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Effects of 3-Trifluoromethyl-4-Nitrophenol (TFM) on Energy Stores and Gill Function in Sea Lamprey (*Petromyzon marinus*) and Rainbow Trout (*Oncorhynchus mykiss*)

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by

Matthew Henry
Bachelor of Science Honours, Wilfrid Laurier University, 2008

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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

The primary method of controlling invasive sea lamprey (*Petromyzon marinus*) populations in the Great Lakes is the application of the pesticide 3-trifluoromethyl-4-nitrophenol (TFM) to larval lamprey nursery streams. Recent evidence suggests that the mode of toxic action of TFM involves the inhibition of oxidative ATP production in the mitochondria. As a result, there is increased reliance on the anaerobic pathways of ATP production which involves the hydrolysis of phosphocreatine (PCr) and anaerobic glycolysis. TFM may also damage the gills, which may disrupt gill-mediated ion regulation and cause internal ion imbalances. While knowledge of TFM toxicity in larval lampreys has been generated in recent years, little is known about how TFM affects the other major stages of the sea lamprey life cycle. Accordingly, a major objective of this thesis was to determine if differences in the basal energy reserves of larval, parasitic and upstream-migrant sea lampreys affected their TFM sensitivity. Exposure of larval, parasitic and upstream migrant sea lampreys to acutely lethal concentrations of TFM [equivalent to the TFM 12-h $LC_{99.9}$], caused significant reductions in liver ATP concentrations in all three life stages examined, but brain ATP concentrations were unaffected. Brain ATP concentrations were likely buffered by the high glycogen and PCr stores found in this organ system, which underwent respective 30-40 % and 40-50 % reductions during TFM exposure. These findings further support the hypothesis that TFM causes death by interfering with ATP production, leading to a mismatch between ATP supply and ATP demand. To test the hypothesis that TFM-induced damage to the gills also contributes to TFM toxicity, the unidirectional movements of Na^+ across the body of larval and upstream-migrant lampreys, and rainbow trout (*Oncorhynchus mykiss*) were

measured using the radio-tracer $^{24}Na^{+}$ during exposure to TFM (12-h $LC_{99.9}$). The effects of TFM on Na^{+} influx, outflux and net flux were minor, and plasma ion concentrations in parasitic and upstream-migrant sea lamprey were unaltered. These findings suggest that disruption to gill-mediated ion exchange is not the proximate cause of death during exposure to TFM. This improved understanding the physiological effects of TFM on sea lamprey and rainbow trout could help to better predict the effects of TFM on target and sensitive non-target organisms during routine TFM treatments in the field.

Acknowledgements

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Chapter 1

History of Sea Lamprey Control and Present Understanding of the Mode of TFM Toxicity

1.1 The Sea Lamprey Life Cycle

Most of the sea lamprey's life cycle (3-7 years) is spent in the soft sediments of streams as blind and toothless larval lamprey (ammocoete) (Figure 1-1; Potter and Gill 2003). This larval stage is characterized by a relatively sedentary life style (Beamish and Potter 1975), low metabolic rate (Lewis and Potter 1976) and a diet that consists of a mixture of algae, organic detritus and bacteria (Moore and Beamish 1973; Sutton and Bowen 1994). During this phase, the larval lampreys deposit large amounts of lipid in their tissues in preparation for their metamorphosis into blood-feeding parasitic lampreys (Beamish and Potter 1975; O'Boyle and Beamish 1977; Youson 2003).

After the larval lamprey have acquired sufficient lipid reserves, metamorphosis is initiated, leading to pronounced morphological, anatomical and physiological changes (Youson 1980, 2003; Youson and Potter 1979). The lipid stores are essential for fueling these changes during the non-trophic (non-feeding) metamorphic phase (Youson 2003). The development of eyes, changes to the dorsal fin and modifications to internal organs (liver and kidney) are some of the changes observed during metamorphosis, and are likely associated with a shift from a relatively sedentary filter feeding larva to a more active, parasitic animal (Youson and Potter 1979; Youson 1980). The development of an oral disc allows parasitic lampreys to attach to fishes and extract their blood by using their rasping tongue to penetrate the hide of the fish (Potter and Gill 2003). The gills are also re-arranged, to allow the animals to breath while attached to their host (prey) because they can no longer use unidirectional ventilation. Instead, the gills are designed

to pump water in and out of several gill pouches through a process called tidal ventilation (Youson 1980).

In the fall, sea lampreys that have completed metamorphosis migrate downstream to the Great Lakes (landlocked populations) or to the Atlantic Ocean to begin their parasitic life stage (Beamish and Potter 1975). Once attached to a fish, the parasitic lamprey begins to consume the blood of its host by penetrating the hide with its rasping tongue and by manipulating the oral disc to create a pressure gradient to "suck" the blood out of the animal (Potter and Gill 2003). To prevent the host's blood from clotting and to aid in digestion, the lamprey secretes a chemical called lamphredin from its buccal glands (Renaud et al. 2009). This parasitism/predation can last several days to weeks and in most cases leads to the death of the host due to blood loss or secondary infections (Farmer et al. 1975). The parasitic phase in landlocked sea lampreys can be completed in less than 12 months (Bergstedt and Swink 1995), but typically lasts 1-2 years (Beamish and Potter 1975). The end of this phase is characterized by the development of the gonads, and the termination of feeding (Beamish and Potter 1975; Youson 1980).

After the parasitic phase, factors such as water temperature, photoperiod and river discharge rates trigger upstream migration in mid- to late spring (Applegate 1950; Beamish and Potter 1975; Youson 1980; Beamish 1980). Because upstream-migrant lampreys do not seek out their natal streams (Bergstedt and Seelye 1995), migratory pheromones released by larval lampreys are thought to attract upstream-migrant lamprey to streams with suitable spawning habitat (Sorensen and Vrieze 2003). Mature males are responsible for selecting spawning sites and construction of U-shaped nests (Larsen 1980). These males, in turn, release a pheromone that attracts females to their spawning

grounds (Li et al. 2007). Following spawning, semelparous sea lamprey die (Larsen 1980).

1.2 Sea Lampreys Invade the Great Lakes

Sea lamprey were first recorded in the Great Lakes (Lake Ontario) in the early 1800s (Lark 1973). Although sea lamprey were originally believed to have invaded Lake Ontario through the Erie canal (Christie and Goddard 2003), more recent evidence suggests that lampreys could have migrated into Lake Ontario via the St. Lawrence River following the last ice age (Waldman et al. 2004; Bryan et al. 2005). However, sea lampreys were prevented from entering the rest of the Great Lakes by Niagara Falls, which created a physical barrier between Lake Ontario and Lake Erie. In the early 1900s the construction of the Welland Canal allowed sea lamprey to bypass Niagara Falls and enter into the remaining Great Lakes. Following the invasion of Lake Erie and the Upper Great Lakes, sea lamprey populations grew at alarming rates, with annual population estimates for Lake Superior averaging approximately 250,000 upstream-migrant lamprey between 1957 and 1961 (Figure 1-2; Heinrich et al. 2003). Because top predators, specifically lake trout (*Salvelinus nemaychus*), lake whitefish (*Coregonus linnaeus*) and lake herring (*Coregonus artedii*) were the primary target of sea lamprey (Lawrie 1970; Smith and Tibbles 1980), the structure and composition of the Great Lakes aquatic community was dramatically altered (Morse et al. 2003). By 1966, only two lake trout populations in Lake Huron remained (Morse et al. 2003) and sea lamprey predation, combined with overfishing, were considered the major cause of the collapse of the lake trout and other fisheries (Lawrie 1970).

In response to the sea lamprey invasion, the Governments of Canada and the United States established the Great Lakes Sea Lamprey Committee in 1946 to coordinate efforts by the two countries to control sea lamprey populations (McDonald and Kolar 2007). In 1955, a bi-national partnership between Canada and the United States was formed, leading to the formation of the Great Lakes Fisheries Commission (GLFC). The primary mandates of the GLFC were to establish means to eradicate sea lamprey populations (GLFC 1955), as well as conduct fisheries research and provide support for fisheries management on the Great Lakes (GLFC 1955).

1.3 Early Methods of Sea Lamprey Control

From the late 1940s to 1960, sea lamprey control efforts primarily comprised mechanical and electrical barriers that were designed to deny of upstream-migrants access to suitable spawning habitat (Lawrie 1970). These barriers were, for the most part, unsuccessful, despite the barriers killing many upstream-migrant lamprey (Christie and Goddard 2003). Because of their limited success and negative impacts on non-target organisms, these barriers were eventually abandoned.

Currently an "integrated pest management" approach is used to control sea lamprey populations (Christie and Goddard 2003). With this approach, a variety of control methods are used to control sea lamprey populations including trapping upstream-migrant adults to reduce the number of animals reaching their spawning grounds (Mullett et al. 2003; Li et al. 2007); sterile male release, in which male upstream-migrants are made infertile with the chemical bisazir and are released onto spawning grounds to compete with fertile males for females (Twohey et al. 2003); low-head barrier dams to prevent access of upstream migrants to spawning habitat (Lavis et al. 2003); and the

application of the lampricides [3-trifluoromethyl-4-nitrophenol (TFM) and niclosamide (Bayluscide®)] to nursery streams to kill larval lamprey (McDonald and Kolar 2007). The application of lampricides at concentrations that kill larval lamprey, with normally minimal effects on non-target organisms, is currently the most effective and widely used method of sea lamprey control (McDonald and Kolar 2007).

1.4 The Search for a Lampricide

The sea lamprey management strategy took a new direction in the 1950s when it was recognized that the larval stage was the most effective life history stage to target because several generations of lamprey could be found in streams concurrently (Applegate 1950). The search for a selective larval lampricide began with the testing of over 6,000 chemicals in a six-year span. In 1956, researchers concluded that of the 10 halogenerated mono-nitrophenols toxic to larval lamprey, TFM was the most suitable because of its effectiveness at low concentrations and relatively low cost (McDonald and Kolar 2007). Another chemical, niclosamide (Bayluscide®), was also found to be toxic to larval lamprey when used with TFM, as well as alone (Dawson 2003). However, Bayluscide® is rarely applied alone because of its greater toxicity to non-target organisms. Instead, Bayluscide® is used in combination with TFM to treat streams with high discharge rates and has proven highly effective in reducing TFM usage (Dawson 2003). TFM remains the main lampricide used to control sea lamprey populations, and it remains the primary method used in the integrated pest management of this invasive species.

1.5 Characteristics of a Typical TFM Treatment

There are four basic steps in the TFM treatment process: i) larval population assessment, ii) collection of water chemistry and flow data, iii) determination of the minimum lethal concentration (MLC) required to kill 99.9% of the larvae and, iv) application of the lampricide (McDonald and Kolar 2007).

To assess the degree of sea lamprey infestation in a stream, the size, age and structure of the lamprey population in the main branch and tributaries of the stream are evaluated. Using body length and mass of larvae collected, the condition factor [CF= (body mass (g)/length (mm))^3 x 10^6; Holmes and Youson 1994] of the larvae is used to predict the number of individuals that will likely metamorphose into parasitic lamprey in the coming year (McDonald and Kolar 2007). This information is then used determine whether or not a stream will be treated.

Using larval lamprey from the assessed stream, an on-site toxicity test or the pH-alkalinity model developed by Bills et al. (2003), is used to determine the MLC. Treatment managers then determine the target concentration, TFM application rates, and the location of booster sites that help maintain appropriate concentrations of TFM throughout the entire stream (B. Stephens, Manager, Sea Lamprey Control Center, Sault Ste. Marie, ON, personal communication).

To achieve a nine-hour block of chemical where the concentration of TFM is 1.5 x MLC, initial application of TFM to the mainstream begins 3 or more hours before the "start" of the nine-hour treatment (McDonald and Kolar 2007). Using the "sprinkler" method, TFM is pumped at a pre-determined rate through a perforated hose at the initial application site on the main branch and tributaries. The "booster sites" are located at

selected sites downstream to compensate for loss of lampricide due to its dilution by water flowing in from tributaries. Throughout the treatment, water samples are continually collected to measure water pH, alkalinity and lampricide concentrations. Finally, if sensitive non-target species such as larval lake sturgeon (*Acipenser fulvescens*) live in the stream, TFM concentrations may be adjusted to 1.2 x MLC in an effort to reduce non-target effects on this TFM-sensitive non-target species (McDonald and Kolar 2007; B. Stephens, Manager, Sea Lamprey Control Center, personal communication). Minimizing non-target effects and reducing the TFM needed for lampricide treatments are ongoing concerns for the GLFC (GLFC 2008).

1.6 TFM Selectivity and GLFC Research Priorities

Toxicity studies have identified a number of highly sensitive non-target organisms including larval lake sturgeon and mudpuppies (*Nectalurus maculosus*) (Boogaard et al. 2003). These species may be killed by TFM concentrations ranging from 1.3 to 1.7 x the MLC. More resistant species such as rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), and lake trout are killed at TFM concentrations ranging from 3-5 x the MLC (McDonald and Kolar 2007). Differences in sensitivity between sea lamprey and more resistant organisms are mainly due to the lampreys' limited ability to detoxify TFM (Lech and Stratham 1975; Kane et al. 1994). In fish, detoxification of TFM occurs within the liver through the process of biotransformation (Lech and Statham 1975; Kane et al. 1993). Biotransformation of TFM involves the process of glucuronidation, in which the enzyme UDP-glucuronyltransferase (UDPGT) is responsible for the addition of UDP-glururonic acid (UDPGA) to the TFM molecule in a process called conjugation (Figure 1-3; Kane et al. 1993). The TFM-conjugate formed is very water-soluble, less toxic and

easy to excrete via the urine. Lech and Stratham (1975) showed that sea lamprey have a limited ability to detoxify TFM via glucuronidation. In addition, Kane et al. (1994) reported that maximum activity of UDPGT (V_{max}) in sea lamprey is significantly lower than in more resistant fish such as rainbow trout and bluegill (*Lepomis mcrochirus*) and the affinity of UDPGT for TFM in sea lamprey is significantly lower compared to rainbow trout and bluegill.

The use of TFM as a control method in the integrative pest management of sea lamprey in the Great Lakes has been very successful at controlling larval lamprey populations, but parasitic lamprey numbers remain above target levels (McDonald and Kolar 2007). Concerns regarding negative impacts to non-target organisms, ongoing public concern regarding pesticide use in the environment, and increasing lampricide costs have increased the need to reduce reliance on lampricides for controlling sea lamprey populations (GLFC 2001).

The Lampricide Control Task Force identified a number of key issues to help improve the effectiveness of lampricide treatments. Of the many issues at hand, two of the most important were *treatment effectiveness* and *non-target effects*. These issues are of utmost importance to the GLFC because lamprey populations in the Great Lakes are still above target levels, the potential for reducing TFM usage and cost savings, and reducing the effects to non-target organisms. Other issues address were the development of new lampricide application methods and a more thorough understanding of the mechanism(s) of TFM toxicity (McDonald and Kolar 2007).

In recent years, light has been shed on the mechanism(s) of TFM toxicity in larval sea lampreys (e.g. Wilkie et al. 2007; Birceanu et al. 2009; Birceanu et al. 2011), but little

is known about the effects that TFM has on other life history stages of the sea lamprey. With the profound changes to the gills, internal anatomy and life style that accompany metamorphosis, TFM sensitivity could vary with life stage in the sea lamprey. In addition, the physiological effects of TFM to non-target fish remain poorly understood. Accordingly, the goal of this M.Sc. thesis was to use *in vivo* techniques to examine TFM toxicity in different life stages of the sea lamprey, and in juvenile rainbow trout.

1.7 Current Understanding of the Mechanism of TFM Toxicity

1.7.1 Does TFM Cause a Mismatch Between ATP Supply and ATP Demand

Oxidative phosphorylation is the process of producing ATP using energy released by the oxidation of nutrients (Voet et al. 2006). In the mitochondria of eukaryotes, oxidative phosphorylation occurs as electrons are passed along the electron transport chain (ETC) in a series of redox reactions. Located in the inner mitochondrial membrane, the ETC consists of four protein complexes (I, II, II and IV). Oxidation of electron donors such as NADH and $FADH_2$ typically begins in complex I (Wallace and Starkov 2000). As electrons are shuttled from complex to complex protons are pumped into the inner membrane space by complex I, III and IV, generating a proton gradient (Figure 1-4). The low permeability of the inner membrane to ions normally prevents the non-specific flow of H^+ (protons) from the inter-membrane space to the mitochondrial matrix. As a result, the H^+ are directed to flow down their electrochemical gradient via ATP synthases (Complex V). The energy released by the H^+ flow is harnessed by the ATP synthases to phosphorylate ADP, leading to ATP generation. Because the formation of ATP is tightly linked to the H^+ gradient and the flow of elections via the ETC this entire process, known

as oxidative phosphorylation, is said to be coupled (Wallace and Starkov 2000; Voet et al. 2006).

The uncoupling of mitochondrial oxidative phosphorylation is primarily caused by drugs or pesticides (Wallace and Starkov 2000), and TFM (Niblett and Ballantyne 1976; Birceanu et al. 2011). Increasing the permeability of the inner membrane to protons and other ions is the most common mechanism of mitochondrial uncoupling. A nonspecific increase in the H^+-permeability of the inner membrane results in the dissipation of the proton gradient, decreasing the H^+-electrochemical gradient and H^+ flux across the inner mitochondrial membrane resulting in less energy release to promote ATP synthesis (Wallace and Starkov 2000; Voet et al. 2006). Thus, in the presence of uncoupling agents, aerobic ATP supply may not meet ATP demand, which would starve tissues of ATP leading to the break-down of ion gradients across the plasma membrane of cells, and eventually death of the organism (see Lutz et al. 2003; Bickler and Buck 2007; for reviews).

Because TFM is structurally similar to the known uncoupler, 2,4-dinitrophenol (DNP; Figure 1-5; Niblett and Ballantyne 1976), it was suspected that TFM worked in a similar fashion (Applegate et al. 1966). Niblett and Ballantyne (1976) provided evidence that 2,4-DNP and TFM induced uncoupling of mitochondrial oxidative phosphorylation, in mitochondria isolated from rat liver. More recently, Birceanu et al. (2011) showed that TFM and 2,4-DNP uncouples both lamprey and rainbow trout mitochondria. Such uncoupling of oxidative phosphorylation by TFM leads to increased reliance on anaerobic ATP production via the process of glycolysis (Wilkie et al. 2007; Birceanu et al. 2009) as the supply of ATP via oxidative phosphorylation may no longer be sufficient

to meet the body's ATP demands. Wilkie et al. (2007) and Birceanu et al. (2009) proposed that such uncoupling would ultimately result in death when glycogen stores were depleted, due to a lack of ATP for the central nervous system.

Evidence supporting the hypothesis of a TFM-induced mismatch between ATP supply and ATP demand was provided by Birceanu et al. (2009), whose work revealed that exposure to TFM reduces both glycogen and ATP concentrations in the brain and liver of larval lamprey. In addition, brain phosphocreatine (PCr), an important anaerobic energy store, was also depleted during exposure to TFM further supporting the hypothesis TFM causes a mismatch between ATP supply and ATP demand. However, Birceanu et al. (2009) exposed larval lamprey to lower concentrations of TFM than the concentrations used in the field, which are typically between 1 and 1.5 times the MLC (see above). As a result a number of questions still remain unanswered regarding the effects that TFM has on ATP supply in larval lamprey and non-target fishes.

One important question regarding the effects of TFM is whether lamprey respond in a similar manner to higher concentrations of TFM as opposed to lower TFM concentrations. It also remains unclear whether or not TFM causes similar reductions in fuel stores in other life stages of the sea lamprey life cycle, when changes in life style, diet and body structure could lead to pronounced changes in patterns of metabolic fuel use. Accordingly, another goal of this thesis was to determine how exposure to TFM concentrations ($LC_{99.9}$) closer to those used for lampricide applications in the field influenced glycogen, ATP and PCr stores in the brain, liver, kidney and muscle of larval, parasitic and upstream-migrant sea lamprey. Based on the marked reductions in these fuels reported in larval lampreys (Birceanu et al. 2009), it was predicted that exposure to

the MLC will result in greater and more rapid reductions of these energy reserves and a faster onset of death.

1.7.2 Possible Effects of TFM on Gill-Mediated Ion Exchange

The gills of fishes are involved in respiration, nitrogenous waste excretion, acid-base regulation and control of ion balance (Evans et al. 2005). Freshwater fishes are hyperosmotic regulators, and are therefore faced with a continual influx of water and continual loss of ions across the gills (Perry 1997; Marshall 2002; Evans et al. 2005). To maintain high ion levels in the blood relative to the environment, freshwater fishes excrete copious amounts of dilute urine and use their gills to actively take-up ions from the water (Marshall 2002). Ion uptake by the gills of freshwater teleost and landlocked sea lamprey involves mitochondrial rich cells (MRC) and pavement cells (PVC), with the former being the most important for gill-mediated ion regulation (Marshall 2002; Bartels and Potter 2004; Evans et al. 2005). For Na^+ uptake to occur, H^+ V-type ATPases found on the apical membrane of MCRs pump H^+ from the ICF to the water, making the apical membrane potential more negative. This process generates an electrochemical gradient that promotes Na^+ uptake from the water via apical Na^+ channels (Figure 1-6; Evans et al. 2005). The uptake of Na^+ from the gill cytosol into the blood is completed at the basolateral membrane via Na^+/K^+-ATPases (Marshall 2002; Bartels and Potter 2004; Evans et al. 2005). Ca^{2+} is taken up via apical Ca^{2+} channels down a Ca^{2+} electrochemical gradient generated by basolateral Ca^{2+}-ATPase (Perry 1997; Marshall 2002). Cl^- uptake occurs in the apical membrane of MRCs and PVCs though Cl^--HCO^-_3 exchange and anion channels in the basolateral membrane. These transport processes can be inhibited by many toxins including metals, changes in water pH, and altered salinity (Rogers et al.

2003; Y. Kara M.Sc. thesis; Wilkie et al. 1998; Bartels and Potter 2004). In many cases, these factors, alone or combination, can lead to disruption of internal ion homeostasis resulting in death of the fish (Marshall 2002; Evans et al. 2005; Niyogi and Wood 2004).

Evidence from gill morphology studies (Christie and Battle 1961; Mallatt et al. 1994) suggests that TFM toxicity may also disrupt gill-mediated ion regulation. Mallatt et al. (1994) reported that MRCs of larval lamprey exposed to TFM showed cellular swelling and enlarged vacuoles. Such damage could impair ion uptake by these cells. However, rainbow trout MRCs were not damaged by exposure to TFM (Mallatt et al. 1994). These findings suggested that there could be TFM-induced reductions in important plasma ions such as Na^+, Cl^- and Ca^{2+} in larval lamprey, but not in non-target rainbow trout. However, Birceanu et al. (2009) reported that there were no significant changes in plasma Na^+, Cl^- or Ca^{2+} of larval lamprey exposed to TFM (12-h TFM LC_{50}). Birceanu's work suggested that the amount of gill damage sustained by MRCs during exposure to the12-h TFM LC_{50} may not be significant enough to impair internal ion balance. It should be noted, however, that Mallatt et al. (1994) exposed lamprey to higher TFM concentrations (9-h TFM LC_{100}), which could have had a greater effect on the gills and plasma ion balance. Accordingly, one goal of this thesis was to examine the effects of TFM upon gill-mediated ion exchange and internal ion balance in the different life stages in sea lamprey life cycle, and in rainbow trout exposed to higher concentrations of TFM than those used by Birceanu et al. (2009).

1.8 Thesis Objectives

The three major objectives of this thesis were to:

(1) determine if different life stages of the sea lamprey were reflected by differences in basal energy reserves in larval, parasitic and upstream-migrant sea lampreys;

(2) establish if differences in basal energy reserves could affect the TFM sensitivity of lampreys;

(3) determine if TFM disrupted gill function and ion balance in different stages of the sea lamprey life cycle, as well as in non-target rainbow trout.

To achieve these goals, I tested the following two hypotheses addressing the mode of TFM toxicity in sea lamprey and rainbow trout:

Hypothesis I. The uncoupling of mitochondrial oxidative phosphorylation by high concentrations of TFM, equivalent to the TFM 12-h $LC_{99.9}$ (MLC), causes rapid reductions in ATP reserves and glycogen stores in the body, and such disturbances are life stage specific (Chapter 2).

Hypothesis II. TFM disrupts gill-mediated ion exchange leading to reductions in Na^+ uptake and corresponding reductions in plasma ions and tissue water balance, in the sea lamprey and non-target rainbow trout (Chapter 3).

The hypothesis that exposure to the MLC of TFM resulted in rapid reductions in ATP and glycogen stores was tested in larval, parasitic and upstream-migrant lamprey by exposing the fish to TFM (12-h $LC_{99.9}$), and collecting tissues (brain, liver, kidney, muscle, gills, blood) at different time intervals for later determination of energy stores and metabolites.

The hypothesis that exposure to the MLC of TFM resulted in marked reductions in ion uptake and internal electrolyte imbalances were tested in larval, parasitic and upstream-migrant lamprey, and rainbow trout. Gill samples were collected from larval, parasitic and upstream-migrant lamprey and analyzed for Na^+/K^+-ATPase activity to determine if TFM directly damaged the ion uptake machinery of the gill. Plasma samples were also collected and analyzed for Na^+, Cl^- and Ca^{2+} content to determine if TFM resulted in internal electrolyte imbalances, and whether there were life-stage or species-specific differences in the responses of lamprey and trout to the lampricide. Unidirectional flux measurements (influx, efflux and net flux) of Na^+ and Ca^{2+} were measured in larval lamprey, upstream-migrant lamprey, and rainbow trout exposed to TFM (12-h $LC_{99.9}$) to determine if TFM interfered with gill-mediated ion uptake processes.

Figure 1-1. The Sea Lamprey Life Cycle.

The sea lamprey's life cycle is characterized by four major life phases: i) larval, ii) metamorphic, iii) parasitic, and iv) upstream-migrant. Figure from Wilkie (2011).

A

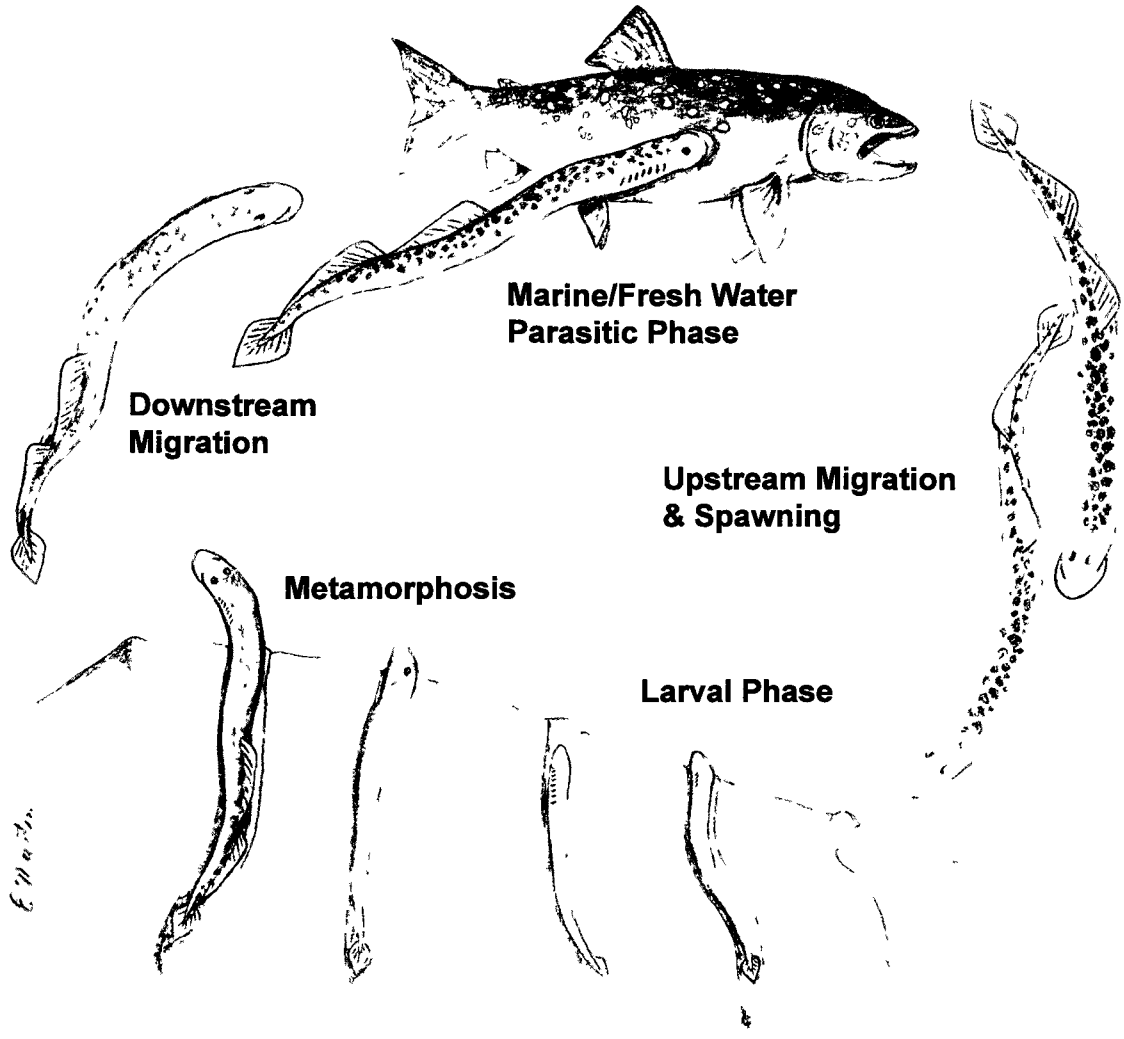

Marine/Fresh Water
Parasitic Phase
Downstream
Migration
Upstream Migration
& Spawning
Metamorphosis
Larval Phase

Figure 1-2. Historical Population Estimates for Sea Lamprey and Lake Trout in Lake Superior.

Sea lamprey predation and overfishing are thought to be the major contributors to the collapse of the lake trout fishery in Lake Superior (USGS Great Lakes Science Centre).

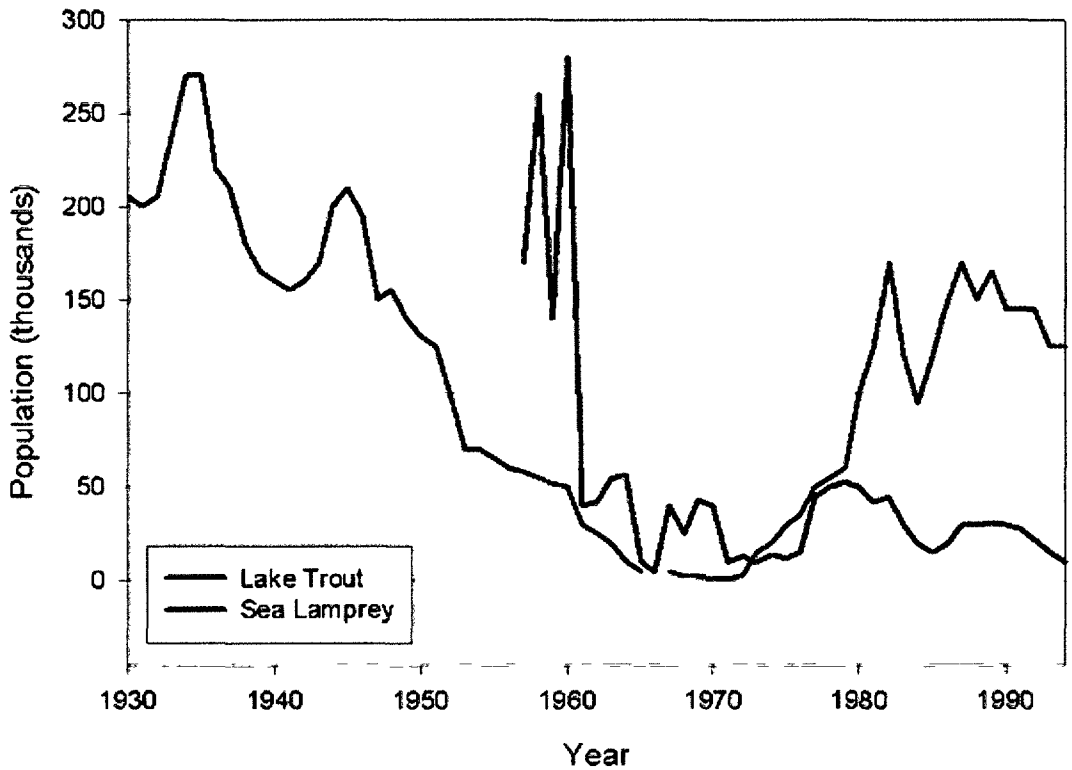
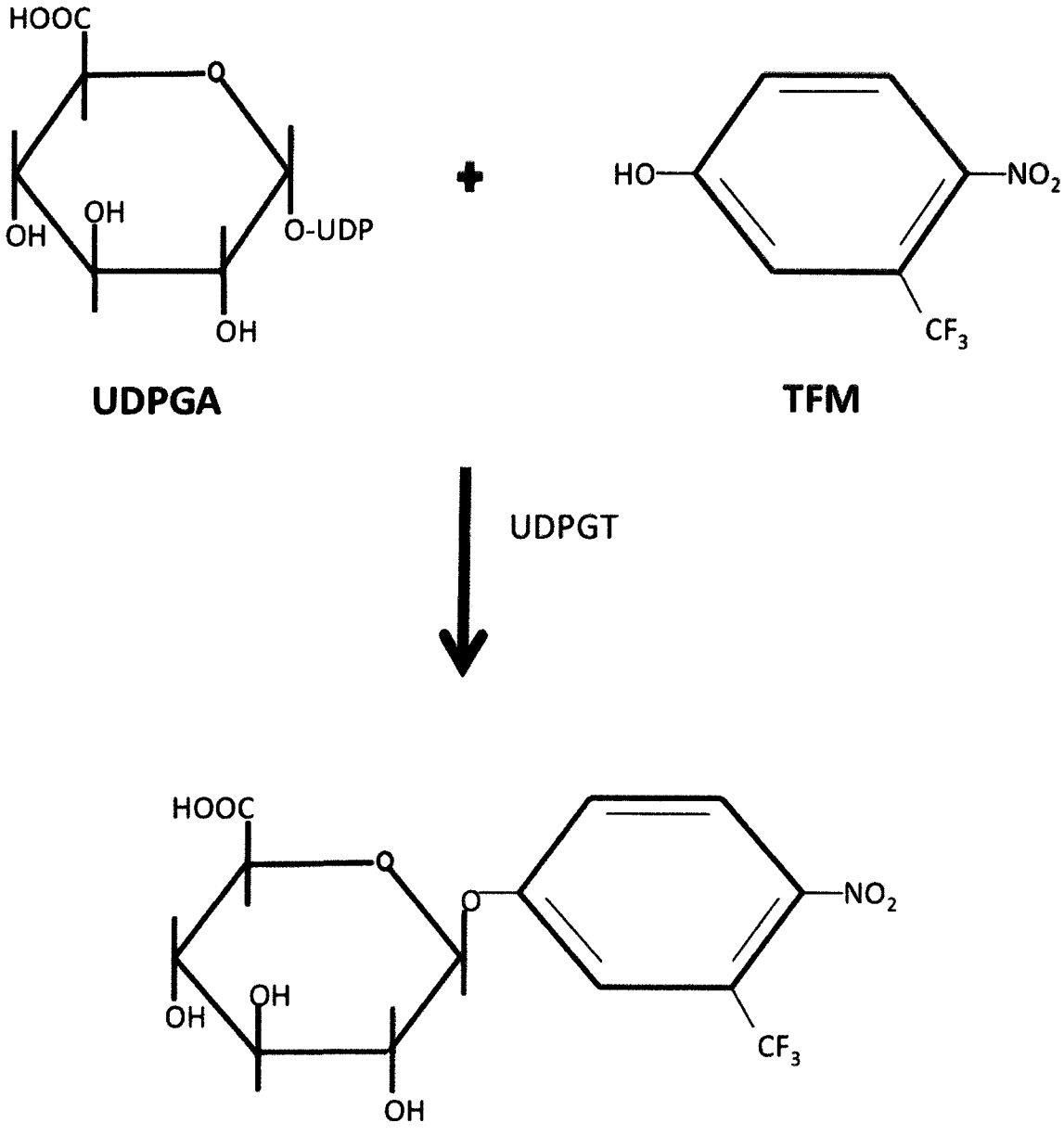

300
250
200
150
100
50
0
Population (thousands)
Lake Trout
Sea Lamprey
1930
1940
1950
1960
1970
1980
1990
Year

Figure 1-3. Conjugation of TFM By Glucuronidation.

Detoxification of TFM mainly occurs via the process of glucuronidation in the liver. The process combines TFM with UDP-glucuronic acid (UDPGA) via the enzyme UDP-glucoronyltransferase (UDPGT), to produce TFM glucuronide. The limited ability of sea lamprey to detoxify TFM via glucuronidation is thought to be the major contributor to the selectivity of TFM to sea lamprey. Figure modified from Kane et al. (1993).



UDPGA **TFM**

TFM glucuronide + UDP

Figure 1-4. Oxidative Phosphorylation In The Mitochondria.

Simplified schematic of oxidative phosphorylation in the mitochondria. Electrons enter the electron transport chain at complex I and are shuttled through complex II, III and IV. H^+ are pumped from the matrix by complex I, III and IV into the inter-membrane space. Oxygen is the final acceptor of the electrons shuttled in the electron transport chain leading to the generation of H_2O. Because the inner mitochondrial membrane is relatively impermeable to these protons, they can only move down their electrochemical gradient via ATP-synthase. The energy released from the protons flowing down their electrochemical gradient is captured by ATP-synthase which drives the phosphorylation of ADP to ATP, thus producing the high-energy molecule that drives most energy consuming processes in the cell. Figure adapted from Voet et al. (2006).

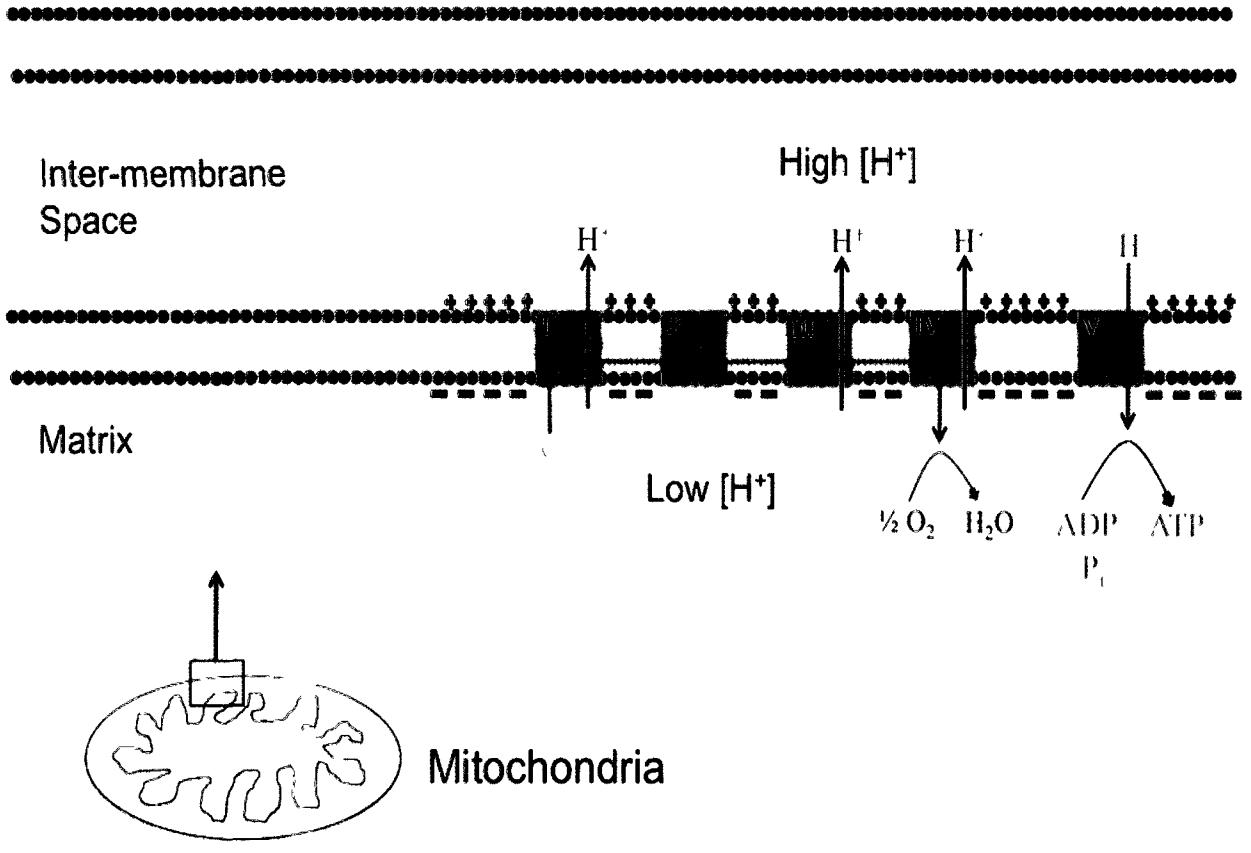
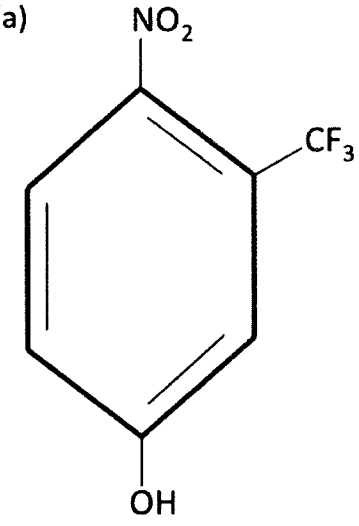

Inter-membrane
Space
High [H+]
H+
H+
H+
H+
Matrix
Low [H+]
½ O2
H2O
ADP
ATP
Pi
Mitochondria

Figure 1-5. Molecular Structure of TFM (a) and 2,4-DNP (b).

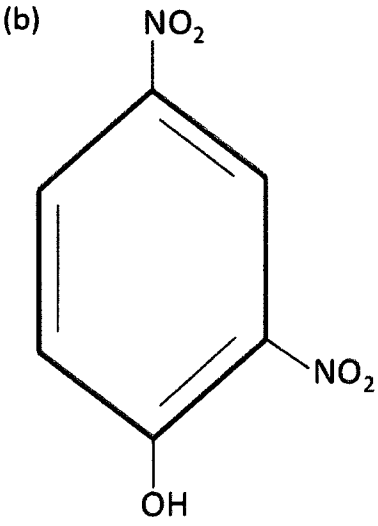
The similarity of (a) TFM to (b) 2,4-DNP, a known uncoupler of mitochondrial oxidative phosphorylation suggests that TFM may exert its toxicity in a similar manner (Applegate et al. 1966).

(a)



TFM

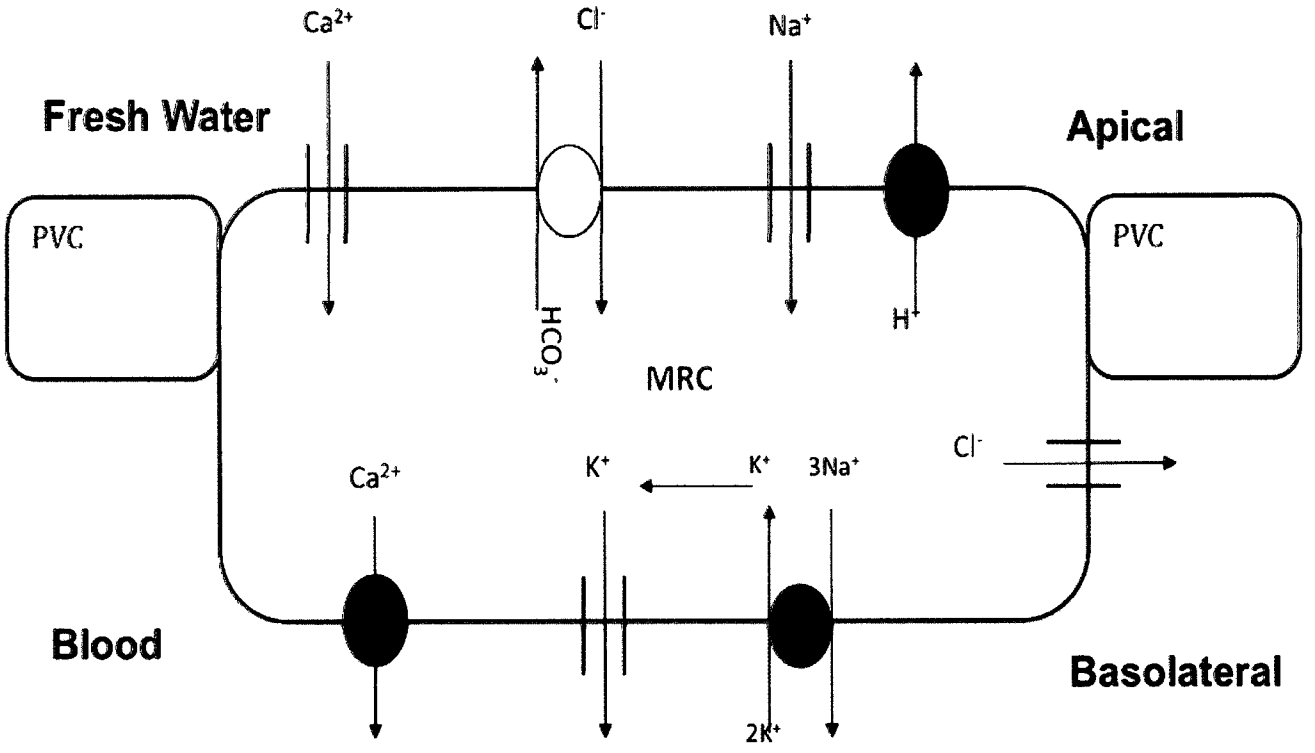
(b)



2,4-DNP

Figure 1-6. Model of Ion Uptake By The Gills of Freshwater Fishes.

The model describes the uptake of ions (Na^+, Cl^- and Ca^{2+}) from fresh water at the apical side of the mitochondrial rich cell (MRC) and the basolateral (blood) side. The solid circles represent active transport of ions (ATP-dependent), open circles are ion exchangers. Ion channels are denoted by parallel lines with arrows indicating ion movement in and out of the MRC. The H^+-ATPases in the apical membrane generates an electrochemical gradient that promotes Na^+ uptake from the water via apical Na^+ channels. Na^+/K^+-ATPase pumps on the basolateral membrane subsequently pump Na^+ from the cytosol into the blood. Ca^{2+} uptake is occurs via apical Ca^{2+} channels, down electrochemical gradients generated by the activity of basolateral Ca^{2+}-ATPase pumps. Cl^- uptake occurs via apical membrane Cl^--HCO_3^- exchange and basolateral anion channels. PVC= pavement cell. Model adapted from Marshall (2002).


Ca2+
Cl-
Na+
Fresh Water
Apical
PVC
PVC
HCO3-
H+
MRC
Cl-
Ca2+
K+
K+
3Na+
Blood
Basolateral
2K+

Chapter 2

Life History Stage Effects the TFM Sensitivity of Sea Lampreys

Abstract

Parasitic sea lampreys continue to plague fisheries in the Great Lakes of North America, which has necessitated continuation of an integrated pest management plan to control these invasive species. The primary method of population control in the Great Lakes is to treat larvae-infested streams with the pesticide 3-trifluoromethyl-4-nitrophenol (TFM). Although TFM lowers brain glycogen energy reserves leading to death in larvae at lower TFM concentrations (12-h LC_{50}), little is known about TFM toxicity in sea lamprey at higher concentrations (12-h $LC_{99.9}$). This study investigated the hypothesis that the uncoupling of mitochondrial oxidative phosphorylation by high concentrations of TFM, equivalent to the TFM 12-h $LC_{99.9}$, causes more pronounced and rapid reductions in ATP reserves and glycogen stores in the body. Exposure of larval, parasitic and upstream-migrant lamprey to TFM caused brain glycogen concentrations to decrease by 36-40% by the end of the exposure, suggesting that the animals increased their reliance on glycolysis to generate ATP in the absence of oxidative ATP production. The reduction in brain glycogen was followed by corresponding increases in brain lactate concentrations. However, changes to brain, kidney and muscle lactate concentrations did not stochiometrically match glycogen depletion in the corresponding tissue. Despite marked, 40-50% reductions in brain phoshocreatine, ATP levels in the brain were unaltered by exposure to TFM. ATP concentrations in the liver and kidney were significantly reduced, however, by approximately 50-90% during exposure to TFM. In conclusion, TFM toxicity at all stages of the sea lamprey life cycle is likely due to an inhibition of ATP supply to the brain, resulting in a mismatch between ATP supply and demand that contributes to death.

2.1 Introduction

Larval sea lampreys generally spend 3-7 years as filter-feeders, burrowed in soft sediments of rivers and streams (Beamish and Potter 1975), primarily feeding on detritus (Sutton and Bowen 1994). When the larvae reach a minimum length of 120 mm and acquire sufficient lipid reserves, they undergo a "true" metamorphosis (transformation) characterized by major changes in their body plan and physiology (O'Boyle and Beamish 1977; Youson 1980; Holmes et al. 1994). The post-metamorphic juveniles then migrate downstream to lakes, where they commence the parasitic life stage, in which they use their oral disc and rasping tongue to attach to host fishes and then feed on the blood of larger fishes. Sea lampreys are primarily considered parasitic fish (King 1980; Potter and Gill 2003), but landlocked sea lamprey can be considered predators because the host fish usually does not survive attacks (Farmer 1980; Bence et al. 2003). After spending 1-2 years in the parasitic phase, sea lamprey stop feeding during the period of sexual maturation (upstream migrant phase; Larsen 1980) and migrate upstream in late spring and early summer to spawn (Larsen 1980).

As a result of modifications to the Welland Canal, sea lampreys were able to invade the upper Great Lakes, which provided access to abundant host organisms such as lake trout (*Salvelinus namaycush*), lake whitefish (*Coregornus clupeaformis*) and lake herring (*Coregonus artedii*) (Christie 1973; Smith and Tibbles 1980; Christie and Goddard 2003, Heinrich et al. 2003). By the mid-1900s, predation by sea lamprey contributed to massive declines in Great Lakes fish stocks, especially lake trout (Applegate 1950; Christie and Goddard 2003). In 1956, the halogenated mononitrophenol, 3-trifluoromethyl-4-nitrophenol (TFM) was applied to larval lamprey

nursery streams in Lake Superior, and it effectively suppressed parasitic sea lamprey populations (Smith and Tibles 1980).

The integrated management of sea lamprey (IMSL) in the Great Lakes continues to rely on the application of TFM to nursery streams for control of parasitic sea lamprey populations. The Great Lakes Fishery Commission, which oversees sea lamprey control, wishes to reduce its reliance on TFM because of the high costs of lampricide treatment and ongoing public concern regarding pesticide use (GLFC 2008). To achieve the goal of lower TFM use, a better understanding of TFM's mechanism(s) of toxicity could help refine TFM treatment protocols, to better assess and manage the risks of TFM on non-target vertebrates and invertebrates. Accordingly, the primary goal of the present study was to determine if different life stages are reflected by differences in energy reserves in different tissues and to establish if these differences affect the TFM sensitivity of larval, parasitic and upstream-migrant lampreys.

Numerous acute toxicity studies have examined the sensitivity of non-target vertebrates and invertebrates to TFM (e.g. Applegate and King 1962; Kawatski et al. 1974; Boogaard et al. 2003). The concentration required to kill more resistant non-target fishes is approximately 3-5 times higher than the concentration needed to kill larval lamprey and the greater sensitivity of lamprey to TFM can be attributed to their limited ability to detoxify TFM via glucuronidation (Olson and Marking 1973; Lech and Statham 1975; Kane et al. 1994). However, only recently has the mechanism of TFM toxicity been examined in larval lamprey (Wilkie et al. 2007; Birceanu et al. 2009). Because of TFM's structural similarity to 2,4-dinitrophenol (2,4-DNP), a known uncoupler of mitochondrial oxidative phosphorylation, Applegate (1966) suggested that TFM might

reduce ATP supply leading to death. Early support for this hypothesis came from a study by Niblett and Ballantyne (1976) demonstrating that TFM at the cellular level causes uncoupling of oxidative phosphorylation in rat mitochondria. Recent evidence indicates that sea lamprey mitochondria experience similar uncoupling as rat mitochondria when exposed to TFM (Birceanu et al. 2011).

Wilkie et al. (2007) exposed larval lampreys to a lethal dose of TFM and reported that whole body concentrations of plasma glucose and phosphocreatine (PCr) were significantly reduced during TFM exposure. More recently, Birceanu et al. (2009) demonstrated that larval lamprey exposed to their 12 h TFM LC_{50} experienced significant reductions in brain and liver glycogen and ATP. This work was the first direct evidence that exposing larval lamprey to TFM forces them to increase their reliance on anaerobic glycolysis to generate ATP when oxidative ATP supply is diminished. Fuel stores such as glycogen and PCr are an important source of ATP when oxidative ATP supply does not meet ATP demand, as is the case during anoxia/hypoxia exposure (see Nilsson 2001; Walsh et al. 2007; Bickler and Buck 2007 for reviews) and exhaustive exercise (see Kieffer 2000; Milligan 2000; Kieffer 2010; Wilkie 2011 for reviews). However, the concentration of TFM used in these earlier studies was less than that used in the field, where Sea Lamprey Control personnel apply TFM at doses between 1 and 1.5 times the $LC_{99.9}$. Moreover, it is unclear if sea lamprey respond to TFM in a similar manner at different life stages in their life cycle. To test the hypothesis that TFM exposure reduces ATP supply and increases reliance on anaerobically generated ATP, larval, parasitic and upstream-migrant lamprey were exposed to their respective 12 h TFM $LC_{99.9}$ and

anaerobic fuels stores such as tissue glycogen and PCr, as well as tissue metabolites (ATP and lactate) were measured in brain, liver, kidney and muscle tissue.

2.2 Materials and Methods

2.2.1 Experimental Animals and Holding

Sea lampreys (*Petromyzon marinus*) in their larval (1-2 g, 90-120 mm) and parasitic phases (~150 g, 30-50 cm) where provided courtesy of the Hammond Bay Biological Station, United States Geological Survey (USGS, Hammond Bay, Michigan), while upstream migrant lampreys were captured in traps on the Humber River, Toronto by Department of Fisheries and Oceans Canada (DFO) (Sault Ste. Marie, Ontario, Canada) personnel, and then transported to Wilfrid Laurier University. At Wilfrid Laurier, larval lamprey were held in tanks with sand (4-5 cm deep) to provide burrowing substrate, while parasitic and upstream-migrant lampreys (non-burrow dwelling life stage) were held in 100-500 L holding tanks. Larval and parasitic lamprey tanks received well water on a flow through basis (pH~ 8.0; titratable alkalinity ~ 200 mg $CaCO_3$ L^{-1}; hardness ~ 450 mg $CaCO_3$ L^{-1}; temperature 10-13 °C), the lampreys were held under a 12 h light and 12 h dark photoperiod. Upstream-migrant lamprey tanks received well water on a flow through basis similar to larval and parasitic lamprey except the water was chilled to approximately 5 °C to prolong survival. Baker's yeast (2 g yeast per larva; Holmes and Youson 1994; Wilkie et al. 1999) was used to feed larval lampreys once a week, but it was not possible to feed the large parasitic lampreys due to an inability to get large enough fish for them to feed upon, and upstream-migrant lampreys do not feed during this terminal phase of their life cycle (Larsen 1980). Lampreys were held in the lab approximately 2-4 weeks before experiments commenced. Experiments and fish

husbandry were approved by the Wilfrid Laurier University Animal Care Committee and followed Canadian Council of Animal Care guidelines.

2.2.2 Experimental Protocols

2.2.2.1 Determination of Acute Toxicity of TFM

It was necessary to run toxicity tests on larval and upstream-migrant lamprey as TFM toxicity has been shown to be effected by a number of different chemical factors such as water pH and alkalinity (McDonald and Kolar, 2007) and the season lampreys were collected (Scholefield et al. 2008). Therefore, to determine the toxicity of TFM to larval, parasitic and up-stream migrant lampreys in Wilfrid Laurier well-water, two range finder toxicity experiments were conducted by exposing the larvae to nominal TFM concentrations of 0.0, 1.0, 3.0, 5.0, 7.0, 10.0 and 20.0 mg l^{-1} for 12 h and the upstream-migrants to nominal TFM concentrations of 0.0, 0.25, 0.5 1.0, 2.0 5.0, 10.0 and 25.0 mg l^{-1} for 12 h. A TFM toxicity test was not conducted on parasitic lamprey because only a limited number (~50 animals) were obtained from the USGS. Prior to the beginning of each range finder toxicity test, larval and upstream-migrant lampreys were acclimated to their respective exposure containers for 12 h prior to the start of the range finder toxicity test. A total of five larvae and six upstream-migrant lampreys were exposed to each respective TFM concentration, which were completed in the dark as larval and upstream-migrant lampreys are negatively phototactic (Rovainen and Schieber 1975) and TFM is light sensitive (Hubert 2003).

Field formulation TFM (Clariant SFC GMBH WERK, Griesheim, Germany), provided courtesy of the DFO, was used for all experiments. The concentrations of TFM in water samples were verified using precision TFM standards, also provided courtesy of

the DFO, by plate spectrophotometry (SpectraMax 190, Molecular Devices, CA) at a wavelength of 395 nm.

2.2.2.2 Effects of TFM on Lamprey Metabolism

To determine the effect of TFM upon fuel stores (glycogen and high energy phosphagens) and metabolites (lactate), different lamprey tissues (brain, liver, kidney and muscle) were taken from larval, parasitic and upstream migrant lamprey at pre-determined intervals (0.5, 1, 2, 3 and 5 h) during exposed to their respective 12 h $LC_{99.9}$.

2.2.2.2.1 Larval, Parasitic and Upstream-migrant Sea Lamprey Exposure to TFM

In the 2-4 weeks upon receiving the larval lamprey and the beginning of experiments, lampreys were not fed. Individual larvae were placed into darkened containers containing aquarium cotton (2 g per container) and 850 mL of flowing, well-aerated water, and left to acclimate overnight. The parasitic and upstream-migrant lampreys were placed into darkened three L boxes receiving well-aerated water on a flow-through basis, and left overnight. The next day, the respective TFM exposures were done under static conditions. Since sea lampreys have very low ammonia excretion rates (20-100 nmol N g^{-1} h^{-1}; Wilkie et al. 2006, 2007a) and ammonia concentrations in individual containers would be well below concentrations known to be toxic to larval sea lamprey (96 h LC_{50} = 3 mmol l^{-1}; Wilkie et al. 1999), exposure water was not changed. Water samples were taken at 0 and 5 h to determine TFM exposure concentration for larval and parasitic lamprey, and at 0 and 3 h for upstream-migrant lamprey.

Larval and parasitic lampreys were terminally sampled at 1, 2, 3, and 5 h of TFM exposure, while upstream-migrant lamprey were terminally sampled at 0.5, 1, 2 and 3 h of TFM exposure. Upstream-migrant lamprey were not sampled at 5 h of TFM exposure as lampreys rarely survived passed 3 h of exposure. Control animals were held under the same conditions as exposed animals, minus the addition of TFM to the water. Lampreys were anaesthetized 5 min prior to sampling using an anaesthetic dose of tricaine methanesulfonate (MS222) [0.5 g^{-1} l^{-1} MS222 (Syndel Labs, British Columbia) buffered with 1.0 g^{-1} $NaHCO_3$.] After 5 min in the anaesthetic, the lampreys were euthanized with 1.5 g^{-1} l^{-1} MS222 buffered with 3.0 g^{-1} $NaHCO_3$. Initially, there were between 7-10 lampreys per TFM exposure time, but the numbers decreased because of mortality prior to the designated sampling time. Only surviving lampreys were sampled. To rule out artifactual changes in fuels and metabolites that could result from the confinement in the exposure containers, control larval and parasitic lamprey were sampled at 0 and 5 h, and control upstream-migrant lamprey were sampled at 0 and 3 h.

After anaesthetization, lampreys were blotted dry with a paper towel, and their mass and length were measured. Immediately following blood collection (within 1 min; see section 3.2.2.3.2 Blood Sampling), the gills, brain, liver, kidney and trunk (larval lamprey) or a filet of muscle (only from parasitic and upstream-migrant lamprey) were removed from each lamprey, snap frozen in liquid nitrogen (Wang et al. 1994a), and stored at -80°C until processed for analysis.

2.2.3 Analytical Techniques

2.2.3.1 Tissue Processing and Analysis

The muscle and liver were processed for metabolite and fuel store quantification by grinding the tissue under liquid nitrogen using the procedure outlined by Wang et al. (1994a) using a mortar and a pestle; kidney and brain were homogenized using a hand-held motorized pestle. Approximately 50-150 mg of powder was then transferred to a 2 ml microcentrifuge tube cooled in liquid nitrogen, its mass recorded, and a volume, four times the tissue mass, of 8% perchloric acid (PCA), containing 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), was added, and the tube left on ice for 10 min. A sub-sample (sub-sample 1), of the slurry (100 µl) was collected for glycogen analysis, while the remainder (sub-sample 2) was centrifuged at 10,000 x *g* for 2 min at 4 °C for metabolite analysis (ATP, PCr and lactate). The supernatant from sub-sample 2 was neutralized by adding 2 M KOH cocktail (containing of 0.4 mol l^{-1} imidazole and 0.4 mol l^{-1} KCl) at 0.5 x the weight of the supernatant, centrifuged at 10,000 x *g* for 3 min at 4 °C and stored at -80°C until analyzed. The same procedure was used for the brain, except that due to the small size of the brain (~3.0 mg), the PCA solution added was 11 times the mass of the brain and homogenized on ice using the hand held motorized plastic pestle (Gerresheimer Kimble Kontes LLC, Dusseldorf, Germany).

Unless noted, all enzymes and reagents were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Tissue ATP, PCr and lactate were quantified on extracts of sub-sample (2), and concentrations expressed in µmol g^{-1} wet tissue. For ATP and PCr analysis, a small volume of sub-sample 2 was added to "ATP/PCr cocktail A" (Triethanolamine-HCl containing Mg, NAD^+, glucose and glucose-6-phosphate

dehydrogenase), followed by the addition of the enzyme hexokinase which consumed ATP in the phosphorylation of glucose to glucose-6-phosphate. The subsequent the conversion of glucose-6-phosphate dehydrogenase to 6-phosphogluconate via glucose-6-phosphate dehydrogenase, led to the reduction of NAD^+ to NADH, which has a specific absorbance at 340 nm. Following the determination of ATP, addition of "ATP/PCr cocktail B" (0.25 M glycine buffer containing ADP and creatine kinase) was added to the microwell plate that allowed for the quantification of PCr from the sample, by driving the formation of ATP, which was quantified as described as above.

For lactate analysis, a small volume of sub-sample (2) was added to a NAD solution (0.2 M hydrazine buffer and NAD) and the addition of the enzyme lactate dehydrogenase (5 units) allowed for the determination of lactate within the sample based on the conversion of lactate to pyruvate resulting in the generation of NADH.

Tissue glycogen was determined using sub-sample (1), to which one part 2 mol l^{-1} acetate buffer to one part tissue was added, followed by 40 units amyloglucosidase to convert the glycogen to glucose. The mixture was incubated at 37 °C for 2 h, and the reaction terminated by adding 70% PCA (25 µl) to the digest, which was then neutralized with 3 mol l^{-1} K_2CO_3. Samples were stored at -80 °C until analyzed for glucose concentration. A second sub-sample of homogenate was taken to determine free glucose in the tissues, which was subsequently subtracted from the total glucose in the tissue to yield the glycogen concentration in µmol glucosyl units g^{-1} wet tissue. Glucose concentrations were determined spectrophotometrically on the homogenates by adding a 25 µl sample to a 500 µl glucose cocktail comprised of 0.250 M TEA-HCl containing Mg, NAD, ATP and glucose-6-phosphate dehydrogenase. Glucose concentration was

then determined following the addition of hexokinase (5 units) to the cocktail measuring the appearance of NADH in the solution at 340 nm due to the conversion of glucose-6-phosphate to glucose-6-phoshophate.

2.2.4 Calculations and Statistical Analysis

Determination of the 12 h $LC_{99.9}$ for the larval and upstream-migrant lampreys exposed to TFM was done using the Two-Point Interpolation method available from the Comprehensive Environmental Toxicity Information System (CETIS) analytical program (Tidepool Scientific Software, Version 1.6.1, California).

To determine whether there were differences in brain glycogen reserves in the three life stages examined, total brain glycogen stores were expressed per unit body mass (mg) using the following equation:

Brain Glycogen per Unit Body Mass (BG/UBM) = BG x MB/ BM, (4)

where BG is brain glycogen concentration in ηmol glucosyl mg^{-1} wet tissue, MB is the mass of the brain (mg) and BM is the body mass (mg) of the lamprey.

Data are presented as the mean ± 1 standard error of the mean (SEM). All comparisons were unpaired and analyzed using one-way analysis of variance (ANOVA). When significant variability was observed, statistical significance between the means was assessed using the Newman-Keuls post-test at the $p < 0.05$ level. When data sets did not meet the assumptions of a parametric design, nonparametric tests (Kruskal-Wallis test) were used to determined statistical significance.

2.3 Results

2.3.1 Determination of Acute Toxicity

The nominal TFM concentrations of 1.0, 3.0, 5.0, 7.0, 10.0 and 20.0 mg l^{-1} for larval lamprey were reflected by measured TFM concentrations of 1.1, 3.3, 5.6, 7.6, 11.4 and 22.0 mg l^{-1}, respectively. The larval lamprey exposed to TFM for 12 h had a $LC_{99.9}$ of 7.6 mg l^{-1} (Table 2-1). Subsequently, larval lampreys were exposed to a nominal TFM concentration of 7.6 mg l^{-1} (measured 8.1 mg l^{-1}) to determine how TFM altered metabolic homeostasis.

The nominal TFM concentrations of 0.25, 0.5 1.0, 2.0 5.0, 10.0 and 25.0 mg l^{-1} for upstream-migrant lamprey were reflected by measured TFM concentrations of 0.9, 1.2, 2.4, 3.5, 7.0, 13.8 and 20.5 mg l^{-1}, respectively. This experiment yielded a 12 h LC_{50} of 2.4 mg l^{-1} and a $LC_{99.9}$ of 5.0 mg l^{-1} (Table 2-1), which was then used as the nominal concentration used in the upstream-migrant (measured 4.8 mg l^{-1}) and parasitic (measured 5.2 mg l^{-1}) lamprey exposure.

2.3.2 Effects of TFM on Fuel Stores and Metabolites

2.3.2.1 Glycogen

Exposure of larval, parasitic and upstream-migrant lamprey to their respective 12 h TFM $LC_{99.9}$ lowered glycogen stores in the brain (Figure 2-1), but not in the liver (Figure 2-2), kidney (Figure 2-3) or muscle (Figure 2-4). Initially after 1 hour of TFM exposure brain glycogen in larval lampreys decreased significantly by 25%, but was then comparable to control levels at 2 and 3 h of exposure. However, after 5 h of TFM exposure, larval brain glycogen had decreased by 37% from control values of 93.0 ± 7.2

μmol glucosyl units g^{-1} wet tissue to 58.6 ± 5.7 μmol glucosyl units g^{-1} wet tissue (Figure 2-1; $p < 0.05$). Parasitic lampreys experienced a 39% reduction in brain glycogen from control values of 65.6 ± 4.4 μmol glucosyl units g^{-1} wet tissue after 5 h of TFM exposure (Figure 2-1; $p < 0.05$). Brain glycogen in control upstream-migrant lampreys were approximately 117.1 ± 4.5 μmol glucosyl units g^{-1} wet tissue and decreased by 37% to 73.8 ± 6.2 μmol glucosyl units g^{-1} wet tissue after 3 hours of TFM exposure (Figure 2-1; $p < 0.05$).

Exposure to TFM had no effect on liver glycogen concentrations (Figure 2-2). Control larval lampreys had liver glycogen concentrations of 8.4 ± 0.89 μmol glucosyl units g^{-1} wet tissue. Parasitic and upstream-migrant lampreys had approximately 1.4 ± 0.30 and 5.1 ± 0.75 μmol glucosyl units g^{-1} wet tissue of glycogen in their liver, which was significantly less than larval lamprey liver glycogen stores (Figure 2-2; Table 2-2).

Kidney glycogen in larval, parasitic and upstream-migrant lamprey were unaffected by TFM exposure. Larval kidney glycogen at all exposure periods was significantly higher than in both parasitic and upstream-migrant kidneys (Figure 2-3). Parasitic and upstream-migrant lamprey kidney glycogen concentrations ranged from 1.2 ± 0.20 and 2.8 ± 0.92 μmol glucosyl units g^{-1} wet tissue (Figure 2-3), while larval kidney glycogen concentrations ranged from 18.4 ± 3.1 to 27.3 ± 2.6 μmol glucosyl units g^{-1} wet tissue (Figure 2-3).

No disturbances were observed in larval, parasitic or upstream-migrant muscle glycogen levels. Control concentrations ranged from 22.2 ± 2.16 to 24.1 ± 1.83 μmol glucosyl units g^{-1} wet tissue in all three life stages (Figure 2-4; Table 2-2).

2.3.2.2 Lactate

The TFM-induced reductions in brain glycogen at each life stage were accompanied by a corresponding increase in brain lactate. Control brain lactate concentrations were approximately 1.3 ± 0.58 μmol g^{-1} wet tissue in larval lamprey, and increased 4-fold after 2 h, and near 9-fold after 5 h (Figure 2-1; $p < 0.01$). Control parasitic and upstream-migrant lampreys had similar brain lactate concentrations of approximately 6.7 ± 0.66 and 6.9 ± 0.71μmol g^{-1} wet tissue, which increased 2 and 2.5-fold after 3 and 5 h, respectively (Figure 2-1; $p < 0.01$).

Exposure to TFM had no effect on liver lactate concentrations in the parasitic and upstream-migrant lampreys (Figure 2-2). However, a 4-fold increase from 2.4 ± 0.63 μmol g^{-1} wet tissue in liver lactate was observed after larval lampreys were exposed to TFM for 5 h (Figure 2-2; $p < 0.05$). Parasitic and upstream-migrant controls had liver lactate concentrations of 2.3 ± 0.23 and 1.3 ± 0.60 μmol g^{-1} wet tissue, respectively (Figure 2-2).

Despite no observed changes in kidney glycogen levels, lactate concentrations in the kidney were altered significantly in the three-life stages. Similar to the liver, control kidney lactate concentrations in the parasitic and upstream-migrant lampreys were approximately 2.4 ± 0.48 to 2.3 ± 0.59 μmol g^{-1} wet tissue, respectively (Figure 2-3; Table 2-2). Control larval kidney lactate levels were slightly lower, approximately 1.0 ± 0.40 μmol g^{-1} wet tissue (Figure 2-3; Table 2-2). Larval and upstream-migrant kidney lactate concentrations increased 7.5 and 3-fold after 5 and 3 h of exposure, respectively (Figure 2-3; $p < 0.01$). Kidney lactate in parasitic lamprey increased approximately 2.5-fold to 6.0 ± 1.6 μmol g^{-1} wet tissue after 5 h of exposure (Figure 2-3; $p < 0.05$).

Muscle lactate concentrations in lampreys were low and ranged from 1.0 ± 0.6 to 1.8 ± 0.6 μmol g^{-1} wet tissue (Figure 2-4). Upstream-migrant lampreys had the most significant changes in lactate concentrations, in which a 35-fold increase to approximately 34.7 ± 5.4 μmol g^{-1} wet tissue was observed after 3 h (Figure 2-4; $p < 0.01$). A 9-fold increase in muscle lactate was observed after 5 h of exposure in the larval lamprey. No changes were observed in parasitic lamprey muscle lactate levels (Figure 2-4; $p < 0.01$).

2.3.2.3 ATP

Despite significant changes observed in brain glycogen and lactate concentrations, ATP levels in the brain were not altered by TFM exposure. Control brain ATP concentrations were approximately 1.4 ± 0.17, 1.2 ± 0.23 and 0.8 ± 0.1 μmol g^{-1} wet tissue in larval, parasitic and upstream-migrant lampreys, respectively (Figure 2-5; Table 2-2).

Liver ATP levels were significantly reduced during TFM exposure in the three-life stages (Figure 2-6). In larval controls, the liver ATP concentrations was 1.3 ± 0.1 μmol g^{-1} wet tissue, and decreased by approximately 57% to 0.56 ± 0.1 μmol g^{-1} wet tissue after 5 h of TFM exposure (Figure 2-6; $p < 0.05$). A similar 70% reduction from 1.25 ± 0.19 to 0.38 ± 0.17 μmol g^{-1} wet tissue in liver ATP was observed in the parasitic lamprey after 5 h of exposure (Figure 2-6; $p < 0.01$). By the 0.5 h and 1 h sampling periods there was a 54% decrease in upstream-migrant liver ATP levels from control concentrations of approximately 1.5 ± 0.23 μmol g^{-1} wet tissue (Figure 2-6). An 88% decrease in upstream-migrant liver ATP was observed by 2 h and 3 h of TFM exposure (Figure 2-6; $p < 0.01$).

Exposure to TFM had no effect on kidney ATP levels in larval lampreys. Control larval and parasitic lamprey kidney ATP concentrations were approximately 1.3 ± 0.23 and 0.9 ± 0.11 μmol g^{-1} wet tissue, respectively (Figure 2-7; Table 2-2). Parasitic lamprey experienced a 65% decrease to approximately 0.31 ± 0.06 μmol g^{-1} wet tissue in kidney ATP after 5 h of TFM exposure ($p < 0.05$). Control kidney ATP in upstream-migrant lampreys were approximately 0.77 ± 0.12 μmol g^{-1} wet tissue and underwent an 86% decrease to 0.11 ± 0.02 μmol g^{-1} wet tissue after 3 h of TFM exposure (Figure 2-7; $p < 0.01$).

For the most part, muscle ATP concentrations were unaffected by TFM exposure. In control larval and parasite lampreys, the muscle ATP concentrations were approximately 3.4 ± 0.39 and 3.5 ± 0.16 μmol g^{-1} wet tissue, respectively (Figure 2-8; Table 2-2). Interestingly, control upstream-migrant ATP levels (1.0 ± 0.17 μmol g^{-1} wet tissue) were approximately 50% lower than 1, 2 and 3 h of TFM exposure (Figure 2-8; Table 2-2; $p < 0.05$).

2.3.2.4 Phosphocreatine (PCr)

The effects of TFM on brain PCr were similar in the larval and parasitic lamprey, while brain PCr in upstream-migrant lampreys was unaltered. The PCr in the brain of larval lampreys underwent a significant 40 % decrease from a control value of approximately 6.8 ± 0.86 μmol g^{-1} wet tissue to approximately 4.2 ± 0.29 μmol g^{-1} wet tissue after 1 h of TFM exposure, and remained near this concentration throughout the experiment (Figure 2-5; $p < 0.05$). The reductions in brain PCr were proportionately greater in the brain of the parasitic lamprey, decreasing more than 50 % from 2.8 ± 0.65 μmol g^{-1} wet tissue in controls to 1.2 ± 0.36 μmol g^{-1} wet tissue after 5 h of exposure

(Figure 2-5; $p < 0.05$). Upstream-migrant brain PCr levels were unaffected by TFM exposure (Figure 2-5).

PCr levels in many samples were below levels of detection in the liver. Control values were approximately 0.3 ± 0.08, 1.1 ± 0.05 and 0.5 ± 0.23 μmol g^{-1} wet tissue for larval, parasitic and upstream-migrant lampreys, respectively (Figure 2-6; Table 2-2). Although liver PCr values appear to be low and very variable, liver PCr in upstream-migrant lamprey was completely depleted after 2 h of TFM exposure (Figure 2-6).

Kidney PCr was below levels of detection in many larval and upstream-migrant lampreys samples, and not detectable in the parasitic lamprey. In control larval and upstream-migrant lamprey, the kidney PCr was approximately 0.3 ± 0.20 and 0.9 ± 0.20 μmol g^{-1} wet tissue (Figure 2-7; Table 2-2). By the 2 h sampling period in the upstream-migrants, kidney PCr was not detectable, however after 3 h of exposure trace amounts of PCr (~ 0.1 ± 0.07 μmol g^{-1} wet tissue) were detected (Figure 2-7).

Life stage dependent differences in PCr concentration were observed amongst the 3 groups of lamprey studied (Figure 2-8; Table 2-2). Control parasitic and upstream-migrant lamprey had large muscle PCr reserves that were approximately 28.6 ± 1.37 and 22.3 ± 2.58 μmol g^{-1} wet tissue, respectively (Figure 2-8; Table 2-2). In contrast, larval lamprey had PCr concentrations that were about 50% of this value (Figure 2-8; Table 2-2). Exposure to TFM resulted in no changes in parasite muscle PCr concentrations, but muscle PCr was markedly reduced by about 25% after 1 h of TFM exposure, and continued to decrease through 3 h when it was 75 % lower than pre-exposure values (Figure 2-8; $p < 0.05$). In larval lamprey muscle, 5 h of TFM exposure resulted in a

significant 70% reduction in PCr compared to control larval muscle PCr concentrations (Figure 2-8; $p < 0.01$).

2.4 Discussion

2.4.1 Life Stage Dependent Differences In Anaerobic Energy Reserves

An added benefit to examining TFM toxicity in more than one sea lamprey life stage is that differences in baseline anaerobic energy reserves and metabolites can be discussed. The present study found many similarities and differences in anaerobic energy reserves and metabolites in the three life stages examined. Brain glycogen concentrations in all three life stages examined were comparable to Rovainen et al. (1969) and Foster et al. (1993) who found lamprey brain glycogen concentrations that were in some instances greater than 100 μmol glucosyl units g^{-1} wet tissue. Animals with large glycogen stores, such as the crucian carp (*Carassius carassius*) and the goldfish (*Carassius auratus*) can withstand hypoxic and anoxic conditions for many weeks (Lutz and Nilsson 1997; Bickler and Buck 2007). Under oxygen-depleted conditions, these animals rely on their large liver glycogen reserves to maintain ATP supply to the nervous system via anaerobic glycolysis (Lutz and Nilsson 1997). Larval lamprey can also tolerate hypoxic conditions for relatively long periods (up to several days), but not anoxia (Potter et al. 1970). This may be necessary in the substrate of some streams where they may experience drastic changes in the dissolved oxygen concentrations in the interstitial water within their burrows (Holmes et al. 1994).

Because of the greater likelihood of hypoxia in their burrows, brain glycogen reserves in the larval stage might be expected to be larger than those observed in the

parasitic and upstream-migrant stage (Wilkie 2011). When comparing total brain glycogen per unit body mass (TBG/UBM) the relationship between body mass and total brain glycogen indicates that the total amount of brain glycogen per milligram body mass decreases consistently with increasing body mass (Figure 2-9). This relationship is more evident with the construction of log-log plots, as the mass exponent describing TBG/UBM was -0.64 (Figure 2-9). The greater amount of total brain glycogen per unit body mass in smaller lamprey compared to larger lamprey suggests that larval lamprey would have a greater capacity to tolerate hypoxia compared to parasitic and upstream-migrant lamprey.

The liver is the primary organ that controls blood glucose levels in vertebrates (LeBlanc et al. 1995; Niswender et al. 1997; Moon 2001). Liver glycogen concentrations in larval lamprey were much higher than observed in parasitic and upstream-migrant lamprey (Table 2-2). However, when compared to many teleost species, larval lamprey have considerably lower levels of glycogen in their liver (Plisetskaya and Kuz'mina 1972; Larsen et al. 2001; Barcellos et al. 2010). With limited glycogen reserves in the liver, it is likely that lamprey primarily rely on large glycogen stores in the brain to supply the nervous system with its main fuel source, glucose (Rovanien et al. 1969; Rovanien 1970; O'Boyle and Beamish 1977). Differences in liver glycogen stores between the life stages could be attributed to fasting. In teleost, starving fish for as little as seven days can completely deplete liver glycogen stores (Larsen et al. 2001; Barcellos et al. 2010). In addition, a study by Sheridan and Mommsen (1991) reported that coho salmon fasted for one and three weeks depleted liver glycogen levels by 50-60%. As mentioned earlier, sea lampreys do not feed during the spawning migration and parasitic

lamprey were not fed and had been starved a minimum of two weeks. Therefore, based on evidence from the aforementioned studies, its seems reasonable to suggest that the parasitic and upstream-migrant lampreys in my study could have depleted their liver glycogen reserves before the beginning of the experiments as a result of starvation (upstream-migrant and parasitic, respectively).

Muscle glycogen stores are proportional to body size in salmonid fishes such as the rainbow trout (Ferguson et al. 1993) and Atlantic salmon (McDonald et al. 1998). As a result, one would expect larger adult lampreys to have greater amounts of resting muscle glycogen per unit wet muscle mass reserves than larval lamprey. Contrary to this, larval muscle glycogens were similar in both parasitic and upstream-migrant lamprey (Table 2-2), a trend also observed by Wilkie et al. (2001).

Analysis of resting tissue lactate concentrations in the three life stages examined suggests that animals were not solely relying on glycolysis for ATP supply under control conditions. Under resting conditions, cells meet their ATP demands via oxidative phosphorylation resulting in low glycolytic rates and lactate concentrations (Moyes and West 1995; Juel 1997; Bickler and Buck 2007). When ATP supply/demand is altered in such away that oxidative phosphorylation cannot meet cellular ATP needs, an increase in glycolytic flux (often referred to as anaerobic glycolysis) ensues to make-up for the ATP shortfall (Juel 1997). Increased glycolysis results in increased tissue lactate concentrations as increased pyruvate generation increases the substrate supply for lactate dehydrogenase (Moyes and West 1995). The low control muscle lactate concentrations in the three life stages examined therefore suggests that lamprey in all three life stages were

in a resting state (Table 2-2), and the values were are in good agreement with those reported by Boutilier et al. (1993) and Wilkie et al. (2001).

Liver lactate concentrations in larval lamprey were relatively low, and comparable to values reported by Birceanu et al. (2009). Parasitic and upstream-migrant lamprey had similar liver lactate concentrations again supporting the premise that the animals were in a relatively stable resting state. Interestingly, larval brain lactate concentrations were significantly lower (Table 2-2) than that of the parasitic and upstream-migrant lamprey. The larval brain lactate concentrations in my study were in good agreement with those found by Rovanien et al. (1969). However, differences in resting brain lactate concentrations between larval, and parasitic and upstream-migrant lamprey may be a result of differences in resting metabolic rate and life style. Parasitic and upstream-migrant lampreys are much more metabolically active (Farmer 1980; Larsen 1980) and have an active life style (Farmer 1980; Larsen 1980; Leblanc et al. 1995), while larval lamprey are sedentary organisms with much lower metabolic rates (Lewis 1980). Animals with higher metabolic rates require higher ATP supply, as their basal ATP demands are greater than animals with lower metabolic rates (Lutz and Nilsson 1997; Bickler and Buck 2007). Higher glycolytic rates may be necessary to meet larger basal ATP demands resulting in elevated resting tissue lactate concentrations. In spite of this, it is unlikely that oxidative phosphorylation in upstream-migrant and parasitic lampreys would be insufficient to meet basal ATP demands, however whether there are life stage differences in tissue glycolytic rates deserves further investigation.

The most important high-energy phosphate molecule to supply cells, tissues and organisms with energy is ATP. Reducing the amount of ATP available to a cell or

organism (i.e. ATP supply) can result in a number of physiological disturbances that can lead to death of the organism (Hochachka, 1991; Bickler and Buck 2007). Resting larval, parasitic and upstream-migrant sea lamprey in my study showed similar ATP concentrations in all four tissues (Table 2-2). Brain, liver and muscle ATP concentrations in larval lamprey were similar to those found by Birceanu et al. (2009). Conversely, muscle ATP concentrations in upstream-migrant lamprey were lower than those reported by Boutilier et al. 1993 (Table 2-2). The lower muscle ATP concentrations observed in upstream-migrant lamprey could be linked to differences in water temperatures. Metabolic demand is reduced during exposure to cold temperatures (Bickler and Buck 2007), which reduces ATP demand and inevitably lowers ATP supply (McCue 2006). In my study upstream-migrants were held at 5^{O}C, while the previously mentioned study held upstream-migrants to 10^{O}C.

When ATP supply is reduced, the hydrolysis of PCr by the enzyme creatine kinase (CK) can help sustain ATP supply (Hochachka 1991). Depletion of PCr when ATP supply is inhibited and/or when ATP demand is high has been demonstrated in a number of oxygen starvation and exhaustive exercise studies (Lutz et al. 1984; Milligan and Wood 1986; Boutilier et al. 1993; Wilkie et al. 1996; Wilkie et al. 2001). Larval lamprey had much higher brain PCr concentrations compared to parasitic and upstream-migrant lamprey (Table 2-2) and were comparable to those of Rovanien et al. (1969) and Birceanu et al. (2009). High brain PCr stores may partially explain why larval lampreys can tolerate hypoxic conditions that lead to reduced ATP supply. As suggested by Rovainen et al. (1971) one way the brain and liver differ is in their PCr content. In larval and upstream-migrant lampreys, only trace amounts of PCr were detected in liver

samples (Table 2-2), which is in good agreement with values reported by Ennor and Rosenberg (1952).

Previous research from this lab (Birceanu et al. 2009) found control larval lamprey brain glycogen concentrations that were approximately 1/5 the values reported in the present study. As mentioned above larval brain glycogen concentrations in my study were similar to those reported by Rovainen et al. (1969). Wang et al. (1994b) has shown that an increase in sampling time decreases tissue glycogen values, likely due continued glycogen breakdown before the tissue is frozen. Despite the same observed trend (reduction in glycogen during TFM exposure) in both studies, differences in absolute values could be at least partially due to improvements in sampling technique that allowed for cleaner and faster removal of the brain and tissue handling. In addition, differences in the time of season the lamprey were collected might have contributed to the observed differences in glycogen reserves. O'Boyle and Beamish (1977) reported that liver glycogen stores are affected by season, which they suggested was due to differences in seasonal food availability. Also, larval lampreys from Birceanu et al. (2009) were held in the lab for a longer period of time compared to the present study. Whether confinement under laboratory holding conditions alters brain glycogen stores in larval lamprey deservers further investigation. Finally, Rovainen et al. (1969) reported that glycogen concentrations in lamprey brains' varied considerably between shipments of animals and among individuals.

2.4.2 TFM Toxicity In Different Life Stages

The present findings support the suggestion that TFM exposure in larval, parasitic and upstream-migrant lampreys causes a mismatch between ATP supply and ATP

demand (Birceanu et al. 2009), as indicated by reductions in ATP, PCr and glycogen in the brain, liver and kidney of the three life stages examined. These findings also support the hypothesis that the inhibition of oxidative ATP production in the mitochrondria (Niblett and Ballantyne 1976; Birceanu et al. 2011) leads to increased reliance on anaerobic fuels such as PCr and anaerobic glycolysis.

2.4.2.1 Brain

Although brain ATP levels were unaltered in the three life stages examined, it is important to note that anaerobic ATP production in the brain likely made-up for short-falls in ATP supply caused by TFM (Lutz and Nilsson 1997; Bickler and Buck 2007). In lampreys and most other vertebrates, anaerobic ATP production would have been sustained by the fermentation of glucose via glycolysis (Rovainen et al. 1971; Polakof et al. 2007) and the hydrolysis of PCr (Hochachka 1991). The latter process occurs in response to decreases in the ATP/ADP ratio via the enzyme creatine kinase (CK) (Hochachka 1991). Because PCr buffers ATP concentrations, PCr will only return to steady-state values when sufficient ATP supplies are capable of reversing the reaction (Ellington 2001). The low level of PCr observed in the brain of larval and parasitic lampreys indicates that ATP supply was indeed impaired throughout the duration of TFM exposure. The large stores of glycogen and PCr in the brain compared to other vertebrates, likely explain the lack of change in ATP during the TFM exposure period (Table 2-3).

Further evidence that the supply of ATP to the brain was impaired by TFM exposure can be seen by the reductions in brain glycogen observed in larval, parasitic and upstream-migrant sea lamprey (Figure 2-1). The availability of glucose in the lamprey

brain is essential for proper nervous system function and its supply is thought to involve the break down of glycogen from the surrounding meningeal tissue (see section 2.4.2.2 **Liver**; Rovainen et al. 1969; Rovainen et al. 1971). Reductions and/or depletion in brain glucose and tissue glycogen stores can be caused by a number of factors including exposure to anoxia/hypoxia, food deprivation and ischaemia (Soengas et al. 1998; Bickler and Buck 2007; Polakof et al. 2007). Ultimately, such glycogen depletion leads to death of the organism because the monomer of glycogen (glucose) is the only substrate capable of supplying ATP via glycolysis (Voet et al. 2006).

As mentioned previously, the crucian carp and the goldfish have large glycogen reserves; however, their glycogen reserves are stored in the liver as opposed to the meninges in the sea lamprey. In the crucian carp and goldfish such reserves contribute to the organism's survival during low environmental oxygen concentrations because it allows for the maintenance of ATP supply via anaerobic glycolysis (Lutz and Nilsson 1997). In addition to high concentrations of fermentable substrate, anoxia/hypoxia tolerant organisms must also suppress whole-body metabolic rate and tolerate acidic metabolic end products (e.g. lactic acid generation; Bickler and Buck 2007). For example, goldfish can suppress their metabolic rate by up to 60% to reduce ATP demand during severe hypoxia exposure (Van Waversveld et al. 1989). Without the suppression of metabolic rate to lower ATP demand, anaerobic glycolysis would be unable to provide sufficient ATP to meet the animals' metabolic requirements. Ultimately, if ATP supply cannot match demand it will lead to the depletion of glycogen, starving the animal of ATP (Bickler and Buck 2007). Because TFM exposure and hypoxia/anoxia exposure cause similar physiological disturbances such as the inhibition of oxidative ATP supply

and reduced glycogen reserves it is reasonable to suggest that the ultimate cause of death in organisms exposed to TFM may be very similar to the cause of death in organisms exposed to hypoxia/anoxia.

Exposure to hypoxia/anoxia in anoxia susceptible organisms and ATP starved anoxia tolerant organisms results in a sequence of toxic events at the neural cellular level that occurs in more or less the following order: i) ATP supply is reduced due to cells relying solely on anaerobic glycolysis. ii) The demand of ATPases that maintain ion gradients cannot be met by ATP supply which results in the loss of K^+ and the gain of Na^+ and Ca^{2+} (depolarization). iii) The release of glutamate from depolarized presynaptic neurons causes an activation of glutamate receptors, most notably NMDA receptors, on post-synaptic neurons and leads to the influx of Ca^{2+} into cells. iv) Intracellular Ca^{2+} accumulation is further enhanced as Ca^{2+} stores are released from the endoplasmic reticulum and mitochondria. v) These events led to loss of ion homeostasis, neuronal swelling, the bulging and rupturing of neuron membranes, and ultimately cell death (Lutz and Nilsson 1997; Walsh et al. 2007).

Another challenge arising from insufficient ATP supply is the generation of metabolic acid arising from increased rates of glycolysis, which can lead to pronounced drops in blood and tissue pH. In order to sustain high glycolytic rates, the end-product of glycolysis, pyruvate is reduced to lactic acid via the enzyme lactate dehydrogenase (Voet et al. 2006). Because of lactic acid's low pK_a (3.9), at physiological pH more than 99% of lactic acid exists in its ionic form (lactate and H^+ in equilmolar amount) (Juel 1997). To avoid self-intoxication the anoxia tolerant crucian carp and goldfish convert lactate to ethanol, an acid-base neutral molecule that can diffuse into the water through the gills

(Shoubridge and Hochachka 1980; Nilsson 1988). In my study brain lactate concentrations increased significantly throughout the duration of the TFM exposure in the three life stages examined, suggesting there may have been substantial metabolic H^+ generated and a pronounced metabolic acidosis. However, this possibility requires further investigation.

The increase in lactate and decrease in glycogen are an indication that lampreys were experiencing diminished oxidative ATP supply and relying on anaerobic glycolysis for ATP production. One would expect, however, that the ratio of lactate accumulation to glycogen disappearance would be 2:1, as seen in previous studies (Milligan and Wood 1986; Schulte et al. 1992; Boutilier et al. 1993; Wilkie et al. 2001). The rate of lactate accumulation in the brain did not match the rate of glycogen disappearance, however (Figure 2-1). On average brain glycogen stores in larval, parasitic and upstream-migrant lamprey dropped ~30 μmol glucosyl g^{-1} wet tissue. Theoretically, brain lactate concentrations on average should increase ~ 60 μmol g^{-1} wet tissue instead of the ~10 μmol g^{-1} wet tissue observed. It should be noted that glycolytic intermediates (e.g. pyruvate, glucose-6-phosphate, fructose-6-phosphate, glycerol-3-phosphate), in addition to lactate could account for the observed changes in glycogen (Pearson et al. 1990). Differences between glycogen disappearance and lactate accumulation could also be attributed the shipping of lactate to muscle tissue for gluconeogenesis/glyconeogenesis (see section 2.4.2.4 **Muscle**; Juel 1997). Furthermore, because TFM likely uncouples rat and lamprey liver mitochondria by increasing the permeability of the inner mitochondrial membrane the flow of electrons via the ETC will increase in an attempt to maintain or restore the proton gradient (Birceanu et al. 2009). As a result, the electron carriers

$FADH_2$ and NADH that are oxidized by the ETC are replaced by increasing the turnover rate of the citric acid cycle (TCA). Because pyruvate can be converted to acetyl coenzyme A, the entry point for the TCA, its reduction to lactic acid can be reduced which could account for the observed difference in glycogen disappearance and lactate accumulation.

Despite their hypoxia tolerance, few studies have addressed how sea lamprey survive hypoxia. The large glycogen reserves found in the meninges of the brain have been identified as one factor that could explain their hypoxia tolerance (Rovainen et al. 1969; Rovainen 1970; Foster et al. 1993). To date, however, no one has examined whether lamprey are capable of depressing their metabolic rate to conserve ATP during hypoxia, or even if they are capable of producing ethanol.

2.4.2.2 Liver

Although Birceanu et al. (2009) showed that larval lamprey exposed to their 12 h TFM LC_{50} experienced a significant reduction in liver glycogen, this study showed that TFM exposure ($LC_{99.9}$) had no effect on liver glycogen in larval, parasitic and upstream-migrant lampreys. Because the liver in vertebrates contains large concentrations of glycogen and high glucose-6-phosphatase activity, one of its many roles is to regulate blood glucose levels. Relative to other vertebrates, sea lamprey liver has a much lower concentration of glycogen (Plisetskaya and Kuz'mina 1972; Larsen et al. 2001; Barcellos et al. 2010) and stores its glycogen reserves in the meningeal tissue surrounding the brain. In addition, glucose-6-phosphotase, an enzyme necessary to export glucose out of a cell (Panserat et al. 2000), activity in lamprey meninges is double that of the lamprey liver (Rovainen et al. 1971). This suggests that meningeal tissue in lamprey may be more

important for supplying the brain and other tissues with glucose than the liver. As a result, it seems reasonable to suggest that TFM exposure would likely have its greatest effect on the lamprey meningeal tissue.

At the cellular level TFM has been shown to uncouple rat and lamprey liver mitochondria (Niblett and Ballanytne 1976; Birceanu et al. 2010), which reduces the ATP yield acquired from oxidative phosphorylation. Not surprisingly, liver ATP concentrations showed significant reductions in the three life stages examined (Figure 2-6). Low levels of pyruvate kinase (PK) and hexokinase (HK) in the liver of lampreys has been reported by LeBlanc et al. (1995) which they suggest reduces the anaerobic capacity of the liver. This conclusion is further supported by the observation that parasitic and upstream-migrant lamprey liver lactate were unaffected by exposure to TFM (Figure 2-2). With oxidative phosphorylation providing low amounts of ATP in the presence of TFM, and the liver's inability to increase rates of anaerobic glycolysis to compensate for reduced ATP production, it suggests that ATP supply does not likely meet the liver's ATP demand. As suggested by Birceanu et al. (2009) a reduction in hepatic ATP supply could then have an effect on TFM detoxification.

In all vertebrates the liver is the main site for detoxifying TFM in a process that utilizes ATP and UDP-glucuronic acid to form TFM-glucoronide, the highly water soluble and less toxic TFM metabolite (Kane et al. 1993). In rat hepatocytes and liver slices, a reduction in ATP supply has been shown to inhibit the glucuronidation of acetaminophen (Aw and Jones 1982; Evdokimova et al. 2001). In addition, Lech and Strathham (1975) could not detect TFM-glucoronide in lamprey tissues exposed to sub-lethal concentrations of TFM. However, the lampreys' limited ability to detoxify TFM

using glucuronidation is likely related to the low efficiency of the glucuronyl transferase enzyme responsible for the formation of TFM- glucuronide and not reduced hepatic ATP supply (Kane et al. 1993). Whether reductions in hepatic ATP supply in non-target organism could further impair TFM detoxification deserves investigation.

2.4.2.3 Kidney

The primary function of the kidney in freshwater fishes is the production of copious amounts of dilute urine (McDonald 2007). Active uptake of electrolytes in the distal tubule and collecting ducts of freshwater lampreys accounts for greater than 95% of the reabsorbed NaCl (Logan et al. 1980). As mentioned above, ion ATPases such as Na^+/K^+-ATPase require a constant supply of ATP to remain functional. Reducing ATP supply in the kidney could prevent the re-absorption of important ions if the ATPases are starved of energy. In this study parasitic and upstream-migrant lamprey kidney ATP concentrations were significantly reduced during exposure to TFM (Figure 2-7). Because TFM is also thought to interfere with gill-mediated ion regulation (Mallatt et al. 1994), reducing the ability of the kidneys to re-absorb ions could further enhance TFM toxicity with regards to ion regulation.

Another function of the kidney is the production of glucose via gluconeogenesis (Gerich 2010). In mammals, kidney gluconeogenesis contributes approximately 25% of the glucose released to the blood stream in the post-absorptive state (Gerich 2010). Furthermore, during extended periods of fasting, the supply of glucose to the body is thought to switch from the liver to the kidney (Mithieux et al. 2005). As most vertebrates have modest glycogen stores in their kidney, lactate is used as the primary gluconeogenic precursor for glucose homeostasis (Gerich 2010). Kidney lactate accumulation in the

three life stages examined increased significantly during exposure TFM (Figure 2-3). It is possible that lactate produced in other tissues (e.g. brain) could be transport to the kidney for glucose production following the physiological disturbance (ie. TFM exposure). The function of the kidney is re-absorption of glucose, amino acids, metabolites and ions, but absorption of lactate by the kidney has also been demonstrated (Jorgensen and Sheikh 1984; Gopal et al. 2004).

2.4.2.4 Muscle

In all vertebrates, muscle glycogen is reserved primarily for locomotion (Wang et al. 1994b), and it is not involved in blood glucose homeostasis (Panserat et al. 2000). The large glycogen and PCr stores found in the muscle of sea lamprey give it an enormous anaerobic capacity comparable to many teleost species (Boutilier et al. 1993; Wilkie et al. 2000). In larval lamprey such reserves are suggested to aid in vigorous burrowing activities (Wilkie et al. 2000), but in parasitic and upstream-migrant lamprey these energy reserves are likely used for pursuing prey/hosts (Farmer 1980) and swimming against strong stream currents during upstream migration and spawning (Boutilier et al. 1993; Wilkie et al. 1998). Because the muscle has a large capacity to supply ATP via anaerobic glycolysis and TFM has been shown to cause a mismatch between ATP supply and ATP demand (Birceanu et al. 2009), one would expect to observe a decrease in muscle glycogen stores as seen in lamprey brain tissue. However, exposure to TFM in the three life stages examined had no effect on muscle glycogen stores (Figure 2-4). The absence of change in muscle glycogen is more than likely related to the reduction in muscle PCr observed in larval and upstream-migrant sea lamprey (Figure 2-8). When an increase in ATP supply is necessary such as during burst exercise (Wood and Perry 1985) or hypoxia

(Bickler and Buck 2007), the first energy store called upon to increase ATP supply is PCr (Dobson and Hochachka 1987). It should be noted that such vigorous swimming was not a factor in the present study because individual animals in each of the three life stages were held in separate, darkened containers to minimize activity. Moreover, larval lamprey were allowed to burrow into cotton, which has been shown to calm larval sea lamprey (thigmokinesis; Rovainen and Schieber 1975). Whether swimming has an effect on TFM toxicity deserves further investigation as larval lamprey have been observed leaving their burrows in an attempt to escape TFM in the water during stream treatments (personal observation, McDonald and Kolar 2007).

The uptake of lactate by resting muscle tissue has been demonstrated in a number of studies (Gladden and Yates 1983; Chin et al. 1991; Gladden 1991; Pagliassotti and Donovan 1991). Following a physiological disturbance such as burst exercise or hypoxia, lactate accumulation in muscle tissue can be used to synthesize glucose or glycogen via gluconeogenesis and glyconeogenesis, respectively (Suarez and Mommsen 1987). The rate of lactate accumulation in the muscle of larval and upstream-migrant lamprey did not match the rate glycogen disappearance. Furthermore, the theoretical yield of lactate in the brain did not match the actual reduction in brain glycogen (see section 2.4.2.1 **Brain**). I suggest that lactate being produced in the lamprey brain during exposure to TFM could be shipped to the muscle in preparation for synthesis of glucose or glycogen upon removal of the physiological disturbance, (ie. exposure to TFM). This conclusion is further supported by the observation that lactate uptake in slow-twitch muscle (oxidative fibers) is greater than in fast-twitch muscle (glycolytic fibers) (Juel 1997). Because lamprey muscle tissue is a mixture of both oxidative (red) and glycolytic fibers (white)

(Peters and Mackay 1961; Meyer 1979) they may have a greater capacity to use lactate compared to more active fish species such as salmonids, in which the bulk of the trunk musculature is primarily fast-twitch (white) fibers, with a thin band of oxidative (red) fibers lying beneath the lateral line.

2.4.3 Factors Affecting TFM Sensitivity In Sea Lamprey

The size of glycogen reserves in the brain may contribute to intra-organism sensitivity to TFM (Birceanu et al. 2009). In anoxia tolerant organisms such as the crucian carp, goldfish and western painted turtle, death ensues when liver glycogen stores are nearly depleted (Lutz and Nilsson 1994; Bickler and Buck 2007). Moreover, tolerance to anoxia is greatly reduced when liver glycogen stores are lower at different times of the year. For instance, the anoxia tolerance of the crucian carp is much lower in the summer compared to winter, when hepatic glycogen stores are much higher (Nilsson and Renshaw 2004). Unlike these anoxia tolerant organisms, the lamprey appears to depend on brain rather than hepatic stores of glycogen. This suggests that temporal differences in brain glycogen stores may explain differences in seasonal TFM sensitivity observed by Scholefield et al. (2008). Because 98% of a larval lamprey diet consists of detritus (Sutton and Bowen 1994), larval lampreys experience reduced food supply during the winter when primary production is low, and increased food supply during the spring and summer when primary production is high. I hypothesize that the increase in sensitivity of larval lampreys to TFM in the spring is reflected by lower glycogen reserves due to reduced food supply during the winter months and higher glycogen reserves from increased food supply in the spring and summer increases resistance to TFM. To determine if the size of glycogen reserves play a role in TFM sensitivity, future

studies should consider comparing brain glycogen reserves in lamprey at different times of the year.

In order to survive anoxia/hypoxia conditions for long periods of time, organisms must reduce ATP demand rather than simply increasing anaerobic ATP production, which only wastes substrate and shortens survival time (Bickler and Buck 2007). Based on the hypothesis that TFM exposure causes increased reliance on anaerobic fuel stores to meet ATP supply, one would expect that the life stage with the lowest ATP demands to be more resistant to TFM. Recall, that parasitic and upstream-migrant lampreys are more metabolically active (Farmer 1980) and have an active life style (Larsen 1980). The ATP demands of animals with high metabolic rates are greater than that of animals with lower metabolic rates (Lutz and Nilsson 1997; Bickler and Buck 2007). Since larval lamprey have high total brain glycogen per unit body mass (Figure 2-9) and have low ATP demands (Lewis 1980), it seems reasonable that this life stage would be more resistant to TFM. The static toxicity tests indicate that larval lampreys were in fact more resistant to TFM compared to upstream-migrant lamprey (Table 2-1).

Differences in life stage sensitivity to TFM could also be attributed to the rate of TFM uptake from the water. Smaller fish have relatively greater surface area for uptake (oxygen, ions) and elimination (ammonia, carbon dioxide) compared to larger fishes (Marshall 2002). In addition, teleost fishes with active life styles are known to have greater gill surface areas compared to less active teleost species (Hughes and Morgan 1973; Wegner et al. 2010a; Wegner et al. 2010b). In spite of this, larval, metamorphosizing and adult stages of *L. fluviatilis* and *L. planeri* have similar gill surface areas (Lewis and Potter 1976) that are comparable to many active teleost species

(Hughes and Morgan 1973). Since surface area is one of the most, if not the most significant contributor to rates of uptake and elimination (Moyle and Cech 2004) one could argue that because the amount gill surface area in larval and adult lamprey is nearly equal, differences in uptake rates may be of little importance in TFM life stage sensitivity. However, very few studies have examined the affects of water pH, the most influential component in TFM toxicity (McDonald and Kolar 2007). Determining TFM uptake rates in lampreys and non-target organisms and how they are affected by water pH could help to minimize non-target effects and reduce TFM usage (McDonald and Kolar 2007).

2.4.4 Conclusion

Until recently, few studies examined the integrated physiological responses of lampreys to TFM. This study and previous work done by Birceanu et al. (2009) provides strong evidence that TFM toxicity in larval, parasitic and upstream-migrant lampreys is linked to an inability to sustain ATP supply at the levels needed to meet basal ATP demands. Ultimately, this reduction in ATP supply forces the animal to rely on PCr and glycogen reserves to prevent a gap between ATP supply and ATP demand that can lead to homoestatic disturbances that culminate in death. However, TFM toxicity may not be solely the result of the depletion of anaerobic energy reserves. Other possibilities include acidosis and a breakdown of internal ion gradients within the nervous system, leading to brain cell necrosis. Finally, differences in life stage sensitivity could be linked to differences in basal metabolic rates, leading to larger gaps between ATP supply and demand in the post-metamorphic (parasitic and upstream migrant) stages compared to larval life stages.

Table 2-1. LC_{50} and $LC_{99.9}$ for Larval and Upstream-migrant Sea Lamprey in Static Toxicity Tests With TFM.

Life Stage	Test Date	Water pH	LC50 (mg l^{-1}) (95% CI)	LC99.9 (mg l^{-1}) (95% CI)
Larval	11-13 Dec 2009	8.2	4.5 (4.1 - 5.1)	7.6 (7.2 - 8.1)
Upstream-migrant	20-22 May 2008	8.1	2.4 (1.7 - 2.9)	5.0 (4.7 - 5.5)

Table 2-2. Comparison of Tissue Metabolites and Fuel Stores In Different Sea Lamprey Life Stages Held Under TFM Free Conditions.

Data presented as the mean ± 1 SEM. Data sharing the same letter are not significantly different ($P > 0.05$). Larval lamprey n= 10, parasitic lamprey n = 12 and upstream-migrant n = 7.

Tissue	Glycogen (μmol glucosyl units g^{-1} wet tissue)			Lactate (μmol g^{-1} wet tissue)		
	Larval	Parasite	Upstream-migrant	Larval	Parasite	Upstream-migrant
Brain	93.0±7.2ab	65.6±4.4b	117.1±4.5a	1.3±0.58a	6.7±0.66b	6.9±0.7b
Liver	8.4±0.89a	1.4±0.29b	5.1±0.75b	2.4±0.63a	2.3±0.23a	1.3±0.59a
Kidney	27.3±2.6a	2.3±0.25b	2.0±0.25b	1.0±0.4a	2.4±0.7a	2.3±0.59a
Muscle	22.8±2.4a	24.1±1.8a	22.2±2.1a	1.8±0.55a	1.8±0.52a	1.0±0.63a

Tissue	ATP (μmol g^{-1} wet tissue)			PCr (μmol g^{-1} wet tissue)		
	Larval	Parasite	Upstream-migrant	Larval	Parasite	Upstream-migrant
Brain	1.4±0.17a	1.2±0.23a	0.83±0.07a	6.8±0.86a	2.2±0.32b	2.8±0.65b
Liver	1.3±0.14a	1.3±0.19a	1.5±0.23a	0.28±0.08a	1.1±0.05b	0.5±0.22b
Kidney	1.3±0.23a	0.86±0.11a	0.77±0.12a	0.28±0.2a	N/A	0.86±0.2a
Muscle	3.4±0.39a	3.5±0.16a	0.98±0.17b	12.4±0.89a	28.6±1.4b	22.3±2.6b

Table 2-3. Brain Glycogen and PCr Stores In Various Animals.

Species	Brain		References
	Glycogen (μmol glucosyl units g^{-1} wet tissue)	**PCr** (μmol g^{-1} wet tissue)	
Larval Sea Lamprey (*Petromyzon marnius*)	45 to 100+	~7	Rovainen 1970; Rovainen et al. 1968; 1971; present study
Parasitic Sea Lamprey (*Petromyzon marnius*)	45 to 100+	~2	Rovainen 1970; present study
Upstream-migrant Sea Lamprey (*Petromyzon marnius*)	35 to 290	~2	Foster et al. 1993; present study
Frog and Turtle	10 to 25	4 to 6	McDougal et al. 1968
Goldfish (Carassius *auratus*)	13 to 20	~3	Ginneken et al. 1996; Lutz and Nilsson 1997
and Crucian Carp (*Carassius carassius*)	4 to 8	~3	Birceanu 2009; Lutz and Nilsson 1994
Rainbow Trout (*Oncorhynchus mykiss*)	~2	~11	Lowry et al. 1964
Mouse (*Mus* sp.)	~7	~2	Klein and Olsen 1947; Noika et al. 1993
Cat (*Felis catus*)			

Figure 2-1. Effects of TFM on Brain Glycogen and Lactate in Larval, Parasitic and Upstream-migrant Lamprey.

Changes in brain concentrations of *(a)* glycogen and *(b)* lactate in resting larval (open bars), parasitic (grey bars) and upstream-migrant (dark bars) sea lamprey (*Petromyzon marinus*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM). Larval lampreys were exposed to nominal TFM concentrations of 7.6 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 7-9). Parasitic lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 4-12). Upstream-migrant lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 0.5 1, 2, and 3 h or under control conditions (n= 7-10). Asterisks "*" indicate significant differences from controls not exposed to TFM ($P < 0.05$).

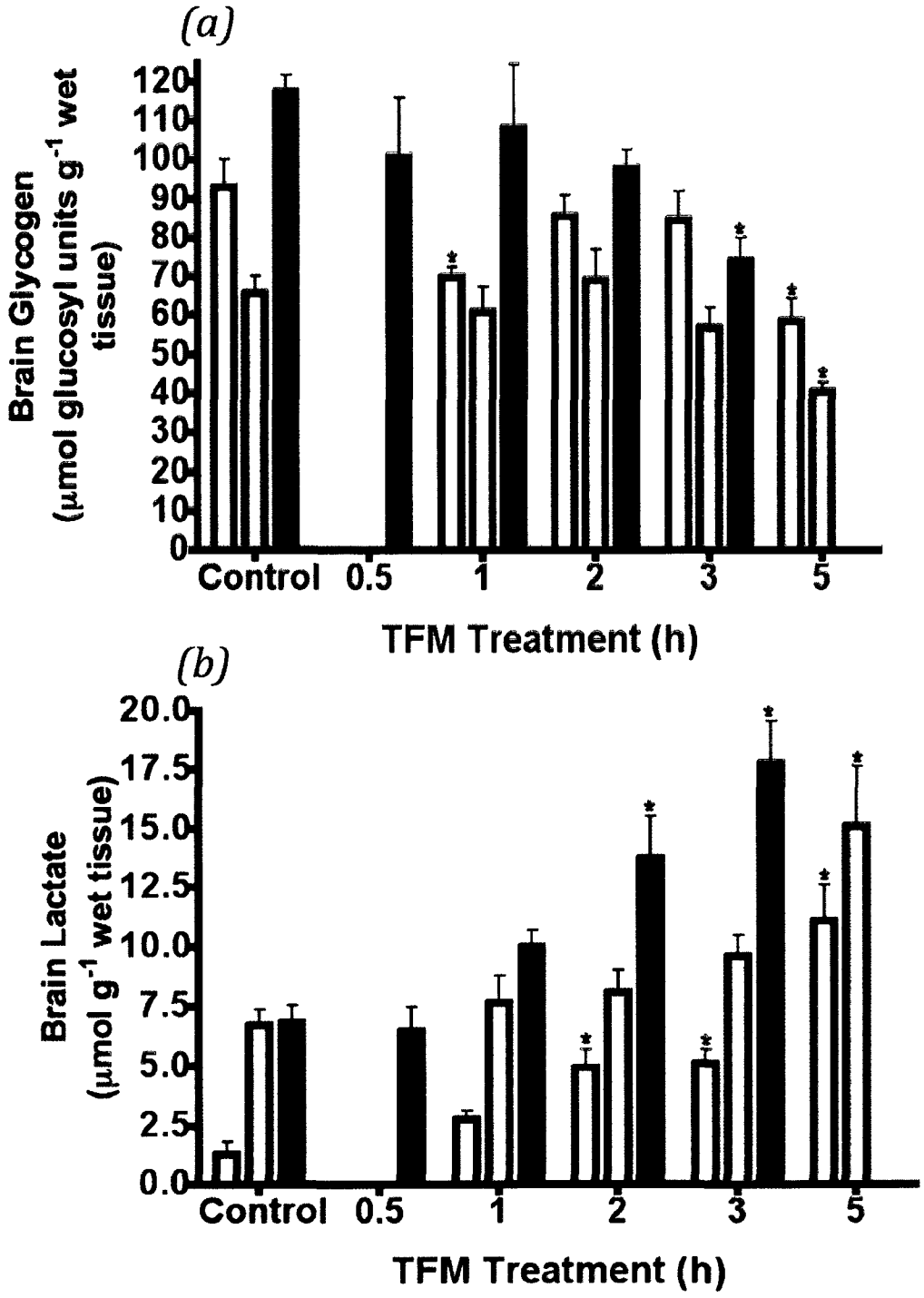

(a)
Brain Glycogen (μmol glucosyl units g-1 wet tissue)
120
110
100
90
80
70
60
50
40
30
20
10
0
Control
0.5
1
2
3
5
TFM Treatment (h)
(b)
Brain Lactate (μmol g-1 wet tissue)
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Control
0.5
1
2
3
5
TFM Treatment (h)

Figure 2-2. Effects of TFM on Liver Glycogen and Lactate in Larval, Parasitic and Upstream-migrant Lamprey.

Changes in liver concentrations of *(a)* glycogen and *(b)* lactate in resting larval (open bars), parasitic (grey bars) and upstream-migrant (dark bars) sea lamprey (*Petromyzon marinus*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM). Larval lampreys were exposed to nominal TFM concentrations of 7.6 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 7-9). Parasitic lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 4-12). Upstream-migrant lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 0.5 1, 2, and 3 h or under control conditions (n= 7-10). Asterisks "*" indicate significant differences from controls not exposed to TFM ($P < 0.05$).

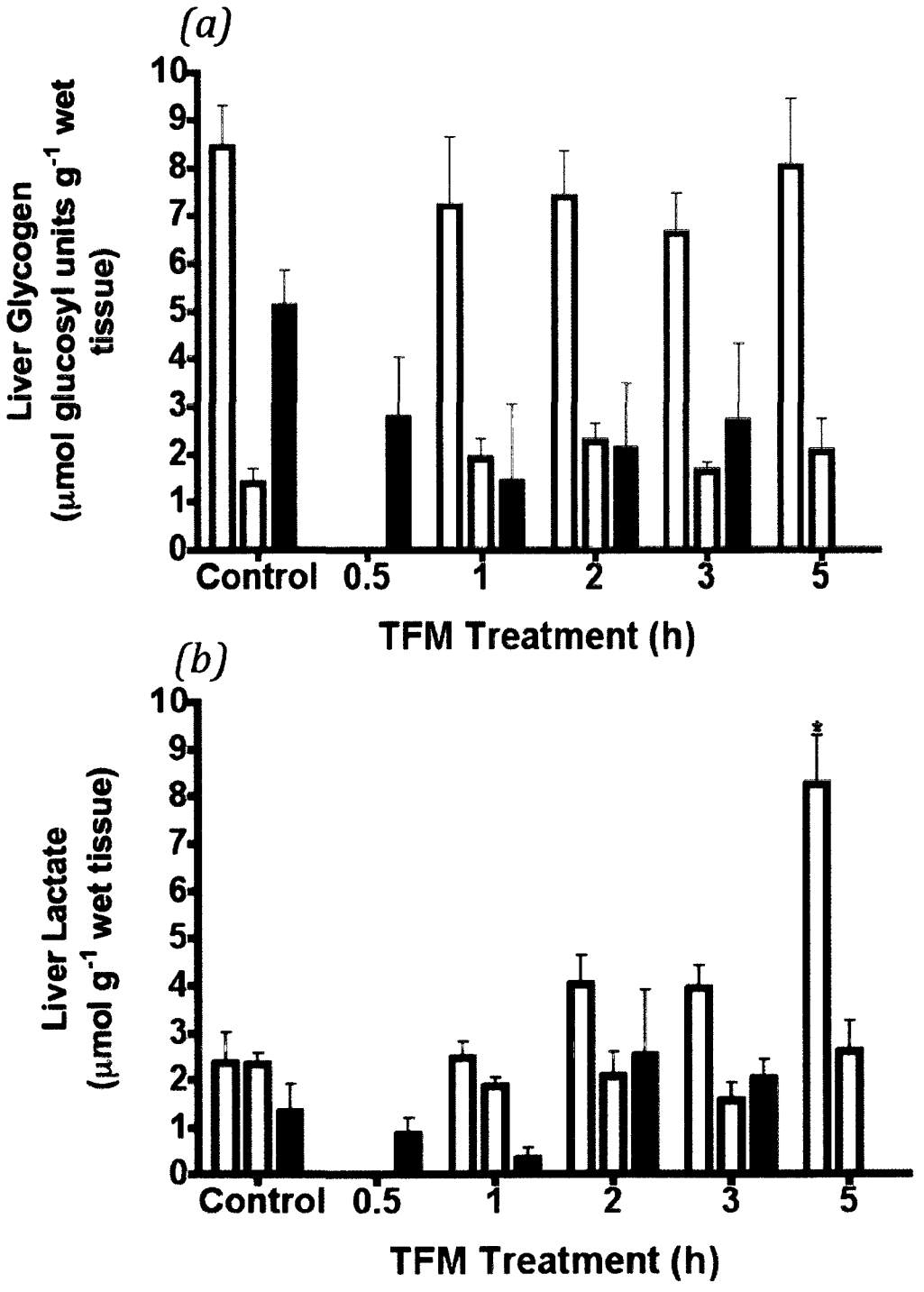

(a)
Liver Glycogen
(μmol glucosyl units g-1 wet
tissue)
10
9
8
7
6
5
4
3
2
1
0
Control
0.5
1
2
3
5
TFM Treatment (h)
(b)
Liver Lactate
(μmol g-1 wet tissue)
10
9
8
7
6
5
4
3
2
1
0
*
Control
0.5
1
2
3
5
TFM Treatment (h)

Figure 2-3. Effects of TFM on Kidney Glycogen and Lactate in Larval, Parasitic and Upstream-migrant Lamprey.

Changes in kidney concentrations of *(a)* glycogen and *(b)* lactate in resting larval (open bars), parasitic (grey bars) and upstream-migrant (dark bars) sea lamprey (*Petromyzon marinus*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM). Larval lampreys were exposed to nominal TFM concentrations of 7.6 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 7-9). Parasitic lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 4-12). Upstream-migrant lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 0.5 1, 2, and 3 h or under control conditions (n= 7-10). Asterisks "*" indicate significant differences from controls not exposed to TFM ($P < 0.05$).

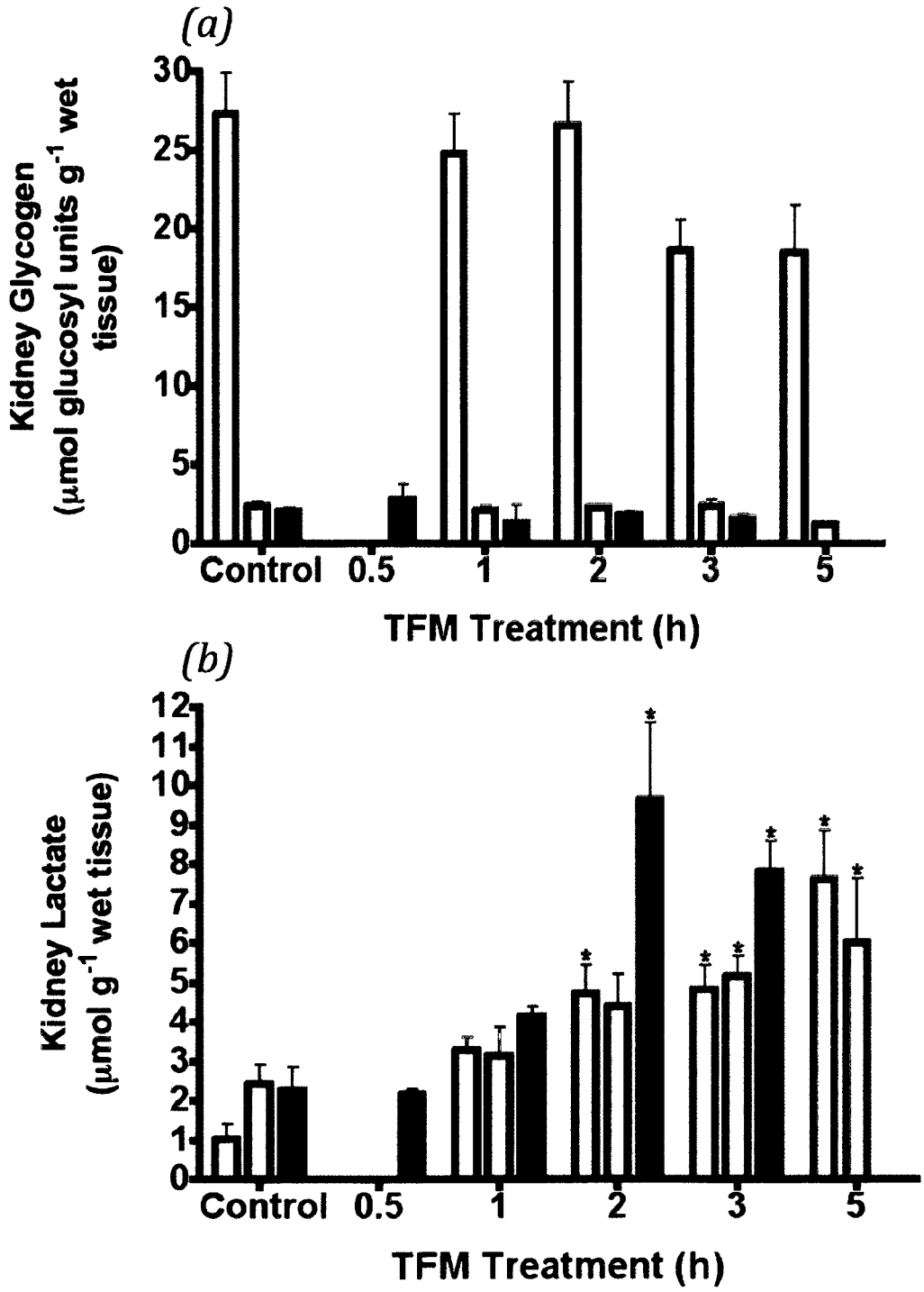

(a)
Kidney Glycogen
(μmol glucosyl units g-1 wet
tissue)
30
25
20
15
10
5
0
Control
0.5
1
2
3
5
TFM Treatment (h)
(b)
Kidney Lactate
(μmol g-1 wet tissue)
12
11
10
9
8
7
6
5
4
3
2
1
0
Control
0.5
1
2
3
5
TFM Treatment (h)

Figure 2-4. Effects of TFM on Muscle Glycogen and Lactate in Larval, Parasitic and Upstream-migrant Lamprey.

Changes in muscle concentrations of *(a)* glycogen and *(b)* lactate in resting larval (open bars), parasitic (grey bars) and upstream-migrant (dark bars) sea lamprey (*Petromyzon marinus*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM). Larval lampreys were exposed to nominal TFM concentrations of 7.6 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 7-9). Parasitic lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 4-12). Upstream-migrant lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 0.5 1, 2, and 3 h or under control conditions (n= 7-10). Asterisks "*" indicate significant differences from controls not exposed to TFM ($P < 0.05$).

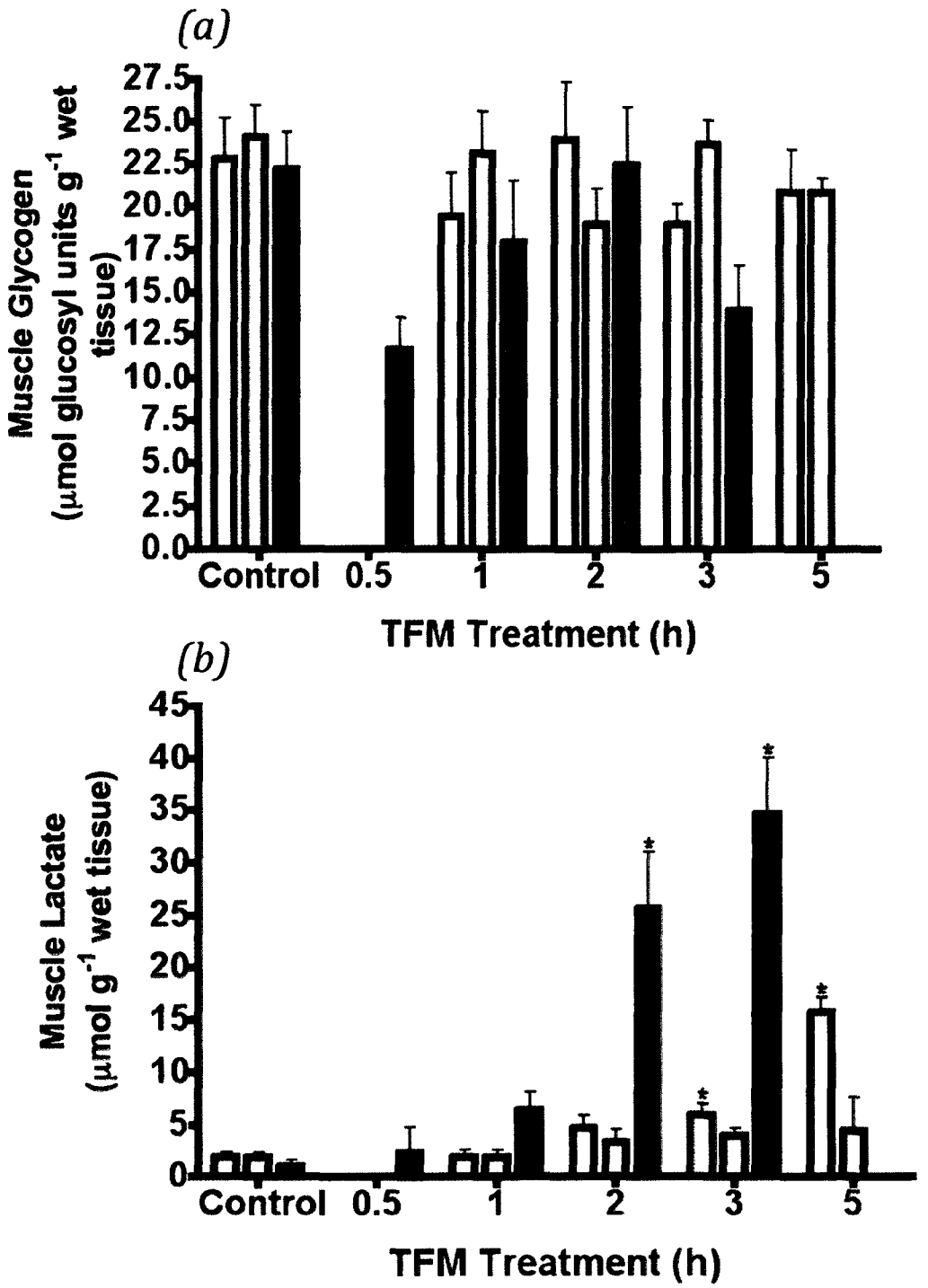

(a)
Muscle Glycogen
(μmol glucosyl units g-1 wet tissue)
27.5
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Control
0.5
1
2
3
5
TFM Treatment (h)
(b)
Muscle Lactate
(μmol g-1 wet tissue)
45
40
35
30
25
20
15
10
5
0
Control
0.5
1
2
3
5
TFM Treatment (h)

Figure 2-5. Effects of TFM on Brain ATP and PCr in Larval, Parasitic and Upstream-migrant Lamprey.

Changes in brain concentrations of *(a)* ATP and *(b)* PCr in resting larval (open bars), parasitic (grey bars) and upstream-migrant (dark bars) sea lamprey (*Petromyzon marinus*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM). Larval lampreys were exposed to nominal TFM concentrations of 7.6 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 7-9). Parasitic lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 4-12). Upstream-migrant lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 0.5 1, 2, and 3 h or under control conditions (n= 7-10). Asterisks "*" indicate significant differences from controls not exposed to TFM ($P < 0.05$).

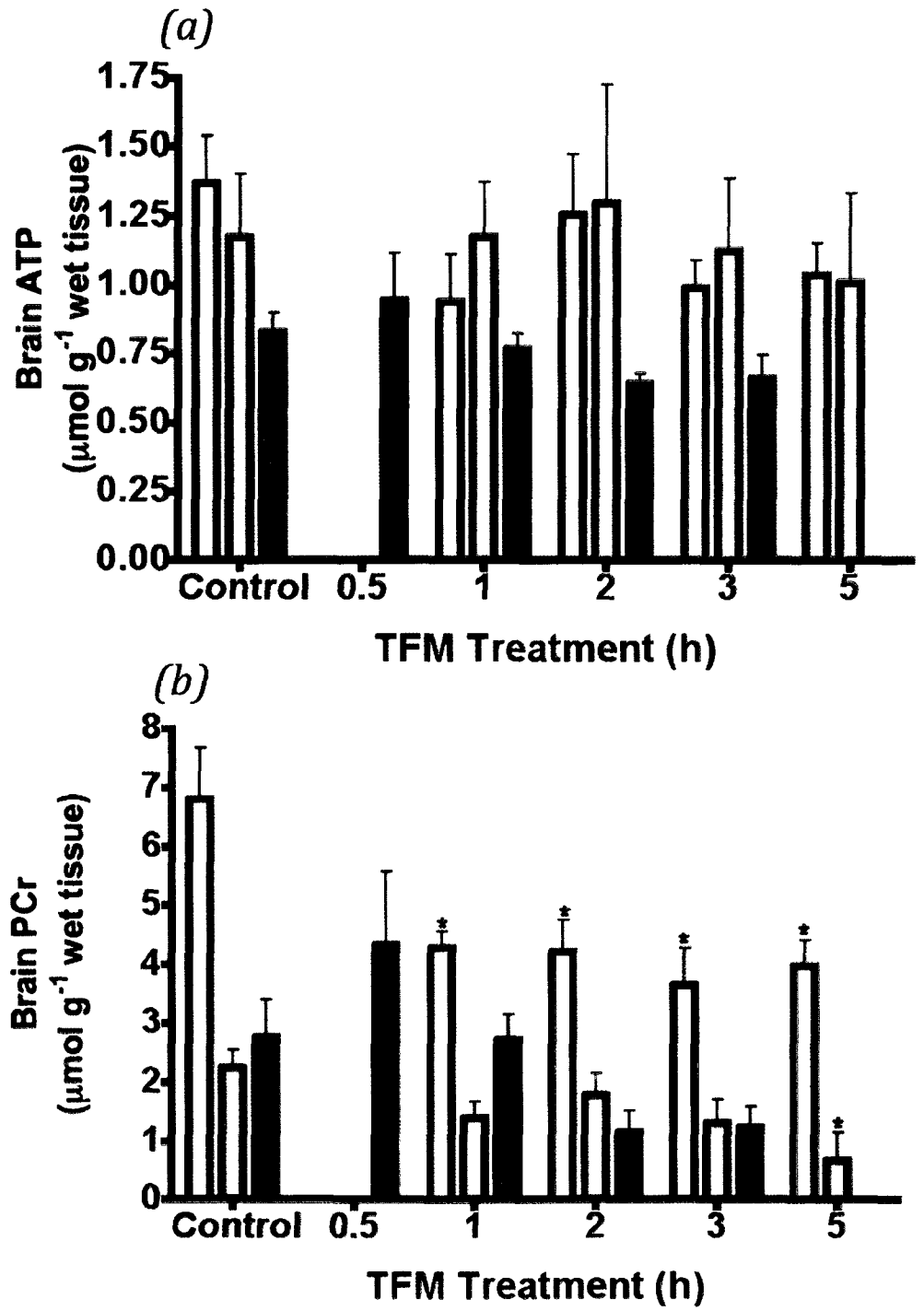

(a)
Brain ATP
(µmol g-1 wet tissue)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Control
0.5
1
2
3
5
TFM Treatment (h)
(b)
Brain PCr
(µmol g-1 wet tissue)
8
7
6
5
4
3
2
1
0
Control
0.5
1
2
3
5
TFM Treatment (h)

Figure 2-6. Effects of TFM on Liver ATP and PCr in Larval, Parasitic and Upstream-migrant Lamprey.

Changes in liver concentrations of *(a)* ATP and *(b)* PCr in resting larval (open bars), parasitic (grey bars) and upstream-migrant (dark bars) sea lamprey (*Petromyzon marinus*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM). Larval lampreys were exposed to nominal TFM concentrations of 7.6 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 7-9). Parasitic lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 4-12). Upstream-migrant lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 0.5 1, 2, and 3 h or under control conditions (n= 7-10). Asterisks "*" indicate significant differences from controls not exposed to TFM ($P < 0.05$).

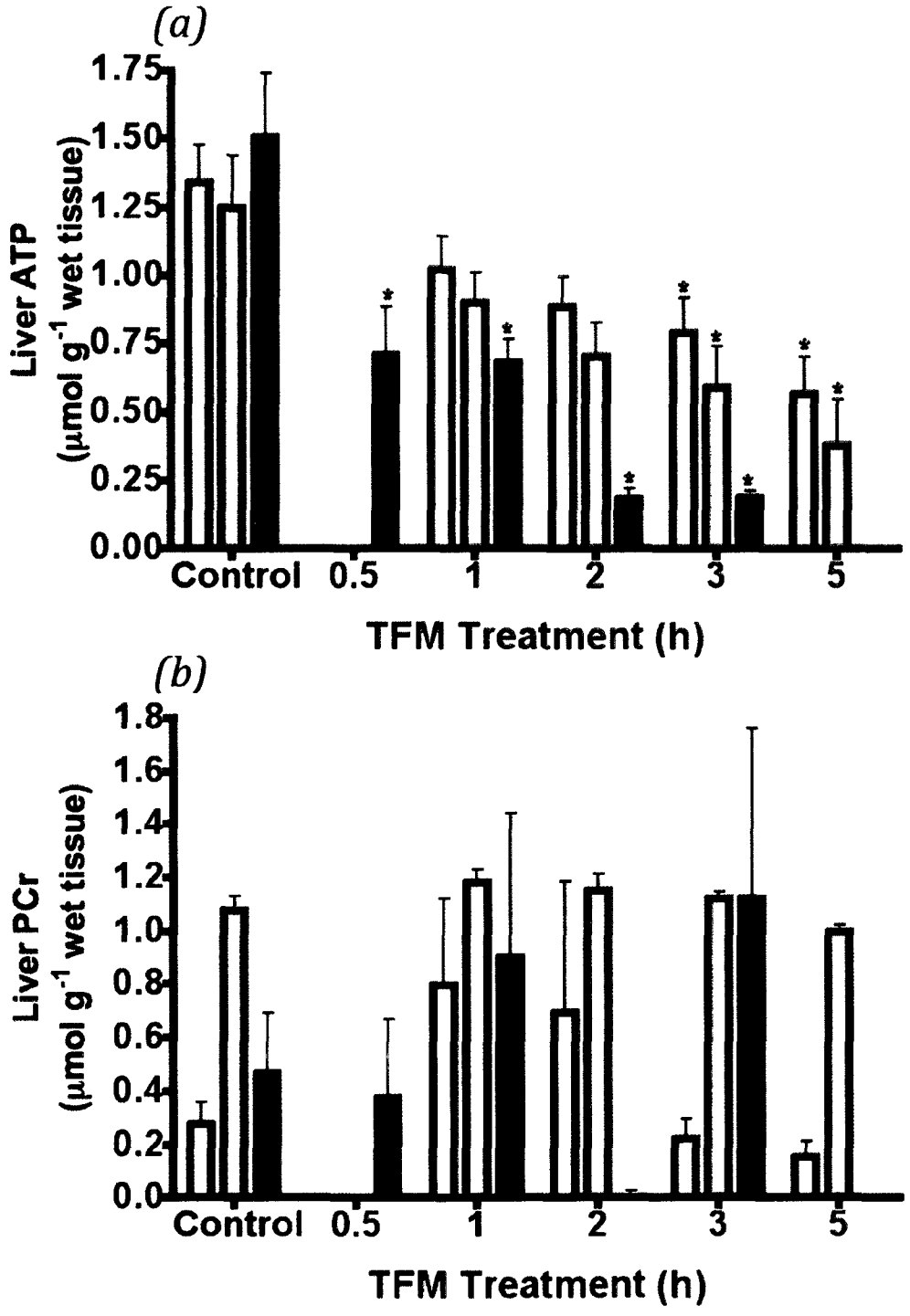

(a)
Liver ATP
(μmol g-1 wet tissue)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Control
0.5
1
2
3
5
TFM Treatment (h)
(b)
Liver PCr
(μmol g-1 wet tissue)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
0.5
1
2
3
5
TFM Treatment (h)

Figure 2-7. Effects of TFM on Kidney ATP and PCr in Larval, Parasitic and Upstream-migrant Lamprey.

Changes in kidney concentrations of *(a)* ATP and *(b)* PCr in resting larval (open bars), parasitic (grey bars) and upstream-migrant (dark bars) sea lamprey (*Petromyzon marinus*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM). Larval lampreys were exposed to nominal TFM concentrations of 7.6 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 7-9). Parasitic lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 4-12). Upstream-migrant lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 0.5 1, 2, and 3 h or under control conditions (n= 7-10). Asterisks "*" indicate significant differences from controls not exposed to TFM ($P < 0.05$).

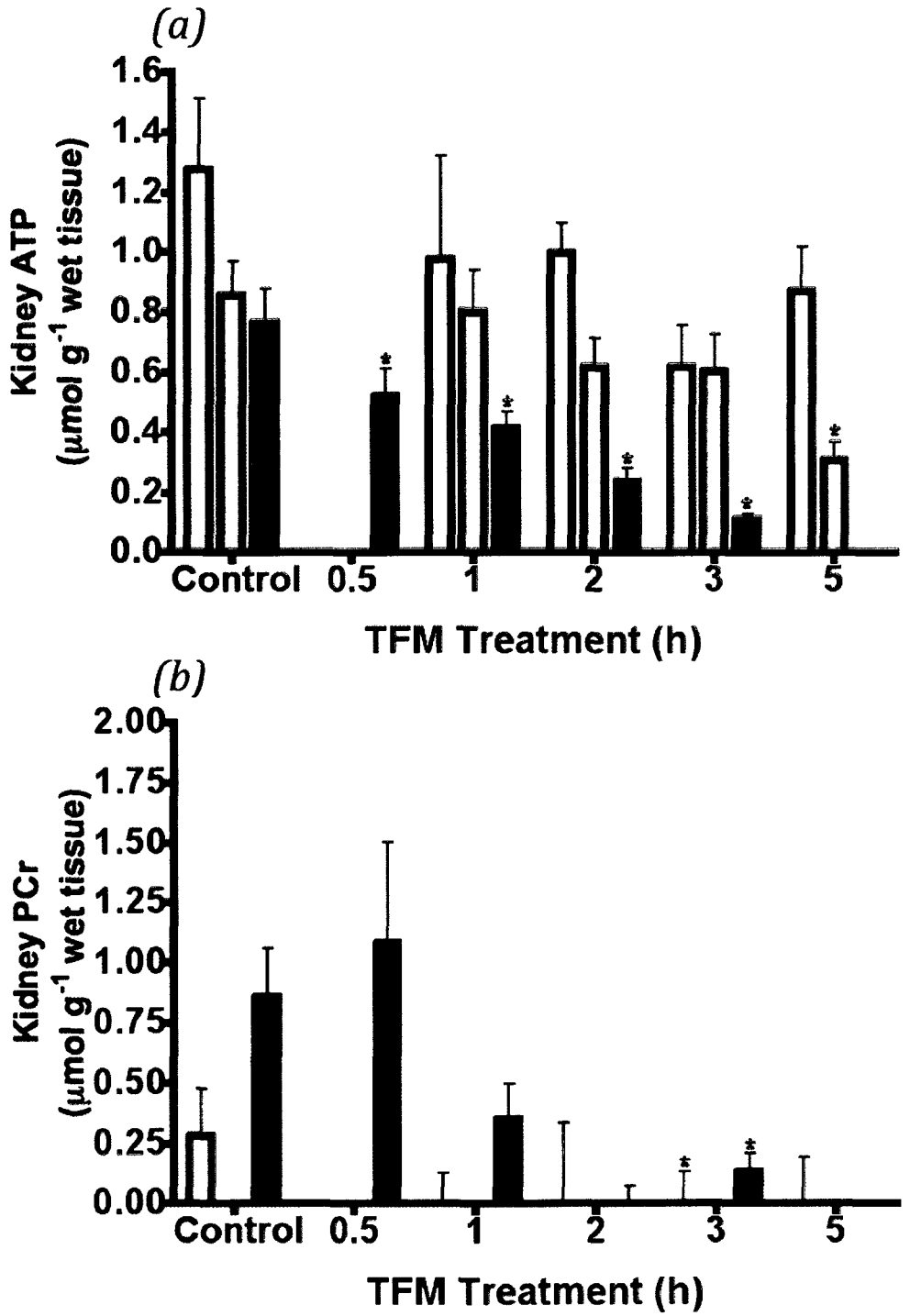

(a)
Kidney ATP
(μmol g-1 wet tissue)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
0.5
1
2
3
5
TFM Treatment (h)
(b)
Kidney PCr
(μmol g-1 wet tissue)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Control
0.5
1
2
3
5
TFM Treatment (h)

Figure 2-8. Effects of TFM on Muscle ATP and PCr in Larval, Parasitic and Upstream-migrant Lamprey.

Changes in muscle concentrations of *(a)* ATP and *(b)* PCr in resting larval (open bars), parasitic (grey bars) and upstream-migrant (dark bars) sea lamprey (*Petromyzon marinus*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM). Larval lampreys were exposed to nominal TFM concentrations of 7.6 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 7-9). Parasitic lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 1, 2, 3 and 5 h or held under control conditions (n= 4-12). Upstream-migrant lampreys were exposed to nominal TFM concentrations of 5.0 mg l^{-1} for 0.5 1, 2, and 3 h or under control conditions (n= 7-10). Asterisks "*" indicate significant differences from controls not exposed to TFM ($P < 0.05$).

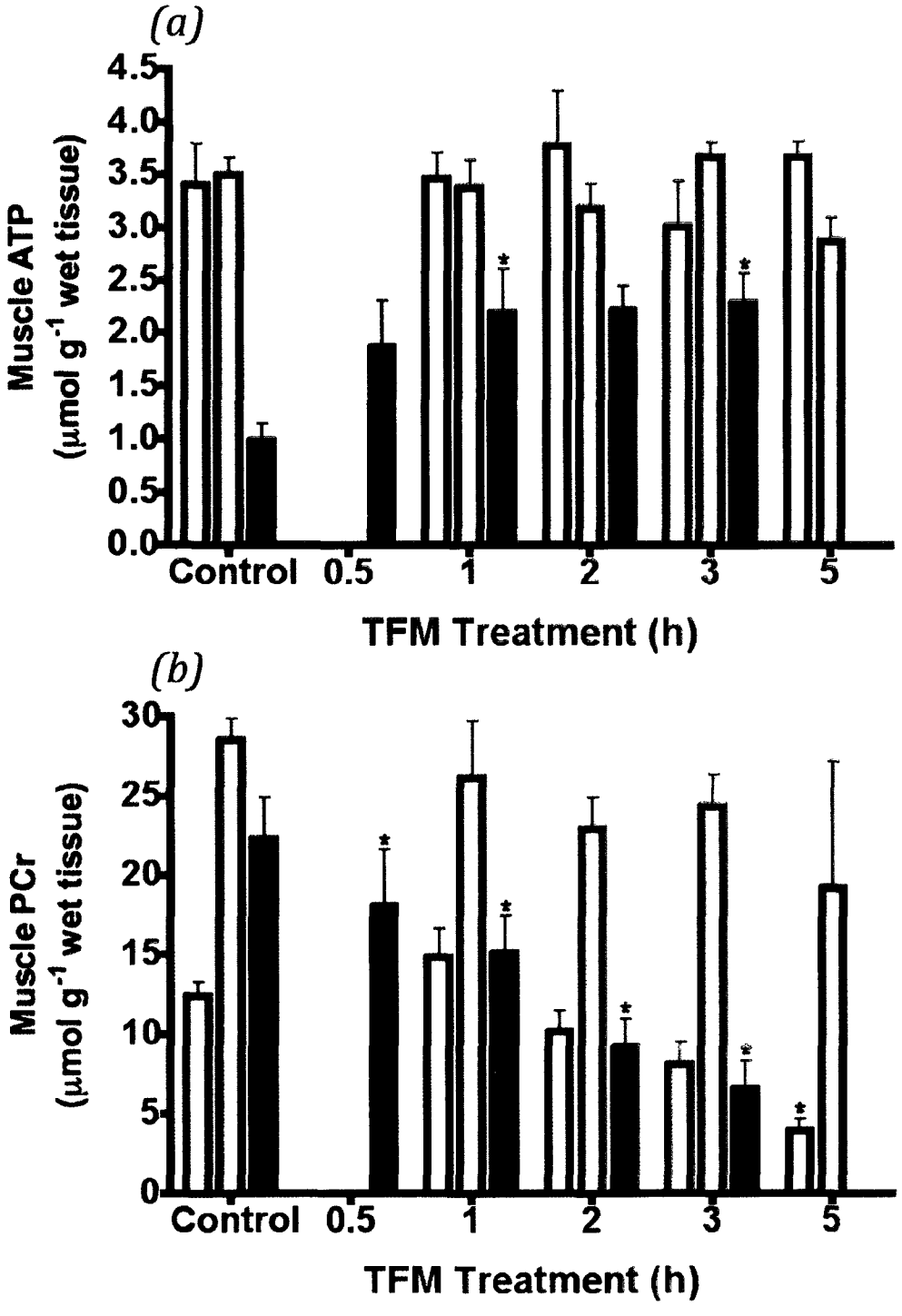
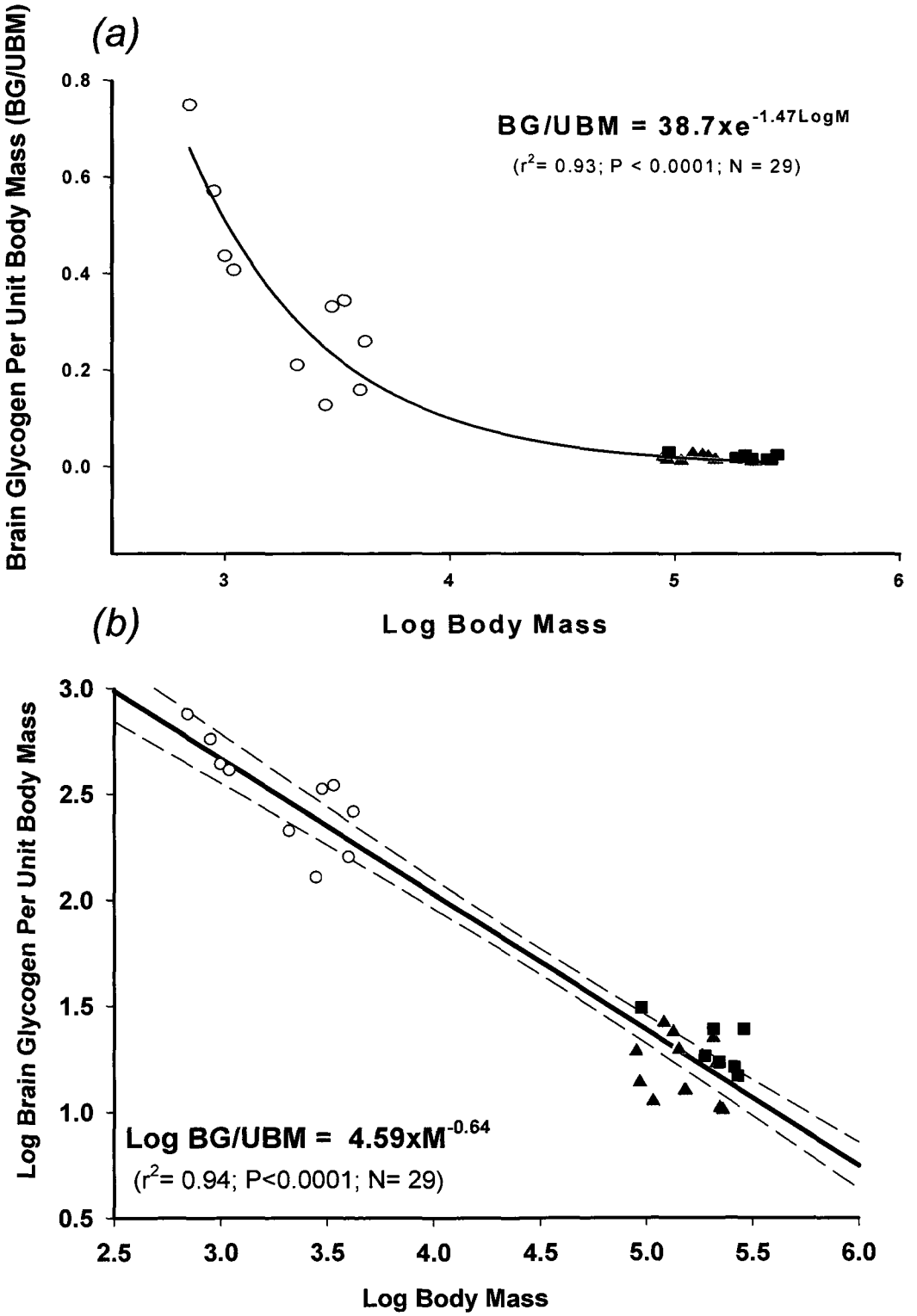

(a)
Muscle ATP
(μmol g-1 wet tissue)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
0.5
1
2
3
5
TFM Treatment (h)
(b)
Muscle PCr
(μmol g-1 wet tissue)
30
25
20
15
10
5
0
Control
0.5
1
2
3
5
TFM Treatment (h)

Figure 2-9. Semi-log plot of *(a)* brain glycogen per unit body mass and *(b)* log-log plot of brain glycogen per unit body mass versus body mass.

Larval lampreys are indicated by open white circles (n=10), parasitic lampreys are indicated by grey triangles (n=12) and upstream-migrant lampreys are indicated by black squares (n=7). The line of best fit in *(a)* is represented by an exponential equation ($BG/UBM = 38.7xe^{-1.47\ Log\ M}$) and the line of best fit in *(b)* was determined using least squares linear regression ($BG/UBM = 4.59 \times BM^{-0.64}$), which was presented ± 95% confidence internal (*dashed lines*).


(a)
Brain Glycogen Per Unit Body Mass (BG/UBM)
BG/UBM = 38.7xe-1.47LogM
(r2= 0.93; P < 0.0001; N = 29)
0.8
0.6
0.4
0.2
0.0
3
4
5
6
Log Body Mass
(b)
Log Brain Glycogen Per Unit Body Mass
Log BG/UBM = 4.59xM-0.64
(r2= 0.94; P<0.0001; N= 29)
3.0
2.5
2.0
1.5
1.0
0.5
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
Log Body Mass

Chapter 3

Effects of TFM upon Gill Mediated Ion Transport in Lamprey and Trout

Abstract

The lampricide, TFM, is added to streams infested with larval sea lampreys to control populations of these invasive pests in the Great Lakes, but its mode of toxicity at TFM concentrations similar to those of stream treatments has not been investigated. Knowledge of the physiological effects of TFM at higher, more environmentally relevant, concentrations is needed for improving the effectiveness of TFM treatments and predicting potential adverse effects on non-target species. To investigate the hypothesis that TFM disrupts gill-mediated ion exchange, larval and upstream-migrant sea lamprey, and rainbow trout were exposed to their respective 12-h TFM $LC_{99.9}$. Exposure to TFM had minimal effects on the influx, outflux and net flux of Na^+ across the body of larval, upstream-migrant sea lamprey and rainbow trout. Control net Na^+ flux rates in larval lamprey were -178 $\mu mol \bullet kg^{-1} \bullet h^{-1}$ while upstream-stream migrant lamprey had control net Na^+ flux rates of -75 $\mu mol \bullet kg^{-1} \bullet h^{-1}$. Rainbow trout had net Na^+ flux rates that were near zero under control conditions, and TFM treatment had only slight, non-significant effects on the net Na^+ flux across the gills. Analysis of total Na^+ losses during exposure to TFM suggests that larval lamprey and rainbow trout experienced 0.1% and 0.9% decreases in their exchangeable respective in their internal exchangeable Na^+ pool. These findings suggest that TFM treatment did not have any physiologically relevant effect on ion balance in either species. In addition, plasma ion concentration in parasitic and upstream-migrant sea lamprey revealed that TFM does not alter plasma ion levels. The results of this study suggest that disruption to gill-mediated ion exchange is not the proximate cause of death in sea lamprey and rainbow trout exposed to TFM.

3.1 Introduction

Currently, the primary method of controlling parasitic sea lampreys (*Petromyzon marinus*) in the Great Lakes is to apply the pesticide 3-trifluoromethyl-4-nitrophenol (TFM) to streams infested with larval sea lampreys (Christie and Goddard 2003; McDonald and Kolar 2007). The use of TFM in the integrated pest management of sea lampreys has led to the restoration of Great Lakes fisheries that were decimated by overfishing and lamprey predation in the mid-1900s (Christie et al. 2003). The success of TFM treatments is mainly due to its selectivity for lamprey (Applegate and King 1962; Lech and Statham 1975; Kane et al. 1993) and, that at any one time, many larval year classes can be found in streams and tributaries connected to the Great Lakes (Potter and Gill 2003). Only recently has the proximate mechanism(s) of TFM toxicity been investigated in detail (Wilkie et al. 2007; Birceanu et al. 2009) and questions regarding its mode(s) of action still exist (McDonald and Kolar 2007).

Wilkie et al. (2007) proposed that TFM causes death by disrupting ATP supply through the depletion of on-board energy reserves in the brain and liver of larval sea lamprey. This hypothesis was supported by Birceanu et al. (2009), and the work completed in Chapter 2 of this thesis. Evidence from gill morphology studies (Christie and Battle 1961; Mallatt et al. 1994) suggests that TFM toxicity may disrupt gill-mediated ion regulation. Wilkie et al. (2007) and Birceanu et al. (2009) found no changes in plasma ions and haematology of larval lamprey exposed to TFM, but these lamprey were exposed to concentrations of TFM that were equivalent to the 12-h LC_{50} for TFM. Typically, sea lamprey control personnel use concentrations of TFM that are 1.0 to 1.5 times the 12-h $LC_{99.9}$ (Christie et al. 2003). Exposure to these higher concentrations of

TFM caused cellular swelling and enlarged vacuoles within the mitochondrial rich cells (MRCs) of larval sea lamprey (Mallatt et al. 1994). The MRCs are the main cell involved in gill-mediated ion uptake in fishes (Beamish et al. 1980; Marshall 2002; Bartels and Potter 2004). In addition, rainbow trout, a more resistant non-target species due to their greater ability to detoxify TFM by biotransformation to TFM-glucoronide (refer to section 1.6 TFM Selectivity and GLFC Research Priorities for detailed description of TFM resistance), showed minimal gill epithelial damage during exposure to TFM at their respective 12-h $LC_{99.9}$ (Mallatt et al. 1994). Despite the evidence suggesting that TFM may interfere with ion exchange in lamprey, only one study has examined the effects of TFM on ion uptake in lamprey (Birceanu et al. 2009). No studies have examined how the unidirectional movements (influx, net flux and outflux) of ions across the gills of lamprey and rainbow trout are affected by TFM exposure.

The goal of the present study was to test the hypothesis that TFM interferes with gill-mediated ion regulation in lampreys but not in rainbow trout by exposing them to a lethal dose of TFM ($LC_{99.9}$) and using the radiotracer $^{24}Na^{+}$ to measure the unidirectional flux of Na^{+} across the gills of lamprey and rainbow trout. Because gill structure changes markedly during the life cycle of the lamprey (Youson 1980s; Bartels and Potter 2003), the effects of TFM on gill-mediated ion exchange were examined in both larval lampreys and upstream migrant lampreys. In addition to measuring unidirectional ion movements, plasma ions, gill Na^{+}/K^{+}-ATPase activity and muscle tissue water were measured to assess the effects TFM had on internal ion balance and the gill ion transport machinery.

3.2 Materials and Methods

3.2.1 Experimental Animals and Holding

Larval lamprey (*P. marinus*, 1-2 g, 90-120 mm) were obtained courtesy of the United States Geological Survey (USGS) from Hammond Bay Biological Station (Millersburg, Michigan, United States). Upstream migrant sea lampreys (~ 150 g), supplied by the Department of Fisheries and Oceans Canada (Sault Ste. Marie, Ontario, Canada), were captured at the beginning of their spawning migration in traps near the mouth of the Humber River, Toronto, and held until delivery in Toronto Region Conservation Authority (TRCA) facilities. Rainbow trout (*Oncorhynchus mykiss*) (30 ± 2.8g) were purchased from the Rainbow Springs Trout Hatchery, Thamesford, Ontario. All animals were held in tanks receiving aerated Wilfrid Laurier University well water on a flow through basis (pH~ 8.0; titratable alkalinity ~ 200 mg $CaCO_3$ l^{-1}; hardness ~ 450 mg $CaCO_3$ l^{-1}; $[Na^+]$ ~ 1.1 mmol l^{-1}; temperature 10-13 °C). Larval and upstream migrant lampreys were held in 500 L fiberglass Living Streams (Frigid Units Incorporated, Toledo, Ohio), while the rainbow trout were held in 120 L polyethylene tanks. The bottom of the tanks holding larval lamprey was lined with sand, to provide burrowing substrate. The larval lampreys were also fed baker's yeast (2 g yeast per larva; Holmes and Youson 1994; Wilkie et al. 1999) once a week, but because upstream-migrant lampreys do not feed during this life stage, no attempts were made to feed these animals. Both the larval and upstream migrant lampreys were held in the lab for approximately 2-4 weeks before experiments commenced. Rainbow trout were fed three times weekly with 3.0 commercial pellets (Corey Feed Mills, Fredericton, New Brunswick) and were held in the lab for 3 months. Approximately 72 h prior to the beginning of experiments, food was

withheld from the larval lamprey and rainbow trout to reduce ammonia excretion rates that could cause ammonia levels in the exposure containers to reach toxic levels. All fish husbandry and experiments were approved by the Wilfrid Laurier University Animal Care Committee and followed Canadian Council of Animal Care guidelines.

3.2.2 Experimental Protocol

3.2.2.1 Determination of the Acute Toxicity of TFM

To determine the toxicity of TFM to larval lampreys, up-stream migrant lampreys and rainbow trout in Wilfrid Laurier well-water, three toxicity range finder experiments were conducted. The TFM sensitivity of larval lamprey was determined by exposing them to nominal TFM concentrations of 0.0, 1.0, 3.5, 5.0 and 8.0 mg l^{-1} for 12 h in 5 l of water. The upstream-migrants were exposed to nominal TFM concentrations of 0, 0.5, 1.5, 2.5, 4.5 and 8.0 mg l^{-1} for 12 h in 6 l of water. Rainbow trout were exposed to nominal TFM concentrations of 8, 10, 12, 16, 20 and 25 mg l^{-1} for 12 h in 15 l of water. All fish were acclimated to their respective TFM exposure containers for approximately 12 h prior to the start of each range finder toxicity test. A total of 5 larvae and rainbow trout, and 6 upstream-migrant lampreys were exposed to each respective TFM concentration. All experiments were completed in the dark as larval and upstream-migrant lampreys are negatively phototactic (Rovainen and Schieber 1975; Binder and McDonald 2008) and TFM photodegrades in the presence of light (Hubert 2003). Field formulation TFM (Clariant SFC GMBH WERK, Griesheim, Germany), provided by the DFO, was used for all experiments. To verify water TFM concentrations, the absorbance of water samples were measured at a wavelength of 395 nm using a 96-well plate

spectrophotometer (SpectraMax 190, Molecular Devices, CA) and precision TFM standards provided by the DFO.

3.2.2.2 ^{24}Na Fluxes in the Presence of TFM

The unidirectional movements (influx, outflux, net flux) of Na^+ across the gills of larval lamprey, upstream-migrant lampreys and rainbow trout during TFM exposure ($LC_{99.9}$) were measured using the radiotracer $^{24}Na^+$. The original objective of these experiments was to measure both $^{24}Na^+$ and $^{45}Ca^{2+}$ to measure the unidirectional movements of Na^+ and Ca^{2+}across the gills. Due to unforeseen technical problems, however, only the unidirectional movements of Na^+ were subsequently determined in this thesis.

3.2.2.2.1 Unidirectional Na^+ Fluxes in Larval and Upstream-Migrant Sea Lampreys, and in Rainbow Trout

Approximately 12 h prior to an experiment, 10 larval lampreys (mass 1-2 g), 8 upstream-migrant lampreys (mass ~ 150 g) or 10 rainbow trout (mass ~ 25 g) were transferred into individual, aerated darkened plastic containers (~ 200, 3500, 1200 ml, respectively) receiving Wilfrid Laurier well water on a flow-through basis. The next day, rates of Na^+ influx, outflux and net flux were measured under control conditions (no TFM, 0-4 h) and at regular intervals (0-2 h, 2-4 h, 4-6 h, 6-8 h, 8-10, 10-12 h) during TFM exposure ($LC_{99.9}$).

At the beginning of each unidirectional flux experiment on larval lamprey, flow to the boxes (flux chamber) was stopped and water levels were adjusted to 114 ml in each flux chamber. The volumes of the flux chambers holding the upstream-migrant lamprey

and rainbow trout were adjusted to 3000 and 1000 ml, respectively, prior to the start of each unidirectional flux. In larval lamprey and rainbow trout, 2.5 μCi of $^{24}Na^{+}$ was added to each chamber, but upstream-migrant lamprey received a dose of 10.0 μCi of $^{24}Na^{+}$ due to the larger volume of their container. After a 10 min mixing period, 10 ml water samples were collected at hourly intervals during a 4 h flux measurement period, from the containers holding larval lamprey. Upstream-migrant lamprey and rainbow trout were sampled at the same time intervals, but 20 ml water samples were collected during each 4 h flux measurement period. At the end of the control flux (no TFM, 0-4 h), the chambers were not flushed, but the water volume was re-adjusted to its respective start volume, and extra isotope was added to the container to compensate for $^{24}Na^{+}$ loss due to radioactive decay and sampling. The TFM was then added to the larval lamprey flux chambers at a nominal concentration of 8.6 mg l^{-1}, (actual concentrations 8.0 mg l^{-1}), while upstream-migrant lamprey exposure were exposed to a nominal concentration of 4.5 mg l^{-1} (actual concentration 4.4 mg l^{-1}). Rainbow trout were exposed to a nominal TFM concentration of 17.8 mg l^{-1} (actual concentration 17.3 mg l^{-1}). No larval lampreys survived beyond 10 h during exposure to their 12 h TFM $LC_{99.9}$, while no upstream-migrant lampreys survived beyond 6 h at their 12 h TFM $LC_{99.9}$. The rainbow trout that survived the 12 h TFM exposure were euthanized by a blow to the head.

Water samples were processed for determination of total counts per minute (CPM) for $^{24}Na^{+}$ by adding 4 ml of aqueous counting scintillant (Amersham Biosciences, USA) to 2 ml sample. CPMs were determined in triplicate using a Beckman-Counter Multi Purpose Scintillation counter (Model LS6500, USA). The remaining water samples were saved for quantification of non-radioactive ("cold") Na^{+}. Due to the short half-life

of $^{24}Na^{+}$ (~15 h) samples mixed with aqueous counting scintillant were not left overnight in an attempt to minimize chemiluminescence as overnight. To determine whether chemiluminescence could affect the measurement of radioactivity in the ion flux samples, the difference in CPM was immediately determined on a subset of water samples spiked with $^{24}Na^{+}$ isotope and the appropriate amount of ACS. The sample was then measured 10 h later and both measurements were back calculated to time zero and indicated there were no significant differences in radio-activity suggesting that chemiluminescence was negligible.

3.2.2.2.2 Determining Rates of Influx, Outflux and Net Flux

The net Na^{+} flux (J^{Na}_{Net}) was determined using the following equation (Wilkie et al. 1999):

$$J^{Na}_{net} = (([Na^{+}]_i - [Na^{+}]_f) \times V) / M \times T, \quad (1)$$

where $[Na^{+}]$ represents the concentration (μmol ml^{-1}) of total non-radioactive ("cold") Na^{+}, measured at the beginning (i) and end (f) of a flux period, M is the fish's mass (kg), V is the volume of the container (ml) and T is the duration of the flux interval (h). Using this approach, positive values represent net *inward* movements or gains of ions, while negative values indicate net *outward* movements or loss of ions. Rates of Na^{+} influx (J^{Na}_{in}) were determined from reductions in water radioactivity during each flux determination period, the known container volume, and the fish's mass, using the following formula (Wilkie et al. 1999):

$$J^{Na}_{in} = ((CPM_i - CPM_f) \times V) / (MSA \times M \times T), \quad (2)$$

where J^{Na}_{In} is the Na^{+} influx rate, CPM is counts per minute, and CPM_i and CPM_f are the ^{24}Na radioactivities (CPM ml^{-1}) at the start and end of the flux determination period, MSA

is the mean water specific activity of $^{24}Na^+$ in the external water relative to the respective "cold" concentrations of $^{24}Na^+$ (CPM μmol^{-1}) as determined by dividing the average CPM (CPM ml^{-1}) by the average total "cold" Na^+ concentration ($\mu mol\ ml^{-1}$) during a given flux period, and M, V and T are as previously stated. Back-flux corrections were not performed because the protocol used was similar to that used by Wilkie et al. (1999), who reported that maximal internal specific activity never exceeded 10% of the water specific activity (Wood 1988). Outward movements of Na^+ (J^{Na}_{out}) were calculated from differences between J^{Na}_{Net} and J^{Na}_{In}, as described by (Wilkie et al. 1999) where:

$$J^{Na}_{out} = J^{Na}_{Net} - J^{Na}_{In} \quad (3)$$

3.2.2.3 Effects of TFM on Larval, Parasitic and Upstream-Migrant Sea Lamprey Gill Na^+/K^+-ATPase Activity, Plasma Ions and Tissue Water

Gill, blood and muscle tissue water samples were collected from separate groups of fish exposed to the same concentrations of TFM (12-h TFM $LC_{99.9}$) described above, but not subjected to the unidirectional Na^+ flux protocol using $^{24}Na^+$. A separate group of lampreys were also exposed to the 12-h TFM LC_{50}, and the gills, plasma and muscle were collected for analysis of gill Na^+/K^+-ATPase activity, and plasma and tissue water and ion concentrations.

3.2.2.3.1 Gill Na^+/K^+-ATPase Activity Determination

To determine the effects of TFM on branchial Na^+/K^+-ATPase activity, gills were processed following the methods described by McCormick et al. (1993) in which Na^+/K^+-ATPase activity was calculated based on the difference between uninhibited ATPase activity and oubain inhibited ATPase activity. The assay solutions consisted of 2.8 mmol

l^{-1} phosphoenolpyruvate (PEP), 3.5 mmol l^{-1} ATP, 0.495 mmol l^{-1} NADH, LDH (4 units ml^{-1}), pyruvate kinase (PK; 5 units ml^{-1}) in imidazole buffer (50 mmol l^{-1}) at pH 7.5. A salt solution containing 189 mmol l^{-1} NaCl, 10.5 mmol l^{-1} $MgCl \bullet 6H_2O$, 42 mmol l^{-1} KCl prepared in de-ionized water was added in a 3:1 ratio to the non-oubain assay solution and the oubain (0.5 mmol l^{-1}) assay solution.

Approximately 25 mg of gill tissue was homogenized on ice in a 4:1 (400µl: 100µl) ratio of SEI buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA and 50 mmol l^{-1} imidazole buffer) to SEID buffer (0.1 g sodium deoxycholate to 50 ml SEI buffer). Total ATPase activity was then measured by adding 25µl of the homogenized tissue to a microwell plate with the addition of 200µl of the non-oubain and oubain assay solution. The protein concentration in homogenates was determined by the Bradford assay (Bradford 1976). The Na^+/K^+-ATPase activity was subsequently based on the difference between the total ATPase activity minus the oubain-inhibited ATPase activity, using the following formula:

$$Na^+/K^+\text{-ATPase activity} = ((WO\text{-}O/SCS)/P)*60 \text{ min}), \qquad (4)$$

where WO and O are Na^+/K^+-ATPase activity without ouabain (WO) and with ouabain (O) (mOD/25µl/min), SCS is the ADP standard curve slope (mOD/nmol ADP), P is the concentration of protein in the homogenate (µg/25µl) and 60 min converts the units from min to h.

3.2.2.3.2 Blood Sampling

To remove blood from larval lampreys, the caudal fin was cut off behind the cloca exposing the caudal vessels. Lampreys were held vertically, allowing the blood to drip from the caudal vein into the capillary tube. Capillary tubes were then centrifuged for 5

min at 10, 000 x g for plasma and frozen at -80 °C for later determination of plasma ions (Na^+, Cl^- and Ca^{2+}). To remove blood from parasitic and upstream-migrant lampreys, a 1 ml insulin syringe coated with heparinized saline was inserted behind the cloca into the caudal vessels. Blood was then transferred into a microcentifuge tube that been coated with heparinized saline and emptied completely to prevent the saline from altering plasma ion concentrations. The microcentrifuge tube was then centrifuged for 5 min at 10, 000 x g for plasma and frozen at -80°C for later determination of plasma ions (Na^+, Cl^- and Ca^{2+}).

3.2.2.3.3 Plasma Ion and Water Na^+ Analysis

Plasma Cl^- was diluted and determined according to the mercuric thiocyanate assay (Zall et al. 1956), while plasma Na^+ and Ca^{2+} were diluted 850 and 100 times (respectively) in de-ionized water (E-pure) to fall within an appropriate range that the flame atomic absorption spectrometry could measure.

Total Na^+ in the water for unidirectional flux samples was also determined by using flame atomic absorption spectrometry, but there was no need to dilute water samples as the flame atomic absorption spectrometry could measure Na^+ at the expected concentration and a large enough volume of sample was taken.

3.2.3 Calculations and Statistical Analysis

Determination of the 12 h $LC_{99.9}$ for the larval and upstream-migrant lampreys, and rainbow trout exposed to TFM was done using the Two-Point Interpolation method available from the analytical program CETIS (Tidepool Sientific Software, Version 1.6.1, California).

All data is presented as the mean ± one SEM. Comparisons between control and experimental fish were made using one-way ANOVA. Where significant variability was observed, significant differences between the means were determined using a Newman-Keuls post-test for flux measurements, plasma ion concentrations and muscle tissue water, and gill Na^+/K^+ ATPase activity at the $p<0.05$ level. When data sets did not meet one or all the three assumptions of a parametric design, a non-parametric test (Kruskal-Wallis) was used to determine significant differences between means.

3.3 Results

3.3.1 Determination of the Acute Toxicity of TFM

The nominal TFM concentrations of 1.0, 3.5, 5.0 and 8.0 mg l^{-1} for larval lamprey were reflected by measured TFM concentrations of 1.6, 3.3, 5.0 and 8.6 mg l^{-1}, respectively. The larval lamprey exposed to TFM for 12 h had $LC_{99.9}$ of 8.6 mg l^{-1} (Table 3-1). Subsequently, larval lampreys were exposed to nominal TFM concentration of 8.6 mg l^{-1} (measured 8.0 mg l^{-1}).

The nominal TFM concentrations of 0.5 1.5, 2.5 4.5, and 8.0 mg l^{-1} for upstream-migrant lamprey were reflected by measured TFM concentrations of 0.6, 1.6, 2.6, 4.7 and 8.7 mg l^{-1}, respectively. This experiment yielded a 12 h TFM $LC_{99.9}$ of 4.5 mg l^{-1}, which was then used as the nominal concentration used in the upstream-migrant TFM exposure experiments (measured 4.8 mg l^{-1}; Table 3-1).

Rainbow trout were exposed to measured concentrations of 8.0, 9.7, 11.6, 13.9, 17.8 and 27.1 mg l^{-1}. It was determined that the 12 h TFM $LC_{99.9}$ of 17.9 mg l^{-1} (Table 3-

1), which was then used as the nominal concentration used in the rainbow trout exposure (measured 17.5 mg l^{-1}).

3.3.2 Effects of TFM on Ion Transport

Under pre-exposure (control) conditions, larval sea lamprey experienced a net loss of -178 ± 54 μmol•kg^{-1}•h^{-1} Na^+ as influx rates were approximately 117.7 ± 24.3 μmol•kg^{-1}•h^{-1} Na^+ and out flux rates were approximately -296.2 ± 59.5 μmol•kg^{-1}•h^{-1} Na^+ (Figure 3-1). Net loss of Na^+ continued in the first 2 h of TFM exposure, followed by a net gain of Na^+ during 2-4 h of TFM exposure, both of which were not significantly different from control rates (Figure 3-1). Outflux during 2-4 h of TFM exposure were not significantly different from control rates (Figure 3-1). By 4-6 and 6-8 h of TFM exposure Na^+ influx, net flux and outflux was comparable to control values. Na^+ influx, net flux and outflux during 8-10 h of TFM exposure were not significantly different from control rates.

Upstream-migrant lamprey not exposed to TFM experienced a net loss of Na^+ at rate of -75 ± 77 μmol•kg^{-1}•h^{-1} (Figure 3-2). However, exposure to TFM did not significantly alter Na^+ influx, net flux or outflux. Upstream-migrant lamprey experienced a similar net loss of Na^+ to controls during the first 4 h of TFM exposure of -84.3 ± 61.6 and -160.9 ± 35.7 μmol•kg^{-1}•h^{-1} Na^+, respectively (Figure 3-2).

Resting rainbow trout not exposed to TFM were in net ion balance. Under pre-exposure (control) conditions trout experienced a net loss of -57 ± 64 μmol•kg^{-1}•h^{-1} Na^+, when influx rates were approximately 245.9 ± 34.2 μmol•kg^{-1}•h^{-1} Na^+ and out flux rates were approximately -302.7 ± 73.9 μmol•kg^{-1}•h^{-1} Na^+ (Figure 3-3). From 0-2 and 2-4 h of TFM exposure rainbow trout continued to lose Na^+ at comparable rates to control

conditions. Although rainbow trout experienced a net gain of 165.2 ± 39.7 μmol•kg^{-1}•h^{-1} Na^+ during 4-6 h of TFM exposure, this was not significantly different from control rainbow trout. Following 4-6 h of TFM exposure rainbow trout net Na^+ movement returned to rates comparable to control conditions.

3.3.2.1 Impact on Gill Na^+/K^+-ATPase Activity

Gill Na^+/K^+-ATPase activity was measured in larval, parasitic and upstream-migrant lamprey exposed to their respective 12 h TFM $LC_{99.9}$. Exposure to TFM had no effect on gill Na^+/K^+-ATPase activity in the larval or upstream-migrant life stages (Table 3-2). Control larval and upstream-migrant lampreys had activities that were approximately 0.21 ± 0.034 and 0.27 ± 0.047 μmol ADP mg-1 prot. h^{-1} (Table 3-2). However, parasitic lamprey did experience changes in gill Na^+/K^+ ATPase when exposed to TFM. By the first hour of exposure gill Na^+/K^+-ATPase activity in parasitic lamprey had increased approximately 50% from the control values of 0.17 ± 0.026 μmol ADP mg^{-1} prot. h^{-1}. Gill Na^+/K^+-ATPase activity remained high throughout the rest of the exposure peaking at 0.38 ± 0.066 μmol ADP mg^{-1} prot. h^{-1} during the 5th hour of TFM exposure (Table 3-2).

3.3.2.2 Plasma Ions

TFM had no effect on plasma ion balance in parasitic or upstream-migrant lampreys. Parasitic and upstream-migrant plasma Na^+ ranged from 112.0 ± 6.0 to 128.1 ± 2.8 mmol l^{-1} (Table 3-3). As expected, plasma Cl^-'s were comparable to plasma Na^+ in each life stage. Plasma Cl^- in parasitic and upstream-migrant lamprey ranged from 104.6 ± 3.4 to 123.2 ± 3.0 mmol l^{-1} (Table 3-3). Parasitic lamprey plasma Ca^{2+} ranged from 2.4

± 0.1 to 3.6 ± 0.7 mmol l^{-1} (Table 3-3), while upstream-migrant plasma Ca^{2+} ranged from 1.5 ± 0.1 to 1.8 ± 0.1 mmol l^{-1} (Table 3-3). Muscle tissue H_20 in the three life stages was unaffected by TFM exposure, ranging from 76.1 ± 2.0 to 80.2 ± 0.7 % water in larval lamprey, 80.2 ± 2.3 to 82.2 ± 1.5 % water in parasitic lamprey and 81.0 ± 0.4 to 81.6 ± 0.6 % water in upstream-migrant lamprey (Table 3-3).

3.3.3 Effects of Lower Level TFM Exposure (LC_{50}) on Upstream-migrant Sea Lamprey Plasma Ion Balance and Gill Na^+/K^+-ATPase Activity

TFM had no effect on plasma ions, tissue water or gill Na^+/K^+-ATPase in upstream-migrant lamprey exposed to their 12 h TFM LC_{50}. Plasma Na^+ in lampreys ranged from 115.1 ± 5.4 to 130.9 ± 5.9 mmol l^{-1} (Table 3-4). Plasma Cl^- were similar to plasma Na^+, ranging from 97.9 ± 10.8 to 121.5 ± 7.1 mmol l^{-1} (Table 3-4). Control plasma Ca^{2+} in upstream-migrants was approximately 1.8 ± 0.2 mmol l^{-1} (Table 3-4). Muscle tissue water was stable, ranging from 81.2 ± 0.6 to 82.7 ± 0.6 % H_2O (Table 3-4). Despite an upward trend in gill Na^+/K^+-ATPase activity, TFM exposure did not significantly alter Na^+/K^+-ATPase activity from control values of 0.09 ± 0.02 μmol ADP mg^{-1} prot. h^{-1} (Table 3-4).

3.4 Discussion

To test the hypothesis that larval and upstream-migrant lampreys, and rainbow trout experience a disruption in gill-mediated ion regulation in the presence of TFM, we examined the unidirectional movement of Na^+. In addition, plasma ion concentrations (Na^+, Cl^-, Ca^{2+}) and gill Na^+/K^+-ATPase activity in larval, parasitic and upstream-migrant lampreys were quantified to determine if TFM interfered with active ion transport processes in the gill and internal electrolyte balance. The current findings suggest that exposure to TFM ($LC_{99.9}$) has little effect on unidirectional Na^+ movements (Figure 3-1; 3-2; 3-3), plasma ion concentrations (Table 3-3; 3-4), with a limited non-pathological effect on gill Na^+/K^+-ATPase activity (Table 3-2; 3-4).

3.4.1 Effects of TFM on Plasma Ions and Gill Na^+/K^+-ATPase Activity

The histological effects of TFM on lamprey and rainbow trout gills have been well documented (Christie and Battle 1962; Mallatt et al. 1985, 1994). Research from such studies indicates that most damage caused by TFM is localized to MRCs, which is characterized by cell and mitochondrial swelling, the appearance of vacuoles in the MRCs, and spaces between adjacent gill epithelial cells. Such damage, however, was only observed in larval lamprey exposed to their TFM LC_{100}, while rainbow trout MRCs were unaffected by TFM.

In freshwater lampreys there are two types of MRCs thought to be involved in the uptake of Na^+ and Cl^- uptake from fresh water (Bartels and Potter 2004; Wilson et al. 2007). As in teleost fishes, lampreys are faced with an outwardly directed ion gradient

favoring ion loss across the gills. In teleosts, it appears that Na^+ uptake is promoted by apical Na^+-channel and V-type-ATPase complexes in which the active extrusion of H^+ by the ATPase generates an electrical gradient across the apical membrane that favours Na^+ entry into the cell, while the basolateral Na^+ /K^+-ATPase pumps Na^+ out of the cytosol to contribute to the electrochemical gradient (Marshall 2002; Evans et al. 2005; Figure 1-6). A similar arrangement is thought to be present in lamprey (Bartels and Potter 2004; Perry 1997), but more experimental evidence is needed. Due to the importance of the MRCs in gill ion regulation, Mallatt et al. (1994) suggested that the damage caused by TFM to MRCs in sea lamprey could cause disruption to gill-mediated ion regulation. They proposed that analysis of blood ions would determine if TFM did disrupt gill-mediated ion regulation. In the present study, sea lampreys (parasitic and upstream-migrant) exposed to their 12 h TFM $LC_{99.9}$ experienced no changes in plasma Na^+, Cl^-, or Ca^{2+} concentrations, which is in good agreement with others who reported that TFM exposure did not affect plasma ion concentration (Wilkie et al. 2007; Birceanu et al. 2009).

Analysis of gill Na^+/K^+-ATPase activity suggests that exposure to TFM does not damage this protein, further suggesting that TFM has minimal effect on gill-mediated ionoregulatory processes. Larval sea lamprey gills showed no changes in Na^+/K^+-ATPase activity, and control rates were comparable to those reported in larval lamprey in previous studies (Reis-Santo et al. 2008; Birceanu et al. 2009). However, Birceanu et al. (2009) found that gill Na^+/K^+-ATPase activity increased after 9 h of TFM exposure, but the lamprey were exposed to a lower concentration of TFM than that used in the present study. As a result, the lamprey studied by Birceanu et al. (2009) had longer survival times, which may have provided the animals with a wider window of opportunity for

increased synthesis and/or activation of Na^+ /K^+-ATPase quantity in the gill. Because ATP is necessary to power Na^+/K^+-ATPase activity (Beamish 1980; Marshall 2002), such a response might have compensated for reduced ATP supply due to presence of TFM, which impairs ATP production (Birceanu et al. 2009).

A limitation of the method used to measure Na^+/K^+-ATPase activity, was that coarsely ground gill filaments, with an excess of ATP, were used to assess enzyme activity. As a result, it was not possible to detect changes in Na^+/K^+-ATPase activity arising from changes in gill ATP supply due to TFM. Further investigation would help determine whether gill ATP supply during TFM exposure effects gill Na^+/K^+-ATPase activity. However, Na^+/K^+-ATPase activity and internal ion balance appear to be robust when faced with challenges that can cause ionic disturbances. For instance, Reis-Santos et al. (2008) reported that when anadromous larval lamprey were exposed to deionized water, plasma ion concentrations were unaltered and gill H^+-ATPase and Na^+/K^+-ATPase activity were unaffected. Deionized water would be expected to put lamprey under ionic and/or osmotic stress because of an increased blood-water electrochemical gradient that would favor ion losses, and a lack of external substrate for ion pumps. Reis-Santos and colleagues suggested that baseline ATPase levels (H^+-ATPase and Na^+/K^+-ATPase) may have been sufficient to maintain proper ion balance in poor-ion water. If apical H^+-ATPase activity were to increase in lampreys under TFM exposure, Na^+ uptake would increase, helping to compensate for any Na^+ loss that lampreys may experience during a TFM exposure.

Although TFM had no effect on larval or upstream-migrant gill Na^+/K^+-ATPase activity, parasitic lamprey gill Na^+/K^+-ATPase activity was affected by TFM. Following

one hour of TFM exposure, gill Na^+/K^+-ATPase activity in parasitic lamprey increased by 100%. Unlike, larval and upstream-migrant sea lampreys, which have a limited ability to increase Na^+/K^+-ATPase activity during exposure to salt water or osmotic stress (Bartels and Potter 2004), recently metamorphosed lamprey have a greater capacity to alter Na^+/K^+-ATPase activity in response to such challenges (Beamish 1980; Reis-Santos et al. 2008). For instance, recently metamorphosized and juvenile anadromous sea lamprey increase gill Na^+/K^+-ATPase activity 13-fold following fresh water-to-salt water challenges, due to the development of salt-water chloride cells (swCC; Bartels and Potter 2004; Reis-Santos et al. 2008). Interestingly, landlocked juvenile sea lampreys are also capable of withstanding direct transfer to full seawater (Mathers and Beamish 1974), indicating that landlocked juvenile lampreys may also have an ability to acclimatize to sudden ionic stresses. Exploring whether landlocked parasitic sea lampreys can increase gill Na^+/K^+-ATPase activity when faced with an osmotic challenge in response to more dilute fresh water, to my knowledge, has not been investigated.

3.4.2 Unidirectional Movement of Na^+ During TFM Exposure

Upstream-migrant lampreys had similar Na^+ influx rates to those measured in previous studies (Wilkie et al. 1998), but the relatively large outlfux of Na^+ suggest that the animals were experiencing some net Na^+ loss under control conditions. Larval and upstream-migrant lamprey exposed to TFM in this study had comparable influx rates, which contrast the findings of Morris and Bull (1968) who found larval sea lamprey Na^+ influx rates 2-3 times higher than the rates of larval and upstream-migrant sea lamprey in this study. Differences in species, collection locations, feeding protocols and water

temperature, or other confounding factors might explain this discrepancy, should be further investigated.

Changes in water pH, ionic content, exposure to metals and other toxicants, and exercise have all been shown to alter the unidirectional movement of Na^+, Cl^-, and Ca^{2+} across the gills of lampreys and other fishes (see Wood 1991; Wood 1992; Niyogi and Wood 2004; Garcia Parra and Baldisserotto 2007 for reviews). Although Mallatt et al. (1994) suggested that damage to MRC due to TFM exposure could alter the influx, net flux and outflux of ions, the present study suggests such effects are minimal.

Larval lamprey exposed to their 12 h TFM $LC_{99.9}$ survived for over 6 h, but then died between 6 and 10 h of TFM exposure. This time course of survival was similar to that reported by Mallatt et al. (1994) during exposure of larval lamprey to their 9-h LC_{100} for TFM. The current findings suggest that lamprey exposed to TFM do not appear to experience challenges in ion regulation. However, Na^+ influx rates did decrease by almost 70 % during 8-10 h of TFM exposure, the change noted was not statistically significant due to the small sample size (n=2). Combined with this reduction in Na^+ influx, was an increase in net Na^+ losses which indicated that Na^+ outflux and therefore gill permeability were increasing with TFM exposure. Using the net Na^+ movement of control larval lamprey as a baseline for determining whether these increased net losses of Na^+ could have detrimental effects on internal ion balance, the change in the total exchangeable Na^+ pool was calculated (e.g Wood 1988). The calculations revealed that 10 h of TFM exposure produced a total net loss of 26.6 μmol/kg of Na^+. This value was only a small fraction (0.1 %) of the reported exchangeable internal Na^+ pool of 23.1

mmol/kg calculated by Thorson (1959) for lamprey, and would therefore have a minimal impact on internal ion balance.

The majority of upstream-migrant lampreys only survived 4 h of TFM exposure, but there was no increase in net Na^+ loss compared to controls during the first 4 h of exposure. However, by 4 h, there was a trend towards a net gain of Na^+, but this was not significant due the small sample size at that time due to TFM-induced mortality. These data further suggest that TFM effects on internal ion balance are not the proximate cause of death in lamprey.

Mallatt et al. (1994) reported that rainbow trout MRCs were not affected by 9 h of exposure to the larval lamprey 9 h TFM LC_{100}, suggesting that the fish would be resistant to TFM-induced ionic disturbances. The present study suggests that this is in fact the case. Exposure of rainbow trout to their 12 h TFM $LC_{99.9}$ had no significant effect on the unidirectional movement of Na^+ across the gills (Figure 3-3). Rainbow trout appeared to be in net Na^+ balance for most of the exposure, despite a trend towards outward movement of Na^+ observed during the later hours of TFM exposure, but this was not significantly different from controls (Figure 3-3). Calculations of total net Na^+ loss suggest that the trout would have lost the equivalent of 392 μmol/kg of Na^+, which would represent a 0.9% decrease in the trout's exchangable internal Na^+ pool of 42.0 mmol/kg (calculated by Wood 1992). Although this study did not measure plasma ions in rainbow trout exposed to their TFM $LC_{99.9}$, Birceanu (2008) reported that plasma ions (Na^+, Cl^-, Ca^{2+}) were unaffected in rainbow trout exposed to their TFM LC_{50}. Although gill Na^+/K^+-ATPase activity in rainbow trout exposed to their $LC_{99.9}$ was not measured in the present study, Birceanu (2009) reported no change in Na^+/K^+-ATPase activity.

There are a variety of mechanisms that cause ionic disturbances in fish while they are exposed to environmental stressors (Wood 1992). For instance, fish exposed to acidic waters experience both an increase in branchial Na^+ efflux and a decrease in Na^+ influx (Garcia Parra and Baldisserotto 2007). The high amount of H^+ in the water results in the loss of Na^+ through the opening of tight junctions of gill epithelia (Gonzalez 1996) and the inhibition of apical Na^+ channel and H^+-ATPase complex preventing the uptake of Na^+ (Garcia Parra and Baldisserotto 2007). Fish exposed to metals can also experience ionic disturbances (Wood 1992), however, unlike heavy metals, which compete for binding sites with important ions such as Na^+ and Ca^{2+} on the gill of fishes (causing ionic disturbances; Niyogi and Wood 2004), TFM uptake is thought to occur via passive diffusion at the apical membrane of the gill (Hunn and Allen 1974, 1975; McDonald and Kolar 2007). If this is the case, TFM could then diffuse along any part of the gill where it is in contact with the lipid membrane. Passive diffusion of TFM would likely eliminate the possibility that TFM would be competing with Na^+ and Ca^{2+} for uptake sites on the gill. However, the speciation of TFM at the gill micro-environment and the effects TFM has on the gill micro-environment have not been thoroughly investigated. For example, if TFM were to decrease the pH of the gill micro-environment one could expect to observe changes in Na^+ influx and efflux similar to that of fish exposed to acidic waters. Further investigation into the effects that TFM has on the gill micro-environment of lampreys would increase our current understanding of TFM on gill-mediated ion regulation.

3.4.3 Conclusion

In conclusion, plasma ion concentrations in parasitic and upstream-migrant lamprey were unaffected by TFM exposure. Larval and upstream-migrant gill Na^+/K^+-

ATPase activity remained comparable to control levels during TFM exposure, however parasitic lamprey gill Na^+/K^+-ATPase activity was elevated. Increased activity in gill Na^+/K^+-ATPase could have helped the animals overcome any loss of Na^+ as a result of the TFM exposure. The unidirectional movement of Na^+ in larval and upstream-migrant lamprey, and rainbow trout suggested that TFM might have caused minor increases in net Na^+ loss, but such losses would have had minimal effects on internal ion balance due to the relatively short time course of TFM exposure. Overall, TFM appears to have little effect on gill-mediated ion regulation despite the damage to MRCs observed by Mallatt et al. (1994).

Future studies should examine the intensity of damage to MRCs necessary to cause disruption in gill-mediated ion regulation. For example, because more of the lipid soluble free phenol is present as the water pH decreases (Hunn and Allen 1974; McDonald and Kolar 2007) TFM could cause more damage to fish gills as more of it is available to across the gills compared to waters with higher pH. In addition, examining whether shorter exposure periods with higher concentrations of TFM, or long exposure periods with lower concentrations of TFM, has a greater or lesser effect on degree of damage to MRCs is important for understanding the potential effects TFM has on gill-mediated ion regulation.

Table 3-1. TFM Toxicity in Larval and Upstream-migrant Sea Lamprey and Rainbow Trout.

12 h TFM LC_{50} and $LC_{99.9}$ and 95% confidence interval.

Life Stage	Test Date	pH	LC50 (mg l^{-1}) (95% CI)	LC99.9 (mg l^{-1}) (95% CI)
Larval	June 10 2010	8.2	5.0 (4.7 – 5.3)	8.6 (7.9 – 8.7)
Upstream-migrant	June 9 2010	8.1	1.6 (0.9 – 2.2)	4.5 (4.2 – 5.5)
Rainbow Trout	August 4 2010	8.1	11.0 (10.6 – 11.5)	17.9 (17.0 – 18.6)

Table 3-2. Gill Na^+/K^+ ATPase Activity in Larval, Parasitic and Upstream-migrant Sea Lamprey Exposed To Their Respective 12 h TFM $LC_{99.9}$.

Data presented as the mean ± 1 SEM (N). Data sharing the same letter are not significantly different. Larval, parasitic and upstream-migrant lamprey were exposed to their respective 12-h TFM $LC_{99.9}$ of 8.1 mg l^{-1}, 5.2 mg l^{-1} and 4.8 mg l^{-1}.

TFM Exposure (h)	Gill Na+/K+ ATPase Activity (μmol ADP mg^{-1} prot. h^{-1})		
	Larval	Parasite	Upstream-migrant
Control	0.21±0.034 (10)[a]	0.17±0.026 (12)[a]	0.27±0.047 (7)[a]
0.5			0.26±0.070 (6)[a]
1	0.35±0.057 (10)[a]	0.34±0.045 (7)[b]	0.23±0.052 (9)[a]
2	0.32±0.023 (10)[a]	0.30±0.027 (7)[a]	0.35±0.060 (8)[a]
3	0.34±0.050 (9)[a]	0.32±0.049 (10)[b]	0.39±0.058 (9)[a]
5	0.32±0.027 (9)[a]	0.38±0.066 (4)[b]	

Table 3-3. The effect of TFM on plasma ions and tissue water in Larval, Parasitic and Upstream-migrant Sea Lamprey.

Data presented as the mean ± 1 SEM (N). Data sharing the same letter are not significantly different. Larval, parasitic and upstream-migrant lamprey were exposed to their respective 12-h TFM $LC_{99.9}$ of 8.1 mg l^{-1}, 5.2 mg l^{-1} and 4.8 mg l^{-1}.

TFM Exposure (h)	Plasma Na^+ (mmol l^{-1})			Plasma Cl^- (mmol l^{-1})			Plasma Ca^{2+} (mmol l^{-1})			Muscle Tissue H_2O (% Water)		
	Larval	Parasite	Upstream -migrant	Larval	Parasite	Upstream -migrant	Larval	Parasite	Upstream -migrant	Larval	Parasite	Upstream -migrant
Control	N/A	120.7± 4.0 (11)[a]	117.8±3. 7 (7)[a]	N/A	108.8± 3.4 (11)[a]	122.2±3. 3 (7)[a]	N/A	2.9±0.2 (11)[a]	1.5±0.1 (7)[a]	76.9± 2.0 (7)[a]	80.9±1. 1 (12)[a]	81.3±0.7 (8)[a]
0.5	N/A	N/A	122.6±6. 3 (7)[a]	N/A	N/A	121.0±5. 4 (7)[a]	N/A	N/A	1.7±0.1 (7)[a]	N/A	N/A	81.6±0.6 (7)[a]
1	N/A	113.4± 4.7 (6)[a]	128.1±2. 8 (8)[a]	N/A	115.8± 3.5 (6)[a]	123.2±3. 0 (8)[a]	N/A	2.4±0.1 (11)[a]	1.8±0.1 (8)[a]	80.2± 0.7 (10)[a]	82.2±1. 5 (7)[a]	81.0±0.9 (9)[a]
2	N/A	112.0± 6.0 (7)[a]	117.1±5. 2 (8)[a]	N/A	101.0± 6.5 (7)[a]	112.2±5. 5 (8)[a]	N/A	2.7±0.2 (11)[a]	1.6±0.1 (8)[a]	77.1± 1.0 (9)[a]	80.3±1. 8 (7)[a]	81.0±0.4 (8)[a]
3	N/A	121.7± 4.4 (9)[a]	123.7±13 .8 (10)[a]	N/A	115.4± 5.0 (9)[a]	115.5±3. 0 (10)[a]	N/A	2.4±0.2 (11)[a]	1.7±0.1 (10)[a]	76.1± 1.4 (9)[a]	81.8±1. 9 (8)[a]	81.3±0.4 (10)[a]
5	N/A	115.5± 2.5 (4)[a]	N/A	N/A	104.6± 3.4 (4)[a]	N/A	N/A	3.6±0.7 (11)[a]	N/A	76.1± 2.0 (7)[a]	80.2±2. 3 (4)[a]	N/A

Table 3-4. The effects of TFM on plasma ions, tissue water and gill Na^+/K^+ ATPase activity in Upstream-migrant Sea Lamprey exposed to their 12 h TFM LC_{50}.

Data presented as the mean ± 1 SEM (N). Data sharing the same letter are not significantly different.

TFM Exposure (h) 2.4 mg g^{-1}	Plasma Na^+ (mmol l^{-1})	Plasma Cl^- (mmol l^{-1})	Plasma Ca^{2+} (mmol l^{-1})	Muscle Tissue H_2O (% Water)	Gill Na+/K+ ATPase Activity (μmol ADP mg^{-1} prot. h^{-1})
Control	115.1±5.4 (7)[a]	109.2±5.2 (7)[a]	1.8±0.20 (7)[a]	82.1±0.70 (7)[a]	0.09±0.02 (7)[a]
1	130.9±5.9 (6)[a]	121.5±7.1 (6)[a]	1.7±0.17 (6)[a]	81.2±0.56 (6)[a]	0.19±0.05 (6)[a]
3	123.5±5.4 (8)[a]	119.1±5.6 (8)[a]	1.9±0.11 (8)[a]	78.8±0.86 (8)[a]	0.23±0.04 (8)[a]
6	105.8±10.7 (6)[a]	97.9±10.8 (6)[a]	1.6±0.10 (6)[a]	82.4±1.10 (6)[a]	0.15±0.04 (6)[a]
9	120.4±0.9 (7)[a]	116.3±10.0 (7)[a]	1.5±0.12 (7)[a]	82.3±0.75 (7)[a]	0.21±0.03 (7)[a]
12	119.6±12.9 (6)[a]	116.0±9.3 (6)[a]	1.4±0.03 (6)[a]	82.5±0.57 (6)[a]	0.26±0.10 (6)[a]

Figure 3-1. Changes In Rates of Na^+ influx, Outflux and Net Flux Across The Body Surface of Larval Sea Lamprey During Exposure To Acutely Toxic Concentrations of TFM ($LC_{99.9}$).

Upward facing open bars denote ion influx (inward movement), downward facing open bars represent ion outflux (outward movement), and shaded bars indicate the net flux (sum of inward and outward movements). Influx, net flux and outflux are presented as the mean. Under control conditions (n=10), 0-2 h (n=8), 2-4 h (n=8), 4-6 h (n=8), 6-8 h (n=8) and 8-10 (n=4). For clarity, the error bars are only shown for influx and outflux data (± 1 SEM). No significant differences ($P>0.05$) were observed in Na^+ influx, net flux or outflux.

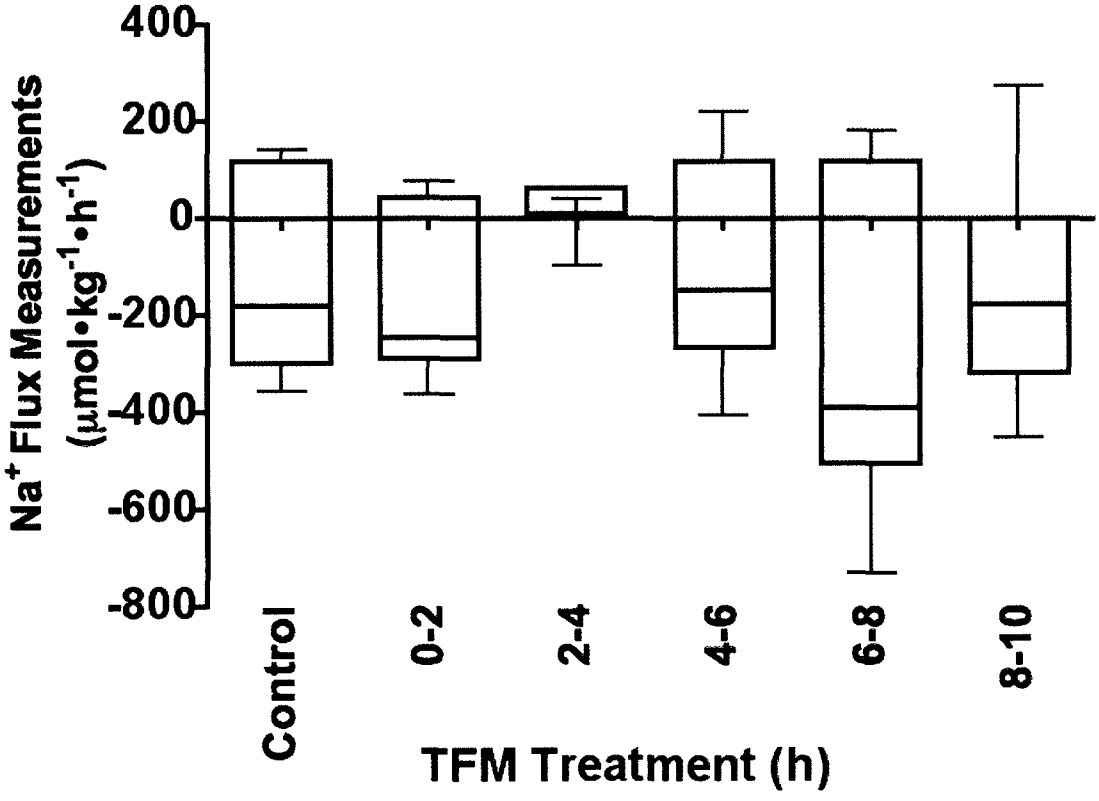

400
200
0
-200
-400
-600
-800
Na+ Flux Measurements
(µmol•kg-1•h-1)
Control
0-2
2-4
4-6
6-8
8-10
TFM Treatment (h)

Figure 3-2. Changes In Rates of Na^+ Influx, Outflux and Net Flux across The Body Surface of Upstream-migrant Sea Lamprey During Exposure To Acutely Toxic Concentrations of TFM ($LC_{99.9}$).

Upward facing open bars denote ion influx (inward movement), downward facing open bars represent ion outflux (outward movement), and shaded bars indicate the net flux (sum of inward and outward movements). Influx, net flux and outflux are presented as the mean. Under control conditions (n=8), 0-2 h (n=8) and 2-4 h (n=3). For clarity, the error bars are only shown for influx and outflux data (± 1 SEM). No significant differences ($P>0.05$) were observed in Na^+ influx, net flux or outflux.

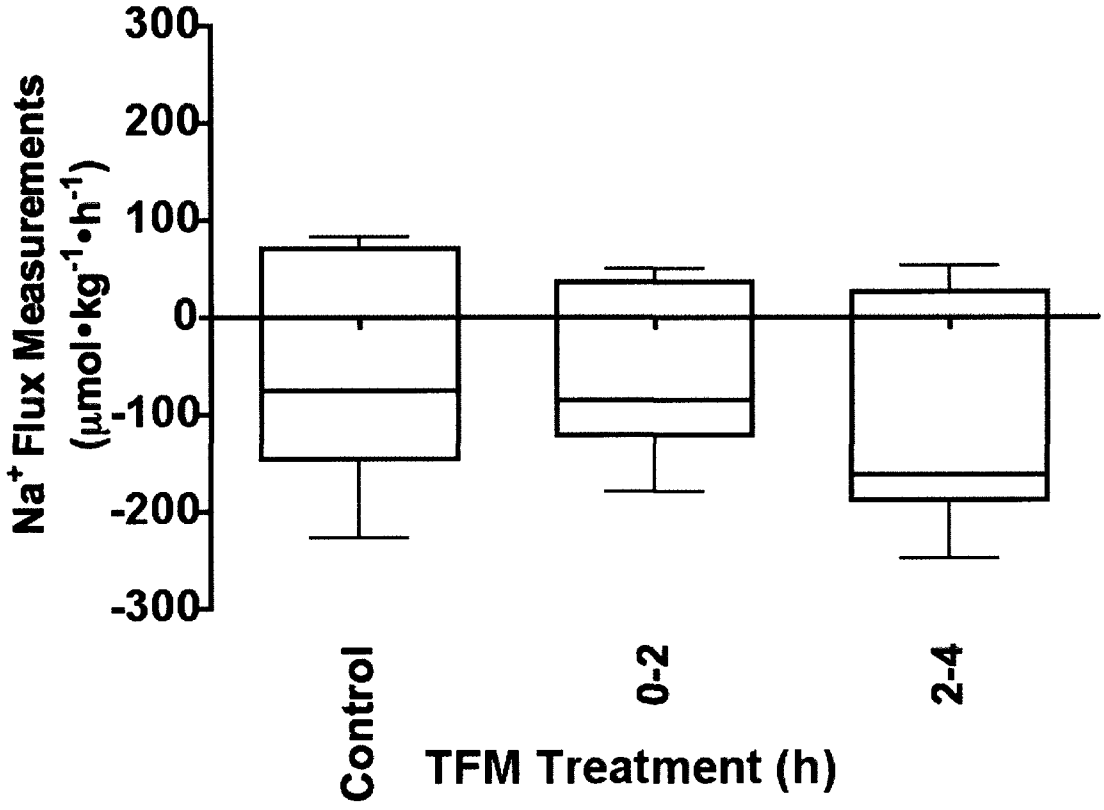
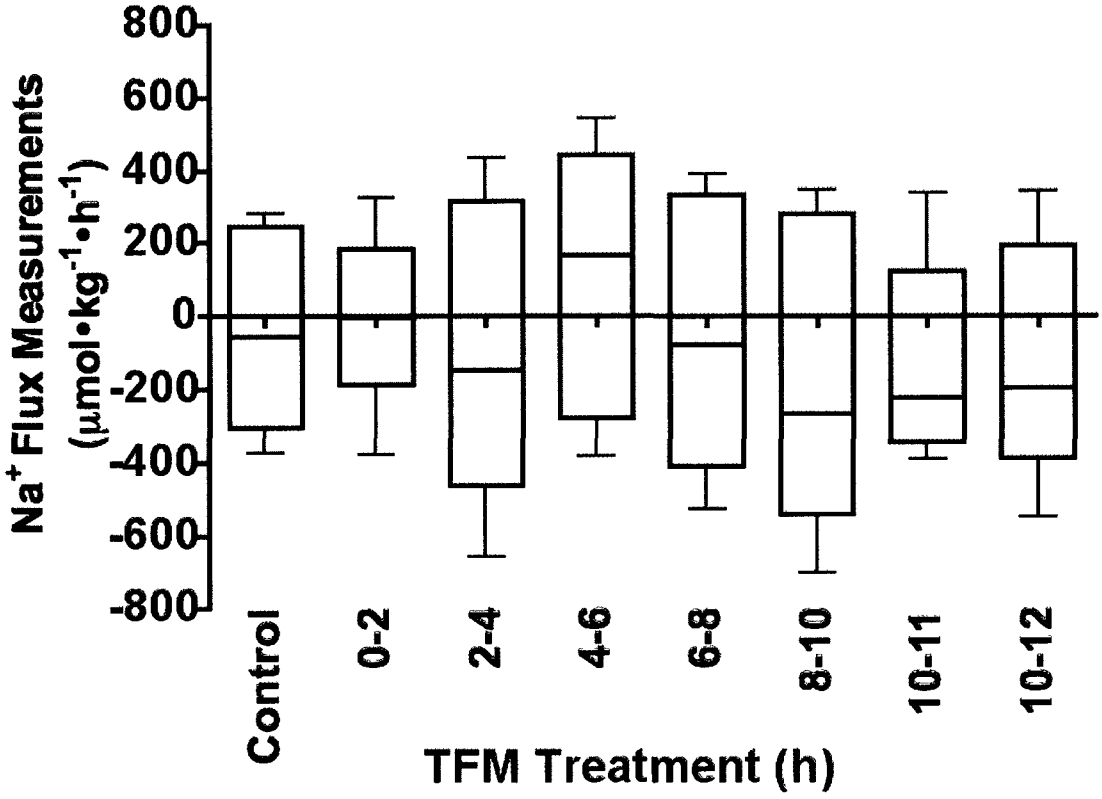

300
200
100
0
-100
-200
-300
Na+ Flux Measurements
(µmol•kg-1•h-1)
Control
0-2
2-4
TFM Treatment (h)

Figure 3-3.Changes In Rates of Na^+ Influx, Outflux and Net Flux Across The Body Surface Of Rainbow Trout During Exposure To Acutely Toxic Concentrations of TFM ($LC_{99.9}$).

Upward facing open bars denote ion influx (inward movement), downward facing open bars represent ion outflux (outward movement), and shaded bars indicate the net flux (sum of inward and outward movements). Influx, net flux and outflux are presented as the mean. Under control conditions (n=9), 0-2 h (n=9), 2-4 h (n=9), 4-6 h (n=9), 6-8 h (n=9), 8-10 h (n=9), 10-11 h (n=2) and 10-12 h (n=3). For clarity, the error bars are only shown for influx and outflux data (± 1 SEM). No significant differences ($P>0.05$) were observed in Na^+ influx, net flux or outflux.


800
600
400
200
0
-200
-400
-600
-800
Na+ Flux Measurements
(µmol•kg-1•h-1)
Control
0-2
2-4
4-6
6-8
8-10
10-11
10-12
TFM Treatment (h)

Chapter 4

An Integrated Model of TFM Toxicity in Sea Lamprey (*Petromyzon marinus*) and Rainbow Trout (*Oncorhynchus mykiss*)

4.1 Synopsis of the Integrative Response of Lamprey and Rainbow Trout to TFM

The structural similarity of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) to 2,4-dinitrophenol (DNP), a known uncoupler of mitochondrial oxidative phosphorylation, suggested that TFM could exert its toxic effects in a similar manner (Applegate et al. 1966). Oxidative phosphorylation, the process that drives ATP production in mitochondria is uncoupled by TFM in isolated rat mitochondria (Niblett and Ballanytne 1976), and as recently demonstrated, in sea lamprey and rainbow trout mitochondria (Birceanu et al. 2011). Previous work by this lab (Birceanu et al. 2009; Birceanu et al. 2011) and the present thesis suggests that the uncoupling of oxidative phosphorylation caused by TFM leads to a reduction in ATP supply, and increased reliance on anaerobic energy pathways such as phosphocreatine hydrolysis, and anaerobic glycolysis. When phosphocreatine and anaerobic glycolysis cannot meet ATP demand and/or glycogen and PCr reserves are too low the organism will likely die. In the nervous system insufficient ATP supply may lead to loss of ion homeostasis across cell membranes, causing cellular swelling and, membrane blebbing and rupture (see Lutz et al. 2003; Bickler and Buck 2007; for reviews), but this possibility awaits further study.

Several studies have suggested that TFM toxicity may be linked to the disruption in gill-mediated ion regulation because of previous reports of TFM-induced histological damage to the gills of trout (Christie and Battle 1961), and to the MRCs in lamprey, which are thought to be involved in ion uptake (Mallatt et al. 1985; Mallatt et al. 1994). However, unidirectional flux measurements of branchial Na^+ influx, outflux, and net flux in lamprey and rainbow trout exposed to TFM (Chapter 3) indicated that larval and

upstream-migrant lamprey experienced at worst, minor changes in Na^+ movement during acute exposure to toxic concentrations of TFM. This was also the case for rainbow trout exposed to acutely toxic concentrations of TFM. The weight of evidence from this thesis, combined with the previous work by Wilkie et al. (2007) and Birceanu et al. (2009), therefore, suggests that disruption of gill-mediated ion regulation is not the proximate cause of TFM toxicity in fishes living in hard, fresh water environments. It remains to be seen if exposure to TFM in softer waters elicits significant disturbances to ion balance in lamprey and non-target fishes.

4.2 More Evidence Supporting the Current Model of TFM toxicity

TFM likely causes toxicity by increasing the permeability of the inner mitochondrial membrane to protons, the most common mechanism of mitochondrial uncoupling (Wallace and Starkov 2000). Birceanu et al. (2011) presented convincing evidence that exposure to TFM decreased the H^+ electrochemical gradient that drives oxidative ATP production. Because of the structural similarity of TFM to 2,4-DNP and the similar response of sea lamprey and rainbow trout mitochondria to TFM as to 2,4-DNP they suggested that TFM is likely a protonophore, which like 2,4-DNP, increases the H^+ permeability of the inner mitochondrial membrane. As a result the H^+ electrochemical gradient that drives the formation of ATP via ATP synthase is dissipated, leading to less ATP production (Wallace and Starkov 2000). My work suggests that this process likely occurs at all three major stages of the sea lamprey life cycle, which forces the lamprey to rely on anaerobic energy pathways such as PCr and anaerobic glycolysis to meet their ATP demands during exposure to TFM. Birceanu et al. (2009) showed that larval lamprey exposed to TFM (12-h LC_{50}) experienced significant reductions in brain

glycogen and PCr, and liver glycogen. Because PCr concentrations are typically smaller, and more short-lived than glycogen reserves, any ATP shortfall must be made-upon by increased reliance on anaerobic glycolysis, which leads to marked reductions in glycogen in the muscle and brain, and the accumulation of lactate (Figure 4-2).

In sea lamprey, glycogen reserves in the meninges of the brain are likely the most important source of glucose for the CNS in the three life-stages of the lamprey life cycle examined. The meninges is a layer of tissue comprising melanocytes, flattened cells and round cells that surrounds the brain (Rovainen et al. 1971). Rovainen (1971) reported that meninges had rich deposits of glycogen which may be an important source of glucose in the parasitic stage, when lampreys might undergo long periods of starvation between feeding bouts. However, the present study suggests that they might also be an important source of glycogen for anaerobic glycolysis in situations where ATP supply does not equal ATP demand such as when lamprey are faced with hypoxia, or during TFM exposure. The reduction in meningeal glycogen stores suggests that the rate of anaerobic glycolysis was increased during exposure to TFM to offset reduced oxidative ATP supply. Thus, meningeal glycogen stores could be a predictor of TFM sensitivity in lampreys.

Parasitic and upstream-migrant lamprey were more sensitive to TFM compared to larval lamprey. Larval lamprey have much larger meningeal glycogen stores relative to their body mass compared to parasitic and upstream-migrant sea lamprey. This would provide larval lamprey with a relatively larger anaerobic energy reserve allowing them to maintain high glycolytic rates for ATP production, a necessary process when oxidative ATP supply is low during exposure to TFM. In addition, differences in basal metabolic

rates could explain differences in the TFM resistance of larval lamprey, compared to parasitic and upstream-migrant lampreys. Because organisms such as larval lampreys have lower metabolic rates, and therefore lower ATP demands (Bickler and Buck 2007), they might be more capable of meeting their basal ATP demands via anaerobic glycolysis, but without depleting their glycogen reserves, than upstream-migrant and parasitic lampreys, which have higher basal metabolic rates. These conclusions are further supported by the fact that larval lamprey are hypoxia tolerant (Potter et al. 1970). Because a common theme between TFM exposure and hypoxia exposure is reduced oxidative ATP supply, if larval lampreys are able to withstand hypoxia, they should be more tolerate to TFM compared to the less hypoxia tolerant parasitic and upstream-migrant sea lamprey, which is what was observed.

4.3 Integrating the Mode of Toxicity into TFM Field Treatments

Because of the increasing costs of lampricides, concerns over impacts to non-target organisms, and ongoing public concern regarding the use of pesticides in the environment, the GLFC has stated that it needs to reduce its reliance on lampricides (McDonald and Kolar 2007). To achieve this goal, a better understanding of TFM's mode of toxicity could provide sea lamprey control personnel with tools to better identify when sea lamprey are most sensitive to TFM. Sea lamprey do appear to exhibit seasonal differences in their sensitivity to TFM (Scholefield et al. 2008), and the present study supports suggestions that such sensitivity may be linked to seasonal differences in glycogen reserves (Birceanu et al. 2009). During the winter months, food availability is much lower compared to the summer months which leads to seasonal differences in liver

glycogen reserves (O'Boyle and Beamish 1977). Whether there are seasonal differences in brain glycogen stores in sea lamprey has not been investigated.

With further evidence that TFM sensitivity is related to internal glycogen stores, it may be possible for TFM treatment supervisors to alter TFM treatment schedules so that southern parts of the Great Lakes are treated first. The overwintering periods are shorter in the southern parts of the Great Lakes and treating this region first may allow sea lamprey control personnel to target larval lamprey before they have had a chance to build-up their glycogen reserves during the spring when primary production starts to increase. Treating streams based on a system that ranks them according to the longest growing season or shortest overwintering period would allow managers to target larval lamprey populations when they are most vulnerable to TFM. This system of treating streams could reduce the use of TFM resulting in significant cost savings and minimizing impacts to non-target organisms.

4.4 Future Directions

Water pH has the most profound effect on the toxicity of TFM to sea lampreys and non-target organisms (McDonald and Kolar 2007). At pH 7, TFM toxicity to sea lamprey is about five times greater than at pH 8, when measured either as the 12 h LC_{50} or the 12 h $LC_{99.9}$ (McDonald and Kolar 2007). Yet,TFM's rate of uptake from the water and how it is affected by water pH has only loosely been investigated (Hunn and Allen 1974). A decrease in pH is thought to increase TFM uptake because a greater portion exists in the lipid soluble free phenol form , which is more permeable across the gills. The effects of water pH on TFM uptake are important because fluctuations in water pH are a common observation during stream treatments (B. Stephens, Manager, Sea Lamprey

Control Center, personal communication; McDonald and Kolar 2007). Understanding how changes in water pH affect sea lamprey and non-target organism's TFM uptake rates would allow sea lamprey control personnel to adjust TFM concentrations accordingly.

Lech and Statham (1975) did report that sea lamprey take up more TFM than rainbow trout based on greater rates of accumulation and steady state concentrations of TFM in the body. This suggests that lamprey may have an increased capacity to transport TFM across the body surface compared to rainbow trout. However, very few studies have directly measured the rate of TFM uptake from the water in sea lamprey and non-target organisms. Understanding how rates of TFM uptake are affected by water pH would further our understanding of how TFM exerts its toxic effects, and provide valuable information to Sea Lamprey Control personnel.

The effects of lampricide treatments on non-target organisms remain an important issue to the GLFC. Many non-target organisms are able to survive lampricide treatments because the target concentration is well below lethal levels. However, the sub-lethal physiological impacts of TFM to non-target organisms exposed to typical stream treatment TFM concentrations have not been thoroughly investigated. For instance, it is unclear whether disturbances in non-target organisms could impair swim performance. If TFM were to reduce swim performance in non-target organisms, they could more vulnerable to predators.

Sensitive non-target organisms, such as larval lake sturgeon and mudpuppies are killed by TFM concentrations ranging from 1.3 to 1.7 x the MLC. Despite this, the mechanism of TFM toxicity in sensitive non-target organisms has not been investigated. Whether glycogen stores are important for TFM resistance in sensitive non-target

organisms is not known. Understanding the importance of glycogen stores to sensitive non-target organisms could help reduce impacts on these species. For instance, if glycogen reserves in sensitive non-target organisms were affected by the seasonal abundance of food, as it is in sea lamprey, treatment managers could avoid TFM applications when glycogen stores are low in sensitive non-target organisms.

4.5 Conclusions

This thesis used an integrative approach to determine how typical stream treatment TFM concentrations affected fuel stores, gill function and ion balance to different life stages of the sea lamprey life cycle, and in the non-target, rainbow trout. Exposure of larval, parasitic and upstream-migrant sea lamprey to TFM causes a mismatch between ATP supply and ATP demand, which leads to increased reliance on anaerobic fuel pathways such as PCr hydrolysis and anaerobic glycolysis. Also, TFM appears to have little affect on gill-mediated ion regulation as the unidirectional movement of Na^+ in larval and upstream-migrant sea lamprey, and rainbow trout were unaffected by TFM exposure. In addition, ion balance in parasitic and upstream-migrant sea lamprey was unaltered by TFM exposure.

Improving our understanding of TFM toxicity will help the GLFC reach its goal of reduced lampricide use and reduce effects to non-target organisms because such knowledge will help managers better predict the vulnerability of both lamprey and non-target organisms. Finally, a better understanding of TFM's mechanism of toxicity should assist the GLFC in re-registration of TFM with the United States Environmental Protection Agency (USEPA) and Health Canada. Continued research on the affects of TFM on sea lamprey and non-target organisms should also contribute to more effective

and safer use of this pesticide in the integrative management of sea lamprey in the Great Lakes.

Figure 4-1. Dissociation equilibria for TFM.

The toxicity of TFM is thought to be closely related to the pK_a (6.38) of TFM. At lower pHs, more TFM is in its lipid soluble free phenol form, and likely more permeable across the gills of lamprey and other fishes than the water-soluble phenolate anion (Hunn and Allen 1974).

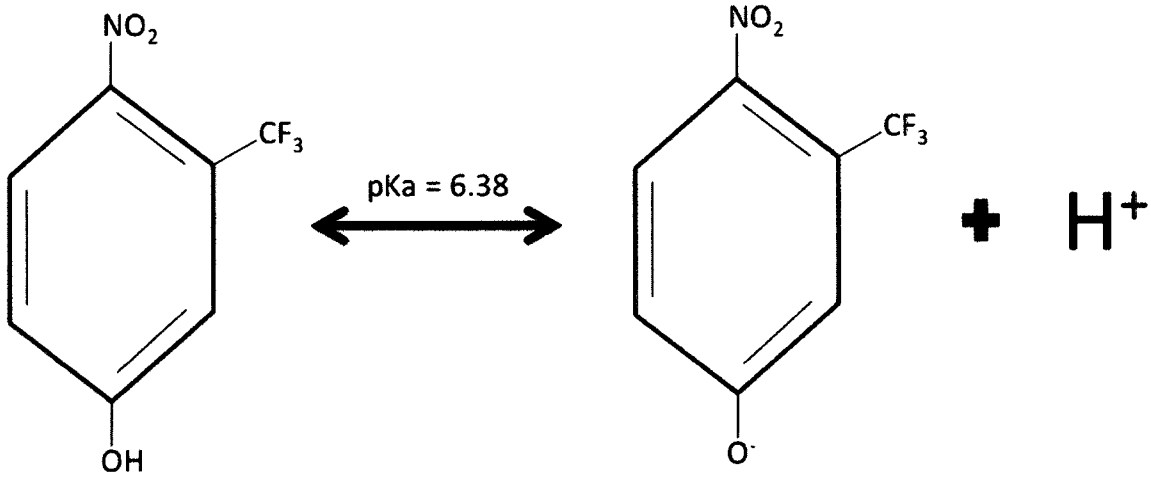

NO2
CF3
OH
pKa = 6.38
NO2
CF3
O-
+
H+

Figure 4-2. *In vivo* Mechanism of TFM Toxicity in Sea Lamprey and Rainbow Trout.

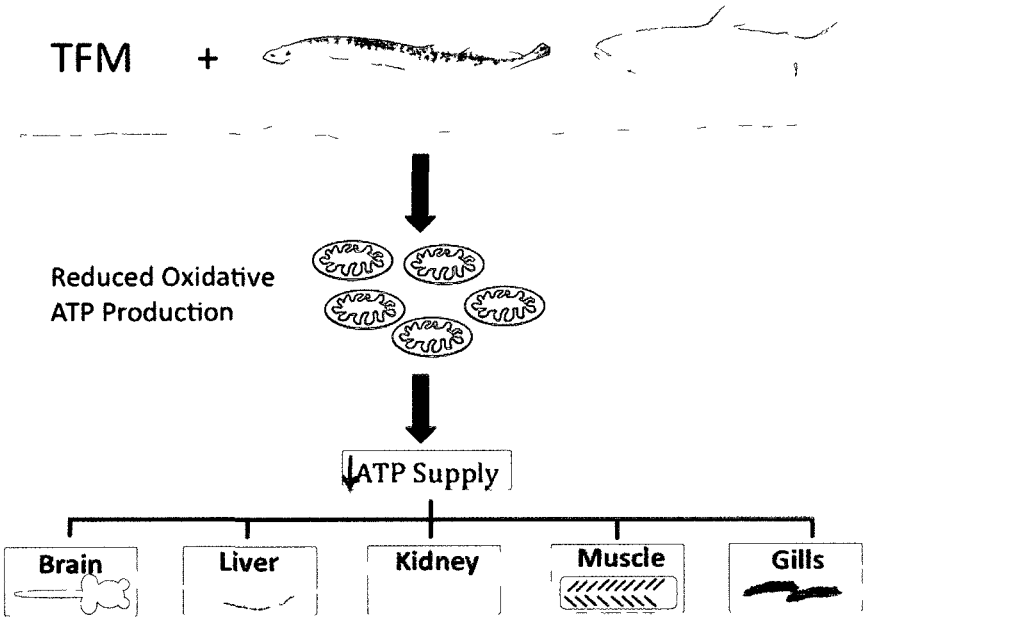
Sea lamprey and rainbow trout exposed to lethal concentrations of TFM accumulate unconjugated TFM and free TFM in their tissues. Because sea lamprey have a limited capacity to detoxify TFM most of it can be found in the sea lampreys' tissue in the unconjugated form. The dissipation of the H^+ electrochemical gradient in the inter membrane gradient reduces oxidative ATP production, which forces sea lamprey to rely on anaerobic ATP production in an attempt to maintain adequate ATP supply to its tissues. As a result, glycogen reserves and PCr stores are utilized to a greater extent. Death will eventually ensue when anaerobic ATP production can longer meet ATP demand. Exposure to TFM had a limited effect on gill-mediated ion regulation in sea lamprey and rainbow trout, suggesting that ionic disturbances do not likely play a major role in TFM-mediated toxicity.

Rainbow trout picture obtained from:

http://pond.dnr.cornell.edu/nyfish/Salmonidae/rainbow_trout

Sea lamprey picture obtained from:

http://pond.dnr.cornell.edu/nyfish/Petromyzontidae/sea_lamprey


TFM
+
Reduced Oxidative
ATP Production
↓ATP Supply
Brain
Liver
Kidney
Muscle
Gills
Sea Lamprey
Brain
↑PCr Hydrolysis
↑Anaerobic Glycolysis
ATP Supply Inhibited
Sea Lamprey
Liver & Kidney
↓ATP Supply
↓Anaerobic Capacity
Sea Lamprey
Gills
Minimal Gill-mediated
Ion disturbances
Rainbow Trout
Gills
Minimal Gill-mediated
Ion disturbances

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