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DELAYED EFFECTS OF SUB-LETHAL LAMPRICIDE, 3-TRIFLUOROMETHYL-4-NITROPHENOL (TFM), EXPOSURE ON THE METABOLIC PHYSIOLOGY OF JUVENILE LAKE STURGEON (ACIPENSER FULVESCENS)

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DELAYED EFFECTS OF SUB-LETHAL LAMPRICIDE, 3-TRIFLUOROMETHYL-4-NITROPHENOL (TFM), EXPOSURE ON THE METABOLIC PHYSIOLOGY OF JUVENILE LAKE STURGEON (ACIPENSER FULVESCENS)

BY

Matthew J. Symbal

THESIS

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Delayed effects of sub-lethal lampricide, 3-trifluoromethyl-4-nitrophenol (TFM), exposure on the metabolic physiology of juvenile lake sturgeon (*Acipenser fulvescens*)

This thesis by Matthew J. Symbal is recommended for approval by the student’s Thesis Committee and Department Head in the Department of Biology and by the Interim Director of Graduate Education

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ABSTRACT

DELAYED EFFECTS OF SUB-LETHAL LAMPRICIDE, 3-TRIFLUOROMETHYL-4-NITROPHENOL (TFM), EXPOSURE ON THE METABOLIC PHYSIOLOGY OF JUVENILE LAKE STURGEON (ACIPENSER FULVESCENS)

BY
Matthew J. Symbal

The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) can be lethal to juvenile lake sturgeon (Acipenser fulvescens) at high concentrations used to control invasive sea lamprey (Petromyzon marinus). Little is known about how TFM would affect juvenile lake sturgeon (age-0) at the sub-lethal concentrations encountered during routine lampricide treatments; specifically, its effects on growth, stress response, and metabolism. Age-0 lake sturgeon were exposed to TFM concentrations at levels 0.0x, 0.5x, and 1.0x the minimum lethal concentration (mlc) of TFM required to kill 99.9% of sea lamprey larvae during a routine stream treatment. The mlc was estimated based on pH and alkalinity of water in the lab and U.S. Fish and Wildlife Service TFM prediction charts. Sub-lethal exposure to TFM did not affect growth or stress response; however, metabolism was significantly reduced (p=0.048) for sturgeon exposed to the 1.0xmlc compared to controls fourteen days after exposure. Additionally, metabolism varied over time due to TFM exposure and was significantly higher (p=0.037) for fish exposed to the 1.0xmlc than controls for up to seven days after exposure. The results of this study suggest that sub-lethal TFM exposure causes prolonged variation in metabolism of juvenile lake sturgeon, which could negatively affect fitness and survival as they age. Future studies should focus on changes in mitochondrial dynamics and densities as a result of exposure to TFM in order to clarify the mechanism of effect.
DEDICATION

This thesis is dedicated to my wife, Kendra Kelley Symbal, and to my children Hunter James and Evelyn Grace Symbal.
ACKNOWLEDGEMENTS

Thank you, Dr. Jill Leonard, my advisor, for all the energy, guidance, and advice you provided throughout the course of this research. Your ability to challenge my thinking and to find ways to motivate me was greatly appreciated and did not go unnoticed. Thanks also go to my Thesis Committee members who have helped tremendously throughout the research process. Specifically, thank you Dr. Erich Ottem, for the stimulating ideas pertaining to mitochondrial dynamics which were greatly appreciated and Dr. Stephen Lantz, thank you for your guidance in conducting lab based research and for sharing your expertise in toxicology.

Thanks goes to the Michigan Department of Natural Resources for providing the lake sturgeon for this study and for granting permission that allowed the Northern Michigan University (NMU) Fish Lab to hold and conduct research with these fish. To the U.S. Fish and Wildlife Service (USFWS) Sea Lamprey Control Program, thank you for providing the use of a variety of equipment and donating the lampricide needed to complete this study. Additional funding support for this project was provided by the NMU Excellence in Education Scholarship which allowed for the purchase of laboratory reagents needed for this study.

Thank you to all those that assisted me in conducting my research, specifically; Allyssa Milam for assisting in what seemed like countless hours of respirometry measurements and fish care. Thanks Joe Susco for providing me with a crash course in sturgeon care and giving me my first batch of fish. Spencer Chicoine and Jake Bowman, thanks for the great discussions, ideas, and feedback you provided me as I completed my
Finally, thanks to the staff of the NMU Fish Lab who kept watch over my fish when I was not available.

Thank you to Shawn Nowicki and Lisa Walter, my USFWS Sea Lamprey Program supervisors, who instigated this challenge and supported me throughout its completion. Also thank you to all my co-workers for your patience, support, advice, and understanding as I completed this research; without my Sea Lamprey Control family this adventure would never have started.

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This thesis is written following the format used in the Canadian Journal of Fisheries and Aquatic Sciences (www.nrcresearchpress.com/page/cjafs/authors).
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### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide m-chlorophenyl hydrazine</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>LC₅₀</td>
<td>lethal concentration yielding 50% mortality</td>
</tr>
<tr>
<td>mlc</td>
<td>minimum lethal concentration</td>
</tr>
<tr>
<td>MO₂</td>
<td>metabolic rate</td>
</tr>
<tr>
<td>MS-222</td>
<td>tricaine methanesulfonate</td>
</tr>
<tr>
<td>NMU</td>
<td>Northern Michigan University</td>
</tr>
<tr>
<td>SMR</td>
<td>standard metabolic rate</td>
</tr>
<tr>
<td>TFM</td>
<td>3-trifluoromethyl-4-nitrophenol</td>
</tr>
<tr>
<td>USFWS</td>
<td>United States Fish and Wildlife Service</td>
</tr>
<tr>
<td>USGS</td>
<td>United States Geological Survey</td>
</tr>
<tr>
<td>VI</td>
<td>visual implant</td>
</tr>
<tr>
<td>xmlc</td>
<td>multiplied by minimum lethal concentration</td>
</tr>
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</table>
INTRODUCTION

The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) is a pesticide that when used at proper concentrations selectively targets invasive sea lamprey (*Petromyzon marinus*) larval populations residing in streams. Killing the larvae in the stream reduces the number of juvenile sea lampreys that metamorphose and migrate to the Great Lakes, ultimately suppressing their predation on fish. Since the 1960’s, the U.S. Fish and Wildlife Service (USFWS) has sought to control sea lamprey populations in the Great Lakes through the use of lampricides. While this method has been successful, little research has been done to better understand the effects of sub-lethal exposure on non-target species, specifically lake sturgeon (*Acipenser fulvescens*).

Lake sturgeon population levels are currently estimated at less than 1% of their historical levels and the species is listed as threatened or endangered in 19 states within its range (Hay-Chmielewski and Whelan 1997, U.S. Fish and Wildlife Service 2015). Once abundant in the Great Lakes, lake sturgeon populations were devastated by commercial harvest, habitat degradation, pollution, and other anthropogenic stressors beginning as early as the 1800’s. The lake sturgeon’s life history strategy has allowed the species to survive since the late cretaceous period, but unfortunately now limits the recovery of today’s populations. Sturgeon put forth most of their energy as juveniles to drive rapid growth in order to avoid size-dependent predation. In doing so, energy is diverted from development of gametes which causes them to become sexually mature later in life. Typically, the first mating is at 15 to 20 years old. It is estimated that once mature, only 20% of the adult lake sturgeon spawn per year which results in production
of small year classes. Additionally, it is hypothesized that this intermittent breeding strategy reduces the risk of inbreeding (Auer and Dempsey 2013). Lake sturgeon are highly fecund; for example, a 25 year old female can produce approximately 250,000 eggs (Auer and Dempsey 2013). High fecundity is a necessity considering natural mortality in wild early life stage sturgeon is nearly 99% in the larval to juvenile (Age 0) life stage (Caroffino et al. 2009).

Historically, lake sturgeon inhabited 97 rivers in the Great Lakes; however, today only 57 are known to support lake sturgeon at some point during their life history (O’Connor et al. 2017). Of the historically known lake sturgeon streams, 46 receive regular lampricide treatments every 3 to 4 years to control larval sea lamprey populations (O’Connor et al. 2017). Lampricide treatment protocols have been established to avoid treating streams with known spawning populations of lake sturgeon until after August 1st to allow juvenile lake sturgeon to reach 100 mm in length (Bills et al. 2000). However, juvenile lake sturgeon may remain in their natal streams and stream mouths until they reach five years of age (Schram et al. 1999). By remaining in these areas, sturgeon are at risk of exposure to sub-lethal concentrations of TFM.

Many studies have demonstrated that fish taxa display a large range of sensitivity to TFM. Boogaard et al. (2003) demonstrated that bluegill (Lepomis macrochirus) and other Centrarchidae have the lowest sensitivity while lake sturgeon and bullhead (Ameiurus spp.) have the highest. Further, lake sturgeon <100 mm in total length were more sensitive to TFM than either very small yolk-sac fry or juveniles >100 mm (Boogaard et al. 2003). Additionally, for lake sturgeon < 100 mm, the LC50, or concentration of toxicant yielding 50% mortality, is less than that of larval sea lampreys.
This puts lake sturgeon at risk even in situations where the lowest possible effective concentrations of TFM are applied (Boogaard et al. 2003).

Studies by Middaugh et al. (2014) and Sakamoto et al. (2016) focused on the effects of sub-lethal TFM exposure on instantaneous growth rates, TFM avoidance, predator avoidance, olfactory responses to food cues, and swimming acceleration for a variety of species, including lake sturgeon. While Middaugh et al. (2014) did not document significant effects due to TFM exposure Sakamoto et al. (2016) reported reduced olfactory responses to food cues, reduced attraction to food scent, and a reduction of swimming acceleration for juvenile lake sturgeon. However, these studies fail to fully address TFM’s effects on metabolism, growth, and stress response, as well as the duration of these effects.

The mechanism of toxicity for TFM is its ability to uncouple mitochondrial oxidative phosphorylation which decreases the supply of adenosine triphosphate (ATP) and results in the inability to meet energy demands (Figure 1; Birceanu et al. 2011). The uncoupling is likely caused by TFM’s ability to act as a protonophore, an agent that can shuttle protons across a biological membrane (Figure 1). In doing so, TFM permits protons to pass through the inner mitochondrial membrane, thus reducing their flow through ATP synthase, resulting in decreased ATP production rates (Figure 1; Birceanu et al. 2011). The fish is then forced to rely on energy stores such as muscle glycogen reserves and anaerobic metabolism to maintain energy levels (Birceanu et al. 2014). Birceanu et al. (2011) documented in both rainbow trout (*Oncorhyncus mykiss*) and sea lamprey that oxidative phosphorylation was impaired even at low TFM concentrations and hypothesized that at sub-lethal concentrations they might experience some metabolic
disturbance. This suggests that sub-lethal TFM exposure could lead to metabolic impairment; however, little work has been done to evaluate this possibility.

The response of lake sturgeon to sub-lethal TFM exposures is of interest to researchers and fishery managers, because of their documented sensitivity to TFM and general imperiled status (Boogaard et al. 2003). Of particular concern is how TFM may affect the recruitment of juvenile lake sturgeon to adult populations. Exposure to TFM and its ability to alter the metabolism in juvenile lake sturgeon could negatively affect its ability for quick growth at the life-stage when it is most critical.

The objectives of this study focused on the effects sub-lethal TFM exposure had on growth, metabolism, and stress response of juvenile lake sturgeon, as well as duration of the effects. I compared the effects of exposure across three treatment concentrations based on the amount of TFM required to create a minimum lethal concentration (mlc) for sea lamprey larvae. The minimum lethal concentration is the amount of TFM required to kill 99.9% of sea lampreys during a stream treatment and is based on pH and alkalinity of the water. Concentrations used in this study were; a high concentration (1.0xmlc), a low concentration (0.5xmlc) and a control (0.0xmlc). Lake sturgeon are likely to be exposed to these concentrations in stream mouths, spring seeps, or backwater areas during typical lampricide treatments. I hypothesized that sub-lethal exposure to TFM would cause a decrease in instantaneous growth, and that stress response and standard metabolic rates would be elevated.
METHODS

Experiment 1 – Long term effects of sub-lethal TFM exposure

Animal Husbandry

The lake sturgeon used in this study were full siblings from a mating of wild parental stock from Black Lake, Michigan. They hatched in early June 2015 and were reared in the Northern Michigan University Aquatics Laboratory located in Marquette, Michigan. Fish were held in a 1,668 L plastic circular tank connected to a recirculating filtration system. The water was maintained at ambient temperature (range; 13.6 to 18.7°C) and fish were fed *ad libitum* rations of thawed chironomid larvae (Omega One, USA). The handling and care of the fish for this project was approved by the Northern Michigan University Institutional Animal Care and Use Committee (IACUC Application #270, approved November 2015) (Appendix A).

Lab Water Chemistry

Marquette City, Michigan municipal water, obtained from the tap in the Northern Michigan University Aquatics Lab, was dechlorinated by aeration for a minimum of twelve hours in a stock tank and was then used to fill all tanks in this study. Generally, the water temperature in the stock tank fluctuated with the air temperature of the lab and ranged from 14.8 to 18.1°C. The pH of the water averaged 8.05±0.02 (±SE) and ranged from 7.96 to 8.09. Alkalinity averaged 45.9±0.5 mg·L⁻¹ CaCO₃, ranging from 44 to 47 mg·L⁻¹ over the course of the experiment.
TFM Concentrations

U.S. Fish and Wildlife Service Sea Lamprey Control Program TFM prediction charts were used to determine the minimum lethal concentrations for this study based on measured pH and alkalinity of the source water (Bills et al. 2003). This approach indicated that a solution of 1.5 mg·L\(^{-1}\) TFM would be required to achieve the minimum lethal concentration (mlc) needed to kill 99.9% of larval sea lampreys in a typical stream treatment. The amount of TFM needed for the solution was estimated using the percent active ingredient found in the liquid TFM formulation (Iofina, Covington, Kentucky, USA).

Three TFM concentrations, a 0.0xmlc (control), 0.5xmlc, and 1.0xmlc, were created for each exposure trial. Desired concentration were estimated using the USFWS TFM prediction chart based on actual pH and alkalinity of the water of the exposure baths measured at the beginning of each trial. The desired 1.0xmlc TFM concentrations averaged 1.6±0.03 mg·L\(^{-1}\) and ranged from 1.5 to 1.7 mg·L\(^{-1}\). Measured 1.0xmlc TFM concentrations averaged 1.36±0.04 mg·L\(^{-1}\) and ranged from 1.19 to 1.85 mg·L\(^{-1}\) over the course of the study.

TFM Exposures

A total of 252 fish were used in the exposures, 84 fish per treatment group. A total of seven TFM exposure trials were completed during this experiment. Trials were completed monthly until the supply of fish was exhausted. A single trial consisted of randomly selecting and exposing 36 fish to the three TFM treatments; 12 fish per treatment group (Figure 2). Exposures were completed over the course of twelve hours
to simulate the time fish would be exposed to TFM had it been a typical lampricide stream treatment.

Treatments were conducted using three 68-L plastic bins filled with 38-L of continuously aerated, de-chlorinated water. Pre-measured TFM solutions were added to the respective tanks and mixed into solution. The TFM concentrations in the tanks were monitored using a portable Hach DR/1900 spectrophotometer (Hach, Loveland, Colorado, USA) following protocols established by the USFWS Sea lamprey Control Program (Technical Operating Procedure: 018.7, US FWS Sea Lamprey Control Program, Marquette Biological Station, Marquette, Michigan, USA). Concentrations were measured at the beginning (t=0 hours), middle (t=6 hours), and end (t=12 hours) of the treatment. Treatment water samples were compared to a three point standard curve bracketing the desired concentrations to determine the actual amount of TFM in solution. The standards for the curve were prepared through appropriate dilution of USFWS field standard solutions (USGS, Hammond Bay Biological Station, Millersburg, Michigan, USA). TFM levels within treatment tanks were stable at the mentioned time periods for each trial. Water chemistry data (pH, dissolved oxygen, and temperature) were monitored at the same interval as the TFM concentration. During the time TFM concentrations were measured, fish were monitored for any signs of extreme stress.

Upon completion of the treatment, the fish were removed from the exposure tanks and placed into fresh aerated water. Total length and weight were measured and each fish was tagged using a uniquely numbered visual implant (VI) tag (Northwest Marine Technology, Inc., Shaw Island, Washington, USA) colored to correspond to the treatment group (Figure 2). Once measured and tagged, the fish were placed into six 76-L tanks
containing fresh, filtered and aerated water at ambient temperature. Two fish from each of the TFM treatment groups (0.0xmlc, 0.5xmlc, and 1.0xmlc) were placed into each of the 76-L tanks. The fish were held for fourteen days following the exposure and fed *ad libitum* using chironomid larvae (Omega One, USA) (Figure 2). Waste and excess feed was removed using a siphon hose and water changes were completed as needed. Each tank was treated uniformly to avoid introducing additional stressors that could affect cortisol levels.

*Growth*

The effect of TFM on growth was determined by comparing the instantaneous growth rates of the fish by length and by weight between treatment groups. Following the fourteen day holding period, all fish were identified by their VI tag and measured for total length (mm) and weight (g) (Figure 2). Growth was estimated for instantaneous change in both length, millimeters of change per day (mm·d\(^{-1}\)) and weight, grams per day (g·d\(^{-1}\)). The equations for growth were:

\[
\text{Inst. Growth (mm·d}^{-1}\text{)} = \frac{(\ln TL_f) - (\ln TL_i))}{dt}
\]

And

\[
\text{Inst. Growth (g·d}^{-1}\text{)} = \frac{(\ln W_f) - (\ln W_i))}{dt}
\]

Where \(\ln TL_i\) is the natural log of the initial total length (\(TL_i\)) measurement in millimeters and \(\ln TL_f\) is the natural log of total length at the final measurement. The number of days between initial and final measurements was symbolized as \(dt\). Instantaneous growth by weight variables was were \(\ln W_i\), the natural log of initial weight in grams of the fish, and \(\ln W_f\) the natural log of the final weight, while \(dt\) was the number
of days between initial and final measurements. A two-way analysis of variance with (ANOVA) of the instantaneous growth by total length and weight was used to determine whether there was a statistically significant difference caused by treatment concentration, age, or whether age interacted with treatment. Tukey Honest Significant Difference (HSD) post hoc analysis was completed to identify significant differences by groups. Statistical significant difference was determined when \( p \leq 0.05 \).

**Stress Response**

Enzyme-linked immunosorbent assay (ELISA) kits were used to determine plasma cortisol levels of the lake sturgeon (Arbor Assays, Ann Arbor, Michigan, USA). The cortisol sensitivity and detection limits for the ELISA kits were 0.0173 and 0.0454 ng·mL\(^{-1}\) respectively (Arbor Assays, Ann Arbor, Michigan, USA). Cross reactivity in the kits was relatively low, less than 1.2% for related steroids such as corticosterone and cortisone (Arbor Assays, Ann Arbor, Michigan, USA). The range of detection was 0 to 3.2 ng·mL\(^{-1}\) for this experiment based on the cortisol dilutions created that developed the standard curve which bracketed expected cortisol levels for lake sturgeon. The ELISA kits were validated using sturgeon plasma with known cortisol levels provided by the McCormick Fish Physiology Laboratory (University Massachusetts, Amherst, USA).

Blood plasma cortisol concentration was used to indicate whether sub-lethal exposures to TFM induced a stress response in juvenile lake sturgeon fourteen days after exposure. Blood was collected from 126 fish, 6 per treatment group per trial, into heparinized capillary tubes using tail ablation. Fish were selected from the tank and bled within five minutes of initial disturbance to avoid any amplification in cortisol response due to handling (Flik et al. 2006), then euthanized in an anesthetic bath of 300 ppm
tricaine methanesulfonate (MS-222). The tubes were centrifuged for five minutes at 10,000 x g to separate the plasma from other blood components. Plasma was transferred into 0.5 mL snap cap vials and stored at -70°C until cortisol concentrations could be measured using ELISA reagents and plates were prepared according to the kit instructions (Arbor Assays, Ann Arbor, Michigan, USA). Plasma was removed from the freezer, thawed, then transferred to 1 mL snap vials and diluted with deionized water at a ratio of 1:5. Samples were then placed in a 70°C water bath and allowed to heat for 1 h. Heating the sample aided in releasing cortisol that may have been bound in plasma proteins (Bates et al. 2014). After heating, samples were prepared as directed in the ELISA kit and plates were read using a plate reader set to 450 nm. A two-way ANOVA was used to determine whether cortisol concentration differed significantly between treatment group, age, and whether age interacted with treatment group. Tukey HSD post hoc analysis was performed to compare groups and identify statistical differences. Statistical significant difference was determined when p≤0.05.

**Standard Metabolic Rate**

Standard metabolic rate in the form of oxygen consumption was measured using Loligo Systems intermittent flow respirometry equipment and methods (Loligo® Systems ApS, Denmark). The 4 chamber respirometer utilizes optical dissolved oxygen sensors to measure oxygen concentration (mg·L⁻¹) within sealed glass respirometer chambers. Sensors for each chamber were calibrated at a low and high dissolve oxygen concentration. Chambers were placed in a solution of sodium sulphite, which binds to oxygen molecules in water, to create a low dissolved oxygen set point. The upper set point was generated by placing the chamber into highly aerated water.
Standard metabolic rate was estimated for the remaining fish (N=126) not used in
the cortisol analysis, 18 fish per trial. Post-absorptive fish (N=3) from the same holding
tank, one from each treatment group, were placed in the respirometer chambers and
allowed to acclimate overnight (12-18h). During the acclimation period, fresh water was
circulated through the system to maintain oxygen levels. Water temperature of the
respirometer apparatus was maintained at approximately 15.0°C using a water
refrigeration unit. The chambers were shaded throughout the acclimation and
measurement period so fish were not disturbed by outside stimuli. Measurements were
made during the morning to avoid activities of other projects occurring in the lab that
may cause excessive noise that could startle the fish in the chamber. The measurement
period was initiated by recirculating the water through the chambers via a closed loop.
The rate of oxygen decline due to respiration of the sturgeon in the chambers was
recorded for 10 minutes followed by a 5 minute flush of fresh water. The flush of fresh
water allowed oxygen levels in the chambers to return to normal levels. This process
was repeated six times for each group of fish.

Standard metabolic rate was estimated using the mass of the fish, volume of water
in the respirometer, and slope of oxygen consumption. The formula for standard
metabolic rate as described by Svendsen et al. (2016) was:

\[ MO_2 = V_{RE} \cdot W_o^{-1} \cdot (dCO_2/dt) \]

where standard metabolic rate is \( MO_2 \), \( V_{RE} \) is the total volume of the respirometer minus
the volume of the lake sturgeon (L), \( W_o \) represents the mass of the sturgeon, and \( dCO_2/dt \)
is slope of the linear decrease in oxygen content of the water in the chamber over time
during the recirculation period.
The calculated slopes used for oxygen consumption were standardized to avoid bias and corrected based on slopes of the control chamber. Standardized slopes were 3 min in length and began 3 min after the water flow was switched to recirculate through the chambers and ended 3 min later. A total of six slopes were developed for each fish over the course of one hour and standard metabolic rate was calculated using all slopes to allow for replication and to capture variability. Two-way repeated measures ANOVA was used to determine if TFM, age, or TFM treatment group age interactions caused significant effects to standard metabolic rate of juvenile lake sturgeon. Tukey HSD was used to identify significant differences between groups. Statistical significance was demonstrated when $p \leq 0.05$.

Upon completion of the tests, all fish were euthanized in an anesthetic bath containing 300 ppm tricaine methanesulfonate (MS-222).

**Experiment 2** – Time frame of TFM metabolic effects

Based on the initial finding of my first experiment which suggested a long-term metabolic effect caused by sub-lethal TFM exposure, a second experiment was conducted designed to evaluate the time frame of TFM effects in greater detail. This experiment is different from Experiment 1 as fish of the same age were exposed to TFM, with growth and metabolic rates measured periodically over a period of twenty-eight days (Figure 2).

**Animal husbandry**

The lake sturgeon used in this study were full siblings, progeny from a mating of wild parental stock located in the Peshtigo River, Wisconsin. They were hatched from
eggs by the Michigan Department of Natural Resources and reared in a sturgeon incubation trailer to an average size of 94 mm before delivery to the Northern Michigan University Aquatics Laboratory in August, 2016. Fish were held in a 1,668 L plastic circular tank connected to a recirculation filtration system. The water was maintained at ambient temperature (range: 13.1 to 19.0°C) and fed *ad libitum* rations of thawed chironomid larvae (Omega One, USA). The handling and care of the fish for this project was approved by the Northern Michigan University Institutional Animal Care and Use Committee (IACUC Application #270, approved November 2015) (Appendix A).

Tap water was dechlorinated by aeration for a minimum of 12 hours and used to fill all tanks in this study. Water temperature was 17.1°C for the NMU Aquatics Lab holding tank.

*TFM Exposures*

TFM concentrations for treatment baths were based on lab water chemistry and created in the same manner as Experiment 1. The pH of the water was 7.9 and alkalinity was 46.8 mg·L\(^{-1}\). Based on USFWS TFM prediction charts the desired TFM concentration to achieve mlc was 1.4 mg·L\(^{-1}\). The measured 1.0xmlc concentration for this experiment was estimated to be 1.31±0.01 mg·L\(^{-1}\) and ranged from 1.25 to 1.35 mg·L\(^{-1}\). As in the first experiment, TFM concentrations were 0.0xmlc, 0.5xmlc, and 1.0xmlc.

A total of 56 fish were used in this experiment. Treatment exposure events were completed daily over the course of five days (Figure 2). Fish were distributed evenly between treatment groups for the first four trials, four fish per treatment group per trial
(48 fish total). The remaining eight fish used during the fifth exposure were distributed between the treatment groups as follows; two fish in the 0.0xmlc and three fish each for the 0.5xmlc and 1.0xmlc treatment groups. As with the first study, TFM exposure was completed over the course of twelve hours to simulate the time fish are exposed to TFM during a typical lampricide stream treatment.

Treatment exposures were conducted using three 24.6L plastic bins filled with 13.5L of continuously aerated, de-chlorinated water. The measured TFM formulation was added to the respective tanks and mixed into solution. TFM concentration, dissolved oxygen, and pH were monitored using the same methods as Experiment 1.

Upon completion of the treatment, the fish were removed from the exposure tanks and placed into fresh, aerated water to be measured. Total length and weight were measured and each fish was tagged using a uniquely numbered visual implant (VI) tag (Northwest Marine Technology, Inc., Shaw Island, Washington, USA) colored to correspond to treatment group. Once sampling and tagging were finished, the fish were placed into six 76-L tanks containing fresh filtered and aerated water at ambient temperature. Four fish from each of the TFM treatment groups, 0.0xmlc, 0.5xmlc, and 1.0xmlc, were placed into each of the 76-L tanks. However, only eight fish were placed into a holding tank following the last exposure. During the holding period fish were fed ad libitum using chironomid larvae (Omega One, USA) (Figure 2). Waste and excess feed was removed using a siphon hose and water changes were completed as needed.
Data collection

Growth and standard metabolic rate data was collected as described in Experiment 1. Growth and metabolic rate data were collected at one, seven, fourteen, and twenty-eight days post exposure to the TFM concentrations (Figure 2). Growth and standard metabolic rate were evaluated as in Experiment 1. Cortisol was not measured for this experiment because fish were handled frequently during placement and removal from the respirometer. This type of handling would likely have increased cortisol levels and confounded the results of a cortisol assay.

Statistical Analysis

Two-way ANOVA with Tukey HSD post hoc test was used to determine whether growth significantly differed by treatment group or days after exposure as well as to determine if growth was affected by the interaction between the two factors. Statistical significance was achieved when $p \leq 0.05$.

A two-way repeated measures ANOVA with Tukey HSD post hoc test was applied to the standard metabolic rate data to determine whether TFM concentration, days post exposure, or the combination of the two caused metabolic rate to differ significantly. Statistical significance was achieved when $p \leq 0.05$. 
RESULTS

Experiment 1 – Long term effects of sub-lethal TFM exposure

Only six out of the 252 fish used in the exposures died over the course of this experiment, the overall mortality rate was 2.4% with each treatment group having two fish die. The mortality rate for each treatment group was also 2.4%. Equal mortality rates between treatment groups suggests that TFM exposure levels used in this experiment did not result in elevated mortality compared to the control group.

The mean initial total length ranged from 104 ±4.7mm (±SE) for the six month age group to 168 ±4.4mm for the twelve month age group, resulting in a 62% increase in length over the course of the study (Figure 3A and 3B). Initial total length did not differ between treatments (Two-way ANOVA; F=1.025, df=2 and 222, p=0.360), nor did the final length (Two-way ANOVA; F=1.36, df=2 and 222, p=0.259) suggesting that TFM exposure did not affect the growth of juvenile lake sturgeon in terms of total length. However, because the fish grew over time, total length differed between ages (Two-way ANOVA; F=171.968, df=6 and 222, p<0.001, Figure 3A and 3B). Further, the effects of TFM exposure on total length did not significantly differ as fish aged (Two-way ANOVA; F=1.094, df=12 and 222, p=0.367). Generally, total length increased over time and plateaued at 11 months (Figure 3A and 3B).

The mean weight of the fish prior to treatment ranged from 5.1±0.9g for the six month old group to 14.8±2.3g for fish twelve months of age, resulting in a 190% increase over the course of the study (Figure 3C and 3D). Initial weight did not differ between
treatment groups (Two-way ANOVA; F=1.929, df=2 and 226, p=0.148). Further, weights measured fourteen days after the treatment were not different (Two-way ANOVA; F=1.91, df=2 and 226, p=0.306), suggesting that TFM did not affect growth in terms of mass. Initial and final weights were significantly different across ages because the fish grew over time (p<0.001), but TFM exposure did not have a significant effect on weight as the fish aged (Two-way ANOVA; F=0.799, df=12 and 226, p=0.651). As with total length, weight increased over time, but slowed at 11 months (Figure 3C and 3D).

Instantaneous growth rates based on total length ranged from 0 to 0.01 mm·d\(^{-1}\) and were not different between treatment groups (Two-way ANOVA; F=1.244, df=2 and 222, p=0.29). Length based growth rates did differ among age groups (Two-way ANOVA; F=107.7, df=6 and 222, p<0.001); however, these rates were not influenced by treatment group (Two-way ANOVA; F=1.134, df=12 and 222, p=0.334; Figure 4A). Further, initial weight applied as a covariate did not change the outcome of the results. Fish comprising the six to eight month age groups demonstrated the highest growth rates, but rates declined with age demonstrating a change in growth rates as fish aged (Figure 4A).

Instantaneous growth rates based on weight ranged from 0 to 0.05g·d\(^{-1}\) over the course of the study. Growth rates were not significantly different between treatment groups (Two-way ANOVA; F=1.236, df=2 and 222, p=0.293; Figure 4B) and there was no significant interaction between the age of the fish and treatment group (Two-way ANOVA; F=1.160, df=6 and 222, p=0.313). Applying initial total length as a covariate did not alter the outcome of the results (F=2.297, df=2 and 221, p=0.103). Instantaneous growth (g·d\(^{-1}\)) was significantly different for each age group throughout the study (Two-
way ANOVA; F=217, df=6 and 222, p<0.001; Figure 4B) supporting the result found with rates based on total length, that growth rates changed as fish grew and aged. Growth rates of fish in the nine month age group differed by treatment group (Figure 4B). In this case, fish exposed to the 1.0xmlc dose of lampricide appeared to grow twice as fast when compared to controls (ANOVA; F=3.68, df=2, 34, p=0.037). However, fish of the 1.0xmlc treatment group were smaller than those of the control and 0.5xmlc, this bias was likely the cause of the significant finding.

Positive growth rates were demonstrated over all ages, but rates tended to decrease as the fish aged (Figure 4). Fish less than 9 months in age tended to grow more quickly than the older age groups (p<0.001). There was an overall 82% reduction in growth from six months to twelve months of age. This trend was unrelated to TFM exposure as demonstrated by the asymptotic change in the mean initial weight of the fish over the course of the study (Figure 3C). Mean weight increased through ten months in age, but tended to plateau at later ages (Figure 3C).

Plasma cortisol concentrations varied over the course of this study with the minimum cortisol level being 0.006 ng·mL⁻¹ and the maximum 11.7 ng·mL⁻¹. Sub-lethal concentrations of TFM did not have a significant effect on cortisol levels of juvenile lake sturgeon fourteen days after exposure (ANOVA; F=0.593, df=2 and 88, p=0.555). Additionally, age did not interact with treatment concentrations (Two-way ANOVA; F=0.751, df=12 and 88, p=0.698). Total length and weight were not significant covariates affecting cortisol concentrations of juvenile lake sturgeon for treatment group or age (p=0.669 and p=0.774). However, cortisol concentrations were significantly different between age group (F=7.101, df=6 and 88, p<0.001) (Figure 5). Specifically,
cortisol levels were elevated in fish from the ten and eleven month age group compared to all other age groups with overall mean cortisol concentrations at 1.65 and 2.76 ng·mL⁻¹ respectively; however, there was no significant difference between treatments within these age groups (p=0.602 and p=0.218). It is likely that environmental conditions within the lab may have caused the elevated cortisol levels for the 10 month age group as a result of minor construction occurring in the lab near the holding tanks during this time period. Additionally, rearing conditions caused additional stress for fish in the 11 month age groups as blood sample were dark brown in color indicating nitrite poisoning likely inducing a stress response. During this time period fish were becoming too large for the tanks and water changes were not frequent enough to control nitrite levels as a result of the excess fish waste. This problem was rectified by the following month.

Standard metabolic rate ranged from 15.8 to 440.1 mg O₂·kg⁻¹·hr⁻¹ over the course of the study. Overall, sub-lethal TFM exposure significantly affected rates fourteen days after exposure (Two-way repeated measures ANOVA; F= 3.351, df= 2 and 82, p=0.048, Figure 6). The mean standard metabolic rate of fish exposed to the 1.0xmlc treatment (153.3 mg O₂·kg⁻¹·hr⁻¹, ±7.27) was 15.8% lower than the unexposed fish (178.4 mg O₂·kg⁻¹·hr⁻¹, ±7.32). The mean rate for fish exposed to the 0.5xmlc (159.9 mg O₂·kg⁻¹·hr⁻¹, ±7.16) was intermediary to the other groups with rates 10.8% lower than the control group, but 4.4% higher than the 1.0xmlc exposed group suggesting a dose dependent response to TFM exposure (Figure 6). Metabolic rates for each age group were significantly different from each other (F=17.956, df=5 and 82, p<0.001); however, there was no significant interaction between treatment and age groups (F=0.884, df=10 and 82, p=0.551). Total length was not a significant covariate (p=0.872). Fish nine months of
age had elevated metabolic rates in relation to all other age groups; however, these fish demonstrated the same overall decreasing trend from 0.0xmlc to 1.0xmlc as the other age groups (p<0.001, Figure 6). The standard metabolic rates for fish exposed to the 1.0xmlc treatment were lower than controls at all ages with the exception of the twelve month age group. Fish younger than ten months of age exposed to the 0.5xmlc tended to have lower rates than control fish, but higher than fish exposed to the 1.0xmlc, further supporting the idea that TFM induces a dose dependent response (Figure 6). Fish eleven and twelve months of age exposed to the 1.0xmlc concentration had rates relatively equal to control fish suggesting that either age or fish size may cause a decrease in sensitivity to TFM.

**Experiment 2 – Time frame of TFM metabolic effects**

Total length of juvenile lake sturgeon prior to exposure ranged from 89 to 147 mm. Initial length did not differ between treatment groups (ANOVA; F=0.583, df=2 and 32, p=0.563). The time after exposure did not significantly affect total length (p>0.05). Additionally, the number of days after exposure did not interact with the treatment groups (F=0.639, df=4 and 32, p=0.639).

Initial weight of juvenile lake sturgeon ranged from 2.7 to 11.0 g for this study. Weight was not significantly different between treatment groups prior to exposure (ANOVA; F=0.275, df=2 and 38, p=0.761). Further, weight measured after exposure was not significantly different by treatment group or days after exposure (p>0.05) suggesting that growth was relatively slow over the course of the experiment.
Additionally, weight was not affected by an interaction between treatment group and days post exposure (Two-way ANOVA, F=0.755, df=4 and 32, p=0.562).

Over the course of the experiment the mean instantaneous growth rates based on weight ranged from 0.002 to 0.009 g·day⁻¹. Growth rates did not differ significantly between treatment groups or days post TFM exposure (F=0.276, df=2 and 32, p=0.76 and F=2.045, df=2 and 32, p=0.146) (Figure 7). Additionally, there was no significant interaction between treatment groups and days post exposure in terms of growth (F=0.40, df=4 and 32, p=0.807). Initial total length was not a significant covariate in this analysis (F=0.179, df=2 and 31, p=0.837).

Mean standard metabolic rate was 186.4 ±5.1 mg O₂·kg⁻¹·hr⁻¹ and ranged from 17.9 to 439.5 mg O₂·kg⁻¹·hr⁻¹ throughout this experiment. Overall, fish exposed to the 1.0xmlc treatment had mean rates of 186.8 ±8.7 mg O₂·kg⁻¹·hr⁻¹ while rates for the control and 0.5xmlc were 184.4 ±8.9 mg O₂·kg⁻¹·hr⁻¹ and 188.0 ±8.9 mg O₂·kg⁻¹·hr⁻¹ respectively. Standard metabolic rate by time period varied significantly (F=18.086, df =3 and 43, p<0.001). The overall mean metabolic rate for fish one day after exposure to the treatments was significantly lower than the all other time periods (F=18.086, df=3 and 43, p<0.001), but did not differ between treatment groups. However, fish exposed to the 1.0xmlc did have metabolic rates 11.2 % higher than that of the control group (Figure 8). The SMR for fish seven days after exposure significantly differed between treatment groups (F=4.385, df=2 and 12, p=0.037), where the metabolic rate for fish exposed to the 1.0xmlc concentration were 18.0% higher than the control and 0.5xmlc treatment groups (p=0.034 and p=0.019, Figure 8). This finding suggests that TFM causes a chronic metabolic response through increased oxygen consumption for at least seven days after
the exposure. The standard metabolic rate for the fourteen and twenty-eight day time periods did not demonstrate significant differences between treatment groups (F=1.248, df=2 and 12, p=0.332 and F=1.280, df=2 and 12, p=0.332). However, similar to Experiment 1, when the 1.0xmlc treatment group reached the fourteen day time period, the metabolic rates dropped and were 9.3% below the control group and 17% below the 0.5xmlc treatment group (Figure 8). Twenty-eight days after exposure the 1.0xmlc treatment group had rates equal to the control group (Figure 8). There was no significant interaction between the treatment group and the number of days post exposure (F=2.002, df=6 and 43, p=0.086). Standard metabolic rate tended to increase with increasing days after the sub-lethal exposure TFM concentrations (Figure 8). The rates for fish exposed to the 1.0xmlc concentration varied over time and fish exposed to the 0.5xmlc continually increased, while the rates for the control group plateaued at fourteen days post exposure (Figure 8). Applying total length as a covariate was not significant in this analysis (p=0.4).

For both experiments, the standard metabolic rate for fish exposed to the 1.0xmlc demonstrated a remarkable decrease in rates 14 days after the treatment compared to the control groups (Figure 6 and Figure 8). In the first experiment, the SMR for the 1.0xmlc treatment group was significantly lower by 16% than that of the average rate for the control group. In the second experiment, while not as significant, likely due to low sample size, the 1.0xmlc group demonstrated metabolic rates 9% less than the control group fourteen days after exposure. The results provided here suggest that TFM alters the metabolism of juvenile lake sturgeon and these effects will linger over a prolonged period of time.
DISCUSSION

Lake sturgeon are a threatened species in the Great Lakes and juvenile lake sturgeon can be sensitive to TFM exposures at levels used to control sea lampreys (Boogaard et al. 2003). At times these fish may be exposed to TFM at levels below lethal doses. The goal of this study was to determine if sub-lethal exposure to TFM had delayed effects on juvenile lake sturgeon. My results demonstrate that acute sub-lethal exposure to TFM does not have detectible chronic effect on the growth or stress response of juvenile lake sturgeon; however, metabolic effects were observed up to fourteen days after exposure, suggesting a sub-lethal long term effect on energy production.

Exposure to TFM is known to kill juvenile lake sturgeon at concentrations used to control sea lamprey populations in the field environment. Lampricide concentrations typically applied to streams range from 1.2 to 1.5xmlc TFM needed to kill 99.9% of larval sea lampreys (Klar and Schleen 2000). Boogaard et al. (2003) found lake sturgeon <100 mm in length are highly sensitive to TFM when exposed to these levels and that mortality decreases as fish grow and age. My results support Boogaard’s previous work as mortality of lampricide exposed fish was 2.4% for the first experiment and 0.0% for the second. My data further supports ideas noted in Boogaard et al. (2003) suggesting that TFM concentrations at 1.0xmlc for sea lampreys would have little effect on the mortality of lake sturgeon larger than 100 mm in length. Because the sturgeon used in this study ranged from 85 to 192 mm in length, it is likely that these fish were less sensitive to TFM due to their size and therefore mortality was negligible.
Size of lake sturgeon is not the only factor influencing the survival of lake sturgeon exposed to TFM. Alkalinity and pH of the treated water also influence survival. Boogaard et al. (2003) noted that lake sturgeon <100 mm were highly sensitive to TFM, at alkalinity levels >100 mg·L⁻¹ CaCO₃. A recent study by O’Connor et al. (2017) discovered a negative relationship between lake sturgeon survival and alkalinity. Specifically, as alkalinity increased, survival of lake sturgeon exposed to TFM decreased (O’Connor et al. 2017). Additionally, as alkalinity rose above ~100 mg/L CaCO₃, the descending slope of the survival curve steepened and survival rates rapidly decreased; however, when alkalinity was ≤60 mg·L⁻¹ CaCO₃ age-0 lake sturgeon demonstrated survival rates >90% (O’Connor et al. 2017). The data in our study supports these findings, since alkalinity was <60 mg·L⁻¹ CaCO₃ and survival rate was 97.6% further suggesting that low alkalinity water results in relatively high juvenile lake sturgeon survival rates. This also suggests that my experiment was very conservative in effect, and depending on alkalinity, non-lethal effects could have been substantially larger than demonstrated here.

As with Middaugh et al. (2014), my study demonstrated that instantaneous growth of lake sturgeon was not affected by exposure to sub-lethal concentrations of TFM. However, while not the focus of this study, instantaneous growth rates were lower for the second experiment when compared to rates of the first. These differences are likely attributed to lower water temperatures of the holding tanks in the second experiment. Mean temperature for tanks during the first experiment was 15.9 ºC (SE ±0.04), but mean temperature for the second experiment was 12.6 ºC (SE ±0.14). Also, different strains of lake sturgeon were used in the two experiments. The Black Lake strain of lake sturgeon
were subject fish of the first experiment while Peshtigo River strain were used in the second, which could also have contributed to the differences in growth rates across experiments.

Changes in water quality or chemistry, such as dissolved oxygen, ammonia, pH, alkalinity, and temperature, can induce stress in fish (Iwama et al. 1999). Exposure to TFM is presumed to be a stressful event and cortisol, the most common stress hormone produced in fish, should be mobilized. Research has demonstrated that elevated levels of cortisol alter osmoregulation and negatively affect growth, reproduction, and immunosuppression (Schreck 2010, Barton 2002, Bonga 1997, Barton and Iwama 1991). TFM applications occurring in the field have a twelve hour duration, therefore, I hypothesized that cortisol levels would be elevated due to exposure should the fish perceive it as a stressor. Peak levels of cortisol may take minutes to hours to be generated from the time of exposure to the stressor (Pankhurst 2011). For most species elevated cortisol levels are typically sustained for approximately six hours after the cessation of the acute stress (Pankhurst 2011). Additionally, Pankhurst (2011) suggests that the duration of the stress can also directly affect how long cortisol will be detected in the fish. Lankford et al. (2003) demonstrated that green sturgeon (Acipenser medirostris) subjected to a 1 min air emersion had peak cortisol levels 15 min after the stress, but levels returned to normal 120 min after being subjected to the stressor. The results of Lankford et al. (2003) suggest that sturgeon cortisol levels return to normal rather quickly after exposure to a stressor. In my study, fish were exposed to TFM for twelve hours and cortisol levels measured fourteen days after TFM exposure. In my study, cortisol levels
were not elevated at the time of sampling, which suggests that if a cortisol spike does occur, it likely happens early and ceases before fourteen days have elapsed.

The mechanism for TFM toxicity lies in its ability to uncouple mitochondrial oxidative phosphorylation causing a shift from aerobic to anaerobic respiration, thereby affecting the metabolism of fish exposed to the chemical. Birceanu et al. (2009) demonstrated that the mechanism of TFM toxicity is the result of impaired ATP production in the mitochondria being unable to meet the ATP demand by the organism. Additionally, Birceanu et al. (2011) found that oxygen consumption of rainbow trout liver mitochondria exposed to TFM was higher than that of controls, indicating that oxygen is being brought in, but is not being used in energy production. Based on this, I hypothesized that metabolic rate would be elevated for fish exposed to the TFM fourteen days after exposure. Results of the second experiment in this study support Birceanu’s finding as metabolic rates tended to be higher in fish exposed to the 1.0μM TFM than that of the control group initially. Metabolic rates demonstrated an increasing trend twenty-four hours post exposure and the increase lasted at least seven days. However, standard metabolic rate of exposed fish was lower than that of the control fish fourteen days post-treatment. This decrease in metabolic rate suggests that fish exposed to TFM were using less oxygen to maintain their metabolic rate at levels equal to the control group. These findings suggest that there is some form of change to the mitochondria due to TFM exposure and that the effect lasts for a prolonged period of time.

Studies have shown that chemicals that uncouple mitochondrial oxidative phosphorylation damage mitochondria by depolarizing the inner mitochondrial membrane potential, blocking mitochondrial fusion, increasing mitochondrial
fragmentation and fission (Twig and Shirihai 2011). Buildup of dysfunctional mitochondria will lead to oxidative stress and impairment of cell function (Twig and Shirihai 2011). The removal of dysfunctional mitochondria is crucial for cell survival (Berezhnov et al. 2016). While little information is available pertaining to the effects of mitochondrial dysfunction in fish, accumulation of dysfunctional mitochondria in humans has been linked to heart disease, obesity, cancer, and diabetes, as well as Alzheimer and Parkinson diseases (Twig and Shirihai 2011).

Mitochondrial fusion maximizes oxidative capacity and compensates for damage caused to mitochondria, particularly in response to toxic stress (Youle and van der Bliek 2012). In the presence of a protonophore, mitochondrial fusion is shut down by dissipating the inner membrane potential. Oxygen consumption is increased as a result of depolarization of the inner membrane potential (Caldeira da Silva et al. 2008, Liesa and Shirihai 2013) as the cells, and whole organism, seek to compensate for the decrease in functionality. Increased respiration attempts to maintain the potential; however, it is not efficient and results in reduced ATP production (Liesa and Shirihai 2013). Studies completed by Legros et al. (2002) and Twig et al. (2008) found that protonophores can cause a greater than 20% reduction in ATP production. In my experiment, oxygen consumption rates were elevated up to seven days after exposure suggesting that membrane potentials were low in the mitochondria of TFM exposed fish and that ATP production could have been reduced.

Inhibition of mitochondrial fusion caused by exposure to protonophores, such as TFM, results in rapid fission resulting in fragmentation of the mitochondria (Legros et al. 2002). Twig and Shirihai (2011) state that increased fission will result in decrease in
mitochondrial energy production and is a result of decreased fusion. Mitochondria with depolarized membranes are considered damaged by the cell and are removed through the process of mitophagy (Youle and Narendra 2011). Through this process, dysfunctional mitochondria are segregated from healthy mitochondria through mitochondrial fission, thereby preserving energy production in the cell using healthy mitochondria (Youle and van der Bliek 2012). Dysfunctional mitochondria are transported to the lysosome of the cell where they are degraded (Youle and Narendra 2011). In lake sturgeon exposed to TFM, it is likely that suppression of mitochondrial fusion and the process of mitophagy result in overall decreased density of functional mitochondria in the cells. The decrease in metabolic rate of fish fourteen days after exposure to TFM observed in my study supports this, as fewer functional mitochondria in the cells of the fish would consume less oxygen.

My study demonstrated fluctuations in standard metabolic rate over a twenty-eight day time span. Metabolic rate, a function of oxygen consumption, had an increasing trend from twenty-four hours, lasting at least seven days after exposure. Dissipation of the mitochondrial membrane potential caused by TFM likely resulted in measurable increases of oxygen consumption as the mitochondria attempted to reestablish the mitochondrial membrane potential. Further the inhibition of mitochondrial fusion limited the amount of healthy mitochondria that could be reproduced. As mitochondrial fission segregated the dysfunctional mitochondria for removal from the cells by mitophagy, mitochondrial density likely decreased. This decrease would result in a reduction of oxygen consumption observed at fourteen days in this study.
Typically, damaged mitochondria repair themselves very efficiently through the use of fusion and fission (Detmer and Chan 2007), and repairs can take place rather quickly. Youle and van der Bliek (2012) demonstrated this by injecting a fluorescent dyed protein into the matrix of a single lab-cultured mitochondria. The dyed protein spread to all mitochondria in the cell within one hour, demonstrating rapid fusion and fission of the mitochondrial population. In human cells, Legros et al. (2002) mentions that three hours after the protonophore carbonyl cyanide m-chlorophenyl hydrazine (CCCP) was washed from the cells the mitochondria were still viable. Further, after four hours, the mitochondria had returned to their original state.

Fish can metabolize TFM and clear it from the body through urine and feces by the process of glucuronidation, which is mediated by the enzyme UDP-glucuronyl transferase (Kane 1994). The efficiency of TFM biotransformation ranges phylogenetically between species, with bluegill (Lepomis macrochirus) being highly efficient and sea lampreys least efficient among those tested (Kane 1994). Le Clair (2014) demonstrated that lake sturgeon can also metabolize TFM through the process of glucuronidation and that lake sturgeon exposed to 0.5xmlc of TFM had cleared the chemical from the body within 6 hours after being placed in fresh water. Based on Le Clair’s 2014 study, the TFM had likely been cleared from the sturgeon in my study, yet metabolic effects of the chemical were still present over fourteen days after exposure. This suggests that mitochondrial repair is a longer process in lake sturgeon exposed to TFM compared to humans cells exposed to protonophores.

Rapid rates of fusion and fission are required to maintain adequate mitochondria in the cell for energy production, but the rates vary in response to metabolism (Youle and
van der Bliek 2012). In my study metabolic rates were elevated at seven days after exposure and reduced at fourteen days suggesting that mitochondrial repair is taking longer in lake sturgeon than described in humans by Legros (2002). Metabolic rate differences between fish and mammals likely explain this observation. Metabolic rates of endotherms are typically higher than those of ectotherms (Berner 1999) and J.R. Brett (1972) described birds and mammals expending energy at rates 10 to 100 that of active fish, such as salmon. Therefore, it is reasonable that vertebrates demonstrating higher metabolic rates would experience faster repair of mitochondrial damage induced by protonophores compared to fish.

The results of this study showed that exposure to sub-lethal concentrations of TFM affect the metabolism of juvenile lake sturgeon for a prolonged period of time. As with all fish, the juvenile life stage of lake sturgeon is a key developmental stage for production of viable adults in the overall lake sturgeon population. Even minor metabolic impairment, in the ranges found in this study, through mitochondrial dysfunction could affect fitness and overall survival of exposed fish. For example, these impairments could limit the ability of fish acclimating to changing temperatures. Guderley and St-Pierre (2002) demonstrated that fish with healthy mitochondria increase mitochondrial density when acclimating to cold water temperatures. Inability to acclimate to changing water temperatures could cause additional stress to the fish and potentially death. Additionally, Sakamoto et al. (2016) found a decrease in swimming acceleration of juvenile lake sturgeon as well as a reduction in response to olfactory food cues when exposed to sub-lethal concentrations to TFM. The results of that study suggest that sturgeon may be unable to escape predators or find forage, which can lead to
mortality or developmental impairment. My study and these others begin to suggest that sub-lethal exposure to TFM may affect the overall health of juvenile lake sturgeon and could lead to long-term problems with survival.

This study documented for the first time changes in metabolic rate over time in juvenile lake sturgeon exposed to realistic sub-lethal TFM concentrations and further demonstrates that these affects are long lasting. Metabolic impairment and mitochondrial dysfunction can negatively affect the health of an organism, as specifically documented in humans. It should be expected that the health of lake sturgeon exposed to TFM is impaired; however, very little information is available pertaining to the long term effects on fish health in response to metabolic impairment and mitochondrial dysfunction. Future research should be directed toward documenting mitochondrial dynamics in fish exposed to TFM over time and demonstrating when complete recovery has occurred. This information could help better plan the timing of TFM treatments in the future ensuring, that fish have an appropriate amount recovery time before a second application begins.
Figure 1. TFM uncouples mitochondrial oxidative phosphorylation by shuttling hydrogen ions (H+) through the inner mitochondrial membrane bypassing ATP synthase. This reduces the production of ATP and degrades the mitochondrial membrane potential. Normal ATP production is the result of the electron transport chain, complexes I, II, III, IV, transporting hydrogen ions across the inner mitochondrial membrane creating an electrochemical gradient (membrane potential). Hydrogen ions travel down the electrochemical gradient and pass through ATP synthase resulting in energy production.
Figure 2. Study design for Experiments 1 and 2. Light gray arrows indicate start of trial by TFM exposure. Dark gray arrows indicate times measurements were recorded. White arrows signify a holding period for fish where no measurements occurred. Black arrows denote end of experiment.
Figure 3. Mean (±SE) total length (mm) (A and B) and weight (g) (C and D) of juvenile lake sturgeon by age for both pre- and post-exposure to sub-lethal concentrations of TFM (xmlc = multiplied by the minimum lethal concentration) during Experiment 1.
Figure 4. Mean (±SE) instantaneous growth rates by total length (mm·d$^{-1}$) (A) and weight (g·d$^{-1}$) (B) for juvenile lake sturgeon ages 6 to 12 months exposed to three sub-lethal TFM concentrations (xmlc=multiplied by the minimum lethal concentration) during Experiment 1. Growth rates were significantly different by age group (p<0.001). There was no significant difference between age groups sharing the same letters within panel. Asterisk in panel (B) indicates that fish exposed to the 1.0xmlc dose grew significantly more than the other treatment groups at that age (F=3.66, df=2 and 32, p=0.037).
Figure 5. Mean (±SE) plasma cortisol concentration (ng·mL⁻¹) for juvenile lake sturgeon ages 6 to 12 months exposed to three sub-lethal concentrations of TFM (xmlc = multiplied by minimum lethal concentration) during Experiment 1. Groups highlighted with the same letters indicate no significant difference.
Figure 6. Mean (±SE) standard metabolic rate (mg O₂·kg⁻¹·hr⁻¹) of juvenile lake sturgeon ages 7 to 12 months exposed to three sub-lethal concentrations of TFM (xmlc = multiplied by minimum lethal concentration) during Experiment 1. Age groups sharing the same letter indicate there was no significant difference. Asterisk denotes significantly lower standard metabolic rate for fish exposed to the 1.0xmlc dose by age (p<0.05). Overall metabolic rate for fish exposed to 1.0xmlc treatment was significantly lower than controls (p=0.048).
Figure 7. Mean (±SE) instantaneous growth rates (g·d⁻¹) of juvenile lake sturgeon measured at seven, fourteen, and twenty-eight day intervals after exposure to sub-lethal TFM concentrations (xmlc = multiplied by minimum lethal concentration) during Experiment 2. Exposure to sub-lethal TFM concentrations did not chronically effect growth rates of juvenile lake sturgeon (F=0.276, df=2 and 32, p=0.76).
Figure 8. Mean (±SE) standard metabolic rate (mg O₂·kg⁻¹·hr⁻¹) of juvenile lake sturgeon (n=55) exposed to sub-lethal concentrations of TFM (xmlc = multiplied by minimum lethal concentration) measured at one, seven, fourteen, and twenty-eight day intervals during Experiment 2. Asterisk denotes significantly elevated standard metabolic rate for fish exposed to the 1.0xmlc concentration (p=0.037). Time periods sharing the same letter indicate no significant difference in average standard metabolic rate by time.
REFERENCES


APPENDIX A

Application to Use Vertebrate Animals in Research, Testing or Instruction

Project Title (If using external funds, enter the title used on the grant application): Effect of sub-lethal TFM exposure on growth and energetics of lake sturgeon

General Instructions
Please check the IACUC website to ensure you are using the current version of the form. All parts of this form must be submitted electronically to the Institutional Animal Care and Use Committee (email: IACUC@nmu.edu) and the relevant Department Head or other departmental designee. Review of this application will commence upon receiving the electronic application, but the project may not begin until all required approval signatures are obtained via Right Signature. Please contact the IACUC chair (email: IACUCchr@nmu.edu) if you have any questions.

Review Dates:
Designated Member Review of applications (appropriate for USDA Use Categories B and C) will be completed within two weeks after receipt of the electronic application.

Full Committee Review of applications will take place on the last Friday of every month. Applications for Full Committee Review must be electronically received by the first Friday of the month. Full Committee Review is required for applications that fall under USDA Use Categories D and E. Applications that fall under USDA Use Categories B and C will receive Full Committee Review if requested by an IACUC member. Detailed procedures on the IACUC review processes are located at the IACUC website.

I. Principal Investigator (Must be a faculty member or Department Head): Jill Leonard

Co-Investigator: Matt Symbal

Department: Biology

Phone number: 227-1619

II. Funding Sources/Course Information and Dates
If the proposed work is for a course, please include the number of the course and title of the course

Funding Sources (External & Internal, if applicable) internal

Additional Funding Pending (click on the correct box)? □ Yes □ No

Project/Course Start Date: October 12, 2015

End Date (three year maximum): 5/1/2017

This application is (check one) □ New □ Modification of an application currently approved by the Institutional Animal Care and Use Committee (a new protocol must be submitted after three years)

III. SPECIES, NUMBER OF ANIMALS, AND USE CATEGORY
Revised June 19, 2014 Check the IACUC website to ensure you are using the most recent form.
SIGNATURE PAGE

IACUC #: 270  PROPOSAL TITLE (From cover page): Effect of sub-lethal TFM exposure on growth and energetics of lake sturgeon

X.  ACKNOWLEDGEMENT BY PRINCIPAL INVESTIGATOR

    I acknowledge responsibility for this project. I have read the Northern Michigan University Principles for the Care and Use of Laboratory Animals and certify that this project will be conducted in compliance with those principles. I assure that I will obtain Institutional Animal Care and Use Committee approval prior to significant changes in the protocol. I assure that this project does not unnecessarily duplicate previous research or instructional projects. I assure that students, staff and faculty on the project are qualified or will be trained to conduct the project in a humane, safe, and scientific manner.

Signature: ____________________________  11/10/2015
Principal Investigator                     Date

XI.  APPROVAL OF SCIENTIFIC MERIT (to be completed by the Department Head)

Before the project is initiated, it must be reviewed and approved on the basis of its scientific merit.

☐ Review conducted by external agency.
    ☐ Governmental Agency: Please specify the reviewing agency or board Federal agency (e.g., NIH, NSF, USDA, etc.) and evidence of approval

☐ Nongovernmental agency (e.g., University review, specify if other):

☒ Departmental Review: I assure that this project has been reviewed and approved for scientific or instructional merit by:

☐ Expert reviewer (Name)

☐ Departmental Committee Review (Committee Name and Chairperson):

☒ Other (Describe): Reviewed by Department Head

Signature: ____________________________  11/10/2015
Department Head/Offer Authorized to Sign
Departmental Designee                     Date

XII. REVIEWS AND APPROVED BY THE IACUC

Signature: ____________________________  11/10/2015
Institutional Animal Care and Use Committee

Signature: ____________________________  11/10/2015
Institutional Animal Care and Use Officer

Following action on this application, copies of approval or denial letters will be sent to the applicant, Department Head, and appropriate College Dean.

Revised June 19, 2014 Check the IACUC website to ensure you are using the most recent form.