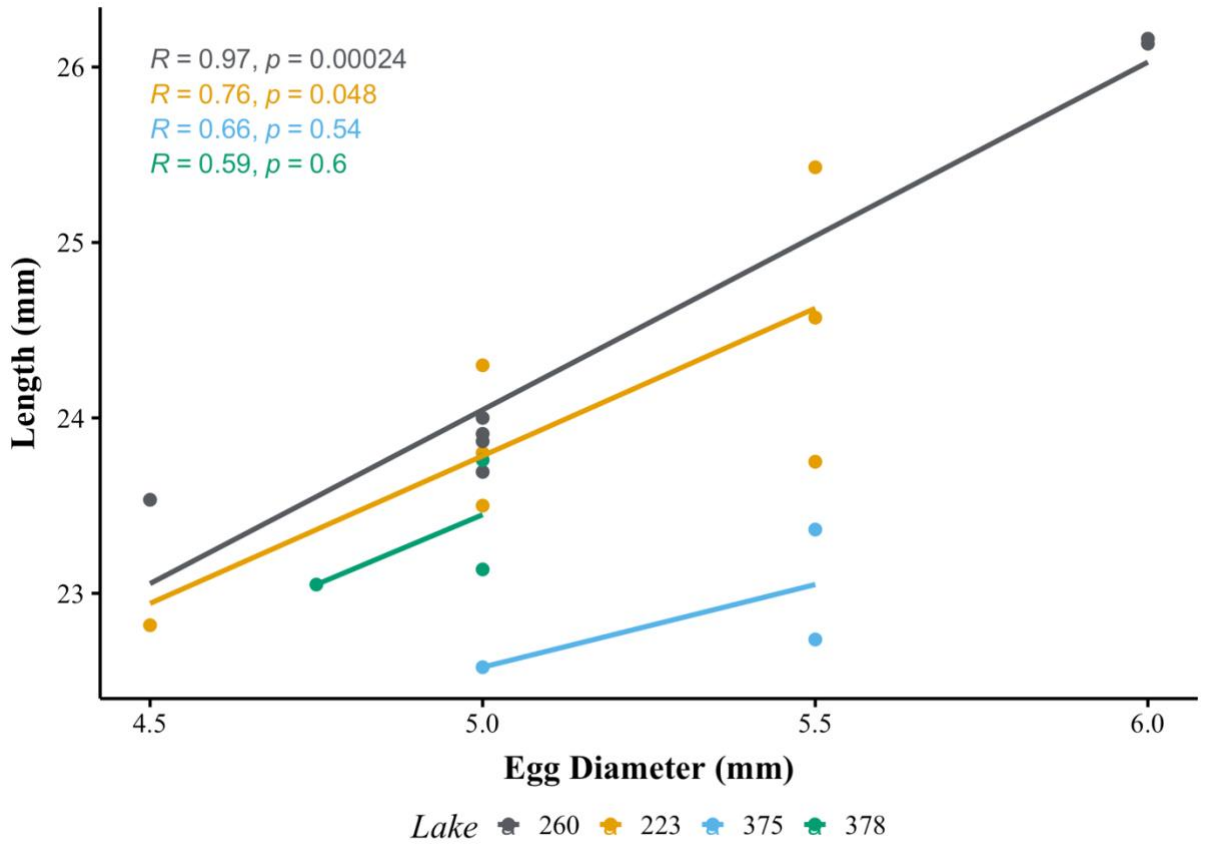


FIGURE A.17. Relationship between mean length in swim-up fry (~800 ATU) and egg diameter in each of the study lakes. Pearson correlation and associated p-values for each lake are included in the matching colour to their corresponding lake ($\alpha = 0.05$).

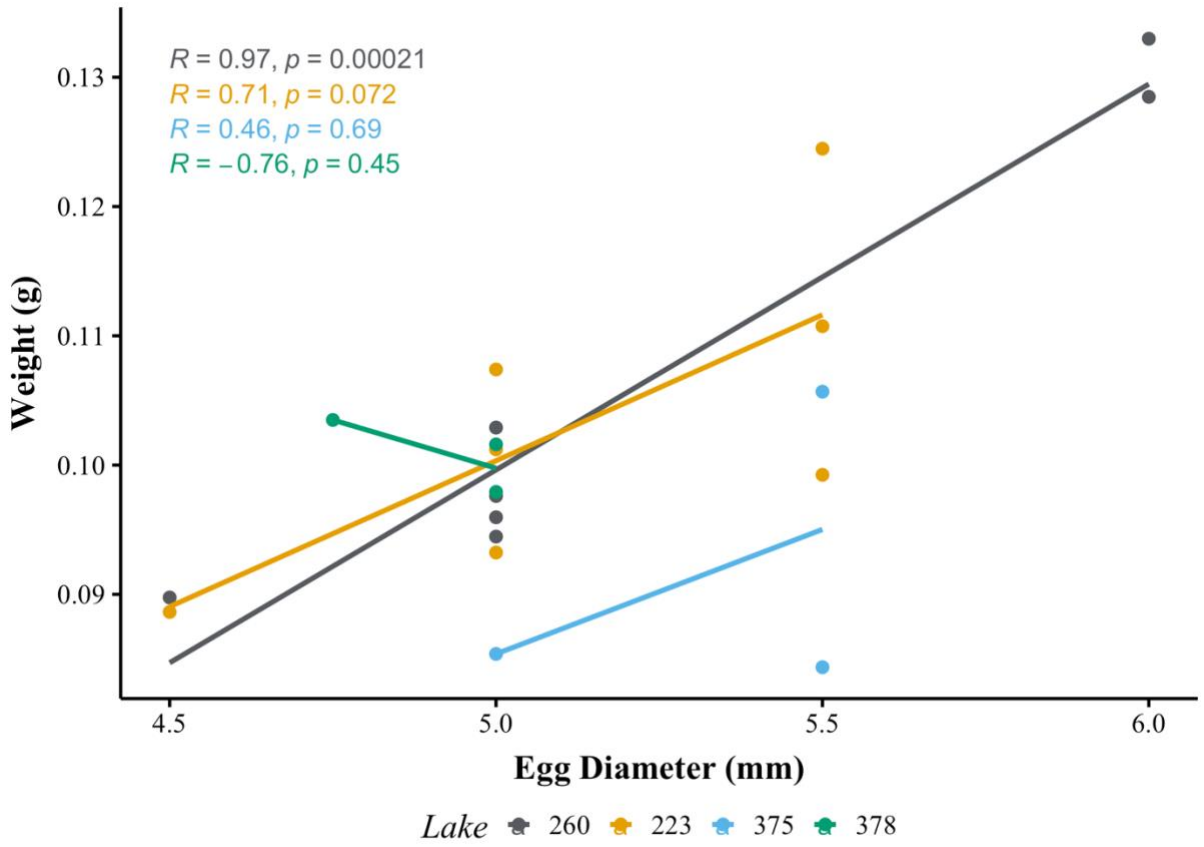


FIGURE A.18. Relationship between mean weight in swim-up fry (~800 ATU) and egg diameter in each of the study lakes. Pearson correlation and associated p-values for each lake are included in the matching colour to their corresponding lake (α = 0.05).

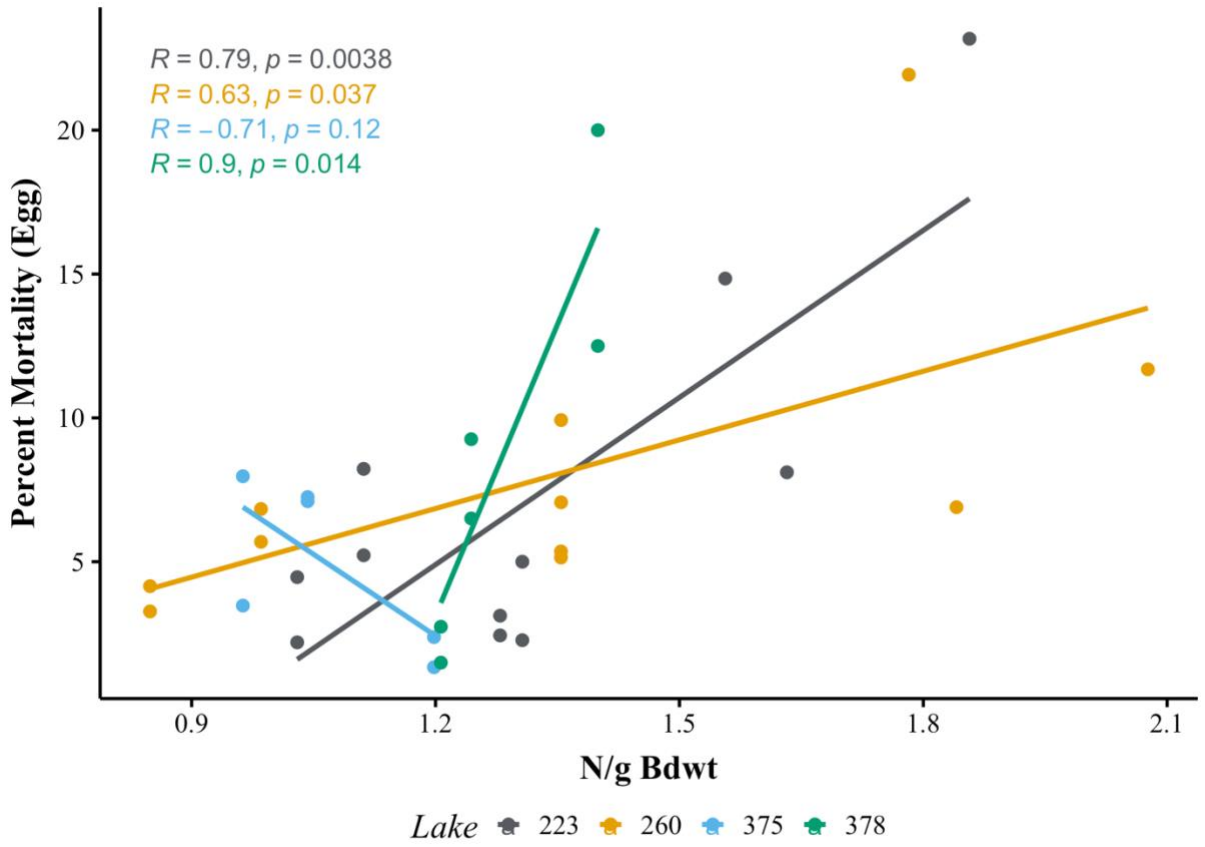


FIGURE A.19. Relationship between egg number per gram weight in the adult female lake trout and percent mortality in the eggs in each of the study lakes. Pearson correlation and associated p-values for each lake are included in the matching colour to their corresponding lake ($\alpha = 0.05$).

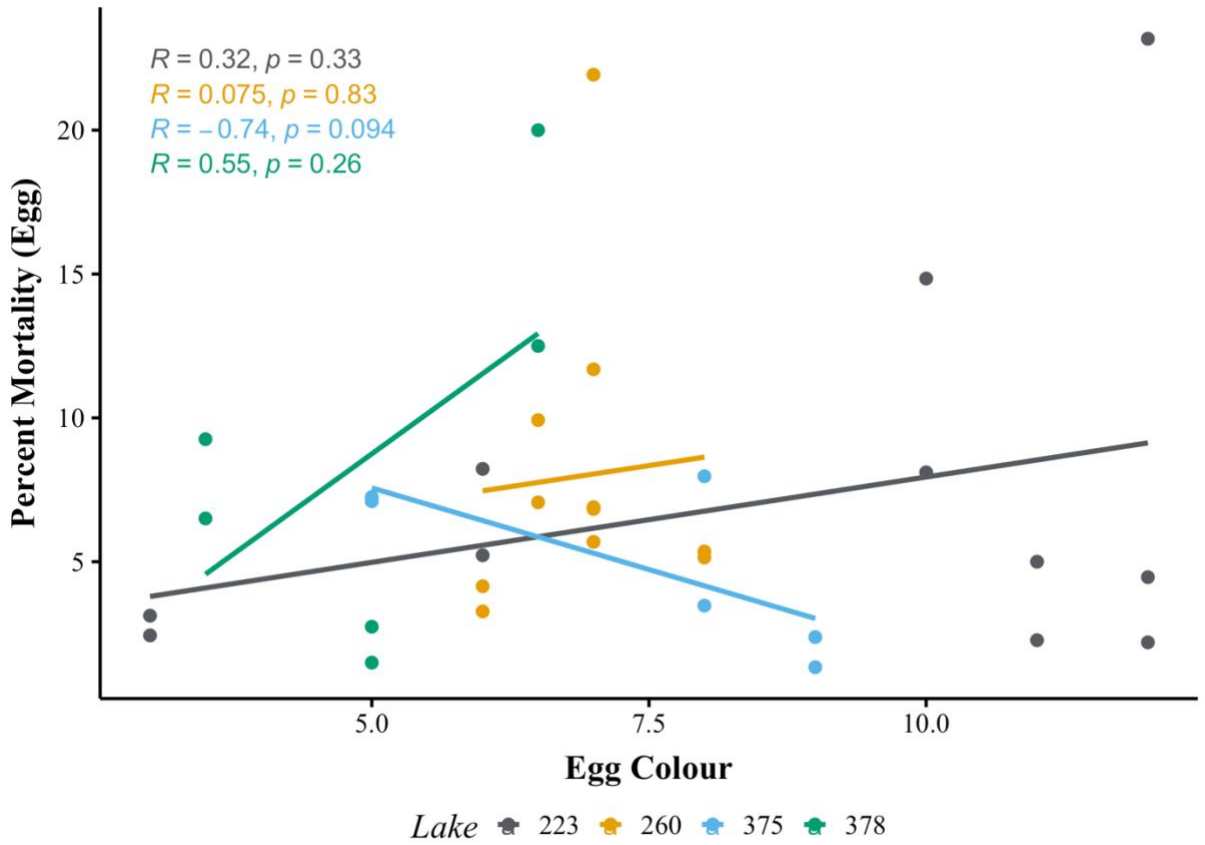


FIGURE A.20. Relationship between egg colour in the adult female lake trout and percent mortality in the eggs. Pearson correlation and associated p-values for each lake are included in the matching colour to their corresponding lake (α = 0.05).

Table A.1. Adult fish capture and egg deployment dates for the in-situ and laboratory sites.

	Adult fish capture date	Egg deployment date
***In-situ* study**		
Lake 260	9-Oct-19	9-Oct-19
Lake 223 LT# 1-3	10-Oct-19	11-Oct-19
Lake 223 LT# 4-7	14-Oct-19	14-Oct-19
Lake 378	11-Oct-19	11-Oct-19
Lake 375	15-Oct-19	16-Oct-19
Laboratory study		
Lake 260	9-Oct-19	10-Oct-19
Lake 223 LT# 1-3	10-Oct-19	11-Oct-19
Lake 223 LT# 4-7	14-Oct-19	15-Oct-19
Lake 378	11-Oct-19	12-Oct-19
Lake 375	15-Oct-19	17-Oct-19

APPENDIX B: DEFORMITY RUBRIC

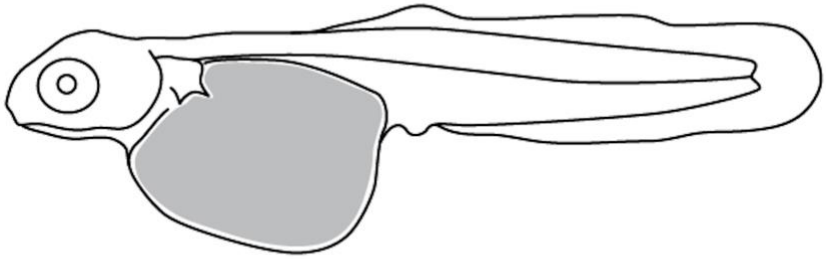
Lake Trout Alevin Deformity Scoring Rubric (~550 TU)

Adapted from: Holm et al., (2003); Rudolph (2006); Covington et al., (2018)

Reference fish



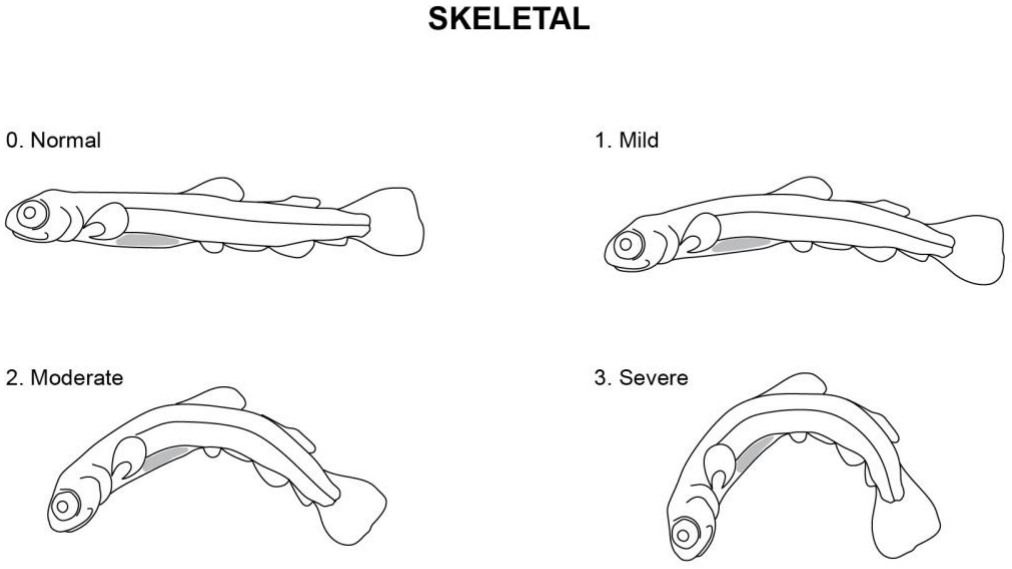
Reference fish (sketch)



Skeletal

Spinal Curves are a deformation which causes a bend in the spine

- 3 types of spinal curves:
  - **Kyphosis:** downward bend of the spine
  - **Lordosis:** upward bend of the spine
  - **Scoliosis:** sideways bend in the spine (will only be evaluated in extreme cases where a deformity is obvious)
- 0 – no curve present, the fish appears straight (<15 degrees)
- 1 – **mild** bend in the spine (15-45 degrees)
- 2 – **moderate** bend in spine (46-90 degrees)
- 3 – **severe** bend in spine (>90 degrees)
- NA – moderate side bends (scoliosis) may have occurred from storage in falcon tubes after fixation



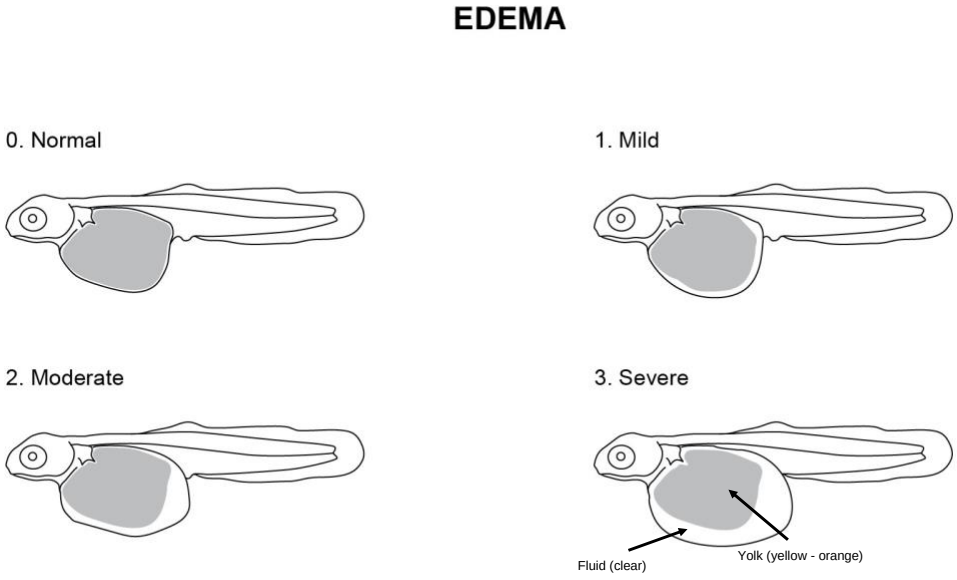
Note: Severity of curvatures in other planes (lordosis, scoliosis) scored using the same criteria.

Edema

Edema is an accumulation of fluid that appears as localized swelling and is characterized by the location of fluid buildup.

- **Yolk Sac Edema:** swelling of coelomic cavity with **clear fluid**

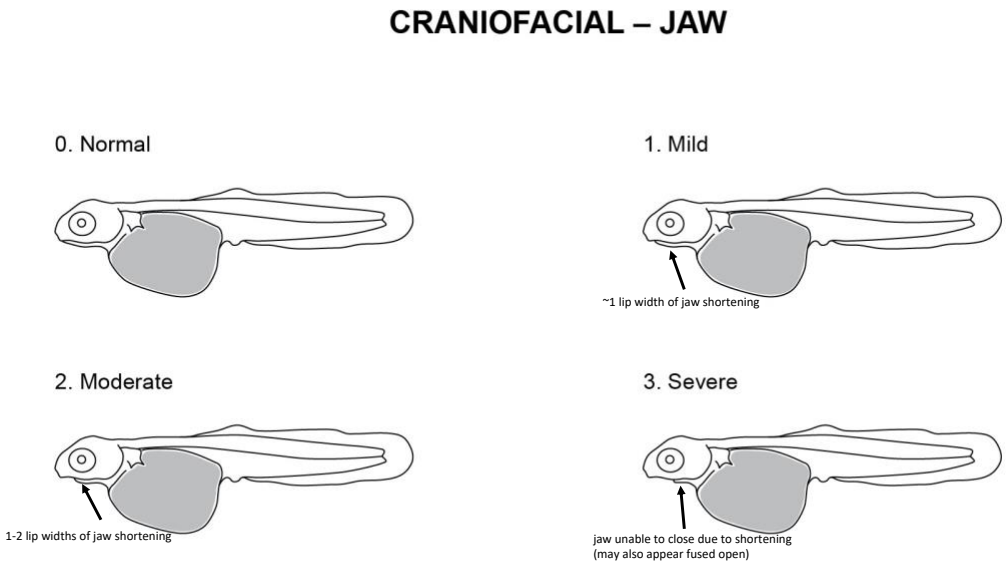
- 0 – no fluid accumulation present
- 1 – mild fluid accumulation (<20% volume relative to yolk)
- 2 – moderate fluid accumulation (20-49% volume relative to yolk)
- 3 – severe fluid accumulation (>50% volume relative to yolk)
- NA – ventral surface is damaged



Note: On the ventral surface of the sketches (i.e. coelomic cavity), grey represents yellow-orange yolk, white represents clear fluid.

Craniofacial

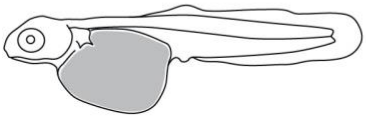
- Craniofacial deformities include abnormalities in the size and functionality of the jaw and eyes
- 0 – normal jaw and eyes
- 1 – mild shortening of jaw (~1 lip width) or eye poorly pigmented
- 2 – moderate shortening of jaw (>1-2 lip widths) or a slightly disfigured jaw or eye poorly developed (small or misshapen)
- 3 – non-functional jaw (e.g. jaw not able to close) or non-functional/missing eyes
- NA – snout, jaw or eyes are obscured, at a poor angle or otherwise damaged



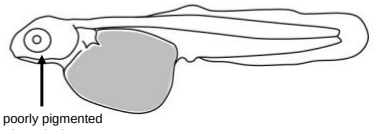
Note: 1 lip width determined to be approximately 1/5 of the length of a normal jaw. Jaw length measured from anterior edge to region where upper and lower jaw meet (roughly parallel to posterior end of eye)

CRANIOFACIAL – EYE

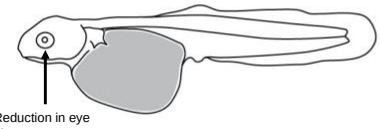
0. Normal



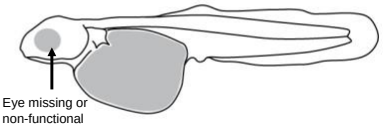
1. Mild



2. Moderate



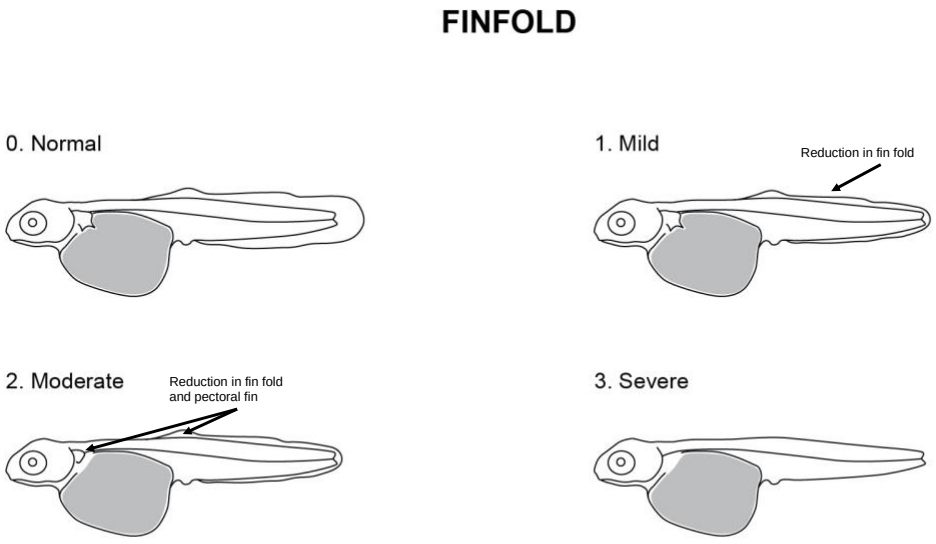
3. Severe



Finfold

- Finfold deformities can manifest as the absence of certain fins, or reduction in their size.

- 0 – all fins present and normal in size
- 1 – 1 fin underdeveloped (either pectoral or fin fold)
- 2 – all fins underdeveloped
- 3 – fins absent
- NA – fin appears folded or is otherwise damaged



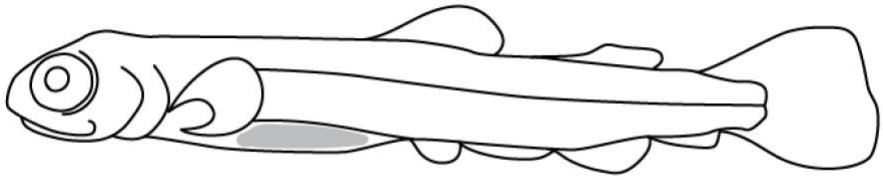
Lake Trout Swim-up Fry Deformity Scoring Rubric (~800 TU)

Adapted from: Holm et al., (2003); Rudolph (2006); Covington et al., (2018)

Reference fish



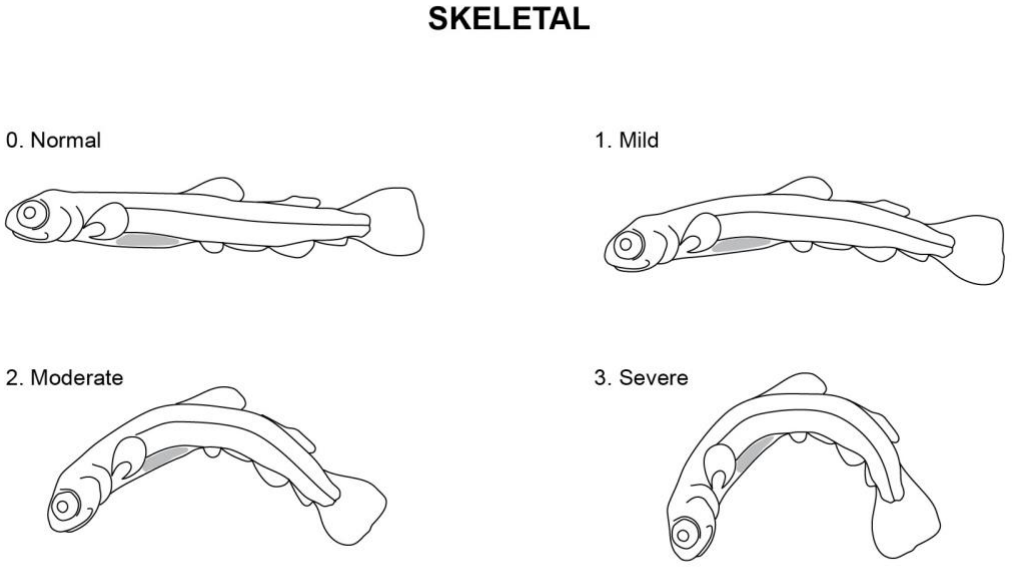
Reference fish (sketch)



Skeletal

Spinal Curves are a deformation which causes a bend in the spine

- 3 types of spinal curves (mutually exclusive for the purposes of this rubric):
  - **Kyphosis:** downward bend of the spine
  - **Lordosis:** upward bend of the spine
  - **Scoliosis:** sideways bend in the spine (will only be evaluated in extreme cases as small bends could have occurred from fixation)
- 0 – no curve present, the fish appears straight (<15 degrees)
- 1 – **mild** bend in the spine (15-45 degrees)
- 2 – **moderate** bend in spine (46-90 degrees)
- 3 – **severe** bend in spine (>90 degrees)
- NA – moderate side bends (scoliosis) may have occurred from storage in falcon tubes after fixation.



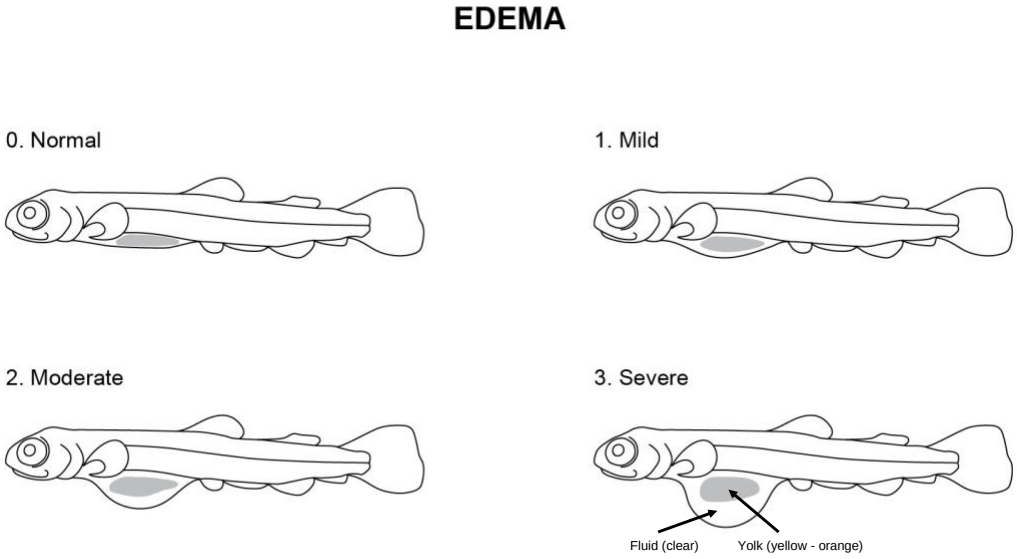
Note: Similar degree range of bends in different orientations (kyphosis, scoliosis) scored the same.

Edema

Edema is an accumulation of fluid that appears as localized swelling and is characterized by the location of fluid buildup.

- **Yolk Sac Edema:** swelling of coelomic cavity with **clear fluid**

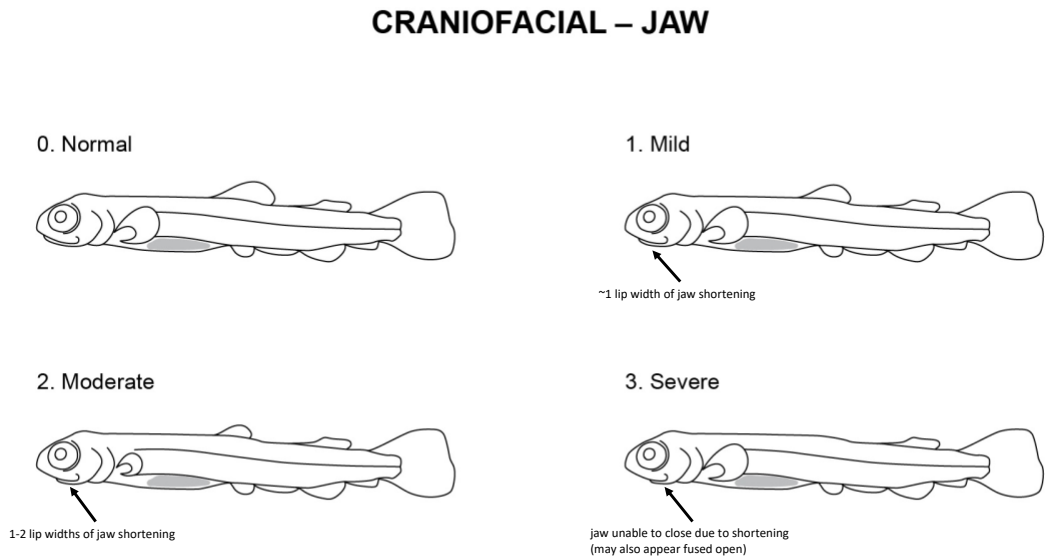
- 0 – no fluid accumulation present
- 1 – mild fluid accumulation (<20% volume relative to yolk)
- 2 – moderate fluid accumulation (20-49% volume relative to yolk)
- 3 – severe fluid accumulation (>50% volume relative to yolk)
- NA – ventral surface is damaged



Note: On the ventral surface of the sketches (i.e. coelomic cavity), grey represents yellow-orange yolk, white represents clear fluid.

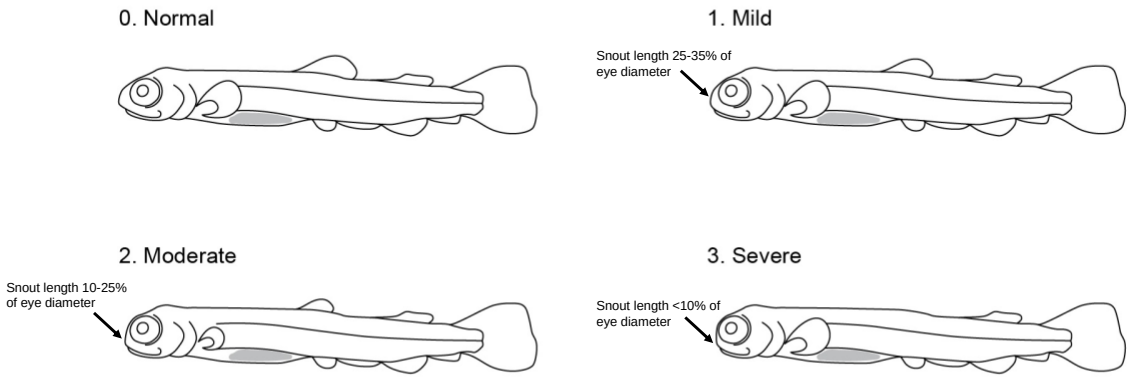
Craniofacial

- Craniofacial deformities include abnormalities in the size and functionality of the jaw, snout and eyes:
- 0 – normal jaw, snout and eyes
- 1 – mild shortening of jaw (<= 1 lip width), slightly blunted snout (26-35% normal eye diameter) or eye poorly pigmented
- 2 – moderate shortening of jaw (>1-2 lip widths) or a slightly disfigured jaw, very blunt snout (11-25% normal eye diameter) or eye poorly developed (small or misshapen)
- 3 – non-functional jaw (i.e. jaw not able to close), flat snout (<10% normal eye diameter), or non-functional/missing eyes
- NA – snout, jaw or eyes are obscured, at a poor angle or otherwise damaged



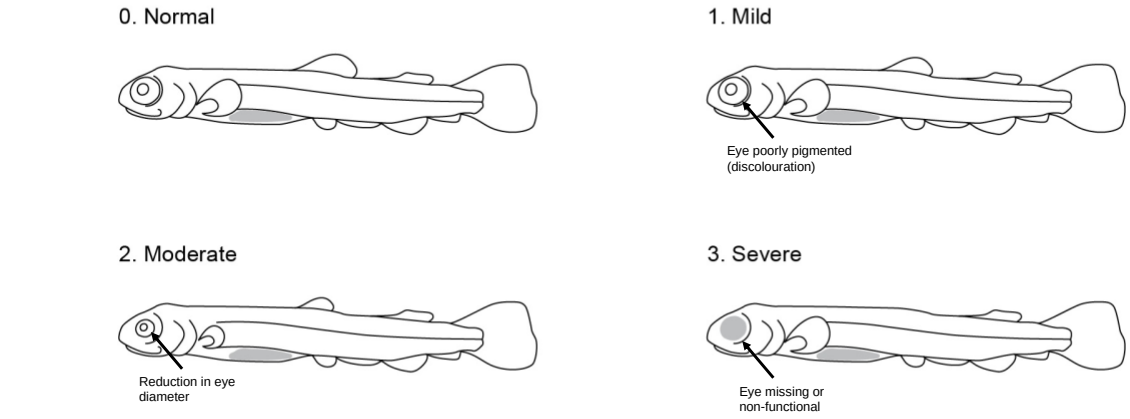
Note: 1 lip width determined to be approximately 1/5 of the length of a normal jaw. Jaw length measured from anterior edge to region where upper and lower jaw meet

CRANIOFACIAL – SNOUT



Note: If eye also reduced in size, then ratio of snout to mean eye diameter of all fish is used to determine score.

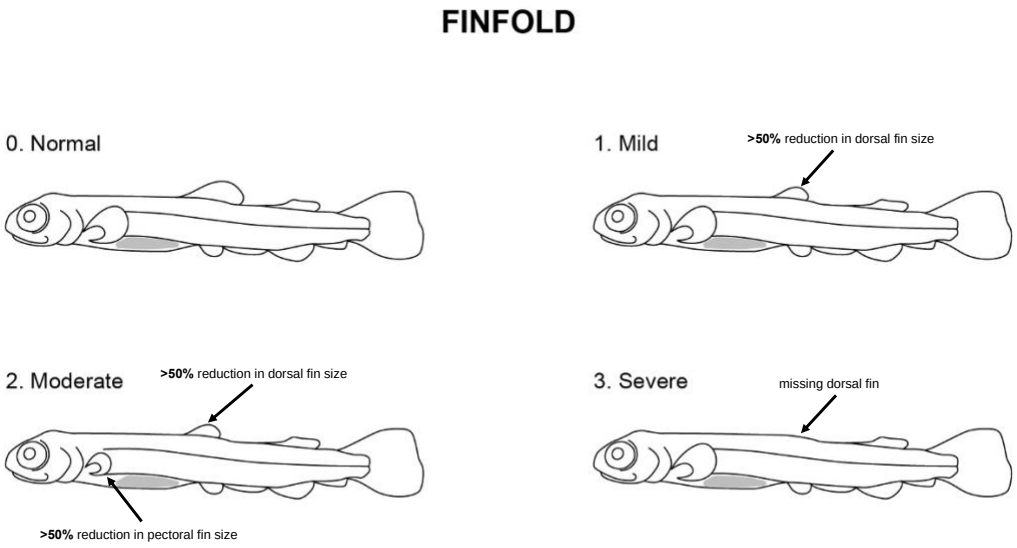
CRANIOFACIAL – EYE



Finfold

- Finfold deformities can manifest as the absence of certain fins, or reduction in their size.

- 0 – all fins present and normal in size
- 1 – 1 fin reduced in size (>50% reduction from normal)
- 2 – 2 fins reduced in size (>50% reduction from normal)
- 3 – 1 or more missing fins OR >2 fins reduced in size (>50% reduction)
- NA – fin appears folded or is otherwise damaged



Note: similar percent reduction or presence/absence in caudal fin, dorsal fin and pectoral fin scored the same. Pelvic, adipose, anal fins only scored if obviously missing (i.e. not a fixation artefact) = finfold score of 3.

APPENDIX C: STATISTICAL ANALYSIS

Condition Factor (K) – Adult Females

ANCOVA Table

Effect	DF (numerator)	DF (denominator)	*F*-value	*p*-value
Fork Length	1	15	74.62	< 0.001
Lake Origin	3	15	2.34	0.12

Weight (g) – Adult Females

Fixed Effects (Full Model):

	Coef.	Std. Error	*t*-value	*p*-value
Intercept (Lake 260)	816.57	33.28	24.54	0.0000
Lake 223	-75.29	47.07	-1.60	0.13
Lake 375	-58.90	60.76	-0.97	0.35
Lake 378	0.76	60.76	0.013	0.99

Model Fit:

DF (numerator)	DF (denominator)	*F*-value	*p*-value
3	16	1.10	0.38

Fork Length (mm) – Adult Females

Fixed Effects (Full Model):

	Coef.	Std. Error	*t*-value	*p*-value
Intercept (Lake 260)	417.14	7.84	53.192	0.0000
Lake 223	-13.71	11.09	-1.24	0.23
Lake 375	-1.48	14.32	-0.10	0.92
Lake 378	-4.81	14.32	-0.34	0.74

Model Fit:

DF (numerator)	DF (denominator)	*F*-value	*p*-value
3	16	0.57	0.64

Egg Diameter (mm)

Fixed Effects (Full Model):

	Coef.	Std. Error	*t*-value	*p*-value
Intercept (Lake 260)	5.21	0.16	31.89	0.0000
Lake 223	-0.07	0.23	-0.31	0.76
Lake 375	-0.12	0.30	-0.40	0.70
Lake 378	-0.30	0.30	-0.99	0.33

Model Fit:

DF (numerator)	DF (denominator)	*F*-value	*p*-value
3	16	0.52	0.68

Egg Number (N) / Female Body Weight (g)

Fixed Effects (Full Model):

	Coef.	Std. Error	*t*-value	*p*-value
Intercept (Lake 260)	0.75	0.07	10.43	0.0000
Lake 223	-0.006	0.10	-0.06	0.95
Lake 375	0.19	0.13	1.47	0.16
Lake 378	0.03	0.13	0.24	0.81

Model Fit:

DF (numerator)	DF (denominator)	*F*-value	*p*-value
3	16	0.57	0.64

Egg Colour

Kruskall-Wallis:

Effect	ChiSq	DF	*p*-value
Lake Origin	5.59	3	0.13

Condition Factor (K) – Adult Males

ANCOVA Table

Effect	DF (numerator)	DF (denominator)	*F*-value	*p*-value
Fork Length	1	16	49.24	< 0.001
Lake Origin	3	16	0.91	0.46

Weight (g) – Adult Males

Fixed Effects (Full Model):

	Coef.	Std. Error	*t*-value	*p*-value
Intercept (Lake 260)	713.00	29.82	23.91	0.0000
Lake 223	41.00	43.65	0.94	0.36
Lake 375	-40.25	51.65	-0.78	0.45
Lake 378	-11.00	66.68	-0.17	0.87

Model Fit:

DF (numerator)	DF (denominator)	*F*-value	*p*-value
3	17	0.84	0.49

Fork Length (mm) – Adult Males

Fixed Effects (Full Model):

	Coef.	Std. Error	*t*-value	*p*-value
Intercept (Lake 260)	401.50	6.68	60.11	0.0000
Lake 223	18.92	9.77	1.94	0.07
Lake 375	-3.00	11.57	-0.26	0.80
Lake 378	-5.00	14.94	-0.34	0.74

Model Fit:

DF (numerator)	DF (denominator)	*F*-value	*p*-value
3	16	1.88	0.17

Condition Factor (K) – Yolk-sac Fry (550 ATU)

Random Effects (Full Model):

	Intercept	Residual
Standard Deviation:	0.11	0.18

Fixed Effects (Full Model):

	Coef.	Std. Error	DF	*t*-value	*p*-value
Intercept (Lake 260)	1.61	0.05	283	35.32	0.0000
Lake 223	0.03	0.06	16	0.52	0.61
Lake 375	0.12	0.08	16	1.45	0.17
Lake 378	0.41	0.08	16	5.02	0.0001

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	3	69.59			
Full Model	6	78.37	3	17.56	0.0005

Length (mm) – Yolk-sac Fry (550 ATU)

Random Effects (Full Model):

	Intercept	Residual
Standard Deviation:	0.60	0.57

Fixed Effects (Full Model):

	Coef.	Std. Error	DF	*t*-value	*p*-value
Intercept (Lake 260)	17.44	0.24	294	73.27	0.0000
Lake 223	-0.29	0.34	16	-0.86	0.40
Lake 375	-0.74	0.43	16	-1.73	0.10
Lake 378	-1.57	0.43	16	-3.65	0.002

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	3	-300.83			
Full Model	6	-295.43	3	10.83	0.013

Weight (g) – Yolk-sac Fry (550 ATU)

Random Effects (Full Model):

	Intercept	Residual
Standard Deviation:	0.01	0.005

Fixed Effects (Full Model):

	Coef.	Std. Error	DF	*t*-value	*p*-value
Intercept (Lake 260)	0.088	0.004	288	19.48	0.0000
Lake 223	-0.004	0.006	16	-0.71	0.49
Lake 375	-0.007	0.008	16	-0.90	0.38
Lake 378	-0.007	0.008	16	-0.91	0.37

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	3	1140.2			
Full Model	6	1140.8	3	1.28	0.73

Yolk Volume (mm^3) – Yolk-sac Fry (550 ATU)

Random Effects (Full Model):

	Intercept	Residual
Standard Deviation:	60.85	50.14

Fixed Effects (Full Model):

	Coef.	Std. Error	DF	*t*-value	*p*-value
Intercept (Lake 260)	351.15	23.92	267	14.68	0.0000
Lake 223	-7.18	33.79	16	-0.21	0.83
Lake 375	-4.74	43.25	16	-0.11	0.91
Lake 378	33.02	43.30	16	-0.76	0.45

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	3	-1561.5			
Full Model	6	-1561.0	3	0.92	0.82

Condition Factor (K) – Swim-up Fry (800 ATU)

Random Effects (Full Model):

	Intercept	Residual
Standard Deviation:	0.03	0.06

Fixed Effects (Full Model):

	Coef.	Std. Error	DF	*t*-value	*p*-value
Intercept (Lake 260)	0.72	0.01	364	66.59	0.0000
Lake 223	0.03	0.02	16	1.79	0.09
Lake 375	0.05	0.02	16	2.37	0.03
Lake 378	0.08	0.02	16	4.11	0.0008

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	3	523.09			
Full Model	6	529.63	3	13.08	0.004

Length (mm) – Swim-up Fry (800 ATU)

Random Effects (Full Model):

	Intercept	Residual
Standard Deviation:	0.78	0.69

Fixed Effects (Full Model):

	Coef.	Std. Error	DF	*t*-value	*p*-value
Intercept (Lake 260)	24.48	0.30	364	80.77	0.0000
Lake 223	-0.46	0.43	16	-1.06	0.30
Lake 375	-1.59	0.55	16	-2.87	0.01
Lake 378	-1.16	0.55	16	-2.11	0.05

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	3	-436.01			
Full Model	6	-431.89	3	8.231	0.04

Weight (g) – Swim-up Fry (800 ATU)

Random Effects (Full Model):

	Intercept	Residual
Standard Deviation:	0.01	0.008

Fixed Effects (Full Model):

	Coef.	Std. Error	DF	*t*-value	*p*-value
Intercept (Lake 260)	0.11	0.005	365	23.00	0.0000
Lake 223	-0.003	0.007	16	-0.39	0.70
Lake 375	-0.01	0.008	16	-1.69	0.11
Lake 378	-0.005	0.008	16	-0.61	0.55

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	3	1254.9			
Full Model	6	1256.3	3	2.82	0.42

Hatching Success – *In-situ*

Random Effects (Full Model):

	Variance	Std. Dev.
Intercept:	1.008	1.004

Fixed Effects (Full Model):

	Coef.	Std. Error	*z*-value	*p*-value
Intercept (Lake 260)	1.23	0.39	3.13	0.002
Lake 223	0.54	0.56	0.97	0.33
Lake 375	-1.60	0.73	-2.20	0.03
Lake 378	0.32	0.72	0.45	0.65

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	2	-83.57			
Full Model	5	-79.70	3	7.73	0.05

Hatching Success – *In-situ* (outlier L375 LT2 removed, reference lakes pooled)

Random Effects (Full Model):

	Variance	Std. Dev.
Intercept:	1.008	1.004

Fixed Effects (Full Model):

	Coef.	Std. Error	*z*-value	*p*-value
Intercept (Lake 260)	1.23	0.36	3.45	< 0.001
Reference Lakes	0.28	0.45	0.62	0.53

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	2	-75.62			
Full Model	3	-75.43	1	0.38	0.54

Percent Unfertilized

Random Effects (Full Model):

	Variance	Std. Dev.
Intercept:	0.71	0.85

Fixed Effects (Full Model):

	Coef.	Std. Error	*z*-value	*p*-value
Intercept (Lake 260)	-2.49	0.34	-7.4	< 0.001
Lake 223	0.66	0.47	1.40	0.16
Lake 375	0.75	0.59	1.25	0.21
Lake 378	-0.61	0.62	-0.98	0.33

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	2	-117.83			
Full Model	5	-115.26	3	5.13	0.16

Percent Egg Mortality

Random Effects (Full Model):

	Variance	Std. Dev.
Intercept:	0.46	0.68

Fixed Effects (Full Model):

	Coef.	Std. Error	*z*-value	*p*-value
Intercept (Lake 260)	-2.48	0.28	-8.94	< 0.001
Lake 223	-0.08	0.39	-0.21	0.84
Lake 375	-0.64	0.51	-1.27	0.21
Lake 378	-0.16	0.51	-0.32	0.75

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	2	-103.99			
Full Model	5	-103.17	3	1.65	0.65

Percent Hatch Success (outlier L223 LT6 removed)

Random Effects (Full Model):

	Variance	Std. Dev.
Intercept:	0.28	0.53

Fixed Effects (Full Model):

	Coef.	Std. Error	*z*-value	*p*-value
Intercept (Lake 260)	1.61	0.22	7.49	< 0.001
Lake 223	0.06	0.32	0.18	0.86
Lake 375	-0.28	0.38	-0.73	0.47
Lake 378	-0.26	0.39	0.66	0.51

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	2	-118.32			
Full Model	5	-117.62	3	1.40	0.71

Total GSI Score – Yolk-sac Fry (550 ATU)

Fixed Effects (Full Model):

	Coef.	Std. Error	*z*-value	*p*-value
Lake 223	0.36	0.35	1.04	0.30
Lake 375	2.10	0.36	5.91	< 0.001
Lake 378	0.86	0.38	2.26	0.02

Threshold Coefficients, *j* (Full Model):

	Coef.	Std. Error	*z*-value
0 | 1	1.52	0.26	5.86
1 | 2	3.22	0.32	10.10
2 | ≥3	4.33	0.40	10.77

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	3	-280.31			
Full Model	6	-258.66	3	43.30	< 0.001

Total GSI Score – Swim-up Fry (800 ATU)

Fixed Effects (Full Model):

	Coef.	Std. Error	*z*-value	*p*-value
Lake 223	0.85	0.37	2.30	0.02
Lake 375	1.38	0.39	3.50	< 0.001
Lake 378	1.28	0.39	3.27	0.001

Threshold Coefficients, *j* (Full Model):

	Coef.	Std. Error	*z*-value
0 | 1	2.29	0.28	8.19
1 | 2	3.80	0.35	10.94
2 | ≥3	4.38	0.40	10.96

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	3	-240.02			
Full Model	6	-231.56	3	16.93	< 0.001

Total PACs in Unfertilized Eggs

Fixed Effects:

	Coef.	Std. Error	*t*-value	*p*-value
Intercept (Lake 260)	9.56	4.63	2.07	0.05
Lake 223	10.08	6.55	1.54	0.14
Lake 375	11.46	8.45	1.36	0.19
Lake 378	-1.16	8.45	-0.14	0.89

Model Fit:

DF (numerator)	DF (denominator)	*F*-value	*p*-value
3	16	1.32	0.30

Alkyl PACs in Unfertilized Eggs (log transformed, outlier L223 LT4 removed)

Fixed Effects:

	Coef.	Std. Error	*t*-value	*p*-value
Intercept (Lake 260)	0.62	0.14	4.54	0.00
Lake 223	0.28	0.20	1.37	0.19
Lake 375	0.39	0.25	1.56	0.14
Lake 378	-0.36	0.25	-1.44	0.87

Model Fit:

DF (numerator)	DF (denominator)	*F*-value	*p*-value
3	16	2.9	0.07

CYP1A expression (log transformed)

Random Effects (Full Model):

	Intercept	Residual
Standard Deviation:	0.33	0.22

ICC: 0.61

Fixed Effects (Full Model):

	Coef.	Std. Error	DF	*t*-value	*p*-value
Intercept (Lake 260)	-1.66	0.13	150	-13.01	0.0000
Lake 223	-0.23	0.18	16	-1.29	0.21
Lake 375	-0.12	0.23	16	-0.52	0.61
Lake 378	-0.29	0.23	16	-1.25	0.23

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	3	-14.99			
Full Model	6	-13.86	3	2.28	0.52

Ratio of oxidized to reduced glutathione (GSH/GSSG) (log transformed)

Random Effects (Full Model):

	Intercept	Residual
Standard Deviation:	0.12	0.13

ICC: 0.51

Fixed Effects (Full Model):

	Coef.	Std. Error	DF	*t*-value	*p*-value
Intercept (Lake 260)	1.14	0.06	72	-20.01	0.0000
Lake 223	-0.04	0.08	14	0.49	0.63
Lake 375	0.13	0.10	14	-1.29	0.22
Lake 378	-0.03	0.10	14	0.35	0.73

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	3	38.06			
Full Model	6	39.57	3	3.04	0.39

Total glutathione (TGSH)

Random Effects (Full Model):

	Intercept	Residual
Standard Deviation:	32.32	60.93

ICC: 0.44

Fixed Effects (Full Model):

	Coef.	Std. Error	DF	*t*-value	*p*-value
Intercept (Lake 260)	447.98	17.65	72	25.38	0.0000
Lake 223	-55.14	24.97	14	-2.21	0.04
Lake 375	48.25	30.58	14	1.58	0.14
Lake 378	5.05	30.58	14	0.17	0.87

Likelihood Ratio Test (Null vs. Full Model)

	DF	LogLik	DF diff.	Chisq	*p*-value
Null Model	3	-510.45			
Full Model	6	-505.48	3	9.93	0.02