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The Influence of Physiological and Abiotic Factors on the Sensitivity of the Sea Lamprey (Petromyzon marinus) to the Lampricide 3-trifluoromethyl-4-nitrophenol (TFM)

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The Influence of Physiological and Abiotic Factors on the Sensitivity of

the Sea Lamprey (*Petromyzon marinus*) to the Lampricide 3-

trifluoromethyl-4-nitrophenol (TFM)

by

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Thesis

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

Invasive sea lamprey (*Petromyzon marinus*) in the Great Lakes are controlled using the pesticide (lampricide) 3-trifluoromethyl-4-nitrophenol (TFM), which is applied to nursery streams containing larval lamprey. The toxicity of TFM to lamprey is affected by various environmental and physiological factors, which can lead to residual lamprey that survive TFM treatment. The goal of this study was to investigate how abiotic (season and temperature) factors interacted with physiological parameters (whole body and tissue energy reserves) to influence TFM sensitivity in sea lamprey. Toxicity tests were conducted at different times of the year (spring, early and late summer, fall) and temperatures (6, 12 and 21 °C) using larval sea lamprey collected from the same stream, the Au Sable River, Michigan, USA. Toxicity tests revealed that TFM tolerance was greatest in late summer, when the 12 h LC_{50} and 12 h $LC_{99.9}$ were 2.0 to 2.5-fold greater than in the spring and fall, when water temperatures were cooler. Toxicity tests conducted the following year on larval sea lamprey collected from the same river, but acclimated and exposed to TFM at different temperatures, revealed that 12 h LC_{50} and 12 h $LC_{99.9}$ increased by 50 % as water temperature increased from 6 °C to 12 °C, and was 2.5-fold greater at 21 °C than at 6 °C. In addition, body composition experiments were conducted on lamprey that were not exposed to TFM to quantify changes in energy stores with season and temperature. Seasonal variation in whole body and tissue energy stores including glycogen, lipid and protein had little influence on the differences in TFM sensitivity. No differences in TFM burden or the TFM metabolite, TFM-glucuronide, were detected in the carcasses of lamprey exposed to TFM during different seasons. Nor could any differences in body condition (condition factor, hepatosomatic index) explain

the differences in TFM sensitivity with season. I conclude that increased water temperature is the primary abiotic factor contributing to the larval sea lamprey's greater ability to withstand TFM during the summer, possibly due to an increase in their capacity to detoxify TFM. These data suggest that it may be prudent to consider seasonal variations in temperature when using current models to select and treat sea lamprey-infested streams with TFM.

Co-Authorship

Work presented in this master's thesis was completed with the cooperation of Dr. Oana Birceanu and Benjamin Hlina. Seasonal and temperature toxicity experiments of larval sea lamprey exposed to 3-trifluoromethyl-4-nitrophenol (TFM) were conducted with the assistance of Benjamin Hlina at the Hammond Bay Biological Station (US-Geological Survey, Millersburg, MI, USA) as a part of his MSc thesis. Some of the laboratory analyses on the larval lamprey body composition were conducted jointly with Dr. Oana Birceanu at Wilfrid Laurier University. I conducted all other experiments and analyses presented in this thesis.

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LIST OF ABBREVIATIONS

ATP Adenosine triphosphate

DFO Department of Fisheries and Oceans

ETC Electron transport chain

GLFC Great Lakes Fisheries Commission

HBBS Hammond Bay Biological Centre

HPLC High Performance Liquid Chromatography

LC Lethal Concentration

SPE Solid Phase Extraction

TFM 3-trifluoromethyl-4-nitrophenol

TFM-G TFM-glucuronide

USFWS United States Fish and Wildlife Service

WLU Wilfrid Laurier University

Chapter 1

The Sea Lamprey Invasion and Chemical Control in the Great Lakes

1.1 Introduction

1.1.1 Invasive Species and the Great Lakes

Invasive species are introduced species that become widespread and have adverse effects on the invaded habitat (Lee, 2002). Species are often introduced for landscape restoration, biological pest control, entertainment, food processing and domestication. Introductions of flora and vertebrate fauna are usually intentional, whereas introductions of invertebrate fauna and microbes are often accidental (Pimentel *et al*. 2005). Most introductions of invasive species have occurred because of human activity (Vitousek *et al*. 1997).

European exploration, settlement and commercial development around the Great Lakes (Ashworth, 1986) resulted in the opening of many waterways linking the Great Lakes to the Atlantic Ocean. With increased commercial trade on the Great Lakes, however, came increased introductions of invasive species. Over one hundred invasive species have been introduced to the Great Lakes in the last two centuries, and the invasion rate has increased in the last 40 years (Mills *et al*. 1993). Some highly publicized invasive species of the Great Lakes include zebra mussels (*Dreissena polymorpha*), Asian carps (*Hypophthalmichthys nobilis*, *Hypophthalmichthys molitrix, Ctenopharyngodon idella* and *Mylopharyngodon piceus*) and sea lamprey (*Petromyzon marinus*). Historically, the sea lamprey has been the most devastating invasive species (MDNR 2014).

1.1.2 Invasion of the Great Lakes by Sea Lamprey

The sea lamprey is a phylogenetically ancient jawless fish related to jawed vertebrates and native to the Atlantic Ocean and the Baltic, western Mediterranean and

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Adriatic seas (Potter, 1980). Although it was unclear if sea lamprey are native to Lake Champlain, Lake Ontario and the Finger Lakes (Eshenroder 2009; Waldman *et al*. 2004; Waldman *et al*. 2009), it is widely accepted that they are not native (Eshenroder 2014). Sea lamprey likely gained access to Lake Champlain, Lake Ontario and the Finger Lakes through the Saint Lawrence River or through the Hudson River via the Erie Canal and other constructed waterways (Bryan *et al*. 2005; Eshenroder 2014) and were first documented in Lake Ontario in 1835 (Lark 1973). From Lake Ontario, sea lamprey circumvented Niagara Falls after modifications to the Welland Canal between Lake Ontario and Lake Erie in the late 1800s and early 1900s. Sea lamprey were documented in Lake Erie in 1921 (Dymond 1922), and then Lake Saint Clair in 1934 (Shetter 1949), Lake Huron in 1937 (Applegate, 1950), Lake Michigan in 1936 (Smith and Tibbles 1980) and Lake Superior in 1946 (Applegate, 1950); the interconnectivity of the Great Lakes waterways made possible the invasion and spread of the sea lamprey (Figure 1.1; Aron and Smith 1971; Morman *et al*. 1980). Currently, the St. Mary's River (Michigan-Ontario) linking Lake Superior to Lake Huron is the largest uncontrolled source of sea lamprey, due to its large volume and ideal spawning habitat for adults and rearing habitat for larvae (Schleen *et al.* 2003).

1.1.3 Consequences of the Sea Lamprey Invasion

Sea lamprey parasitism combined with water pollution and overfishing caused dramatic reductions of populations of large predatory fish, such as walleye (*Sander vitreus*), deepwater cisco (*Coregonus johannae*, *Coregonus nigripinnis*) and lake trout (*Salvelinus namaycush*) (Schneider *et al*. 1996; Smith and Tibbles 1980). Lake trout catch in Lake Huron declined from 1,545,454 kg in 1937 to a virtual collapse in 1947.

Catches in Lake Michigan declined from 2,500,000 kg in 1946 to 167,272 kg in 1953. In Lake Superior, catches declined from an average of 927,802 kg to 167,272 kg in 1961 (Scott and Crossman 1973), and the effects cascaded through the food web, causing large-scale disruptions of the aquatic community and contributing to the collapse of regional commercial, aboriginal and recreational fisheries (Smith and Tibbles 1980). After millions of dollars in economic losses to fisheries and the tourism industry (Fetterolf 1980; Fuller *et al.* 1999; Hansen and Jones 2008), it became evident that sea lamprey control was required to protect Great Lakes fisheries from sea lamprey.

1.2 Sea Lamprey Life History and Spawning

Sea lamprey have a complex transformational life history (Figure 1.2). During the larval phase, sea lamprey typically spend three to seven years burrowed in the sediment of tributaries of the Great Lakes. This phase is characterized by a relatively sedentary life style (Beamish and Potter 1975), a low metabolic rate (Lewis and Potter 1976; Wilkie *et al.* 2002; Tessier *et al.* 2018) and a filter-feeding diet (Moore and Beamish 1973; Sutton and Bowen, 1994), consisting mostly of diatoms and detritus (Creaser & Hann, 1929; Moore & Beamish, 1973; Moore & Potter 1976). Larvae accumulate large amounts of lipid and glycogen reserves in preparation for metamorphosis (Youson 1980, 2003), which involves distinct morphological, anatomical and physiological changes. Metamorphosis takes place over 3-4 months in the summer and early fall, during which lamprey develop eyes, change external body colouration from brown to a darker silver-black, and take on a more stream-lined shape with the development of more pronounced dorsal fins (transformers are metamorphosing lamprey; Youson 1980, 2003). The largest

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changes take place in the organization of the respiratory-feeding apparatus, in which the oral-hood is transformed into an oral disc, and a rasping tongue is formed, which allows the animal to attach to a fish and puncture its hide to draw blood for feeding. The gills switch from a uni-directionally to a tidally ventilated gill to facilitate breathing while attached to their host. In the fall, juvenile sea lamprey migrate downstream to the Great Lakes or the Atlantic Ocean, where they parasitize and prey on other fish **(B)**. Following a 12-20-month parasitic phase, adult sea lamprey migrate upstream in the spring **(C)**; Applegate 1950; Beamish and Potter 1975; Sorensen and Vrieze 2003); they are semelparous (Hardisty and Potter 1971) and hence die after spawning (GLFC 2013). Anadromous populations migrate to sea (Potter and Beamish 1975; Youson 1980).

Both landlocked and anadromous sea lamprey juveniles parasitize and prey on other fishes. Using their oral disk, sea lamprey attach onto fishes and feed on the blood of their hosts (Figure 1.3; hematophageous feeding; Beamish and Potter 1975), often resulting in death of the fish either directly from the loss of fluids, or indirectly from secondary infection of the wound (Farmer 1980; Phillips *et al*. 1982). Following a 12-20-month parasitic phase (Swink 2003), increases in water temperature, photoperiod, river discharge rates and pheromones produced by larval lamprey prompt the upstream spawning migration of adults in the spring (Applegate 1950; Beamish and Potter 1975; Li *et al*. 2007; Siefkes 2017; Sorensen and Vrieze 2003). Sea lamprey do not home like some fish, but rather exhibit regional panmixia, selecting the most 'suitable river' in which to spawn through the detection of bile salts (petromyzolol sulfate and allocholic acid) secreted by resident larval sea lamprey (Li *et al*. 1995; Polkinghorne *et al*. 2001; Waldman *et al*. 2008).

Ideal spawning habitat includes suitable substrates of sand and gravel, moderate water velocities, shallow water depths and temperatures ranging from 10 to 26 °C (Applegate 1950). Breeding occurs at the end of June and early July; the male (and sometimes the female) builds a redd (nest) by depositing rocks to form a crescent-shaped mound. The monocyclic female lays tens of thousands of nonbuoyant and adhesive eggs, which the male fertilizes; an estimated 86 % of eggs are not deposited in the redd and are instead consumed by predators. For eggs deposited in the redd, fertilization and survival is approximately 90 % (Applegate 1950; Manion and Hanson 1980). Because sea lampreys are semelparous (Hardisty and Potter 1971), they die after spawning. There are other lamprey and non-parasitic lamprey species native to the Great Lakes, including silver lamprey (*Ichthyomyzon unicuspis*) and American brook lamprey (*Lampetra appendix*). However, the other parasitic species have not caused such devastating effects as sea lamprey.

1.3 Integrated Sea Lamprey Control

In 1955, the Canadian and United States governments formed the Great Lakes Fishery Commission (GLFC), to develop and coordinate research and sustainable management plans for native fishes and sea lamprey control in the Great Lakes (Sorensen and Hoye 2007). Because current control techniques do not allow for complete eradication (Christie *et al*. 2003), the integrated sea lamprey control program relies on several techniques to control sea lamprey populations. Velocity barriers, electrical barriers and adjustable-crest barriers block adult sea lamprey migration to spawning tributaries with minimal effects on non-target organisms (Hunn and Younges 1980;

McLaughlin *et al*. 2007). Cages are also used to trap sea lamprey during migration to remove spawning adult sea lampreys from tributaries. Male sterilization programs are used to target adult lamprey by injecting males with the chemosterilant bisazir, which reduced reproductive potential when they attempt to spawn with females (Bergstedt *et al*. 2003; Twohey *et al*. 2003a, 2003b). Trial testing of the potential to use mating pheromones and alarm cues to guide lamprey towards barriers and traps is also underway (Johnson *et al*. 2005; Siefkes 2017). However, chemical control using the selective lampricide 3-trifluoromethyl-4-nitrophenol (TFM) remains the primary means for sea lamprey control in the Great Lakes (Krueger and Marsden 2007; Li *et al*. 2007; McDonald and Kolar 2007).

1.4 Discovery and Use of 3-trifluoromethyl-4-nitrophenol (TFM)

The lampricide, 3-trifluoromethyl-4-nitrophenol (TFM; Chemical Abstracts Service Registry Number 88-30-2) is a yellow-orange, crystalline solid that has a solubility of approximately 5,000 mg L^{-1} and a pKa of 6.07 at 25 °C. It is an aromatic, fluoro-containing, m-substituted phenol (Figure 1.4) that is chemically and biologically very stable. Other physical and chemical properties and methods of chemical preparation are documented in Schnick (1972).

In 1946, the United States Congress directed the Fish and Wildlife Service to develop measures for the control of sea lamprey in the Great Lakes (Van Oosten 1949). In 1953, studies at the Hammond Bay Biological Station (HBBS; Millersburg, Michigan) were conducted to identify a chemical that would be acutely toxic to larval sea lamprey, but non-toxic to other aquatic biota. Tests on more than 4, 300 compounds (see

Applegate *et al*. 1957) indicated that halogenated mononitrophenols were selectively toxic to larval sea lamprey (Applegate *et al*. 1958). Eventually, TFM was identified and sold to the Fish and Wildlife Service as Lamprecid 2770 by the Progressive Color and Chemical Company of New York (Moffett 1958). The first field testing of TFM was done on the Mosquito River, Michigan on May 14, 1958, and on the Silver River, Michigan on June 11, 1958 (Moffett 1958). The trials were successful, and marked the end of research efforts in the field. In Canada, experimental field work was completed on the Pancake River, Ontario, August 26 to 27, 1958, and on the West Davignon River, Ontario, November 5, 1958 (Fisheries Research Board of Canada 1958; Johnson 1959; Johnson and Tibbles 1962).

Currently, the application of TFM to tributaries of the Great Lakes targets larval sea lampreys in the early spring to fall, when water temperatures range from 0.5 °C to 23 °C (USGS 2013). However, the variability in the toxicity of TFM to larval sea lampreys is affected by the ratio of un-ionized to ionized TFM (Bills *et al*. 2003; Hlina *et al*. 2017; Hunn and Allen 1974). When TFM is un-ionized at lower pH, it is lipid soluble and can easily cross epithelial membranes but at higher pH, it becomes less lipid-soluble and therefore harder to uptake (Hlina *et al*. 2017; Hunn and Allen 1974; McDonald and Kolar 2007). Variability in TFM toxicity is also affected by factors such as temperature, conductivity and dissolved oxygen of the tributary water (Applegate *et al*. 1961; Johnson *et al*. 1999). Scholefield *et al*. (2008) suggested that seasonal variations in feeding activity, habitat, nutrition and lipid content could also affect sensitivity of larval sea lamprey to TFM.

The amount of TFM used in treatments is based on the 9 h minimum concentration of TFM that is lethal to 99.9 % of the larval lamprey, referred to as the $LC_{99.9}$ or minimum lethal concentration (MLC). These determinations are often done using a TFM toxicity prediction chart, commonly referred to as the pH-alkalinity model (Johnson and Morse 1999; Bills *et al*. 2003), which accounts for the differences in TFM toxicity at higher pH compared to lower pH (Bills *et al*. 2003; Lech and Statham 1975; McDonald and Kolar 2007).

Historical data from previous applications of TFM are also used to determine the application rate (Scholefield *et al*. 2008). Field applications of TFM use concentrations that are 1.2 to 1.5 times the MLC (McDonald and Kolar 2007) and treatments usually last 10 to 12 h (Bills *et al*. 2003). Sometimes multiple treatments are necessary as water flow dilutes the TFM concentration. Due to economic and operational constraints, only 200 of the approximately 433 tributaries containing larval sea lamprey are treated with TFM on a regular cycle (GLFC 2018).

1.5 Toxicity of 3-trifluoromethyl-4-nitrophenol (TFM)

TFM's mechanism of toxicity is by uncoupling oxidative phosphorylation (Birceanu *et al*. 2011; Niblett and Ballantyne 1976). Oxidative phosphorylation leads to the formation of ATP in the mitochondria when a proton electrochemical gradient (proton motive force) is established between the mitochondrial matrix and the intermembrane space as electrons are passed along different protein complexes on the inner mitochondrial membrane. Three of these protein complexes (complexes I, III, IV) pump protons from the mitochondrial matrix into the inter-mitochondrial space to establish the

proton motive force. The protons then flow down their electrochemical gradient through another protein complex, the ATP synthase, which harnesses the energy released by the H^+ movement to phosphorylate ADP to ATP (Figure 1.4; See Voet *et al*. 2006, Wallace and Starkov 2000 for reviews).

Acting as a protonophore, TFM leads to the dissipation of the proton electrochemical gradient, which interferes with mitochondrial ATP production because fewer protons pass through the ATP synthase, resulting in increased reliance on anaerobic glycolysis and increased mitochondrial oxygen consumption (Birceanu *et al*. 2011; Niblett and Ballantyne 1976). In turn, there is increased reliance anaerobic pathways of ATP generation, including the increased deposphorylation of high energy phosphagens such as phosphocreatine, and on glycolysis which results in reduced glycogen and increased lactate in the brain, liver and muscle (Birceanu *et al*. 2011; Clifford *et al*. 2012). When ATP supply can no longer match demand, this likely starves the central nervous system and other physiological processes of ATP, eventually leading to death. It is not known, however, how variation in energy stores throughout the year affects the lamprey's sensitivity to TFM.

The toxicity of TFM depends on its rate of uptake, detoxification and elimination, which vary with environmental factors such as temperature (McDonald and Kolar 2007; Scholefield *et al*. 2008). TFM is most likely taken up as un-ionized TFM down diffusion gradients across the gills. This suggests that factors such as the rate of uptake could be dependent on metabolic activity, which increases with temperature (Holmes and Lin 1994; Lewis 1980). Thus, at higher temperatures, rates of TFM uptake could be higher, leading to greater toxicity.

However, toxicity will also depend on how efficiently TFM is detoxified and eliminated by the fish. In non-target fishes such as rainbow trout (*Oncorhynchus mykiss*), TFM is conjugated to TFM-glucuronide (Kane *et al*. 1994; Lech 1974; Lech and Statham 1975), which detoxifies the lampricide and makes it more water soluble and easier to excrete via the bile or urine (Clarke *et al*. 1989). In sea lamprey, the activity of the enzyme that metabolizes TFM to TFM-glucuronide, UDP-glucuronosyltransferase (UDP-GT), is much lower compared to non-target fishes (Kane *et al*.1994; Lech and Statham 1975), which makes them much more susceptible to TFM toxicity than non-target fishes. This was thought to be the sole basis for the lampricide's selective effects on lamprey (Lech and Statham 1975; Kane *et al*. 1994; Statham 1974), but more recent work indicates that other pathways of TFM detoxification are also present in lamprey and non-target fish. Recent work by Bussy *et al*. (2018a,b) demonstrated that conversion of TFM to TFM-sulfate, via sulfotransferase enzymes was another route of TFM detoxification, and that other pathways involved in the reduction and oxidation of TFM might also play a role in TFM detoxification.

1.6 Seasonal Variation in the Toxicity of 3-trifluoromethyl-4-nitrophenol (TFM)

Applegate *et al*. (1961) first speculated that the sensitivity of TFM varied seasonally and described TFM tolerance increasing during spring, and even more so in late summer. More recently, Scholefield *et al*. (2008) collected sea lamprey from a number of different streams in the spring and later summer, and ran rigorous acute toxicity tests which demonstrated that tolerance was up to 2-fold greater during the summer. In other words, the amount of TFM required to treat streams in the summer was

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greater. This could have important implications for sea lamprey control because underestimates of TFM requirements during a treatment could lead to "residual" sea lamprey that survive treatment, complete metamorphosis, and migrate down to the Great Lakes as parasitic lamprey. Moreover, use of greater amounts of TFM could make non-target fish species more vulnerable to non-target adverse effects or mortality. However, the reasons for seasonal variations in TFM sensitivity have not yet been explained, which is the goal of this thesis.

1.7 Research Objectives

The overarching goal of this M.Sc. was to determine how season affected larval sea lamprey sensitivity to TFM, and to determine what physiological and abiotic factors best explained observed differences in TFM toxicity.

Objective 1: Effects of Season on Sea Lamprey Sensitivity to TFM

My first objective was to determine if sea lamprey collected from the same stream during different seasons exhibit differences in their sensitivity to TFM. Based on previous research (Scholefield *et al*. 2008; Robinson *et al*. unpublished data), I hypothesized that larval sea lamprey collected from the same stream will tolerate exposure to higher concentrations of TFM in the late summer than in mid-spring or early summer. To address this objective, larval sea lampreys were collected from the Au Sable River, Michigan, USA. in the spring, summer and fall and returned to the lab at the HBBS. The animals were then subjected to acute toxicity tests to determine if the 12 h LC_{50} and the 12 h $LC_{99.9}$ (MLC) of TFM varied between the spring, summer and fall.

Objective 2: Effects of Temperature on Sea Lamprey Sensitivity to TFM

My second objective was to determine if seasonal differences in TFM sensitivity were due to temperature effects, independent of seasonal changes in the biology of the animal. Accordingly, the effects of temperature on larval lamprey sensitivity were measured in animals collected from the same stream (Au Sable River) at the same time of year, but acclimated and exposed to TFM at different temperatures in the lab. I hypothesized that temperature will affect larval sea lamprey sensitivity to TFM more than season because it would directly influence metabolic rate, and therefore affect rates of TFM uptake, detoxification and elimination. For instance, at higher temperatures, increased metabolic rate could result in increased oxygen consumption by larval sea lamprey, which would create a positive feedback loop that would accelerate TFM uptake by the gills due to increased ventilation rates, and increase toxicity. Alternatively, greater metabolic rate could also increase rates of TFM detoxification, counter-balancing increased TFM uptake, resulting in greater tolerance to TFM.

Objective 3: Influence of Fuel Stores, Body Composition and Condition on the TFM Sensitivity of Larval Sea Lamprey

My final objective was to quantify how seasonal variation in internal fuel stores, body composition and condition affected larval sea lamprey sensitivity to TFM. Due to increased nutrient availability, I predicted that energy reserves would be highest in the summer, leading to greater TFM tolerance. In the spring, however, I predicted that these reserves would be much lower due to over-wintering and decreased nutrient supply and feeding. This objective was addressed by measuring internal energy stores (glycogen, lipid, protein), dry ash and water content, and body condition (condition factor, hepatosomatic index) in lamprey collected at different times of the year (spring, summer,

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fall) or subjected to the different temperature acclimation regimens described under objectives 1 and 2.

FIGURES

Figure 1.1 The Great Lakes waterways

The interconnectivity of the Great Lakes waterways made possible the spread of the sea lamprey (Morman *et al*. 1980).

Source: US Army Corps of Engineers 2013.
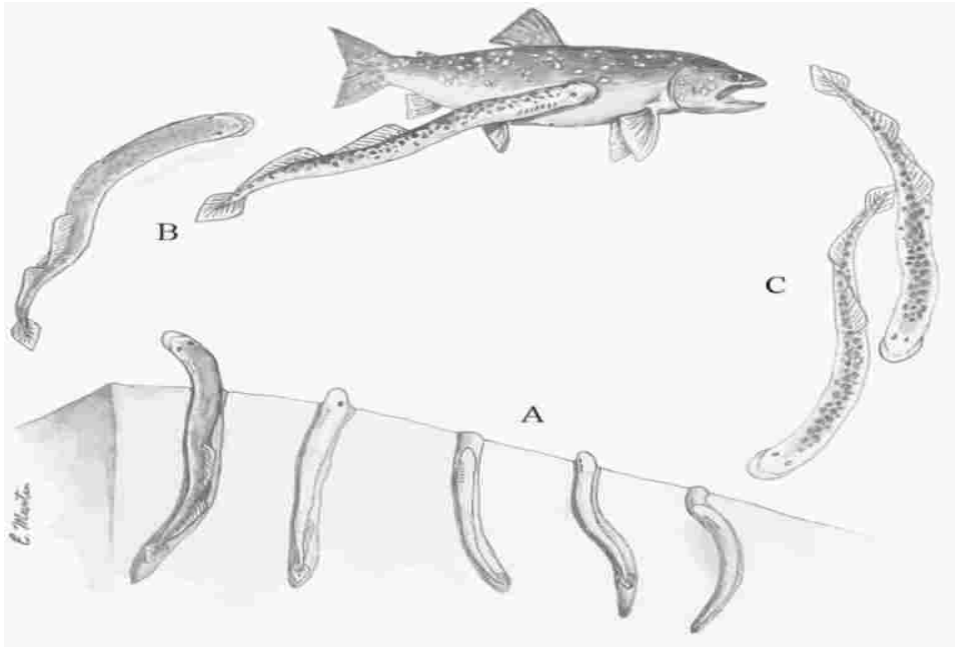
Figure 1.2 Life History of the Sea Lamprey

During the larval phase, sea lamprey typically spend three to seven years burrowed in the sediment of tributaries to the Great Lakes, and accumulate large amounts of lipid and glycogen reserves in preparation for metamorphosis (Youson 1980, 2003). Metamorphosis takes place over 3-4 months in the summer and early fall. **(B)** In the fall, juvenile sea lamprey migrate downstream to the Great Lakes or the Atlantic Ocean, where they parasitize and prey on other fish **(C)**. Following a 12-20-month parasitic phase, adult sea lamprey migrate upstream in the spring (Applegate 1950; Beamish and Potter 1975; Sorensen and Vrieze 2003); they are semelparous (Hardisty and Potter 1971) and hence die after spawning (GLFC 2013). Figure courtesy of Emily Martin (2014).

Figure 1.3 Hematophagous Feeding

The sea lamprey (*Petromyzon marinus*) uses an oral disk to attach onto a host fish and feed on its bodily fluids (Beamish and Potter 1975). Host fish are parasitized for several weeks to several months, and each sea lamprey can eradicate up to 10-20 kg of fish during the adult parasitic phase (Bower 1998; Swink 2003). Source: Minnesota Sea Grant 2011.
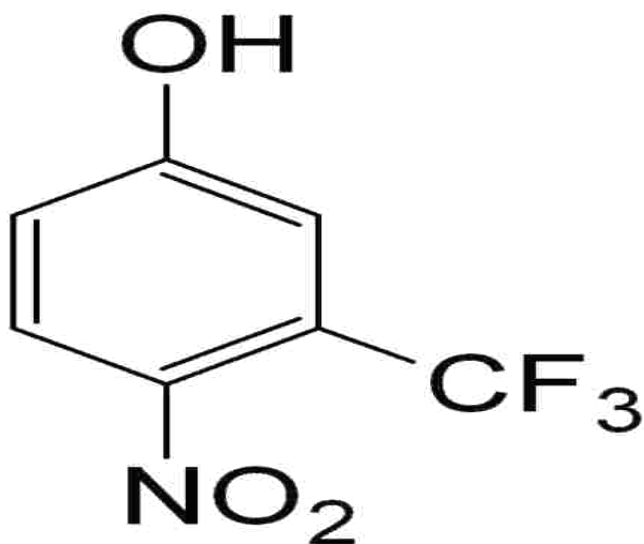
Figure 1.4 Chemical Structure of 3-trifluoromethyl-4-nitrophenol

Chemical structure of 3-trifluoromethyl-4-nitrophenol (TFM), a selective lampricide used to control invasive larval sea lamprey. TFM (Chemical Abstracts Service Registry Number 88-30-2) is a yellow-orange, crystalline solid that has a solubility of approximately 5,000 mg L^{-1} and a pKa of 6.07 at 25 °C. It is an aromatic, fluoro-containing, m-substituted phenol that is chemically and biologically very stable.

Figure 1.5 Schematic Diagram of the Electron Transport Chain (ETC) in the Mitochondria

(**A**) Electron donors such as NADH and $FADH_2$ pass through the ETC in a series of redox reactions; protons (H^+) are pumped (green arrows) into the inner membrane space (dark orange), generating a proton gradient. The low permeability of the inner membrane normally prevents non-specific flow of protons into the mitochondrial matrix (light orange). As a result, protons pass along the electrochemical gradient via ATP synthases. The energy released by proton flow is used by the ATP synthases to phosphorylate ADP, generating ATP. This entire process is coupled since the formation of ATP is linked to the proton gradient and the flow of electrons via the ETC (Wallace and Starkov 2000; Voet *et al*. 2006). (**B**) The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) works by uncoupling oxidative phosphorylation; as a protonophore, TFM leads to the dissipation of the proton electrochemical gradient and interferes with mitochondrial energy production (Niblett and Ballantyne 1976, Birceanu *et al*. 2011; Clifford *et al*. 2013), resulting in increased reliance on anaerobic glycolysis and increased oxygen consumption.
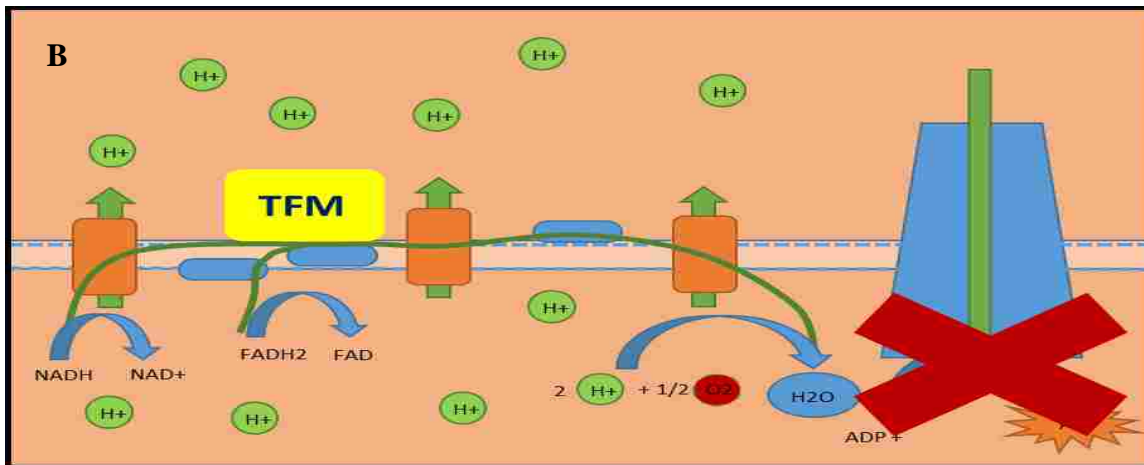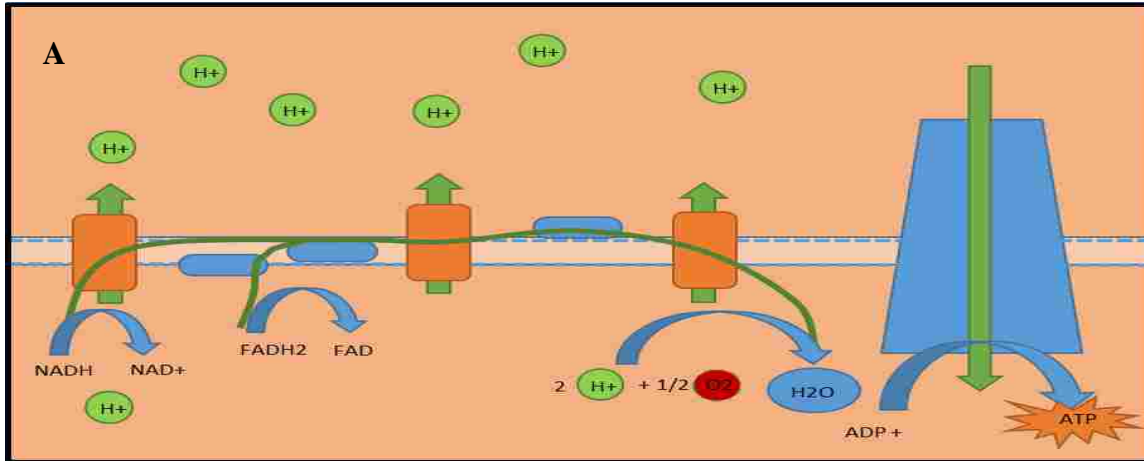
Chapter 2:

Contribution of physiological and abiotic factors to seasonal differences in the sensitivity of sea lamprey (*Petromyzon marinus*) to the lampricide 3-trifluoromethyl-4-nitrophenol (TFM)

2.1 Introduction

The piscicide, 3-trifluoromethyl-4-nitrophenol (TFM), has been used to control invasive sea lamprey (*Petromyzon marinus*) populations in the Great Lakes since the early 1960s (Applegate *et al*. 1961; Hubert 2003; McDonald and Kolar 2007). Applied at regular intervals to nursery streams and rivers containing larval sea lamprey, TFM specifically targets the animals in their burrows, where they live as relatively sedentary, suspension feeders (Beamish and Potter 1975; Moore and Mallatt 1980; Sutton and Bowen 1994). Current treatment protocols are based on the minimal lethal concentration (MLC) of TFM, which is defined as the amount of TFM needed to kill 99.9 % of larval sea lamprey over 9 h (Bills *et al*. 2003). In practice, the concentrations used in treatments range from 1.2–1.5 times the MLC to ensure that treatment residuals (i.e., sea lamprey that survive TFM exposure) are minimal (McDonald and Kolar 2007).

TFM exerts its mode of action by uncoupling mitochondrial oxidative phosphorylation, interfering with ATP production in both target and non-target fishes (Birceanu *et al*. 2011). Therefore, exposure to TFM induces a mismatch between energy supply and demand in the body, forcing the fish to rely on anaerobic pathways for survival. Energy reserves, such as glycogen and high energy phosphagens (e.g., phosphocreatine) become depleted, and once ATP supply cannot keep up with ATP demand, the animal dies (Wilkie *et al*. 2007, Birceanu *et al*. 2009, 2014, Clifford *et al*. 2012). While the mechanism of TFM action is similar amongst different aquatic organisms (Viant *et al*. 2001, Wilkie *et al*. 2007, Birceanu *et al*. 2009, 2014; Clifford *et al*. 2012; Henry *et al*. 2015), the specificity of TFM is related to the reduced ability of larval sea lamprey to detoxify TFM using glucuronidation (Lech and Statham 1975; Kane

et al. 1994). In non-target fishes, this Phase II detoxification process converts TFM to TFM-glucuronide via conjugation, which is more water soluble and easier to excrete via the gastrointestinal or urinary tract (Dutton and Montgomery 1958; Dutton 1980; Clarke *et al*. 1991).

Biotic factors such as life stage and body mass affect TFM sensitivity in sea lamprey. Henry *et al*. (2015) demonstrated that TFM sensitivity was highest in sexually mature adults compared to the earlier larval life stages. Tessier *et al*. (2018) demonstrated that rates of TFM uptake were inversely proportional to body size in lamprey, and that larger larval sea lamprey survived for longer periods when exposed to the MLC of TFM, suggesting that larger larvae are a potential source of treatment residuals. Other factors, such as larval sea lamprey abundance, year class and size structure may also influence the effectiveness of TFM treatments (Hansen *et al*. 2003, Dunlop *et al*. 2017).

Abiotic factors, such as differences in stream discharge and water chemistry, particularly pH and alkalinity, also influence TFM treatment success (Bills *et al*. 2003, Hansen *et al*. 2003). Because TFM toxicity decreases with increasing water pH and alkalinity, it is sometimes necessary to determine the MLC using stream-side toxicity tests and/or standard tables to calculate the MLC from on-site measurements of water pH and alkalinity (Bills *et al*. 2003). Previous treatment history and water discharge rates are also considered (P. Sullivan, Sea Lamprey Control Centre, Fisheries and Ocean's Canada, pers. comm.). Scholefield *et al*. (2008) reported that the TFM sensitivity of larval sea lampreys was markedly higher in the spring compared to late summer, suggesting that season and/or temperature have a marked influence on TFM toxicity.

Given that TFM depletes energy reserves such as glycogen (see above), which are also known to fluctuate seasonally in the sea lamprey (O'Boyle and Beamish 1977), the goal of this study was to investigate how abiotic (season and temperature) factors interacted with physiological parameters (whole body and tissue energy reserves) to influence TFM sensitivity in sea lamprey. To this end, larval sea lamprey were collected at different times in 2013 (spring, early and late summer, fall) to determine if variations in energy stores explained the seasonal differences in the TFM sensitivity of sea lamprey. A second set of experiments, using lamprey collected from the same river but at the same time of year (July 2014), were conducted to determine if differences in water temperature contributed to seasonal differences in TFM sensitivity. The concentrations of TFM and TFM-glucuronide (Lech and Statham 1975; Kane *et al*. 1994; Hubert 2003) in the whole bodies of sea lamprey were also measured, to determine if seasonal differences in TFM detoxification capacity were an indicator of TFM sensitivity. In addition, body composition experiments were conducted on lamprey that were not exposed to TFM, to quantify changes in energy stores with season and temperature.

2.2 Material and Methods

2.2.1 Collection Site, Animals and Experimental Holding

Larval sea lampreys were collected from the Au Sable River, Michigan using pulsed-DC backpack electrofishing (ABP-2 Electrofisher, Electrofishing Systems, LLC, Madison, WI, USA) by US Fish and Wildlife personnel in April, June, August and October 2013 and in June 2014. The river (Oscoda Charter Township, MI, USA) is a tributary of Lake Huron and runs approximately 200 km through the northern Lower

Peninsula of the state. The Au Sable River is treated with TFM on a three-year cycle by US Fish and Wildlife Service personnel, and underwent TFM treatment following our last collection of sea lamprey in 2014. Following collection, the larval sea lampreys were transported to the Hammond Bay Biological Station (HBBS; Millersburg, MI) in coolers containing aerated river water. For the 2013 experiments, upon arrival at HBBS animals were transferred to 30 L glass aquaria (N = ~100 per aquaria), continuously receiving aerated Lake Huron water (pH 7.8 ± 0.4; hardness = 150 mg L^{-1} as $CaCO_3$; dissolved oxygen ≥ 80 % saturation), with a 4-5 cm deep layer of sand lining the bottom of the aquaria to provide the larval sea lamprey with burrowing substrate. In 2014, animals were held in three 200 L plastic tanks (N = ~250 animals per tank), filled with sand as described above. Immersion chillers or heaters were used to set and maintain water temperature in the aquaria at the ambient temperature of the river at the time of collection (seasonal experiment) or at select experimental temperatures (temperature experiment; nominal temperatures = 6 °C, 12 °C, 21 °C). The larval sea lamprey were not fed during this period. All animal holding conditions and experimental methods were approved by the Wilfrid Laurier University Animal Care Committee and followed Canadian Council of Animal Care (CCAC) guidelines.

2.2.2 Experimental Protocols

2.2.2.1 Seasonal and Temperature Variation in the Toxicity of TFM to Larval Sea Lampreys

The toxicity of TFM to larval sea lampreys was determined in two toxicity tests lasting 24 h. The animals were then acclimated to the water temperature at which they

were collected or experimental temperature(s) for 7 to 10 d before performing acute toxicity tests, which comprised a preliminary range-finder toxicity test to determine the approximate TFM concentration ranges to be used to determine the 12 h LC_{50} and 12 h $LC_{99.9}$ of the fish in the subsequent larger scale tests. Each range-finder toxicity test comprised nine glass aquaria (18 L) filled with aerated Lake Huron water (16 L) to which the appropriate amounts of TFM were added (Appendix, Figure A1.1) The information from these ranger-finder tests was used to select the concentrations of TFM used in the acute toxicity tests. These tests were conducted in triplicate, at six TFM concentrations (N = 3, plus one control; 19 aquaria in total; see Table A1.1 for concentrations) using a similar set-up, but larger aquaria (30 L). The purpose of the control was to access the health of the animals without exposure to TFM and assure that mortality was <10 %, as outlined in the American Society and Testing Materials (ASTM; 2007) guidelines; survival during all experiments was 100 %.

Twelve hours prior to the range-finder or acute toxicity tests, each aquarium was filled with Lake Huron water and placed in a Living Stream (108"L x 24"W x 22"D, 190 gallons; Frigid Units Inc., Toledo, OH) partially filled with re-circulating water that was maintained at the appropriate temperature using either a chiller or immersion heater. At this time, the aquaria, containing no animals, were dosed with sufficient amounts of field grade TFM (35 % active ingredient dissolved in isopropanol, Clariant SFC GMBH WERK, Griesheim, Germany) to yield the appropriate target concentrations in each aquarium [0 (control), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0 and 7.0 mg L^{-1}]. The range of TFM concentrations selected for the acute toxicity tests were based on the information generated in the range-finder tests, and varied according to season and temperature. For

both the range-finder and the acute toxicity tests, water samples were collected immediately after adding TFM, and again the next morning prior to adding animals to the aquaria, and the amounts of TFM verified using spectrophotometric assays. The day of testing, the larvae were randomly distributed to each aquarium (N = 10 per aquaria in the range-finder tests; N = 15 for toxicity tests), and temperature, pH and dissolved oxygen were recorded immediately after the animals were added (0 h), and at 12 h and 24 h of exposure. The TFM concentration of water samples (5 mL) collected at 0 h, 12 h and 24 h of the exposure was measured to confirm that TFM concentrations remained constant during the exposures. Survival was monitored hourly from 0-12 h, and at 24 h; when animals appeared dead (immobile, no visible ventilation), survival was tested by gently pinching the caudal fin with tweezers. Unresponsive (dead) animals were immediately removed from the tanks, at which time body length and mass were measured, and the whole bodies flash frozen in liquid N_2 and stored at -80 °C. Surviving lamprey were euthanized with an overdose of tricaine methanesulfonate (1.5 g L^{-1} buffered with 3.0 g L^{-1} of $NaHCO_3$; MS-222; Syndel Labs, Port Alberni, BC, Canada) before storage. The frozen carcasses from the seasonal range-finder toxicity tests were transported to Wilfrid Laurier University (WLU) on dry ice for subsequent measurement of whole-body TFM and TFM-glucuronide (preliminary range-finder toxicity test larval sea lamprey). The toxicity tests followed American Society and Testing Materials (ASTM; 2007) guidelines and the results were used to calculate lethal concentrations (12 h LC_{50} or 12 h $LC_{99.9}$).

2.2.2.2 Variation in the Proximate Body Composition of Sea Lamprey with Season or Temperature

To determine how energy stores and proximate body composition of larval sea lamprey changed with season or temperature acclimation and how this could impact TFM toxicity, tissues were collected from the appropriate groups of larvae not exposed to TFM. The night before sampling, lamprey (N = 48) were distributed into twelve containers (750 mL) receiving aerated lake water. Each container contained 2 g of diffuse aquarium cotton to provide burrowing substrate (e.g. Wilkie *et al*. 1999, 2001). Temperature, pH and dissolved oxygen were recorded immediately after the animals were added (0 h) and at 12 h. After 12 h, the lamprey were euthanized, one container at a time by cutting off water flow to the container, and adding a slurry of buffered tricaine methanesulfonate sufficient to anaesthetize the animals (0.5 g L^{-1} buffered with 1.0 g L^{-1} of NaHCO$_3$; MS-222), before transferring them one at a time to container containing a lethal dose (1.5 g L^{-1} buffered with 3.0 g L^{-1} of NaHCO$_3$) of the anaesthetic. Lampreys were weighed, body length was measured, and then dissected for collection of brain and liver. The brain, liver and remaining carcass were snap frozen in liquid nitrogen and stored at -80 ℃ until transported to WLU on dry ice. Upon arrival, tissues and carcasses were stored at -80 ℃ until further analysis.

2.3 Analytical Techniques

2.3.1 Quantification of TFM Concentrations in Water

Quantification of TFM concentration in water during range-finder and acute toxicity tests were completed using a spectrophotometric assay following Standard Operating Procedures of the Sea Lamprey Control Centre (IOP: 012.4, Fisheries and Oceans Canada, Sault Ste. Marie, ON). Briefly, the absorbance of freshly collected water samples (analyzed within 1 h of collection) and freshly prepared TFM standards (0, 0.5,

1.0, 2.0, 3.0, 5.0 and 7.0 g L^{-1}) were read at a wavelength of 395 nm using a Genesys 6 spectrophotometer (at HBBS; Thermo Electron Corporation, MA, USA), and the data used to calculate water TFM concentration.

2.3.2 Solid Phase Extraction and High-Performance Liquid Chromatography

Whole-body TFM concentrations were determined using high performance liquid chromatography (HPLC), following solid phase extraction (SPE; Appendix, Figure A1.2) as described by Hubert *et al*. (2001), in Birceanu *et al*. (2014). Larval sea lamprey from the seasonal range-finder toxicity tests were randomly selected from aquaria with 2.0 mg L^{-1} TFM.

Briefly, quantification of TFM was done using a Varian HPLC set-up, comprised of a Varian ProStar 410 auto-sampler, ProStar 230 solvent delivery module and Prostar 310 UV-VIS detector (Varian, Inc., Palo Alto, CA, USA), fitted with a reverse phase HPLC C-18 column (Kinetex 2.6 μm XB-C18 100A 100 x 3.00 mm; Phenomenex Inc., CA, USA). TFM standards (0.015, 0.050, 0.250, 0.500, 1.500, and 5.000 μg mL^{-1}) were prepared from analytical grade TFM (Sigma Aldrich, St. Louis, MO, USA) in 20 mM sodium borate buffer (pH 8.5 ± 0.2). Standards and samples were injected (0.1 mL) via a mobile phase comprised of 83 % 20 mM sodium borate buffer (pH 8.5 ± 0.2) and 17 % acetonitrile, which yielded a TFM retention time of approximately 4.66 min. Percent recovery of TFM was determined to be 58 ± 9 %, which made it necessary to correct values for TFM and TFM-glucuronide by multiplying raw values by 1.42. Chromatographs were generated using Varian Star 5.51 software (Appendix, Figure A1.3; Varian, Inc., Palo Alto, CA, USA). Solutions were prepared with HPLC-grade chemicals and filtered reverse osmosis water (0.20 μm Millipore filters, Millipore, ME,

USA). Control samples were fortified with a 100 ng mL^{-1} TFM solution (Sigma Aldrich, St. Louis, MO, USA) to determine percent recovery. As above, corrected values for TFM and TFM-glucuronide were reported after raw values were multiplied by 1.42.

2.3.3 Glucose and Glycogen Analysis

Larval sea lamprey carcasses (N = 10 – 15 per experiment) were homogenized by mortar and pestle under liquid N_2, and approximately 100 mg of the powder was transferred into a 1.5 mL microcentrifuge tube containing four volumes of 8 % PCA and 1 mmol L^{-1} EDTA solution, vortexed, placed on ice for 10 minutes and centrifuged for 5 minutes at 4 °C and 10,000 g. The supernatant (100 µL) was then neutralized with 15-30 µL 3 M K_2CO_3, vortexed, and frozen in liquid nitrogen. To determine glycogen concentration, the supernatant was separated into two 100 µL aliquots; the first, for background glucose, was neutralized with approximately 5 µL of 3M K_2CO_3, and the second incubated with 20 µL of amyloglucosidase solution to hydrolyze the glycogen into glucose (glucosyl units) (pH 4.5 – 4.6) for 2 h in a water bath at 37 °C. After incubation, 70 % PCA was added to terminate the reaction, followed by neutralization of the sample with approximately 3M K_2CO_3. The free glucose in the background glucose aliquot and the amyloglucosidase treated aliquot was quantified using the LiquiColor® enzymatic method (Reference No. 1070-125, StanBio Laboratory, TX, USA) in 96-microwell plates at 500 nm (NovaSpec II spectrophotometer; Pharmacia Biotech, Cambridge, England, UK) after 10 min incubation at 37 °C.

2.3.4 Brain and liver analysis

Brain and liver glycogen (N = 10 – 12 per experiment) were determined using the same method as in Section *2.3.3 (Glucose and Glycogen Analysis)*. For livers, a 20 µL

aliquot was taken during liver protein analysis as outlined in Section 2.3.3 (*Protein*), and 1 part (20 μL) 16 % PCA was added for a final concentration of 8 % PCA in the microcentrifuge tube; for brains, 10 parts PCA was added.

2.3.5 Lipid analysis

Lipid content in the carcass was determined gravimetrically (N = 8 – 11 per experiment), using the chloroform:methanol extraction method (Lauff and Wood, 1996). Briefly, after grinding the carcasses to a fine powder under liquid N_2, approximately 100 mg of tissue was added to 10 mL of chloroform:methanol (2:1) in a 20 mL glass scintillation vial and left to incubate for 12 h at 4 °C. After incubation, 2.6 mL of 0.9 % NaCl solution was added and the samples were again left to incubate for 12 h at 4 °C. Next, a 5-mL syringe fitted with a 25 G needle was used to collect the 4-mL chloroform phase into a pre-weighed glass culture tube and chloroform was evaporated to dryness under a stream of nitrogen gas. The culture tubes were then transferred to a desiccator for 1 h to ensure that any residual chloroform had evaporated and were then re-weighed to determine the mass of lipid in the tube. Lipid was calculated as outlined in Section 2.4 (*Calculation and Statistics*).

2.3.6 Protein analysis

Larval sea lamprey carcasses (N = 8 – 11 per experiment) were homogenized as described above, and four parts 50 mM tris(hydroxymethyl)aminomethane buffer (pH 7.4) were added to a 1.5 mL microcentrifuge tube. The slurry was further broken down with a hand-held pestle (PowerGen model 125 Homogenizer, Fisher Scientific, Mississauga, ON), vortexed and put on ice. The samples were then diluted 50 times for protein analysis, which was quantified spectrophotometrically using the bicinchoninic

acid (BCA) assay (Smith *et al*. 1985), with bovine serum albumin for standards. Samples were incubated at 37 ºC for 30 minutes and the absorbance was determined at 562 nm in a 96-well plate, using a NovaSpec II spectrophotometer (Pharmacia Biotech, Cambridge, England, UK).

Livers (N = 11 – 12 per experiment) were analysed using the same method except that they were homogenized in five parts TRIS (hydroxymethyl) aminomethane buffer (50 mM, pH 7.4) containing protease inhibitor (1 mg, Sigma). Sample preparation and analysis were done as previously described.

2.3.7 Water content and dry ash analysis

Percent water content and dry ash in larval sea lamprey carcasses (N = 9 – 10 per experiment) were determined gravimetrically using standard methods. First, percent tissue water was determined in the carcasses (whole body minus brain and liver) of lamprey not exposed to TFM by grinding the carcass to a fine powder under liquid N_2. Approximately 50 mg of ground carcass was then placed in pre-weighed crucibles and dried to constant mass in a laboratory oven (Barnstead Thermolyne, 48000 furnace) at 60 °C, over 48 h. The amount of dry ash was determined by combusting the dried tissue at 750 °C for 4 h. Water content and dry ash were calculated as outlined in Section 2.4 (*Calculations and Statistics*).

2.4 Calculations and Statistics

2.4.1 Glucose and glycogen

Glycogen was determined spectrophotometrically and calculated as follows:

Glycogen (μmol g^{-1} ww) = glycogen (μmol g^{-1} ww) – background glucose (μmol g^{-1} ww)

$$(1)$$

Results for glucose are presented in $\mu mol\ g^{-1}$ ww (wet weight) and results for glycogen are presented in μmol glucosyl units g^{-1} ww (wet weight). Modifications to the calculations were made as necessary for calculation of brain and liver glycogen.

2.4.2 Lipid

Lipid was determined gravimetrically; glass culture tubes were weighed before assay (empty) and after assay, with remaining lipid (g). Lipid was calculated as follows:

Lipid in aliquot (g) = [empty culture tube (g) + lipid (g)] – empty culture tube (g)   **(2)**

Lipid in sample (g) = [lipid in aliquot (g)] x [organic phase volume (mL)] / [aliquot volume mL)]   **(3)**

Where organic phase volume (mL) is the volume of the chloroform phase which separates from the methanol (approximately 7 mL) and the aliquot volume is the volume of chloroform phase transferred into the pre-weighed glass culture tube and evaporated (approximately 4 mL).

Lipid (mg lipid mg^{-1} ww) = lipid in sample (mg) / wet tissue (mg)   **(4)**

Where tissue mass is the mass of the ground tissue used for the analysis (approximately 100 mg). Results for lipid are presented in mg lipid mg ww^{-1}.

2.4.3. Protein

Protein was determined spectrophometrically and calculated as follows:

[Protein] = [protein]$_{sample}$ (mg mL^{-1}) x dilution volume (mL) x initial DF / wet tissue (g)

(5)

Where [Protein] is expressed in mg protein g^{-1} ww, $[protein]_{sample}$ is the protein concentration in the analyte calculated from the standard curve, dilution volume is the total combined volume of homogenate and distilled water (approximately 2.5 mL) and initial DF (5) is a dilution factor calculated as follows:

Initial DF = tissue mass (mg) + TRIS-HCl (µL) / tissue mass (mg)   **(6)**

Where wet tissue mass is the mass of ground sample in grams. Results for protein are presented in mg protein mg ww^{-1}. Modifications to the calculations were made as necessary for calculation of liver protein.

2.4.4 Water content and dry ash

Percent tissue water was calculated as follows:

% tissue water = [wet tissue (mg) – dry tissue (mg)] / wet tissue (mg) x 100 %   **(7)**

Percent dry ash was equivalent to the amount of matter remaining in the crucible after combustion in the muffle furnace divided by the wet tissue mass according to the following equation:

% dry ash = [dry ash (mg) / wet tissue (mg)] x 100 %   **(8)**

2.4.5 Hepatosomatic index (HSI) and condition factor (CF)

The HSI was based on the wet liver mass divided by body mass according to the following formula:

HSI = liver mass (mg) / body mass (mg) x 100   **(9)**

Whereas, CF was calculated according to Holmes *et al.* (1994) using the equation below:

$$CF = ([\text{mass (g)}] / \text{length (mm)}]^3 \times 10^6 \tag{10}$$

Probit analysis, linear regression (MLE) and maximum likelihood estimates were used to calculate lethal concentrations (LCs) using Comprehensive Environmental Toxicity Information System software (CETIS, Tidepool Scientific Software, McKinleyville, CA, USA, v1.8.5). Values for the 12 h LC_{50} and 12 h $LC_{99.9}$ between seasons or temperature were considered significantly different if the 95 % confidence intervals (CI) did not overlap (Wheeler *et al*. 2006).

Differences in larval lamprey length, mass, proximate body composition (glucose and glycogen, lipid, protein, water and dry ash), brain and liver glycogen, liver protein and hepatosomatic index (HSI) were analysed using analysis of variance (ANOVA), Kruskall-Wallis test, Welch's ANOVA or Dunns Test, as appropriate, depending on assumptions of normality and homogeneity of variance. To determine where the significant differences lie, post hoc Tukey's HSD, Multiple comparison test after Kruskal-Wallis, Dunn's Test of Multiple Comparisons Using Rank Sums or Games Howel test were used, as appropriate. Statistical analyses were conducted using R (version 3.1.3), R Studio (version 3.2.3) and figures were produced using Excel. Statistical significance was assessed at $\alpha = 0.05$.

2.5 Results

2.5.1 Effects of Season and Temperature on the Acute Toxicity of TFM

There were significant differences between the 12 h LC_{50} among acute toxicity tests run at 6 °C in April, 20 °C in June, 23 °C in August, and 12 °C in October. The sensitivity of sea lamprey to TFM was greatest in colder (5.6 °C) water, during the spring

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(April), when the 12 h LC_{50} of TFM was 1.18 mg L^{-1} (95 % CI = 1.15 – 1.23; pH 7.68 ± 0.02). However, TFM tolerance increased markedly during the summer with the LC_{50} increasing more than two-fold by June (20.6 °C), to a value of 2.55 mg L^{-1} (95 % CI = 2.41 – 2.69; pH 7.96 ± 0.04), then peaked in August, when the 12 h LC_{50} was 3.15 mg L^{-1} (95 % CI = 3.04 – 3.26; pH 8.30 ± 0.01) and the water was warmest (23.5 °C). This was followed by an approximately 50 % reduction in the 12 h LC_{50}, to 1.64 mg L^{-1} (95 % CI = 1.59 – 1.69; pH 8.26 ± 0.01) in October, at which time the water temperature was 12 °C (Figure 2.1). Similar, but more variable trends were observed when the acute toxicity data were expressed as the minimum lethal concentration (MLC) of TFM (12 h $LC_{99.9}$). The MLC increased more than two-fold from 1.65 mg L^{-1} (95 % CI = 1.48 – 2.11; pH 7.68) in April to 3.98 mg L^{-1} (95 % CI = 3.44 – 5.95; pH 7.96 ± 0.04) in June, peaked in August to 4.91 mg L^{-1} (95 % CI = 4.49 – 5.71; pH 8.30 ± 0.01) before falling to 2.32 mg L^{-1} (95 % CI = 2.16 – 2.60 pH 8.26 ± 0.01) in October (Figure 2.1A).

Temperature markedly influenced TFM sensitivity, resulting in significant differences between the 12 h LC_{50} amongst fish subjected to acute tests at 6, 12 or 21 °C. TFM tolerance was lowest at 6 °C, when the 12 h LC_{50} was 1.41 mg L^{-1} (95 % CI = 1.37 – 1.46; pH 7.85 ± 0.03), increased to 1.96 mg L^{-1} (95 % CI = 1.90 – 2.01; pH 8.05 ± 0.03) at 12 °C and then to 3.27 mg L^{-1} (95 % CI = 3.04 – 3.26; pH 8.24 ± 0.01) at 21 °C (Figure 2.1B). Similarly, there were significant differences in the MLC, such that the $LC_{99.9}$ at 6 °C was significantly lower than at 21 °C. Overall, the 12 h $LC_{99.9}$ increased with temperature from 2.07 mg L^{-1} (95 % CI = 1.90 – 2.37; pH 7.85 ± 0.03) at 6 °C, to 2.85 mg L^{-1} (95 % CI = 2.65 – 3.20; pH 8.05 ± 0.03) at 12 °C, and then to 5.02 mg L^{-1} (95 % CI = 4.50 – 6.41; pH 8.24 ± 0.01) at 21 °C (Figure 2.1B).

2.5.2 Effects of Season and Temperature on TFM Accumulation and Detoxification

In the larval sea lamprey sampled at the 12 h LC_{50} from the range-finder toxicity tests, the whole-body TFM burden was not significantly different in larval sea lamprey that suffered mortality in May, June, August and October, with values ranging from 28.4 to 144.4 nmol TFM g^{-1} ww. TFM-glucuronide concentration ranged from 0.3 to 6.1 nmol TFM-glucuronide g^{-1} ww (Table 2.1).

Range-finder results and acute toxicity test, TFM measured aquaria concentrations and water chemistry data are provided in Supplementary Data, Section 2.1.A. (*Preliminary Range-Finder Toxicity Test Results and Acute Toxicity Test Water Chemistry*) and Supplementary Data Tables 1.3A – 1.9A.

2.5.3 Effects of Season and Temperature on the Proximate Body Composition of Sea Lamprey

2.5.3.1 Glucose and Glycogen

Season had no effect on the concentrations of carcass (whole body minus brain and liver) glucose in larval sea lamprey, which averaged 7.2 ± 0.2 µmol g^{-1} ww, 7.3 ± 0.1 µmol g^{-1} ww, 7.2 ± 0.1 µmol g^{-1} ww and 7.3 ± 0.1 µmolg^{-1} ww in April, June, August and October, respectively (Kruskal-Wallis chi-squared = 4.85, DF = 3, P = 0.18). There were however, differences in glycogen (Kruskal-Wallis chi-squared = 7.04, DF = 3, P = 1.80 x 10^{-7}), which averaged 24.2 ± 5.4 µmol g^{-1} ww in April, 21.1 ± 4.4 µmol g^{-1} ww and 19.5 ± 3.5 µmol g^{-1} in June and August, before significantly decreasing to 11.31 ± 2.8 µmol g^{-1} ww in October (Figure 2.2A). Mean brain glycogen exhibited similar, but more pronounced trends (Kruskal-Wallis chi-squared = 13.017, DF = 3, P = 1 x 10^{-12}), averaging 131.2 ± 20.0 µmol g^{-1} ww and 128.0 ± 8.8 µmol g^{-1} ww in April and June, respectively. By August, however, brain glycogen had significantly decreased to 83.3 ±

7.4 µmol g^{-1} ww, where it remained through October (85.4 ± 9.5 µmol g^{-1} ww; Figure 2.2B). The opposite trend was observed in the liver, which was lowest in April, at 6.4 ± 1.2 µmol g^{-1} ww, and then doubled in June to 12.8 ± 1.3 µmol g^{-1} ww, followed by a slight drop to 9.6 ± 0.9 µmol g^{-1} ww in August, before peaking at 15.5 ± 1.5 µmol g^{-1} ww in October (Figure 2.2C; Kruskal-Wallis chi-squared = 19.59, DF = 3, P = 1.00 x 10^{-12}).

Temperature acclimation slightly affected mean carcass glucose concentration, which averaged 8.8 ± 1.3 µmol g^{-1} ww, 7.4 ± 0.3 µmol g^{-1} ww, and 6.9 ± 0.1 µmol g^{-1} ww in larval lamprey at 6, 12 and 21 °C, respectively (Kruskal-Wallis chi-squared = 14.56, DF = 2, P = 1.00 x 10^{-3}). Mean carcass glycogen was more variable (Kruskal-Wallis chi-squared = 4.03, DF = 2, P = 1.30^{-2}), however, averaging 9.0 ± 1.5 and 10.4 ± 2.1 µmol g^{-1} ww at 6 °C and 12 °C, but it was significantly lower at 21 °C, where it averaged 6.0 ± 1.2 µmol g^{-1} ww (Figure 2.3A). Mean brain glycogen was inversely proportional to acclimation temperature, averaging 173.9 ± 24.1 µmol g^{-1} ww at 6 °C, significantly decreasing to 147.6 ± 24.1 µmol g^{-1} ww at 12 °C and 58.0 ± 5.0 µmol g^{-1} ww at 21 °C (Figure 2.3B; Kruskal-Wallis chi-squared = 20.08, DF = 2, P = 1.00 x 10^{-12}). There were no significant effects of temperature on mean liver glycogen, which averaged 11.2 ± 1.0 µmol g^{-1} ww, 11.1 ± 0.8 µmol g^{-1} ww and 11.6 ± 1.0 µmol g^{-1} ww at 6, 12 and 21 °C, respectively (Figure 2.3C; Kruskal-Wallis chi-squared = 1.76 x 10^{-2}, DF = 2, P = 0.99).

2.5.3.2 Whole-body lipid

Season had a pronounced effect on mean carcass lipid in larval sea lamprey (ANOVA, F = 6.68, DF = 3, P = 1.06 x 10^{-3}), which decreased in a stepwise fashion between April, when carcass lipid averaged 160 ± 29 mg g^{-1} ww, decreased to 115 ± 16

and 114 ± 18 mg g^{-1} ww in the summer (June and August), followed by a further decline in October to 47 ± 11 mg g^{-1} ww (Figure 2.4A). However, acclimation temperature had no significant effect on mean carcass lipid which averaged 109 ± 9, 102 ± 19, and 99 ± 17 mg g^{-1} ww, at 6, 12, and 21 °C, respectively (Figure 2.4B; ANOVA, F = 0.38, DF = 2, P = 0.69).

2.5.3.3 Whole-body and liver protein

Mean carcass protein concentrations did not change with season in larval sea lampreys (ANOVA, F = 0.49, DF = 3, P = 0.69), averaging 85.7 ± 7.7 mg mg^{-1} ww in April, before dropping to 63.5 ± 4.9 mg mg^{-1} ww and 51.8 ± 5.5 mg mg^{-1} ww in the summer, and then increasing to 72.4 ± 4.5 mg mg^{-1} ww in the fall (Figure 2.5A). Temperature acclimation had no significant effect on mean carcass protein, which averaged 75.5 ± 12.4, 59.7 ± 3.4 and 60.9 ± 12.6 mg g^{-1} ww at 6, 12, and 21 °C, respectively (Figure 2.5B; Welch's ANOVA, F = 0.71, num DF = 2, denom DF = 10.54, P = 0.52).

Liver protein concentrations varied with season (ANOVA F = 7.356, DF = 3, P = 4.38 x 10^{-4}), but not with temperature (Welch's ANOVA, F = 0.19743, num DF = 2.00, denom DF = 18.47, P = 0.82). Mean liver protein averaged 127.0 ± 7.2 mg mg^{-1} ww in April and was reduced by approximately 25% to 95.5 ± 4.9 mg mg^{-1} ww and 88.6 ± 8.1 mg mg^{-1} ww in the summer. However, liver protein rebounded to a concentration of 113.3 ± 5.1 mg mg^{-1} ww in the fall (Figure 2.6A). Mean liver protein concentrations in the temperature experiments averaged 106.3 ± 5.0, 102.4 ± 3.4, and 108.4 ± 8.7 mg mg^{-1} ww at 6, 12 and 21 °C, respectively (Figure 2.6B).

2.5.3.4 Water content and dry ash

The mean water content of larval sea lamprey, based on measurements of the whole-body in April was 77.7 ± 1.8 %, but dropped significantly in the summer to 72.7 ± 1.3 % and 74.4 ± 1.1 % in the summer (June, August, respectively), before significantly increasing to 81.4 ± 0.7 % in October (Figure 2.7A; Kruskal-Wallis chi-squared = 18.00, DF = 3, P = 1.00 x 10^{-3}). Acclimation to different temperatures had no significant effect on the carcass water content of larval lamprey, which averaged 77.0 ± 1.0 %, 77.9 ± 0.5 %, and 77.6 ± 0.9 % at 6, 12 and 21 °C, respectively (Figure 2.7B; Kruskal-Wallis chi-squared = 0.30, DF = 2, P = 0.86).

Whole-body dry ash also varied seasonally, averaging 0.85 ± 0.02 % in April, and then slightly decreasing to 0.79 ± 0.02 % and 0.80 ± 0.02 % in June and August. By October, mean dry ash was lowest, at 0.69 ± 0.04 % (Figure 2.7A; Kruskal-Wallis chi-squared = 14.44, DF = 3, P = 1.00 x 10^{-3}). Temperature acclimation resulted in greater variation in mean dry ash of larval sea lamprey. However, there were no significant differences among the animals acclimated to 6, 12 and 21 °C, which averaged 0.84 ± 0.01 %, 0.79 ± 0.02 % and 0.96 ± 0.09 %, respectively (Figure 2.7B; Kruskal-Wallis chi-squared = 5.48, DF = 2, P = 0.06).

2.5.3.5 Hepatosomatic Index Analysis

Mean hepatosomatic index (HSI) changed with season in larval sea lampreys, averaging 1.21 ± 0.06 in April, before dropping to 1.00 ± 0.05 and 0.79 ± 0.07 in the summer (June and August) and then again increasing to 1.09 ± 0.05 (ANOVA, F = 11.18 DF = 3, P = 1.4 x 10^{-5}). Temperature also had a pronounced effect on mean HSI in larval sea lamprey, which averaged 1.34 ± 0.04 at 6 °C, decreased significantly to 1.09 ± 0.07,

and then decreased again to 0.99 ± 0.04 (Table 2.2; Welch's ANOVA, $F = 9.64$, DF = 2, $P = 1.00 \times 10^{-3}$).

Larval lamprey length and mass, brain and liver mass and water chemistry data are provided in Appendix A, Supplementary Data, Tables A2.10 – A2.13).

2.6 Discussion

2.6.1 Effects of Season and Temperature on the Acute Toxicity of TFM

It has long been known that the sensitivity of larval sea lamprey varies with season (Applegate *et al*. 1961; Scholefield *et al*. 2008), but the underlying mechanisms were poorly understood. The present study demonstrates that the greater tolerance of sea lamprey to TFM in the summer is due primarily to corresponding increases in water temperature. Applegate *et al*. (1961) first reported that the 'biological activity of TFM varies seasonally', with the maximum toxic effects of TFM occurring during 'the late fall, winter and early spring, and then declining through later spring and summer, with greatest TFM tolerance during July and August'. Using sea lamprey collected from streams in Michigan, Scholefield *et al*. (2008) demonstrated that TFM toxicity was greatest in the spring (May to June) compared to late summer (July to August), when the 9 h LC_{50} and $LC_{99.9}$ were 2- to 3-fold greater in a given stream. They also reported that spring 9 h $LC_{99.9}$ test values were similar to those predicted by pH–alkalinity charts (104% to 117% of the chart values), which are used to calculate TFM application amounts based on water pH and alkalinity measurements (Bills *et al*. 2003). In contrast, the corresponding 9-h $LC_{99.9}$ test values measured in the summer were 32-170 % higher than those predicted by the charts. They also noted that the discrepancies from the charts

in the summer were unrelated to differences in alkalinity or pH, each of which affect the bioavailability of un-ionized TFM, which is the main determinant of TFM accumulation in lamprey (Hunn and Allen 1974; Hlina *et al*. 2017).

The findings of the present study strongly suggested that the 2.5-3.0-fold greater tolerance of sea lamprey to TFM in the summer was due to warmer water temperatures, rather than differences in energy reserves. This conclusion is supported by the strong trend between temperature and the acute toxicity of TFM (12 h LC_{50}, 12 h $LC_{99.9}$). At first glance, this observation seems counter-intuitive because rates of TFM uptake and accumulation increase in direct proportion to water temperature (Hlina *et al*. 2017). Sediment or dissolved organic carbon absorption and adsorption of TFM are not factors in the current study, since there was no sediment in the aquaria. However, it should be noted that under such conditions the detoxification of TFM would be more efficient at warmer temperatures because like other metabolic processes, the reactions involved in TFM detoxification would proceed more quickly (see Hochachka and Somero 2002 for review). Because the lamprey were collected from the same sections of the Au Sable River, differences in local water chemistry and nutrient supply can also be ruled out as factors affecting TFM sensitivity. Moreover, all animals were acclimated to the same Lake Huron water prior to experiments at the HBBS.

In non-target fishes such as rainbow trout, the detoxification of TFM relies on reactions corresponding to Phase II metabolism that include glucuronidation (Lech and Statham 1975; Kane *et al*. 1994), sulfation (Bussy *et al*. 2018a), and possibly phase I processes including the reductive amination of TFM (Bussy *et al*. 2018a,b). The lamprey's lower capacity to bio-transform TFM using glucuronidation explains its greater

sensitivity to TFM (Lech and Statham 1975; Kane *et al*. 1994; Bussy *et al*. 2018b). While their overall capacity to detoxify TFM is lower than most non-target fishes, these studies demonstrate that lampreys do exhibit limited capacity to detoxify TFM (Kane *et al*. 1994; Bussy *et al*. 2018a,b). Moreover, Bussy *et al*. (2018b) recently demonstrated that other processes including sulfation, reductive and oxidative metabolism may be quantitatively important in TFM metabolism (Figure 2.11). Therefore, I propose that at higher acclimation temperatures, the combined higher activities of these enzymes may be sufficient to increase the sea lamprey's capacity to detoxify TFM by forming greater amounts of TFM-glucuronide, sulfated-TFM, and reduced amino TFM, which leads to the generation of glutathione and acetylated metabolites (Figure 2.8). While the quantitative importance of these newly identified metabolites has not yet been defined, these products are generally associated with detoxification. In the sea lamprey, the reduced amino metabolite of TFM is also more abundant than the sulfated and glucuronide conjugates, suggesting that it may play a more important role in TFM detoxification. Future studies, addressing the role of these metabolites in TFM detoxification, or toxicity, and how their production is influenced by increased temperature would be very informative.

TFM is a phenolic compound. It is notable that the toxicity of two other phenols known to uncouple oxidative phosphorylation, 4-nitrophenol and 2,4-nitrophenol, decreased in rainbow trout (*Oncorhynchus mykiss*) with increases in water temperature (Howe *et al*. 1994). Howe *et al*. (1994) also reasoned that an increased capacity to detoxify these compounds in warmer waters explained the greater survival of rainbow trout. Phenol toxicity to silver perch (*Bidyanus bidyanus*), rainbow trout (*Oncorhynchus*

mykiss), rainbowfish (*Melanotaenia duboulayi*) and western carp gudgeon (*Hypseleotris klunzingerii*) also decreased with increases in temperature that were well within the thermal tolerance ranges of these fishes (Patra *et al*. 2015). However, toxicity increased as water temperatures approached the thermal tolerance thresholds of each fish, when the combined effects of toxicant exposure and thermal stress likely made the fish more vulnerable. It should be noted that the maximum temperatures to which the sea lamprey in the present study were exposed, 21 °C, was well below their upper critical thermal tolerance limits of 29.5 – 31°C (Potter and Beamish 1975).

2.6.2 Effects of Season and Temperature on Proximate Body Composition

Exposure to TFM leads to a reduction of glycogen reserves in the brain and liver of sea lamprey and non-target fishes (Wilkie *et al*. 2007; Birceanu *et al*. 2009, 2014; Clifford *et al*. 2012; Henry *et al*. 2015) due to lower ATP production rates arising from TFM interference with mitochondrial oxidative phosphorylation (Birceanu *et al*. 2011). As a result, the animals rely on anaerobic energy reserves such as glycogen and phosphocreatine to make up for the shortfall in ATP supply, which likely culminates in death when these reserves are unable to meet ATP demands (Birceanu *et al*. 2009; Clifford *et al*. 2012). If glycogen reserves were lower in the spring due to decreased nutrient supply during the winter months, larval lamprey would be more vulnerable to TFM. This hypothesis was not supported, however. In fact, glycogen reserves were highest in the brain and carcass in the spring-early summer, before dropping markedly through the late summer and fall, suggesting that seasonal differences in these energy stores had little impact on TFM tolerance.

Unlike other vertebrates, the brain and meningeal tissue of the sea lamprey has very high glycogen reserves (e.g. Rovainen *et al*. 1971, Murat *et al*. 1979, Foster *et al*. 1993, Clifford *et al*. 2012). Indeed, glycogen levels in lamprey brain and meningeal tissue are at least four times those found in other vertebrates (Plisetskaya 1968, Rovainen *et al*. 1969, 1971), which may explain the relative unimportance of the liver as a site of glycogen storage in larval sea lamprey. These brain glycogen stores provide the lamprey with a large reservoir of glucose, an essential fuel for the central nervous system of chordates (Hochachka *et al*. 1993). Glucose supply to the brain is via glycogenolysis, which yields glucose-6-phosphate, which in turn is converted to glucose via the enzyme glucose-6-phosphatase (Rovainen *et al*. 1971). In other vertebrates, the liver fulfills this role, which in the lamprey appears to be less important. Because brain glycogen can drop by more than 50 % (Clifford *et al*. 2012) following TFM exposure, it was predicted that lower initial brain glycogen concentrations would make sea lamprey more susceptible to lampricide exposure. However, brain glycogen was well maintained through the winter, which argues against this prediction.

Liver glycogen, on the other hand, was lower in the spring, which might be because it was needed to sustain blood glucose through the winter. Indeed, carcass glucose stores were remarkably stable throughout the year (Figure 2.4A). Liver glycogen stores had more than doubled by the fall, but the liver glycogen storage patterns were not suggestive of any role in TFM tolerance. Nor were the patterns in carcass glycogen reserves, which steadily decreased. Because the bulk (~60 %; Thorson 1958; Wilkie *et al*. 2001) of the whole body is muscle, most of the glycogen likely reflects intramuscular stores, which would be essential for fueling burst swimming or burrowing (Boutilier *et*

al. 1993; Wilkie *et al*. 2001). Because metabolic rate and activity levels were higher in the warmer, summer months, it is possible that steady state glycogen stores were lower for this reason. Similarly, O'Boyle and Beamish (1977) reported comparable declines in muscle glycogen through the late spring and summer in non-metamorphosing sea lamprey. The reductions in glycogen stores in the carcass were not likely a consequence of changes in tissue water content, which could have resulted in lower wet weight glycogen concentrations. Instead, carcass water actually decreased between early spring and late summer, before increasing markedly in the fall, which is also consistent with previous observations made in non-metamorphosing sea lamprey (Lowe *et al*. 1973).

Also arguing against any interaction between tissue glycogen stores and TFM tolerance were the decreases in brain glycogen concentration though the spring and summer, when the 12 h LC_{50} and 12h $LC_{99.9}$ were highest. Although glycogen concentration, particularly in the brain, is affected by TFM exposure, the present findings indicate that glycogen stores are not a reliable predictor of TFM tolerance in sea lamprey.

Season had a pronounced effect on whole body lipid in larval sea lamprey but temperature did not. Lipid concentrations were highest in April, and continuously declined through the summer and fall. These observations were similar to much earlier work by Lowe *et al*. (1973), who made similar observations in larval sea lamprey studied over a one year period. The current studies suggest that higher lipid stores in the early spring were probably due to the onset of feeding following the spring thaw, followed by increased lipid consumption with warming waters. Indeed, this was noted by Kao *et al*. (2010) who showed that larval lamprey acclimated to 21 °C had total lipid amounts in the liver and kidneys that were more 30 % lower than in larvae acclimated to 13 °C.

Relatively lower lipid contents in the liver and kidneys of 21 °C-acclimated lamprey primarily resulted from a reduction in stored lipid reserve, triacylglycerol, but not structural lipid, phospholipid (Kao *et al*. 2010). Nevertheless, it may be worth additional investigation to study whether lipid stores additional amounts of TFM, in larger pre-metamorphic lamprey that have much higher lipid reserves (Lowe *et al*. 1973; O'Boyle and Beamish 1977; Kao *et al*. 1997), and thus increases TFM tolerance.

Lowe *et al*. (1973) previously reported that whole-body protein does not vary seasonally in larval lampreys, as shown in the present study. The protein composition (5.1 – 8.5 % ww) measured was slightly lower than reported by Lowe *et al*. (1973), which averaged (about 10.5 to 12.5% of the wet weight). These differences could be explained by a range of factors, including that the present lamprey generally had higher lipid reserves than those studied by Lowe *et al*. (1973), and the animals were generally smaller as well.

Seasonal variations in liver protein were evident, but temperature had no effect on liver protein. The reduced liver protein in the summer (June and August) coincided with a lower HSI at this time, which could be attributed to increased metabolic demands associated with living at warmer temperatures. There was also considerable glycogen accretion in the liver during the summer, which could have reduced the relative proportion of protein in the liver. The protein content of larval lamprey is in general not as high as in teleost fishes, lying at the extreme lower end of values reported for the class *Actinopterygii* (Brett, Shelbourne and Shoop 1969, Groves 1970, Beamish 1972, Niimi 1972). Protein content in lamprey also does increase with metamorphosis (Lowe *et al*. 1973), but as mentioned the lampreys in this study were not approaching metamorphosis,

so the observed trend cannot be attributed to pre-metamorphosis or metamorphosis. Although low whole-body and liver protein coincides with higher TFM tolerance in the late summer (August), it is unlikely it would not be a preferred source of ATP production during TFM exposure, because the oxidation of amino acids arising from proteolysis relies on oxidative phosphorylation to generate ATP. Nevertheless, it would be informative to determine the relative fuel use patterns of sea lamprey during the larval phases to better determine how they manage their energy stores during this trophic phase of the sea lamprey life cycle.

The ranges in water content values observed (72 – 81 %) are consistent with the literature when compared to the earlier work on larval sea lamprey by Lowe *et al.* (1973). Seasonal changes to water content have also been documented in many other fish species in winter and fall (marine fish, see Boran and Karaçam 2011; freshwater fish, see Sreenivasa *et al.* 1964). Dry ash in the seasonal experiments was significantly less in October than in April, June and August and significantly greater in April than in June while in the temperature experiments, there were no significant differences in dry ash of larval sea lamprey. These changes may be in response to physiological changes associated with, spawning, migration, and starvation or heavy feeding (Boran and Karaçam 2011). In general, season seems to have more effect on larval sea lamprey water content than temperature, although no trend can be observed between water content, dry ash and TFM sensitivity.

Body mass throughout the present experiment was similar in all groups, and the condition factor was greater than 1.5, suggesting that the animals were in good health. The slightly higher CF in the spring larvae coincided with a high carcass water content,

which could have increased the mass of the animals, leading to a slight overestimate of this value. That lipid, glycogen and protein were highest at this time suggests that even in the spring, the animal's nutrient reserves were not compromised. Similarly, because acclimation to different temperatures had little effect on CF, body mass and energy reserves, it demonstrates that the animals subjected to higher temperatures readily coped.

Tessier *et al*. (2018) reported that TFM uptake rates of larval sea lampreys were inversely related to the body mass of sea lamprey, with smaller lamprey (0.6 g) clearing TFM four times faster than larger lamprey (2.4 g). It was concluded that while there is a very strong correlation between metabolic rates and sensitivity to TFM, body size may only be indirectly related to toxicity. Rather, metabolic rates of species are more likely a driving factor for toxicity compared to body size (Baas and Kooijman 2015). This observation strongly suggests that acclimation to cooler or warmer temperatures did not compromise the physiology of the larval lampreys, providing greater confidence in the conclusion that the greater tolerance of sea lamprey to TFM in the summer compared to the spring and fall is a function of water temperature, and not the condition of the lamprey.

2.7 Conclusion

In conclusion, the tolerance of sea lamprey to TFM is lowest in spring, and then markedly increases through summer, when water temperatures and presumably food availability are greatest, before dropping in the fall. The hypothesis that TFM tolerance was related to greater energy stores was not supported, however, because lipid and glycogen reserves were in fact lowest during late summer, when TFM tolerance was

greatest. Rather, there appeared to be a cause and effect between water temperature and TFM tolerance, which led to 2.5-fold increases in the 12 h LC_{50} of the lampricide. The greater TFM tolerance at higher temperatures is likely due to a higher capacity to detoxify TFM via enzyme-mediated phase II pathways which biotransform TFM to its glucuronide and/or sulfate conjugates, and perhaps by phase I reduction and oxidation pathways. Although the activity of TFM-G is relatively low in sea lamprey, temperature-induced increases in the activities of these chemical reactions, and those involved in sulfation, and phase I reduction and oxidation paths, due to Arrhenius effects may be sufficient to increase survival. Testing this hypothesis should be a priority of future studies. Another priority would be conduct toxicity tests at the same experimental temperature using larval lamprey collected at different times of the year.

These observations could also have important implications for the sea lamprey control program in the Great Lakes. For instance, it may be prudent to incorporate season and water temperature into models that are used to evaluate the amounts of TFM required for lampricide applications. It may also be possible to reduce total TFM requirements by treating large streams or rivers earlier or later in the year, when sea lamprey are most sensitive to TFM. It will also be important to validate the results from these laboratory studies, by performing similar tests under natural stream conditions. Finally, sea lamprey control efforts in the Great Lakes could be further complicated by future increases in mean and peak water temperatures arising from climate change, which would increase the amounts of TFM required for lampricide applications. Thus, it is imperative to better understand how TFM uptake, distribution and elimination will be affected by further

increases in water temperature and how this could potentially affect ongoing efforts to control this invasive species in the Great Lakes.

TABLES & FIGURES

Table 2.1. Effects of TFM exposure (12 h LC$_{50}$; 2.0 mg L^{-1}) on the concentrations of 3-trifluoromethyl-4-nitrophenol (TFM) and TFM-glucuronide (TFM-G) in larval sea lamprey collected from the Au Sable River at different seasons in 2013, and the concentration of TFM in the whole body sea lamprey that either survived or experienced mortality during exposure to 2.0 mg L^{-1} TFM regardless of season. The limit of quantification (LOQ) was 7.5 x 10^{-3} mg L^{-1} and the limit of detection (LOD) was 3.7 x 10^{-3} mg L^{-1}. Data presented as mean ± standard deviation. Data sharing the same letters are not statistically different.

Season	Sample Size, N	TFM nmol g^{-1} ww	Sample Size, N	TFM-G nmol g^{-1} ww
April (5.6 °C)	6	82.8 ± 27.8 (A)	7	1.9 ± 0.5 (a)
June (20.6 °C)	6	79.1 ± 26.0 (A)	6	1.7 ± 0.6 (a)
August (23.5 °C)	6	56.7 ± 4.90 (A)	7	1.7 ± 0.4 (a)
October (11.7 °C)	7	52.0 ± 9.60 (A)	6	2.0 ± 0.5 (a)

Table 2.2 Hepatosomatic index (HSI) of larval sea lamprey in the proximate body composition experiments (12 containers, N = 3 – 5 per container, no 3-trifluoromethyl-4-nitrophenol added) by season and temperature. Seasonal proximate body composition experiments were conducted at the ambient stream temperature at the time of larval lamprey collection (shown in brackets); temperature proximate body composition experiments were conducted in July 2014. Data presented as mean ± standard deviation. Data sharing the same letters are not statistically different.

Experiment	Sample Size, N	HSI
April (5.6 °C)	12	1.21 ± 0.06 (A)
June (20.6 °C)	12	1.00 ± 0.05 (B)
August (23.5 °C)	12	0.79 ± 0.07
October (11.7 °C)	12	1.09 ± 0.05 (A,B)
6 °C	10	1.34 ± 0.04
12 °C	11	1.09 ± 0.07 (a)
24 °C	12	0.99 ± 0.04 (a)

Figure 2.1 The effects of (A) season and (B) water temperature on the 12h LC_{50} and minimum lethal concentration (12 h $LC_{99.9}$; MLC) of 3-trifluoromethyl-4-nitrophenol. Seasonal acute toxicity tests were conducted at the water temperature at which the larval lampreys were collected (in brackets). Data presented as the 12 h LC_{50} (hatched bars) or the MLC [solid bars; N = 3 with lamprey = 247 – 313 per season and temperature, ± 95 % confidence interval (CI)]. Bars where CIs do not overlap are significantly different from one another.
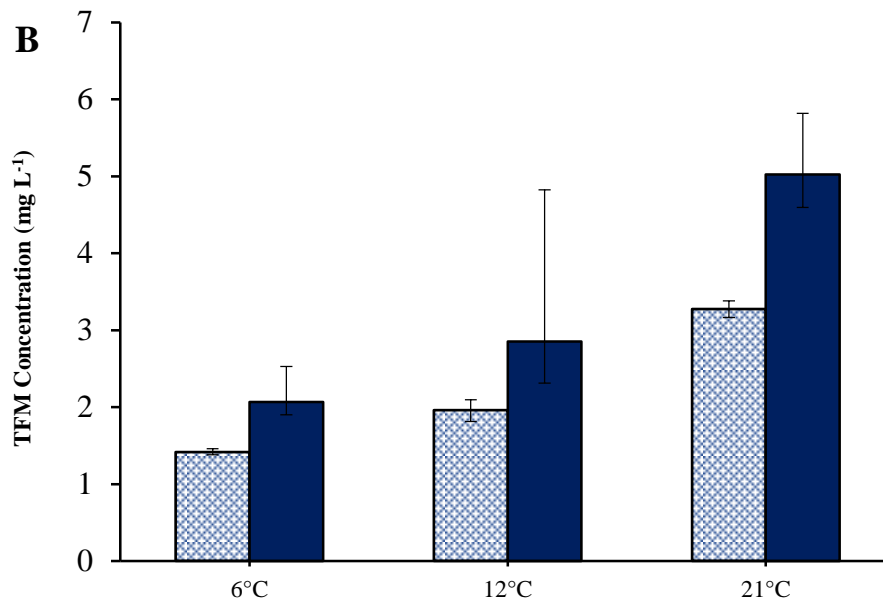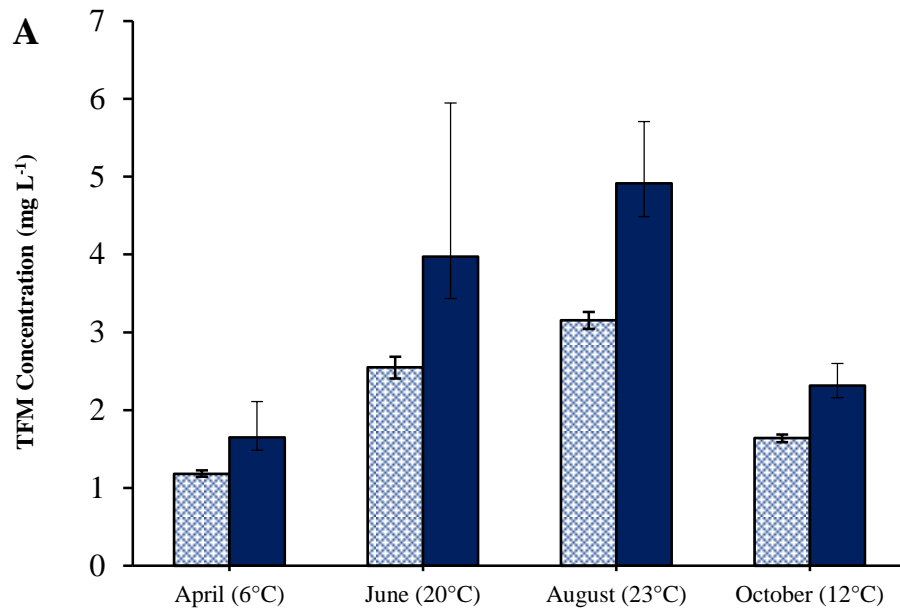
Figure 2.2 Influence of season on glucose (hatched fill) and glycogen (solid fill) in larval sea lamprey **(A)** carcass, **(B)** brain and **(C)** liver collected in the spring (April), early (June) and late (August) summer, and the fall (October). Values in brackets correspond to the to the water temperature at which the animals were collected and held prior to sampling. Data presented as the mean + 1 SEM, N = 11 – 12 per experiment. Bars sharing the same uppercase letters denote glycogen values that were not statistically significant from one another and identical lowercase letters denote glucose values that are the same.
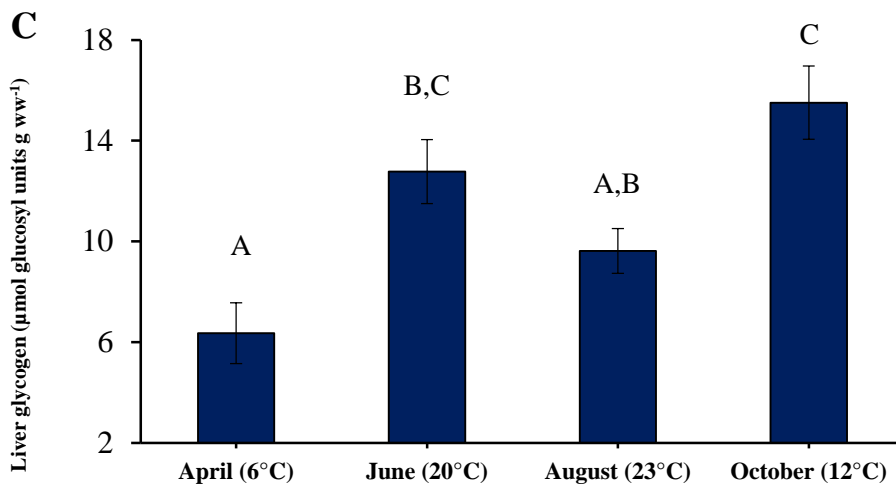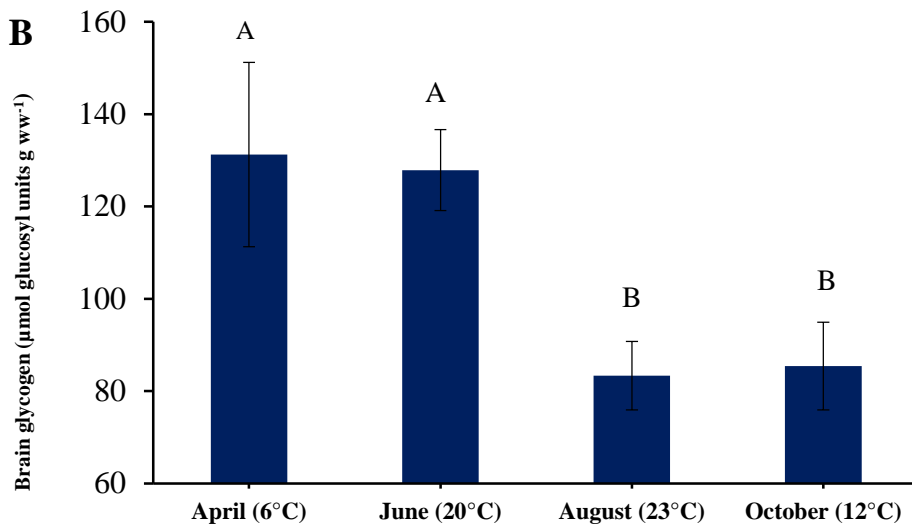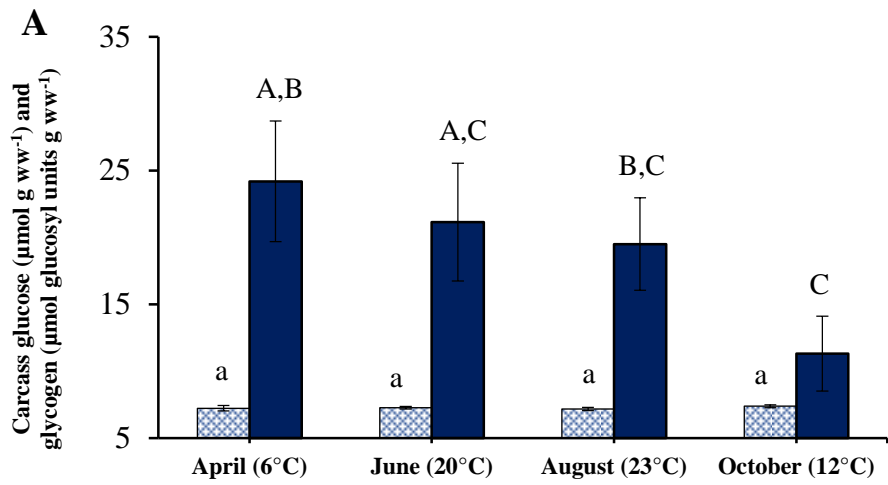
Figure 2.3 Influence of acclimation temperature on glucose (hatched fill) and glycogen (solid fill) in larval sea lamprey (A) carcass, (B) brain and (C) liver. Animals were captured from the Au Sable River, Michigan, temperatures correspond to the to the water temperature at which the animals were collected and held prior to sampling. Data presented as the mean ± 1 SEM, N = 8 – 11 per temperature. Bars sharing the same uppercase letters denote glycogen values that were not statistically significant from one another and identical lowercase letters denote glucose values that are the same.
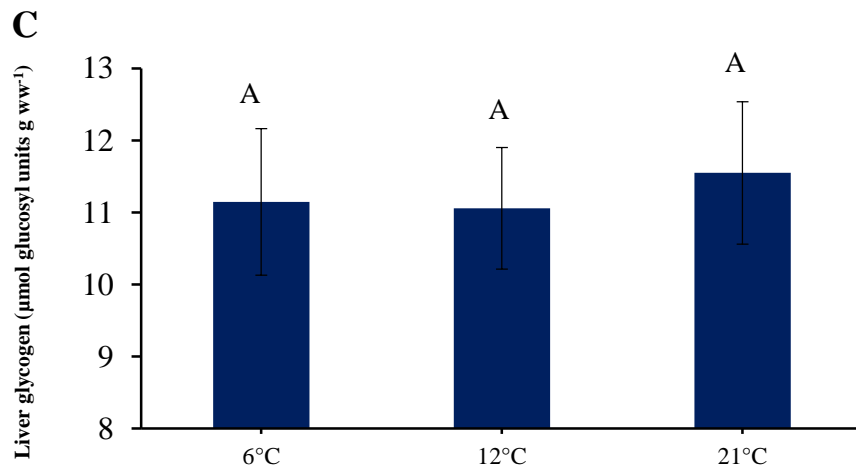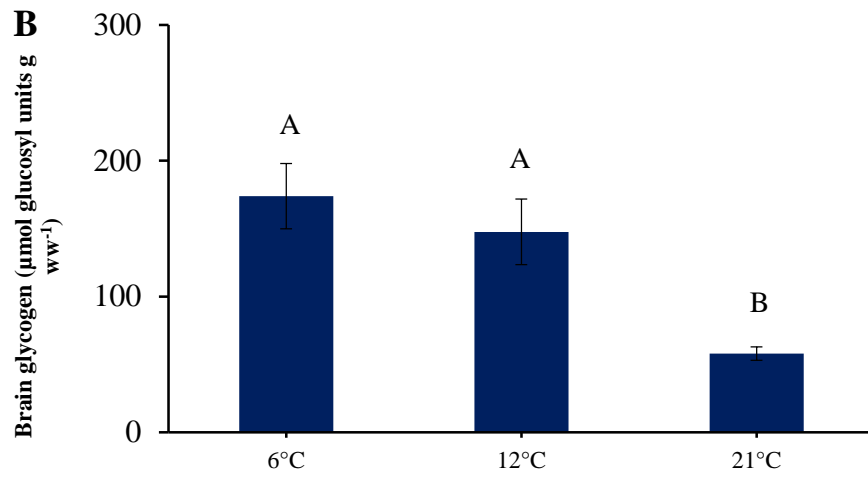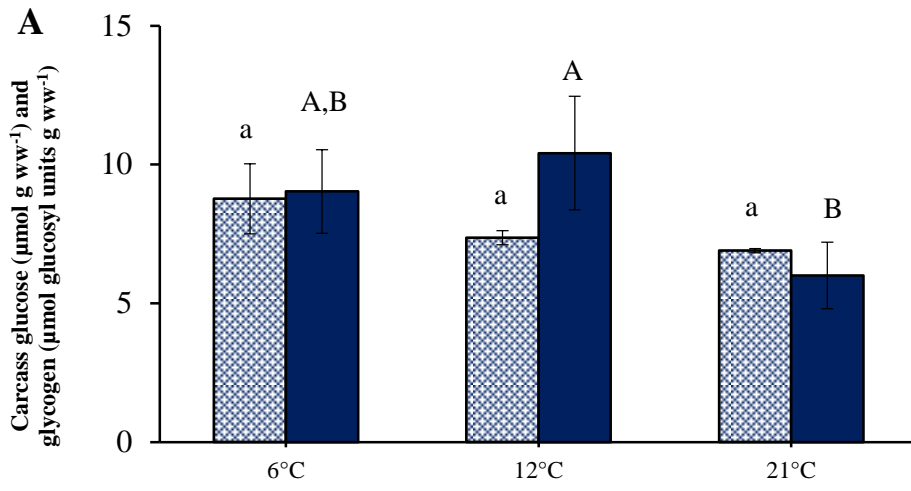
Figure 2.4 Influence of **(A)** season and **(B)** acclimation temperature on carcass lipid in larval sea lamprey captured from the Au Sable River, Michigan. Temperatures correspond to the to the water temperature at which the animals were collected and held prior to sampling. Data presented as the mean + 1 SEM, N = 8 – 11 per season, and N = 8 at each temperature. Bars sharing the same letters denote values that were not statistically significant from one another.
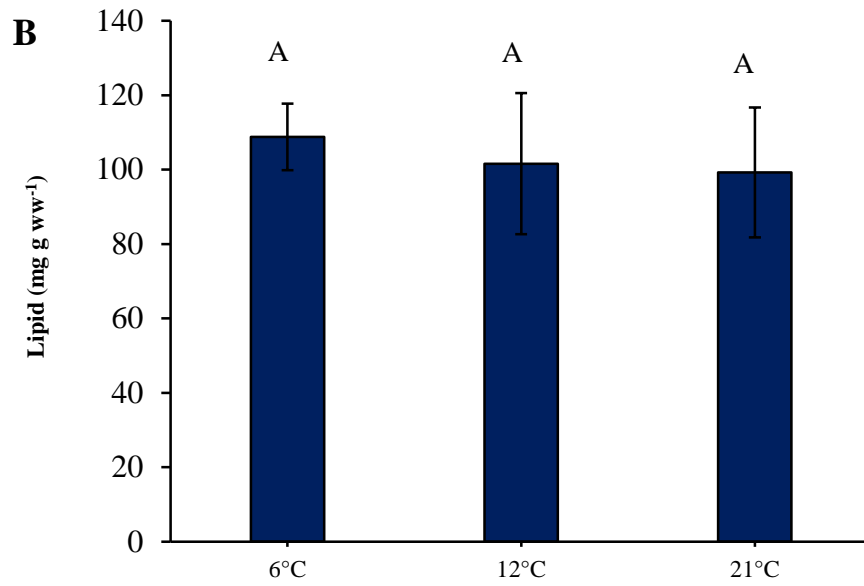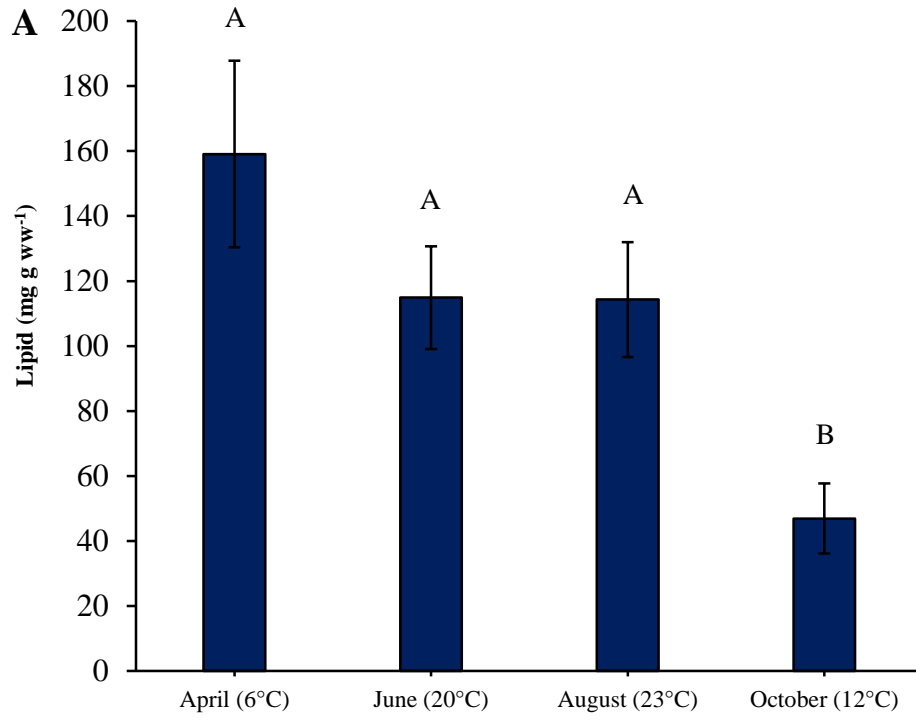
Figure 2.5 Influence of **(A)** season and **(B)** temperature on whole-body protein in larval sea lamprey. Seasonal proximate body composition experiments were conducted at the ambient stream temperature at the time of larval lamprey collection (shown in brackets). N = 8 – 11 per season. Bars represent the standard error of the mean (SEM). **B** Temperature proximate body composition experiments were conducted in July 2014. N = 7 – 8 per temperature. There were no significant differences in mean protein.
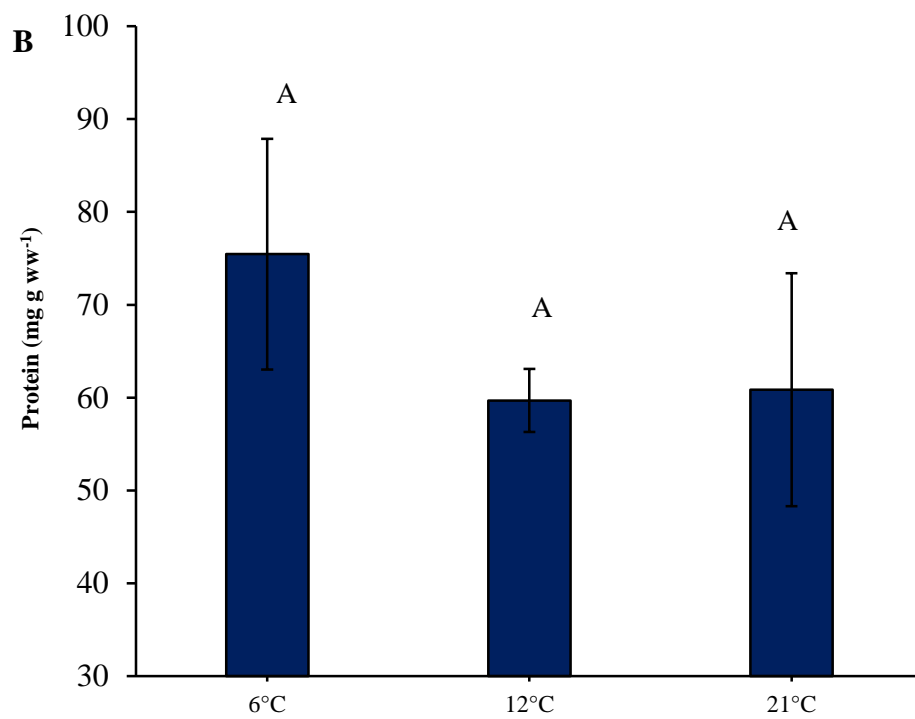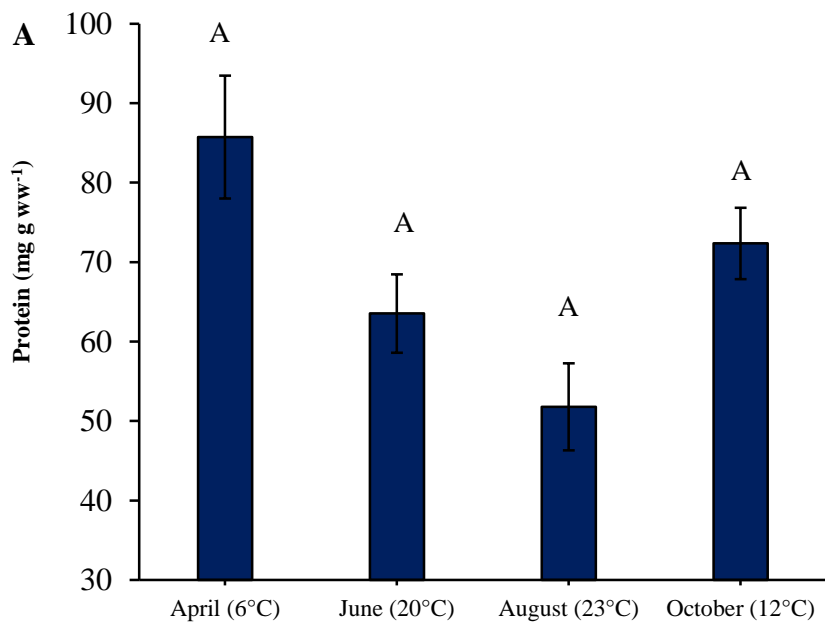
Figure 2.6 Influence of **(A)** season and **(B)** acclimation temperature on liver protein in larval sea lamprey captured from the Au Sable River, Michigan. Temperatures correspond to the to the water temperature at which the animals were collected and held prior to sampling. Data presented as the mean + 1 SEM, N = 11 – 12 animals per season, and N = 10 – 12 per temperature tested. Bars sharing the same letters denote values that were not statistically significant from one another.
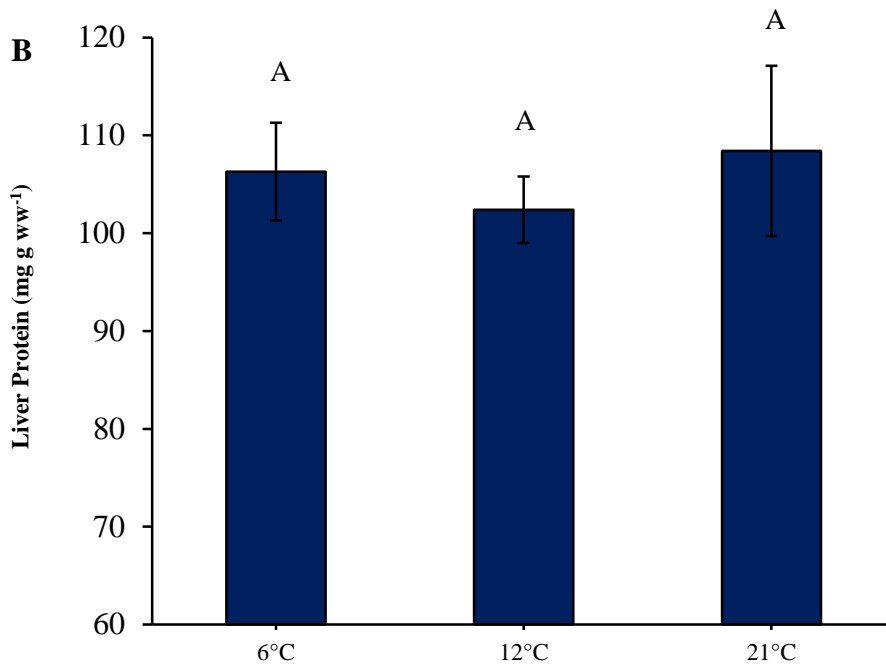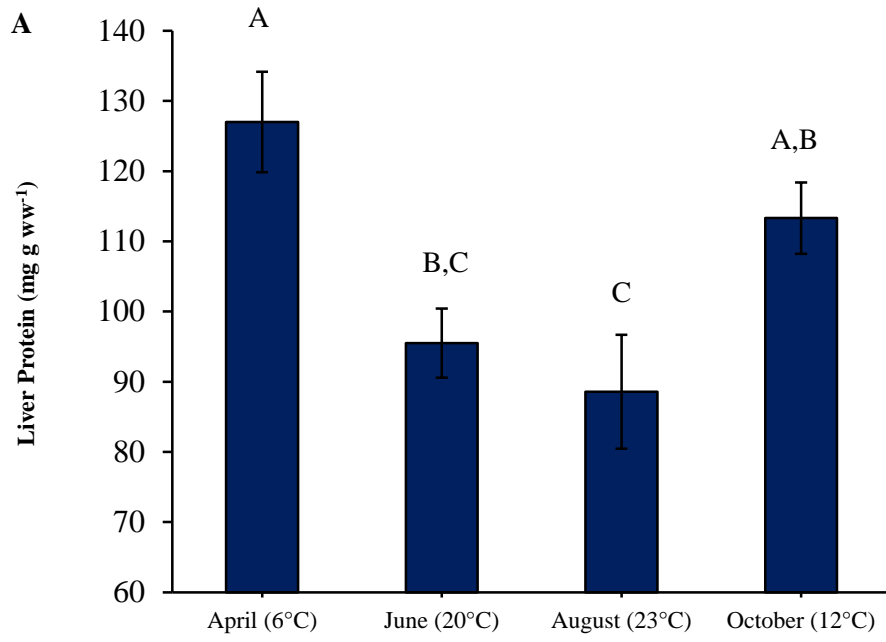
Figure 2.7 Influence of **(A)** season and **(B)** temperature acclimation on carcass water content (hatched fill) and dry ash (solid fill) of larval sea lamprey. Data presented as the mean ± 1 SEM, N = 8 – 11 carcasses per sample period (season or temperature) Bars sharing the same uppercase letter denote water content values that are not statistically different, whereas data sharing same lowercase letters represent dry ash measurements that are not statistically different.

A

B
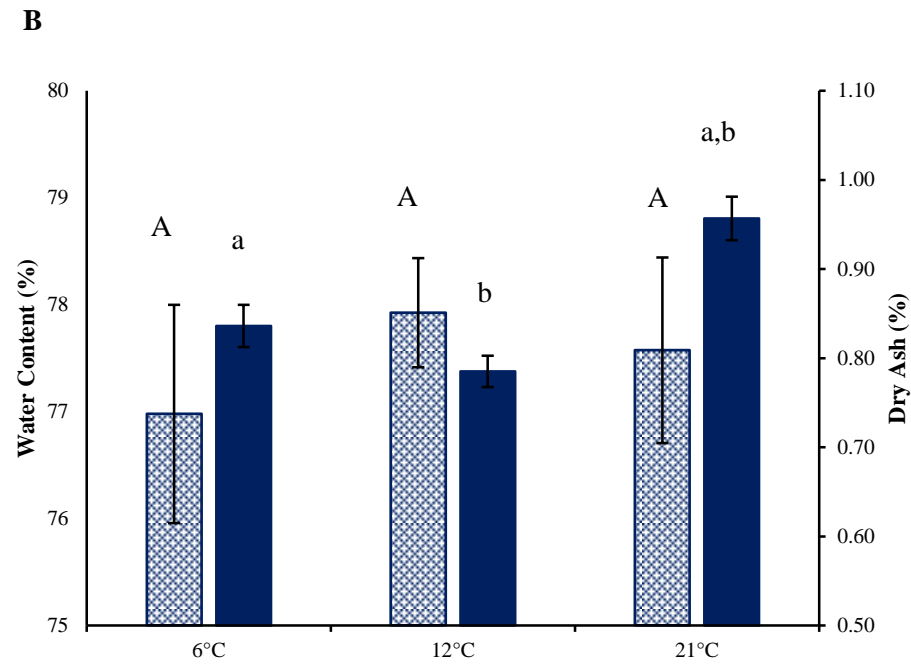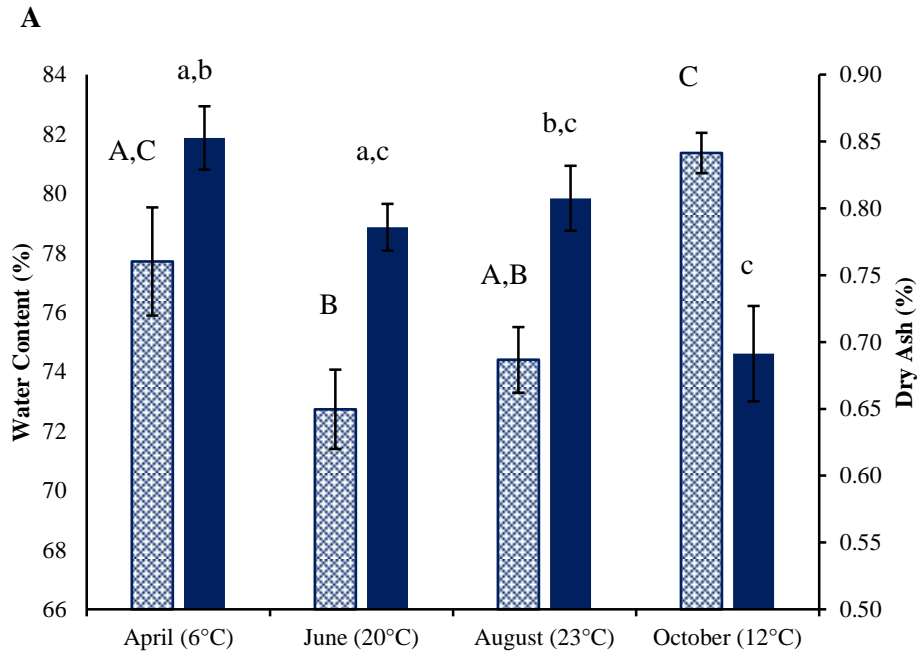
Figure 2.8 TFM metabolism in fish as proposed by Bussy *et al*. (2018). UDPGT, uridine diphosphate glucuronosyltransferase; UDPGA, uridine5′-diphosphoglucuronic acid; PST, phenol sulfate transferase; GST, glutathione S transferase; GSH, glutathione and NAT N-acetyltransferase (Source: Bussy *et al*. 2018).
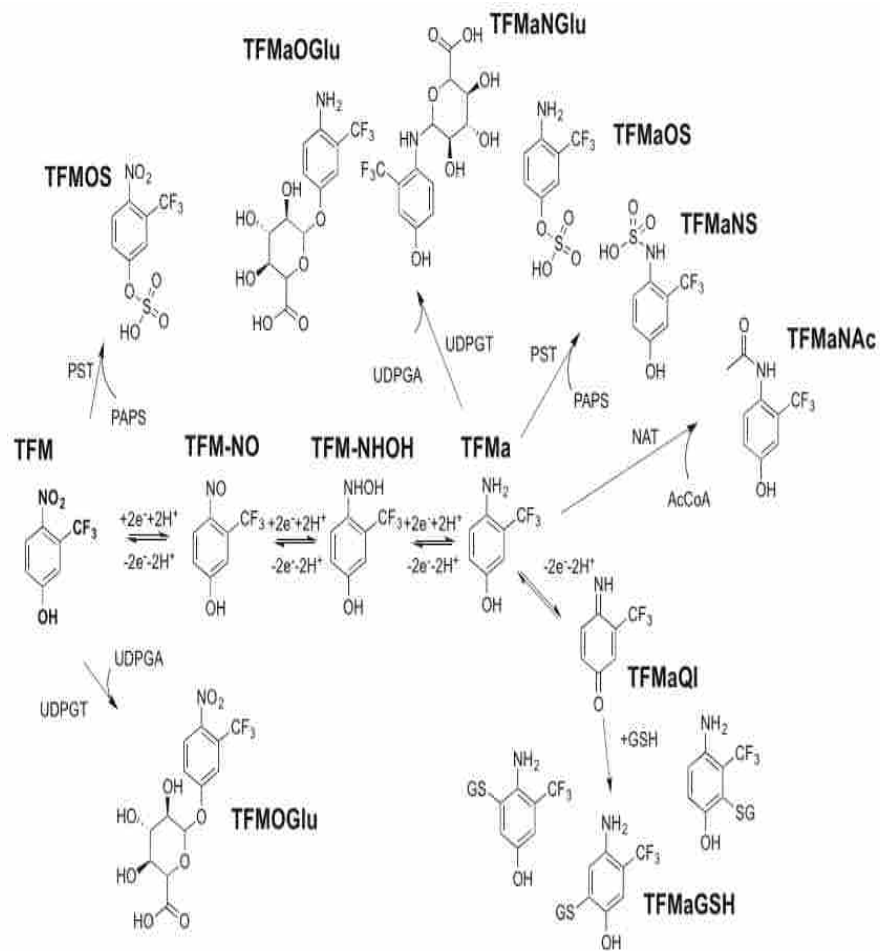
Chapter 3:

An integrated model of the effects of season and temperature on sea lamprey (*Petromyzon marinus*) sensitivity to the lampricide 3-trifluoromethyl-4-nitrophenol (TFM)

3.1 Introduction

The application of TFM to tributaries of the Great Lakes targets the invasive larval sea lamprey in the early spring to fall. Although it has been used for over 60 years, there remains a need to better understand why treatment residuals occur and how to reduce them. It is well known that TFM sensitivity is strongly influenced by biotic and abiotic variables, but the underlying reasons for this are poorly understood. The present study provided insight on the underlying factors that result in variations in TFM sensitivity. Although a direct statistical comparison between LC_{50}s and $LC_{99.9}$s (which is defined as the amount of TFM needed to kill 50 % and 99.9 % of larval sea lamprey, respectively) and proximate body composition was not established, there was a relationship between temperature and TFM sensitivity. Thus, the seasonal variations in TFM sensitivity reported here and in other studies (Applegate *et al*. 1958; Scholefield *et al*. 2008) were mainly due to corresponding changes in water temperature. Thus, it may be advisable to incorporate water temperature into predictive models of sea lamprey sensitivity to TFM. In this chapter, the results of the current study are discussed in context of current chemical control methods for sea lamprey, and the implications of this information on future control efforts of sea lamprey in the Great Lakes are also discussed.

3.2 Integrated Pest Management of the Sea Lamprey in the Great Lakes

Invasive sea lamprey became a threat to the Great Lakes in the 1900s, resulting in the collapse of major fisheries within the Great Lakes by the 1940s and 1950s (Siefkes *et al*. 2013). The integrated sea lamprey control program relies on several techniques to control sea lamprey, including velocity barriers, electrical barriers, adjustable-crest

barriers and cages to block or remove adult sea lamprey with minimal effects on non-target organisms (Hunn and Younges, 1980; McLaughlin *et al*. 2007). These physical control methods may eventually be used in combination with trial testing of mating pheromones and alarm cues, which could be used to guide lamprey towards barriers and traps (Johnson *et al*. 2005; Siefkes 2017). With chemical treatment, streams containing larval sea lamprey are treated with TFM every two to four years, resulting in decreased parasitic juvenile sea lamprey populations in the Great Lakes, which has led to the recovery of some game and commercial fisheries such as lake trout and whitefish (McDonald and Kolar 2007; Siefkes *et al*. 2013). However, residual sea lamprey that survive TFM treatment can undermine the effectiveness of sea lamprey control efforts. This thesis reveals that temperature is a key variable that needs to be considered when TFM is applied to sea lamprey infested streams.

3.3 Methods for Selecting Streams for Lampricide Treatment

Using an adaptive management approach ("science informs management"), the methods used to select streams for lampricide treatments are frequently evaluated and updated, resulting in a flexible treatment program that can be tailored to changes in lamprey distribution, abundance or abiotic variables including stream flows, and even time of year. The basis of this approach is prior assessment of larval density and stream productivity – the predicted number of transforming sea lamprey the following year – which is evaluated to select and prioritize streams for lampricide applications (Christie *et al*. 2003; Slade *et al*. 2003; Hansen and Jones 2008).

The two current stream survey methods are the quantitative assessment sampling (QAS) method and the rapid assessment (RA) approach (Hansen and Jones 2008). Using the QAS method, data on the density, size, distribution, and habitat (Slade *et al*. 2003) are considered. The densities are then used to rank streams for chemical treatment using a computer model (the Empiric Stream Treatment Ranking [ESTR] system). The objective is to maximize the cost: benefit ratio of lampricide treatment resources (Hansen and Jones 2008). Data collected using the QAS method are costly because it involves intensive assessment of larval sea lamprey populations in the streams (Slade *et al*. 2003). The RA method evaluates the first type of habitat encountered at a site, as opposed to the best available habitat in QAS, and compares cost per kill for larvae > 100 mm. The assumption is that larval habitat, larval densities, and control strategies (i.e., above or below a sea lamprey barrier; Slade *et al*. 2003) are relatively homogenous. TFM treatments are typically deemed successful when they eradicate 95% to 100% of the larval sea lampreys in the tributary (Smith and Swink, 2003).

3.4 The influence of physiological and abiotic factors on TFM sensitivity

By better understanding the effects of season and temperature on TFM toxicity in larval sea lamprey, it may be possible to reduce the use of TFM by explicitly incorporating temperature in current models used to determine TFM concentrations (pH-alkalinity model). For example, during the period from 1979 to 1989, an average of 52,904 kg active ingredient (kg year^{-1}) of TFM was applied annually to 316 streams. This was reduced to an average of 38,698 kg year^{-1} for the decade of 1990 to 1999. This reduction was enhanced in the years 1995 to 1999 to an average of 34,120 kg year^{-1}.

Changes in streams selected for treatment and scheduling treatments during low stream discharge accounted for about 26% of the reduction in annual TFM use (Brege *et al*. 2003). Based on current research, larval sea lampreys were less sensitive to TFM in the summer compared to the spring and fall, mainly due to the effects of water temperature rather than physiological status of the animals. For instance, the decrease in the LC_{50} in October was an unexpected finding because lamprey that had fed throughout the summer were expected to have greater glycogen stores which would be expected to increase TFM tolerance. Moreover, lower temperature in October would presumably lower larval sea lamprey metabolic rates and lower TFM uptake, leading to greater survival (Hlina *et al*. 2017). However, the opposite occurred. At warmer temperatures TFM tolerance was greater, and this may have been due to an increased capacity of the animals to detoxify TFM at this time of the year. Support for this hypothesis is the recent discovery that TFM detoxification pathways are more complex than previously thought. It was known that sea lamprey have the enzymes required to biotransform TFM to TFM-glucuronide, but that the activities of these enzymes were lower than in non-target fishes studied including rainbow trout (Lech and Statham 1975; Kane *et al*. 1994). However, the recent discovery that sea lamprey may possess other enzymes that metabolize TFM suggest that they have other routes of TFM detoxification including oxidation and reduction pathways (Bussy *et al*. 2018a,b). I propose that at higher temperatures the activities of the enzyme pathways involved in TFM detoxification are higher, which increases lampreys' ability to survive exposure to TFM in the summer.

3.5. Implications for Sea Lamprey Control

Re-scheduling TFM treatments to earlier or later in the year might be beneficial for large streams or rivers to decrease the total amount of TFM required and to reduce the risk of residual sea lamprey that survive treatments, complete metamorphosis, and subsequently parasitize economically and culturally significant fish in the Great Lakes.

Moreover, to reduce use of TFM, temperature needs to be incorporated in current models used to determine TFM concentrations (e.g., pH-alkalinity model), perhaps to create a pH-alkalinity-temperature model. Toxicity tests at various pH, alkalinity and temperature ranges could be conducted to develop a chart of TFF LC99.9 concentrations. Temperature is especially important as water temperatures increase with climate change; this would also allow for the spatial spread of sea lamprey to previously colder tributaries. Currently, TFM treatments are done in September and October to avoid non-target effects on sturgeon, which is an optimal treatment time for larval sea lamprey because TFM toxicity is greatest at lower temperatures. Incorporating temperature into the TFM treatment model would allow for the most efficient use of TFM and reduce the number of lamprey that survive TFM treatments (residuals) and reduce the amount of TFM needed to treat the streams.

3.6. Future Control Methods

As this research was conducted, there have been advances in other methods of sea lamprey control. One such advance is the sea lamprey mating pheromone, called 3-keto ketromyzonol sulfate (3kPZS). In 2015, 3kPZS was registered as the first ever vertebrate pheromone biopesticide with the U.S. Environmental Protection Agency. During registration, several factors, including human health and occupational risks, and

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environmental considerations are considered (Health Canada, 2017). The North American Free Trade Agreement (NAFTA) Technical Working Group on Pesticides serves as a focal point for addressing pesticide issues arising in the context of liberalized trade among the NAFTA countries and cooperation has included undertaking collaborative scientific work, collaborating on risk assessment or compliance methods and carrying out joint reviews, among others. The registration of 3kPZS had been many years in the making and was a collaborative effort with the U.S. Geological Survey, Michigan State University and Bridge Organics Company. This research has also provided a path for chemosensory compounds to be registered to control other vertebrate species, including aquatic species.

Another advance is the use of nonphysical barriers, which are being tested for future use. Nonphysical barriers do not impound water and, in some cases, may be the only option in areas where physical barriers are impractical or would cause an unacceptable change in a waterway. Also, temporary nonphysical barriers could be used to control lamprey during migration times or even on a diel basis (Johnson *et al*. 2016) to limit impacts on nontarget species. The Bioacoustic Fish Fence (BAFF) marketed by Ovivo USA was tested for potential use in "pushing" and "pulling" sea lamprey in specific patterns, to prevent their upstream movement and spread. Miehls *et al*. (2017) could not provide definitive conclusions about the effectiveness of the BAFF, and further research will be needed to better understand the impact of various light and sound intensities.

Finally, there has also been research into RNA interference to develop a sea lamprey-specific lampricide. This technique would cause increased mortality of the

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larvae and could be used to kill filter-feeding larvae within streams, following development of a slow-release formulation. This technique is at proof-of-concept stage and has been made possible due to sequencing of the entire sea lamprey genome in 2012 (Kuraku *et al*. 2013).

3.7 Conclusion and Future Directions

In conclusion, the tolerance of sea lampreys to TFM is lowest in spring. There was a relationship between water temperature and TFM tolerance, which resulted in increases in greater TFM sensitivity at lower temperatures. These observations could also have important implications for the sea lamprey control program in the Great Lakes. Incorporating season and water temperature into models that are used to predict the amounts of TFM required for lampricide applications is important, it may be possible to reduce total TFM requirements by treating large streams or rivers earlier or later in the year, when sea lamprey are most sensitive to TFM. It will also be important to validate the results from these laboratory studies, by performing similar tests under natural stream conditions. Finally, it is imperative to better understand how TFM uptake, distribution and elimination will be affected by further increases in water temperature due to climate change and how this could potentially affect ongoing efforts to control this invasive species in the Great Lakes.

There is a movement away from physical controls and removal of many dams due to effects on migrating fishes and also to restore the natural riparian environment (Miehls *et al*. 2017; Poff *et al*. 1997). However, this would result in increased use of TFM (Lavis *et al*. 2003) due to greater access to spawning habitat and ultimately, increased sea

lamprey production. Caution should therefore be exercised when making such decisions because traps and barriers, along with TFM, are key components of the integrated sea lamprey control program. Although there will be further developments in the understanding of sea lamprey biology, and potentially promising new methods of sea lamprey control in the future, for now TFM remains an essential tool for controlling invasive sea lamprey in the Great Lakes.

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APPENDICES

Appendix A – Supplementary Data

A1.0 – Supplementary Data

A1.1 Preliminary Range-Finder Toxicity Test Results and Acute Toxicity Test Water

Chemistry

The seasonal range-finder toxicity tests suggested that the 12 h LC_{50} in April, June, August and October was between 1.00 – 1.50, 2.50 – 3.00, 2.50 – 3.00 and 1.30 – 2.00 mg L^{-1}, respectively. The temperature preliminary range-finder toxicity tests suggested that the 12 h LC_{50} at 6, 12 and 21 °C was between 1.25 – 1.75, 1.75 – 2.25 and 3.0 – 3.50 mg L^{-1}, respectively. Based on these results, the nominal concentrations of TFM were selected for the seasonal (Tables A1.1, A1.3 – A1.6) and temperature (Table A1.1, A2.7 – A2.9) acute toxicity tests. The resulting 12 h LCs are presented in Figure 2.3A (season) and 2.3B (temperature). The resulting 12 h LTs are presented in Figure 2.4A (season) and Figure 2.4B (temperature).

Mean temperature in acute toxicity tests in April, June, August and October were 5.6 (± 0.1 SEM, N = 57), 20.6 (± 0.1 SEM, N = 57), 23.5 (± 0.1 SEM, N = 57) and 11.7 °C (± 0.1 SEM, N = 57), respectively. Mean measured temperatures in the temperature experiments were 6.4 (± 0.1 SEM, N = 63), 12.3 (± 0.7 SEM, N = 63) and 24.1 °C (± 0.01 SEM, N = 63), respectively. As expected, there were differences in mean temperature of the toxicity tests (Kruskal-Wallis chi-squared = 60.463, DF = 6, P = 3.624 x 10^{-11}). Temperature in April was significantly lower than in June (observed difference = 40.89, critical difference = 39.69), August (observed difference = 78.58, critical difference = 39.69) and 23 °C (observed difference = 71.82, critical difference = 38.74) temperature experiments. Temperature in August was significantly greater than in

October (observed difference = 62.63, critical difference =39.69) and 6 (observed difference = 56.56, critical difference = 38.74) and 12 °C (observed difference = 45.68, critical difference = 38.74) temperature experiments.

Mean pH in April, June, August and October was 7.68 (± 0.02 SEM, N = 57), 7.96 (± 0.04 SEM, N = 57), 8.30 (± 0.01 SEM, N = 57) and 8.26 (± 0.01 SEM, N = 57), respectively. Mean pH in the temperature experiments was 7.85 (± 0.03 SEM, N = 63), 8.05 (± 0.03 SEM, N = 63) and 8.24 (± 0.01 SEM, N = 63), respectively. There were differences in mean pH of the toxicity tests (Kruskal-Wallis chi-squared = 119.7, DF = 6, $P < 2.2 \times 10^{-16}$). The pH in April was significantly lower than in August (observed difference = 109.87, critical difference = 39.69), October (observed difference = 93.63, critical difference = 39.69) and at 12 (observed difference = 49.63, critical difference = 38.74) and 23 °C (observed difference = 84.53, critical difference = 38.74) temperature experiments. The pH in June was significantly lower than in August (observed difference = 73.55, critical difference = 39.69), October (observed difference = 57.32, critical difference = 39.69) and at 23 °C (observed difference = 48.22, critical difference = 38.74) temperatures experiments. The pH in August was significantly higher than pH at 6 (observed difference = 89.64, critical difference = 38.74) and 12 °C (observed difference = 60.24, critical difference = 38.74) temperatures experiments. The pH at 6 °C was significantly lower than pH in October (observed difference = 73.41, critical difference = 38.74) and at 23 °C (observed difference = 64.31, critical difference = 37.76) temperature experiments and pH at 12 °C experiments was significantly lower than in October (observed difference = 44.00, critical difference = 38.74).

Mean dissolved oxygen in April, June, August and October was 106.5 ($\pm$ 0.4 SEM, N = 57), 89.9 ($\pm$ 0.5 SEM, N = 57), 87.5 ($\pm$ 0.1 SEM, N = 57) and 91.9 % ($\pm$ 0.7 SEM, N = 57). Mean dissolved oxygen in the temperature experiments was 103.5 ($\pm$ 0.7 SEM, N = 63), 98.9 ($\pm$ 0.5 SEM, N = 63) and 88.1 % ($\pm$ 0.8 SEM, N = 63), respectively.

Temperature, pH and dissolved oxygen in acute toxicity tests are presented in Tables 2.1A – 2.5A (seasonal experiments) and Tables 2.1 A, 2.6A – 2.8A (temperature experiments), respectively.

A.1.2 Proximate Body Composition Experiment Larval Sea Lamprey Sample Size, Length, Mass, Condition Factor and Water Chemistry

Larval sea lamprey sample size, length, mass and condition factor in the proximate body composition experiments are presented in Table A2.10. A random subset dissected for collection of brain and liver from each experiment, with sample sizes and mass presented in Tables 2.11A and 2.12A, respectively. Proximate body composition water chemistry measured at start (0 h) is presented in Table A2.13.

A.1.3 Solid Phase Extraction and High-Performance Liquid Chromatography

Larval lampreys were ground under liquid nitrogen and approximately 300 mg of tissue collected in a polypropylene centrifuge tube. Next, 4 mL of 80 % methanol was added, and the sample was shaken (MaxQ 2000 orbital shaker, Thermo Fisher Scientific Inc., MA USA) and centrifuged (1228 $\times$ *g*; IEC MediLite 12, Thermo Electron Corporation, MA, USA) for 10 minutes each; supernatant was removed and placed in another polypropylene centrifuge tube. The process was repeated twice more, and the

supernatant was then evaporated to approximately 8 mL in a 55 °C water bath using a nitrogen gas evaporator (N-EVAP Analytical Evaporator, Organomation, MA, USA).

The supernatant was then eluted through 1 cm of high-density glass filter beads (Empore Filter Aid 400, 3M, St. Paul, MN, USA) contained in Bond Elute LRC-C18 SPE columns (; Bond Elute LRC-C18 OH, 500 mg, Agilent Technologies, Santa Clara, CA, USA), previously conditioned with 10 mL of 100 % methanol and 10 mL of 70 % methanol. The pH of the collected eluent was adjusted to 9.5 ± 0.2 using 10 N NaOH, prior to a second elution using Empore SDB-XC SPE columns (10mm diameter/6 mL volume, 3M, St. Paul, MN, US), which had been conditioned with 10 mL of 100 % methanol, followed by 10 mL of pH 9.5 water. The resulting eluent was collected in clean polypropylene centrifuge tubes, evaporated and pH adjusted to 4.0 ± 0.2 using 12.1 N HCl. The treated eluent was again filtered through the Bond Elute LRC-C18 columns, which had been conditioned with 10 mL of 100 % methanol and 10 mL of 24.6 mM acetate buffer (pH 4.0 ± 0.2). This was followed by elution with 12 mL of 60 % 24.6 mM acetate buffer:methanol (pH 4.0 ± 0.2), resulting in the extraction of TFM-glucuronide, for which the eluent was collected in a clean glass tube. A second elution using 6 mL of 75 % methanol was then performed to isolate the TFM, which was collected in a second clean glass tube. The tubes containing TFM were evaporated to approximately 1 mL and then 1 mL of 40 mM sodium borate buffer (pH 8.5 ± 0.2) was added to the samples, resulting in a final volume of 2 mL; whole body TFM concentrations were determined using HPLC (described below).

To the tubes containing TFM-glucuronide, 1 mL of solution of β-glucuronidase (1,644,000 unitsg^{-1} from bovine liver; Sigma Aldrich, MO, USA) dissolved in 400 mM

potassium phosphate buffer (pH 6.8 ± 0.2) was added. Samples were incubated in a water bath at 35 °C for 18 h to allow the enzyme to hydrolyze TFM-glucuronide to TFM, after which the reaction was terminated by the addition of 12.1 N HCl. The solution was then processed as described above for parent TFM, followed by HPLC quantification of TFM, which served as an indirect measure of TFM-glucuronide.

Quantification of TFM was done using a Varian HPLC set-up, comprised of a Varian ProStar 410 auto-sampler, ProStar 230 solvent delivery module and Prostar 310 UV-VIS detector (Varian, Inc., Palo Alto, CA, USA), fitted with a reverse phase HPLC C-18 column (Kinetex 2.6 μm XB-C18 100A 100 x 3.00 mm; Phenomenex Inc., CA, USA). TFM standards (0.015, 0.050, 0.250, 0.500, 1.500, and 5.000 μg mL^{-1}) were prepared from analytical grade TFM Sigma Aldrich, St. Louis, MO, USA) in 20 mM sodium borate buffer (pH 8.5 ± 0.2). Standards and samples were injected (0.1 mL) via a mobile phase comprised of 83 % 20 mM sodium borate buffer (pH 8.5 ± 0.2) and 17 % acetonitrile, which yielded a TFM retention time of approximately 4.66 minutes. Percent recovery of TFM was determined to 58 ± 9 %.

TABLES & FIGURES

Table A1.1 Summary of acute toxicity test nominal TFM concentrations (19 aquaria: 6 concentrations in triplicate and control(s)) and water chemistry measured at 0, 12 and 24 h by experiment. Data presented as mean ± SEM. Seasonal toxicity tests were conducted at the ambient stream temperature at the time of larval lamprey collection (shown in brackets); temperature toxicity tests were conducted in July 2014. Statistical significance is indicated by letters.

Experiment	Nominal [TFM] (mg L^{-1})	Mean Temperature (°C) ± SEM	Mean pH ± SEM	Mean Dissolved Oxygen (%) ± SEM
April (5.6 °C)	0.00, 0.50, 0.80, 1.00, 1.20, 1.50, 2.00	5.6 ± 0.1 **(A)**	7.68 ± 0.02 **(A)**	106.51 ± 0.36
June (20.6 °C)	0.00, 1.25, 1.75, 2.25, 2.50, 2.75, 3.00	20.6 ± 0.1 **(B)**	7.96 ± 0.04 **(C)**	88.99 ± 0.49
August (23.5 °C)	0.00, 2.00, 3.00, 3.75, 4.50, 4.75, 5.00	23.5 ± 0.1 **(B, C)**	8.30 ± 0.01 **(B, D, E)**	87.51 ± 0.10
October (11.7 °C)	0.00, 1.00, 1.30, 1.60, 1.80, 2.00, 2.30	11.7 ± 0.1 **(D, F)**	8.26 ± 0.01 **(B, D, H, J)**	91.90 ± 0.71
6 °C	0.00, 1.25, 1.50, 1.75, 2.0, 2.25, 2.5	6.4 ± 0.1 **(D, F)**	7.85 ± 0.03 **(F, G)**	103.47 ± 0.69
12 °C	0, 1.50, 1.75, 2.0, 2.25, 2.50, 2.75	12.3 ± 0.7 **(D, F)**	8.05 ± 0.03 **(B, F, I)**	98.95 ± 0.55
24 °C	0.00, 3.00, 3.25, 3.50, 3.75, 4.00, 4.25	24.1 ± 0.1 **(B, E)**	8.24 ± 0.01 **(B, D, H)**	88.08 ± 0.84

Table A1.2 Summary of larval sea lamprey sample size, length, mass and condition factor in the acute TFM toxicity tests (19 aquaria: 6 concentrations in triplicate and control(s)) by season and temperature. Data presented as mean ± SEM. Seasonal proximate body composition experiments were conducted at the ambient stream temperature at the time of larval lamprey collection (shown in brackets); temperature toxicity tests were conducted in July 2014. Statistical significance is indicated by letters.

Experiment	Lamprey Mortality, N	Surviving Lamprey, N	Total Lamprey, N	Mean Length (mm) ± SEM	Mean Mass (g) ± SEM	Condition Factor[**] ± SEM
April (6 °C)	168	122[*]	290[+]	69.2[*] ± 1.1 (A)	0.62[*] ± 0.03 (A)	1.70[*] ± 0.02 (A,B)
June (20 °C)	107	184[*]	291[+]	61.1[*] ± 1.2 (B)	0.44[*] ± 0.02 (B)	1.88[*] ± 0.06
August (23 °C)	234	79	313[+]	59.6 ± 0.7 (B)	0.39 ± 0.01 (B)	1.74 ± 0.02 (B)
October (12 °C)	146	101	247[+]	68.6 ± 1.1 (A)	0.56 ± 0.03 (A)	1.63 ± 0.02 (A)
6 °C[#]	209	106	315	74.0 ± 1.6	0.69 ± 0.03	1.59 ± 0.02 (b)
12 °C[#]	170	144	314[+]	70.0 ± 1.2	0.61 ± 0.03	1.63 ± 0.02 (a,b)
21 °C[#]	198	116	314[+]	65.6 ± 1.1	0.52 ± 0.02	1.66 ± 0.02 (a)

[*]Surviving lamprey were not measured and weighted; values are for mortalities during 24 h toxicity test
[+]Total sample size (N) varied per aquaria during the toxicity tests
[#]Experiments conducted with 2 additional control aquaria from seasonal experiments
[**]Condition factor = (mass (g))/length (mm))^3 x 10^6 (Youson *et al*. 1993)

Table A1.3 April acute toxicity test nominal TFM concentrations (19 aquaria: 6 concentrations in triplicate and one control) and water chemistry measured at 0, 12 and 24 h by aquaria. Data presented as mean ± SEM.

Aquaria	Nominal [TFM] (mg L^{-1})	Mean Measured [TFM] (mg L^{-1}) ± SEM	Mean Temperature (°C) ± SEM	Mean pH ± SEM	Mean Dissolved Oxygen (%) ± SEM
A	2.0	1.90 ± 0.02	5.2 ± 0.28	7.58 ± 0.11	102.6 ± 3.05
B	1.2	1.16 ± 0.02	5.1 ± 0.28	7.50 ± 0.09	110.5 ± 1.63
C	0.5	0.45 ± 0.01	5.1 ± 0.24	7.51 ± 0.01	106.2 ± 1.19
D	1.2	1.85 ± 0.68	5.1 ± 0.32	7.63 ± 0.01	107.2 ± 1.69
E	2.0	1.88 ± 0.03	5.2 ± 0.27	7.69 ± 0.03	107.0 ± 2.47
F	1.5	1.51 ± 0.04	5.4 ± 0.12	7.70 ± 0.02	105.0 ± 0.49
G	0.5	0.50 ± 0.04	5.3 ± 0.35	7.67 ± 0.02	106.8 ± 0.89
H	0.8	0.80 ± 0.02	5.4 ± 0.30	7.65 ± 0.03	107.9 ± 2.00
I	1.5	1.55 ± 0.12	5.5 ± 0.18	7.70 ± 0.00	106.3 ± 1.77
J	0.8	0.78 ± 0.03	6.0 ± 0.10	7.75 ± 0.01	105.2 ± 1.92
K	1.0	1.00 ± 0.04	5.9 ± 0.15	7.77 ± 0.01	107.4 ± 0.59
L	1.5	1.48 ± 0.03	6.0 ± 0.19	7.80 ± 0.02	105.3 ± 1.70
M	2.0	2.02 ± 0.04	5.9 ± 0.22	7.78 ± 0.09	105.7 ± 1.00
N	1.0	0.93 ± 0.02	5.9 ± 0.18	7.73 ± 0.05	107.0 ± 1.28
O	0.8	0.80 ± 0.04	5.9 ± 0.12	7.75 ± 0.03	106.4 ± 0.98
P	0.5	0.46 ± 0.01	5.9 ± 0.15	7.71 ± 0.07	107.0 ±0.90
Q	1.0	0.92 ± 0.03	5.9 ± 0.06	7.76 ± 0.04	105.4 ± 0.30
R	1.2	1.17 ± 0.07	5.8 ± 0.12	7.77 ± 0.05	107.4 ± 0.85
S	0.0	0.00 ± 0.00	5.7 ± 0.12	7.48 ± 0.07	107.5 ± 0.83

Table A1.4 June acute toxicity test nominal TFM concentrations (19 aquaria: 6 concentrations in triplicate and one control) and water chemistry measured at 0, 12 and 24 h by aquaria. Data presented as mean ± SEM.

Aquaria	Nominal [TFM] (mg L^{-1})	Mean Measured [TFM] (mg L^{-1}) ± SEM	Mean Temperature (°C) ± SEM	Mean pH ± SEM	Mean Dissolved Oxygen (%) ± SEM
A	3.00	2.90 ± 0.02	19.9 ± 0.38	7.28 ± 0.06	89.07 ± 3.86
B	1.75	1.77 ± 0.03	19.8 ± 0.35	7.74 ± 0.06	94.10 ± 3.14
C	1.25	1.22 ± 0.01	19.8 ± 0.32	7.84 ± 0.04	90.93 ± 0.35
D	2.75	2.69 ± 0.04	19.9 ± 0.36	7.90 ± 0.02	87.50 ± 0.82
E	2.50	2.43 ± 0.03	19.9 ± 0.31	7.99 ± 0.03	90.80 ± 2.27
F	2.25	2.28 ± 0.02	19.9 ± 0.31	8.02 ± 0.04	87.47 ± 2.92
G	1.25	1.14 ± 0.00	21.1 ± 1.34	8.00 ± 0.03	88.80 ± 3.04
H	0.00	0.00 ± 0.00	21.1 ± 1.41	8.01 ± 0.04	92.03 ± 1.91
I	1.75	1.86 ± 0.07	21.1 ± 1.44	8.02 ± 0.04	88.90 ± 1.82
J	3.00	2.95 ± 0.04	21.3 ± 1.66	8.05 ± 0.05	88.70 ± 2.46
K	2.50	2.45 ± 0.04	21.2 ± 1.62	8.05 ± 0.02	88.27 ± 5.20
L	2.75	2.86 ± 0.05	21.2 ± 1.59	8.04 ± 0.03	88.30 ± 4.07
M	2.25	2.29 ± 0.03	21.2 ± 1.63	8.04 ± 0.02	87.93 ± 3.30
N	1.25	1.22 ± 0.05	20.6 ± 0.07	8.03 ± 0.01	85.60 ± 3.89
O	3.00	3.09 ± 0.09	20.5 ± 0.20	8.04 ± 0.01	85.90 ± 4.59
P	2.50	2.45 ± 0.06	20.5 ± 0.23	8.05 ± 0.01	86.77 ± 2.84
Q	2.75	2.77 ± 0.05	20.5 ± 0.23	8.04 ± 0.02	90.43 ± 2.65
R	1.75	1.73 ± 0.05	20.5 ± 0.20	8.05 ± 0.01	90.77 ± 2.58
S	2.25	2.20 ± 0.02	20.5 ± 0.20	8.05 ± 0.02	88.47 ± 1.43

Table A1.5 August acute toxicity test nominal TFM concentrations (19 aquaria: 6 concentrations in triplicate and one control) and water chemistry measured at 0, 12 and 24 h by aquaria. Data presented as mean ± SEM.

Aquaria	Nominal [TFM] (mg L^{-1})	Mean Measured [TFM] (mg L^{-1}) ± SEM	Mean Temperature (°C) ± SEM	Mean pH ± SEM	Mean Dissolved Oxygen (%) ± SEM
A	4.50	4.31 ± 0.01	23.8 ± 0.38	8.18 ± 0.08	91.2 ± 1.94
B	4.75	4.68 ± 0.03	23.8 ± 0.35	8.26 ± 0.04	89.2 ± 2.83
C	3.00	2.99 ± 0.02	23.7 ± 0.35	8.27 ± 0.03	85.0 ± 2.52
D	5.00	4.91 ± 0.01	23.7 ± 0.37	8.29 ± 0.01	92.4 ± 2.25
E	3.75	3.82 ± 0.02	23.7 ± 0.36	8.30 ± 0.03	93.6 ± 1.58
F	2.00	1.90 ± 0.02	23.7 ± 0.36	8.24 ± 0.04	78.2 ± 1.43
G	4.50	4.44 ± 0.01	23.6 ± 0.26	8.30 ± 0.03	92.6 ± 0.55
H	2.00	2.15 ± 0.03	23.6 ± 0.23	8.30 ± 0.03	89.4 ± 1.80
I	3.00	2.88 ± 0.04	23.6 ± 0.26	8.30 ± 0.02	89.0 ± 0.62
J	0.00	0.06 ± 0.01	23.6 ± 0.26	8.31 ± 0.02	86.7 ± 3.65
K	5.00	4.94 ± 0.01	23.5 ± 0.26	8.33 ± 0.02	84.0 ± 1.67
L	3.75	3.72 ± 0.03	23.6 ± 0.23	8.32 ± 0.03	90.2 ± 1.87
M	4.75	4.79 ± 0.02	23.5 ± 0.26	8.34 ± 0.02	87.7 ± 2.41
N	2.00	1.99 ± 0.01	23.2 ± 0.23	8.27 ± 0.03	82.2 ± 2.07
O	4.75	4.72 ± 0.02	23.2 ± 0.23	8.31 ± 0.03	79.2 ± 1.98
P	3.75	3.66 ± 0.04	23.2 ± 0.23	8.32 ± 0.02	89.8 ± 1.30
Q	4.50	4.43 ± 0.01	23.1 ± 0.26	8.33 ± 0.02	87.5 ± 0.58
R	3.00	2.90 ± 0.09	23.2 ± 0.23	8.31 ± 0.02	84.7 ± 5.53
S	5.00	4.91 ± 0.04	23.1 ± 0.26	8.33 ± 0.02	89.9 ± 0.85

Table A1.6 October acute toxicity test nominal TFM concentrations (19 aquaria: 6 concentrations in triplicate and one control) and water chemistry measured at 0, 12 and 24 h by aquaria. Data presented as mean ± SEM.

Aquaria	Nominal [TFM] (mg L^{-1})	Mean Measured [TFM] (mg L^{-1}) ± SEM	Mean Temperature (°C) ± SEM	Mean pH ± SEM	Mean Dissolved Oxygen (%) ± SEM
A	1.80	1.80 ± 0.01	12.4 ± 0.09	8.16 ± 0.03	109.4 ± 9.8
B	1.00	0.95 ± 0.03	12.3 ± 0.03	8.17 ± 0.08	99.1 ± 2.1
C	2.00	1.96 ± 0.02	12.2 ± 0.03	8.25 ± 0.01	99.4 ± 1.2
D	1.30	1.24 ± 0.03	12.2 ± 0.03	8.26 ± 0.01	101.1 ± 0.3
E	1.30	1.40 ± 0.03	12.1 ± 0.13	8.26 ± 0.03	100.4 ± 0.6
F	2.30	2.26 ± 0.00	12.1 ± 0.15	8.29 ± 0.02	98.6 ± 1.2
G	1.30	1.22 ± 0.04	11.5 ± 0.17	8.27 ± 0.02	99.0 ± 1.4
H	1.80	1.81 ± 0.03	11.5 ± 0.19	8.27 ± 0.02	101.4 ± 1.0
I	2.00	1.93 ± 0.02	11.4 ± 0.15	8.27 ± 0.02	99.6 ± 1.7
J	0.00	0.00 ± 0.00	11.4 ± 0.15	8.28 ± 0.01	100.2 ± 1.2
K	1.00	1.07 ± 0.03	11.5 ± 0.19	8.28 ± 0.02	100.0 ± 1.9
L	1.60	1.64 ± 0.03	11.5 ± 0.19	8.27 ± 0.02	101.8 ± 0.9
M	2.30	2.23 ± 0.02	11.4 ± 0.18	8.29 ± 0.01	101.3 ± 0.6
N	2.00	2.01 ± 0.01	11.6 ± 0.27	8.27 ± 0.00	100.9 ± 0.6
O	1.60	1.52 ± 0.00	11.5 ± 0.8	8.27 ± 0.02	97.7 ± 1.4
P	2.30	2.34 ± 0.03	11.6 ± 0.27	8.26 ± 0.02	99.3 ± 1.2
Q	1.00	1.05 ± 0.03	11.6 ± 0.27	8.25 ± 0.03	100.9 ± 0.5
R	1.30	1.39 ± 0.05	11.6 ± 0.27	8.27 ± 0.02	100.4 ± 1.2
S	1.80	1.80 ± 0.04	11.6 ± 0.28	8.27 ± 0.02	91.9 ± 11.0

Table A1.7 Acute 6 °C toxicity test nominal TFM concentrations (19 aquaria: 6 concentrations in triplicate and controls) and water chemistry measured at 0, 12 and 24 h by aquaria. Data presented as mean ± SEM.

Aquaria	Nominal [TFM] (mg L^{-1})	Mean Measured [TFM] (mg L^{-1}) ± SEM	Mean Temperature (°C) ± SEM	Mean pH ±SEM	Mean Dissolved Oxygen (%) ± SEM
A	2.25	2.25 ± 0.06	6.43 ± 0.52	8.13 ± 0.03	102.7 ± 2.9
B	0.00	0.00 ± 0.00	6.37 ± 0.48	8.08 ± 0.02	104.7 ± 1.5
C	1.75	1.81 ± 0.07	6.33 ± 0.57	8.02 ± 0.02	109.0 ± 3.3
D	2.50	2.64 ± 0.02	6.33 ± 0.62	7.97 ± 0.03	105.7 ± 1.2
E	2.00	2.01 ± 0.03	6.47 ± 0.53	7.85 ± 0.01	103.6 ± 0.4
F	1.50	1.54 ± 0.04	6.47 ± 0.48	7.77 ± 0.04	105.3 ± 0.8
G	1.25	1.26 ± 0.06	6.60 ± 0.50	7.76 ± 0.04	102.2 ± 1.4
H	2.50	2.53 ± 0.03	7.03 ± 0.09	7.75 ± 0.15	92.2 ± 6.4
I	1.50	1.49 ± 0.02	7.03 ± 0.07	7.86 ± 0.07	100.9 ± 0.9
J	2.00	2.04 ± 0.03	6.90 ± 0.06	7.83 ± 0.10	103.3 ± 0.3
K	1.75	1.74 ± 0.03	6.97 ± 0.03	7.83 ± 0.08	103.8 ± 1.0
L	1.25	1.16 ± 0.05	6.97 ± 0.09	7.86 ± 0.05	103.1 ± 1.3
M	0.00	0.00 ± 0.00	7.13 ± 0.09	7.89 ± 0.05	103.5 ± 0.5
N	2.25	2.30 ± 0.02	7.20 ± 0.06	7.92 ± 0.07	102.6 ± 0.8
O	0.00	0.00 ± 0.00	5.73 ± 0.03	7.84 ± 0.04	106.4 ± 1.8
P	1.75	1.76 ± 0.04	5.73 ± 0.07	7.77 ± 0.01	106.9 ± 0.9
Q	2.50	2.53 ± 0.01	5.70 ± 0.06	7.47 ± 0.23	104.6 ± 1.2
R	1.25	1.23 ± 0.06	5.80 ± 0.15	7.72 ± 0.00	102.8 ± 0.5
S	1.50	1.48 ± 0.02	5.83 ± 0.07	7.76 ± 0.00	102.9 ± 0.7
T	2.25	2.38 ± 0.04	5.83 ± 0.12	7.84 ± 0.00	103.9 ± 1.0
U	2.00	1.94 ± 0.05	6.03 ± 0.07	7.86 ± 0.02	102.7 ± 1.9

Table A1.8 Acute 12 °C toxicity test nominal TFM concentrations (19 aquaria: 6 concentrations in triplicate and controls) and water chemistry measured at 0, 12 and 24 h by aquaria. Data presented as mean ± SEM.

Aquaria	Nominal [TFM] (mg L^{-1})	Mean Measured [TFM] (mg L^{-1}) ± SEM	Mean Temperature (°C) ± SEM	Mean pH ± SEM	Mean Dissolved Oxygen (%) ± SEM
A	0.00	0.00 ± 0.00	13.17 ± 0.38	7.97 ± 0.05	103.5 ± 2.6
B	1.75	1.69 ± 0.02	13.17 ± 0.43	7.94 ± 0.06	102.1 ± 1.4
C	2.25	2.25 ± 0.04	13.13 ± 0.45	7.90 ± 0.06	99.2 ± 1.0
D	1.50	1.55 ± 0.06	13.13 ± 0.49	7.89 ± 0.07	99.0 ± 1.7
E	2.50	2.47 ± 0.06	13.17 ± 0.47	7.90 ± 0.07	97.7 ± 0.6
F	2.75	2.84 ± 0.06	13.23 ± 0.44	7.90 ± 0.06	99.0 ± 1.3
G	2.00	2.05 ± 0.04	13.23 ± 0.44	7.90 ± 0.06	97.4 ± 0.6
H	2.50	2.55 ± 0.04	11.67 ± 0.22	8.10 ± 0.05	103.8 ± 1.6
I	0.00	0.00 ± 0.00	11.70 ± 0.25	8.07 ± 0.02	103.3 ± 1.0
J	1.50	1.50 ± 0.03	11.50 ± 0.26	8.08 ± 0.03	99.0 ± 0.9
K	1.75	1.75 ± 0.03	11.57 ± 0.23	8.05 ± 0.01	98.6 ± 1.4
L	2.75	2.78 ± 0.05	11.47 ± 0.23	8.09 ± 0.02	94.2 ± 2.1
M	2.25	2.27 ± 0.02	11.43 ± 0.24	8.06 ± 0.03	96.2 ± 1.8
N	2.00	2.06 ± 0.08	11.57 ± 0.27	8.03 ± 0.03	96.4 ± 1.2
O	2.50	2.54 ± 0.07	12.23 ± 0.18	8.24 ± 0.06	97.8 ± 2.8
P	2.75	2.78 ± 0.05	12.20 ± 0.17	8.18 ± 0.00	98.4 ± 2.8
Q	1.50	1.53 ± 0.03	12.17 ± 0.15	8.22 ± 0.10	98.0 ± 0.5
R	2.00	2.07 ± 0.03	12.10 ± 0.17	8.19 ± 0.08	98.9 ± 0.8
S	2.25	2.33 ± 0.07	12.13 ± 0.18	8.15 ± 0.11	96.1 ± 0.8
T	0.00	0.00 ± 0.00	12.17 ± 0.20	8.18 ± 0.07	100.3 ± 0.3
U	1.75	1.81 ± 0.04	12.17 ± 0.20	8.14 ± 0.07	99.0 ± 1.6

Table A1.9 Acute 21 °C toxicity test nominal TFM concentrations (19 aquaria: 6 concentrations in triplicate and controls) and water chemistry measured at 0, 12 and 24 h by aquaria. Data presented as mean ± SEM.

Aquaria	Nominal [TFM] (mg L^{-1})	Mean Measured [TFM] (mg L^{-1}) ± SEM	Mean Temperature (°C) ± SEM	Mean pH ± SEM	Mean Dissolved Oxygen (%) ± SEM
A	3.00	3.10 ± 0.05	24.47 ± 0.28	8.25 ± 0.10	81.2 ± 2.8
B	3.75	3.80 ± 0.03	24.43 ± 0.32	8.25 ± 0.06	87.2 ± 3.3
C	0.00	0.00 ± 0.00	24.47 ± 0.33	8.28 ± 0.06	87.5 ± 0.5
D	4.00	4.12 ± 0.05	24.47 ± 0.28	8.28 ± 0.06	86.7 ± 1.5
E	3.50	3.58 ± 0.03	24.47 ± 0.28	8.26 ± 0.06	87.5 ± 1.1
F	3.25	3.33 ± 0.05	24.43 ± 0.27	8.23 ± 0.05	86.5 ± 1.7
G	4.25	4.23 ± 0.06	24.43 ± 0.27	8.27 ± 0.03	85.4 ± 2.5
H	4.25	4.44 ± 0.00	23.63 ± 0.15	8.24 ± 0.11	98.7 ± 8.9
I	3.00	3.10 ± 0.06	23.70 ± 0.12	8.18 ± 0.07	83.4 ± 2.1
J	3.50	3.62 ± 0.01	23.70 ± 0.12	8.22 ± 0.03	92.6 ± 0.5
K	0.00	0.00 ± 0.00	23.67 ± 0.09	8.21 ± 0.04	92.9 ± 2.0
L	3.25	3.35 ± 0.04	23.67 ± 0.09	8.21 ± 0.03	90.5 ± 2.8
M	3.75	3.78 ± 0.02	23.67 ± 0.09	8.21 ± 0.02	83.9 ± 1.6
N	4.00	4.11 ± 0.03	23.67 ± 0.09	8.23 ± 0.01	86.7 ± 1.1
O	3.00	3.17 ± 0.03	24.00 ± 0.17	8.27 ± 0.02	85.7 ± 1.1
P	4.00	4.22 ± 0.02	24.03 ± 0.15	8.26 ± 0.01	86.7 ± 1.7
Q	3.50	3.73 ± 0.04	24.03 ± 0.15	8.27 ± 0.01	91.7 ± 0.9
R	4.25	4.54 ± 0.05	24.07 ± 0.18	8.27 ± 0.00	90.9 ± 2.5
S	3.25	3.42 ± 0.03	24.07 ± 0.18	8.26 ± 0.03	86.0 ± 1.5
T	0.00	0.00 ± 0.00	24.07 ± 0.18	8.25 ± 0.03	88.7 ± 1.0
U	3.75	3.91 ± 0.01	24.07 ± 0.18	8.25 ± 0.00	89.3 ± 0.5

Table A1.10 Summary of larval sea lamprey sample size, length, mass and condition factor in the proximate body composition experiments (12 containers, N = 3 – 5 per container, no TFM added) by season and temperature. Data presented as mean ± SEM. Seasonal proximate body composition experiments were conducted at the ambient stream temperature at the time of larval lamprey collection (shown in brackets); temperature proximate body composition experiments were conducted in July 2014.

Experiment	Sample Size, N[+]	Mean Length (mm) ± SEM	Mean Mass (g) ± SEM	Condition Factor[**] ± SEM
April (5.6 °C)	38	98 ± 4	1.60 ± 0.20	1.45 ± 0.38
June (20.6 °C)	44	90 ± 3	1.19 ± 0.12	1.50 ± 0.20
August (23.5 °C)	47	82 ± 2	0.84 ± 0.06	1.48 ± 0.29
October (11.7 °C)	49	82 ± 1	0.78 ± 0.03	1.38 ± 0.17
6 °C	50	86 ± 2	0.88 ± 0.05	1.30 ± 0.14
12 °C	49	87 ± 2	0.88 ± 0.05	1.29 ± 0.15
24 °C	45	83 ± 2	0.76 ± 0.05	1.30 ± 0.12

[+]Total sample size (N) varied per container during proximate body composition experiments
[**]Condition factor = (mass (g))/length (mm))^3 x 10^6 (Youson *et al.* 1993)

Table A1.11 Larval sea lamprey brain sample size and mass in the proximate body composition experiments (12 containers, N = 3 – 5 per container, no TFM added) by season and temperature. Data presented as mean ± SEM. Seasonal proximate body composition experiments were conducted at the ambient stream temperature at the time of larval lamprey collection (shown in brackets); temperature proximate body composition experiments were conducted in July 2014. A random subset dissected for collection of brain from each experiment.

Experiment	Sample Size, N	Mean Brain Mass (mg) ± SEM
April (5.6 °C)	11	3.32 ± 0.44
June (20.6 °C)	10	3.34 ± 0.50
August (23.5 °C)	8	2.60 ± 0.26
October (11.7 °C)	9	2.78 ± 0.62
6 °C	8	2.45 ± 0.40
12 °C	9	2.26 ± 0.33
24 °C	9	2.38 ± 0.35

Table A1.12 Larval sea lamprey liver sample size and mass in the proximate body composition experiments (12 containers, N = 3 – 5 per container, no TFM added) by season and temperature. Data presented as mean ± SEM. Seasonal proximate body composition experiments were conducted at the ambient stream temperature at the time of larval lamprey collection (shown in brackets); temperature proximate body composition experiments were conducted in July 2014. A random subset dissected for collection of liver from each experiment. Statistical significance is indicated by letters.

Experiment	Sample Size, N	Mean Liver Mass (mg) ± SEM
April (5.6 °C)	12	24.60 ± 4.49
June (20.6 °C)	12	13.54 ± 2.17
August (23.5 °C)	12	7.48 ± 0.74
October (11.7 °C)	12	9.22 ± 0.99
6 °C	10	12.60 ± 1.45
12 °C	11	10.92 ± 1.37
24 °C	12	7.38 ± 0.94

Table A1.13 Summary of proximate body composition (12 containers, N = 3 – 5 per container, no TFM added) water chemistry measured at start (0 h) by season and temperature. Data presented as mean ± SEM. Seasonal proximate body composition experiments were conducted at the ambient stream temperature at the time of larval lamprey collection (shown in brackets); temperature proximate body composition experiments were conducted in July 2014.

Experiment	Temperature (°C)	pH	Dissolved Oxygen (%)
April (5.6 °C)	6.1	7	105.96
June (20.6 °C)	19.7	.9	95.77
August (23.5 °C)	22.6	.3	90.72
October (11.7 °C)	11.3	8.3	98.47
6 °C	7.3	7.8	105.60
12 °C	11.2	8.0	102.30
24 °C	22.0	8.2	90.71

Figure A1.1 Acute Toxicity Testing of TFM.

The acute toxicity (12 h LC_{50} and the MLC) of TFM to larval sea lamprey was determined using static exposure systems comprising glass aquaria (18 L) filled with aerated Lake Huron water (16 L), to which the appropriate amounts of TFM were added.

Figure A1.2 Solid phase extraction (SPE)

Three solid phase extractions were performed on sea lamprey tissues to determine TFM and TFM-glucuronide concentrations.

~ 300 mg ground tissue

15 ml falcon

Repeat 3 X total

Pellet

Add 4ml 80% methanol

| Shaker @ 499 for 10 mins |

| Centrifuge @ 3000 rpm for 10 mins |

| Transfer supernatant to new labelled test tube (T.T.) |

1ST SPE

| N_2 Evaporate in 55°C H_2O bath (Until ~ 8 ml left) |

| Condition column with 10 ml pure methanol and let it go to waste |

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| Condition column with 10 ml 70% methanol and let it go to waste |

N$_2$ Evaporate in 55°C H$_2$O Bath
(until ~ 4.4 ml left)

Adjust sample pH to 9.5 with 1 N NaOH
(~ 5.5 - 17.5 ul)

2ND SPE

Condition column with 10 ml pure methanol
and let it go to waste

Condition column with 10 ml pH 9.5 water
and let it go to waste

Add sample to column and collect in new 15
ml T.T.

Elute original T.T. with 2.5 ml 95 % pH 9.5
water and go to sample

Elute column with 1 ml 95 % pH 9.5 water
and go to sample (Vol$_f$ = 7.9 ml)

N$_2$ Evaporate in 55°C H$_2$O Bath
(lose ~ 0.175 ml methanol)

Adjust sample pH to 4.0 with 1 N NaOH
(~ 5.5 - 17.5 ul)

3RD SPE

Condition column with 10 ml pure methanol
and let it go to waste

Condition column with 10 ml 24.6 mM
acetate buffer and let it go to waste

Add sample to column and let it go to waste

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                          ┌─────────────────────────────────┐
                          │  Elute original T.T. with 3 ml 24.6 mM │
                          │   acetate buffer and go to waste  │
                          └─────────────────────────────────┘
                                        │
                          ┌─────────────────────────────────┐
                          │  Elute column with 3 ml 24.6 mM acetate │
                          │      buffer and go to waste       │
                          └─────────────────────────────────┘
                                        │
                          ┌─────────────────────────────────┐
                          │  Elute TFM-glucuronide with 2 6ml portions │
                          │   of 60% 24.6 mM acetate buffer: methanol │
                          │      into new labelled 10 ml T.T. │
                          └─────────────────────────────────┘
```

Glucuronide   **TFM**

Glucuronide	TFM
Cap and Remove T.T.	Add new T.T. to collect TFM
N_2 Evaporate in 55°C H_2O Bath (until ~ 6 ml is left)	Elute TFM with 3 2 ml portions of 75% methanol into 8 ml T.T.
Add 1 ml of β-glucuronidase solution to sample	N_2 Evaporate in 55°C H_2O Bath (until ~ 1.5 ml left)
Incubate ~ 18-h in mixer @ 35°C H_2O Bath	Add 0.5 ml of 40 mM borate buffer
Adjust pH between 2.5-3 with 0.6 N HCl (~190 ul lamprey, 300 ul trout)	Adjust vol_f to 2.0 ml using E-pure

4TH SPE   **HPLC**

```
┌─────────────────────────────────┐
│  Condition column with 10 ml pure methanol │
│       and let it go to waste      │
└─────────────────────────────────┘
                 │
┌─────────────────────────────────┐
│   Condition column with 10 ml 4.92 mM │
│     acetate buffer and go to waste │
└─────────────────────────────────┘
                 │
┌─────────────────────────────────┐
│  Add sample to column and let it go to waste │
└─────────────────────────────────┘
                 │
┌─────────────────────────────────┐
│  Elute original T.T. with 3 ml 4.92 mM │
│    acetate buffer and go to sample │
└─────────────────────────────────┘
```

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Elute column with 3 ml 4.92 mM acetate buffer

↓

Elute TFM with 3 2 ml portions of 75% methanol into 8 ml T.T.

↓

N_2 Evaporate in 55°C H_2O Bath (until ~ 1.5 ml left)

↓

Add 0.5 ml of 40 mM borate buffer

↓

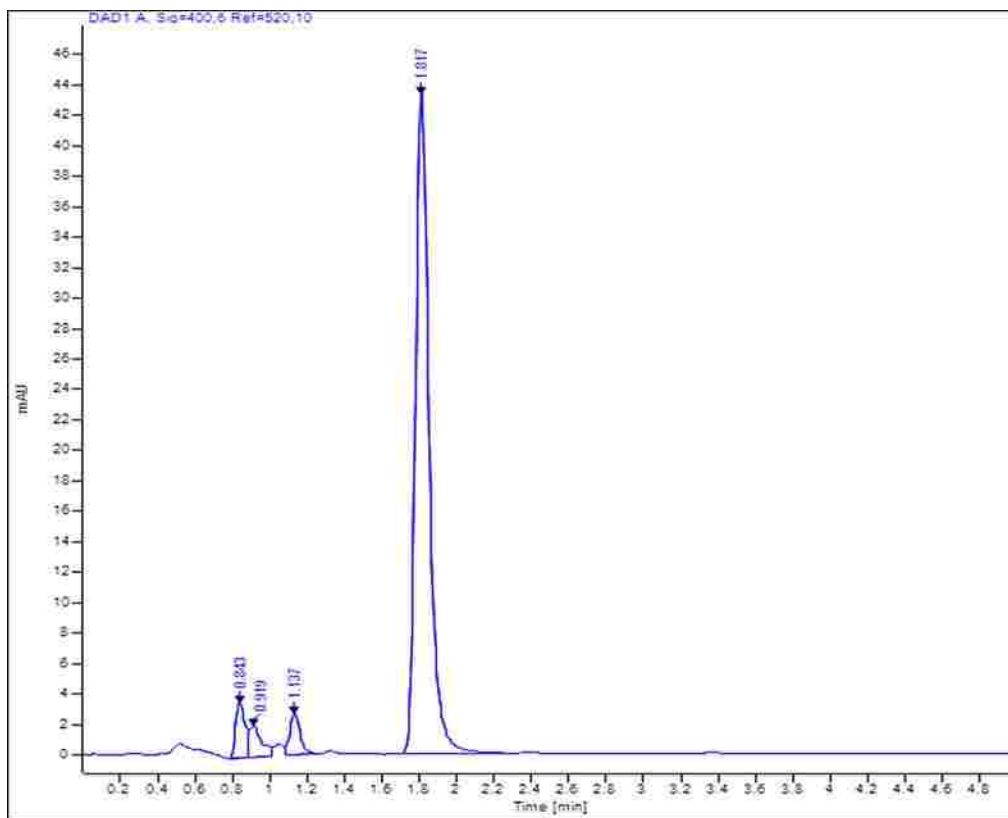Adjust vol_f to 2.0 ml using E-pure

↓

HPLC

Figure A1.3 TFM Chromatograph

Quantification of TFM was done using a Varian HPLC set-up, comprised of a Varian ProStar 410 auto-sampler, ProStar 230 solvent delivery module and Prostar 310 UV-VIS detector (Varian, Inc., Palo Alto, CA, USA), fitted with a reverse phase HPLC C-18 column (Kinetex 2.6 μm XB-C18 100A 100 x 3.00 mm; Phenomenex Inc., CA, USA). TFM retention time was approximately 4.66 minutes.